

Genetic variability of aflatoxin B₁ producing *Aspergillus flavus* strains isolated from discolored rice grains

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Abstract Twenty-two aflatoxin B₁ (AFB₁) producing *Aspergillus flavus* strains were isolated from 1,200 discolored rice grain samples collected from 20 states across India and tested their potential to produce AFB₁ on different agar media. Further these isolates were characterized through randomly amplified polymorphic DNA method. All the strains of *A. flavus* were produced AFB₁ on yeast extract sucrose agar media and none of the strains on *A. flavus* and *A. parasiticus* agar. Among the 22 strains, two strains from Tamil Nadu (DRAf 009) and Maharashtra (DRAf 015) produced high amount of AFB₁ in all the media tested. To assess the genetic variability in *A. flavus*, the isolates were analyzed by using random amplified polymorphic DNA markers. Isolates showed 17–80% similarity with standard culture of *A. flavus* (MTCC 2799).

Keywords Rice · Genetic variability · AFB₁ · TLC · RAPD · PCR

Introduction

Rice (*Oryza sativa* L.) is the most important staple food crop in India and bulk of rice is grown in kharif or wet season. During the wet season, sun drying practiced by most of the farmers may not adequately reduce the moisture content in grains. Thus, grain with moisture content higher than the desired level enters the storage system. As a result,

invasion of both field and storage fungi take place. The harmful effects of such fungal invasion are grain discoloration, loss in viability, quality and toxin contamination. Infection of *Aspergillus* spp. was found on most paddy samples collected from different rice growing areas in India (Reddy et al. 2004). In these rice samples, AFB₁ producing fungi were isolated (Reddy et al. 2005). However, mycotoxin producing fungi is less commonly reported for rice than for many other cereal crops (Tanaka et al. 2007) but rice represents a very good substrate for fungal growth and toxinogenesis since it is used as an ideal culture medium to test the toxigenic potential of isolated strains (Bars and Bars 1992). The aflatoxigenic species *A. flavus* Link and *A. parasiticus* Speare can produce the potent carcinogen aflatoxin and can pose a significant human health threat (Reddy and Raghavender 2007). The nonaflatoxigenic species *A. oryzae* (Ahlburg) Cohn and *A. sojae* Sakaguchi & Yamada are widely used for the production of food-grade amylase and the fermentation of sake, miso and soy sauce. All four species belong to the *Aspergillus* section Flavi and have many phenotypic similarities (Lee et al. 2004). Current methods for the identification of these species still depend primarily on cultural and morphological characteristics. However, it is often difficult to differentiate these species because the phenotypic differences are not distinct and are easily affected by the environment and are also confused by the high degree of intra- and interspecies variation. Because of the economic value of members of the *Aspergillus* section Flavi and the importance of differentiating them, several molecular methods have been developed to distinguish these strains. DNA relatedness of these species in *Aspergillus* section Flavi was studied by Kurtzman et al. (1986) with the DNA reassociation method. The randomly amplified polymorphic DNA (RAPD) method has been used to differentiate *A. parasiticus* from *A. sojae* with

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three screened decamer primers (Yuan et al. 1995). In this paper we described that the potential of AFB1 production by the strains *A. flavus* on different agar media and their genetic variability by RAPD-PCR.

Materials and methods

Collection of seed samples from different rice growing areas in India

A total of 1,200 rice samples were collected from 43 locations covering 381 varieties in 20 rice growing states across India (Table 1). The quantity of the seed varied from 1 to 2 kg. The seeds collected were either from areas exposed to different weather conditions or stored at various storage conditions, viz., seeds from the standing crop or from the crop exposed to heavy rain or seeds exposed to rain and floods or seeds from the submerged or damp conditions or seeds stored in the godown for 1–4 years or seeds from the rice mills or from the grain market. To avoid the sampling error due to highly heterogeneous nature of fungal distribution, each 2 kg composite sample collected from one storehouse was a mixture of ten sub-samples (200 g each). The ten such sub-samples were collected from five diagonal on each of the upper, middle and lower layers of each storehouse (Liu et al. 2006).

