TITLE: MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC AND NON-AFLATOXIGENIC *ASPERGILLUS* ISOLATES

By: Ms PHAKAMILE TRUTH MNGADI

Submitted in partial fulfillment of the requirements for the Masters in Technology, in the Department of Biotechnology & Food Technology, Faculty of Engineering, Science and the Built Environment, Durban University of Technology. March 2007.

***SUBMISSION APPROVED FOR EXAMINATION**

_____ _____

SUPERVISOR **DATE**
DR ROSHINI GOVINDEN

_____ _____

SUPERVISOR **DATE**
PROF BHARTI ODHAV

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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Engineering, Science and the Built Environment, Durban University of Technology, South Africa, under the supervision of **Dr Roshini Govinden** and **Prof Bharti Odhav**.

_____

Student's signature

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DEDICATION

I dedicate this work to everyone that has brought JOY and HAPPINESS into my life and for those that STILL put a SMILE on my face. I'm truly grateful to ALL of you!

ABSTRACT

A demand exists for rapid and reliable techniques to detect mycotoxins. Conventional plating out methods are used to indicate specific viable fungi from a sample. Analysis of the pure cultures (e.g. by chromatography) then provide information regarding the ability to produce mycotoxins. Currently, molecular methods hold promising avenues for determining "true" evolutionary relationships. High performance liquid chromatography (HPLC) was used to detect aflatoxin production in 12 *Aspergillus* isolates. These isolates were also subjected to randomly amplified polymorphic DNA (RAPD) and dendogram analysis to assess genetic similarities. Polymerase chain reaction (PCR) was used to prepare the probe for hybridization and check for the presence/absence of the *afl*R gene among the isolates. Restriction fragment length polymorphism (RFLP) analysis was also performed in preparation for Southern blotting/hybridization in order to detect polymorphisms.

Seven of the 12 isolates analyzed were found to be aflatoxigenic (*A. flavus* ATCC 32592, *A. parasiticus* ATCC11970, PPRI 2885, 3641, 5063, 5183 and 5990). Aflatoxin B_2 (AFB_2) was produced by seven of the 12 isolates; aflatoxin B_1 (AFB_1) by four of the 12 isolates; aflatoxin G_1 (AFG_1) by one of 12 isolates and aflatoxin G_2 (AFG_2) by three of the 12 isolates. Dominance of AFB_2 instead of AFB_1 could mean that there was loss of AFB_1 production during sub-culturing or conditions were unfavourable. The remaining five isolates: *A. oryzae* ATCC 3151, *A. niger* MLST, *A. tamarii* MLST1 and two *A. flavus* isolates (i.e. PPRI 5065 and 7052) were found to be non-aflatoxigenic. RAPDs and dendograms produced different genetic profiles. Four of the 14 primers (i.e. UBC204, 246, 285 and 287) produced profiles that exhibited 60-100% similarity between *A. oryzae* 3151 and *A. flavus* 5065. This means that these two isolates are more genetically similar to each other than to the rest of the isolates. PCR produced a band corresponding to a portion of the *afl*R gene of approximately 450bp (nine of the twelve isolates) and it was used as a probe during hybridization. The three known non-aflatoxin producers (i.e. *A. oryzae* ATCC 3151, *A. niger* MLST, *A. tamarii* MLST1) did not produce a PCR band (450bp). Even though the hybridization conditions were optimized, 'no signal' or negative results were obtained for hybridization.

This analysis showed that non-aflatoxigenic *A. oryzae* is genetically more similar to *A. flavus.* Also, isolates can 'lose' their ability to produce aflatoxins. The presence/absence of an *afl*R gene can be used to detect for potential aflatoxigenic isolates.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
PPRI	Plant Protection Research Institute
DUT	Durban University of Technology
MLST	M. L. Sultan Technikon
SMKY	Sucrose Magnesium sulphate Potassium nitrate Yeast extract
rpm	runs per minute
ml	millilitre
HPLC	High Performance Liquid Chromatography
µl	micro liter
min	minute
vol	volume

µg	microgram
g	gram
U	Unit
µM	micro molar
M	molarity
mM	milli molar
DNA	De-oxy Nucleic Acid
RAPD	Randomly Amplified Polymorphic DNA
RT	Room Temperature
NaOH	Sodium Chloride
HCl	Hydrochloric Acid
bp	base pair
kb	kilo base
GFX	Glass Fiber Matrix
DIG	Digoxigenin
DMF	Dimethylformamide
SD	Standard Deviation
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RFLPH	Restriction Fragment Length Polymorphism with Hybridization
UBC	University of British Columbia
V	Volts
mg	milligram
NTP	Nucleotide Tri-phosphate
dNTP	de-oxy Nucleotide Tri-phosphate
w/v	weight per volume
SSC	Sodium chloride Sodium Citrate
SDS	Sodium Dodecyl Sulphate
cm^2	square centimetre
ng	nanogram
GE	Genetically Engineered

ST	Sterigmatocystin
OMST	*O*-methyl sterigmatocystin
DHOMST	Dihydro-*O*-methyl sterigmatocystin
NA	Norsolorinic Acid
AVN	Averantin
AVNN	Averufanin
VAL	Versiconal
VB	Versiconal B
AIDS	Acquired Immune Deficiency Syndrome
RACE-PCR	Rapid Amplification of cDNA Ends Polymerase Chain Reaction
TAIL-PCR	Thermal Asymmetric Interlaced Polymerase Chain Reaction
UV	Ultraviolet
mtDNA	Mitochondrial DNA
ºC	degrees Celsius
TFA	Trifluoroacetic Acid
EDTA	Ethylene diamine tetracetic acid
CSIR	Council of Scientific and Industrial Research

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

For decades the genus *Aspergillus* (of fungi) has been classified based on morphological and growth criteria. Members of the *Aspergillus* section *Flavi* are economically valuable and methods of differentiating them are thus very important. Several molecular methods have been developed to distinguish these strains. Also, a number of biochemical and genetic studies have been used in order to provide a better means of classification (Lee *et al*., 2004).

Aflatoxins, the most frequently studied mycotoxins, are produced by certain *Aspergillus* species/strains/isolates of fungi. The aflatoxin biosynthetic pathway studies have led to a number of discoveries. Several structural and regulatory genes (and their enzymes) involved in the biosynthesis of aflatoxins have been discovered and purified (Trail *et al*., 1995).

Aflatoxin production and contamination of agricultural crops are major causes of economic losses in agriculture. Thus, better methods of characterization/differentiation are required for both aflatoxigenic and non-aflatoxigenic isolates. Molecular biology is one of the current tools used to differentiate between these isolates. Polymerase Chain Reaction (PCR)-based randomly amplified polymorphic DNA (RAPD) analysis has been used successfully in the analysis of DNA relatedness of species of fungi, bacteria, plants and animals. Dendograms which evaluate/assess the likeness between different isolates has also been used (Martinez *et al.,* 2001). Restriction fragment length polymorphism (RFLP) analysis has been applied to a number of studies to detect differences between fungi and to establish relationships between them.

Therefore, the scope of this study was to investigate RAPD analysis (with dendograms) and detection of RFLPs by hybridization as molecular methods that can distinctly differentiate or characterize the aflatoxigenic and non-aflatoxigenic *Aspergillus* isolates. Aflatoxin production was also quantitatively analysed using high performance liquid chromatography (HPLC). Results obtained were assembled

together in order to try and find a rapid method of differentiating aflatoxigenic *Aspergillus* isolates from the non-aflatoxigenic ones.

1.1 Description and natural habitats of *Aspergillus*

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in a variety of substrates under a diverse range of environmental conditions as shown in Table 1. It is commonly isolated from soil, plant debris, indoor environment and it plays a very important role in the deterioration of grains (St-Germain and Summerbell, 1996). As a food contaminant *Aspergillus* is able to tolerate or thrive under situations of high temperature and reduced water availability. It is also known to produce secondary metabolites. Some species are capable of growing in the animal body and are responsible for aspergillosis (St-Germain and Summerbell, 1996).

Table 1: Habitats for some important *Aspergillus* species (St-Germain and Summerbell, 1996).

Species	Occurrence
A. flavus	Alkali-tolerant, animal dung, soil and decomposing organic material
A. fumigatus	Tropical and subtropical soils, plant products such as ground nuts and maize
A. niger	Cosmopolitan but particularly in the tropics
A. parasiticus	Insect pathogen, saphrophyte on plant products
A. versicolor	Soil, mature cheeses, cured meats, decaying vegetation

1.2 Interspecies diversity

The genus *Aspergillus* has long had one of the better taxonomic descriptions found among fungi (Raper and Fennell, 1965). It is inevitable that such taxonomic structure should give rise to hypotheses concerning the evolution of the genus. The major macroscopic features remarkable in species identification are growth rate, colour of colony and thermotolerance. The basic morphology is the same for all species. However, some microscopic structures are unique to certain species and constitute the key for species identification together with the surface colour of the colony (St-Germain and Summerbell, 1996).

This genus is characterised by the production of the aspergillum (a conidiophore with an aseptate stipe terminating in a vesicle). The vesicle bears conidiogenous cells (primary and secondary phialides and metulae) on which there are long chains of small, dry, single-celled conidiospores with different pigmentation and ornamentation (Samson, 1992). The *Aspergillus* conidiophore structure is shown in Fig 1.

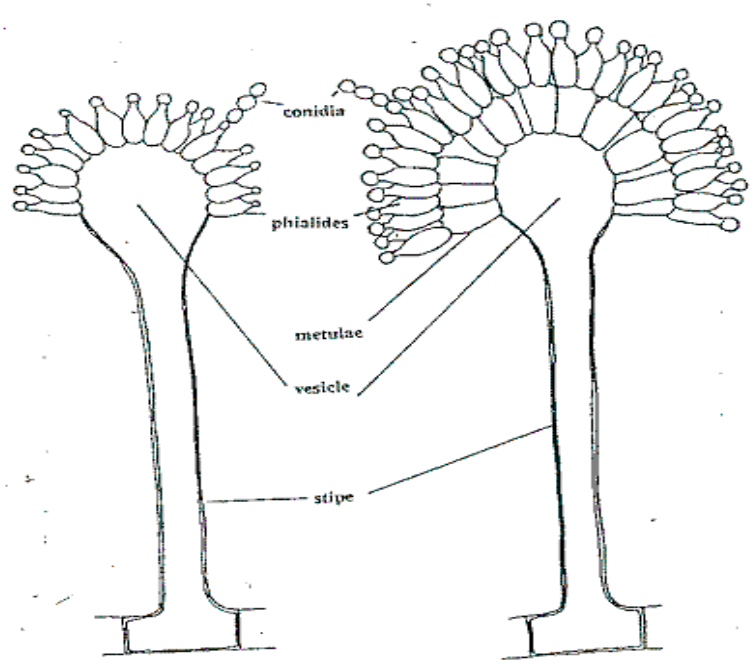


Figure 1: *Aspergillus* conidiophore structure (Samson and van Reenen-Hoekstra, 1988).

