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MOLECULAR IDENTIFICATION OF THE FUNGAL BIOMASS ISOLATED FROM CONTAMINATED SOIL USING 18S rRNA SEQUENCING

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ABSTRACT

Soil is a living ecosystem teeming with multitudes of invisible residents. One cup of native soil supports billions of microscopic organisms, including bacteria and fungi. These unseen creatures have great influence; can be beneficial, neutral or harmful. The present paper deals with isolation and identification of fungal biomass from contaminated soil using 18S rRNA based molecular technique. The fungal species was isolated and characterized and confirmed using a molecular approach. Comparison of this gene sequence with known sequence in NCBI database, it is considered that isolate is closely related to members of the *Aspergillus spp.,* Phylogenetic and molecular evolutionary analyses were conducted using 18S rRNA sequencing. The fungal strain was identified as *Aspergillus flavus.* (Gene Bank accession No: FJ537130.1)The sequence when submitted to nrdatabase of NCBI using BLAST showed 99 – 100% maximum identity and E - value equal to 0 for all closely related taxa.

INTRODUCTION

There are approximately 70,000 to 80,000 species of fungi, while filamentous fungi are identified using mainly morphological characteristics, such as their ability to utilize carbon and nitrogen compounds. However these methods of identification are often problematic as there can be different biotypes within a single species. It is also a time consuming and requires a great deal of skill. Henceforth to overcome this problem molecular approach for Fungal Identification using Ribosomal Internal Transcribed Spacer (ITS) analysis was employed.

More recently, the Internal Transcribed Spacer (ITS) regions of the ribosomal operon have been used for fungal systematics and classification. There are 2 ITS regions in the fungal rRNA operon. The first, ITS1, is found between the18S and 5.8S rRNA genes. The second, ITS2, is located between the 5.8S and the 28S rRNA genes. The entire rRNA operon is transcribed; however, after transcription, the 2 ITS sequences are excised and are therefore not used for any functional purpose.

Since the ITS sequences are important enough as spacer regions to be maintained by the cell, but not used for any functional purpose, they are allowed to accumulate mutations at a faster rate than the 5.8S, 18S, and 28SrRNA genes. It is this slightly increased rate of accumulated mutations which allows the ITS sequences to provide an improved level of resolution. It is generally accepted to sequence the entire stretch of ITS1-5.8S-ITS2 for use in fungal classification. However, for the purposes of routine identification, a study has found that the use of ITS2 alone is usually sufficient for species level identification.

In this method a partial region of the subunit of the fungal rRNA gene is sequenced and compared with the known fungal DNA sequences .In this study isolated fungal biomass was identified using 18S rRNA sequencing.

One of the major limitations in investigating fungal diversity in soil, in which the extracted DNA pool constitutes DNA from a diverse range of eukaryotic and prokaryotic organisms, has been the suitability of available PCR primers. The challenge has been to design PCR primers that amplify as broad a taxonomic range of fungi as possible, but at the same time to prevent co-amplification of closely related eukaryotic DNA. The primary target for the development of PCR primers for assessing fungal diversity in soil has been the rRNA gene cluster and, despite its limitations, the 18S rRNA gene has been the most widely used, exploiting both the conserved and the variable regions contained within it [1-4.]. In addition, the internal transcribed spacer (ITS)

region located between the 18S rRNA and 28S rRNA genes, and incorporating the 5.8S rRNA gene, has also been targeted [1, 5-6]. Non-/coding rDNA spacer regions, such as the ITS, benefit from a fast rate of evolution, resulting in greater sequence variation between closely related species compared with the more conserved coding regions of the rRNA gene cluster. Thus, fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions [7, 8.]

MATERIALS AND METHODS

Sample Collection

Soil sample was collected at different sites of the field using sterile scalpel and transferred to sterile polythene bags for further analysis.

Isolation of fungal biomass

10 g soil sample were added into 90ml sterile distilled water and agitated for uniform microbial suspension. Serial dilution were made up to 10^{-7} .10 ml were poured into 15-20 ml sterile Sabourd Dextrose agar medium (Hi-media) supplemented with chlortetracycline(10mg/L).Plates were incubated at room temperature for 3-5 days. Fungal isolates were identified using the characteristic structures seen in culture which includes colonial morphology, hyphae, asexual spores, reproductive bodies and conidia arrangement. Slide culture techniques were used to observe morphological characteristics of fungi. Isolates were used for genomic DNA isolation and were identified further by 18S rRNA gene analysis.

The culture was examined by morphological and microsopical characteristics.

