

## Fungal Identification method by rDNA sequence analysis: Molecular approach to reveal the role of microbial community in vermicomposting

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**Abstract:** Internal transcribed spacer is a special sequence that present in between sequence of ribosomal DNA. It is present in multiple copies and act as a conservative sequence. This sequence is unique for particular living being there for it is used as tool for identification. Present study carried out to identification of floral degrading fungi by using of PCR method. This process was done with the help of specific primer. The length of primer was different for different group of fungi. There were four fungi which identified were *Aspergillus flavus*, *A. fumigatus*, *A. terreus* and *Alternaria alternata*.

**Key words:** ITS, PCR, vermicomposting, rDNA, electrophoresis.

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### I. Introduction

Fungi are the group of heterotrophic filamentous organisms that found everywhere on the earth. Their diverse nature affects the activity of ecosystem. Among these fungi, genus *Aspergillus* and *Alternaria* are the most common fungi that have a great impact on living being. The *Aspergilli* are a ubiquitous group of filamentous fungi spanning over 200 million years of evolution. Among over the 185 *Aspergilli* are several that have an impact on human health and society, including 20 human pathogens as well as beneficial species used to produce foodstuffs, composting process and industrial enzymes (Timberlake and Marshall, 1989). Several fungi have been pointed for their key role in vermicomposting of different organic wastes. (Taiwo & Oso, 2004; Peters et al., 2000). The group of fungi mostly characterized on the basis of morphology, physiology and genomic sequencing. The 18S rRNA sequence is generally used to identify the eukaryotic organism because of less change occurred in this sequence. The ribosomal DNA (r RNA genes) acquires characteristics which are appropriate for the recognition of microorganisms at the species level. This rDNA is highly stable and show a variety of conserved and diverse regions within the genome (Hibbett, 1992). They also occur in multiple copies in per haploid genome (Bruns, et al., 1991; Yao et al., 1992) arranged in tandem repeats, each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan et al., 1995).

Therefore we focused on the ITS regions of ribosomal genes (Figure 1) for the identification of floral waste degrading fungi. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *Fusarium* (O'Donnell, 1992) and *Verticillium* spp. (Nazar et al., 1991). Moller et al. (1999) also developed polymerase chain reaction (PCR) to identify *Gibberella fujikuroi* anamorphs in maize kernels using primer pairs based on sequences of RAPD fragments, and were specific for *Fusarium moniliforme* and *Fusarium subglutinans*. Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. The objective of our investigation was to develop a reliable and sensitive PCR assay for the selective detection of composting fungal species and also find out their evolutionary correlation between them.

### II. Materials And Methods

#### Fungal isolates

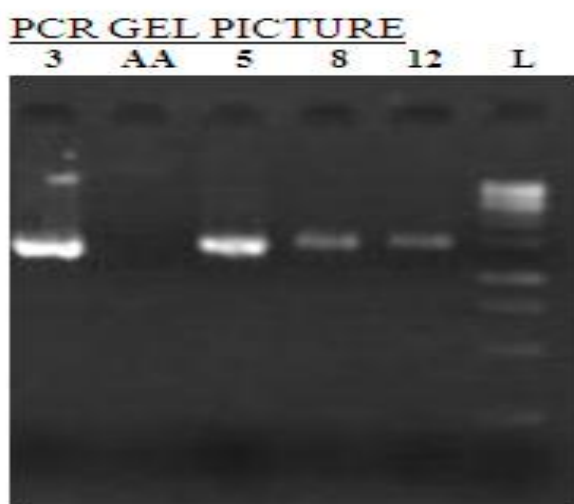
In the present study, following four isolates were used i.e. *Aspergillus flavus*, *A.fumigatus*, *Alternaria alternata* and *A.terreus*. The isolates originated from floral waste vermicomposting process done in Govt. MVM Ujjain (M.P.), India.