Aspergillus colonies are fluffy to powdery in texture. The surface colour may vary depending on the species. The reverse is uncoloured to pale yellow in most of the isolates. However, the reverse colour may be purple to olive in some strains of *A. nidulans* and orange to purple in *A. versicolor* (St-Germain and Summerbell, 1996). The colour of the colony in various *Aspergillus* species is shown in Table 2.

Table 2: The colour of the colony of *Aspergillus* species (St-Germain and Summerbell, 1996).

SPECIES	SURFACE	REVERSE
A. clavatus	Blue-green	White, brownish with age
A. flavus	Yellow-green	Goldish to red brown
A. fumigatus	Blue-green to gray	White to tan
A. glaucus group	Green with yellow areas	Yellowish to brown
A. nidulans	Green, buff to yellow	Purplish red to olive
A. niger	Black	White to yellow
A. terreus	Cinnamon to brown	White to brown
A. versicolor	White at the beginning, turns to yellow, tan, pale green or pink	White to yellow or purplish red

Aspergillus fumigatus is a thermotolerant fungus and grows well at temperatures over 40°C. This property is unique to this fungus among the *Aspergillus* species (St-Germain and Summerbell, 1996). With the exception of *A. nidulans* and *A. glaucus* (they grow very slowly), the growth rate in *Aspergillus* species is moderately rapid to rapid. These variations in growth rate help in species identification (St-Germain and Summerbell, 1996).

Aspergillus section *Flavi* has attracted worldwide attention for economic and public health reasons because of its industrial use and toxigenic potential (Samson *et al*., 2000). This section consists of two groups of species; one includes *A. flavus*, *A. parasiticus* and *A. nomius* – all of which have toxigenic potential. The second group includes *A. oryzae*, *A. sojae* and *A. tamarii* – which have been used for production of traditionally fermented foods in Asia. Identification of the species has relied mainly upon the morphological characters used as taxonomic criteria (Samson *et al*., 2000). It is, however, difficult to identify section *Flavi* species because of morphological divergence among isolates of the same species.

Molecular genetic techniques have been used as tools with which to study the phylogeny and classification of *Aspergillus* section *Flavi* (Samson *et al*., 2000). It has

been reported that analysis of Taka-amylase A gene can divide the species of section *Flavi* into several groups. It can also help in detecting intraspecies variations within *A. nomius, A. tamarii* and *A. flavus* strains. On the basis of the sequence data of several protein-coding genes, Geiser *et al*. (1998) failed to find any evidence that *A. flavus* and *A. oryzae* are independent species. It was suggested that *A. oryzae* have evolved by domestication from *A. flavus*. Other genetic attempts have been made to classify the section *Flavi* with other genes. Some methods have provided useful information on the phylogenetic relationship among species of *Aspergillus* section *Flavi.*

Aspergillus caelatus is the latest species assigned to *Aspergillus* section *Flavi* (Horn, 1997). The species has been isolated from agricultural soils, tea plantations and from peanut seeds (McAlpin *et al*., 2005). *A. caelatus* shares several morphological characteristics with *A. tamarii,* but the metabolite profiles of the two species are quite different.

1.3 Pathogenecity and clinical significance of *Aspergillus*

Aspergillus species can cause severe opportunistic human disease. Around 20 *Aspergillus* species have been reported as causative agents of infections in humans. Immunosuppression is the major factor predisposing to development of an infection (Larone, 1995). In immunocompromised hosts, the inhalation of conidia may provoke pulmonary, invasive and disseminated infections associated with high mortalities (Heinemann *et al.,* 2004). The most common aetiologic agent of aspergillosis is *A. fumigatus,* but *A. flavus* and *A. niger* and some other thermotolerant species of the genus are also involved in this disease (Denning, 1998). *Aspergillus* spp. infections in immunocompetent patients are less frequent. Cases of post-operative infections by *A. fumigatus, A. flavus* or *A. terreus* (Collazos *et al.,* 2001) have nevertheless been described, particularly after cardiac surgery. Outbreaks of aspergillosis are most often hospital acquired (Van den Bergh *et al.,* 1999).

1.4 Mycotoxins

Mycotoxins are classified by chemists as ‘natural products’ and by biologists as ‘secondary metabolites’ produced by filamentous fungi. Why filamentous fungi produce mycotoxins is still unclear. Mycotoxins are produced under special conditions of moisture and temperature. These mycotoxin-producing fungi are aerobic

(use oxygen), microscopic and may colonise many kinds of food in the field (Yu *et al.*, 1995).

Not all fungi can produce mycotoxins. In addition, some fungi are able to produce mycotoxins only under special conditions. Even those with the ability to produce mycotoxins may not produce them all the time. The absence of mycotoxins doesn't ensure the absence of fungal spores. Mycotoxins are also very resistant to temperature treatments and conventional food processes such as freezing (Reddy and Waliyar, 2005).

There are more than 300 species of fungi with the ability to produce mycotoxins. Only about 20 mycotoxins produced by the five genera of fungi (i.e. *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps*) are found periodically in food at levels posing threats to humans (Benbrook, 2005). Mycotoxins have also become part of the global debate over the benefits of genetically engineered (GE) crops. Studies have shown that GE insect protected field maize is less prone to mycotoxin contamination than conventional maize (Benbrook, 2005).

1.4.1 Mycotoxin diversity

The complex ecology of mould growth and mycotoxin production can give rise to more than one mycotoxin produced by one organism resulting in mixtures of different mycotoxins (Smith *et al*., 1994). Most fungal species can produce two to four mycotoxins. However, some species can produce as many as seven or twelve toxins. For example, *A. flavus* has the potential to produce up to nine different toxins. Some species use a single biochemical pathway to produce related toxins. Other fungi, such as *A. flavus,* utilise two or more different biochemical pathways to produce chemically diverse mycotoxins. The structures of some molecules are very simple while others are complex. Some related metabolites are biosynthesised via interconnected metabolic grids and a metabolic flux (Smith *et al*., 1994).

Co-production of mycotoxins has additive toxic effects and increases the toxigenic potential of the fungus. Contamination with several mycotoxins results in "synergism". Mycotoxin-mycotoxin synergism is defined as the combined effects of multiple mycotoxin ingestion in an animal. The summation of these effects is more

severe than would be predicted based upon the known effects of the individual mycotoxins. Mycotoxin-mycotoxin synergism can also be regarded as the combined action of multiple mycotoxin exposure that results in a unique condition in affected animals (Smith *et al*., 1994).

1.4.2 Aspergillus toxins

Some of the most frequently studied mycotoxins belong to the genus *Aspergillus*. These toxins are structurally diverse and sometimes they are produced in combination by a single species. Toxicity of these toxins is also different for each species and/or strain (Cotty *et al*., 1994).

1.4.2.1 Aflatoxins

Aflatoxins (***A****spergillus* ***fla****vus* **toxins**) are naturally occurring mycotoxins that are produced by many species of *Aspergillus*; but most notably *A. flavus* and *A. parasiticus* (Trail *et al.,* 1995). Aflatoxins are toxic and carcinogenic to animals, including humans. Aflatoxins often occur in crops in the field prior to harvest. Post-harvest contamination can occur if crop drying is delayed and during storage if water exceeds the critical value for mold growth (Trail *et al.,* 1995).

At least 18 different types of aflatoxins are produced in nature (Reddy and Waliyar, 2005). Aflatoxin B_1 (AFB_1) (Figure 2a) is considered the most toxic and is produced by both *A. flavus* and *A. parasiticus*. Aflatoxin B_2 (AFB_2) (Figure 2b) is less toxic that AFB_1 and is also produced by both *A. flavus* and *A. parasiticus*. Aflatoxin G_1 (AFG_1) (Figure 3a) and aflatoxin G_2 (AFG_2) (Figure 3b) are produced exclusively by *A. parasiticus* (Reddy and Waliyar, 2005). While the presence of *Aspergillus* in food products does not always indicate harmful levels of aflatoxins, it does imply a significant risk in consumption of that product. After entering the body, aflatoxins are metabolised by the liver to an intermediate reactive aflatoxin M_1 (Reddy and Waliyar, 2005).

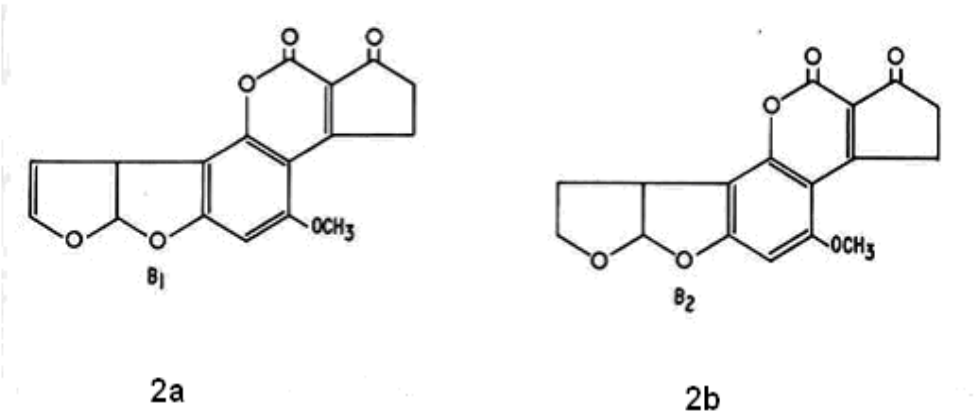


Figure 2a and 2b: Aflatoxin B_1 and aflatoxin B_2 structures (Reddy and Waliyar, 2005).

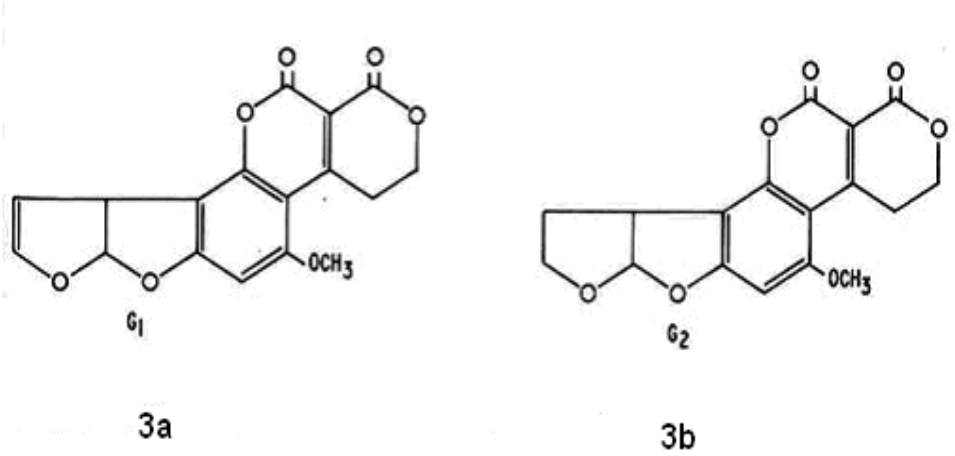
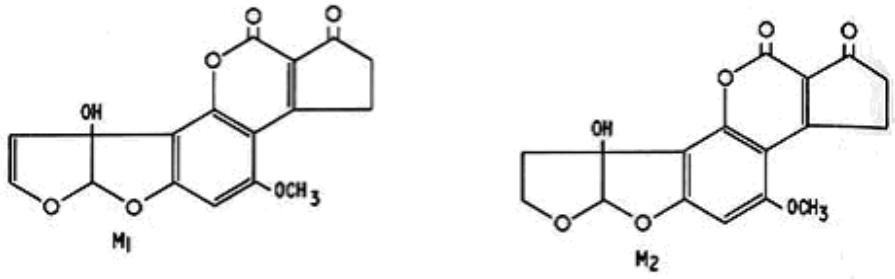


Figure 3a and 3b: Aflatoxin G_1 and aflatoxin G_2 structures (Reddy and Waliyar, 2005).

