



## Analysis of genetic variability for the fungal strains isolated from contaminated soil of Kasur

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### ABSTRACT

Present study focused on detection of *Aspergillus* and *Curvularia* species from contaminated peri-urban agricultural area of Kasur by soil serial dilution method. Genetic variability among the fungi was determined by RAPD technique. Genomic DNA was extracted and amplified with 8 RAPD markers which were previously designed for *Aspergillus* species. Amplified products of DNA were analyzed electrophoretically and fragments ranging in 250-10000bp were generated. Clustering analysis was accomplished and a significant genetic relation was found in the isolates of these two genera which would be helpful in devising management approaches appropriate to bioremediation of contaminated soils.

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### Introduction

Soil is the habitat of countless organisms comprising of algae, bacteria, fungi, protozoa and viruses. Approximately 30,000 species of bacteria, 60,000 species of algae, 3000 earthworms and 1,500,000 fungal species are found in soil (Pankhurst, 1997). Soil contamination is a buildup of noxious chemicals, salts, pathogens or radioactive substances that affect plants growth and animals' health (Singer and Munn, 1999). Release of industrial effluents and sewage has largely contributed to heavy metal infectivity of agricultural soils. Effluents directly enter the agricultural fields and consequently enormous toxic chemicals and heavy metals are included in soil. Excessive use of pesticides, nitrogen fertilizers and raw organic matter comprising heavy metal residues may percolate or runoff into the water resources originating the soil, air and water pollution in these regions (Evan, 2002).

Different methodologies using molecular markers are widely used to analyze the pattern of variation within natural population. Random amplification of polymorphic DNA (RAPD) is a modification of the polymerase chain reactions (PCR) in which a single primer is capable to anneal and prime at multiple locations throughout the genome and generates a spectrum of amplification products that are characteristics of the template DNA. RAPD markers have been found with a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Aim of the study was to detect the fungal species from heavy metal contaminated peri-urban agricultural soil of Kasur and determination of genetic diversity analysis in accordance with bioremediation.

### Materials and Methods

#### Collection of samples

A survey was conducted in metal contaminated agricultural areas of Kasur and soil samples were accumulated erratically from 3-5cm depth. Samples were mixed systematically and brought in laboratory for detection of fungi.

### Detection of fungi

Soil samples were assessed with isolation process by using soil dilution plate method as described by Waksman, (1992). A mixture was created by adding 1g of soil in 10mL of distilled water and 1/1000 dilutions of soil samples were formulated. Resultant assortments were inoculated on potato dextrose agar medium and plates were incubated at 28°C. Colonies were distinguished morphologically and preserved on PDA slants for further investigation.

#### DNA Extraction

Genomic DNA was isolated by Phenol extraction method as described by Alves *et al.*, (2004) with minor modifications. For this purpose, 50mg of mycelium was crumbed from 1 week old culture. Mycelium was squeezed with glass rod suspended in 1000µl of DNA extraction buffer (Tris-HCl, EDTA, NaCl and SDS). After homogenization, 500µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added in mycelium and crushed for few seconds. Mixture was transferred to 1.5ml eppendorff tube and centrifuged for 10min at 10,000rpm. After that, supernatant was separated circumspectly from pellet and added 500µl of isopropanol and 50µl of sodium acetate (3M, pH4.8) in pellet and mixed smoothly until the emergence of a thread in mixture and centrifuged subsequently. Then pellet was separated and rinsed with 500µl of 70% EtOH followed by centrifugation. Pellet was detached from wash buffer and samples were put on tissue paper for air drying. After complete drying of two hours, samples were suspended in 200µl of TE (Tris-HCl and EDTA pH 8.0) buffer and incubated at 35°C for 30min. Isolated DNA was purified by adding 1µL of RNase (100µl/m) to each sample.

#### PCR amplification

RAPD reactions were carried out in 25µL volume containing 2.5µL of Taq buffer, 3µL of MgCl<sub>2</sub>, 4µL of dNTPs, 2µL of primer, 2µL of DNA template, 0.2µL of Taq DNA polymerase, 11.3 µL of distilled water. The amplification reactions were performed in thermal cycler programmed as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 72°C, annealing at 36°C for 1 min, extension at 72°C for 2 min, final extension at 72° C for 10 min. PCR

products were stored at 4°C. DNA amplified products were separated on 1% agarose gel and stained with ethidium bromide. DNA fragments were envisioned under UV light and fragment sizes were determined by comparison to 10kb DNA marker.

**Table 1. List of RAPD-PCR primers used in this study.**

Primer	Sequence
OPB 1	5' GTTTCGCTCC 3'
OPB 2	5' TGATCCCTGG 3'
OPB 3	5' CATCCCCCTG 3'
OPB 4	5' GGA CTGGAGT 3'
OPB 5	5' TGCGCCCTTC 3'
OPB 6	5' TGCTCTGCC 3'
OPB 7	5' GGTGACGCAG 3'
OPB 8	5' GTCCACACGG 3'

#### RAPD data analysis

RAPD fragments were scored from the gel image as presence or absence for all isolates. Clustering analysis was performed based on genetic relationship of the isolates and a dendrogram was constructed by Minitan software.

