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Research Paper

Molecular characterisation of *Aspergillus flavus* isolates from peanut fields in India using AFLP

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Abstract

Aflatoxin contamination of peanut, due to infection by *Aspergillus flavus*, is a major problem of rain-fed agriculture in India. In the present study, molecular characterisation of 187 *Aspergillus flavus* isolates, which were sampled from the peanut fields of Gujarat state in India, was performed using AFLP markers. On a pooled cluster analysis, the markers could successfully discriminate among the 'A', 'B' and 'G' group *A. flavus* isolates. PCoA analysis also showed equivalent results to the cluster analysis. Most of the isolates from one district could be clustered together, which indicated genetic similarity among the isolates. Further, a lot of genetic variability was observed within a district and within a group. The results of AMOVA test revealed that the variance within a population (84%) was more than that between two populations (16%). The isolates, when tested by indirect competitive ELISA, showed about 68.5% of them to be atoxigenic. Composite analysis between the aflatoxin production and AFLP data was found to be ineffective in separating the isolate types by aflatoxigenicity. Certain unique fragments, with respect to individual isolates, were also identified that may be used for development of SCAR marker to aid in rapid and precise identification of isolates.

Key words: aflatoxin, AMOVA, ELISA, genetic diversity, groundnut, PCA.

Introduction

Peanut (*Arachis hypogaea* L.), also known as groundnut, is an important oilseed and ancillary food crop worldwide. In addition to the expulsion of oil, it is also used for production of peanut-butter and as a component of various food products. India possesses the largest peanut cultivation area in the world and is the second largest producer after China. The major Indian states, which collectively account for about 90% of the national area for peanut farming, include Gujarat, Andhra Pradesh, Tamil Nadu, Rajasthan, Karnataka and Maharashtra; Andhra Pradesh and Gujarat raking at first positions, in terms of cultivation area, and production, respectively. In Gujarat, about 80% of the peanut cultivation is concentrated in Junagadh, Rajkot, Porbandar, Amreli and Jamnagar districts of Saurashtra region (Anonymous, 2012; Misra and Thirumalaisamy, 2012).

Among the various parameters for quality assessment, aflatoxin contamination constitutes one of the major non-tariff trade barriers, especially, in the international peanut trade market (Misra and Thirumalaisamy, 2012). Aflatoxins are secondary metabolites, which are produced by *Aspergillus flavus* group of fungi, and are known to be carcinogenic and mutagenic (Abbas *et al.*, 2004). Aflatoxin contamination of peanut, due to invasion by *Aspergillus*, is a major problem of the rain-fed agricultural cultivation conditions in India (Misra and Thirumalaisamy, 2012). The fungi are wide spread in light sandy soils, which are most suitable for the peanut cultivation (Kumar *et al.*, 2005). Although the aflatoxin contamination does not affect peanut production, but it causes serious health risks in humans and cattle (Horn *et al.*, 1994).

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Owing to the sensory properties, Indian peanuts are in great demand across the world. However, the export of peanut from India is hampered by aflatoxin contamination. The European Union (EU) has set a stringent maximum permissible limit (2 ppb) for aflatoxin in directly consumed peanuts (Wu *et al.*, 2013). Due to aflatoxin contamination, recently, several consignments have been rejected at the destination ports in the EU (Misra and Thirumalaisamy, 2012).

Peanut pods, when come in direct contact with the spores of *A. flavus* in soil, get frequently invaded before the harvest. The mode and extent of infection by the fungus depends on the population density of *A. flavus* in the soil, soil moisture and soil temperature during the pod development till maturity (Smith *et al.*, 1995). Once the kernels are contaminated, the elimination of aflatoxins is not possible by routine cooking or processing practices. Roasting, however, appreciably reduces the level of aflatoxin in the peanuts. Therefore, the best strategy to counteract this problem would be the prevention rather than decontamination (Misra and Thirumalaisamy, 2012).

The aflatoxins are produced by the *Aspergillus* species, belonging to the section *Flavi*, such as, *A. flavus, A. parasiticus* and the others, like, *A. nomius, A. minisclerotigenes*, *A. pseudocaelatus*, etc. (Varga *et al.*, 2011). Since, not all the isolates of *Aspergillus* are toxigenic (Desai *et al.*, 1991), the characterisation of the isolates, for their toxigenicity in the major agro-ecological zones of peanut production system in India, is the need of the hour.