Aflatoxin M_1 (AFM_1) (Figure 4a) and aflatoxin M_2 (AFM_2) (Figure 4b) were first isolated from milk of lactating animals fed aflatoxin preparations; hence the M designation (Eaton and Groopman, 1994). The B designation of AFB_1 and AFB_2 resulted from the exhibition of blue fluorescence under UV light, while the G designation refers to the yellow-green fluorescence of the relevant structures under UV light (Eaton and Groopman, 1994).

These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring compounds. Their molecular formulas as established from elementary analyses and mass spectrometric determinations are: AFB_1- $C_{17}H_{12}O_6$; AFB_2- $C_{17}H_{14}O_6$; AFG_1- $C_{17}H_{12}O_7$; and AFG_2- $C_{17}H_{14}O_7$. $AFB_2$and AFG_2were established as the dihydroxyl derivatives of $AFB_1$and AFG_1, respectively. Whereas AFM_1 is 4-hydroxyl $AFB_1$and AFM_2 is 4-dihydroxyl AFB_2 (Eaton and Groopman, 1994).



4a 4b

Figure 4a and 4b: aflatoxin M_1 and aflatoxin M_2 structures (Reddy and Waliyar, 2005).

1.4.2.2 Biosynthetic pathway: enzymes and genes

Due to health and economic significance, impacts of aflatoxin contamination, the chemistry, enzymology and genetics of the pathway in *A. flavus* and *A. parasiticus* have been widely studied (Figure 5) (Bhatnagar *et al.*, 2003; Yu *et al.*, 2004a). Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* led to the cloning of 25 genes within a clustered 70kb DNA region (Yu *et al.*, 1995; Yu *et al.*, 2004a). Regulatory elements such as *aflR* and *afl*S (Meyers *et al.*, 1998; Chang, 2003), nutritional and environmental factors, fungal development and sporulation have also been found to affect aflatoxin formation (Yu *et al.*, 2004b).

Aflatoxin production usually begins during idiophase, upon completion of nutrients necessary for primary metabolism, although other conditions for induction exist (Trail *et al*., 1995). The main conditions/parameters that induce aflatoxin production are: moisture, pH, temperature and nutrients (Furtado *et al.*, 2005). Regulatory

mechanisms involved in the induction of aflatoxin biosynthesis are complex and not always fully understood. To study the regulation of the aflatoxin biosynthetic pathway at the molecular level, much effort has been focussed on purifying the enzymes involved. Isolating the genes which encode these enzymes has also been the focal point (Trail *et al*., 1995). The biosynthetic pathway consists of more than 18 enzymes.

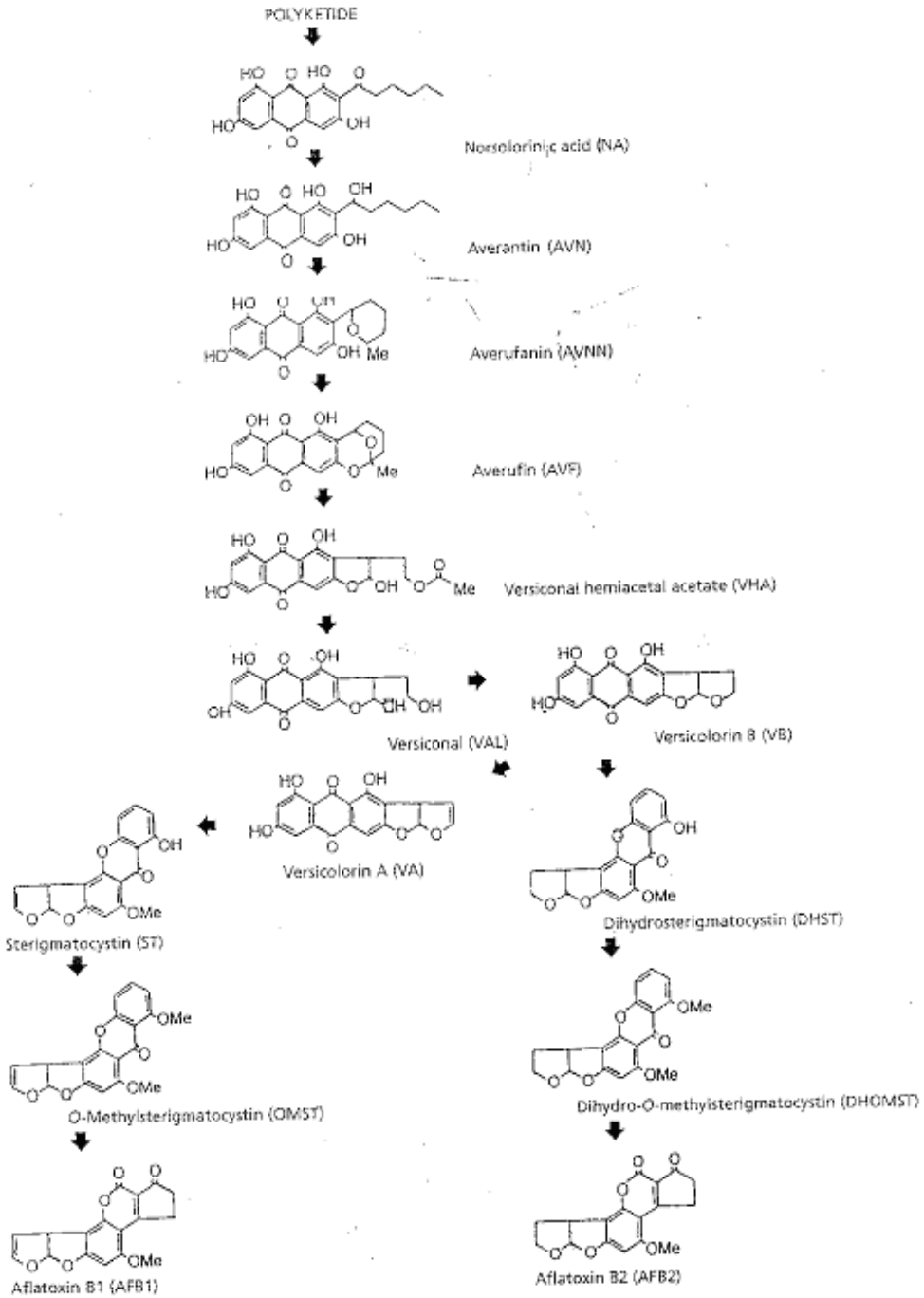


Figure 5: Aflatoxin B_1 and aflatoxin B_2 biosynthetic pathway (Trail *et al.*, 1995).
(key: **arrows** represent enzymatic reactions as seen in more detail in Figure 6)

Several enzymes involved in the aflatoxin pathway are reported to have been purified to homogeneity (Figure 6) (Trail *et al*., 1995). They include two separate *O*-methyltransferases involved in the conversion of sterigmatocystin (ST) to *O*-methylsterigmatocystin (OMST). Norsolorinic acid (NA) reductase or possibly two separate enzymes are involved in the reversible conversion of NA to averantin (AVN) (Trail *et al*., 1995). Cyclase is involved in the conversion of versiconal to versicolorin B. Two versiconal hemiacetal acetate reductases (VHA reductase I and II; probably isozymes) catalyse the reaction from versiconal hemiacetal acetate to versiconal acetate (Matsushima *et al*., 1994).

The purified enzymes provided important tools for cloning of genes. Cloning of genes involved in aflatoxin biosynthesis is the key to understanding the molecular biology of the pathway. Some genes coding for enzymes involved in the aflatoxin biosynthesis pathway have been cloned. These include: (i) the *pks*A gene which codes for a polyketide synthase (Yu *et al*., 1995); (ii) the *nor*1 gene which codes for a reductase that converts NA to AVN; (iii) the *ver*1 gene involved in the conversion of versicolorin A to ST; and (iv) the *omt*A gene (previously named *omt*1), coding for an *S*-adenosylmethionine-dependent *O*-methlytransferase that converts ST to OMST. Dihydrosterigmatocysin is then converted to dihydro-*O*-methlysterigmatocystin (DHOMST). The *omt*A gene has been cloned and characterized for both *A. parasiticus* and *A. flavus* (Yu *et al*., 1993).

Liu and Chu (1998) have successfully obtained both monoclonal and polyclonal antibodies against several key enzymes/proteins in the aflatoxin biosynthetic pathway. Effective immunochemical methods for sterigmatocystin methyltransferase (ST-MT), and norsolorinic acid reductase have also been established.

A putative fatty acid synthase gene, *uvm*8, potentially involved in polyketide backbone synthesis has been cloned. Another gene, *aad,* homologous to aryl-alcohol dehydrogenase potentially involved in an intermediate step of aflatoxin biosynthesis was isolated by the reverse genetics approach (Trail *et al*., 1995). This procedure was also used to clone the *omt*A gene from *A. flavus* (Yu *et al*., 1993).

In addition to these structural genes, a regulatory gene, *afl*R (previously named *afl*2 for *A. flavus* and *apa*2 for *A. parasiticus*), that codes for a regulatory factor (AFLR protein) has been cloned (Chang *et al*., 1993; Payne *et al*., 1993). It was shown to be involved in the activation of the transcription of pathway genes (Chang *et al*., 1993). The pathway genes: *nor*1, *ver*1 and *uvm*8 and the one regulatory gene, *afl*R were isolated using the genetic complementation approach (Trail *et al*., 1995).

To confirm the role of these genes in aflatoxin biosynthesis, recombinational inactivation (gene disruption) has been conducted on toxigenic strains of *A. parasiticus* (Trail *et al*., 1994; Liang and Linz, 1994). Disrupted strains retained their ability to produce low levels of aflatoxin. This supported the hypothesis that there is one or more alternative pathway routes (or enzymatic activities) in the aflatoxin pathway to synthesize AVN from NA (Yabe *et al*., 1993).

1.4.2.3 The aflatoxin gene cluster

Linkage of aflatoxin pathway genes was first evidenced in an *A. parasiticus* cosmid clone, NorA, that contained both *nor*1 and *ver*1 genes. A physical and transcriptional map of the 30 kb genomic DNA insert in cosmid NorA suggested that several genes are involved in the early stages of AFB_1 biosynthesis. These genes are clustered on one chromosome (Yu *et al*., 1995). However no complete physical maps involving all the identified aflatoxin pathway-related genes from both *A. flavus* and *A. parasiticus* have been reported (Yu *et al*., 1995).