#### Results and Discussion

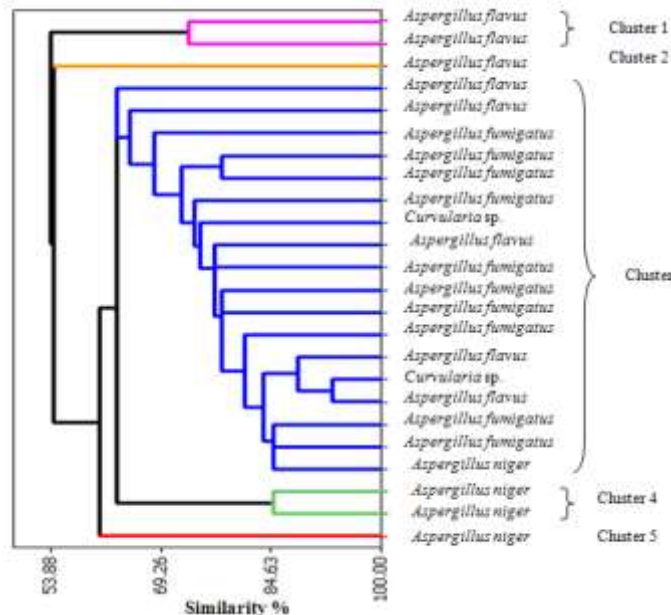
In current investigation, four fungal species (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Curvularia* sp.) were isolated from agricultural areas of district Kasoor. Peri-urban areas are contaminated with heavy metals and resources are sewage, tanneries and industrial waste and it can be disputed that these areas are not valuable with uppermost level of pollution (Bever *et al.*, 1996). Diversity in species suggests that fungal pathogens pursue different policies to ascertain symbiosis and *Aspergillus* sp. was found frequently in soil samples. Certain fungal species in soil are better acclimatized to the trouble created by the heavy metals that conquer the stress conditions and complete their life cycle. Relation between the genetic diversity with the fungal populations and heavy metal stress in soil escort to increase in diversity (Giller *et al.*, 1998). Understanding of the mechanisms involved in fungal diversity predominantly of the fungal adaptation and lenience to metals could be functional in the management and bioremediational programs (Comis, 1996).

Present research work was conducted to study the genetic variations of *Aspergillus* and *Curvularia* isolates that were isolated from contaminated soil of peri urban agricultural area of Kasur. All the primers showed amplification bands with *Aspergillus* and *Cuvularia* isolates ranging in 250-1000bp. Extraction of DNA is the first step for all molecular marker type. DNA can be extracted either from fresh, lyophilized, preserved or dried samples but for obtaining good quality DNA fresh material is recommended. In present study DNA was isolated from the fungal mycelium, and cell wall was ruptured by the liquid nitrogen. (Semagn *et al.*, 2006).

The method presented in present research eliminates much of the laborious and time consuming steps of most other protocols. DNA was isolated immediately from mycelium. In this procedure cell walls were broken by liquid nitrogen, the amount and quality of DNA obtained from this method was good and sufficient for PCR amplification and gel electrophoresis process. Similarly Bolano *et al.* (2001) reported that many of these protocols are apparently suitable for certain groups or morphological forms of fungi but may not be versatile and efficient for extracting nucleic acids from diverse groups of filamentous fungi.

Any contamination in reaction mixture leads to the failure of DNA analysis. Material contamination can occur on several levels, and for this reason, the contamination risk should be

minimized as much as possible. In the previous research, a number of practical problems are encountered in using molecular biology methods, e.g. false positivity resulting from contamination or false negativity resulting from presence of PCR-inhibitors, both of which can lead to an incorrect interpretation of the findings (Loeffler *et al.*, 1999). Isolates were discriminated into five groups and a low genetic variability was found among the isolates (figure 1).



**Fig. 1 Dendrogram showing genetic relationship among the isolates.**

RAPD technique analysis is technically fast and simple. As according to prior study, RAPD analysis can detect minute variation among strains even a single nucleotide mismatch in the priming region may prevent annealing and the absence of a characteristic band on gels. Small differences in any aspect of PCR conditions that affect binding of the primer may have similar effects. This problem can be minimized if strains under study are treated identically. When multiple strains are compared, the same PCR buffer, the master mix (includes all four nucleotides, primers, appropriate ions, and DNA polymerase) and the same thermal cyclor and PCR running program should be used at the same time (Xu, 2002)

In PCR reaction mixture, 3µl MgCl<sub>2</sub> was used. MgCl<sub>2</sub> stabilizes primer annealing; therefore, the concentration of MgCl<sub>2</sub> has a large effect on the specificity and yield of a reaction. Too little Mg<sup>2+</sup> can decrease the yield while excess Mg<sup>2+</sup> results in non-specific amplification as a result of reduced enzyme fidelity (Meunier *et al.*, 1993)

In this study PCR conditions are in the line of a previous study parameters including temperatures, particularly the annealing and the extension temperatures, and time period of different steps are vital for optimum amplification of DNA (Smith and Devey, 1994), therefore, requires a longer period of denaturation. For example, Lu *et al.* (1995) applied temperature of 94° C for 3 min to allow for maximum denaturing of DNA. Chosen suitable primers is very important process for PCR-RAPD to get clear and good bands. Eight primers had been used and amplification showed differences banding pattern (Parenrengi, 2000). Similarly, quantity and purity of extracted genomic DNA also plays crucial role for analysis of molecular diversity and optimization of different parameters for PCR (Staub *et al.*, 1996).

The RAPD technique is easier and faster than other. In this work, optimal conditions like precise extraction and constant amplification conditions obtain reproducible results. The generation of DNA fingerprints using the randomly amplified polymorphic DNA techniques (RAPD) is particularly useful because no prior genetic knowledge of the target organism is required (Delye et al., 1997). This investigation has provided a very useful tool to understand and distinguish the fungal species. Use of waste water for irrigation is the major source of contamination in peri- urban agricultural fields because fresh water is not easily accessible. RAPD technique is rapid, easy and very useful in determining genetic diversity of the natural populations. Further studies like tolerance and biosorption are required in accordance with bioremediation of contaminated soil.

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