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NATURAL INCIDENCE OF AFLATOXINS, MYCOLOGICAL PROFILE AND MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC STRAINS IN CHICKPEA FLOUR

SAIRA MUSHTAQ[1], ABIDA AKRAM[1], NAFEESA QUDSIA HANIF[2], RAHMATULLAH QURESHI[1*], ZAHID AKRAM[3], SHAISTA AKHUND[1] AND BRIAN GAGOSH NAYYAR[1]

[1]Department of Botany, Pir Mehr Ali Shah Arid Agriculture university Rawalpindi, Pakistan
[2]Romer Labs, Pakistan, Rawalpindi, Pakistan
[3]Department of Plant Breeding ad Genetics, PMAS-Arid Agriculture university Rawalpindi, Pakistan
[*]Corresponding author: rahmatullahq@yahoo.com, rahmatullahq@uaar.edu.pk

Abstract

The mycological profile of retail chickpea flour (locall called *Baisan*), sold in the markets in the Rawalpindi district was studied. All the samples were tested for the contamination with aflatoxins. A total of 13 fungal species isolated from the flour and out of which, *Aspergillus flavus* was recorded the most common species (100%), followed by *Rhizopus oryzea* (50%), *Aspergillus niger* (40%), *Penicilium digitatum* (30%), *Cladosporium cladosporoides, Fusarium oxysporium, Mucor recemosus*, *M. petrinsularis* and *Rhizopus arrhizus* (20% each), *Aspergillus oryzea*, *Botritus cinerea*, *Mucor circineloides* and *Penicillium* sp. (10% each). Aflatoxin B_1 was found in only 20% of the samples ranging from 3.03-4.24ppb. The molecular characterization was carried out by using PCR using simple sequence repeats (SSR) primers. The SSR amplification pattern clearly showed the genetic variability among the 10 strains of *A. flavus.* A dendrogram was generated through MVSP software program. Genotype AF04 was most diverse among all genotypes. The similarity value was ranged between 0.538 (53.8%)-0.938 (93.8%).

Key words: Chickpea, Molecular characterization, Aflatoxins, Dendrogram, PCR, SSR.

Introduction

Chickpea flour, locally known as *Baisan*, is one of the alternatives of wheat flour. It is rich in carbohydrates, protein and gluten free (Turcotte, 2010). Flour is generally considered to be free from the contamination, because it is mainly a low active commodity. Many pathogens that contaminate flour may survive for a long time (Cabanas, 2008). Chickpea is the most important crop of Indo-Pak region (Sethi *et al.,* 1997). In Pakistan, it is cultivated over an area of 1053.8 hectares with its production of about 470.7 kg/hectare (Anon., 2010-2011). It is normally sown as Rabi crop in tropical and subtropical areas of the world (Islam, 1991). Mainly two types of chickpea are grown in this region, i.e. the brown seeded called as desi and white seeded called as kabuli (Dawar *et al.,* 2007).

The chickpea is exposed to the fungal diseases and so far, many fungal species have been isolated from chickpea in Pakistan. These include *Absidia glauca*, *Alternaria porri*, *A. alternata, Aspergillus amsteldomi, A. flavus, A. fumigatus, A. niger, A. wentii, Botrytis cinerea, Cladosporium* sp., *Curvularia lunata, Fusarium equiseti, Macrophomina phaseolina* (Dawar *et al.,* 2007). Amongst them, three main genera *Aspergillus*, *Fusarium* and *Penecillium* are considered to produce mycotoxins during crop growth, harvesting and storage (Al-Hazmi, 2010). Of them, *Aspergillus flavus* is responsible for aflatoxin production, a potential threat to humans caused by direct contamination through grains and grain products (Bushby & Wogan, 1984; Kotsonis *et al.,* 2001).