The organisation and arrangement of the aflatoxin pathway genes responsible for early and later stages of biosynthesis are clustered on a 60 kb region of DNA (Yu *et al*., 1995). Based on restriction enzyme analysis, maps have been generated for *A. parasiticus* and *A. flavus* (Yu *et al*., 1995). Karyotyping studies have also confirmed that the aflatoxin pathway genes are clustered. Karyotype analysis of *A. parasiticus* confirmed that the *nor*1, *ver*1 and *omt*A were located on the same chromosome (Yu *et al*., 1995).

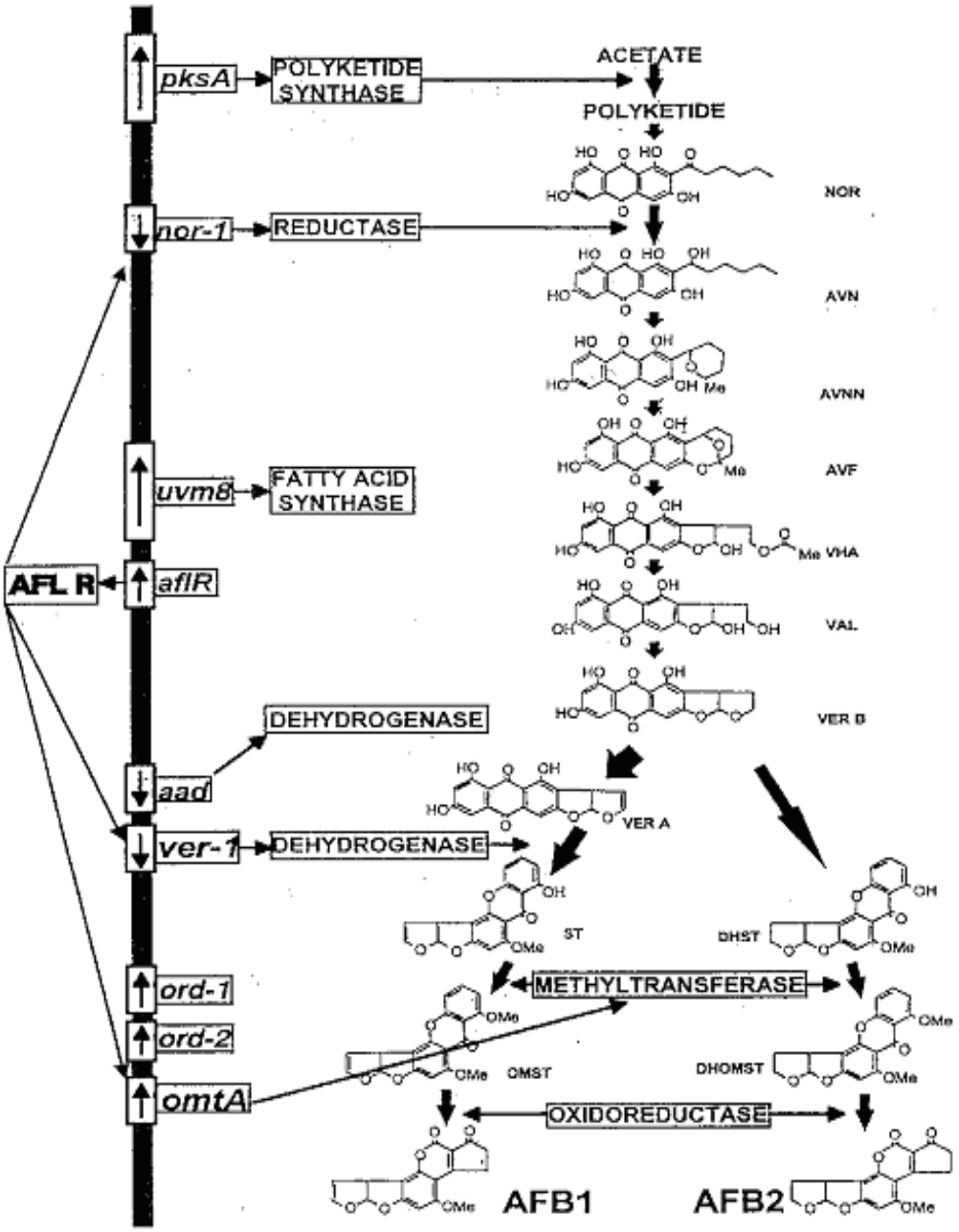


Figure 6: Enzymes and genes involved in $AFB_1$and AFB_2 biosynthesis (Yu *et al.*, 1995).

Studies of aflatoxigenic aspergilli indicate that these fungi have six to eight chromosomes ranging from 3 to >7 Mb. The order of most of the genes characterized in the gene cluster is co-incident with the order of the enzyme activities in the aflatoxin biosynthesis pathway encoded by these genes (Yu *et al*., 1995).

Studies on the expression and regulation of the aflatoxin biosynthetic regulatory and pathway genes involving *afl*R, *nor*1, *ver*1 and *omt*A showed that the transcription of *nor*1, *ver*1 and *omt*A is activated by the *afl*R gene product, AFLR. In other words, the transcription of the structural genes is dependent on the transcription of the *afl*R gene (Yu *et al*., 1995). The significance of the tight cluster of aflatoxin-biosynthetic gene is not known. No evidence has been obtained to suggest that the presence of the genes organized in a cluster confers any selective advantage for the survival of the organism. Aflatoxins do not deter the growth of competing organisms and also do not increase the ability of the organism to invade its hosts (Yu *et al*., 1995).

In *A. flavus* there are eight chromosomes with an estimated genome size of about 33-36 Mbp that harbour an estimated 12 000 functional genes (Yu *et al.,* 2004b). The global regulatory control of aflatoxin formation, *aflR* controlling gene and *aflS* expression, signal transduction, on/off aflatoxin production, genes involved in pathogenesis and survival of the fungus in nature still remain unknown (Yu *et al.,* 2004b).

1.4.2.4 Duplication of aflatoxin genes

In physical mapping studies of cosmid NorA, it became apparent that there are at least two copies of the *ver*1 gene, *ver*1A and *ver*1B, located in separate regions in the *A. parasiticus* genome (Liang and Linz, 1994). By comparing the restriction enzyme polymorphisms present in these two chromosomal copies with the cloned *ver*1 gene, it was confirmed that the gene cloned originally was *ver*1A. The *ver*1B gene was subsequently cloned and its nucleotide sequence determined. These genes were found to share 93% nucleotide sequence identity (Trail *et al*., 1995). A stop codon was identified near the middle of the predicted *ver1*B gene transcript suggesting that it may encode a truncated polypeptide that has little or no function (Trail *et al*., 1995).

A duplicated chromosomal region extending approximately 12 kb upstream from *ver*1A and *ver*1B was identified which also contains an additional copy of *afl*R (Liang and Linz, 1994). Duplication of *ver*1 and *afl*R genes in *A. parasiticus* may explain the higher stability of toxin production in *A. parasiticus* as compared to *A. flavus* where such duplication is not apparent. More than 90% of *A. parasiticus* isolates produce aflatoxins whereas 50% (or less) of *A. flavus* isolates are toxigenic.

1.4.2.5 Biosynthetic relationship between B- and G-group

It has been hypothesized that B-group and G-group aflatoxins are formed independently (Figure 7). The data obtained in a study by Yabe *et al*. (1999) supported this hypothesis. AFG_1 and AFG_2 were produced from OMST and DHOMST, respectively. Neither AFG_1 nor AFG_2 were produced from AFB_1 or AFB_2 in the cell-free systems used. These results indicated that the B-group aflatoxins are not the precursors of the G-group aflatoxins. The G- and B-group aflatoxins are independently produced from the same substrate (OMST for AFB_1 and AFG_1, and DHOMST for AFB_2 and AFG_2). They are also produced through different pathways from a common branching point although the branch point is not known (Yabe *et al*., 1999).

The *ord*1 and *ord*A genes were isolated from *A. flavus* (Prieto and Woloshuk, 1997) and *A. parasiticus* (Yu *et al*., 1998), respectively. These genes were found to be required for the conversion of OMST to AFB_1. AFB_1 and AFG_1 contain dihydrobisfuran rings whereas AFB_2 and AFG_2 contain tetrahydrobisfuran rings (Yabe *et al*., 1999).

1.4.2.6 Presence of the aflR gene in A. sojae and A. oryzae

While *A. flavus* and *A. parasiticus* are fungal contaminants of food and feed, and produce aflatoxins, in contrast, *A. sojae* and *A. oryzae* are used for industrial enzyme production. They are also used for fermented food production such as sake (rice wine), miso (bean paste), and shoyu (soy paste) in Eastern Asia (Matsushima *et al*., 2001b).