At present, DNA fingerprinting is relatively economical and allows discrimination of the fungal strains from the genus down to the clone level (Berbee and Taylor, 2001). Various types of molecular markers have been successfully employed to aid in detection of genetic variability in several *Aspergillus* species. Amplified Fragment Length Polymorphism (AFLP) is a technique which uses the benefits of both restriction digestion and PCR based selective amplification. AFLP has been widely used for molecular characterisation of *Aspergillus* spp. (Montiel *et al.*, 2003; Lee *et al.*, 2004). Till date, limited information, from India, is available on the prevalence and variability across the isolates of *Aspergillus* belonging to the section *Flavi* in respect to their toxigenicity (Desai *et al.*, 1991; Rajarajan *et al.*, 2013). Hence, the present investigation was an attempt towards detailed molecular characterisation of *A. flavus* isolates, collected from different peanut cultivation fields in Gujarat. The ultimate aim of this study was to analyse the genetic association among the *Aspergillus flavus* isolates, with respect to their toxigenicity, in one of the major peanut producing set-up in India.

Materials and Methods

Fungal isolation and identification

A total of 187 fungal isolates, analysed in this study, were originally collected from the farmers' fields, which were utilised for peanut farming, from 10 districts of Gujarat state (Table 1). Soil samples were collected from the groundnut fields and at each sampling, 5 randomly se-

Table 1 - Location and toxigenicity details of *Aspergillus flavus* isolates collected from soil samples under groundnut production system from Gujarat.

S. No.	AccessionNo.*	Aflatoxin content (ppb)	S. No.	AccessionNo.	Aflatoxin content (ppb)	S. No.	AccessionNo.	Aflatoxin content (ppb)
1	01009	0.00	64	02040	5.22	127	08006	1.00
2	01012	0.00	65	03005	0.00	128	08007	1.76
3	01016	1.12	66	03007	0.23	129	08008	0.71
4	01018	0.00	67	03024	0.00	130	08009	0.00
5	01025	6.37	68	03026	11.32	131	08010	2.65
6	01026	0.00	69	03027	56.54	132	08011	0.00
7	01031	5.24	70	03028	0.00	133	08012	6.59
8	01032	8.11	71	03029	0.00	134	08013	0.12
9	01035	5.24	72	03030	3.16	135	08014	39.96
10	01036	0.00	73	03031	8.77	136	08015	10.71
11	01038	0.00	74	03032	6.76	137	08016	0.00
12	01039	0.00	75	03037	2.40	138	08017	2.46
13	01040	0.00	76	04005	875.54	139	08018	0.73
14	01041	0.00	77	04010	698.47	140	08019	0.79
15	01043	0.00	78	05005	725.36	141	08020	2.17
16	01045	0.00	79	05010	145.02	142	08021	0.46
17	01046	0.00	80	05011	471.27	143	08022	1.55
18	01047	0.20	81	05016	0.98	144	09001	3.28

Table 1 - Cont.

S. No.	AccessionNo.*	Aflatoxin content (ppb)	S. No.	AccessionNo.	Aflatoxin content (ppb)	S. No.	AccessionNo.	Aflatoxin content (ppb)
19	01048	0.00	82	05017	5.11	145	09002	0.00
20	01049	0.00	83	05018	3.02	146	09005	3.78
21	01051	0.00	84	05019	4.71	147	10001	0.91
22	01052	0.84	85	05020	0.00	148	10002	1.66
23	01053	1.41	86	05021	3.93	149	10003	58.21
24	01055	5.98	87	05022	3.02	150	10004	0.00
25	01056	3.02	88	05023	16.04	151	10005	0.44
26	01057	26.73	89	05024	168.05	152	10006	0.00
27	01058	0.00	90	05025	0.00	153	10007	0.00
28	01059	0.62	91	05026	1.81	154	10008	0.00
29	01060	0.00	92	05027	17.22	155	10009	4.67
30	01061	7.13	93	05028	0.42	156	01003	1.05
31	01062	0.00	94	05029	0.00	157	01007	3.68
32	01063	0.00	95	05030	2.13	158	01028	9.25
33	01064	0.60	96	05031	35.61	159	01037	0.00
34	01065	0.00	97	05032	6.48	160	01044	1.67
35	01066	7.82	98	05033	0.00	161	01054	6.25
36	01067	0.00	99	05034	31.12	162	02019	0.00
37	01068	4.50	100	05035	1.10	163	02025	0.00
38	01069	0.67	101	05036	0.00	164	02028	0.61
39	01070	25.14	102	06001	214.25	165	02041	2.62
40	01071	1.27	103	06002	254.14	166	03003	8.99
41	01073	1.08	104	06003	715.23	167	03015	0.00
42	01074	3.60	105	06004	657.14	168	03019	0.00
43	01075	5.36	106	06006	0.71	169	03020	0.00
44	01076	4.23	107	06007	0.39	170	03025	0.00
45	01077	0.00	108	06008	128.29	171	03034	0.00
46	02002	0.00	109	06009	347.18	172	06005	0.25
47	02004	25.24	110	06011	239.47	173	06010	267.84
48	02007	50.24	111	06012	314.80	174	06014	132.35
49	02008	0.00	112	06013	0.05	175	06017	257.24
50	02009	5.39	113	06015	0.54	176	01042	6.14
51	02011	0.35	114	06016	0.00	177	02026	0.00
52	02013	7.29	115	06018	213.58	178	02027	0.00
53	02014	0.00	116	06019	0.18	179	02033	0.00
54	02017	0.00	117	06020	247.36	180	02036	0.00
55	02021	81.68	118	06021	14.28	181	02042	1.72
56	02024	0.29	119	07001	8.17	182	02043	4.72
57	02029	0.00	120	07002	0.00	183	03033	3.05
58	02031	0.00	121	07004	4.60	184	03035	0.00
59	02034	9.43	122	08001	2.50	185	03036	0.00
60	02035	37.13	123	08002	0.00	186	07003	1.75
61	02037	3.02	124	08003	0.00	187	09004	118.15
62	02038	6.01	125	08004	0.00			
63	02039	0.00	126	08005	0.00			