### DNA isolation

Total genomic DNA was isolated from fresh mycelium according to a miniprep protocol described by Ceniz (1992) and Abd-Elsalam (2003). In this method potato dextrose broth medium (Hi-media) was inoculated with fungal mycelium and left at room temperature for three days. After that, tube allowed to centrifuge at 10,000 rpm for 5 min, so that mycelial mat was pelleted. Now Pellet was washed with 500 µl Tris-EDTA buffer. The mat was then homogenized by hand in 300 µl of extraction buffer for 5 min. One hundred and fifty micro liters of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20°C for 10 min. Fungal debris was pelleted by centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 10,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was resuspended in Tris-EDTA.

### PCR amplification of ribosomal DNA regions

The ITS and the inverting 5.8S coding rDNA were amplified by PCR using the primers ITS described by White et al. (1990). Each PCR reaction mixture contained 10 ng of genomic DNA, 1µM each of the primers ITS, reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH 8.3] 0.1 mg/ml bovine serum albumin), 3 mM MgCl<sub>2</sub>, 200µM each of dNTP and 2.5 U of Taq DNA polymerase (Promega, Mannheim, Germany) in a total volume of 50 µl. The PCR profile was enetration at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min. ITS bands of interest were excised from agarose gels and re-amplified by PCR using the same primer pair that was used for generating the ITS bands (Fig.1).



**Fig 1.** PCR amplified Gel Picture

### Fungal rDNA sequencing:

PCR products were purified to remove excess primer using microconcentrators and after obtaining the ITS band, it was taken out from the agarose gel was directly sequenced with the Di- Deoxy Terminator sequencer. Obtained sequences are as follow.

#### 1) *Aspergillus fumigatus*:

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>ATATGAGGAGGCTCGCCGAAGAATGCGAAGGATTCAGCGGTGCGGATTTGGGAAGTTTGCTACG
CCGTGCCGGTTATTCTGCAATCAAAAGACGCGATCAGATCAGCTTTGAGGACTTCGTTGCCGCTAA
GGCTTTCATTCGGCCTAGTGTTACCGATCTCAAAAAGTATGAGAAGCTCAGGAGAGAGTGGAGCG
GCGGCGTGTTGTAGATATCCGACCAGTGTGGCATACAAAGTCAACGAGAGCATTGTGTACGTACG
GGATAATGGTCATATACAAAAGCTTTGCATGTGCGGATGCTGACAATCTCCTCGCTTGCTTGGAT
GTTTGTACTCAATGAGTCTACCAATCGTTCGTTCTTCCACATAATACCTCCAAGAATTAGACTTGAT
ATCGGGATGCCAGGTTGCTTGGACCATCAGACGAGGGCTTATTTTAAAGGAAACAGTTTCTATAAG
GACTATAACTTTTATAAAGCGGATATATCTCTTGACGAAAACATTGATGCACACCAAGACGCCTCT
CATGTAGACATGACCTGATAAATTAACAGTTGGAAATCCCAATATGTACGCTTCGAATCGACGTGT
GATTACATCCAGAGACGGCAAGTT
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#### 2) *Aspergillus flavus*:

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>ATTCCTGAATTCCCTCCCTCACCTCCACGATGGTTGACCATATCTCCCCCGGGCATCTCCCGGACC
GATCCGTTCCCTCCAGACTCGCCGCGCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTC
AAAAGTGCGATGCACCAAGGAGAAACCGGCCGTGTGCTCGGTGTATCGAACGTGGTCTTGCCCTGTC
AATACATGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCAGTCCCCTTGATTCAACTCGGC
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GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGTA  
ACGCCTCATGCTCATAACGACAGGCCACACTCATGCTCATTCTCATCCGCAACCGCATCC  
CACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCACCCCAATGGTAGCAGT  
AGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGGGCCTT  
GGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCCTGTCTTCCCTAACAGTCGATTTCG  
GAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCGAG  
TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAA GTAGGGACCCCATGATCGACCCGTTCC  
TCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTAT TGCTGCTTTTCGCTAGCACTA  
CAAACACTGACCCACCTCTTCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGGACG  
GTGAGGACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGG AACAAGAGGG  
CTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGCT  
GAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA  
CCCA GTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGT  
CCCGCCACCGTGTCAGTGGCTGTCTGACGGAAGAGCGGTGCTGCACCTCCCTAGTATG  
ATGGGGCAGGATTGTG TGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCG  
AGTGAAGTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCT GCAA.
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### 3) *Alternaria alternata*