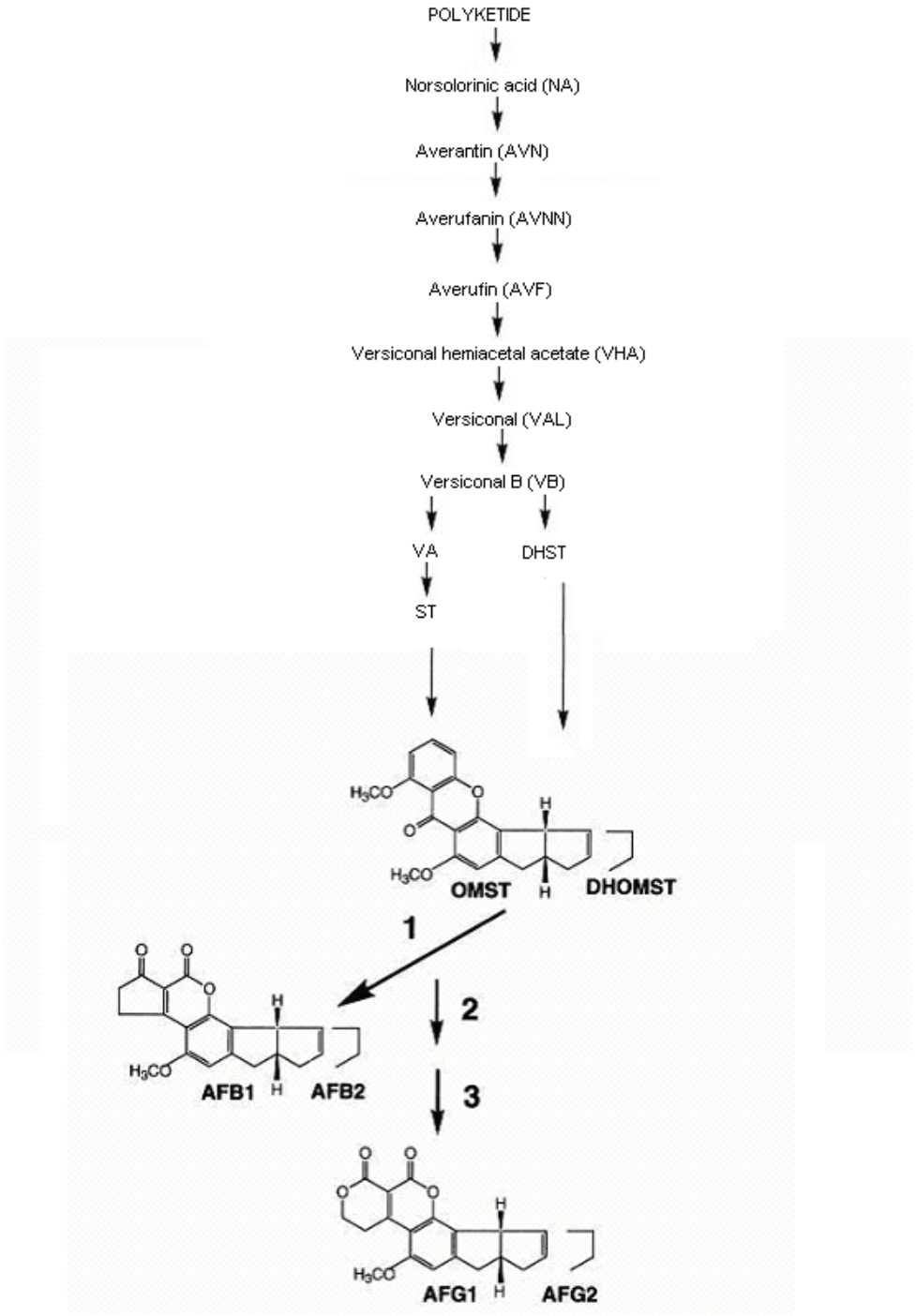


Figure 7: Metabolic pathways for formation of AFG_1 from OMST and formation of AFG_2 from DHOMST. At least three enzyme reactions may be commonly present in both pathways. Reaction 1 may be catalyzed by microsome cytochrome P-450 oxygenase. A transient intermediate formed during this pathway may be subsequently converted to another substance in reaction 2 and finally converted to the final product, AFG_1 or AFG_2, in reaction 3. Reactions 2 and 3 may be catalyzed by an unstable microsome enzyme and a 220-kDa cytosol protein (Yabe *et al*., 1999).

It is generally accepted that *A. sojae* and *A. oryzae* never produce aflatoxins under any culture conditions. However, because of their taxonomical similarity to aflatoxin-producing fungi, it is necessary and important to determine why food-grade *Aspergillus* species do not produce aflatoxins (Matsushima *et al*., 2001b).

The presence of *afl*R and *uvm*8 in the non-aflatoxin-forming *A. sojae* isolates may indicate that these mRNA are involved in some role related to the onset of secondary metabolism not directly connected to aflatoxin production (Klich *et al.,* 1997). Southern blots indicated that aflatoxin-pathway genes are present in the DNA of *A. sojae* and non-aflatoxigenic *A. parasiticus* (Klich *et al.,* 1995; Klich *et al.,* 1997). Results of the Northern blots indicate that these pathway genes are transcriptionally blocked in the *A. sojae* and non-aflatoxigenic *A.parasiticus* isolates (Klich et al., 1997).

In aflatoxigenic *Aspergillus* species *afl*R is the main transcriptional regulatory gene. The *afl*R deficiency causes the loss of expression of aflatoxin biosynthesis-related genes leading to lack of aflatoxin production (Chang *et al*., 1995). It has been concluded that the loss of *afl*R expression in *A. sojae* is likely responsible for the inability of these species to produce aflatoxins.

The *afl*R sequence of *A. oryzae* is identical to that of aflatoxigenic fungi (Watson *et al*., 1999), however, it also lacks the *afl*R transcription regardless of the presence or absence of the aflatoxin gene cluster (Kusumoto *et al*., 1998; Kusumoto *et al*., 2000). Lack of *afl*R transcription in *A. sojae* and *A. oryzae* has been suggested to be due to a pre-mature stop codon that de-activates *afl*R transcription (Matsushima *et al*., 2001a).

Watson *et al*. (1999) reported two characteristic features in the *afl*R homologue of *A. sojae* (when compared with the *afl*R of *A. parasiticus*). The *afl*R homologue of *A. sojae* contains: (i) a duplication of the histidine and alanine residues at positions 111-114 (HAHA motif), and (ii) a C→T transition that replaces Arg-385 with a stop codon, leading to truncation of the carboxyl-terminal region by 62 residues. The truncation in the *A. sojae afl*R may hamper its effectiveness as a transcriptional activator (Matsushima *et al*., 2001b).

1.5 Quantification of aflatoxins

1.5.1 High Performance Liquid Chromatography (HPLC)

Chromatography comprises all separation techniques in which analytes partition between different phases that move relative to each other or where the analytes have different migration velocities (Neue, 1997). Preparative HPLC refers to the process of isolation and purification of compounds. The degree of solute purity and throughput (amaount of compound produced per unit time) is important. This differs from analytical HPLC, where the focus is to obtain information about the sample compound (Wellinder *et al.,* 1995). HPLC comprises all liquid chromatographic techniques and it requires the use of elevated pressures to force the liquid through a packed bed of the stationary phase.

The degree or extent of separation is mostly determined by the choice of stationary phase and mobile phases (Fente *et al.,* 2001). Stationary phases for aflatoxins (B and G derivatives) are mainly C18 materials. In practice, there is only a limited choice of solvents for optimizing selectivity in reversed-phase HPLC. Methanol, acetonitrile, and tetrahydrofuran generally provide enough selectivity changes to deal with most samples (Neue, 1997). Methanol/water/acetonitrile is the common choice of mobile phase for the detection/quantification of aflatoxins (Cary *et al.,* 2005; Fente *et al.,* 2001).

Some substances cannot be detected by HPLC because they do not contain the necessary fluorophoric groups. However, this problem can be overcome by inducing derivatization reactions (add reagents for fluorometric detection). These rections produce stable derivatives rapidly (Mitamura, 2001). Derivatization reaction is often required in order to increase sensitivity and selectivity of the method. Derivatization can be achivied by use of detection methods such as fluorescence or absorption in visible light, at long wavelength. HPLC derivatization plays an important role in the detection of aflatoxins. For the detection of aflatoxins, derivatisation is performed with strong acids and oxidants resulting in an increase of fluorescence. Fluorescence detection is possible for aflatoxin B_1 at 474nm (emission) and 365nm (excitation) (Cary *et al.,* 2005). It is therefore important to quantify these compounds in a sensitive and specific way (Mitamura, 2001).

The migration of the compounds and contaminants through the column need to differ so that the the pure desired compound can be collected without incuring any other undesired compounds (Wellinder *et al*., 1995).

Identification of compounds by HPLC is crucial and a detector must be selected first; optimal detection settings and a separation assay must be developed. The parameters of this assay should be such that a clean peak of known sample (standard) is observed from the chromatogram. A standard must be utilized in order to assure to identification of the unknown compound. Quantification is the process of determining the unknown concentration of a compound in a known solution (Wellinder *et al*., 1995).

HPLC has also become a method of choice in the pharmaceutical industry. From basic research to the release of the final product, HPLC is interlaced in the chain of analyses more than any other technique. HPLC derives its strength from its versatility, the ease with which it can be learned and applied, its ruggedness and the relative ease with which it can be interfaced with a wide variety of detection schemes (Neue, 1997).

Fente *et al* (2001) used HPLC to confirm the presence aflatoxin in strains of *A. flavus, A. parasiticus* and *A. nomius.* They added cyclodextrin to common media to enhance the natural fluorescence of aflatoxins. Aflatoxin production co-incided with the presence of a blue-green fluorescent area surrounding colonies when observed under UV light. The presence of aflatoxins was confirmed by extracting the medium with chloroform and examining the extracts by HPLC. Cary *et al*. (2005) also used HPLC to confirm and quantify aflatoxins. They characterized aflatoxin production by fungi that do not belong to the *Aspergillus* section *Flavi.* HPLC results showed that the aflatoxin B_1 production was two-fold less for *A. ochraceoroseus* than that observed in the section *Flavi.*

1.6 Molecular analysis

A variety of molecular techniques have been used to classify the *Aspergillus* genus. These methods include: characterization of secondary metabolites, DNA complementarity, restriction fragment length polymorphism (RFLP) and randomly

amplified polymorphic DNA. Some of these techniques have been used successfully and thus improved the understanding of the *Aspergillus* genus.

1.6.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) has rapidly become one of the most widely used techniques in molecular biology. It is rapid, inexpensive and simple. It produces large numbers of copies of DNA molecules from minute quantities of source DNA material (Bloom, 1999). This process is called amplification. The PCR method was devised by Mullis and colleagues (Mullis, 1990). PCR is versatile. Most PCR uses DNA as target, rather than RNA, because of the stability of the DNA molecule. The essential criterion for any DNA sample is that it contains at least one intact DNA strand. This strand should encompass the region to be amplified (Bloom, 1999).

Medical research and clinical medicine are profiting from PCR mainly in two areas: detection of infectious disease organisms and detection of variations/mutations in genes. PCR can easily distinguish among the tiny variations in DNA, this method is also leading to new kinds of genetic testing. PCR is also useful when searching out disease organisms that are difficult to culture, such as many kinds of bacteria, fungi and viruses. For example, PCR can detect the AIDS virus sooner – during the first few weeks after infection (Zaman *et al.,* 2002).

Many authors have reported detection of *Aspergillus* nucleic acids by PCR for improved diagnosis of invasive aspergillosis (Rantakokko-Jalava, 2003). Detection of circulating *Aspergillus* DNA has shown high sensitivity but poor specificity in the screening of high risk patients for the development of this disease.

The nested PCR assay is a feasible and specific method to detect *Aspergillus* DNA in blood samples of mice when fungi are present in large numbers in parenchymal organs. The sensitivity of the PCR assay varies and depends on the fungal burden (Hummel *et. al.,* 2004). PCR also detects fungal DNA independently of viability or phagocytosis. PCR assay have been found to be more sensitive compared to cultures of blood in detecting *Aspergillus* in disseminated invasive aspergillosis (Hummel *et. al.,* 2004). In a study by Motomura *et al.* (1999) cloning of the *dmtA* gene (from *A. parasiticus* NIAH-26) was accomplished by using PCR strategies. These strategies

are: conventional PCR based on the *N*-terminal amino acid sequence of the purified enzyme, 5' and 3' rapid amplification of cDNA ends PCR (RACE) and thermal asymmetric interlaced PCR (TAIL-PCR).

1.6.1.1 Randomly amplified polymorphic DNA (RAPD) analysis

Randomly amplified polymorphic DNA (RAPD) analysis is a PCR-based technique that was developed over two decades ago (Welsh and McClelland, 1990) to detect polymorphisms in genomic DNA. This technique relies on the presence of priming sites on the genome, close enough to permit PCR amplification using single primers of arbitrary nucleotide sequence. RAPD have shown grest potential for identifying many pathogens. This technique, unlike the traditional PCR analysis, does not require any specific knowledge of the DNA sequences of the target microorganism/organism (Power, 1996).

The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR (Bardakci, 2001). Amplification products are generally separated on agarose gels and stained with ethidium bromide. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites (Bardakci, 2001).

Single base variations in the DNA where the primer binds or deletion/insertions between the primer and the binding sites give an altered PCR pattern and therefore a different DNA fingerprint. Resolving the fragments on a gel allows side-by-side comparison of different individual genotypes (Qiagen, 2001).

