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RESEARCH ARTICLE

MOLECULAR BIOLOGY

A FIRST REPORT OF MOLECULAR TYPING BASED ON *aflR* GENE POLYMORPHISM, RFLP, RAPD AND MICROSATELLITE TYPING OF *ASPERGILLUS FLAVUS* ISOLATED FROM THE PEANUTS COLLECTED IN TAMIL NADU, INDIA.

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ABSTRACT

Aflatoxins are potent carcinogenic compounds that occur naturally as secondary metabolites produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus, Aspergillus nomics and Aspergillus tamarii. Aspergillus flavus* is second to *A. fumigatus* as a cause of aspergillosis. In the present study, we compared various molecular typing methods namely, restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA analysis, analysis of the polymorphism of aflR gene by PCR-RFLP and microsatellite/oligonucleotide fingerprinting for *A. flavus* strains isolated from peanuts. A total of 20 strains and a standard strain were used for comparison. The typing methods were evaluated with respect to discriminatory power (D), reproducibility and typeability to determine their performance and utility. RAPD with primer R-108 produced nine types with a discriminatory power of 92.5, PCR-RFLP of aflR gene failed to discriminate those 20 isolates. The microsatellite fingerprinting with pms2 produced eight types with a discriminatory power of 93. Typeability was 100% in all methods. The reproducibility with repeated runs of the same DNA preparation or with different DNA preparation of the same strain was good for all the methods. RAPD and microsatellite finger printing were found to be useful methods for the molecular typing of *A. flavus.* A high degree of genetic variation was observed in the test population. No clustering of particular group of strains was observed with any of the typing methods.

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KEYWORDS

Peanuts, *Aspergillus flavus, aflR* gene, Molecular typing

INTRODUCTION

Aflatoxins are potent carcinogenic compounds that occur naturally as secondary metabolites produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus, Aspergillus nomics and Aspergillus tamarii.* Aflatoxin contamination of agricultural commodities, such as maize, peanuts and cottonseeds is a serious risk to human and animal health, and has a significant economic impact. Over 30 years of investigation in to the aflatoxin biosynthetic pathway has identified more than 20 enzymes involved. Most of the aflatoxin-related genes are clustered within a 75 kb region of the genome[1]. The aflR gene, which regulates the clustered genes, has been identified in *A. flavus, A. parasiticus, A. sojae* and *A.oryza*[2]. The predicted aflR protein contains GAL4-type zinc-finger motif that transcriptionally activates most of the structural pathway genes, such as ver-1 and nor-1 [3,4.]

The *aflR* promoter contains an aflR binding motif, resulting in a positive feedback system, known as autoregulation [2]. Autoregulation results in an explosive expression of *aflR* upon induction, which in turn leads to instantaneous high expression of the genes found in the AF gene cluster. *aflR* and *aflJ* shares a bidirectional promoter but only the expression of *aflR* is affected by binding of aflR**.** The autoregulation scheme presents serious problems when induction needs to be stopped, two factors has been identified which might work as off-switches: (1) the phosphorylation state of aflR[5,6] and (2) the existence of a yet to be characterized repressor factor [4].

It is very important to detect the variability in the aflR gene, which is the key determinant in the expression of aflatoxin in these isolates. aflR gene typing is thus important in case of strains isolated from food stuff contaminated with *A.flavus*. It is also important gene which can be utilized for the differentiation of isolates from Aspergillus section *Flavi*. It is interesting to detect the correlation of aflR gene types with existing typing methods of *A.flavus*. These differentiations can segregate the aflatoxin producing isolates in an epidemiological point of view. Their easy detection is thus of outmost in food safety. In the genus Aspergillus, *A.fumigatus* is considered as the most pathogenic species followed by *A.flavus*. Since *A. flavus* is genetically and taxonomically poorly defined, a detailed and systematic study of this organism is required in relation to aflatoxin.

Molecular typing of strains isolated from aflatoxin producing peanuts, other environmental isolates can bring out vital information regarding the origin and spread of toxin producing and non-toxic producing strains in the environment. But a well-established system for the molecular typing of *A. flavus* is lacking. In case of *A.fumigatus* there are some established methods available, which include microsatellite fingerprinting[7], restriction fragment length polymorphism (RFLP)[8], RFLP with hybridization using specific probes[9,10,11], Random Amplified Polymorphic DNA (RAPD)[12,13] etc. Some of these methods had been tried in case of *A. flavus* as well [14]. But a large-scale study to evaluate various methods has not been conducted. The use of such a method in case of aflatoxin producing strains is also rarely done. Therefore, the present study was aimed at molecular typing of *A. flavus* strains producing aflatoxins from peanuts. Various methods were evaluated for their discriminatory power, reproducibility.

The molecular methods evaluated in this study include (a) RAPD using arbitrary primer R-108, (b) Analysis of the polymorphism of aflR gene by PCR-RFLP, (c) Analysis of the polymorphism of microsatellite/oligonucleotide fingerprinting of *A. flavus* strains.

MATERIALS AND METHODS

Sample collection

A total of 25 samples of groundnuts suggestive of fungal contamination were collected from different parts of the Chennai city used in this study. The peanuts with kernels were sealed in polypropylene bags and stored at 4^0C until used. Sealed bags of peanuts were removed from the refrigerator and tampered at the ambient temperature (25 to 30^0C) before opening.

A.flavus isolates

The *A.flavus* isolated by culturing the 25 samples of peanuts on Sabouraud Dextrose Agar (SDA, Hi-Media, India) was used in this study. A standard strain of *A.flavus* ATCC16883 was also used as a control sample in this study.