*Where Accession numbers were NRCG Accession numbers. S.No. 1-155; 156-175 and 176-187 are group 'A', 'B' and 'G' isolates respectively. The district from which the isolates were collected is as follows Junagadh (S.No. 1-30, 34-41, 43-45, 147-153, 156-161, 176); Porbandar (31-33, 42, 154-155); Amreli (46-64, 162-165, 177-182); Bhuj (65-75, 166-171, 183-185); Anand (76-77); Bhavnagar (78-101); Sabar Kantha (102-118, 172-175); Jamnagar (119-121); Surendranagar (112-143, 186) and Rajkot (114-146, 187).

lected spots, at 0-10 cm of depth, from between the plants and individual samples were pooled for each plot. The interval between soil samples was 100-300 m at any single location, the pair-wise distance between populations was about 5-30 km, whereas, the pair-wise distance between the districts was approximately 50-430 km. The fungal isolation was done by the dilution plate method as previously described by Horn and Dorner (1998) and the cultures were purified using the single spore isolation technique and maintained as single spore cultures on agar slants. All the isolates were cultured on *Aspergillus flavus/parasiticus* agar (AFPA; Sigma-Aldrich), which is a selective identification medium for the detection of *A. flavus* group strains, (Pitt *et al.*, 1983) for 3 to 5 days at 25 °C in dark, to confirm identification at the section level by reverse colony colour. Further, the morphological and growth characteristics of all the isolates were analysed on solid medium, the Czapek's Dox agar (CZ), and identification of the species was done on the basis of the colour of the colonies, *i.e.* yellow-green for *A. flavus* and dark green or nearly Ivy green for *A. parasiticus*.

Indirect competitive-enzyme linked immunosorbent assay (ELISA)

Indirect competitive ELISA was performed for the quantitative screening of the collected isolates. *Aspergillus flavus* strains were grown at 30 °C for 7 days on PDA plates (three replicates per isolate) as described by Waliyar *et al.* (2009). Aflatoxin B_1-bovine serum albumin (AFB_1-BSA) conjugate was prepared in carbonate coating buffer (100 ng mL^{-1}) and 150 µL was added to each well. The plates were then incubated at 37 °C for 1 h, after which the toxin was collected and stored. The wells were washed with PBST (*Phosphate Buffered Saline* supplemented with Tween 20) followed by incubation with BSA solution (0.2% BSA prepared in PBST) (200 µL per well) for 1 h at 37 °C. Antiserum diluted in BSA solution was added to the wells and incubated for 45 min at 37 °C. After appropriate blocking, the wells were washed with PBST.

Extract of healthy seed of peanut variety J-11 was taken as the negative control and for the positive control, the AFB_1 standard was diluted (1:10) with peanut extract at concentrations ranging from 100 ng to 10 pg (100 µL per well). Then, 50 µL of the anti-serum (Sigma-Aldrich) was added to each dilution of aflatoxin standard (100 µL) and the peanut seed extract (100 µL). The plates having aflatoxin samples and antiserum were incubated at 37 °C for 1 h and subsequently washed with PBST.

Alkaline phosphatase (ALP) labelled goat anti-rabbit IgG (1:1000 dilution; volume 150 µL) was then added to each well and incubated at 37 °C for 1 h. The ELISA wells were washed with PBST and 150 µL of the substrate solution (p-nitrophenyl phosphate prepared in 10% diethanolamine buffer, pH 9.8) was added and incubated for 1 h at room temperature. The absorbance was measured at 405 nm in an automatic ELISA reader. A standard curve for AFB_1 was prepared for estimation of aflatoxin content in the test samples. The detection limit for aflatoxin was 0.05 ppb.

Fungal DNA isolation

The isolates were cultured on potato dextrose agar (PDA) slants for isolation of genomic DNA. Conidia were harvested from 7-days old slant cultures, grown at 28 °C and inoculated into 50 mL of Yeast extract-Peptone-Dextrose broth followed by incubation at 25 °C for 48-72 h with shaking at150 rpm. After appropriate growth, the mycelial suspension was filtered through a Buchner funnel with sterile Whatman No. 1 filter paper. Mycelium was rinsed twice with sterile distilled water, transferred into a 50 mL centrifuge tube and froze at -80 °C.