Isolation of *A. flavus* from rice grains

Using agar plate method (ISTA 1966), *A. flavus* was isolated from all the seed samples. From each sample, 400 seeds were plated on one-half strength potato dextrose agar (PDA) medium containing Rose Bengal at 50 ppm (Cotty 1994). The plates were incubated at room temperature and the presence of *A. flavus* was observed after 6 days. The *A. flavus* isolated from samples were further purified individually by sub culturing PDA slants. They were then identified according to Raper and Fennell (1965) and Klich (2002).

Isolation and extraction of AFB1 from *A. flavus*

Fifty-two strains of *A. flavus* isolated from rice grain samples were grown on sterilized different agar media (*A. flavus* and *A. parasiticus* agar, Czapeks agar, PDA, yeast extract sucrose (YES) agar, Sodium chloride glucose dichloran agar and Sucrose peptone dichloran agar) for 5 days at $25 \pm 2^\circ\text{C}$. Three replications were maintained for each isolate for each media. Aflatoxins were extracted by grinding the moldy agar (20 g) in Waring blender for 5 min with chloroform (100 ml) (Lee 1965). The mixture was centrifuged for 15 min at 3,000 rpm and was filtered

Table 1 States and locations from where the seed was collected and the number of varieties covered

S. No.	State	Locations	No. of samples	Varieties
1	Andhra Pradesh	6	257	87
2	Andaman & Nicobar	1	34	34
3	Assam	3	40	29
4	Bihar	2	24	14
5	Chhattisgarh	1	11	7
6	Delhi	1	122	–
7	Gujarat	2	12	11
8	Jarkhand	2	21	18
9	Jammu & Kashmir	2	27	6
10	Karnataka	4	92	27
11	Kerala	2	294	5
12	Madhya Pradesh	1	19	14
13	Maharashtra	1	7	7
14	Meghalaya	1	9	3
15	Puducherry	2	47	16
16	Rajasthan	1	10	8
17	Tamil Nadu	4	85	14
18	Tripura	2	11	5
19	Uttaranchal	1	28	26
20	West Bengal	4	50	50
Total		43	1,200	381

through cheesecloth into a separator funnel. The chloroform layer was treated with anhydrous sodium sulphate (25 g) to remove water. Sodium sulphate was collected on a Buchner funnel, and the clarified filtrate was concentrated in vacuum on a rotary evaporator to 1 ml and identified on thin layer silica gel chromatography (TLC). Then the dried residue was dissolved minimum quantity of methanol and estimated by indirect competitive ELISA.

Identification of AFB1 on TLC

Aflatoxin production was evaluated by standard TLC to confirm the presence or absence of AFB1. Two replicates were analyzed by spotting crude extract of aflatoxins. The TLC plates used were coated with silica gel 60 F₂₅₄ on aluminum sheet, 20 cm × 20 cm and developed in chloroform/methanol (97:3) (Shotwell et al. 1966). The plates were then observed under UV light at 365 nm and compared with standard aflatoxin spotted on the same plate.

Materials for ELISA

Aflatoxin B₁, AFB1–bovine serum albumin (BSA) conjugate, goat anti-rabbit IgG–ALP conjugate, *p*-Nitrophenyl phosphate and BSA used were from Sigma, St Louis, USA, and microtiter plates (Maxi-sorp F96) were from Nunc

(Nalge Nunc International, Denmark). All other chemicals were of reagent grade or chemically pure. Highly specific polyclonal antibodies for AFB1 were purchased from International Crop Research Institute for the Semi Arid Tropics, Patancheru, India (Devi et al. 1999).