The four most common aflatoxins produced are AFB_1, AFB_2, AFG_1, and AFG_2 with AFB_1which are the most potent carcinogen (Zinedine & Manes, 2008) and the International Agency for Research on Cancer has classified such aflatoxins in class 1A carcinogen for human being (IARC, 1993). *Aspergillus flavus,* a group consists of phylogenetically similar species, either aflatoxin producers or non-aflatoxin producers showing extreme genetic diversity. This genetic variability is due to several differences in morphology, mycotoxin production and toxigenicity (Bayman & Cotty 1993; Horn *et al.,* 1996). Even the toxigenic strains don't necessarily produce toxins. It depends upon favorable environmental conditions like temperature and humidity (Correa, 2000). Hence, to prevent mycotoxin production a comprehensive view of population study of *A. flavus* focused on its genetic diversity is required. Molecular characterization aims at obtaining relatedness of the strains isolated from chickpea flour. Three molecular methods have been proposed for *A. flavus* typing: restriction fragment length polymorphism (James *et al.,* 2000: Moody & Tyler, 1990), randomly amplified polymorphic DNA (Rath, 2001), and random amplified microsatellite polymorphism analysis (Guarro *et al.,* 2005). Recent studies have described various polymorphic microsatellite markers that may be used to type aflatoxin-producing *A. flavus* and *A. parasiticus* (Tran-Dinh & Carter, 2000; Hadrich *et al.,* 2010). In the present study chickpea flour was screened for incidence of mycoflora and aflatoxin contamination. In addition, isolated strains of *A. flavus* were characterized to explore the genetic diversity among them.

Materials and Methods

Sampling: Ten Samples of chickpea flour (250 g) were collected randomly from different retailers and shops of Rawalpindi. All samples were stored in airtight polythene bags at 4˚C till further analysis.

Moisture: For moisture content, samples were dried at 100-105°C for overnight or until their weights remain constant and the difference in their weight was calculated as described by ISTA (1993).

Mycological analysis

Isolations: The dilution plating method was modified as described by Aziz *et al.* (1991). All dilutions were prepared in sterilized distilled water. A 10 g of flour was mixed thoroughly in 90 ml sterilized distilled water on shaker for one hour. Tenfold serial dilutions of concentration 1/10, 1/100 and 1/1000 were prepared in three replicates. A 4ml suspension was platted on potato dextrose agar (PDA) media and incubated at 30˚C for 3-5 days. After incubation period the average frequency of each species was calculated and fungi were identified by following references (36; 27: 11). Strains of *A. flavus* were preserved in Malt extract agar (MEA) slants at 4˚C for future use.

Mycotoxin analysis: Samples were extracted and analysis for total aflatoxins was performed by HPTLC according to the method described by (Hanif *et al.,* 2006). About 12.5g of the sample was homogenized with 50 ml methanol: water (60:40 v/v) and shaked for one hour and filtered through Whatman filter paper No. 1. The filtrate was then loaded on AflaStar® imunoaffinity columns (Romer Labs Inc., USA) with the flow rate of 2 ml per minute. Later, washing of the column was completed with 20 ml distilled water until antibody gel was free from any pigment following flow rates of 3 ml per minute. Aflatoxins were then eluted with 3 ml of Methanol at a flow rate of 0.5 ml per minute. Eluted extract was evaporated with the help of Romer Evap® System (Romer Labs Inc., USA) at a temperature of 60°C. Toxin was then reconstituted in Toluene: Acetonitrile (95:5 v/v). After redissolving, 40ul of the sample was spotted along with aflatoxins reference standard (Biopure, Austria) on the silica gel 60 plate (Merck, Germany). Upon spotting completion, the plate was developed in Chloroform: Acetone (9:1 v/v). Later on, the plate was derivatized by dipping/spraying in 10% H_2SO_4 (v/v). Aflatoxins were observed under the UV light (365nm) by comparing the size, intensity and RF of sample with standard spots.

Molecular characterization: Mycelia were grown in malt extract broth (Rodrigues *et al.,* 2007) and collected by filtration through Whatman filter paper 2, rinsed twice with 1% NaCl solution, centrifuged and placed in aliquots of 1g at -80 ºC for later analyses.

