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Molecular Identification of *Aspergillus flavus* Using Inter Transcribed Spacers (ITS)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Infections caused by *Aspergillus* have grown in importance in the last decade. Outbreaks which are caused by *Aspergillus flavus* appeared to be related to the single or closely related fungal strains. The fungi produce a compound known as aflatoxin which is reputed to be the most toxic and powerful hepatocarcinogenic compound. Most of the recent studies are mainly based on, the most abundance species in the genus. However, *A. flavus* is the second major reason for invasive aspergillosis and direct infections in most part of the world especially tropical countries. This study is focused on the molecular identification of *A. flavus.* For this purpose, spores were collected from air sample. Molecular identification of the fungi was done by using universal primers ITS -1F and ITS-4R. Phylogenetic tree demonstrated that the fungal spores collected from the air lie in the clad with *Aspergillus*, indicating its close relationship with the group.

Keywords: Aspergillus; universal primers; and phylogenetic tree.

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1. INTRODUCTION

Fungi play an important role in the soil ecosystem as they are plant major decomposers, releasing nutrients that maintain and fuel plant growth in the process [1,2]. Some fungi have antagonistic properties towards plant pathogens [3]. They act as plant pathogens, mycorrhizal symbiotic relationship and most importantly they are major decomposers of organic materials Fungi also represent a main part of the soil microflora in terms of biomass [2].

Scientist named "Link" first define *Aspergillus flavus* as a group of closely related fungal species. After *A. fumigatus*, *A. flavus* came in second place to cause invasive aspergillosis in human [4]. Many of infections in insect are caused by *A. flavus* [5]. Moreover, major economical crops, such as, peanut and maize are more susceptible to the fungi species as it produces potent mycotoxins in the crop and causes diseases.

The sequencing of *A. flavus* demonstrated the rich source of comparative data. It indicates that *A. flavus* has eight chromosomes with a genome size of 36.3Mb and 13,071 predicted genes. *A. flavus* is found worldwide, like other *Aspergillus* species. This is most probably because they produce numerous airborne conidia which spread by air movement and insects. Humidity in air is an advantageous factor for the growth of mould [6]. A water activity ranging from 0.86 and 0.96 demonstrate more favorable condition for *A. flavus* growth [7]. The fungi grow at optimal temperature of 37°C but it has been observed that it grows usually at a temperature between 12 to 48°C. The similarity between such high temperature range and optimal temperature of human contributes *A. flavus* frequent infections in humans.

Different studies show that *A. flavus* is predominant in the air tropical countries [8-11]. One of the major features of tropical counties is rainfall which contributes in high air humidity. This type of air composition majorly contributes to the abundance of *A. flavus* in the air. *A. flavus* prevalence in the air is a main risk for both allergic and invasive aspergillosis [12]. It is observed that many invasive aspergillosis outbreaks have been resulted because of the construction work in or around the hospital areas [13,14]. These activities majorly enhance the spore count in the air which eventually result in aspergillosis outbreak. By molecular typing method, the relationship between infection caused by *A. flavus* and environmental contamination has been clearly demonstrated [15,16]. Studies from Iran demonstrated that when hospital wards air was inspected, *A. flavus* was the most abundant fungal stain found in the air [17,18].

A. flavus has been particularly prevalent in the air of some tropical countries [8-11]. Climatic conditions markedly influence the prevalence of *A. flavus* in outdoor air. The presence of *Aspergillus* in the air is a major risk factor for both invasive and allergic aspergillosis [12]. Accordingly, several outbreaks of invasive aspergillosis have been associated with construction and/or renovation activities in and around hospitals, activities that markedly increase the number of spores in the air. Also, in several studies, the link between infection by *A. flavus* and the contamination of the environment was clearly demonstrated by molecular typing methods [13-16].

Fungal growth in drinking water may change the taste and smell of the water. The condition causes many health-related problems such as, allergy, direct infection and mycotoxin exposure. Fungal contamination has a tendency to rise from surface reservoirs rather that deep ground wells [19]. This differentiation basically arose from different variables such as temperature pattern of water (surface versus well), pattern of water treatment and maintenance. In addition to that, it was studied that fungi can survive the water cleanliness treatment by means of system leaks or from contact with water surface with airin distribution system reservoirs. Furthermore, it can also by-pass the chlorine disinfection [20]. This study is mainly focus on the collection of *A. flavus* spores from air and molecular identification using universal primers, ITS1 and ITS4.