Upon treatment with liquid nitrogen, the frozen mycelial mats were ground to fine powder using a mortar and pestle. Approximately, 20 mg of the homogenised mycelial powder was suspended in 600 µL of lysis buffer (100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 20 mM EDTA and 2% SDS) and incubated for 10 min at 60 °C in a water bath. Subsequently, DNA was extracted from the samples by incubation with equal volumes of phenol/chloroform (1:1), followed by chloroform/isoamyl alcohol (24:1) treatment. DNA from the samples was precipitated with 0.7 volume of chilled ethanol and vacuum dried. Finally, the DNA pellets were re-suspended in TE Buffer (pH 8.0) and subjected to treatment with RNase A at 37 °C for 1.5 h, to remove RNA contamination. DNA concentration and purity was determined by measurement of absorbance at 260 nm and 280 nm using Nano Drop, while the integrity of DNA was examined by agarose gel (0.8%) electrophoresis.

AFLP reactions

AFLP analysis system for microorganisms (Invitrogen-Corporation, Carlsbad, CA) was used as previously described by Lee *et al.* (2004). Approximately, 500 ng of genomic DNA, from each isolate, was subjected to restriction digestion with *Eco*RI and *Mse*I restriction enzymes (Invitrogen-Corporation, Carlsbad, CA), and the restricted fragments were ligated to the double-stranded restriction site-specific ligation adaptors supplied with the kit. A preselective PCR (94 °C for 30 s, 20 cycles of 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s; and 72 °C for 5 min; final hold at 4 °C) was carried out in a 25 µL (final volume) mixture. For the selective PCR, 5 µL of the 1:5 dilution of the first PCR product was amplified in a 25 µL (final volume) mixture using the selective primers.

Initially, 30 different primer combinations of *Eco*RI/*Mse*I were used, of which five different primer combinations, *viz*. *Eco*RI-AA/*Mse* I-A, *Eco*RI-AC/*Mse* I-G, *Eco*RI-AC/*Mse* I-A, *Eco*RI-AC/*Mse* I-T and *Eco*RI-C/*Mse* I-CAG showed more polymorphism than the others and

were used for the selective amplification. The PCR program, for selective AFLP amplification, included one cycle of 94 °C for 60 s and one cycle of 94 °C for 60 s, 65 °C for 60 s, and 72 °C for 90 s; this cycle was followed by nine cycles in which the annealing temperature ranged from 64 °C to 56 °C, and decreased by 1 °C for each cycle. Following that, 23 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s were performed, with the final extension at 72 °C for 5 min., and indefinite hold at 4 °C in a thermal cycler (Eppendorf). Thereafter, 5 µL of the reaction product was mixed with 3 µL of 6x loading dye (Fermentas). The amplification for each primer-combination was performed twice, independently, following the same procedure, in order to ensure the fidelity of the AFLP markers.

AFLP fragments were resolved in denaturing 6% polyacrylamide gel with 1X Tris-borate EDTA buffer (pH 8.0) in both the gels. The gels were run at 25 W and stained by silver staining with slight modifications, as described by Benbouza *et al.* (2006), and scanned using the UMAX Mirage II gel scanner (Type H5K0). After digitisation of the gel pictures, the DNA bands were scored and analysed using the software Gel Compare II (Applied Maths, Kortrijk, Belgium).

Genetic distance and cluster analysis of AFLP data

Similarity matrix, using the AFLP polymorphism of *A. flavus* isolates, was measured by Jaccard similarity coefficient, which was subjected to cluster analysis by Neighbour Joining method. FreeTree software (Pavlícek *et al.*, 1999) was employed for construction of dendrogram, on the basis of distance data and for bootstrap analysis of the robustness of the trees. The colour separation in the dendrogram was done using the Interactive Tree of Life (Itol) software (Letunic and Bork, 2007). The allelic information was generated using the softwares, GenAIEx 6.501 and Gel Compare II. The AFLP data were subjected to a hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992), using the three hierarchical levels, *i.e.* individual, population and their regions. The GenAIEx software was used to calculate the principal co-ordinates analysis (PCoA) that plots the relationship between the distance matrix elements based on their first two principal co-ordinates (Peakall and Smouse, 2001).

Results and Discussion

Identification and toxigenicity of the isolates

In the present investigation, *Aspergillus flavus* populations were examined for their afla-toxigenicity. Amongst the isolates, belonging to *Aspergillus* section *Flavi*, 71% were characterised as *A. flavus* (n = 184) and the remaining 29% as *A. parasiticus* (n = 75). Our results are in concordance with the previous findings from studies on *A. flavus* population from the peanut cropping system in India (Patil 1985, Reddy 2007). All the *A. flavus* isolates were selected as population and used for the AFLP analysis.