DNA extraction: For DNA extraction, method as described by (Rodrigues *et al.,* 2007) was followed. Briefly, one gram mycelia were placed in pre cooled pestle and mortar and crushed finely into powder in liquid nitrogen. The grounded sample was suspended in 1.5 ml of preheated (at 60˚C) extraction buffer. It was mixed thoroughly until mixture got homogenized. The mixture was then transferred in eppendorf tubes and incubated at 60-65˚C for 1 hour. Afterwards, for 2 or 3 times after every interval of 15 minutes, the mixture was mixed and then cooled for 5 minutes. The lysate was extracted by adding an equal volume of chloroform: isoamylalcohol (24:1; v/v) mixed thoroughly and centrifuge at 12000 rpm for 10 minutes. The middle transparent layer was transferred very carefully to a new eppendorf tube. DNA was recovered by isopropanol precipitation. The DNA thread formed was carefully hooked and was washed first with 70% ethanol and then with absolute ethanol. The pellet was dried under vacuum and re-suspended in Tris-Ethylenediamine tetra acetic acid (TE) buffer. For further purification, dissolved DNA was treated with 10µl of 4M sodium acetate and 50µl of chilled absolute ethanol. The DNA was hooked again, washed and re-suspended in TE buffer.

Quantification of DNA: The DNA concentration was quantified by gel electrophoresis in 1% agarose gel (0.4g of agarose in 40ml 1X TAE buffer). Dilutions (1/5th) were made of the DNA samples for subsequent SSR analysis.

SSR analysis: Genetic diversity among 10 strains of *A. flavus* assessed using microsatellite markers (Table 1) as described by Tran-Dinh & Carter (2000) and Hadrich *et al.* (2010). The PCR (Eppendorf Mastercycler® Gradient, Germany) reaction was carried in 10µl volume. The PCR reaction contained 1µl template DNA(50ng/µl), 1µl forward (F) and 1µl reverse (R) respective primers (1µM), 0.2µl dNTPs (10mM dNTPs mix), 1µl taq buffer, 0.8 µl $MgCl_2$ (25mM stock), 0.2µl taq polymerase enzyme (5 units/µl). Total reaction volume was made up to 10µl by using nuclease free water. In order to ensure the reproducibility of reaction appropriate negative controls (without DNA template) were also run in parallel.

On completion of PCR amplification cycle, PCR products were analyzed through gel electrophoresis in the presence of specific bands and analyzed to achieve SSR data for analysis. Each single band was considered as a single locus. For every primer which was used for amplification of fungal strains, the incidence of a specific band of genotype was scored as '1' and absence as '0' for each of the primer used. Bands having a similar mobility or having same size were considered as identical bands. The positions of these bands were compared with molecular weight standards. The fungal strains were compared with each other using their SSR-PCR pattern and bands of DNA. Data were analyzed by using MVSP software package. After analyzing the gel images, genetic similarity index was produced based on similarity coefficients. By using UPGMA (unweighted pair group method with arithmetic mean) clustering algorithm (Nei & Li, 1979) a dendrogram was constructed based on these similarity coefficients.

Table 1. SSR oligonucleotide sequences for the forward and reverse primers.

Count	Oligo names	Sequences	Tm
1	AFPM1 F	CCCAGTCACGACCATTAC	54
2	AFPM1 R	GGTTCGTAGGTGGATAGAG	54
3	AFPM2 F	CCACGCTCCTCAAATACG	54
4	AFPM2 R	CTGGACGGAGATCACGAC	54
5	AFPM3 F	CACCACCAGTGATGAGGG	54
6	AFPM3 R	CCTTTCGCACTCCGAGAC	54
7	AFPM4 F	TCTTGCTATACATATCTTCACC	53
8	AFPM4 R	AGCGATACAGTTTTAACACC	53
9	AFPM5 F	CCATTATGACATGTGGTTAAGAG	54
10	AFPM5 R	TCCTACCCGAGAGAGTCTG	54
11	AFPM6 F	CTCAACGCAAGTCAGGTACGC	60
12	AFPM6 R	CGAAAGGCAGTTGTGAAGGC	60
13	AFPM7 F	CAAATACCAATTACGTCCAACAAGGG	60
14	AFPM7 R	TTGAGGCTGCTGTGGAACGC	60
15	AFLA1 F	CGTTGGCATGTTATCGTCAC	54
16	AFLA1 R	CTACTGAATGGCGGGACCTA	54
17	AFLA2 F	GAGCACGTGCGATTTAGTCA	54
18	AFLA2 R	TATCTACTCCGGCCAACTCG	54
19	AFLA3 F	CTGAAAGGGTAAGGGGAAGG	54
20	AFLA3 R	CACGCGAACTTATGGGACTT	54

Tm: Melting temperature

Table 2. Moisture content of chickpea flour samples.