2. MATERIALS AND METHODS

2.1 Culturing of *Metarhizium anisopliae* on Selected Media

Aspergillus flavus spores were collected from the air by placing wet bread in open air for 2 days. The fungus was streaked on PDA media at 27 or 30°C in the dark for 10–19 days. Growth of *A. flavus* was observed during the culture time.

2.2 DNA Extraction

For DNA extraction, an autoclaved pestle and mortar was pre-cooled at -80°C. Approximately

0.4-0.5 g fungi spores from fungi culture and grounded in fine powder by using liquid nitrogen (-80). Lysis buffer (200 mM Tris- HCl [pH=8.5], 250 mM NaCl, 0.5 mM EDTA [pH=8.0], 0.5% [w/v] SDS) was added at the ration of 10 mL per gram and homogenized gently. The homogenized mixture was put into the eppendorf tubes and heated on water bath at 68°C for 45min with occasional mixing. The eppendorf tubes were centrifuged at 13000 rpm for 20 min at 4°C. The supernatant was transferred into fresh eppendorf carefully and one volume of cold 4M sodium acetate (pH=5.2) added to precipitate the polysaccharides and proteins. After mixing it properly the solution was incubated at -20°C for 20 min. the solution was then centrifuged at 13000 rpm for 15 min at 4°C and clean supernatant was transferred to a fresh eppendorf tube. To get the DNA pellet, one volume of chilled iso-propanol was added to it and mixed gently and incubated at -20°C for 10 min. After incubation, eppendorf tubes were centrifuged at 13000 rpm for 15 min at 4°C. 30 uL of ultra-pure (UP) water and 30 uL Roti phenol were added to get clean pellet. Dissolved pellet was centrifuged at 13000 rpm for 10 min. Supernatant was taken and again centrifuged at 13000 rpm for 10 min. The supernatant was discarded and DNA pellet was washed with 1.0 mL of cold 70% ethanol and centrifuged at 13000 rpm for 10 min at 4°C. The pellet was air dir dried and dissolved in 30-50 uL of TE buffer (10mM Tris-HCl [pH=8.5], 1.0 mM EDTA [pH=8.0]) depending upon the pellet size by tapping and stored at -20°C. The quality and quantity were analyzed by resolving the DNA sample on gel electrophoresis.

2.3 Amplification of Full Length ITS Region

2.3.1 Primer design

The amplification of TS region was carried out using universal primer ITS1 as a forward primer (CTT GGT CAT TTA GAG GAA GTA A) [Gardes and Bruns, 1993] and ITS4 as reverse primer (TCC TCC GCT TAT TGA TAT GC) [21].

2.3.2 PCR profile

Reaction mixture 25 µL ($MgCl_2$ 25 mM, BSA 0.5 mg, forward primer 0.2 µM, reverse primer 0.2 µM, dNTPs 0.2 mM, Taq Polymerase 0.04 U), prepared for ITS region amplification was initially denatured at 95°C for 7 min followed by 35 cycles consisting of denaturation at 95°C for 50 sec; primer annealing at 60°C for 1 min and primer extension at 72°C for 1 min and finally extension at 72°C for 10 min in a thermal cycler.

2.3.3 Agarose gel electrophoresis

Amplified PCR products of 16S ribosomal RNA gene were separated on 1% agarose gel in 1X TAE buffer containing ethidium bromide (20 mg/mL). 1 kb DNA ladder (Fermentas) was used as a size marker. The gels were viewed under UV light and photographed using gel documentation system (GelDoc-It 310 Imaging system P/N 97-0266-02).

2.3.4 Purification of PCR products

An equal volume of autoclaved UP water and double volume of 100% ethanol were added to amplified ITS region. The solution was incubated at -20 for overnight. After incubation, the solution was centrifuged at 13000 rpm for 10 min. The supernatant was discarded and pellet was re-suspended in 200 ul of 70% ethanol. The solution was centrifuged at 13000 rpm for 5 min. the supernatant was discarded and pellet was air dries in laminar flow. Pellet was dissolved in autoclaved UP water.