(i) Culture characterization (ii) Microscopic Observation

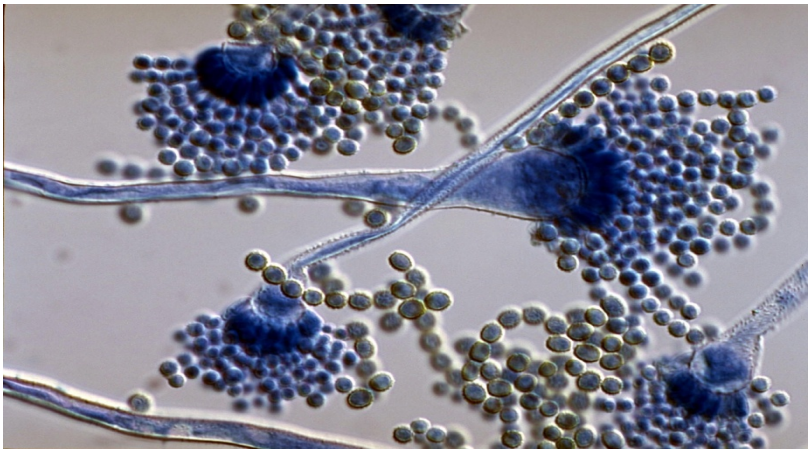

Figure 1: Microscopic Image of *Aspergills Flavus*

Source of the Photo: http://www.clt.astate.edu/mhuss/Aspergillus%20flavus%20pict.jpg

Fungal DNA extraction

1 g (fresh weight) of mycelium is placed in a mortar pre-cooled at -80 ºC and is ground to a fine powder with liquid nitrogen. The powder is suspended in 1.5 mL of lysis buffer (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] SDS) and heated at 68 ºC for 15 minutes, with occasional gentle mixing. Centrifuged at 13 000 rpm for 15 minutes (4 ºC), the supernatant was transferred to a new tube and polysaccharides and proteins are precipitated by adding 750 µL of cold 4 M sodium acetate, at pH 5.2. This solution was gently mixed by inversion, placed at -20 ºC for 20 minutes and centrifuged at 13 000 rpm for 15 minutes (4 ºC). Clean supernatant was transferred to a new tube and precipitated with one volume of cold isopropanol (-20 ºC). This was gently mixed by inversion for a few minutes, incubated at -20 ºC for at least 10 minutes and centrifuged at 13 000 rpm for 15 minutes (4 ºC). DNA pellet is washed with 1.0 mL of cold 70% ethanol, centrifuged at 13 000 rpm for 10 minutes (4 ºC) and air dried. DNA is resuspended in 100 to 200 µL of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0), depending on the yield, and stored at -20 ºC. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 µg/µL). A single intense band with slight smear was noted. The extracted genomic DNA was used as template DNA for amplification of the 18S rRNA gene.

Agarose Gel Electrophoresis

10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with ethidium bromide (0.5µg/µL) as per the standard protocols [9] at 80V/cm and the reaction product was visualized under Gel documentation System (Alpha Innotech).

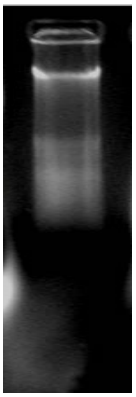

Figure 2: 0.8 % Agarose gel electrophoresis showing band of genomic DNA

PCR amplification of 18S rRNA gene

PCR amplifications[10] are performed on 50 µL of a reaction mixture containing MgCl2-free reaction buffer, 3 mM MgCl2, 2.5 U of Taq polymerase, 200 µM of each dNTP, 10P moles of each primer and template DNA(100 ng). Amplification of the ITS region was performed using Universal primers ITS 1 and ITS 4. ITS1 (5′TCC GTA GGT GAA CCT TGC GG 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′)[11] The amplification was carried out in a Master cycler® Thermocycler (Eppendorf, Germany).PCR is carried out as follows: 1) 1 step at 94 ºC for 3 min; 2) 35 cycles of the following three steps: 1 min 94 ºC, 1 min at annealing temp (specific for each primer pair, usually at or close to 55 ºC), 1 min 72 ºC; and 3) one final 10 min step at 72 ºC. PCR products are separated by electrophoresis on a 1.5% agarose gel with 0.5 % ethidium bromide in 1x TAE buffer (40 mM Tris base, 40 mM acetic acid, 1.0 mM EDTA, pH 8.0) and visualized under UV light. PCR product was purified to remove unincorporated dNTPS and Primers before sequencing.

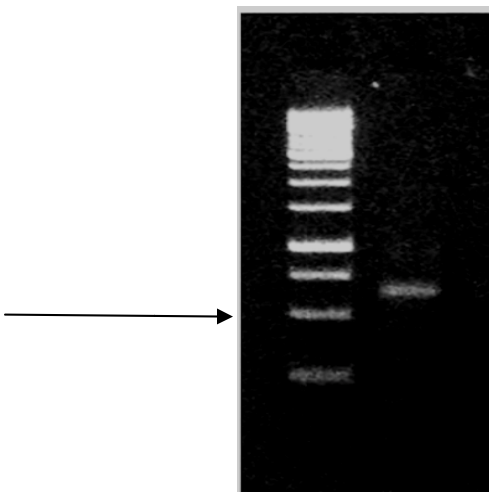

Figure 3: 1 % Agarose gel electrophoresis showed PCR Product of ~600bp,
Lane M: Marker, Lane 1: PCR Product.