Sr. No.	Sample name	Moisture content
1.	CF 1	4.60
2.	CF2	5.40
3.	CF3	7.31
4.	CF4	6.10
5.	CF5	15.00
6.	CF6	4.67
7.	CF7	7.20
8.	CF8	6.00
9.	CF9	7.10
10.	CF10	6.25
Total	10	69.63
Mean		6.96

Results

Moisture content: The moisture levels in the examined samples were ranging from 4.6-15.0%, with the mean value of 6.96% (Table 2). This finding is in the range described by (Duncan & Hagler, 2008). According to their findings, aflatoxins produce at a temperature of 12-40ºC and at the moisture level of 3-18%.

Isolation of mycoflora: A total of 13 different fungal species, namely *Aspergillus flavus* Link ex Gray *1821, Aspergillus niger* van Tieghem 1867, *Aspergillus oryzae* (Ahlburg) Cohn *1884, Botrytis cinerea* Pers. Ex Nocca & Balb. 1821, *Cladosporium cladosporoides* (Fres.) de Vries 1952, *Fusarium oxysporum* Schlecht. 1824 emend. Sny. & Hans. 1940 pro maxima parte, *Mucor circineloides* f. *griseo-cyanus* (Hagem) Schipper 1976, *Mucor petrinsularis* Naumov, 1915. Petersb. Pilze, Petersburg, *Mucor recemosus* Fres. 1850 f. *racemosus, Penicillium digitatum* (Pers. Ex St.-Am.) Sacc. 1882, *Penicillium* spp., *Rhizopus arrhizus* Fischer, 1892. Rabentorst Kryptog, *Rhizopus oryzae* Went & Prinsen Geerligs 1895, were isolated. Among these, *Aspergillus flavus* was found to be the most predominant specie (100%) isolated from all samples followed by *Rhizopus oryzea* (50%) as can be seen in Table 3.

Mycotoxin analysis: The collected chickpea flour samples (n=10) were then subjected to aflatoxins analysis (AFB_1, AFB_2, AFG_1, and AFG_2). Out of these only 20% samples were found positive for AFB_1 contamination with the range from 3.03-4.24 ppb with the mean value of 3.63 ppb (Table 4). Besides, no sample was found positive for AFB_2, AFG_1 and AFG_2. The mean value of AFB_1 was near to the permissible limits as recommended by European Commission (2010).

Molecular characterization: For the identification of polymorphism in DNA, a total of 10 SSR primers were screened for 10 strains of *Aspergillus flavus.* Out of these 10 markers, only 5 showed clear polymorphic bands. These 5 markers were selected to amplify the whole 10 strains. Only apparent and clear bands were selected for scoring, while light bands were not selected further study. Each band was scored as a single allele for the genetic analysis. The alleles were scored as 1 and 0. The band sizes of PCR products ranged from 200 bp to 1500 bp in our case. Different primers showed dissimilarity in number of polymorphic bands. Strain 5, 8 and 9 were amplified with all 5 primers and strain 1, 2, 3, 4, 6, 7 and 10 with four pair of primers. Effectiveness of these 5 primers to do amplification of the 10 genotypes ranged from 5 genotypes by primer AFPM 7, 8 genotypes by primer AFPM 6, and all genotypes by primer AFLA 1, AFLA 3 and AFPM 4 (Figs. 1-5).

Table 3. Isolation and occurrence (%) of mycoflora from chickpea flour.

Name of fungi	Samples infected	Mean ± S.E	Range of average frequency (%)
Aspergillus flavus	10	10.56 ± 2.25	2.94 – 23.52
A. niger	4	3.68 ± 1.75	6.36 – 15.71
A. oryzea	1	0.29 ± 0.30	0 – 2.88
Botrytis cineria	2	1.08 ± 0.85	2.94 – 7.84
Cladosorium cladosporoides	2	1.89 ± 1.367	7.14 – 11.46
Fusarium oxysporum	2	1.89 ± 1.05	8.96 – 9.99
Mucor circineloides	1	0.39 ± 0.41	0 – 3.92
M. petrinsularis	2	0.95 ± 0.90	0.90 – 8.64
M. recemosus	2	4.26 ± 3.32	11.81 – 30.76
Penicillium digitatum	3	2.95 ± 1.36	6.89 – 11.76
Penicillium sp.	1	0.14 ± 0.15	0 – 1.42
Rhizopus arrhizus	2	2.97 ± 2.08	13.79 – 15.43
R. oryzea	5	5.03 ± 1.89	5.43 – 12.74