Isolation of A. flavus from peanut samples

The peanuts were aseptically removed from the pods and were surface sterilized. The seeds were first soaked in sterile water for about 30 mins. The water is decanted and 95% of ethanol is added to it and soaked for 5 minutes. Ethanol is decanted and 10% Sodium hypochlorite is added to it and soaked for 5 minute. Sodium hypochlorite is then decanted and the seeds were rinsed with sterile water. The peanuts were then dried on blotting paper and placed aseptically on the SD agar plate and incubated at 25^0 C for three days.

The cultures were examined daily for fungal growth after a period of three days. Suspected colonies were subcultured on sabouraud's dextrose agar. Whenever there was a fungal growth, the isolates were identified by cultural characters and microscopic morphology on slide cultures. Isolates identified as *A.flavus* were included in the study.

Isolation of whole cell DNA from A. flavus isolates

Whole cell DNA from the mycelial form of each isolate was extracted following a slightly modified protocol of small-scale fungal DNA extraction method by Lee and Taylor [15]. Each isolate of *A. flavus* was grown on SDA slope for three to four days at 37^0C. Conidia collected by sterile glass bend rod from SDA slopes by washing the slope with normal saline and were inoculated into Sabouraud's dextrose broth and incubated at 30^0C in a rotary shaker (150rpm) for two to three days. Mycelial mat was recovered by filtration through a Tarson filtration assembly (Tarson, India). It was then washed with sterile distilled water and dried in the filtration apparatus itself. About 0.3-0.5 g of mycelial mat was taken in a clean mortar and liquid nitrogen was added to it. It was quickly ground to make fine powder using a pestle adding more liquid nitrogen if necessary. Approximately 0.2-0.3 g of ground powder was transferred to sterile 1.5-ml microfuge tube. It was added with 600 μl lysis buffer (100 mM Tris HCl, pH 8.0, 50 mM EDTA, 3% SDS) and vortexed briefly. Tubes were then incubated at 65^0C for 20 minutes to one hour with occasional shaking. To this 650μl of buffered phenol: chloroform (1:1) was added and vortexed again briefly. The tubes were then centrifuged at room temperature for 15 minutes at maximum speed in a micro centrifuge (~12,000Xg). Immediately after centrifugation the top aqueous phase was transferred to another micro centrifuge tube without disturbing the interface. Equal volume of chloroform: isoamyl alcohol (24:1) was added to it.

The tubes were centrifuged at room temperature for five minutes at maximum speed in a micro centrifuge (~12,000Xg). Approximately 350μl of the aqueous phase was removed after centrifugation to a fresh tube (without disturbing the inter phase). 10μl of 3M sodium acetate was added to the aqueous phase followed by addition of 200μl of isopropanol (0.6 volumes). The tubes were inverted gently to mix the contents and to see threads of precipitated DNA. It was then centrifuged in a micro centrifuge (~12000Xg) for two minutes at room temperature. Without disturbing the pellet, the supernatant was discarded. The pellet was washed twice with

approximately 50µl of ice cold 70% ethanol. Tubes were kept inverted on a paper towel till the ethanol evaporates completely. The ethanol free DNA pellet was re-suspended in 100µl TE buffer. The DNA preparation was kept in a 65^0C oven for fifteen minutes after finger-vortexing to dissolve it completely. To this DNA preparation added 1µl of 10mg/ml RNase and incubated at 37^0C to digest RNA. After removing the RNA, the DNA was quantified in a spectrophotometer and stored at -20^0C for further use. This DNA preparation was used for all further studies.

Based on the epidemiological data, an extensive nucleic acid information search for aflatoxin in *A.flavus* submissions has been conducted in the public domain resource, National Centre For Biotechnology Information (NCBI) data bank (Gen Bank). Nucleic acid sequences for the aflR gene have been pulled out for available strains.

A multiple sequence alignment programme CLUSTAL W has been utilized for align the available sequences. The aligned sequences are as follows

Primer Designing:

CLUSTAL X (1.81) multiple sequence alignment

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SRRC            -------TAGACAATCCTTGGGCCAAGTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
CA5             -------TAGACAATCCTTGGGCCAAGTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
CA44            -----GCTAGACAATCCTTGGGCCAACTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
CA43            -----GCTAGACAATCCTTGGGCCAACTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
NPL             ------------CATCCTTGGGCCAACTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
NPLGS10-18s     ---CAGCTAGACAATCCTTGGGCCAAGTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
BCRC            GCACAGCTAGACAATCCTTGGGCCAAGTCAGAACCCCTCAGCTGGTGACAGGAGTGTACA
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SRRC            TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
CA5             TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
CA44            TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
CA43            TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
NPL             TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
NPLGS10-18s     TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
BCRC            TACATTTAGGTCTAAGTGCGAGGCAACGAAAAGGGCGGGCTACTCTCCCGGAGAAAGCCT
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SRRC            TCACA-TTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
CA5             TCACA-TTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
CA44            TCACAATTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
CA43            TCACAATTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
NPL             TCACAATTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
NPLGS10-18s     TCACAATTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
BCRC            TCACA-TTGTGTGTTTTCTTTCCGCTTTCAATTGAGAATTCCTGAATTCCTTCCTCACCT
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SRRC            CCACGATGGTTGACCATATCTCCCCCAGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
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CA5             CCACGATGGTTGACCATATCTCCCCCAGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
CA44            CCACGATGGTTGACCATATCTCCCCCCGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
CA43            CCACGATGGTTGACCATATCTCCCCCCGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
NPL             CCACGATGGTTGACCATATCTCCCCCCGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
NPLGS10-18s     CCACGATGGTTGACCATATCTCCCCCCGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
BCRC            CCACGATGGTTGACCATATCTCCCCCCGGGCATCTCCCGGACCGATCCGTTCCTCCCAGA
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SRRC            CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
CA5             CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
CA44            CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
CA43            CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
NPL             CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
NPLGS10-18s     CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
BCRC            CTCGCCGCGCCCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGCTCAAAAGTGCGAT
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SRRC            GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
CA5             GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
CA44            GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
CA43            GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
NPL             GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
NPLGS10-18s     GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
BCRC            GCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAACGTGGTCTTGCCTGTCAATACA
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SRRC            TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
CA5             TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
CA44            TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
CA43            TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
NPL             TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
NPLGS10-18s     TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
BCRC            TGGTCTCCAAGCGGATGGGCCGCAATCCGCGCGCTCCCAGTCCCCTTGATTCAACTCGGC
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SRRC            GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
CA5             GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
CA44            GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
CA43            GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
NPL             GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
NPLGS10-18s     GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
BCRC            GACCATCAGAGAGTCTTCCTTCAGCCAGGTCGGAACAGGGACTTCCGGCGCATAACACGT
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SRRC             ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
CA5              ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
CA44             ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
CA43             ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
NPL              ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
NPLGS10-18s      ACTCAACGCCTCATGCTCATACCCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
BCRC             ACTCAACGCCTCATGCTCATACGCAGGCCCACACTCATGCTCATTCTCATCCGCAACCGC
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SRRC             ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
CA5              ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
CA44             ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
CA43             ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
NPL              ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
NPLGS10-18s      ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
BCRC             ATCCACAATCTCATCCTCAATCGAATCAACCACCACACGCTCTGCCCACCCCCAATGGTA
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SRRC             GCAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
CA5              GCAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
CA44             GTAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
CA43             GTAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
NPL              GTAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
NPLGS10-18s      GTAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
BCRC             GCAGTAGCGTCTCCGCCATCTTTTCTCATCAGAGTCCGCCGCCACCCGTGGAGACCCAGG
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SRRC             GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
CA5              GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
CA44             GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
CA43             GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
NPL              GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
NPLGS10-18s      GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
BCRC             GCCTTGGAGGAGATCTGGCTGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAGTCGATT
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SRRC             CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
CA5              CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
CA44             CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
CA43             CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
NPL              CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
NPLGS10-18s      CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
BCRC             CGGAATTCGGGGGCTCTTTGCAGTCAATGGAACACGGAAACCATGCCGATTTCTTGGCCG
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SRRC             AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
CA5              AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
CA44             AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
CA43             AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
NPL              AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
NPLGS10-18s      AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
BCRC             AGTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACCCCCATGATCGACCCGT
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SRRC             TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
CA5              TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
CA44             TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
CA43             TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
NPL              TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
NPLGS10-18s      TTCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
BCRC             TCCTCGAGTCGGCCCCACTACCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCAC
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SRRC             TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
CA5              TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
CA44             TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
CA43             TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
NPL              TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
NPLGS10-18s      TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
BCRC             TACAAACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTACGGCTGACGG
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CA43             ACGGTGAGGACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAGA
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NPLGS10-18s      ACGGTGAGGACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAGA
BCRC             ACGGTGAGGACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAGA
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CA44             GGGCTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGC
CA43             GGGCTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGC
NPL              GGGCTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGC
NPLGS10-18s      GGGCTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGC
BCRC             GGGCTACCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTACTTGC
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CA44             TGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA
CA43             TGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA
NPL              TGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA
NPLGS10-18s      TGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA
BCRC             TGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCATGGTATGCTGCGGCAGCAGGCA
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CA5              CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGTC
CA44             CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGTC
CA43             CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGTC
NPL              CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGTC
NPLGS10-18s      CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGTTGTAGCAACAGTC
BCRC             CCCAGTGTACCTCAACGGCGGCGGGTGGAGAAACCAACAGTGGCAGCTGTAGCAACAGTC
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CA5              CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGG
CA44             CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGA
CA43             CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGA
NPL              CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGA
NPLGS10-18s      CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGA
BCRC             CCGCCACCGTGTCCAGTGGCTGTCTGACGGAAGAGCGCGTGCTGCACCTCCCTAGTATGA
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SRRC             TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
CA5              TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
CA44             TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
CA43             TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
NPL              TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
NPLGS10-18s      TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGTGGCACAGCTTGTTCTGA
BCRC             TGGGCGAGGATTGTGTGGATGAGGAAGACCAGCCGCGAGTGGCGGCACAGCTTGTTCTGA
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SRRC             GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
CA5              GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
CA44             GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
CA43             GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
NPL              GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
NPLGS10-18s      GCGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
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BCRC            GTGAACTGCACCGAGTCCAGTCGCTGGTGAACCTATTGGCCAAGCGCCTGCAAGAAGGTG
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SRRC            GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
CA5             GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
CA44            GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
CA43            GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
NPL             GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
NPLGS10-18s     GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
BCRC            GAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGT
************************************************************

SRRC            TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCCTCCGACATTATTG
CA5             TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCCTCCGACATTATTG
CA44            TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCTTCCGACATTATTG
CA43            TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCTTCCGACATTATTG
NPL             TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCTTCCGACATTATTG
NPLGS10-18s     TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCTTCCGACATTATTG
BCRC            TTAGTGGCCTCGAAGCAAATCTCCGCCACCGCTTGCGCGCCGTGTCCTCCGACATTATTG
******************************************** *************