Estimation of AFB1 by ELISA

The AFB1 was estimated by the method of Devi et al. (2001). Microtiter plates were coated with 100 ng/ml of AFB1-BSA in sodium carbonate buffer, pH 9.6 (150 µl/well) and left overnight at 4°C. They were then washed in PBST, added with BSA (0.2%) and allowed to stand at 37°C for 1 h. ELISA plates were again washed with PBST and added with 100 µl AFB1 standards ranging from 25 ng to 10 pg/ml. Preincubation was carried out with 50 µl antiserum diluted in PBST-BSA (1:6000) and held for 45 min at 37°C. Filtrate samples extracted from rice grains with aqueous methanol–KCl as described earlier were added to wells at 1:10 dilution in PBST-BSA, i.e. 100 µl/well. Goat antirabbit immunoglobulins conjugate to alkaline phosphatase were used at a 1:4000 dilution to detect rabbit antibodies attached to AFB1-BSA. *p*-Nitrophenyl phosphate was used as a substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Bio-Rad-680) after incubation at 28°C for 45–60 min. Standard curves were obtained by plotting log₁₀ values of AFB1 dilutions at A₄₀₅. The AFB1 (ng/ml) in samples was determined from the standard curves as: AFB1 µg/kg of agar or milled rice = [aflatoxin (ng/ml) in sample × buffer (ml) × extraction solvent (ml)]/sample weight (g). In order to test the recovery of AFB1, 20 g healthy rice grain was mixed with pure AFB1 (Sigma, St Louis, USA) to give concentrations ranging from 5 to 100 µg/kg. Rice samples were extracted and assayed as for unknown samples.

Culture methods for DNA extraction

Aspergillus flavus cultures for genomic DNA isolation were grown as follows. Conidia were harvested from 7-day-old PDA slant cultures growing at 28°C and inoculated into 100 ml of potato dextrose broth in 250 ml conical flask and then incubated at 25°C for 48 h without shaking (Lee et al. 2004). Filtered with Whatman filter paper and dried mycelial mat for DNA extraction.

DNA extraction

The mycelial mats were ground with a mortar and pestle to fine powder in liquid nitrogen. Approximately 20 mg of homogenized cell powder was mixed with 800 µl of lysis buffer (100 mM, Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM

EDTA and 2% SDS) and incubated at 60°C for 30 min. The DNA was extracted sequentially with an equal volume of phenol/chloroform (1:1), and chloroform/isoamyl alcohol (24:1). Total nucleic acids were precipitated with 0.7 vols of isopropanol and vacuum dried. Finally the pellets were re-dissolved in 1 × TE buffer and treated with RNase at 37°C for 1.5 h to remove RNA, and the RNase was inactivated at 60°C for 1 h. Final DNA concentrations were determined by measuring with a spectrophotometer and the integrity of each DNA sample was examined with an agarose gel.

RAPD analysis

Four primers AP12 h, 5'-CGG CCC CTG T-3' (Williams et al. 1990), R108, 5'-GTA TTG CCC T-3', R151, 5'-GCT GTA GTG T-3' (Aufauvre et al. 1992), and A1, 5'-GAT AGA TAG ATA GAT A-3' (Rath 2001) obtained from Imperial Biomedic were used for PCR based amplification of the template DNA of the isolates. The amplification reactions were performed in a volume of 25 µl containing 2.5 µl of 10 × *Taq* DNA polymerase buffer, 2.5 U *Taq* polymerase (Bangalore Genei, India), 3.0 µl of 1.25 mM dNTPs each, 15 ng of a single primer and 50 ng of genomic DNA. The thermo-cycling profile consisted 1 cycle of initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 34°C for 1 min, extension at 72°C for 2 min, followed by a final extension at 72°C for 3 min on the thermocycler model PTC 100–60 (MJ Res. Inc., USA). To visualize the profile of thus amplified products, a 15-µl aliquot from each of the reaction products was electrophoresed on 1.8% agarose gel (w/v) in 0.5 × TBE, stained in ethidium bromide (10 mg/ml) and photographed using a UV transilluminator with the Polaroid camera attachment. One kilo-base DNA ladder (Stratagene, USA) was used as the molecular size marker.

Results and discussion

AFB1 production by *A. flavus* isolates

Out of 52 strains of *A. flavus* studied, 22 strains produced AFB1 on TLC compared with standard at *R_f* 0.47. Other strains of *A. flavus* did not produce any toxin on any media tested. The results on production of AFB1 by the strains of *A. flavus* by ELISA revealed that the capacity to produce in the range of 0.2–40 µg/g of agar. All the strains of *A. flavus* (22) were produced AFB1 on YES media and none of the strains on AFPA. Two isolates of *A. flavus* isolated from Tamil Nadu (DRAf 009) and Maharashtra (DRAf 015) produced maximum amount of AFB1, 40 µg/g at 25°C, on YES agar media (Table 2). Sixty-eight per cent of the isolates produced AFB1 on Czapeks and PDA agar media,