Table 4. Occurrence of aflatoxin B1 in chickpea flour

Toxins	Positive samples	Mean	Minimum (ppb)	Maximum (ppb)	Detection limits
B_1	2	3.63	3.03	4.24	<0.1
B_2	ND	-	-	-	<0.5
G_1	ND	-	-	-	<0.1
G_2	ND	-	-	-	<0.5

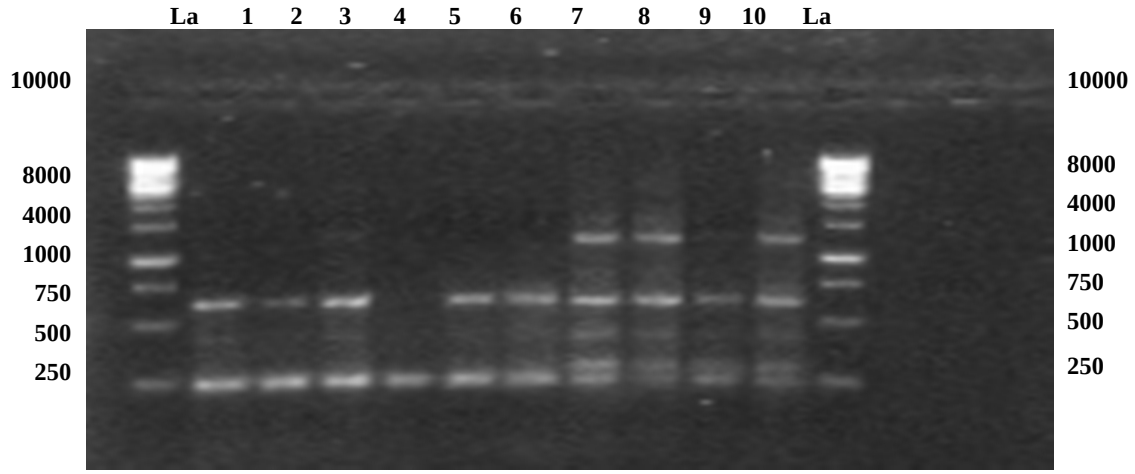

Fig. 1. SSR Amplification pattern of primer AFLA 1.

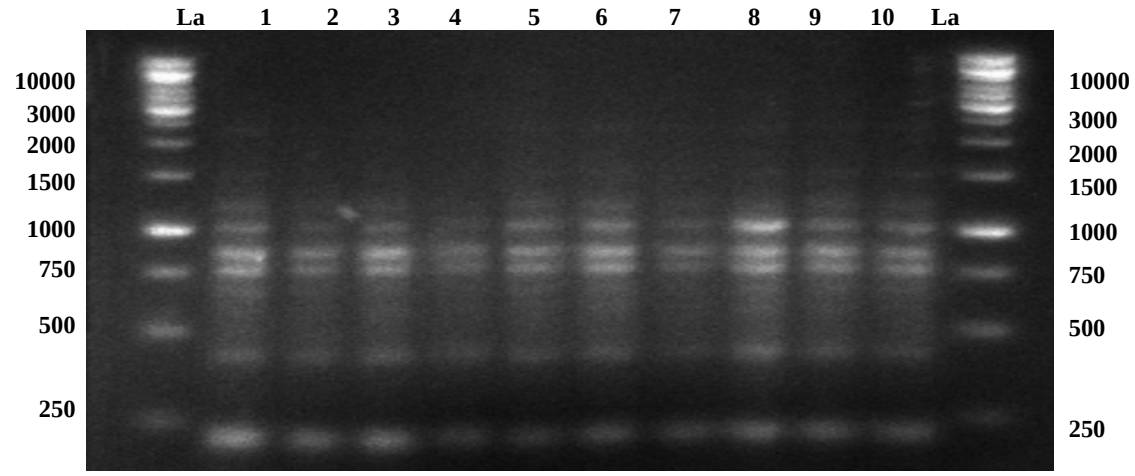

Fig. 2. SSR Amplification pattern of primer AFLA 3.