SRRC            ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
CA5             ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
CA44            ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
CA43            ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
NPL             ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
NPLGS10-18s     ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
BCRC            ATTACCTGCATCGAGAATGAAGAAAAGCCCCACCGCCAGGAGCAGATGACAGGCCGGTTT
************************************************************

SRRC            CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
CA5             CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
CA44            CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
CA43            CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
NPL             CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
NPLGS10-18s     CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
BCRC            CCTCTCCATTAAAATTGGAATGATATCGACATGATATCAGCTCACCCGCTGCCCCTCACC
************************************************************

SRRC            CCCTTGCGATTAGTGTTTTTGCGCCTTTTTGGGTGCAGGGGGGGGGCAGCAAATCGGG-C
CA5             CCCTTGCGATTAGTGTTTTTGCGCCTTTTTGGGTGCAGGGGGGGGGCAGCAAATCGGG-C
CA44            CCCTTGCGATTAGTGTTTTTGCGCCTTTTTAAGTGCAGGGGGGG---AGCAAATCGG---
CA43            CCCTTGCGATTAGTGTTTTTGCGCCTTTTTAAGTGCAGGGGGGG---AGCAAATCGG---
NPL             CCCTTGCGATTAGTGTTTTTGCGCCTTTTTAAGTGCAGGGGGGG---AGCAAATCGTGGC
```

```
NPLGS10-18s      CCCTTGCGATTAGTGTTTTTGCGCCTTTTTAAGTGCAGGGGGGG---AGCAAATCGTGGC
BCRC             CCCTTGCGATTAGTGTTTTTGCGCCTTTTTGGGTGCAGGGGGGG----GCA---------
******************************  ************    ***

