Molecular diagnostics of human pathogenic *Aspergillus* species

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Eight species of *Aspergillus* (*A. hiratsukae, A. flavus, A. fumigatus, A. terreus, A. niger, A. sydowii, A. clavatus* and *A. flavipes*) were found associated with clinical samples causing human diseases. The nonspecific symptoms and lack of rapid diagnostics is a major problem in treating patients suffering from invasive aspergillosis. Molecular markers based on RAPD primers and diversity in ITS region of 5.8S rRNA gene developed for rapid diagnosis facilitated early treatment of patients suffering from aspergillosis related disorders. Direct sequencing of amplified ITS products revealed significant single nucleotide polymorphism, which facilitated identification of pathogenic *Aspergillus* species associated with aspergillosis. The complete sequences of eight *Aspergillus* species were submitted to NCBI database, USA, which were assigned new Gen accession numbers EU515147 to EU515154 and now available in public domain for comparison.

**Keywords:** *Aspergillus*, aspergillosis, genetic markers, RAPD, 5.8S rRNA gene sequencing

**Introduction**

The genus *Aspergillus* is represented by about 132 species and is widely distributed throughout the world. It is one of the most important genera of fungi having the maximum species and has substantially contributed to the taxonomic chaos. Most of *Aspergillus* species commonly grow on fruits, vegetables, pickles, jams, jellies and other food stuffs. They are also found on paper, leather, textile, soil and even in air. Some species of *Aspergillus* are parasitic to plants causing stem and root rot diseases. *A. flavus* contaminates groundnut and other dry food stuffs and produces aflatoxins, which are carcinogenic in nature. Around 20 species have so far been reported as causative agents of opportunistic infection, allergic state and toxicoses in humans. These infections may be present in a wide spectrum, varying from local involvement to dissemination and as a whole called “aspergillosis”. Among these, *A. fumigatus, A. flavus, A. clavatus, A. niger, A. terreus, A. sydowii, A. flavipes* and *A. hiratsukae* are more common and found associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis, invasive infection, onychomycosis, sinusitis, cerebral aspergillosis, meningitis, endocarditis, myocarditis, pulmonary aspergillosis, osteomyelitis, endophthalmitis, cutaneous aspergillosis, hepatosplenic aspergillosis and nosocomial disease. It can be local colonizers in previously developed lung cavities due to tuberculosis, sacoidosis, bronchiectasis and pneumoconiosis. Occurrence of aspergillosis due to catheters and other devices are also common. The most severe disease caused by aspergillosis occurs in immunocompromised patients with invasive pulmonary infection, followed by rapid dissemination. The frequency of invasive mold infection has increased due to the increasing number of patients receiving aggressive chemotherapy regimens and immunosuppressive agents. The nonspecific symptoms and the lack of rapid diagnostic assays to detect these infections have been a major problem in treating patients with invasive disease, particularly those with invasive aspergillosis. Early recognition of invasive fungal infection and treatment with appropriate antifungal therapy shall provide key to reduce the mortality associated with disseminated disease. The diagnosis is often delayed because of slow growth of a large number of pathogenic fungi in routinely used culture media and, therefore, early intervention is not always possible and the patient’s life is often lost.

The identification of *Aspergillus* species based on morphological methods are inadequate and misleading as some microscopic characters are homogenous to many species within the genus. Clinical aspergilli sometime manifest atypically with
slow sporulation and aberrant conidiophore formations. Additionally, members of the section *fumigate* have overlapping morphological characteristics with several genetically distinct species existing with in a single morphospecies.

The recent molecular techniques with species specific genetic markers and direct sequencing of conserved genes can facilitate quick identification of species, which is of permanent clinical significance to treat patients suffering from aspergillosis with specific drugs. Randomly amplified polymorphic DNA (RAPD) uses only short primer with an arbitrary sequence and binds at many complementary sites in genomic DNA, amplifying a variety of differentially sized fragments that can be separated by electrophoresis to give a very specific banding pattern. It is widely used to distinguished strain and individuals both at inter and intraspecies levels including *Aspergillus* species. The ribosomal rRNA gene (rDNA) of fungi are located on single chromosome and are present as repeated subunits of tandem array of transcribed and non-transcribed stretches of DNA, which appear highly conserved.