Table 2 AFB1 production ($\mu\text{g/g}$ of agar) by *A. flavus* isolates at $25 \pm 2^\circ\text{C}$

Strains	Place of seed collection	Czapeks	YES	PDA	SGDA	SPDA	AFPA
DRAf 001	Tamil Nadu	–	1.0	–	–	–	–
DRAf 002	Tamil Nadu	–	0.5	–	1.0	–	–
DRAf 003	Andhra Pradesh	0.5	1.0	0.5	–	–	–
DRAf 004	Tamil Nadu	1.0	1.0	2.0	2.0	0.5	–
DRAf 005	Kerala	0.5	2.0	1.0	–	–	–
DRAf 006	Tamil Nadu	1.0	1.0	0.5	1.0	–	–
DRAf 007	Andhra Pradesh	–	1.0	–	1.0	1.0	–
DRAf 008	Karnataka	1.0	0.5	1.0	–	0.5	–
DRAf 009	Tamil Nadu	4.0	40.0	4.0	4.0	4.0	–
DRAf 010	West Bengal	2.0	0.5	2.0	0.5	–	–
DRAf 011	Tamil Nadu	0.5	1.0	1.0	–	–	–
DRAf 012	Puducherry	–	1.0	0.5	–	–	–
DRAf 013	Jarkhand	1.0	0.5	0.5	0.5	–	–
DRAf 014	Rajasthan	1.0	0.5	–	–	–	–
DRAf 015	Maharashtra	4.0	40.0	4.0	4.0	4.0	–
DRAf 016	Andhra Pradesh	1.0	1.0	0.5	–	–	–
DRAf 017	Tripura	0.5	0.5	–	–	–	–
DRAf 018	Delhi	–	1.0	0.5	1.0	–	–
DRAf 019	Bihar	1.0	0.5	–	–	1.0	–
DRAf 020	Meghalaya	–	0.5	0.5	1.0	–	–
DRAf 021	Andhra Pradesh	–	0.2	0.5	–	–	–
DRAf 022	Chhattisgarh	0.5	0.2	–	–	0.2	–
CD ($P = 0.05$)		0.9	7.4	0.6	0.4	0.4	–
CV (%)		18.5	26.8	15.8	30.7	32.5	–

Czapeks Czapeks Dox agar, YES yeast extract sucrose agar, PDA potato dextrose agar, SGDA sodium chloride glucose dichloran agar, SPDA sucrose peptone dichloran agar, AFPA *Aspergillus flavus* and *A. parasiticus* agar

45% of the isolates on SGDA and 32% on SPDA agar media. *A. flavus* is known to produce AFB1 on rice grain substrate (Reddy et al. 2005). Fente et al. (2001) evaluated different agar (Czapeks, YES, coconut agar, aflatoxin producing ability (APA) media, coconut extract agar and coconut cream agar) media for aflatoxin production by *Aspergillus* spp. They reported more aflatoxin production in YES media compared to others. In our investigation we found that YES agar media is the best media for AFB1 production by *A. flavus*.

Manisha and Sandip (2003) isolated *A. flavus* strains from rice mill surrounding air and tested aflatoxin producing capability in Czapeks agar, APA media and CAM agar. They found that the high aflatoxin production in Czapeks media produced by *A. flavus* compared to other media. In this study 68% of the *A. flavus* strains produced AFB1 on Czapeks agar. Carballo and Miguel (1987) collected 133 samples (mixed feeds and cereal grains) were examined for the incidence of strains of the *A. flavus* group. They tested the ability to produce aflatoxin in all strains isolated on cracked rice, APA medium and glucose–yeast extract agar (GYA) medium. Ten of the 67 isolates were

aflatoxin-producing strains in rice and GYA medium; only three were aflatoxin-positive on the APA test. Of those isolated, 95% were identified as *A. flavus*. Reddy et al. (2005) had reported that high aflatoxin production on rice grain substrate than on agar media.

