Molecular variation analysis of *Aspergillus flavus* using polymerase chain reaction-restriction fragment length polymorphism of the internal transcribed spacer rDNA region

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Abstract. *Aspergillus flavus* is the second most common disease-causing species of *Aspergillus* in humans. The fungus is frequently associated with life-threatening infections in immunocompromised hosts. The primary aim of the present study was to analyze the genetic variability among different isolates of *A. flavus* using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP). A total of 62 *A. flavus* isolates were tested in the study. Molecular variability was searched for by analysis of the PCR amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA using restriction enzymes. PCR using primers for ITS1 and ITS4 resulted in a product of ~600 bp. Amplicons were subjected to digestion with restriction endonucleases *Eco*RI, *Hae*III and *Taq*I. Digestion of the PCR products using these restriction enzymes produced different patterns of fragments among the isolates, with different sizes and numbers of fragments, revealing genetic variability. In conclusion, ITS-RFLP is a useful molecular tool in screening for nucleotide polymorphisms among *A. flavus* isolates.

Introduction

Human infections involving *Aspergillus* species are being characterized with growing frequency in immunocompromised hosts; *Aspergillus fumigatus* causes ~80% of invasive aspergillosis, and the second most common pathogenic species is *Aspergillus flavus*, followed by *Aspergillus niger* and *Aspergillus terreus* (1). *A. flavus* is a mold that exists worldwide. Environment and geographical conditions are significant determinants of the local frequency of *A. flavus* infections (2). The identification of *A. flavus* is not simple because of its similarities with species that are closely related (3).

Variability exists in the phenotype of *A. flavus*, for example, isolates with the potential to produce aflatoxin have been reported (4). Therefore, the ability to distinguish between different strains of *A. flavus* is valuable for diagnosis. The genomic analysis of DNA using polymerase chain reaction (PCR)-based methods is a sensitive, fast and reliable approach for the determination of genetic connections between microorganisms (5,6). The internal transcribed spacer (ITS) region is an effective target for phylogenetic analysis in fungi (7); the ITS region is frequently variable between different isolates of the same species (8,9).

The development of molecular methods for the genetic differentiation of fungal species has advanced their taxonomy as a result of increased sensitivity and specificity. PCR amplification of ITS regions of ribosomal DNA (rDNA) (10,11), combined with the sequencing of amplified regions and the analysis of these by comparing them with sequences that are deposited in GenBank, has been commonly employed for the detection of fungal species (11). However, variations in a sequence of DNA could be recognized using restriction fragment length polymorphism (RFLP), which can distinguish minor differences in nucleotides that may not be expressed at the protein level. RFLP may be able to identify changes in noncoding regions of DNA, recognize closely related organisms using DNA fingerprints and infer phylogenetic relations.

In the current study, the genetic variability among *A. flavus* isolates was analyzed. The samples included were reference strains, and clinical and environmental isolates. RFLP of the PCR fragments of the ITS region were used to analyze the isolates. The primary aim of the present study was to genetically distinguish numerous strains of *A. flavus*, isolated from various sources, using PCR and RFLP.

Materials and methods

Isolates of A. flavus. A total of 62 *A. flavus* isolates were used in the present study. Ten reference strains, including

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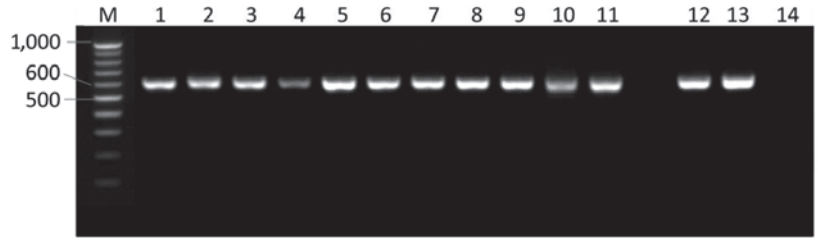