Present research reported herein was aimed at development of molecular markers and to detect variation in ITS 1 and ITS 2 nucleotide sequences of medicinally important *Aspergillus* species (*A. hiratsukae*, *A. flavus*, *A. fumigatus*, *A. terreus*, *A. niger*, *A. sydowii*, *A. clavatus*, & *A. flavipes*) for quick identification and treatment of patients suffering from acute aspergillosis.

### Materials and Methods

Eight species of *Aspergillus*, viz., *A. hiratsukae* (urinary track infection; male 78 yr), *A. flavus* (lung infection; male 52 yr), *A. fumigatus* (pulmonary disease; female 52 yr), *A. terreus* (skin infection; male 33 yr), *A. niger* (chronic ulcer; male 56 yr) *A. sydowii* (vaginitis; female, 40 yr) *A. clavatus* (ear infection; male 10 yr) and *A. flavipes* (nail infection; female 25 yr) were collected from different clinical laboratories of Jodhpur. These fungal cultures were maintained on slopes of PDA media and were subcultured on to 100 mL Erlenmeyer flasks containing 50 mL Czapeck broth media (NaNO₃ 2 g, KH₂PO₄ 1 g, MgSO₄ 0.5 g, FeSO₄ 0.1 g and sucrose 30 g, in 1 L distilled water) for 10 d under stationary conditions in BOD incubator at 25±2°C.

Total genomic DNA was extracted from approximately 100 mg of fungal mycelium, crushed with a micro-pestle in conical 1.5 mL micro-centrifuge tubes with liquid nitrogen. DNeasy® plant minikit protocols of Qiagen GMBH (Germany) were used for DNA isolation. The quality of DNA was checked by electrophoresis on 0.8% agarose gel with 1× TAE buffer and analyzed after staining with ethidium bromide. The purity of the extracted DNA was checked by spectrophotometer at 260 nm and 280 nm using UV spectrophotometer (Pharma Spec UV-1700, Shimadzu Company) and stored at –20°C until used.

RAPD analysis was performed using 8 decamer arbitrary primers supplied by Sigma Genosys, USA, namely, RUF-203, RUF-204, RUF-210, RUF-211, RUF-214, RUF-215, RUF-216 and RUF-220 (Table 1). Amplification was performed in 25 μL reaction mixture, each mixture contained decamer primer, 2 μL (50 pmol/μL); dNTP mix, 2 μL (2 mM each from MBI, Fermentas); MgCl₂ 1 μL (25 mM, MBI, Fermentas); Taq DNA polymerase, 1 μL (5 U/μL, Bangalore Genei); 10× PCR buffer, 2.5 μL (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 250 mM KCl) and 16.5 μL of dH₂O. Taq DNA polymerase, 1 μL (5 U/μL, Bangalore Genei); 10× PCR buffer, 2.5 μL (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 250 mM KCl) and 16.5 μL of dH₂O. To this 4 μL of genomic DNA (approx 40-60 ng) was added and RAPD-PCR amplification was performed in a Thermal Cycler (Eppendorf Master Cycler) with lid heating option at 110°C with initial denaturation step of 94°C for 3 min, followed by 36 amplification cycles of 94°C for 40 sec, 50°C for 40 sec and 72°C for 2 min, and final elongation at 72°C for 10 min. PCR amplification products were electrophoretically separated in 1.6% agarose gel (Sigma Chemicals) prepared in 1× TAE for 3 h at 45 V. The staining was done with ethidium bromide, visualized under 300 nm UV light and photographed. The photographs were scored for presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationships among the isolates, similarity coefficient were calculated between the species and dendogram drawn using UPGMA Algorithm.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Primer sequence (5' - 3')</th>
<th>Tₘ (°C)</th>
<th>GC (%)</th>
<th>Total no. of amplified bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RUF-203</td>
<td>GGC GGA GGT T</td>
<td>38.1</td>
<td>60</td>
<td>08</td>
</tr>
<tr>
<td>2</td>
<td>RUF-204</td>
<td>CCA CAT CGG T</td>
<td>33.9</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>RUF-210</td>
<td>TGA CGA GCT G</td>
<td>42.4</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>RUF-211</td>
<td>GGG TAA CGC C</td>
<td>38.7</td>
<td>70</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>RUF-214</td>
<td>GAA GCA CGA T</td>
<td>40.0</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>RUF-215</td>
<td>GCT GGC TGA C</td>
<td>35.6</td>
<td>70</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>RUF-216</td>
<td>CAG CGA ACT A</td>
<td>26.2</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>RUF-220</td>
<td>GGG TGA ACC G</td>
<td>38.9</td>
<td>70</td>
<td>11</td>
</tr>
</tbody>
</table>