Purification of PCR Product by Exosap-IT

The PCR product is subjected to purification by using Exosap-IT, it is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that removes left over primers and free nucleotides from the PCR reaction. To 5 µl of PCR product add 2 µl of Exosap .Further incubated at 37ºC for 15 minutes to allow the degradation of primers and free nucleotides. Tube was transferred to 80ºC water bath and incubated for 15 minutes to inactivate the Exosap-IT enzyme. The sample is ready for sequencing reaction.

Identification of the isolated fungi by sequencing of the amplified 18S rRNA gene

The most powerful tool to identify the unknown microorganism is to sequence the gene (DNA) coding for 18S rRNA .The gene coding for the 18S rRNA is to be amplified using the PCR and the amplified product has been subjected to sequencing and the sequence obtained has been compared with the known sequence from the Nucleotide Database of NCBI.

Sequencing

The 18S rRNA purified PCR product (100ng concentration) was subjected for the sequencing using ABI DNA 3730 XL sequencer (Applied Biosystem Inc). Sequencing of the 18S rRNA gene of the fungal isolate was done from both the directions. The sequence so obtained was compared with already reported results from the public databases (NCBI) and the assembled sequence of the18S rRNA gene (DNA) of the unknown fungi was determined.

>AACGACCACCACCAAACACCCCGCCGGCTGGTGTGCATGACCTTGACGCTGCCCCC
CGATGCCGGGCCATTGCTTCAAGACCGTGATCCATGACTTTGCAATCACTACTACCG
TGCTTTTCATCGAGCGACCAAATCATTGTTGACCGTTTGATGATGTATTTAGACTCGA
TGCATCACTCTCGGCTGAATTCGTGTCCCGGCGCTGCCCCGGGGGTTCCCAGCCTAG
CTACAATTTATGATTTCATGGTGGGGGTGGGCGCCTGGAGGCAGCCCGCACTCAGTA
ATGATCCTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGTGCGGGCTGCCTCCG
GGCGCCCAACCTCCCACCCGTGAATACCTAACACTGTTGCTTCGGCGGGGAACCCCC
TCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTG
AGTCTGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATC
GATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAGTCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCG
AGCGTCATTGCTGCCCATCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCGGG
GGACGGGCCCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGC
TTTGTCACCCGCTCGACTAGGGCCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTT
TCTTCAGGTGACCTCGGATCACGTACGCTGCCCGTCATTC<

Figure 4: *Aspergillus flavus* isolate South-west0063 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

Computational analysis

BLAST (Basic Local Alignment Search Tool) is a web based program that is able to align the search sequence to thousands of different sequences in a database and show the list of top matches. This program can search through a database of thousands of entries in a minute. BLAST [12] performs its alignment by matching up each position of search sequence to each position of the sequences in the database. For each position BLAST gives a positive score if the nucleotides match, it can also insert gaps when performing the alignment. Each gap inserted has a negative effect on the alignment score, but if enough nucleotides align as a result of the gap, this negative effect is overcome and the gap is accepted in the alignment. These scores are then used to calculate the alignment score, in "bits" which is converted to the statistical E- value. The lower the E-value, the more similar the sequence found in the database is to query sequence. The most similar sequence is the first result listed. Finally a phylogenetic tree was constructed(Fig 5).