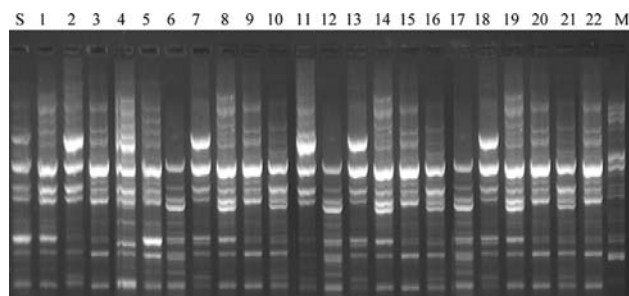
In this study, however, neither AFB2 nor AFG1 was detected and the bulk of aflatoxins produced by *A. flavus* isolates from discolored rice grains belonged to only AFB1 on different agar media. It is therefore, apparent that *A. flavus* in tropical rice mostly produce AFB1. Bulk production of AFB1 on YES agar media by high aflatoxin producing *A. flavus* strains will help to generate pure standards at an economical price and to produce antibodies for ELISA detection and estimation of AFB1 contamination in rice intended for domestic and export markets.

Recovery of AFB1 from rice samples

The effectiveness of the extraction procedure was confirmed by adding pure AFB1 to ground rice and extracted in 70% methanol–KCl. Recoveries from rice samples estimated by ELISA were greater than 95% (Table 3).

Table 3 Recovery of AFB1 from artificially contaminated rice sample as determined by ELISA

Concentration of AFB1 used for spiking ($\mu\text{g/kg}$)	Concentration of AFB1 estimated ($\mu\text{g/kg}$)	Percent recoveries of AFB1 in spiked samples
5	4.8 ± 0.39	96.0 ± 8.1
10	9.6 ± 0.82	96.0 ± 8.6
50	49.2 ± 0.88	98.4 ± 1.8
100	104 ± 3.92	103 ± 4.0

**Fig. 1** Randomly amplified polymorphic DNA patterns of *A. flavus* isolates with R108 primer

RAPD polymorphism

Of the four primers tried, one primer (AP12 h) failed to effect any amplification in the isolates tested, while two

other primers (R151 and A1) showed amplification. Primer R108 showed the highest amplification in all the isolates compared to others. A total of six alleles were produced with R108 primer. All the strains produced 1 to 12 bands (Fig. 1).

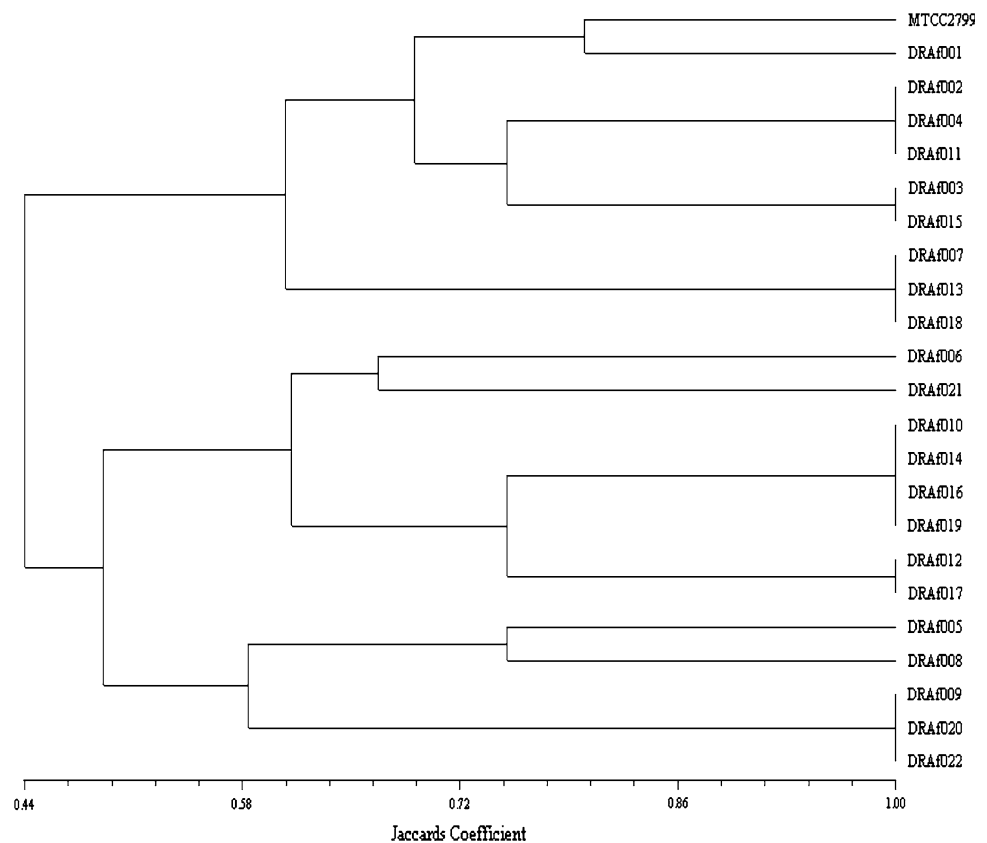
Genetic similarity among the *A. flavus* isolates