In this study, about 68.5% of the isolates were found to be atoxigenic, as tested by indirect competitive ELISA (Table 1), which is in conformity with the results of Chourasia and Sinha (1994). Bio-control by competitive exclusion has been regarded as the most promising means of controlling aflatoxin contamination of peanuts. It was observed that when competitive atoxigenic strains were applied to the soil, they produced large numbers of conidia than the toxigenic isolates (Alaniz Zanon *et al.*, 2013). Since, both occupy the same niches as the naturally occurring toxigenic populations, and aflatoxin contamination is subsequently reduced in the crops (Dorner, 2004). Such atoxigenic strains may be used for successful management of toxigenic *Aspergilli* in soil (Dorner and Lamb, 2006).

Dendrogram analysis

In the present study, five selected AFLP primer-pair combinations produced a complex, but well-resolved fingerprint pattern (Figure 1). This analysis provided novel data on the molecular composition of *A. flavus* populations present in the peanut growing fields of Gujarat (India).

A composite dendrogram was generated based on all the five AFLP primer combinations using GenAIEx and Gel Compare II softwares, where all 187 isolates could be divided into 15 different clusters (I-XV; Figure 2). Moreover, the results of PCoA analysis were also comparable to the cluster analysis. Based on the morphological characterisation, the isolates were grouped into three distinct groups, *i.e.* group A, B and G. The dendrogram showed clear partitioning of ‘A’, ‘B’ and ‘G’ groups of the isolates into 14 (I-VIII and XII-XV), 02 (IX and X) and 01 (XI) clusters, respectively (Figure 2), which indicated that there were more scorable polymorphisms within the group ‘A’ isolates than either the ‘B’ or the ‘G’ groups of isolates. Our results are in agreement with Barros *et al.* (2007), who analysed the *Aspergillus* isolates by AFLP. Similar results have also been obtained through the other molecular methods (Tran Dinh, 1999; Wang *et al.*, 2001).

From the cluster pattern based on the composite AFLP analysis, it has been inferred that most of the isolates of the same district could be clustered together. A few isolates of the other districts, like, Junagadh were also found to be clustered with the isolates of the other districts. This might be due to the dissemination of the contaminated seed material, being cultivated in one district, to another or it could be because of a similar genetic make-up of those isolates. Such result agrees with a number of previous findings in *Flavi* section of *Aspergillus* (Montiel *et al.*, 2003; Barros *et al.*, 2007; Baird *et al.*, 2006). Thus, by using these five AFLP primer combinations, the *A. flavus* isolates could be grouped according to their morphological groups (*e.g.* Group ‘A’, ‘B’ and ‘G’) and, to some extent, to their geographical location too.

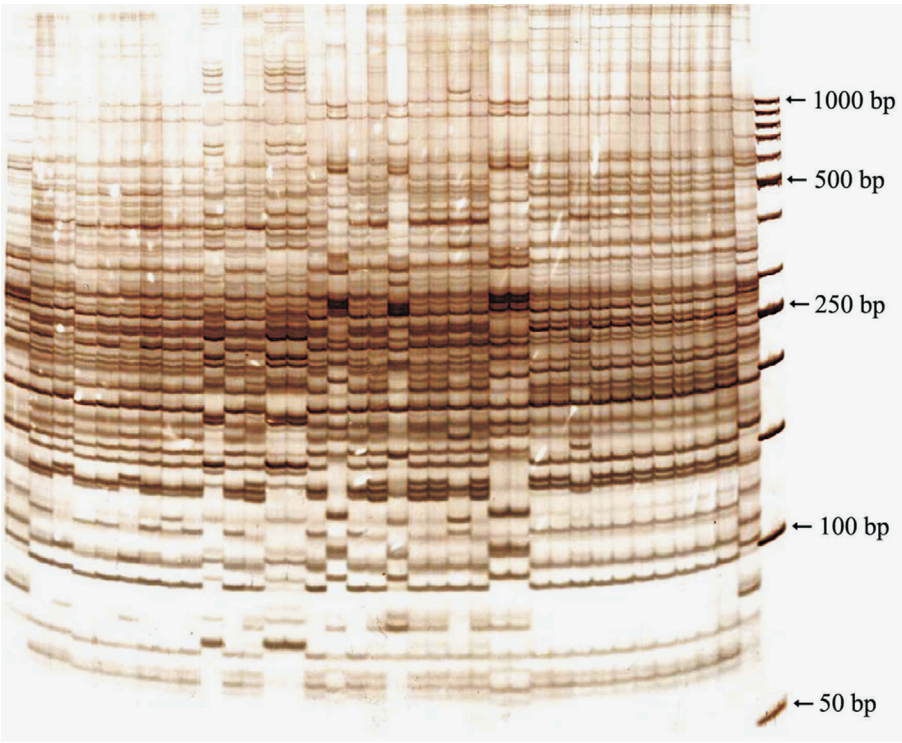

Figure 1 - A representative AFLP profile of *A. flavus* isolates (primer combination E-C/M-CAG).