Figure 1. Internal transcribed spacer (ITS) regions of *Aspergillus flavus* isolates were amplified by polymerase chain reaction using ITS1 and ITS4 primers and the products were separated by agarose gel electrophoresis. M, 100 bp ladder; lane 1, Kh4 isolate; lane 2, Kh5 isolate; lane 3, Kh6 isolate; lane 4, Kh9 isolate; lane 5, Kh10 isolate; lane 6, Kh11 isolate; lane 7, M25 isolate; lane 8, M26 isolate; lane 9, M27 isolate; lane 10, M28 isolate; lane 11, M29 isolate; lane 12, M32 isolate; lane 13, M33 isolate; lane 14, no template control.

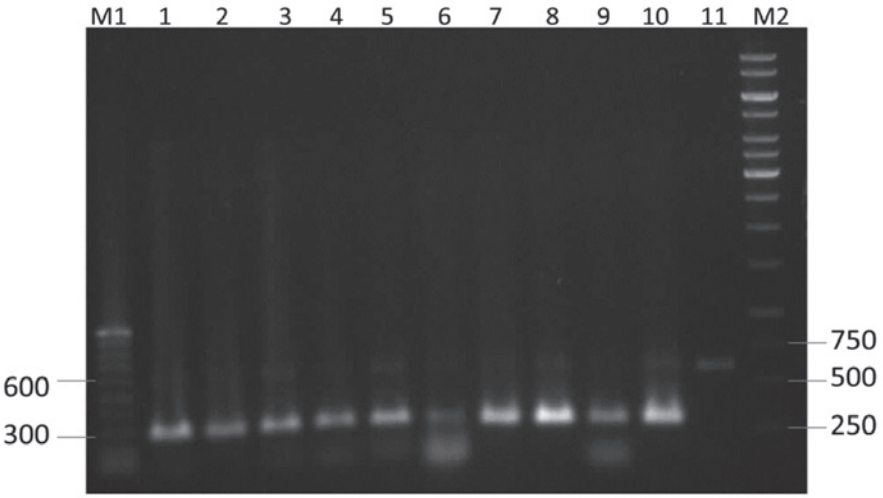

Figure 2. Restriction fragment pattern of internal transcribed spacer (ITS) polymerase chain reaction (PCR) products of *Aspergillus flavus* digested with *Eco*RI. Lane M1, 100 bp ladder; lane 1, Z7 isolate; lane 2, Z8 isolate; lane 3, Z9 isolate; lane 4, Z10 isolate; lane 5, PFCC101 isolate; lane 6, PFCC126 isolate; lane 7, PFCC159 isolate; lane 8, PFCC209 isolate; lane 9, PFCC170 isolate; lane 10, PFCC173 isolate; lane 11, undigested ITS PCR product; lane M2, 1 kb ladder.

A. flavus PFCC101, PFCC123, PFCC124, PFCC125, PFCC126, PFCC159, PFCC209, PFCC170, PFCC173 and PFCC106-139, and 25 clinical and 27 environmental isolates of *A. flavus* were included. Reference strains were obtained from the Pasteur Institute of Iran (Tehran, Iran). The clinical isolates were kindly provided by Dr Hossein Zarrinfar (Mashhad University of Medical Sciences, Mashhsad, Iran), Dr Sadegh Khodavaisi (Tehran University of Medical Sciences, Tehran, Iran) and Dr Parvin Dehghan (Isfahan University of Medical Sciences, Isfahan, Iran). The environmental isolates were obtained from soil or air samples collected in Ahvaz, Iran. The isolates were kept on Sabouraud dextrose agar (Merck KGaA, Darmstadt, Germany) at room temperature. All *A. flavus* isolates were identified by morphology. Isolates were subcultured three times to obtain a pure culture and stained with lactophenol aniline blue. The conidial arrangement, philiades, vesicles and conidiophores were observed under a light microscope for morphological characterization.