A cluster analysis was performed using similarity coefficient matrices. To generate a dendrogram of the 22 strains with UPGMA algorithm using NTSYS-PC version 2.0 (Rohlf 2000), the marker alleles were converted to binary scores based on their presence or absence as 1 and 0, respectively. A pair-wise similarity index (SI) was calculated (Table 4) and the dendrogram obtained is depicted in Fig. 2. The genotypes were grouped into two major clusters, i.e. Cluster I and Cluster II. The Jaccard's similarity coefficient based on RAPD markers loci ranged from 0.44 to 1.00. In pair-wise comparison, the maximum similarity was obtained between MTCC2799 (standard strain) and DRAf 001, 002, 004 and 011 with a SI of 0.800 (80%), whereas DRAf 006 showed least similarity with a SI of 0.167 (17%). All these strains isolated from the seed samples collected from Tamil Nadu, but the strains showed the variability in similarity with standard culture. The isolates (DRAf 002, 004 and 011) showed the 100% similarity between the isolates from Tamil Nadu. Cluster I

Table 4 Similarity index (SI) between the isolates of *A. flavus*

Strains	MTCC2799	DRAf001	DRAf002	DRAf003	DRAf004	DRAf005	DRAf006	DRAf007	DRAf008	DRAf009	DRAf010	DRAf011	DRAf012	DRAf013	DRAf014	DRAf015	DRAf016	DRAf017	DRAf018	DRAf019	DRAf020	DRAf021	DRAf022
MTCC2799	1.00																						
DRAf001	0.80	1.00																					
DRAf002	0.80	0.60	1.00																				
DRAf003	0.60	0.75	0.75	1.00																			
DRAf004	0.80	0.60	1.00	0.75	1.00																		
DRAf005	0.60	0.75	0.40	0.50	0.40	1.00																	
DRAf006	0.17	0.20	0.20	0.25	0.20	0.25	1.00																
DRAf007	0.60	0.40	0.75	0.50	0.75	0.20	0.25	1.00															
DRAf008	0.50	0.60	0.33	0.40	0.33	0.75	0.50	0.17	1.00														
DRAf009	0.40	0.50	0.50	0.67	0.50	0.67	0.33	0.25	0.50	1.00													
DRAf010	0.50	0.60	0.60	0.75	0.60	0.40	0.50	0.40	0.60	0.50	1.00												
DRAf011	0.80	0.60	1.00	0.75	1.00	0.40	0.20	0.75	0.33	0.50	0.60	1.00											
DRAf012	0.33	0.40	0.40	0.50	0.40	0.50	0.67	0.20	0.75	0.67	0.75	0.40	1.00										
DRAf013	0.60	0.40	0.75	0.50	0.75	0.20	0.25	1.00	0.17	0.25	0.40	0.75	0.20	1.00									
DRAf014	0.50	0.60	0.60	0.75	0.60	0.40	0.50	0.40	0.60	0.50	1.00	0.60	0.75	0.40	1.00								
DRAf015	0.60	0.75	0.75	1.00	0.75	0.50	0.25	0.50	0.40	0.67	0.75	0.75	0.50	0.50	0.75	1.00							
DRAf016	0.50	0.60	0.60	0.75	0.60	0.40	0.50	0.40	0.60	0.50	1.00	0.60	0.75	0.40	1.00	0.75	1.00						
DRAf017	0.33	0.40	0.40	0.50	0.40	0.50	0.67	0.20	0.75	0.67	0.75	0.40	1.00	0.20	0.75	0.50	0.75	1.00					
DRAf018	0.60	0.40	0.75	0.50	0.75	0.20	0.25	1.00	0.17	0.25	0.40	0.75	0.20	1.00	0.40	0.50	0.40	0.20	1.00				
DRAf019	0.50	0.60	0.60	0.75	0.60	0.40	0.50	0.40	0.60	0.50	1.00	0.60	0.75	0.40	1.00	0.75	1.00	0.75	0.40	1.00			
DRAf020	0.40	0.50	0.50	0.67	0.50	0.67	0.33	0.25	0.50	1.00	0.50	0.50	0.67	0.25	0.50	0.67	0.50	0.67	0.25	0.50	1.00		
DRAf021	0.33	0.40	0.40	0.50	0.40	0.20	0.67	0.50	0.40	0.25	0.75	0.40	0.50	0.50	0.75	0.50	0.50	0.50	0.75	0.25	1.00		
DRAf022	0.40	0.50	0.50	0.67	0.50	0.67	0.33	0.25	0.50	1.00	0.50	0.50	0.67	0.25	0.50	0.67	0.50	0.67	0.25	0.50	1.00	0.25	1.00