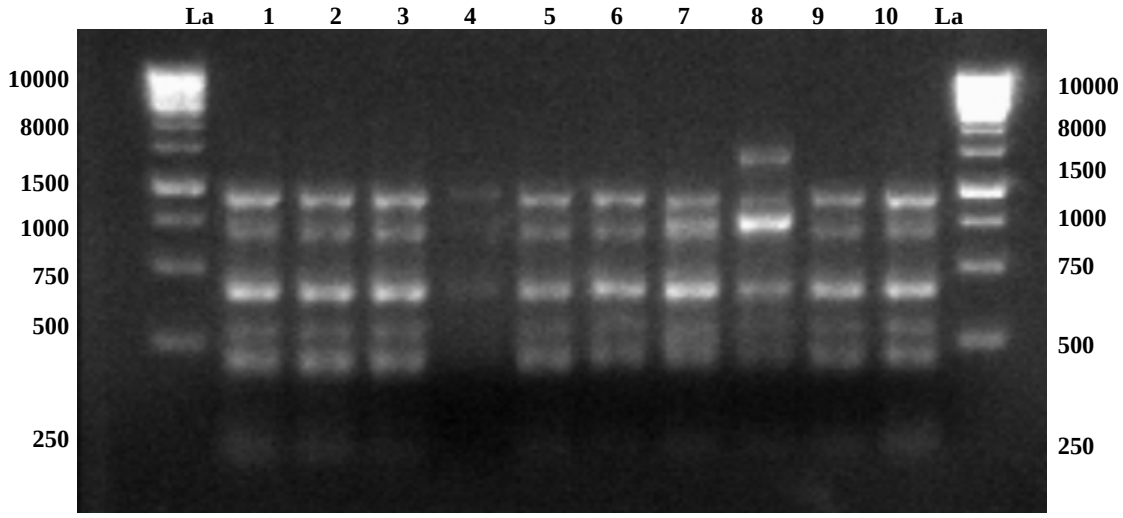

Fig. 3. SSR Amplification pattern of AFPM 4.

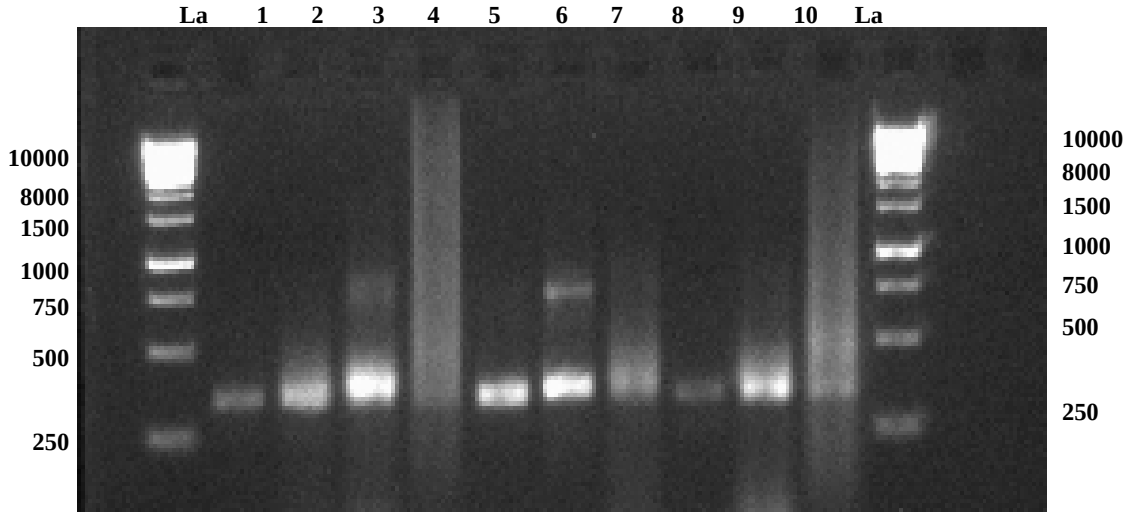

Fig. 4. SSR Amplification pattern of AFPM 6.