DNA extraction. Thick spore suspension (1 ml) from each isolate was transferred to an Erlenmeyer flask with 50 ml yeast extract peptone dextrose medium (Merck KGaA). Following inoculation, the flasks were kept at 200 rpm under agitation at 37˚C for 48 h in order to allow for mycelia growth. The mycelia were harvested with filters, washed with 0.5 M ethylenediamine tetraacetic acid (EDTA) and sterile distilled water (dH_2O) and freeze-dried at -70˚C for DNA extraction. The mycelia were then ground into a fine powder using a pestle and mortar. The powder (~100 mg) was then transferred into a 1.5-ml sterile tube, and 400 μl lysis buffer (100 mM Tris-HCl, pH 8.0, 30 mM EDTA, pH 8.0 and sodium dodecyl sulfate 5% w/v) was added.

The microtubes were kept at 100˚C for 20 min, and 150 μl 3 M acetate potassium was added to each tube. The suspension was kept at -20˚C for 10 min, and centrifuged at 14,000 x g and 4˚C for 10 min. Following transfer of the supernatant to a 1.5-ml Eppendorf tube, 250 μl phenol-chloroform-isoamyl alcohol (25:24:1, v/v) was added, and the solution was briefly was vortexed and centrifuged at 14,000 x g for 10 min. The upper aqueous phase was transferred to a new 1.5 ml microtube and 250 μl chloroform-isoamyl alcohol (24:1) was added. The samples were then briefly vortexed and centrifuged at 4˚C and 14,000 x g for 10 min. The supernatant was transferred to another microtube, an equal volume of iced-cold 2-propanol was added, and samples were kept in -20˚C for 10 min and then centrifuged at 14,000 x g for 10 min. The upper aqueous phase was discarded and the pellet was washed with 300 μl 70% ethanol. Following the removal of ethanol, DNA pellets were air dried and dissolved in 50 μl dH_2O.

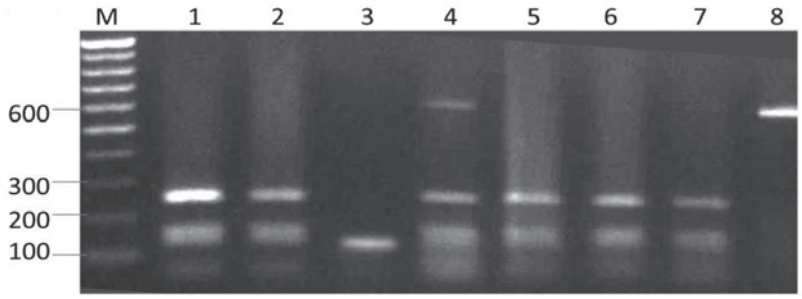

Figure 3. Restriction fragment pattern of internal transcribed spacer (ITS) polymerase chain reaction (PCR) products of *Aspergillus flavus* digested with *Taq*I. Lane M, 100 bp ladder; lane 1, Z9 isolate; lane 2, Z10 isolate; lane 3, D1 isolate; lane 4, D2 isolate; lane 5, M29 isolate; lane 6, M32 isolate; lane 7, M33 isolate; lane 8, undigested ITS PCR product.