Fig. 2 UPGMA dendrogram based on the Jaccard's coefficient illustrating the genetic relationships among *A. flavus* isolates



consisting of DRA# 006, 021, 010, 014, 016, 019, 012, 017, 005, 008, 009, 020 and 022 had 44% similarity with rest of the cultures cluster II (Fig. 2). Teresa et al. (2000) isolated three strains of *A. flavus* and characterized by RAPD and suggested that this technique is a rapid and reliable tool to ascertain the epidemiology of infections caused by *A. flavus*. Moody and Tyler (1990) reported that the use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *A. flavus* group: *A. flavus*, *A. parasiticus* and *A. nigrinus*. Rath (2001) genotypically characterized the four *Aspergillus* spp. (*A. fumigatus* $n = 8$, *A. flavus* $n = 8$, *A. niger* $n = 4$, and *A. nidulans* $n = 5$) by four methods (PAGE, RAPD, determination of patterns in assimilation assay and Immunoblot). In the RAPD assay four out of eight primers gave interpretable patterns with 3–20 bands. None of the primers showed sufficient discriminatory power when used alone, However when combining the results of two of the primers all strains except two *A. fumigatus* strains could be clearly separated from each other. In our investigation the single primer (R108) alone showed the good amplification in all the isolates.

Rath et al. (2002) reported that RAPD patterns of whole-cell lysates from 21 *A. ustus* isolates, including 11 reference strains and 10 patient and environmental strains from one hospital, were investigated. The RAPD assay

discriminated between all reference strains. Comparison of hospital isolates showed identical RAPD patterns in some of the patient and environmental isolates. The data indicate that the RAPD technique is useful for fingerprinting of *A. ustus*. With RAPD, it was possible to differentiate all these strains for at least one primer. Therefore, it was concluded that the method was valuable for identification and fast differentiation of strains. In our study also RAPD helped to confirm the identity of isolates as *A. flavus*. None of them studied the genetic variability between the isolates of *A. flavus* isolated from discolored rice grains. This is the first comprehensive report on the collection of large number of rice grain samples from across the country, isolation of different strains of *A. flavus*, identification of suitable media for maximizing AFB1 production and studies on genetic variability of *A. flavus* isolates by RAPD.

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References

- Aufauvre BA, Cohen J, Holden DW (1992) Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991–2993