The simplicity, speed, efficiency and low cost of the RAPD technique has led to a wide range of applications in many areas of biology. One of the applications in the identification of markers linked to traits of interest without the necessity for mapping the entire genome. Another application is to establish whether isolates of a given

microorganism from different or from a single patient are related (San-Millan *et al.*, 1997).

RAPD analysis has been successfully used in examining DNA relatedness of species of animals, plants, fungi and bacteria. A possible relationship between the ability of *A. fumigatus* strains to invade tissues and genetic polymorphism was studies by RAPD analysis (Mondon *et al.*, 1995). A 100 randomly designed oligonucleotide decamers were examined with DNA of three reference strains, eight environmental isolates and 21 isolates from two distinct clinical situations (non-invasive and invasive aspergillosis). One primer was found to generate a reproducible amplification product that enabled distinction between two groups according to the presence or absence of a 0.95kb fragment. This fragment correlated with the nature of the infection and immune status of the patient.

A. parasiticus and *A. sojae* have been distinguished by RAPD analysis (Yuan *et al*., 1995). Single primers with arbitrary sequences were used to generate RAPD markers from strains of these two species. Three decamers, OPA-04, OPB-10 and OPR-01, allowed adequate discrimination between strains of *A. parasiticus* and *A. sojae* in RAPD analyses. *A. sojae* was further separated into group A and group B when amplified with OPA-04 and OPR-01 primers (Yuan *et al*., 1995). Some previously misidentified and misclassified strains were identified on the basis of RAPD patterns and morphological characteristics (Yuan *et al*., 1995).

The principal limitations of RAPD technique arise from its sensitivity to reaction conditions. Slight changes in the PCR conditions may affect the reproducibility of the amplification products. The most important factor for reproducibility of RAPD profile has been found to be the result of inadequately prepared template DNA. Differences between the template DNA concentration of two individuals' DNA samples result in the loss or gain of some bands (Bardakci, 2001).

1.6.2 Dendograms

Dendograms, also called tree diagrams, are one of the preferred graphical tools for presenting the results of genetic diversity studies. These studies generally involve comparing genetic fingerprints from a set of individuals and obtaining a genetic

similarity matrix. They also involve applying a hierachical clustering algorithm to this matrix to obtain the estimated dendogram (Martinez *et al.*, 2001).

These clusters may be grouped into larger sets and so on, until all points are eventually united into one cluster. The higher the level of aggregation the less similar are the objects in the respective cluster. These methods for cluster analysis are called hierarchical. The results of hierarchical classification can be represented graphically by a dendogram. Dendograms are computer-based techniques (Vandev, 1996).

The analysis of dendograms is usually based on data matrix of the form where the rows refer to individuals and the columns refer to attributes of the individuals. The nature of the data may be very broad, and the data may be continuous, discrete, or qualitative (Masumbuko and Bryngelsson, 2000).

Measurement of similarities can be done in a number of ways: (1) Single linkage – minimum distance between points in different clusters. It tends to produce long chains of clusters; (2) Complete linkage – maximum distance between points. It tends to produce compact, spherical clusters; (3) Mean linkage – average of all distances. Compromise between single and complete linkage; (4) Distance between centroids – each cluster is represented by the cluster centroid. Single, complete and mean linkages produce monotonic dendograms (Speed, 2003).

1.6.3 Restriction Fragment Length Polymorphism (RFLP) analysis

DNA consists of nucleotide bases that comprise genes (coding regions) and the region between genes (non-coding regions). Variation in the DNA sequence composition of individuals can be detected by restriction endonucleases (RE). These enzymes recognize specific short (four-to-eight) nucleotide base sequences of DNA and cleave the DNA strands at these points (Silver, 1995). Using gel electrophoresis, an electric current is produced across the length of an agarose gel. With most organisms the individual fragments cannot be seen after electrophoresis because there are so many of them, thus producing a smear in the agarose gel (Silver, 1995).

Ethidium bromide staining is used to reveal the fragments under UV (260nm) light. Differences result from base substitutions, additions, deletions or sequence rearrangements within RE recognition sequences (Avise, 1994).

RFLP analysis has been used in recent years to type isolates of *A. fumigatus*. Semighini *et al*. (2001) evaluated RFLP markers identified from PCR products amplified by the RAPD primer R108. These markers provided additional probes for *A. fumigatus* typing. Both the R108-primed RAPD analysis and RFLP assays were used to determine the genetic relatedness of clinical isolates of *A. fumigatus*.

Mitochondrial DNA (mtDNA) RFLPs have been described for 64 isolates representing 11 species of *Aspergillus* Section *Flavi*. mtDNA haplotypes were identified following digestion of total cellular DNA with REs *Hae*III, *Ase*I or *Dra*I (Quirck and Kupinski, 2002). Isolates of the same species possessed identical mtDNA haplotypes. Mitochondrial DNA (mtDNA) haplotypes complemented traditional morphological and growth criteria in making taxonomic decisions within *Aspergillus* Section *Flavi* (Quirck and Kupinski, 2002).

RFLP assays have been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridization, is, in general, time consuming and laborious (Bardakci, 2001).

Genetic divergence in the GTMAS:gk (germplasm with resistance to aflatoxin contamination) maize population has been assessed using RFLP DNA markers (Guo *et al.,* 2002). Eleven self inbred lines were assayed for DNA polymorphism using 113 RFLP markers. Considerable variations were detected with RFLP markers. Three polymorphic groups were distinguished by cluster analysis (Guo *et al.,* 2002).

With so many REs available for RFLP analysis, one should choose very carefully which RE they want to use. Obviously, cost is an important consideration. Another consideration is whether the enzyme is optimally active with genomic DNA. However, a critical consideration is the rate at which RFLPs can be detected based on the enzyme that is chosen (Silver, 1995).

1.6.3.1 Southern blotting

Southern blotting is named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. Southern blotting is also known as RFLP with hybridization (RFLPH). Southern blotting/hybridization is the identification of DNA sequences by separation on gels and the binding of specific complementary probe sequences by base-pairing (Alcamo, 1999; Brown, 1998a; Brown, 1998b; Morange 1998; Tait, 1997). Southern blotting is designed to locate a particular sequence of DNA within a complex mixture. For example, Southern blotting could be used to locate a particular gene within an entire genome pairing (Alcamo, 1999; Brown, 1998a; Brown, 1998b; Morange 1998; Tait, 1997). The amount of DNA needed for this technique is dependent on the size and specific activity of the probe. Short probes tend to be more specific.

RFLPH may be used to determine if isolates have regions with similar DNA sequences (Figure 8a). After electrophoresis the bands appear as a smear because there are so many, the DNA fragments are then denatured into single strands and then transferred to a nitrocellulose membrane. A previously prepared sample of DNA (termed the probe) is then radiolabelled. This probe is then mixed with the membrane bound DNA under conditions which favour hybridization between fragment with identical or very similar DNA sequences. The membrane is then washed to remove excess probe and regions of hybridisation visualized by autoradiography (Alberts *et al.*, 1998).

Traditionally, a nitrocellulose membrane is used, although nylon or a positively charged nylon membrane may be used. Nitrocellulose typically has a binding capacity of about 100µg/cm, while nylon has a binding capacity of about 500µg/cm pairing (Alcamo, 1999; Brown, 1998a; Brown, 1998b; Morange 1998; Tait, 1997). Most scientists feel nylon is better since it binds more and is less fragile.

Southern blotting experiments are usually intended to determine not only the presence of a particular DNA fragment, but also the size of this fragment. The proportion of common bands in an RFLPH can be used as a measure of relatedness between isolates pairing (Alcamo, 1999; Brown, 1998a; Brown; 1998b; Morange 1998; Tait, 1997).

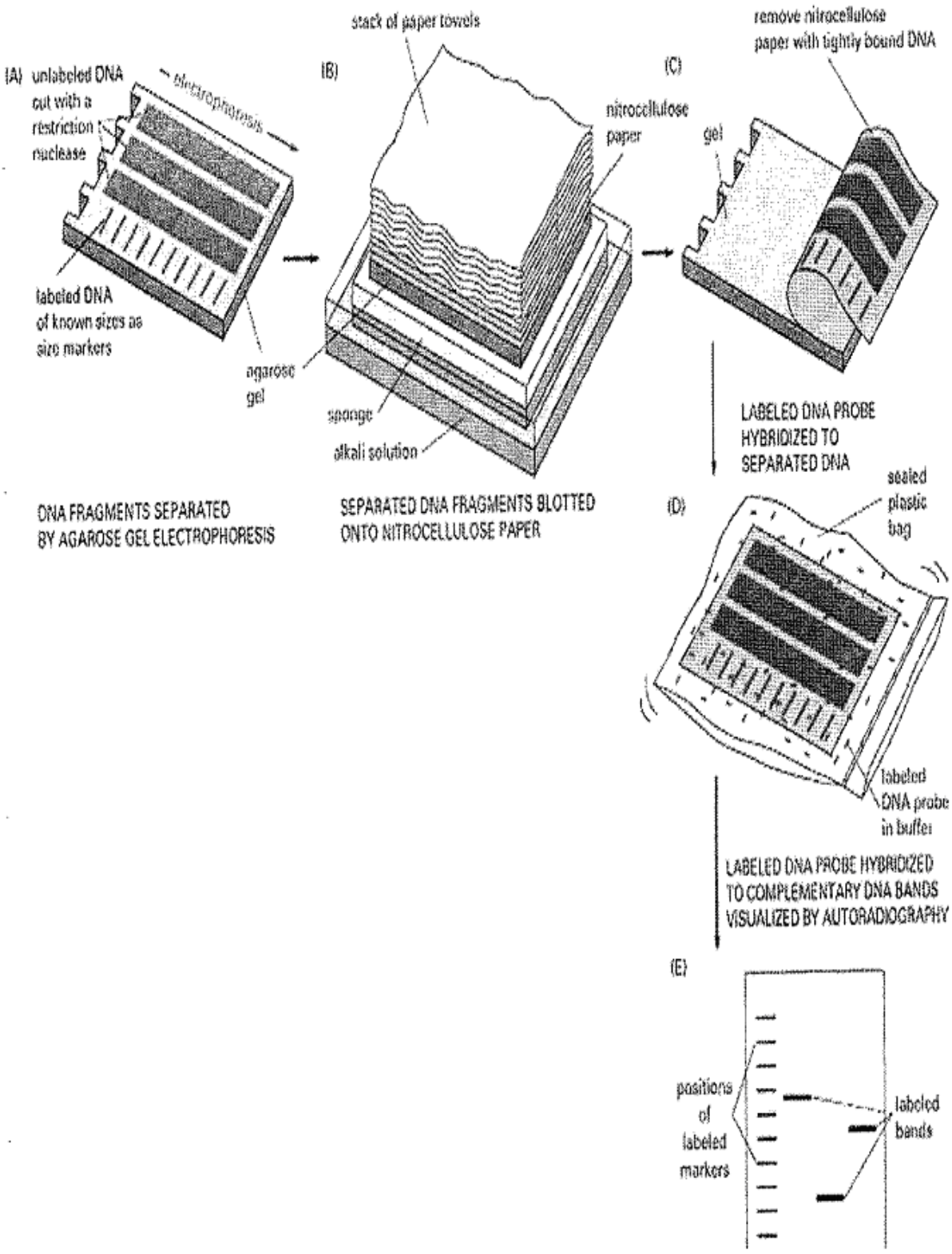


Figure 8a: Southern blotting: Gel transfer (Alberts *et al.*, 1998).