Details of genetic diversity of different *A. flavus* populations

While analysing the genetic diversity details of each population of *A. flavus,* it was observed that the maximum number of bands were amplified, and percentage polymorphic bands were recorded for Junagadh district (360 and 99.17%, respectively), followed by Amreli district (325 and 89.26%, respectively). Likewise, the number of different alleles and the number of effective alleles were also the highest for the isolates that were collected from the Junagadh district. This clearly indicated that the maximum diversity was recorded for Junagadh and minimum for Anand district (Table 2). Certain differences could be attributed to the size of fungal population that was studied from any location, *i.e.* the larger is the population, more

Table 2 - Genetic diversity details about each population of *A. flavus* collected from different parts of groundnut growing fields of Gujarat.

Population (District)	No of isolates				Total No of bands	Polymorphic loci (%)	Na	Ne
	Group of isolates			Total				
	'A'	'B'	'G'					
Junagadh	45	6	1	52	360	99.17	1.983 ± 0.01	1.541 ± 0.015
Amreli	19	4	6	29	325	89.26	1.788 ± 0.032	1.503 ± 0.018
Bhavnagar	24	0	0	24	311	85.40	1.711 ± 0.037	1.505 ± 0.019
Surendranagar	22	0	0	22	320	87.88	1.760 ± 0.034	1.489 ± 0.017
Sabar Kantha	17	4	0	21	305	83.47	1.675 ± 0.039	1.473 ± 0.019
Bhuj	11	6	3	20	283	73.28	1.512 ± 0.044	1.431 ± 0.020
Porbander	9	0	0	9	232	58.95	1.229 ± 0.05	1.338 ± 0.018
Rajkot	3	0	1	4	201	28.10	0.835 ± 0.044	1.188 ± 0.018
Jamnagar	3	0	1	4	208	31.40	0.887 ± 0.045	1.221 ± 0.019
Anand	2	0	0	2	190	16.80	0.691 ± 0.039	1.119 ± 0.014
Total/ Mean	155	20	12	187	2735	65.37	1.407 ± 0.014	1.381 ± 0.006

Where: Mean ± SE; Na = No. of different alleles; Ne = No. of effective alleles.