- Bars LJ, Bars LP (1992) Fungal contamination of aromatic herbs, aflatoxinogenesis and residues in infusions. *Microbiol Aliment Nutr* 10:267–271
- Carballo M, Miguel JA (1987) Rapid detection of aflatoxin-producing strains of the *Aspergillus flavus* group isolated from mixed feed and cereal grain in Spain. *J Sci Food Agric* 40(1):11–15. doi:10.1002/jsfa.2740400103
- Cotty PJ (1994) Comparison of four media for the isolation of *Aspergillus flavus* group fungi. *Mycopathologia* 125(3):57–62. doi:10.1007/BF01146521
- Devi KT, Mayo MA, Reddy KLN, Delfosse P, Reddy G, Reddy SV et al (1999) Production and characterization of monoclonal antibodies for aflatoxin B₁. *Lett Appl Microbiol* 29:284–288. doi:10.1046/j.1472-765X.1999.00685.x
- Devi KT, Mayo MA, Reddy G, Emmanuel KE, Larondelle Y, Reddy DVR (2001) Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India. *Food Addit Contam* 18(9):830–835. doi:10.1080/02652030110044921
- Fente CA, Ordaz J, Vazquez QI, Franco CM, Cepeda A (2001) New additive for culture media for rapid identification of aflatoxin producing *Aspergillus* strains. *Appl Environ Microbiol* 67(10):4858–4862. doi:10.1128/AEM.67.10.4858-4862.2001
- TA IS (1966) International rules for seed testing. *Proc Int Seed Test Assoc* 31:49–85
- Klich MA (2002) Identification of common *Aspergillus* species. *Centraal Bureau Voor Schimmel cultures, AD Utrecht*, p 116
- Kurtzman CP, Robnett MJ, Wicklow DT (1986) DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. *Mycologia* 78:955–959. doi:10.2307/3807436
- Lee WV (1965) Quantitative determination of aflatoxin in groundnut products. *Analyst (Lond)* 90:305–307. doi:10.1039/an9659000305
- Lee CZ, Liou GY, Yuan GF (2004) Comparison of *Aspergillus flavus* and *A. oryzae* by amplified fragment length polymorphism. *Bot Bull Acad Sin* 45:61–68
- Liu Z, Gao J, Yu J (2006) Aflatoxins in stored maize and rice grains in Liaoning province, China. *J Stored Prod Res* 42:468–479. doi:10.1016/j.jspr.2005.09.003
- Manisha RD, Sandip G (2003) Occupational exposure to airborne fungi among rice mill workers with special reference to aflatoxin producing *A. flavus* strains. *Ann Agric Environ Med* 10:159–162
- Moody SF, Tyler BM (1990) Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group. *Appl Environ Microbiol* 56:2441–2452
- Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins, Baltimore, p 686
- Rath PM (2001) Phenotypic and genotypic characterization of reference strains of the genus *Aspergillus*. *Mycoses* 44:65–72. doi:10.1046/j.1439-0507.2001.00620.x
- Rath PM, Petermeier K, Paul E, Ansorg R (2002) Differentiation of *Aspergillus ustus* strains by random amplification of polymorphic DNA. *J Clin Microbiol* 40(6):2231–2233. doi:10.1128/JCM.40.6.2231-2233.2002
- Reddy BN, Raghavender CR (2007) Outbreaks of aflatoxicoses in India. *Afr J Food Agric Nutr Dev* 7 (5): http://www.ajfand.net/Issue16/PDFs/Reddy_2750.pdf Accessed 4 May 2008
- Reddy CS, Reddy KRN, Raja Kumar N, Laha GS, Muralidharan K (2004) Exploration of aflatoxin contamination and its management in rice. *J Mycol Pl Pathol* 34(3):816–820
- Reddy KRN, Reddy CS, Muralidharan K (2005) Characterization of AFB1 produced by *A. flavus* isolated from discolored rice grains. *J Mycol Pl Pathol* 35(3):470–474
- Rohlf FJ (2000) NTSYS-PC: numerical taxonomy and multivariate analysis system, version 3.21. Exeter Publications, New York
- Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG (1966) Production of aflatoxin on rice. *Appl Microbiol* 14:425–428
- Tanaka K, Sago Y, Zheng Y, Nakagawa H, Kushiro M (2007) Mycotoxins in rice. *Int J Food Microbiol* 119:59–66. doi:10.1016/j.ijfoodmicro.2007.08.002
- Teresa MD, Mellado E, Manuel CE, Lourdes G, Navarro JIV, Rodriguez T (2000) Genetic similarity among one *A. flavus* strain isolated from a patient who underwent heart surgery and two environmental strains obtained from the operating room. *J Clin Microbiol* 36(6):2241–2248
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531–6535. doi:10.1093/nar/18.22.6531
- Yuan GF, Liu CS, Chen CC (1995) Differentiation of *Aspergillus parasiticus* from *Aspergillus sojae* by random amplification of polymorphic DNA. *Appl Environ Microbiol* 61:2384–2387