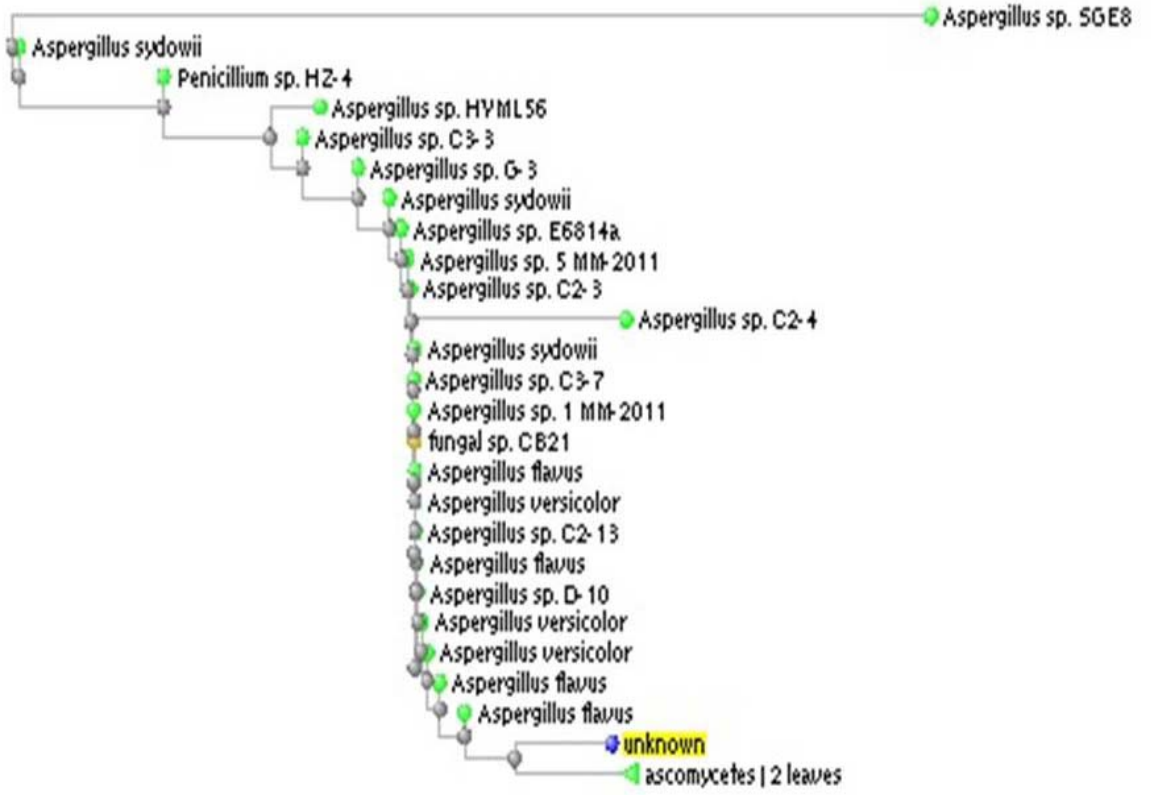

Figure 5: Phylogenetic Analysis by BLAST for *Aspergillus flavus*.

RESULTS AND DISCUSSION

The traditional identification of microorganism on the basis of phenotypic characteristics (Fig 1) is generally not as accurate as identification based on genotypic methods. Comparison of the

fungal 18S rRNA gene sequence has emerged as a preferred genetic technique. The sequence of the 18S rRNA gene has been widely used as a molecular clock to estimate relationships among microorganisms (phylogeny), but more recently it has also become important as a means to identify unknown microorganisms to the genus or species level[13]. The use of 18S rRNA gene sequences to study phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons.

The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains. The presence of genomic DNA isolated from the soil sample was confirmed on 0.8% agarose gel stained with etidium bromide. (Fig.2). an intense single band was seen along with the DNA marker. The extracted DNA was used as template for amplification of 18S rRNA gene. The universal primers ITS 1 and ITS 4 were used for the amplification and sequencing of the 18S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band was visible on 1% agarose gel stained with ethidium bromide (Fig. 3).The PCR product was subjected to sequencing using BDT V3.1 cycle sequencing kit on ABI 3730XL genetic analyzer from both forward and reverse directions. The sequences (Fig. 4) obtained were compared with the nrdatabase of NCBI gene bank database using BLAST search program (http;//www.ncbi.nlm.nih.gov)[14]**.** The percentages of sequence matching were also analyzed. The determined fungal isolate was found to be *Aspergillus flavus* isolate South-west0063 (Gene Bank accession No: FJ537130.1). Phylogenetic tree derived (Fig. 5) from 16S rRNA gene sequences showing the position of *Aspergillus flavus.*

Aspergillus flavus is predominately a saprophyte and grows on dead plant and animal tissue in the soil. For this reason it is very important in nutrient recycling. However, *Aspergillus flavus* can also be pathogenic on several plant and animal species, including humans and domestic animals. *Aspergillus flavus* is the most widely known species of the genus Aspergillus which is known as a species in 1809 and first reported as a plant pathogen in 1920. Like other Aspergillus species, this fungus has a worldwide distribution due to its numerous conidia production, which easily disperses by air movements and possibly by insects. *Aspergillus flavus* plays a major role as a nutrient recycler, supported by plant and animal debris and contaminates a wide variety of agricultural products in the field, storage areas, processing plants, and during distribution. The ability of *Aspergillus flavus* to survive in unfavorable conditions allows it to easily out-compete other organisms for substrates in the soil or plant.

CONCLUSION

The use of 18S rRNA gene sequences to identify new strains of fungal is gaining momentum in recent years. We showed the use of 18S rRNA gene sequence to characterize the fungal species isolated from the soil and was found to be *Aspergillus flavus* isolate South-west0063. Thus, the genotyping method using 18S rRNA gene sequence is both simple and effective in strain identification.

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