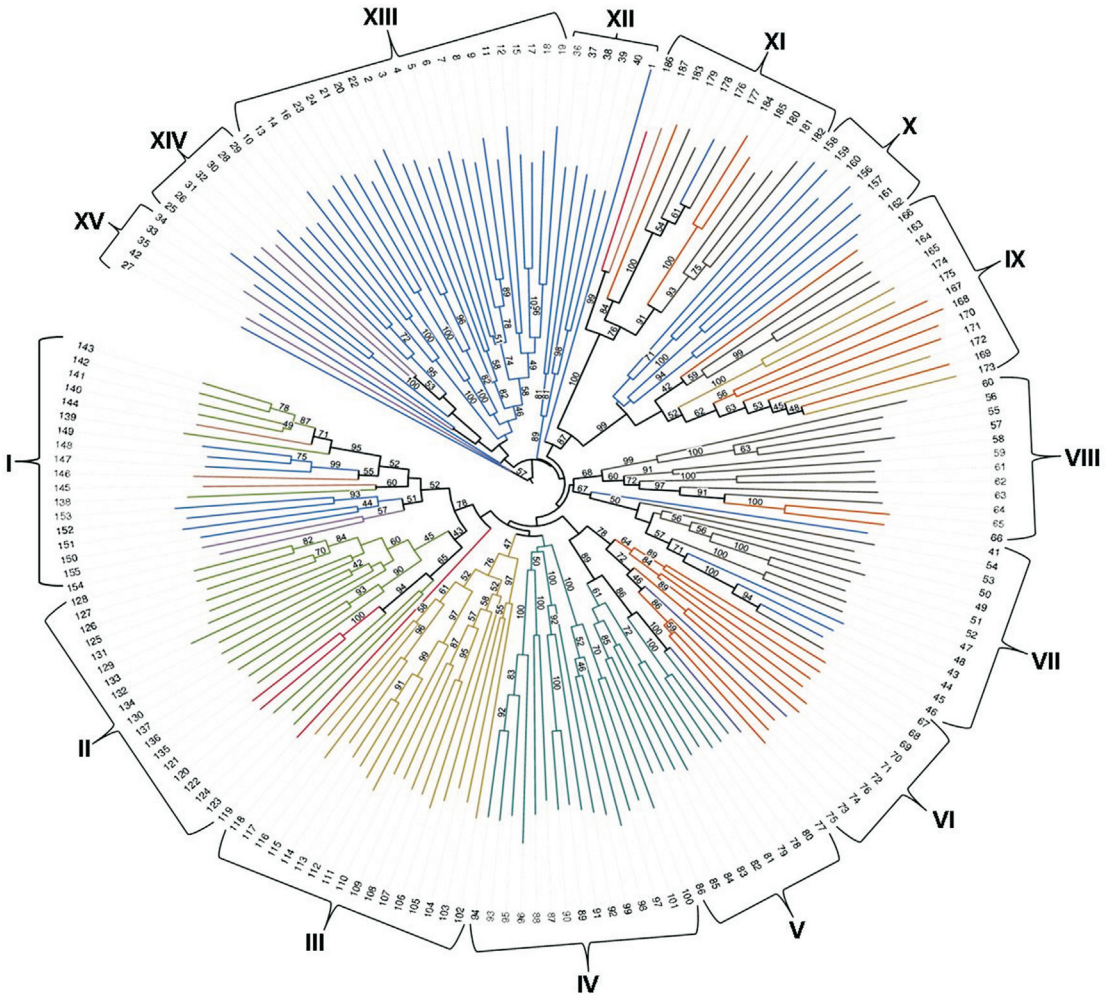

Figure 2 - Clustering pattern of 187 *A. flavus* isolates based on AFLP analysis. Where, group 'A', 'B' and 'G' isolates were clustered in 12 (I-VIII and XII-XV); 02 (IX-X) and 01 (XI) clusters respectively.

pronounced are its genetic diversity details. Genetic diversity details of all the populations studied have been presented in the Table 2.

AMOVA revealed that the variance within the population was much more (84%) than that between the populations (16%). A higher genetic diversity within the *A. flavus* populations indicates that there could be many discreet populations that possess unique genotypes at any location. However, on the population basis, at any two locations, variations are not so high. Thus, high level of genetic diversity observed within the populations of *A. flavus* could be attributed to the evolutionary factors, such as gene-flow, random genetic drift and the anthropogenic activities, such as a specific peanut cropping pattern followed in Gujarat, which needs further investigation. The results of various previous studies on *A. flavus* (Montiel *et al.*, 2003; Barros *et al.*, 2007; Baird *et al.*, 2006) are in tune with our investigation findings.

Analysis of the principal co-ordinates of *A. flavus* populations, which were collected from the fields of 10 peanut growing districts of Gujarat, revealed that the isolates collected from the districts of Junagadh, Sabar Kantha, Surendranagar, Bhavnagar, Porbander and Amreli are closer to each other. However, the isolates collected from Jamnagar, Rajkot, Anand and Bhuj district are quite diverse from each other (Figure 3).

Identification of different groups of isolates

The AFLP primer combinations used in the present study showed certain specific fragments, which may be specifically used for the identification of isolates from each other. For certain isolates, 14 unique bands were identified (Table 3). The 200 bp fragment, amplified by E-AA/M-A primer combination, was absent in each of the 'A' group isolates, whereas, it was present in all of the 'B' and the 'G' group isolates. A fragment of 400 bp, amplified by E-AC/M-A primer combination, was present in all the 'G' group isolates, but absent in all the 'B' and the 'A' group isolates. The same primer combination could amplify a fragment of 525 bp that was present only in the 'B' group

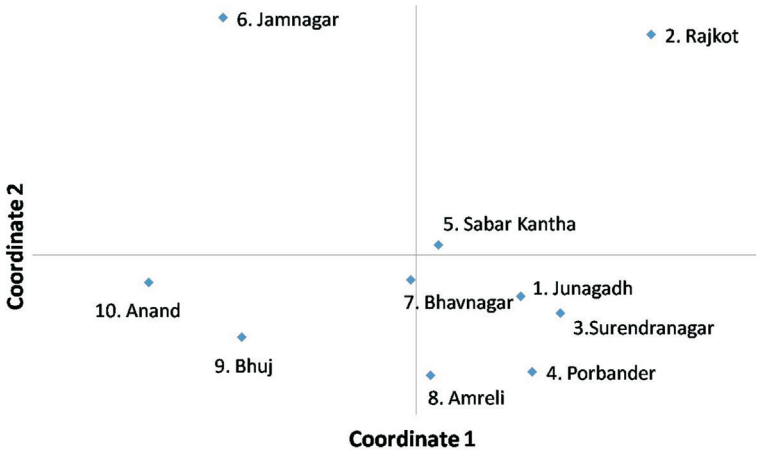

Figure 3 - Principal coordinates analysis of *A. flavus* populations, collected from the fields of ten peanut growing districts of Gujarat state.

Table 3 - Amplicons that can be used for SCAR development against specific isolates and/or group of isolates.