Total no. of amplified bands: 107
(Unweighted Pair Group Method Using Arithmetic Averages) of NTSYS-PC, Version 2.02h program. The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the ITS region of ribosomal DNA, which encompasses the 5.8S rRNA gene and both ITS-1 and ITS-2 regions. Amplification by PCR was performed in a total volume of 50 µL containing 0.2 µL Taq DNA polymerase (5 U/µL of Promega), 5 µL of 10× PCR buffer (10 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂ Sigma Chemicals) 0.4 µL dNTP mix, 2.0 mM each A,T,C,G (MBI Fermentas), 1 µL of each ITS-1 and ITS-4 primers, 2 µL of 5% glycerol, 4 µL of genomic DNA and 36.4 µL dH₂O. The reaction were performed in a thermal cycler with following conditions: 1 min denaturation at 95°C, 30 sec annealing at 50°C, 1 min 20 sec elongation at 72°C, repetition 34 times with a final elongation step of 10 min at 72°C.

The PCR products were visualized on 1% Agarose gel in Tris-acetic acid EDTA (1× TAE) buffer at 60 V for 100 min. Agarose gels were stained with ethidium bromide and photographed under UV light using Syngene gel documentation system for amplified products.

PCR product of ITS amplified region containing ITS1, 5.8S rRNA gene and ITS 2 were directly sequenced using ITS-1 (forward primer) and ITS-4 (reverse primer) by big dye terminator method on ABI Prism DNA Sequencer. The sequence data obtained from ITS-4 reverse primer was inversed using Genedoc software and clubbed with sequence data of ITS-1 to obtain complete sequence of amplified ITS products. Nucleotide sequence comparisons were performed by using Basic Local Alignment Search Tool (BLAST) network services against the National Centre for Biotechnology Information (NCBI) database. The fungal species were designated to the sequenced cultures analysed based on similarity with the best aligned sequence of BLAST search. Multiple 5.8S rRNA gene alignment was performed using Clustal X1.83 software. The complete rDNA sequences of ITS1 and ITS2 encompassing 5.8S of different Aspergillus species were submitted NCBI database and Gen accession numbers obtained.

Results

Under the present investigation eight species of genus Aspergillus were subjected to RAPD analysis. Initially 15 primers were tested for their polymorphism, reproducibility and capacity to differentiate these species; of which 8 primer were selected to access the genetic diversity. The other primers gave indistinct, subtropical and monomorphic amplification products. The eight selected primers, viz., RUF-203, RUF-204, RUF-210, RUF-211, RUF-214, RUF-215, RUF-216 and RUF-220, generated a total of 107 scored amplicons with high degree of polymorphism (Table 1). The number of PCR amplified products formed ranged from 8 (RUF 203) to 17 (RUF 211 and RUF 215) with an average of about band of 13 per primer. The RAPD profiles generated by most informative random primers (RUF 215 & 210) are shown in Figs 1 and 2. The


Fig. 2—RAPD profiles of 8 Aspergillus species using random primer RUF-210 (Lane 1: A. hiratsukae, lane 2: A. flavus, lane 3: A. fumigatus, lane 4: A. terreus, lane 5: A. niger, lane 6: A. sydowii, lane 7: A. clavatus, & lane 8: A. flavipes)
UPGMA dendrogram from cumulative cluster analysis of 8 primers scored loci using Jaccards coefficient clearly delineated all the 8 species of genus *Aspergillus* from each other. The comprehensive dendrogram of the 8 random primer data metrix delineated 8 species into 2 major clusters. Cluster 1 included *A. hiratsukae*, *A. fumigatus*, *A. flavus* and *A. clavatus*, whereas cluster 2 included *A. terreus*, *A. flavipes*, *A. niger* and *A. sydowii*. Both the cluster showed the maximum genetic diversity of about 80% from each other (Fig. 3).