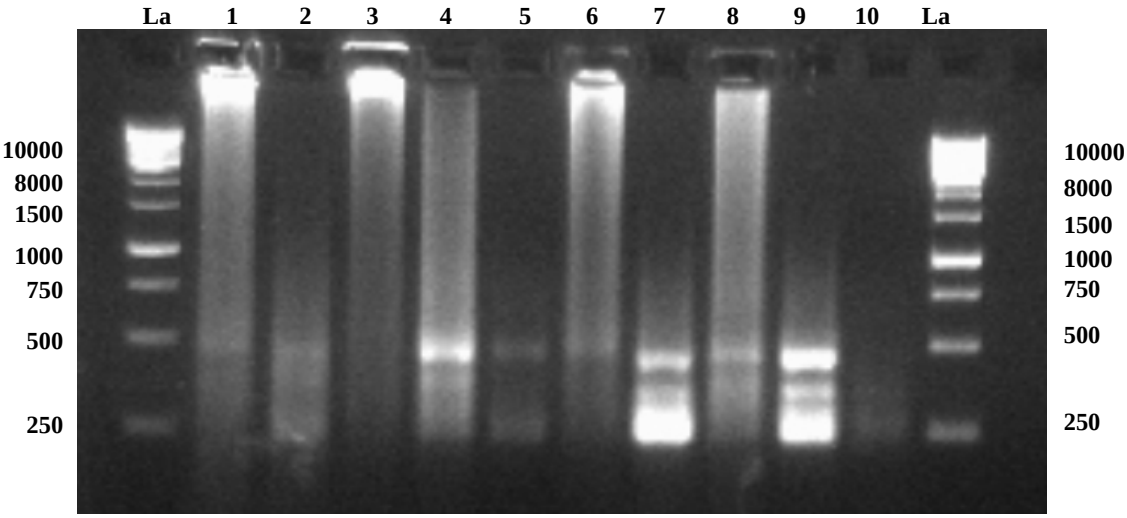

Fig. 5. SSR Amplification pattern of AFPM 7.

Similarity index: The similarity index was produced from SSR amplified fragments data to calculate approximately the genetic distance and genetic similarity between 10 fungal genotypes. After the analysis of the gel photos, the pairwise similar values form similarity index were counted with a similarity co-efficient. The similarity index was then transformed into dendrogram by using UPGMA clustering software to reveal the variation in genetics and similarity of different fungal genotypes (Table 5).

Dendrogram analysis: Dendrogram presents the accurate and defined analysis of the distance in the genetics of fungal strains by groupings the genotypes on the basis of the presence of any kind of similarity and differences in between them. The data calculated on the basis of presence and absence of specified bands was used to access approximately the genetic diversity between the genotypes through UPGMA software. Dendrogram constructed on the basis of the data provided by SSR markers clearly indicate two main clusters A and B. Dendrogram generated from the calculation of genetic distances is shown in Fig. 6.

Table 5. UPGMA Nei and Li's coefficient similarity matrix.

	AF-01	AF-02	AF-03	AF-04	AF-05	AF-06	AF-07	AF-08	AF-09	AF-10
AF-01	1									
AF-02	0.929	1								
AF-03	0.933	0.929	1							
AF-04	0.583	0.636	0.583	1						
AF-05	0.933	0.929	0.867	0.667	1					
AF-06	0.933	0.929	0.933	0.583	0.933	1				
AF-07	0.788	0.774	0.788	0.667	0.788	0.727	1			
AF-08	0.824	0.813	0.824	0.571	0.824	0.765	0.919	1		
AF-09	0.882	0.813	0.824	0.643	0.882	0.824	0.919	0.895	1	
AF-10	0.938	0.867	0.875	0.538	0.875	0.875	0.857	0.889	0.889	1