Amplicons (bp) which may be used for SCAR development	Primer combination used	Isolates S.No./ Group of Isolates that can be identified
265	E-AA/M-A	16, 23, 24, 31, 42, 61, 97, 98, 99, 100 and 101
125	E-AA/M-A	16, 23, 24, 31, 42, 61, 97, 98, 99, 100 and 101, 115, 116, 117, 118, 151 and 152
200	E-AA/M-A	Group 'B' and Group 'G'
375	E-AC/M-A	10, 13, 14, 42, 55, 89, 90, 91 and 92
400	E-AC/M-A	Group 'G'
525	E-AC/M-A	Group 'B'
390	E-AC/M-G	10, 13, 14, 89, 90, 91 and 92
375	E-AC/M-G	Group 'B'
225	E-AC/M-T	Group 'B'
210	E-C/M-CAG	10, 13, 14, 93, 94, 95, 96 and 102
185	E-C/M-CAG	10, 13, 14, 34, 89, 90, 91 and 92
180	E-C/M-CAG	10, 13, 14, 34, 89, 90, 91 and 92
250	E-C/M-CAG	Group 'B'
190	E-C/M-CAG	Group 'B'

isolates and absent in all the 'A' and the 'G' group isolates. Some unique fragments, which were either present or absent in isolates, may also serve as specific markers for identification of respective isolates or group of isolates.

Some specific isolates, *i.e.* NRCG 02037, 02021 and 05024, may be identified by using the specific primer combinations. Apart from the individual isolates, a set of isolates and a group of isolates have also produced specific fingerprint and can be identified by using the primer combinations used in our study. Group 'B', 'G' and 'A' isolates produced different fingerprints and thus, could be differentiated from each other. Since, the PCR diagnostics could be of great value in ecological and epidemiological studies, where vast numbers of isolates have to be screened in a short duration (Schmidt *et al.*, 2004), therefore these diagnostic/specific fragments could be useful in establishment of a PCR-based diagnostic assay (Barros *et al.*, 2007), by development of sequence characterised amplified regions (SCARs). In the current investigation, certain specific amplicons were identified which may be used for the development of SCARs for identification of specific isolates (Table 3).

Association of AFLP markers with toxigenicity

The molecular mechanisms leading to the loss of aflatoxin production in atoxigenic *A. flavus* have been investigated intensively by various researchers across the world (Jiang *et al.*, 2009; Criseo *et al.*, 2008). In the present investigation, AFLP analysis was found to be ineffective for differentiation of isolate types on the basis of aflatoxigenicity, as both toxigenic and atoxigenic forms were inter-mixed within the groups with no clear demarcation. Our result is in concurrence with the previous studies on *Aspergillus* section *Flavi*, in view of RAPDs (Tran Dinh, 1999), quadru-

plex PCR (Wang *et al.*, 2001), AFLP (Montiel *et al.*, 2003), and DNA amplification fingerprinting (Baird *et al.*, 2006). Moreover, the earlier studies have also shown that with AFLP, no genotypic difference could be established between the toxin producers and the non-producers (Barros *et al.*, 2007; Schmidt *et al.*, 2004; Perrone *et al.*, 2006).

As toxin production is a very complex trait and is unlikely to be acquired independently, Tran *et al.* (1999) suggested that in the absence of sexual recombination, non-toxigenicity has been lost multiple times by different isolates. Geiser *et al.* (1998) proved the recombination in *A. flavus*, which means, non-toxigenicity may have passed laterally between isolates of different genetic backgrounds.

It has been reported that the analysis of deletions within the aflatoxin biosynthetic gene cluster, could be a more effective marker for differentiation of toxigenic and atoxigenic isolates (Jiang *et al.*, 2009; Chang *et al.*, 2005). However, the loss of aflatoxin production may be not result only due to deletions in the gene cluster (Wang *et al.*, 2001; Criseo *et al.*, 2008). Criseo *et al.* (2008) reported that 36.5% of atoxigenic strains have the complete aflatoxin gene cluster; however, the exact mechanism of loss of aflatoxin production is still unknown. In the atoxigenic strain AF36, a defect causing a premature stop codon in the coding sequence of the aflatoxin biosynthesis gene *pksA* was reported (Ehrlich and Cotty, 2004). This suggests that there is a need to further characterise the non-aflatoxigenic strains, which have been identified in the present investigation, to find the exact cause of their atoxigenicity.

Based on our findings, we may conclude that the AFLP technique can provide the required genetic information about the *A. flavus* isolates from the peanut cropping systems in India. Moreover, it can also be used as a powerful molecular tool to study the genetic diversity in *A. flavus.* The information generated, from this study, could be used for the prevention of aflatoxin contamination of peanut crop, at field level, by increasing the relative concentration of the atoxigenic strains, in one of the major peanut cultivation area in India, which will ultimately help in improving the crop's export. Further, the molecular characterisation of atoxigenic strains that were identified in the study would be useful to unveil the basis of their atoxigenicity.

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