CHAPTER 2: MATERIALS AND METHODS

2.1 List of materials used in the study

All materials and methods in this study were handled/carried out in accordance to Good Laboratory Practise (GLP) so as to avoid accidents and contamination. The companies where the materials were purchased from are found within the text of this chapter.

1. *Aspergillus* fungal cultures	26. Aflatoxin standards	51. Whatmann paper
2. SAB Agar/Broth	27. Printer	52. Glass pipettes
3. SMKY broth	28. Mortar and pestle	53. Glass plates
4. Shaking incubator	29. Biofuge and microtubes	54. Paper towel
5. Separating funnels	30. Phenol; isoamyl alcohol	55. Incubator (37ºC)
6. Methanol; Hexane	31. Micropipettes and tips	56. PCR primers
7. Dichloromethane; chloroform	32. Weighing scale	57. GFX PCR Gel band purification kit
8. Abolute ethanol; ethanol	33. UV spectrophotometry	58.Razor blade; spatula
9. RNaseA; liquid nitrogen	34. Agarose	59. Vortex
10. Water bath	35. Power pack	60. DIG DNA labeling and detection kit
11. Rotary evaporator	36. Ethidium bromide	61. DIG Easy hybridization buffer
12. Volumetric flasks	37. Latex gloves; goggles	62. Fume cupboard
13. Measuring cylinders	38. UV transluminator	63. pH meter; Petri dishes
14. Microscope	39. COHU camera	64. SDS; Tween 20
15. Slides and covers	40. RAPD primers	65. Dimethyformamide
16. Vac-Elut system	41. Scion image software	66. Foil and cotton wool
17. Sep-Pak cartridges	42. PCR machine; dNTPs	67. Distilled water; Ice
18. Acetone; Toluene	43. Sodium hydroxide	68. Aotuclave; Microwave
19. Maleic acid; EDTA	44. T*aq* buffer and enzyme	69. Hybridization oven
20. Acetonitrile	45. Dendogram software	70. Fridge/Freezer; Cold room
21. Glass vials	46.Restriction enzymes	71. Round-bottom flasks
22. Erlenmeyer flasks	47. Hydrochloric acid; Tris	
23. Nitrogen gas	48. Sodium chloride	
24. Trifluoroacetic acid	49. SSC	
25. HPLC system	50. Nylon membrane	

2.2 Fungal isolates

The fungal isolates used in this study all belong to the genus *Aspergillus.* They were obtained from Plant Protection Research Institute (PPRI), American Type Culture Collection (ATCC) and the M.L. Sultan Technikon (now known as Durban University of Technology) Department of Biotechnology and Food Technology culture collection. They were sub-cultured on sabouraud dextrose agar (Biolab, SA) for 5-7 days at 25°C. These were later stored at 4°C. The fungal isolates that were used in this analysis are listed in Table 3.

Table 3: *Aspergillus* isolates used in the study

ISOLATE	DESIGNATION	HOST	LOCALITY
Aspergillus niger	MLST	Unknown	Unknown
A. oryzae	ATCC 3151	Unknown	Unknown
A. tamarii	MLST1	Unknown	Unknown
A. flavus	ATCC 32592	Unknown	Unknown
A. flavus	PPRI 5065	Facial cream	Gauteng (Pretoria)
A. flavus	PPRI 7052	CSIR 1413 vd Riet	Unknown
A. parasiticus	ATCC 11970	Unknown	Unknown
A. parasiticus	PPRI 2885	*Watsonia marginata* (flower)	Unknown
A. parasiticus	PPRI 3641	Paper (old photo)	Mpumalanga (Botshabelo)
A. parasiticus	PPRI 5063	*Chrysomelidae* (beetle)	Limpompo (Lapalala Nature Reserve)
A. parasiticus	PPRI 5183	*Aspalathus linearis* (rooibos)	Western Cape (Clanwilliam)
A. parasiticus	PPRI 5990	*Arachis hypogaea* (peanuts/groundnuts)	North West (Potchefstroom)

2.3 Aflatoxin analysis

2.3.1 Extraction

The sucrose magnesium sulphate potassium nitrate yeast extract (SMKY, pH 6.0) broth (100ml) (Appendix 5) was inoculated with a conidial spore suspension (1ml x 10^6 spores). The spore suspension was prepared in sterile water and calculated under microscope using a 0.100mm (depth) x 0.0025 (area) slide (Technicolor, South Africa). This mixture (SMKY with spore suspension) was incubated for seven days at room temperature (25ºC) with continuous shaking at 100rpm. Extractions were done in duplicate for all 12 isolates.

After incubation the culture was poured into a one litre separating funnel, 300ml methanol and 200ml dichloromethane were added. The mixture was shaken for one minute and the layers were allowed to separate. The dichloromethane layer (bottom) was transferred to a second one litre separating funnel containing 200ml distilled water. Extraction with dichloromethane was repeated in the first separating funnel and the dichloromethane layer was added to the second separating funnel. The mixture in the second separating funnel was shaken for 5-10 minutes and the layers were allowed to separate. The dichloromethane layer was collected and evaporated (in a water bath at approximately 60ºC) using a rotary evaporator (Buchi, Switzerland) until approximately 10ml of the sample was left in the flask.

Purification of the samples was carried out using the Vac-Elut system with Sep-Pak cartridges (Waters, Ireland). During the whole purification process, the cartridges were not allowed (at anytime) to run dry. This was achieved by stopping the flow of the solvent when there was only a few milliliters of solvent left in the cartridge. The process was carried out at a flow rate of 1.5ml/min. The cartridges were pre-conditioned with toluene (1ml), which was collected in a glass vial and discarded. Ten milliliters of the extracted sample was passed through the cartridge and the residue that did not bind to the cartridge was colleted in a glass vial and discarded. The cartridge was then washed with 10ml of toluene, collected in a glass vial and discarded. Ten milliliters of toluene:acetone (95:5) was also passed through (collected in a glass vial and discarded); followed by a washing with 6ml diethylether:hexane (3:1), collected in a glass vial and discarded. The toxin was then collected (in a clean/new 20ml glass vial) in 10ml chloroform:methanol (97:3) and this was retained.

The collected purified aflatoxin was concentrated by drying under a gentle stream of nitrogen gas (Afrox, SA) using the Reacti-Vap Evaporating unit (Pierce, USA). Dried aflatoxin samples were stored at 4ºC.

2.3.2 HPLC Analysis

2.3.2.1 The HPLC System

Quantification of aflatoxins was also done in duplicate for all 12 isolates. Aflatoxin quantification ws carried out by the method of Takahasi and Beebe (1979) with some modifications. HPLC was used for aflatoxin detection using the: La chrom D-7000 HPLC system manager, a L-7480 fluorescent detector, stainless steel column, in-line reverse phase C-18 guard column (2µm), autosampler (L-7200 Robot) and 150W Xenon lamp and pump (L-7100). The mobile phase was water:acetonitrile:methanol (75:15:10) and this was vacuum filtered with 0.45µm water membrane (Millipore Co-orporation, France) before it was used.

HPLC was initialized via the computer and the method was set up in the D-7000 HMS programme. The detector was set at a wavelength of 365nm (excitation) and 400nm (emission). The system was purged and flushed with ultra-pure water until a steady base line was obtained. The system was equilibrated at a flow rate of 1.5ml/min.

All the readings/chromatograms obtained were printed out (LaserJet printer, Japan) analyzed, calculated and the average (mean of the two results for each isolate) aflatoxin levels were obtained.

2.3.2.2 Preparation of aflatoxin standards for HPLC analysis

Aflatoxin standards (Sigma, Germany) of known concentrations were dissolved in chloroform and 20µl of the solution was dried under nitrogen gas in 10ml volumetric flasks. Hundred microliters TFA was added and this was mixed gently for a few minutes for the reaction to go to completion. The mixture was dissolved, diluted with five milliliters water:acetonitrile (9:1) and mixed well. Three hundred and fifty microliters of each standard was injected into the HPLC. The standard solutions were stored in the fridge when not in use.

For quantification, the standard curves of AFB_1, AFB_2, AFG_1 and AFG_2 (concentrations of 5, 10, 15, 20, 25, 30 and 35 ng/ml) were made.

2.3.2.3 Preparation of samples for HPLC analysis

Samples were also prepared in duplicate. The method for sample preparation is by Takahashi and Beebe (1979) with modifications. To the dry samples, 200µl hexane was added to dissolve the residues/dry sample and then 50µl of trifluoroacetic acid (TFA) (Sigma, Germany) was also added to completely reconstitute the residues. TFA is frequently used as a buffer in liquid chromatography for separation of organic compounds. Five milliliters of water:acetonitrile (9:1) was also added. All solvents added to the sample were then mixed well together using a vortex for approximately 60 seconds. Three hundred and fifty microliters of this solution was then injected into the HPLC for analysis (Merck Hitachi, Tokyo, Japan).

2.4 Molecular Analysis

2.4.1 Isolation of genomic DNA from filamentous fungi

Genomic DNA was extracted from the *Aspergillus* isolates in duplicate using the method of Raeder and Broda (1985) with some modifications. Two hundred and fifty milliliters of Sabouraud dextrose broth (Biolab, SA) was inoculated with spores (1ml x 10^6) of appropriate fungal isolate. The spore suspension was prepared in sterile water and calculated under microscope using a 0.100mm (depth) x 0.0025 (area) slide (Technicolor, South Africa). The mixtures (SAB broth with spore suspension) were grown for 2-3 days at 25ºC on a shaking incubator at 100rpm (Labcon, Maraisburg).

Mycelia were harvested, rinsed with de-ionized water, dried and frozen in liquid nitrogen (Afrox, SA). The frozen mycelia were ground with a chilled pestle until a fine powder formed. The mycelial powder was weighed (Mettler PC 2000 Balancer, Switzerland), transferred into pre-weighed 2.5ml microcentrifuge tubes (Plastibrand, Germany) and suspended in extraction buffer [appendix 6 (1ml/0.1g dry mycelia)]. This was mixed gently (no vortexing) for 2 min. The mixtures were then gently extracted with 0.7 x vol phenol (Sigma, Germany) for 2 min. A 0.3 x vol chloroform:isoamyl alcohol [(24:1) Saarchem, SA] was added and mixed gently for 2 min.