SRRC             AACCGCCTCC-
CA5              AACCGCCTCCC
CA44             ----------
CA43             ----------
NPL              AACCGCCAT--
NPLGS10-18s      AACCGCCATCG
BCRC             ----------
```

A region of 1500 base pairs (bp) were compared and for all the species and representative sequence which is present in all the species (20bp) has proposed as forward primer. This sequence is further analyzed for its suitability as a primer by using another program "Gene Runner". Its melting point, GC content, molecular weight , primer dimer formation, loop structures (hair pin, internal & bulge) etc were studied (Table 1).. Similarly a reverse primer also proposed and all the parameters were analyzed. Both the primers together analyzed for their compatibility in a PCR reaction. Various combination have been tried and a 20 mer portion starting from 370 from *NPL strain* has been chosen as forward primer and named as aflf and a 20 mer portion ending at 1385 has been chosen for reverse primer and named as aflR. These primers have complete similarity with all other strains compared.

Table 1
Properties of Forward and Reverse primer

Properties	Forward primer	Reverse primer
Sequence	$^{5'}$GCAATCCGCGCGCTCCCAGT$^{3'}$	$^{5'}$CCCTGCTGCATCGTCTCCAC$^{3'}$
Molecular weight	6119	6045
Tm	78.9^{0}C	62.2^{0}C
%GC	70%	65%
Hairpin loops	0	0
No.Of Dimers	3	3

PCR Amplification of aflR gene:

A standard PCR was run using a template DNA(100ng), Forward Primer-$^{5'}$GCAATCCGCGCGCTCCCAGT$^{3'}$, Reverse Primer-$^{5'}$CCCTGCTGCATCGTCTCCAC$^{3'}$, dNTP mix (dATP, dCTP, dGTP, dTTP), 10X PCR buffer, Taq polymerase. An optimized PCR program starts with an initial denaturation step at 94^{0}C for 5 minutes followed by 30 cycles of denaturation at 94^{0}C for 2 minutes, primer annealing at 50^{0}C for 1 minute and polymerization at 72^{0}C for 2 minutes and a final extension at 72^{0}C for 7 minutes. Verifying PCR Amplification were subjecting to agarose gel electrophoresis on 1% agarose with ethidium bromide (5µg %) and the bands were visualized in a gel documentation system (Bio-Rad) and the results were recorded.

Restriction Enzyme analysis for aflR gene:

The sequences of the expected PCR product has been downloaded and matched by using Clustal W multiple alignment tool with all the available sequences in the genbank and confirmed their uniformity across the groups. A standard restriction map was

generated by using the genomic data of aflR gene needed for the development of the primers by using the software gene runner.

A total of 418 cut sites observed using 287 restriction enzymes. 381 cut sites from 140 enzymes met search criteria (Table 2).

Table 2
Various restriction enzymes used to digest aflR amplicon DNA

S.No	Restriction Enzyme	Cutting Site	Temperature
1	*Msp*I (*Hpa*II)	5' C↓CGG 3'	37^0C
2	*Bgl*II	5' A↓GATCT 3'	37^0C
3	*Eco*RI	5' G↓AATTC 3'	37^0C

Restriction fragment length polymorphism (RFLP) analysis of aflR gene:

For evaluating RFLP analysis as a typing method for the selected 20 strains, batteries of restriction endonucleases were used. Separate reactions were set up for each enzyme according to its properties. The properties of the enzymes used in this study are given in the table No.3.

Table 3
Basic identification of the samples

Sl.No.	Sample Number	Result
1.	PS1	+
2.	PS2	+
3.	PS3	-
4.	PS4	+
5.	PS5	+
6.	PS6	+
7.	PS7	+
8.	PS8	+
9.	PS9	+
10.	PS10	+
11.	PS11	-
12.	PS12	-
13.	PS13	+
14.	PS14	+
15.	PS15	+
16.	PS16	+
17.	PS17	+
18.	PS18	+
19.	PS19	+
20.	PS20	+
21.	PS21	+
22.	PS22	+
23.	PS23	+
24.	PS24	-
25.	PS25	-

Approximately 5µg of DNA amplicon was subjected to digestion by 10 units of each enzyme (Genei, India) in a 30µl of reaction volume at 1X buffer concentration. The incubation time and other reaction conditions were standardized for individual enzymes.

The digestion was terminated either by adding one volume of stop buffer or kept in the boiling water bath for few minutes and suddenly cooled to ice. The RFLP pattern was found out by separating the reaction mixture by electrophoresis through a native agarose gel of 1 %(w/v) concentration. The electrophoresis was performed for two and half-hours at 75V in a GNA100 electrophoresis apparatus (Bio-Rad, USA Biotech). A concentration of 6X DNA loading dye was used to load the DNA samples in to the gel. After the electrophoresis the gels were stained with ethidium bromide (5mg/l) in a dark room. The image of the ethidium bromide stained DNA bands were digitized using a gel documentation system (Bio-Rad, USA). Isolates were considered similar when the pattern obtained was same.

Random amplification of polymorphic DNA (RAPD) assay

RAPD is carried out with the following primer R108.

R108 -5' GTATTGCCCT 3' [16]

