

Molecular Characterization of Seven Different Species of *Aspergillus* through Random Amplified Polymorphic DNA (RAPD) and Enzyme Analysis

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Abstract The aim of this study was to characterize seven species of *Aspergillus* at molecular level, using random amplification of polymorphic DNA (RAPD). RAPD-PCR conditions were optimized for two primers of series B, GL Decamer B-09 and GL Decamer B-10 out of 10 total primers. RAPD results were evaluated by a statistical software Minitab and a phylogenetic tree was prepared. GL Decamer B-09 showed 38 bands and GL Decamer B-10 gave 46 bands, showing 50% and 57% similarity respectively, among species. Biochemical characterization was done by screening of zones production with particular enzyme activity of each specie resulted in particular substrate degradation.

Keywords *Aspergillus*, RAPD, Minitab, Pectinase activity, Phylogeny

1. Introduction

There are an estimated 1.5 million fungal species of which around 70,000 have been described so far[1]. *Aspergillus* is a filamentous and ubiquitous fungus found in nature and is identified at species level by using the differential culture media. A total of 205 *Aspergillus* species are reported which includes 153(75%) environmental and 52 (25%) clinical *Aspergilli*[2].

Aspergillus niger is economically important fermentation organism used for the production of citric acid and it represents highest yield bioprocess, currently in use by industry[3]. *Aspergillus fumigatus* is considered to be the most frequent isolate from clinically immunocompromised patients, but other important species include *A. flavus*, *A. niger* and *A. terreus* as a cause of opportunistic infections[4]. Phenotypic and genotypic correlations provide strong evidence for differentiation of *A. flavus* from *A. oryzae*, though there are very minor phenotypic and genotypic differences[5]. Bacterial strains are effective in reducing soil populations of mycotoxigenic fungi; thereby reduce fungal spore formation and crop plant infection via airborne transmission[6].

PCR-based technique, involving the random amplification of polymorphic DNA (RAPD) has been used for assessing genomic variability among a wide range of culture collection

strains of *Aspergillus* and related species[7]. The utility of DNA markers as RAPD-DNA employ it as well established sample molecular marker tool for detecting genetic variability for many phytopathogenic fungi[8].

DNA Polymorphisms based on differences in DNA sequences, have advantages over protein polymorphisms[9]. The application of the random amplification of polymorphic DNA (RAPD) assay to the human pathogen *A. fumigatus* was described in the year 1993[10]. RAPD fingerprinting is used to gain rapid and precise information about genetic similarities and dissimilarities of different *Aspergillus* species. RAPD fingerprints of *A. niger*, *A. flavus* and *A. parasiticus* revealed polymorphism in 37, 59, 51% of the analyzed *Aspergillus* species[11].

RAPD polymorphism results from a nucleotide base change, an insertion or deletion that alters the primer binding sites. This product can be polymorphic and may be used as genetic markers for extensive genetic variation analysis[12]. One advantage of this technique is that the primers are universal and they can be used for genomic analysis of a wide variety of species[13].

The research project of analyzing genetic diversity by RAPD PCR is very useful to detect similarities and differences in different fungal species. Random Amplified Polymorphic DNA, more or less randomly distributed in the whole genome[14]. They span the majority of the chromosome and map both proximal and distal to the centromeres and are also able to map novel chromosomal regions. In the present study we have characterized seven different *Aspergillus* strains i.e *A. niger*, *A. nidulans*, *A. parasiticus*, *A. flavus*, *A. japonicus*, *A. oryzae* and *A. fumigatus*. The aim of

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the present study was to characterize seven different species of fungi at molecular level that leads to further elucidation of genetic diversity.

2. Material and Methods

2.1. Source of Fungi

The fungal samples were obtained from First Fungal Culture Bank of University of the Punjab Lahore at Department of Mycology and Plant Pathology (MPPL) and maintained in test tubes as slants and stored at 4°C. Seven different species of *Aspergillus* with their accession numbers, *A. japonicus* (503), *A. niger* (706), *A. parasiticus* (174), *A. nidulans* (722), *A. flavus* (647), *A. oryzae* (01) and *A. fumigatus* (651) were grown at 24-25°C.