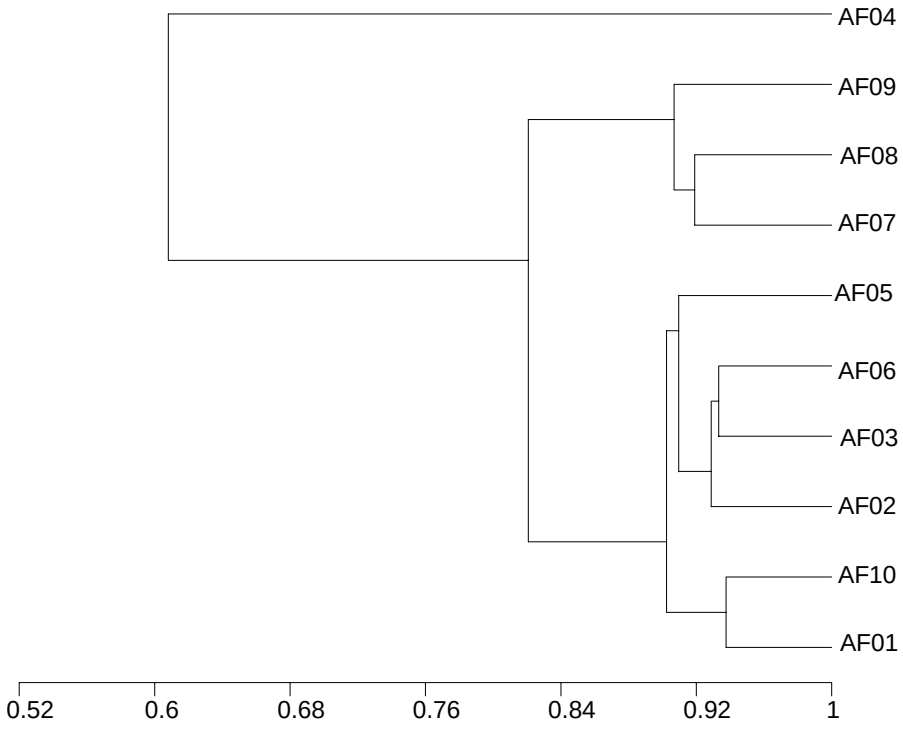

Fig. 6. Dendrogram Obtained by SSR Analysis of 10 Strains of *Aspergillus flavus* using UPGMA.

Discussion

Microorganisms attack different commodities mostly with dust, during harvesting, transportation and storage and food processing (Klich, 2002). Fungal spores usually present in flour may remain there for several years, therefore some careful measurements should be taken in the storage of flour samples (Christensen & Cohen, 1950). To the best of our knowledge, this study is the preliminary study showing the isolation of mycoflora and aflatoxin contamination analysis from chickpea flour and first report from Pakistan showing the genetic diversity of *A. flavus* strains from this region. In the present study, chickpea flour is found to be infected with a variety of storage fungi. Although *Aspergillus flavus* was surprisingly found in all chickpea flour samples, but out of these only 2 samples were contaminated with AFB1. Interestingly these two samples (3 and 5) had a high moisture content. The above findings represent the correlation of moisture contents for the production of aflatoxins. The relatively high moisture content in AFB1 contaminated samples might be responsible for aflatoxin production. According to Eyles *et al.* (1989), low water content of flour can also support the production of mycotoxins which can cause contamination. The changes in level of moisture from 1% or 2% can be enough for contamination with mycotoxins (Weidenborner *et al.,* 2000). The optimal temperature conditions for aflatoxin production is in line with the results presented by Schindler *et al.* (1967), who observed that the temperature range for the production of aflatoxin is between 25-35˚C. The use of different characters which were initially studied with the help of morphology and taxonomy usually is not trustworthy for detecting differences among different strains. Several molecular techniques proved successful to reveal polymorphism in fungi (Mitina *et al.,* 2008). In our study analysis for molecular characterization of the strains of *Aspergillus flavus* was performed by using SSR markers. In this study, 10 different SSR primers were used for observing the genetic diversity in 10 strains of *A. flavus.* These markers are highly polymorphic for *A. flavus.* Combination of 5 markers (AFLA 1, AFLA 3, AFPM 4, AFPM 6, and AFPM 7) revealed complete polymorphism among the 10 strains of *A. flavus*. All strains were non responsive to primers AFPM 1, AFPM 2, AFPM 3, AFPM 5 and AFLA 2. These results are not in line with the results presented by Hadrich *et al.* (2010) and Tan-Dinh & Carter (2000). This difference might be due to the difference in the climatic conditions of distinct geographical regions. Besides that complete heterogeneity was observed by Hadrich *et al.* (2010) in all of the strains as none of their strain was similar to another one. While, in the present study, most of the strains were having genetic similarity of up to 93.8%. This high degree of similarity might be due to reason that all of the strains were isolated from the samples collected from the same ecological zone. Therefore, due to less ecological barrier, maximum similarity was observed among the strain.

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