A fifty microliter reactions were set up with 100ng of genomic DNA, 3U of *Taq*DNA polymerase (Bangalore Genei, India), 200µM deoxy nucleotide tri phosphate mix (dNTP mix), 2.5mM magnesium chloride and 50pmoles of primer R-108 were used in a 1X PCR buffer (all reagents from Bangalore Genei, India). The reaction mixture was subjected to an initial denaturation of 94^0C for 5minutes, followed by 30 cycles of 94^0C -1min, 36^0C -1min and 72^0C -1min. A final extension step of 72^0C was also included. Thermal cycling was conducted in an ABI 9700 (Applied Biosciences, USA). The amplicons were resolved by electrophoresis through an agarose gel of 1- %(w/v) concentration. The electrophoresis was performed for two and half-hours at 75V in a GNA100 electrophoresis apparatus (Biorad, USA Biotech). A concentration of 6X DNA loading dye was used to load the DNA samples in to the gel. After the electrophoresis the gels were stained with ethidium bromide (5mg/l) in a dark room. The image of the ethidium bromide stained DNA bands were digitized using a gel documentation system (Bio-Rad, USA). Isolates were considered similar when the pattern obtained was same.

Microsatellite fingerprinting with primer pms2

A fifty micro liter reactions were set up with 100ng of genomic DNA, 3U of *Taq*DNA polymerase (Bangalore Genei, India), 200µM deoxy nucleotide tri phosphate mix (dNTP mix), 2.5mM magnesium chloride and 100pmoles of primer pms2 were used in a 1X PCR buffer (all reagents from Bangalore Genei, India). The reaction mixture was subjected to an initial denaturation of 94^0C for 5minutes, followed by 35 cycles of 94^0C -1min, 50^0C -1min and 72^0C -1min. A final extension step of 72^0C for 10min was also included. Thermal cycling was conducted in an ABI 9700 (Applied Bioscience, USA). The amplicons were resolved by electrophoresis through an agarose gel of 1- %(w/v) concentration. The electrophoresis was performed for two and half-hours at 75V in a GNA100 electrophoresis apparatus (Bio-Rad, USA). A concentration of 6X DNA loading dye was used to load the DNA samples in the gel. After the electrophoresis the gels were stained with ethidium bromide (5mg/l) in a dark room. The image of the ethidium bromide stained DNA bands were digitized using a gel documentation system (Bio-Rad, USA). Isolates were considered similar when the pattern obtained was same.

Evaluation of various molecular typing methods

Various methods were evaluated for their suitability for typing the entire collection of *A. flavus* by their typeability, reproducibility and discriminatory power. Typeability is defined as the ability of a test method to produce result analyzing a strain. Reproducibility is the ability

to assign an identical type to the same isolate by a repeat assay. The reproducibility of each of the typing method was examined by running the same DNA preparation repeatedly and by analysis of a second DNA preparation from the isolate either at the same laboratory or a different laboratory. Discriminatory power of a typing method is the mathematical probability that two unrelated isolates chosen at random from a test population can be shown to belong to different groups. Discriminatory power as Simpson's index was calculated in the manner described by Hunter [17]. When all the isolates tested were a different type, D is equal to 100. Conversely, D is equal to zero when all isolates are the same type. Formula for the determination of Simpson's Index of Diversity (D) is given below.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j-1}^{s} nj\,(nj-1)$$

Where N- is the total number of strains in the test population, s- is the number of strain types observed, and nj- is the number of strains in the population that belong to the j^{th} strain type.

RESULTS AND DISCUSSION

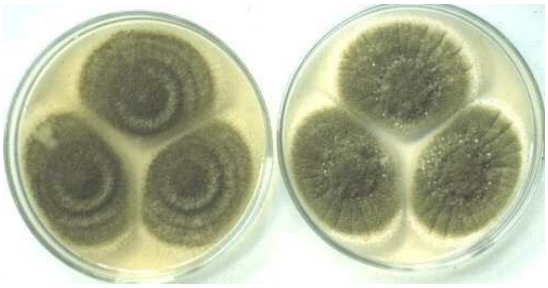
A. flavus isolates

A total of 25 samples of peanuts collected from different fields in the outskirts of Chennai were included in this study. The peanuts were surface sterilized and placed onto the SD Agar and incubate at 25°C. The different cultures of fungal species were found and the culture with A.flavus is considered to be positive and 20 samples were found to produce *A.flavus, which was identified,* by microscopic morphology and cultural characters (Table 3).

Cultural characters and microscopic morphology identified all the strains. Colonies on SD agar showed variable growth rate ranging from rapidly growing (6-7cm in diameter) to moderately growing (3-4 cm in diameter) over 10 days at room temperature. Colonies contained a thin but close textured basal mycelium, which in some strains remained submerged in the marginal 1.0 to 1.5 cm. Most strains produced abundant conidial structures directly from the substrate mycelium. Young conidial heads had often yellow shades near strontium yellow or yellowish citrine, quickly changing through bright to dark yellow green shades near mignonette or light elm green, and finally becoming deep grape green to jade green or cress green (figure1). Colony reverses had commonly uncolored to pinkish drab appearance. Sclerotia produced in many strains, particularly in fresh isolates, sometimes dominating the colony appearance. They were variable in form, dimensions and pigmentation, arising as white mycelial tufts, characteristically globose to subglobose and gradually changing from white through dark red brown to near black. Size varied from 400 to 700µm and rarely exceeding 1mm in diameter, but uniformly smaller in some strains, less deeply pigmented in others, and vertically elongate or of indeterminate apical growth in still others.