2.3.5 Sequencing of full length ITS region

Cloned PCR products were sequenced commercially by MACROGEN. The gene sequences were compared with others in the Gene Bank databases using the NCBI BLAST at. Http://www.ncbi.n1m.nih.gov/blast/Blast.cgi.

2.3.6 Sequence analysis and phylogenetic analysis

The sequence data were assembled and analyzed with the help of Lasergene package of sequence analysis software (DNAStar Inc., Madison, WI, USA). Sequence similarity searches (Blast) were performed by comparing the sequence to other microbes sequencing in the database (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed by ClustalX and phylogeny was determined by neighbor-joining method.

3. RESULTS

3.1 Culturing of *A. flavus* on Selected Media

The colony morphology of *A. flavus* showed that it is a fast-growing fungus. The fungus had taken 1 week to grow its fullest (Fig. 1).

3.2 DNA Extraction and Amplification

Genomic DNA of *A. flavus* was successfully extracted by using the earlier mentioned method. A 700 bp fragment of the r-DNA ITS region was amplified and sequenced, the results were submitted to gene bank databases (Fig. 2).

3.3 Taxonomic Identification of Entomopathogenic Fungus by ITS Sequencing

The DNA coding for the ribosomal RNA gene complex (rDNA) in fungus consists of genes encoding the 18S, 5.8S, and 28S fungal rRNA subunits that are separated by ITS sequences. The region that separates the 16S from the 5.8S subunit is referred to as ITS1; ITS2 separates the 5.8S from the 28S subunit. The section of DNA that encompasses the 18S-ITS1-5.8S-ITS2-28S complex is defined as a repeat unit. This region of DNA is ideal as a target for molecular characterization.

Fig. 1. Growth levels of *A. flavus* on SDA

3.4 Multiple Sequence Alignment and Phylogenetic Tree construction

Ribosomal ITS1 sequence was generated from the genomic DNA of the entomopathogenic fungal isolated from *A. flavus*. Many studies have been carried out to characterize entomopathogenic fungi targeting Internal Transcribed region. PCR amplicon size was almost 700bp but cleaned sequence product size obtained after sequencing was done with forward primer.

The ITS DNA sequence was aligned with the previously published sequences from the genera *Metarhizium, Aspergillus, Davidiella* sp and *Beauveria* retrieved from NCBI nucleotide search engine (Table 1). ClustalX alignment tools were used to construct the alignment. Due to small sequence size of isolate when sequences from NCBI were aligned against it, alignment was not accurate. Extra sequences which were notaligned accurately were omitted from the analysis.

Fig. 5 shows the Phylogenetic analysis of isolated fungal ITS region to other retrieved sequences. Phylogenetic tree demonstrated that our fungus lied in clad with *Aspergillus* indicating its close relationship with this group. While *Metarhizium* and *Beauveria* made separate clad revealing that isolated fungus is less related to these fungal groups. After blasting in the Genbank fungi with ITS sequences similarity 99% could be considered as the same species; with sequence similarity from 95% to 99% could be identified as same genus; with sequence similarity 95% could be identified as family [22]. Isolated fungus lied more close to *Aspergillus flavus* in dendrogram but BLAST search came out with only 93% identity with this species. Thus it may be concluded that the isolated fungus belongs to Aspergillus family.

Table 1. Entomopathogenic fungi used for phylogenetic analysis of *A. flavus*

Serial number	Length (bp)	Strain name	Accession number
1	506	*Metarhizium anisopliae 18S ribosome*	AF134150.1
2	469	*Metarhizium anisoplie*	AF516295.1
3	568	*Aspergillus oryzae*	KC341712.1
4	538	*Aspergillus sp*	KC834797.1
5	556	*Aspergillus flavus*	KC787034.1
6	576	*Aspergillus minisclerotigenes*	JX456193.1
7	633	*Davidiella sp*	JX164075.1