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>GCGTCAGTAACAAATTAATAATTACAACCTTCAACAACGGATCTCTTGGTTCTGGCATCG  
ATGAA GAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGA  
ATCTTTGAAC GCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTCGAGCGTC  
ATTTGTACCCTCAAGCTTTGCTTGGTGTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACT  
CGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAAGTCGACTCT  
CTATCAGCAAAGGTCTAGCATCCATTAAGCC TTTTTCAACTTTTGACCTCGGATCAGGT  
AGGGATACCCGCTGAACTTAAGCATATCAATAAGCGG AGG.
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### 4) *Aspergillus terreus*

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>ATCTTTATGGCCACCTCCCACCCGTGACTATTGTACCTTGTGCTTCGGCGGGCCCCGCC  
AGCGTTG CTGGCCGGCGGGGGGCGACTCGCCCCGGGCCCCGTGCCCGCCGGAGACCCCA  
ACATGAACCCTGT TCTGAAAGCTTGCAGTCTGAGTGTGATTCTTTGCAATCAGTTAAA  
ACTTTCAACAATGGATCTCTTG GTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATA  
ACTAATGTGAATTGCAGAATCCAGTGAA TCATCGAGTCTTTGAACGCACATTGCGCCC  
CTGGTATTCCGGGGGCGCATGCCTGTCCGAGCGTCA TTGCTGCCCTCAAGCCCGCTTGT  
GTGTTGGGCCCTGTCCCCGGCTCCCCGGGGACGGGCCCGA AAGGCAGCGGGCACCGG  
TCCGGTCCCGTATGGGGCTTCGTCTCCGCTCCGTAGGCC GGCCGGCGCCCGCCGACG  
CATTTATTTGCAACTTATTTATTCCA GGTGACCTCGGATCAGTAGGGAA
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## III. Results

In this present study, special types of oligonucleotide probes were used for identification of ITS sequence present in fungal rDNA. This process performed by PCR amplification method. After amplification, PCR products were separated by agarose gel electrophoresis (Fig. 1). On the gel, four different bands were obtained. Using the universal fungal primers (ITS), PCR products were generated from all of the four different fungal species. The base pair of these sequences were determined with the help of control bands. Figure 1 illustrates the different sizes obtained for the full ITS region amplified from a selection of different *Aspergillus* and *Alternaria* species. Oligonucleotide probes were designed from ITS sequences available in this study; the full ITS region was sequenced from each species. One sequencing reaction was performed on each strand. Sequence result showed that the length of base pairs of ITS were in between 398 - 1216 bp. The shortest length of bp was 398 of *Alternaria alternata* while largest length of bp was 1216 of *Aspergillus flavus*. In this method known primer were used that was species specific. The primers showed good specificity for ITS sequence of fungal species. Due to this specificity, fungal species were identified.

## IV. Discussion and Conclusion

The rRNA genes, commonly used in identification and taxonomic studies, were confirmed in the present study to be particularly appropriate for the purpose of providing target sequences for molecular detection. Differences in the nucleotide composition of the variable ITS region have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar et al., 1991; Moukhamedov et al., 1994; Schilling et al., 1996; Moricca et al., 1998). O'Donnell (1992) found a surprising level of divergence for ITS sequences within the species of *F. sambucinum*. We used ITS primers to amplify the entire 18S rDNA gene. The amplified DNA was sequenced to develop a genus-specific PCR assay

for the rapid identification of *Aspergillus* and *Alternaria* genera, isolated from floral waste vermicomposting mixture.

Nevertheless, the current approach of testing known related species is justified primarily by the use of rDNA as the basis for specificity. The nucleotide sequence analysis of rDNA region has been widely accepted to have phylogenetic significance, and is therefore useful in taxonomy and the study of phylogenetic relationships (Hibbett, 1992). This approach, designing primers from the rDNA region has far superior reliability compared to the use of random non-defined probes or primers.

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