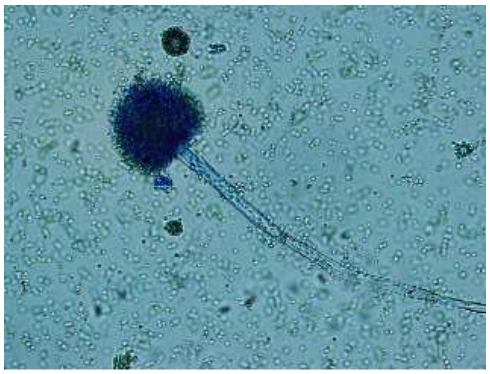
Figure 1

Plates showing Aspergillus flavus growth after 7 days.



Conidial heads had typical radiation, splitting in to several poorly defined columns, rarely exceeding 500 to 600µm in diameter, most commonly 300 to 400µm, smaller heads occasionally columnar up to 300µm by 50µm. Conidiophores was heavy walled, uncolored, coarsely roughened, usually less than 1mm in length but in occasional strains up to 2.0 to 2.5 mm with stalk diameters immediately below the vesicles ranging from 10 to 20µm (figure 2). Vesicles were elongate when young, later becoming subglobose or globose, varying from 10 to 65µm in diameter but most commonly 25 to 45µm; sterigmata on normal vesicle either uniseriate or biseriate with the two conditions rarely occurring in the same head; primaries usually 6.0 to 10.0µm by 4.0 to 5.5µm but sometimes up to15 or 16µm long and infrequently swollen to 8.0or 9.0µm in diameter, secondaries 6.5 to 10.0µm by 3.0 to 5.0µm , uniseriate sterigmata variable in size from 6.5 to 14. 0 µm by 3.0 to 5.5 µm consistently produced on small vesicles. Conidium forming tips usually had phialiform conidia typically globose to subglobose, conspicuously echinulate, variable from 3.0 to 6.0 µm in diameter, but mostly 3.5 to 4.5 µm, sometimes elliptical when first formed, infrequently remaining so and then measuring about 4.5 to 5.5 µm by 3.5 to 4.5µm.

Figure 2
Microphotograph showing typical conidial head of A.flavus (400X)



Identifications were confirmed after studying the colony characteristics on SD agar. Microscopic morphology was confirmed by slide culture on SD agar. Strains were maintained by subculture method on SDA and stored at 4°C. Subcultures were done every month.

DNA extraction form Aspergillus flavus isolates

DNA extraction from filamentous fungi isolated from peanuts and a standard strain ATCC 16883 was carried out by the modified method of Lee and Taylor (1990). They were confirmed by using Agarose gel electrophoresis with 0.8% agarose and 0.5 µg/ ml EtBr. The DNA bands were observed in gel documentation system. (Bio-Rad).

RAPD with primer R-108

Primer R-108 differentiated the 20 isolates into nine types of which four were unique to a single isolate and three were limited to pairs. The other two types contained five isolates each (Table 4). The discriminatory power was found to be 95.2%. The *A. flavus* ATCC 16883 strain used alongside gave a matching pattern with one of the isolates (No. 18 unique type) (Figure 5). ATCC 13073 strain of *A. fumigatus* gave a distinct profile.

Table 4
RAPD pattern of Aspergillus flavus isolates with R-108

Type	Primer
	R-108
A	1,11,16,17,19
B	2,10
C	4,9
D	5
E	6,14
F	7
G	8
H	3,12,13,15,20
I	18
J	-

Figure 3
Young mycelial growth of A.flavus on SDB for DNA extraction

Figure 4
Showing whole genomic DNA isolated from Aspergillus flavus

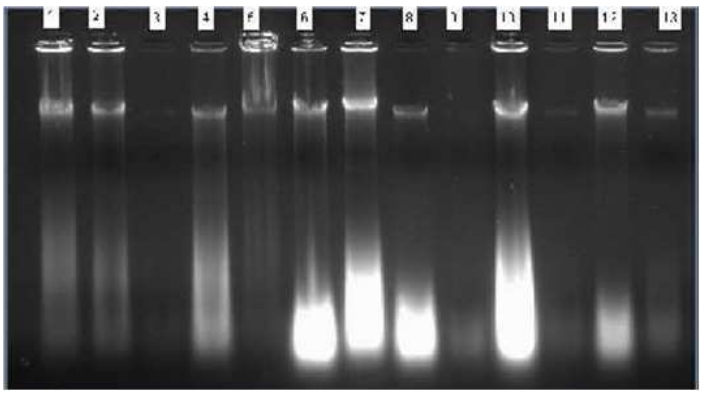
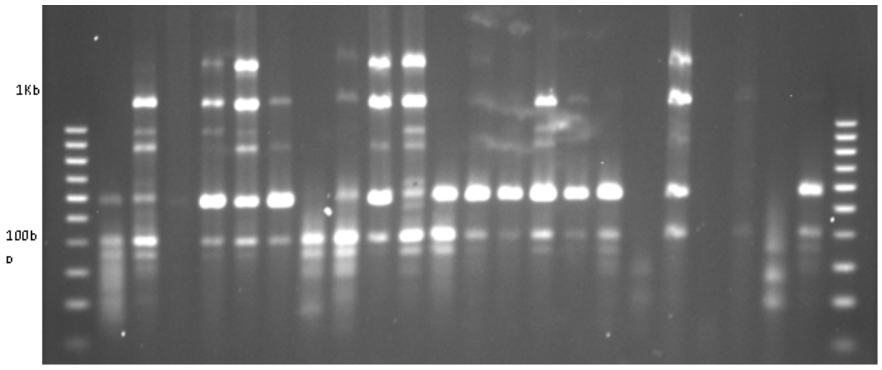


Figure 5
Agarose gel electrophoresis showing RAPD pattern of A.flavus using primer R-108

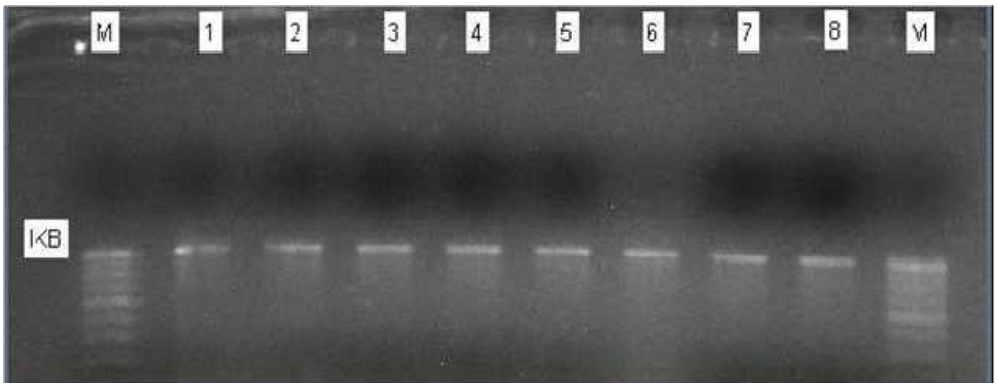


RAPD results with R-108 had produced nine types with a discriminatory power of 95.2%. There was no considerable clustering of the isolates into a particular type. However various controls are to be included in each set of experiments to have reproducible results. In an earlier study it was shown that a 0.95Kb DNA band produced by R108- primer acts as a marker of invasive ability of the organism. In our study we could not draw such a conclusion from our data, were reported, it is difficult to prove or disprove such hypothesis from the present study.

PCR of aflatoxin genes:

PCR amplification for the aflR gene was done using the forward and reverse primers which were designed based on the multiple sequence alignment and the total length of the gene was about 1kb. The PCR amplicon was observed using Agarose gel (Figure 6).

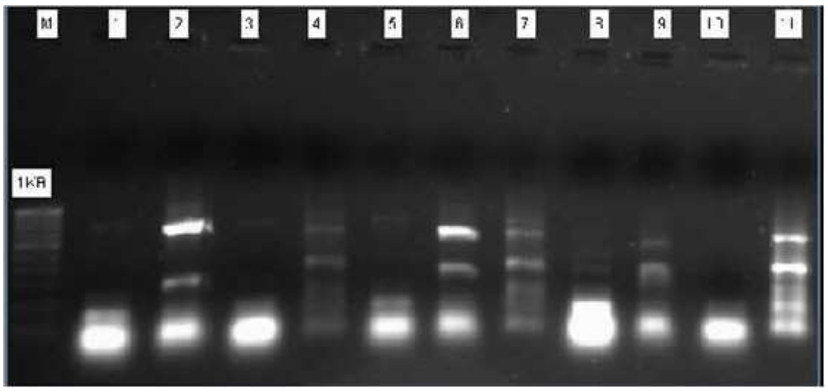
Figure 6
Agarose gel Electrophoresis showing the amplification of aflR gene from A.flavus



PCR-RFLP of aflatoxin genes:

As it was expected, the aflR gene was amplified and the total length of the gene was about 1kbp. There was no polymorphism observed in the total length of that gene for any of the isolates. Restriction enzyme digestion of the amplicon with *EcoRI, BglII and mspI* produced multiple bands at regions of 150bp and 170bp but there were no appreciable differences observed among the twenty isolates (Figure 7). *A. flavus* ATCC 16883 strains was exactly similar to that of other isolates whereas the *A. fumigatus* ATCC 13073 strain was distinctly different from the rest of the isolates.