2.2. Preparation of Culture

Slants prepared in test tubes containing 5 ml of autoclaved medium (1 g yeast, 1 g peptone, 1 g starch and 1 g agar in 100 ml water) were inoculated with their respective cultures with the help of loop and incubated at 37°C for 3-4 days. When growth appeared, they were stored at -20°C. A single colony from the starter culture was inoculated in 30 ml of autoclaved broth (0.01 g Fe SO₄, 0.50 g KCl, 0.50 g Mg SO₄, 1 g K₂HPO₄, 3 g NaNO₃ and 20 g sucrose in one liter of water) in a 100 ml conical flask. Flask was covered with cotton plug and aluminum foil and placed in incubator for 4 days at 27°C. Growth appeared after one week.

2.3. RAPD PCR Amplifications

Total DNA of fungal samples were extracted manually by CTAB method[15]. Optimized primer GL DecamerB-09: 5' TGGGGGACTC 3' and Primer GL DecamerB-10: 5' CTGCTGGGAC 3' were used for PCR of isolated DNA. 50 µl reaction mixture, containing 10X PCR buffer (5 µl), 5 mM dNTPs mix (2 µl), 5 U/µl Taq DNA polymerase (0.6 µl), template DNA (5 µl), 25 mM MgCl₂ (4 µl), 50 pM Primer (1 µl) and PCR water (32.4 µl) each of it in a Bio-rad Thermal Cycler for 40 cycles, each for 1 min at 94°C, 1 min at 35°C and 2 min at 72°C and the final extension for 5 min at 72°C.

2.4. Statistical Analysis

In order to access overall distribution of genetic diversity data was analyzed using Minitab software.

2.5. Biochemical Characterization

For biochemical characterization Czapek Dox media (20 g agar, 20 g sucrose, 0.01 g FeSO₄, 0.50 g KCl, 0.50 g Mg SO₄, 01 g K₂HPO₄ and 3 g NaNO₃) supplemented with 2% pectin for *A. niger*, *A. japonicus* and *A. parasiticus*, glucose for *A. nidulans*, starch for *A. flavus* and *A. oryzae* are added with starch to observe activity of α -amylase specifically and cellobiose for *A. fumigatus* respective substrate was inoculated with 5.10⁶ spores/ml and incubated at 30°C for 24 h. Plates were stained with Ruthenium red (0.05%) solution for 1 hour

or with Congo red dye. Destaining was done by 1 M NaCl and tap water. Plates were observed for their biochemical and enzymatic reaction as by the formation of coloured zone in each case.

3. Results and Discussion

Figures 1 and 2 shows polymorphism among seven *Aspergillus* species, which means a particular primer, gives different bands to evaluate species with minor genetic differences. A total of 46 bands with primer GL-Decamer B-09 (Figure. 1)

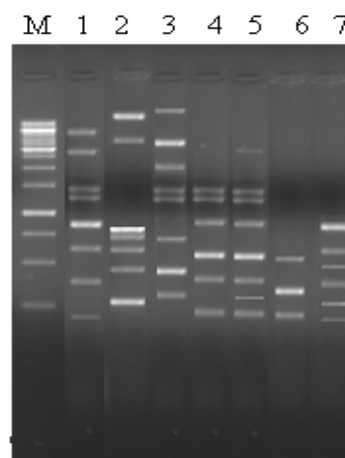


Figure 1. RAPD analysis of *Aspergillus* specie by primers GL-Decamer B-09. M, 1 kb Ladder; Lane 1, *A. niger*; lane 2, *A. nidulans*; lane 3, *A. parasiticus*; lane 4, *A. japonicus*; lane 5, *A. fumigatus* lane 6, *A. oryzae* and lane 7, *A. flavus*

Total 38 bands with primer GL decamer B-10 (Figure. 2) have been identified.