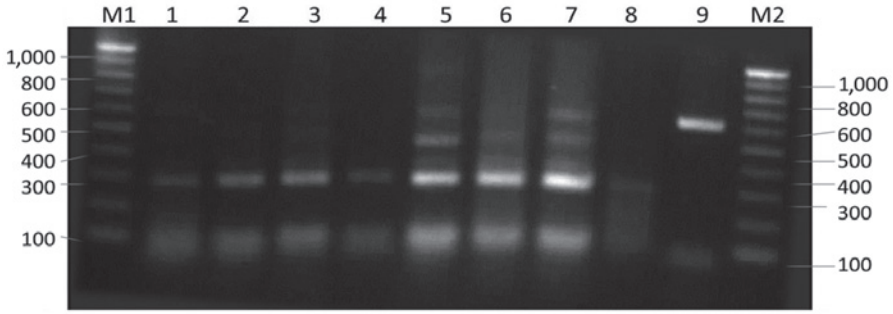

Figure 4. Restriction fragment pattern of internal transcribed spacer (ITS) polymerase chain reaction (PCR) products of *Aspergillus flavus* digested with *Hae*III. Lane M1, 100 bp ladder; lane 1, M2 isolate; lane 2, M4 isolate; lane 3, Kh1 isolate; lane 4, Kh2 isolate; lane 5, Kh4 isolate; lane 6, Kh5 isolate; lane 7, Kh6 isolate; lane 8, Kh8 isolate; lane 9, undigested ITS PCR product; lane M2, 100 bp ladder.

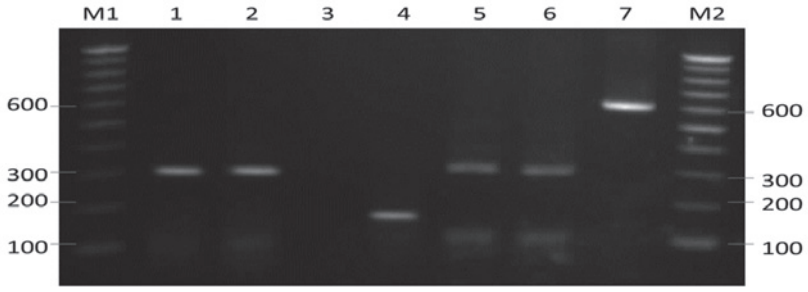

Figure 5. Restriction fragment pattern of internal transcribed spacer (ITS) polymerase chain reaction (PCR) products of *Aspergillus flavus* digested with *Hae*III. Lane M1, 100 bp ladder; lane 1, M1 isolate; lane 2, M3 isolate; lane 3, Kh3 isolate; lane 4, Kh7 isolate; lane 5, Z9 isolate; lane 6, Z10 isolate; lane 7, undigested ITS PCR product; lane M2, 100 bp ladder.

PCR amplification. Molecular identification of the ITS region of each *A. flavus* isolate was performed using the ITS1 (5'-TCC GTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCT TATTGATATGC-3') primers. PCR reactions were performed using a final volume of 50 μl, containing reaction buffer, 2.2 mM $MgCl_2$, 200 μM each dNTP (dATP, dCTP, dGTP and dTTP), 2.5 units *Taq* DNA polymerase (all CinnaGen, Tehran, Iran), 100 ng template DNA and 50 pmol of each primer. The amplification conditions were as follows: Initial denaturation at 94˚C for 5 min; 35 cycles of denaturation at 94˚C for 2 min, annealing at 53˚C for 2 min and extension at 72˚C for 2 min; and final extension at 72˚C for 30 min. The PCR products were separated by 1.2% agarose gel electrophoresis in a Tris base, acetic acid and EDTA buffer, and stained with ethidium bromide. PCR amplification of the ITS region yielded a 595-bp band.

Restriction site analysis of PCR products. Following amplification, the PCR products were digested with the restriction endonucleases *Hae*III, *Eco*RI and *Tag*I (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction for each enzyme was performed in a total volume of 20 μl containing 10 units enzyme, 2 μl buffer (500 mM KCl and Tris-HCl, pH 8.4), 8 μl PCR product and ultrapure water. The fragments were separated on a 1.2% agarose gel by electrophoresis and stained with ethidium bromide .

A number of amplicons were submitted for direct sequencing (Bioneer Corporation, Daejeon, South Korea). The obtained sequences were searched for in the NCBI database (http://www.ncbi.nlm.nih.gov/). The sequences had 100% identity with *A. flavus* sequences deposited in the NCBI database. The computer software package MEGA5

(http://www.megasoftware.net) was used for alignment of sequences.