Figure 7
Agarose gel Electrophoresis of PCR-RFLP of aflR gene of Aspergillus flavus with EcoRI



All the isolates studied have shown the presence of aflR gene. There was no length polymorphism in the total aflR amplicon. So further RFLP has been tried after an extensive analysis of the sequence of amplicon. The RFLP of this gene with 3 commonly used restriction enzymes did not show any polymorphism (Table 5). This shows the gene is highly conserved in *A.flavus.*

Table No.5.
PCR-RFLP of aflR gene of Aspergillus flavus isolates

Restriction enzymes used	***Bgl* II**	***EcoR*I**	***Msp*I**
Amplicon size	1016bp	1016bp	1016bp
Restriction fragment produced	656,360	173,843	450,556
Variation	Not detected	Not detected	Not detected

Microsatellite finger printing with primer pms2

Primer pms2 differentiated the 20 isolates into eight types of which four were unique to a single isolate. Two types contained three isolates each. Another type contained two isolates and the major type contained four isolates (Table 6). The discriminatory power was found to be 93%. *A. flavus* ATCC 16883 strains used alongside gave a matching pattern with one of the isolates. *A. fumigatus* ATCC 13073 gave a distinct profile (Figure 8).

Figure 8
Agarose gel Electrophoresis showing the microsattelite fingerprinting of A.flavus using primer pms2

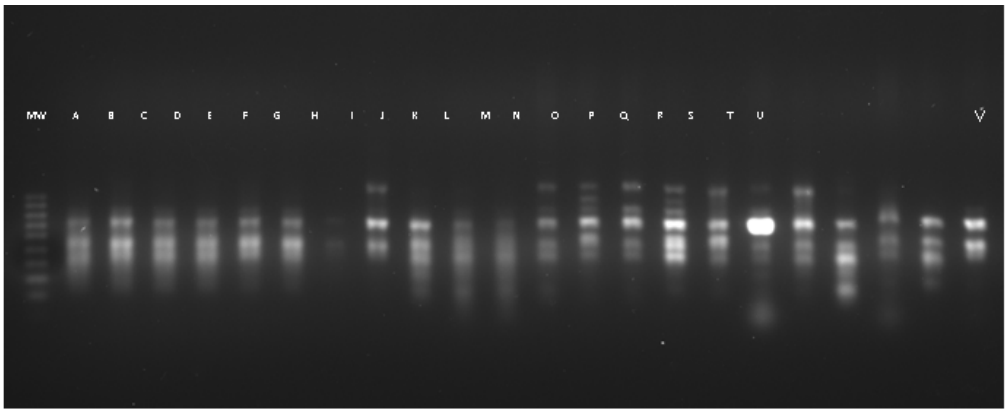


Table 6
Microsatellite fingerprinting pattern of A. flavus with primer pms2

Type	Strain numbers
A	1,3,11,12
B	2
C	4
D	5
E	6
F	7,8,19
G	9,15
H	16,17,18

In our study also we found that this method giving discriminatory power of 93% with based typing methods.

Evaluation of the molecular typing methods

Usefulness of techniques was evaluated by their discriminatory power, reproducibility and typeability. All the methods were found to be reproducible in repeated experiments with the same DNA samples as well as a different DNA sample extracted from the same organism after a period of six months. All the isolates were found to be typable with the methods used. Different methods varied in their discriminatory power. Microsatellite finger printing and RAPD showed maximum discrimination. Overall RAPD showed discriminatory power ranging from 86.3 to 95.2 and microsatellite fingerprinting showed 59 to primer pms2. This was slightly lower than that of RAPD, but much higher than that of rDNA 93. Therefore, it was decided to use those two techniques with all the primers to type entire collection of *A. flavus* strains. It was also decided to perform the PCR-RFLP of *aflR* region of rDNA as this technique is very specific and not been evaluated elsewhere.

Evaluation of molecular typing methods for A. flavus

The merits and demerits of any typing method is evaluated by three basic criteria namely typeability, reproducibility and discriminatory power. All the techniques that we have tried were found to be reproducible and all the isolates used were found to be typeable, the ultimate criterion for the selection of the method for typing of all the strains was discriminatory power (Table 7).

Table 7
***Summary of the various typing methods used to standardize molecular typing methods for A. flavus* strains.**

Method		No of types obtained	Discriminatory power
RAPD	R-108	9	95.2
Analysis of aflR genes			
	BglII	One group	No discrimination
	EcoRI	One group	No discrimination
	MspI	One group	No discrimination
Microsatellite / Oligonucleotide finger printing			
	pms2	8 types	93

Among all these methods, RAPD with primer R-108 and microsatellite fingerprinting with primer pms2 had highest discriminatory power with a D value of 95.2 and 93. Considering the inherent deficiency of RAPD as a typing method in its reproducibility and specificity, we performed also microsatellite typing with pms2. Though usefulness of PCR-RFLP of the aflR gene for strain typing was limited, we evaluated this method for all strains as this gene was never evaluated earlier for *A. flavus* strain typing. The specificity of this method was another reason for consideration.

The results of this study proved that microsatellite fingerprinting with primer pms2 and RAPD with primer R-108 are the better methods for strain typing of *A. flavus.* Further in aflatoxin producing *A.flavus* strains, the aflR gene exist as conserved across the study area. There is no particular clustering observed while using other generalized typing techniques. However a correlation between the levels of expression of aflatoxin would be meaningful while considering the aflR gene polymorphism. Thus it may be extrapolated that all strains are potentially aflatoxigenic and contamination with this fungus pose a real threat to the mankind.

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