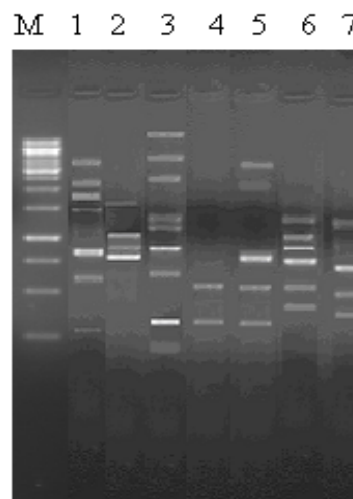


Figure 2. RAPD analysis of *Aspergillus* specie by primers GL-Decamer B-10. M, 1 kb Ladder; Lane 1, *A. niger*; lane 2, *A. nidulans*; lane 3, *A. parasiticus*; lane 4, *A. japonicus*; lane 5, *A. fumigatus* lane 6, *A. oryzae* and lane 7, *A. flavus*

Dendrogram of primer GL Decamer B-09 (Figure. 3) shows that *A. oryzae* and *A. flavus* are 83% similar to each other. *A. parasiticus* and *A. fumigatus* are 80% similar. *A.*

niger is 82% similar to *A. parasiticus* and *A. fumigatus*. These two groups are 75% similar to each other. *A. nidulans* shows 60% similarity to *A. oryzae*, *A. flavus*, *A. parasiticus*, *A. fumigatus* and *A. niger*. *A. japonicus* is 50% similar to all *Aspergillus* strains. This result of similarity corresponds to the work of Carbone[16] with respect to *A. parasiticus*.

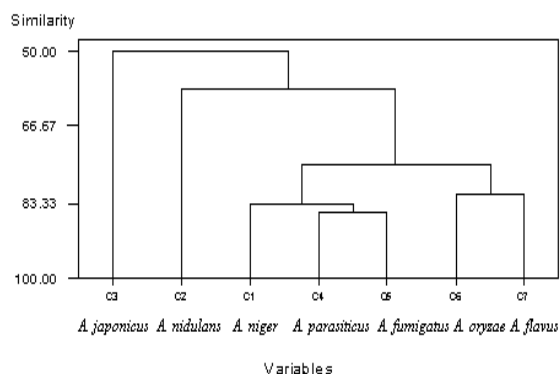


Figure 3. Dendrogram showing genetic diversity with primer GL- De-camer B-09. C1, *A. niger*, C2, *A. nidulans*, C3, *A. japonicus*, C4, *A. parasiticus*, C5, *A. fumigatus*, C6, *A. oryzae*, C7, *A. flavus*

The dendrogram at figure. 4 shows that *A. parasiticus* and *A. fumigatus* are 80% similar to each other. *A. oryzae* and *A. flavus* 87% similarity while *A. japonicus* is 68% similar to *A. oryzae* and *A. flavus*. *A. niger* is 55% similar to the above three species[17].

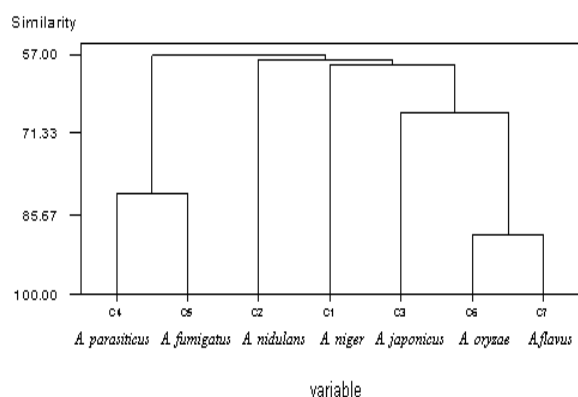


Figure 4. Dendrogram showing genetic diversity with primer GL-- De-camer B-10. C1, *A. niger*, C2, *A. nidulans*, C3, *A. japonicus*, C4, *A. parasiticus*, C5, *A. fumigatus*, C6, *A. oryzae*, C7, *A. flavus*

A. nidulans is 56% similar with the four species and *A. parasiticus* and *A. fumigatus* is 57% similar with all other *Aspergillus* strains. This data of current research work correlated the result of Pan[18] who analyzed *A. oryzae*, *A. flavus* and *A. sojae* as control by RAPD technology.