Results

Molecular variation analysis of A. flavus isolates. Using ITS1 and ITS4 primers, a unique band of ~595 bp was obtained for all tested *A. flavus* isolates (Fig. 1). The results following digestion with restriction enzymes indicate that *A. flavus* isolates vary in the ITS region. The results suggest the existence of variation among *A. flavus* isolates. The pattern of the ITS-RFLP bands obtained following the cleavage of the PCR products with the restriction enzymes *Eco*RI, *Hae*III and *Taq*I showed genetic variability among the isolates that varied in the size and number of fragments (Figs. 2-5).

Digestion of the ITS amplicons with *Eco*RI produced the expected 300-bp fragment for 59 of the 62 isolates (Fig. 2). Three clinical isolates did not present any fragments following digestion with *Eco*RI.

Restriction maps of the PCR product of the ITS region fragments allowed the identification of a restriction endonuclease, *Taq*I, which could be used to differentiate *A. flavus* isolates. Following PCR amplification of the ITS region cut with a *Taq*I enzyme, the PCR product produced two fragments, ~150 and 250 bp in size, for 59 of the 62 isolates. Three isolates, including 2 clinical and 1 environmental isolate, showed one band, ~150 bp in size (Fig. 3). Digestion of the ITS amplicons with *Hae*III resulted in more restriction patterns, as compared with *Taq*I and *Eco*RI (Figs. 4 and 5).

The PCR products of the ITS region of 3 isolates were sequenced and aligned with references in the NCBI database. The sequences had 100% identity with *A. flavus* sequences deposited in the NCBI database.

Discussion

Methods in molecular biology have been efficiently employed for the rapid identification of microorganisms, and for overcoming the limitations associated with conventional direct culture analysis (12). Fungal rDNA has been demonstrated to include regions that are variable within genera. The ITS region of nuclear rDNA, including the intervening 5.8S rRNA gene, ITS1 and ITS2, has been extensively used to investigate the variability in fungal species and subspecies.

Restriction enzyme map analysis of the ITS regions has been employed to study the genetic diversity among the fungal isolates of various types (13,14). Variations in DNA sequences can be identified using PCR-RFLP, which is able to identify minor differences in nucleotides (11). Henry *et al* (7) reported that ITS1 and ITS2 are required to accurately identify the species of *Aspergillus*. Huang *et al* (15) reported interspecies variability in the ITS2 region, and used this dissimilarity to design microarray probes for the detection of pathogenic fungi.

In the present study, isolates of *A. flavus* were analyzed using ITS-RFLP to evaluate the genetic variability among them. A total of 62 *A. flavus* isolates were tested for genetic variability in the ITS regions. The primers ITS1 and ITS4 amplified successfully all the ITS region isolates tested using conventional PCR. Sequence analysis indicates that the restriction enzymes *Eco*RI, *Hae*III and *Taq*I can cleave the PCR products into fragments that are useful tools for the detection of specific strains.

Numerous techniques have been developed for the systematic investigation of fungi, including random amplified polymorphic DNA, and diagnosis based on specific PCR primers (16) and sequencing (17,18). However, the techniques used are frequently based on rRNA (or rDNA) gene analysis sequences that are universal and include conserved and variable regions, and permit the discrimination of fungi at different taxonomic levels (18,19).

Analysis of PCR-amplified rDNA sequences with restriction enzymes has been shown to be an appropriate approach for taxonomic studies in several *Aspergillus* and *Fusarium* species (20-23). RFLP analysis of ITS regions has demonstrated that the quantity of the carcinogenic metabolite aflatoxin B_1 produced by isolates of *A. flavus* ranges between 1.9 and 206.6 ng/ml, with the variability being suggested to be due to differences in genetic composition (24).

In conclusion, the present study demonstrated that restriction fragments of the amplified ITS regions of *A. flavus* isolates are effective for the identification of different strains. The restriction enzyme found to be the most effective in the discrimination of isolates in the current study was *Hae*III, followed by *Taq*I and *Eco*RI.

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