The PCR amplified products of ITS regions including 5.8S rDNA gene of all the 8 species of *Aspergillus* studied are depicted in Fig. 4. It is clear from results that there is slight variation in ITS profiles, which ranged between 479 to 520 bp (Table 2). It is practically impossible to distinguish different species on the basis of gel photographs due to a few base pair differences among the *Aspergillus* species studied. Nevertheless, upon direct sequencing of the amplified ITS products revealed significant single nucleotide polymorphism (SNP) in both ITS-1 and ITS-2 regions by way of insertion, deletion and replacement by another nucleotide base. A high level of uniformity within the conserved 5.8S rDNA region was observed with 157 bp in all the 8 species of *Aspergillus*. The molecular data of sequences obtained using ITS-1 and ITS-4 primers were aligned and common portions deleted. The complete sequences were blasted with available sequences in NCBI data base and species of *Aspergillus* identified on the basis of the maximum percent identities. Subsequently, all the 8 sequences of genus *Aspergillus* were submitted to NCBI database and Gen accession numbers obtained which are now available in public domain for comparison (Table 2).

The multiple sequence alignment using Clustal X1.83 software program yielded an unrooted phylogram and delineated all the species tested. The phylogram based on multiple sequence alignment generated 3 major clusters. The cluster 1 included *A. hiratsukae* and *A. fumigatus* with *A. clavatus* and *A. flavus* as an outgroup. Cluster 2 included *A. terreus* and *A. flavipes*, whereas cluster 3 included *A. niger* and *A. sydowii* (Fig. 5).

**Table 2—ITS1-5.8S-ITS2 regions of Aspergillus species with Gen Accession Number**

<table>
<thead>
<tr>
<th>No.</th>
<th>Aspergillus species</th>
<th>Size (bp) of ITS1-5.8S-ITS2 region</th>
<th>Gen acc. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>A. hiratsukae</em></td>
<td>187 + 157+ 171 = 515</td>
<td>EU515147</td>
</tr>
<tr>
<td></td>
<td>(Teleomorph N. hiratsukae)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td><em>A. flavus</em></td>
<td>181 + 157+ 169 = 507</td>
<td>EU515148</td>
</tr>
<tr>
<td>3.</td>
<td><em>A. fumigatus</em></td>
<td>184+ 157+ 168 = 509</td>
<td>EU515149</td>
</tr>
<tr>
<td>4.</td>
<td><em>A. terreus</em></td>
<td>186+ 157+ 177 = 520</td>
<td>EU515150</td>
</tr>
<tr>
<td>5.</td>
<td><em>A. niger</em></td>
<td>185+ 157+ 169 = 511</td>
<td>EU515151</td>
</tr>
<tr>
<td>6.</td>
<td><em>A. sydowii</em></td>
<td>154+ 157+ 168 = 479</td>
<td>EU515152</td>
</tr>
<tr>
<td>7.</td>
<td><em>A. clavatus</em></td>
<td>187+ 157+ 168 = 512</td>
<td>EU515153</td>
</tr>
<tr>
<td>8.</td>
<td><em>A. flavipes</em></td>
<td>182+ 157+ 160 = 499</td>
<td>EU515154</td>
</tr>
</tbody>
</table>
Discussion

Presently, taxonomy of the genus *Aspergillus* is based on morphological and microscopic characteristics. Under present study, we observed that the selected RAPD primers have clearly delineated all the 8 species under study and can be of clinical importance. Aiat used arbitrary 5 base pair primer and revealed polymorphism among three species of *Aspergillus* (A. niger, A. flavus & A. parasiticus). Melchers et al. developed a PCR assay for detection of *Aspergillus* species and found genus specific sequences in V7 and V9 region of 18S rRNA. Hanry et al. used ITS regions 1 and 2 for identification of *Aspergillus* species with ATCC accession and clinical isolates of A. flavus, A. fumigatus, A. nidulance, A. niger, A. terreus and A. ustus. They demonstrated that both single nucleotide differentiation and short length of sequence diversity due to insertion and deletion existed in the ITS1-5.8S-ITS2 regions among the pathogenic *Aspergillus* species. The result of present investigation also found substantial single nucleotide polymorphisms and sequences diversity in the invasive aspergillosis in immunocompromised clinical samples. All the 8 *Aspergillus* species associated with clinical samples under present study exhibited one to several base pair differences in the total length of ITS region (ITS1-5.8S-ITS2) and that can be of significance in species designation. Further, the combined dendrogram based on RAPD profiles of informative primers, and PHYLIP phylogram generated using multiple alignment of ITS sequences, clearly delineated all the 8 pathogenic species of *Aspergillus* and yielded similar genetic relationship that can serve as useful genetic markers to facilitate correct diagnosis and appropriate treatment to the patient.

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References