Biochemical characterization elucidated that specific enzyme secreted by particular specie which degrades its respective substrate. This was also mentioned by Carpita[19] that *A. niger* produces pectinase as major enzyme to degrade pectin though there are many other as glucose oxidase, lipase, xylanase, also secreted but the prominent one is pectinase[20]. *A. niger*, *A. japonicus* and *A. parasiticus* was provided with pectin as sole source of carbon substrate and was stained with Ruthenium red dye solution (0.05%) for one

hour. After one hour tap water destaining light pink demarcation at the zones showed that pectinase enzyme was secreted by these species which hydrolyzed the respective substrate source where growth had been occurred (Table 1).

Table 1. Enzyme activity of different *Aspergillus* species in response to particular substrate

Sr. no.	Species	Substrates	Enzyme Activity (+ve)
1	<i>A. niger</i>	Pectin	Pectinase +ve
2	<i>A. japonicus</i>	Pectin	Pectinase +ve
3	<i>A. parasiticus</i>	Pectin	Pectinase +ve
4	<i>A. nidulans</i>	Glucose	Glucose oxidase +ve
5	<i>A. fumigatus</i>	Cellobiose	β -glucosidase +ve
6	<i>A. oryzae</i>	Starch	α -amylase +ve
7	<i>A. flavus</i>	Starch	α -amylase +ve

* +ve positive

A. nidulans was grown in glucose as a source of carbon substrate. It was stained with Congo red dye solution (0.1%) for one hour and destained with 1M NaCl for one hour. Finally the light yellow color of the zones appeared that means glucose oxidase enzyme from the specie brought oxidation of glucose, the respective substrate source (Table 1) as mentioned by Luque in 2004[21].

A. fumigatus secretes β -glucosidase that was analysed by adding cellobiose as substrate.

A. oryzae and *A. flavus* have been identified to produce α galactosidase and two distinct β -glucosidases which were analysed by adding starch in growth medium. *A. oryzae* and *A. flavus* were grown in starch as a source of carbon substrate, then stained with Congo red dye solution (0.1%) for one hour and destained with 1M NaCl for one hour further. Finally the light yellow color of the zones appeared that means α -amylase enzyme from both species brought hydrolysis of starch contents that was added as carbon source, the respective substrate of α - amylase (Table 1).

Genetic analysis using RAPD is attractive as no prior knowledge of the DNA sequence is available and primers are designed randomly with the sole constraint being GC contents. It permits simultaneous investigation of multiple loci in a single PCR reaction[22]. Like all other techniques, the RAPD-PCR has limitations; the prominent shortcoming of RAPD is its low band repeatability and occurrence of pseudo bands. But it can be improved by applying suitable conditions to remove the impurities in extracted DNA and keeping the amplification conditions stable. It is concluded that the use of DNA marker can increase the efficiency of analyzing genetic diversity among different fungal isolates, as described by Rath[23].

According to our current research work the data generated by this RAPD study is useful in estimating distances between and within same species and might help future programs of management and conservation. Results indicated that genetic differences between species of the same genus maintain genetic diversity within this population. This study may provoke future research in a way that more and more closely related species of not only fungi but also of any other organism can be characterized at their molecular and bio-

chemical level. The present research work paved a way of finding minor differences of closely resembling species at genetic level and more and more evaluation are welcoming in this regard.

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