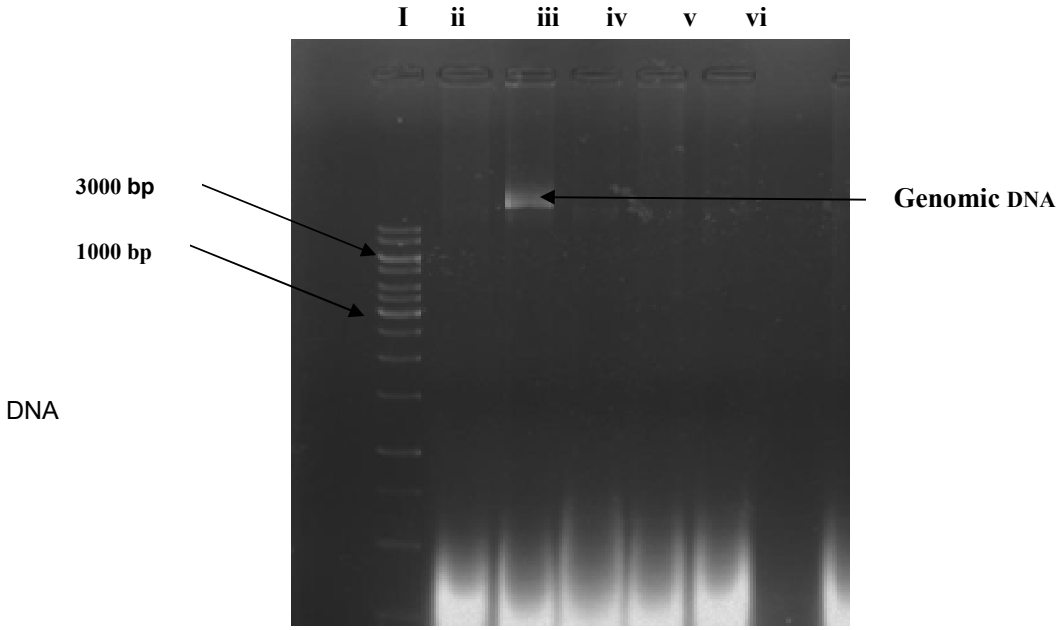

Fig. 2. Genomic DNA extraction: i: 1KB leader iii: Genomic

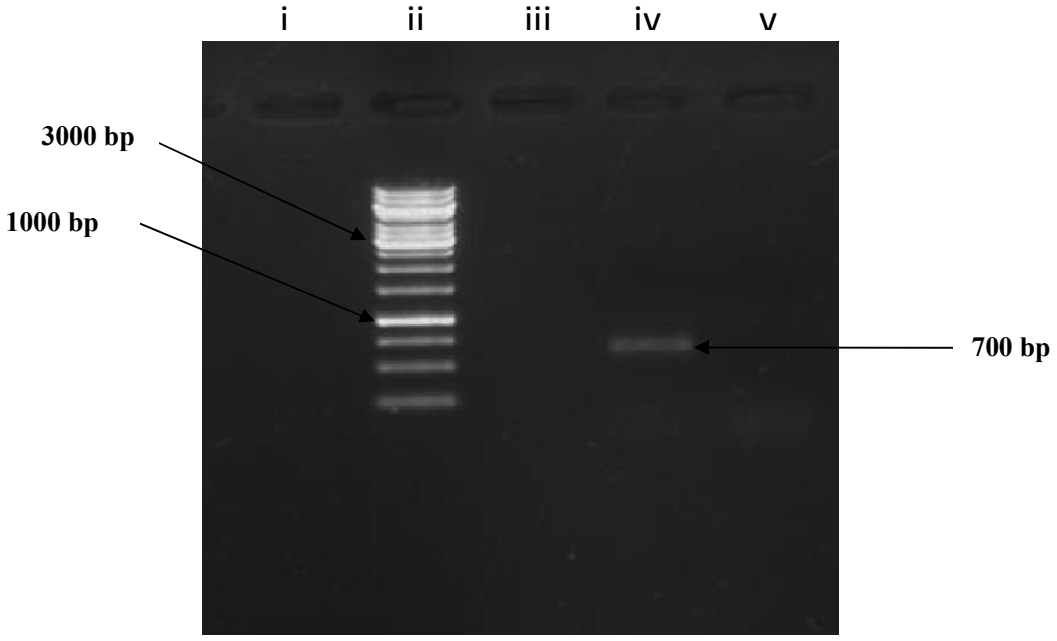

Fig. 3. PCR amplification of ITS gene region: ii: 1KB leader, iv: PCR product

4. DISCUSSION

Fungi cause many pathogenic infections in human, animal and plant [23]. In this study, environmental fungal strain *A. flavus* was collected and cultured using PDA media. Identification of the strain was first achieved by culture morphology which then further confirmed using more definie molecular technique; PCR amplification. Genomic DNA of the fungal strain was successfully extracted using method [24]. To get the successful PCR amplification, it is necessary to ensure that the genome is extracted successfully and without any

contamination especially inhibitors which create hurdle during PCR using amplification. These inhibitors can be eliminate either increase amount of DNA polymerase or addition of BSA which provide resistance to inhibitors [25]. Hence, certain amount of BSA was added in this during PCR amplification to get more efficient PCR results.

Techniques in molecular biology have been rapidly used for precise and accurate identification of microbes which are far better than the convention culture morphological analysis [26]. DNA amplification and sequencing are gaining more attention as tools for fungal identification and characterization [27]. In this study, an inexpensive and accurate method was used for the extraction of fungal genomic DNA. For PCR based amplification, primers were designed using 28S, 18S and 5.8S rDNA regions (ITS region). Most common ITS primer that use in molecular approaches for fungal identification was studied in the 90s which was mainly aimed to characterized broad range of fungal communities from environment [21].

Phylogenetic tree demonstrated that our fungus lied in clad with *Aspergillus* indicating its close relationship with this group. Fungi having 99% of ITS sequences resemblance could be identified as the same species as with sequence similarity from 95% to 99% could be considered as same genus; with sequence similarity 95% could be identified as family [22].

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>MG857638.1 Aspergillus flavus, internal transcribed spacer 1, 5.8S
ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCC
TAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCG
CCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCT
GAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAAC
GCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGC
GCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTT
GGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGC
GTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAG
GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCA
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Fig. 4. Shows the nucleotide sequence of *Aspergillus flavus*

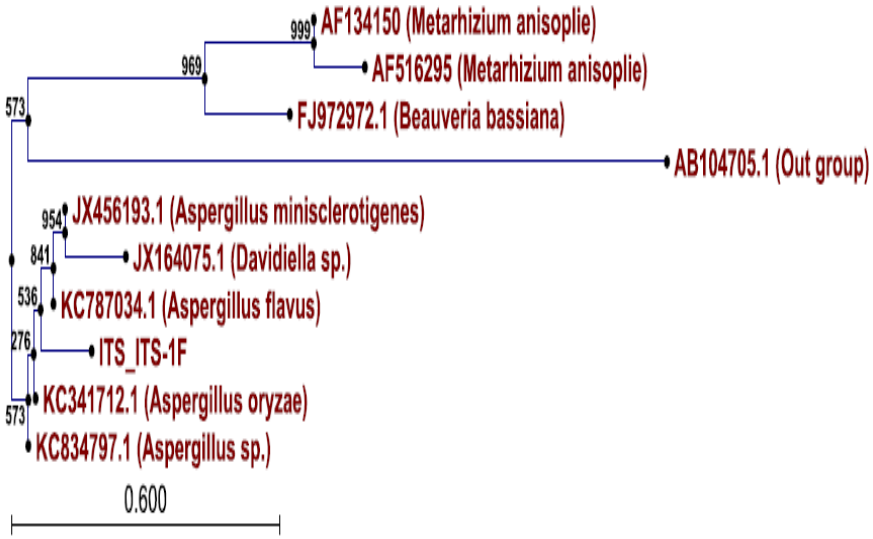

Fig. 5. Established phylogenetic tree of fungal isolate and its related genera based on ITS sequences. ITS sequences were aligned using CLC Bio alignment tools. The phylogenetic tree construction was conducted with neighbour-joining method packaged in software ClustalX

Bootstrap = 1000. Plocamiumtelfairiae chloroplast was used as the out group. The Scale bar shows nucleotide substitutions. Bootstrap values (%) are displayed at branching points

5. CONCLUSION

In conclusion, this study shows that fungal rDNA regions are effective to identify *A. flavus* isolates.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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