The resulting mixtures were centrifuged in a Biofuge (Heraeus Instruments, Germany) for 30 minutes at 13 000 x *g*. The aqueous supernatants were transferred into clean 2.5ml microcentrifuge tubes and treated with 0.05 x vol RNaseA (appendix 7) (Roche, Germany). The mixtures were incubated at 37°C for 20 min then extracted with 1 x vol chloroform:isoamyl alcohol and centrifuged for 10 min at 13 000 x *g*. The upper aqueous supernatant was transferred to a clean microcentrifuge tube and precipitated with 2 x vol 100% absolute ethanol (Analytical Reagents, SA). The resulting DNA pellets were washed once with 2 x vol 70% ethanol, placed on ice for 5 min, centrifuged for 5 min, the excess fluid was removed and the DNA pellets were air dried.

The dry DNA pellets were dissolved in sterile TE buffer [appendix 8 (0.2ml/0.1g dry mycelia)]. DNA concentration was determined at 260nm and 280nm using the UV-Visible Spectrophotometer (Cary-Varian, Australia) and the Cary Win UV Simple Reads computer application. Electrophoresis was carried out at 80 V (2197 Power Supply – LKB Bromma, Sweden) for approximately an hour using 0.8% agarose gel (Appendix 11) (Conda Laboratories, SA). Gels were stained in 0.5µg/ml ethidium bromide [appendix 14 (Sigma, Germany)] for 15-20 min, de-stained under a steady stream of running water (for approximately two minutes). Stained gels were viewed under the UV transilluminator (Bachofer Laboratoruimsgeräte, West Germany) and photographed using a COHU High Performance CCD Camera (Japan) and the Scion Image Beta 4.0.2 computer package (Scion Corporation, Frederick).

2.4.2 Random Amplified Polymorphic DNA (RAPD) analysis

The primers (10 bases long) and other PCR materials used for RAPD analysis were purchased from Roche Molecular Biochemicals (South Africa). The primers that were used are listed in Table 4. PCR was carried out (in duplicate) with a Genius Thermal Cycler (Techne, England). Amplification was performed in 50µl reaction mixtures containing 100 µM of each deoxynucleotide triphosphate (dNTPs), 0.2 µM of primer, 1 U of T*aq* DNA polymerase, 1X T*aq* buffer and 10 ng of genomic DNA. PCR mixtures were subjected to 45 cycles (denaturation at 92ºC for 1 min, annealing at 35ºC for 1 min and extension at 72ºC for 2 min). A 10µl portion of each PCR product was eletrophoresed using 2% agarose gel (Appendix 12) and DNA marker IV (Roche,

SA) for approximately an hour at 100V. The gels were stained and de-stained, visualized and photographed (as described in section 2.4.1) for further analysis.

Table 4: RAPD primers used in the study

PRIMER	SEQUENCE
UBC 211	5'-GAAGCGCGAT-3'
UBC 213	5'-CAGCGAACTA-3'
UBC 204	5'-TTCGGGCCGT-3'
UBC 220	5'-GTCGATGTCG-3'
UBC 229	5'-CCACCCAGAG-3'
UBC 230	5'-CGTCGCCCAT-3'
UBC 246	5'-TATGGTCCGG-3'
UBC 270	5'-TGCGCGCGGG-3'
UBC 286	5'-CGGAGCCGGC-3'
UBC 285	5'-GGGCGCCTAG-3'
UBC 284	5'-CAGGCGCACA-3'
UBC 287	5'-CGAACGGCGG-3'
UBC 295	5'-CGCGTTCCTG-3'
UBC 300	5'-GGCTAGGGCG-3'

2.4.3 Dendograms

The gel images generated during RAPD analysis were analyzed using Gelcompar: 'Comparative Analysis of electrophoresis patterns', Version 4.0, Applied Mathematics (Kortrijk, Belgium). These analyses were performed at the University of Kwa-Zulu Natal (Molecular Biology Department – Medical School campus).

2.4.4 Restriction Analysis

The restriction endonuclease enzymes used for this study were purchased from Roche Molecular Biochemicals (South Africa) and are listed in Table 5. Restriction analysis was performed (in duplicate with all five restriction enzymes) in 15µl reaction mixtures containing 10µg of genomic DNA, 1U of restriction enzyme and 1.5µl of restriction buffer. The digestion or restriction process was allowed to take place overnight (approximately 20 hours) at 37ºC. The digested DNA was electrophoresed

in 0.8% agarose gel using DNA marker III (Roche, SA). The gels were stained, de-stained, visualized and photographed (as described in section 2.4.1).

Table 5: Restriction enzymes used in the study

RESTRICTION ENZYME	RECOGNITION SEQUENCE
*EcoR*I	G/AATTC
*EcoR*V	GAT/ATC
*Dra*I	TTT/AAA
*Hind*III	A/AGCTT
*Bgl*I	GCCNNNN/NGGC

2.4.5 Southern Blotting

After photographing, the restricted gels were rinsed in water, soaked in 200ml 200mM HCl for 10 min at RT (25ºC) then rinsed three times in water. The gels were soaked with occasional shaking in 200ml 0.5M NaOH/1.5M NaCl for 30 min at RT, rinsed in water and soaked (as above) in 200ml 1M Tris (pH 7.5)/1.5M NaCl. The gels were then rinsed in water and soaked in 200ml 20X SSC (appendix 17) for 5 min. The Hybond-N nylon membrane (Amershan Pharmacia Biotech, UK) was soaked in water for 5 seconds, then in 100ml 20X SSC for 5-10 min. One milliliter of 20X SSC was dropped on a clean glass plate, the gel was inverted onto the glass plate, the membrane was placed on top of the gel. The air bubbles were removed by rolling a clean 5ml glass pipette over the wet membrane (on top of the gel) until the air bubbles had disappeared. Three sheets of 3MM Whatman papers (Whatman International Ltd., England) were soaked in 100ml 2X SSC (appendix 15) for 1 min then placed on top of the membrane. A stack of paper towels (folded at least 10-15 times) were placed onto the Whatman papers and then another glass plate was placed on top. The transfer was allowed to take place overnight. The membrane was rinsed in 100ml 5X SSC (appendix 16), air dried and cross-linked (i.e. exposing the membrane to UV light for approximately 5 minutes).

2.4.6 PCR for Probe Preparation

Based on a study done by Chang *et al.*, (1995), two 20-bases long primers were chosen for probe preparation. One forward (5'-TCGGTACGTAAACAAGGAAC-3')

and one reverse primer (5'-TCTGATGGTCGCCGAGTTGA-3') were selected because they were specific for the *afl*R gene and produce a 450bp PCR product.

Primers were purchased from Integrated DNA Technologies, Inc. (Coralville). All 12 *Aspergillus* template genomic DNA were subjected to PCR (in duplicate) and all reagents were provided within the Roche Molecular Biochemicals (South Africa) PCR kit (except for sterile water). PCR amplification was carried out in a 50µl reaction mixture containing: 100µM dNTPs, 0.2µM forward primer, 0.2µM reverse primer, 1U T*aq* DNA polymerase, 1X T*aq* buffer and 10ng genomic template DNA. PCR mixtures were subjected to 45 cycles (denaturation at 92ºC for 1 min, annealing at 50.5ºC for 1 min and extension at 72ºC for 2 min).

After amplification, approximately 10µl of the PCR product was electrophoresed in 0.8% agrose, stained and de-stained (as described in section 2.3.1). The gel was visualized and photographed (as described in section 2.3.1). The gel band from the *A. parasiticus* PPRI 5990 isolate was then purified.

2.4.6.1 Probe/DNA Purification

Purification of DNA from the *A. parasiticus* PPRI 5990 isolate was done using the GFX PCR DNA and Gel band Purification Kit (Amershan Biosciences, England). An empty 1.5ml microfuge tube was weighed, and then using a clean razor blade, a slice of agarose containing the DNA to be purified was excised. This (cutting) was done as close to the DNA as possible. The slice was then cut into smaller pieces and transferred to the pre-weighed 1.5ml microfuge tube. This was then weighed.

All reagents (except for water and absolute ethanol) were provided within the GFX PCR DNA and Gel band Purification Kit (Amershan Biosciences, England). The purification process was carried out as per Kit's instruction manual. Ten microlitres of capture buffer (buffered solution containing acetate and chaotrope) for each 10mg of gel slice was added to the gel slice. The tube was then closed and this was mixed vigorously by vortexing for approximately 60 seconds. The mixture was incubated at 60ºC until the agarose was completely dissolved (approx. 5 min). The mixture/sample was transferred to a GFX column (placed in collection tube) then incubated at RT

(25ºC) for 1 min. The sample was centrifuged in microfuge at full speed for 30 seconds. The flow-through was discarded by emptying the collection tube.

The GFX column was placed back inside the collection tube. Five hundred microlitres of wash buffer (TE buffer, pH 8.0 diluted with absolute ethanol) was added to the column and this was centrifuged as previously mentioned in this section. The collection tube was discarded and the GFX column was transferred to a new/fresh 1.5ml microfuge tube. Approximately 25µl of elution buffer (double-distilled water) was added directly to the top of the glass fiber matrix in the GFX column. The sample was then incubated at room temperature for 1 min then centrifuged at full speed for 1 min for recovery of the purified DNA. The purified DNA was kept frozen when not in use.

2.4.6.2 Probe labelling

Labeling of the probe was carried out using the DIG DNA Labeling and Detection Kit purchased from Roche Molecular Biochemicals. 10 ng to 2g (as per Kit instruction manual) of purified DNA/probe and autoclaved double-distilled water were added up to a final volume of 15µl in a reaction vial. For a control labeling reaction, 5µl of control DNA and 10µl of double distilled water were added up in a reaction vial. The DNA in the both vials was denatured by heating in boiling water bath (100ºC) for 10 minutes and quickly chilled in ice/water bath. To the freshly denatured probe and control DNA: 2µl hexanucleotide mix, 2µl dNTP labeling mix and 1µl Klenow enzyme was added. This solution was mixed and centrifuged briefly (13 000 x *g* for 30 seconds). The mixture was incubated overnight (approximately 20 hours) at 37ºC. After incubation the reaction was stopped by adding 2µl of 0.2M EDTA (pH 8.0).

2.4.6.3 Determination of labeling efficiency

A dilution series of the labeled probe and control DNA was prepared as shown in Table 6. The additional reagents (not provided within the kit) that were used in this this labelling are shown in Appendix 18 and 19. Buffer volumes (approximately 20ml or enough to cover the membrane completely during all steps) were used during direct detection. A 1µl spot of tubes 2-9 from the labeled probes and the labeled control DNA was applied to a small strip of nylon membrane. The nucleic acid was then fixed to the membrane by cross-linking with UV light (3-4 min). The membrane was

transferred into a plastic container with 20ml maleic acid buffer and was incubated under shaking for 2 min at 15-25ºC. After 2 min the membrane was incubated for 30 min into 20 ml blocking working solution then incubated for 30 min in 20 ml antibody solution. The membrane was then washed for 2 x 15 min in 20ml washing buffer.

The membrane was equilibrated for 2-5 min by incubating in the 20ml detection buffer then incubating in 20ml freshly prepared colour substrate solution in a dark-coloured container with a lid. The contents were stored in the dark and not shaken during colour development. The membrane was only exposed to light for short periods of time to monitor colour development. The reaction was stopped (when desired spot intensities were achieved) by washing the membrane for 5 min with sterile double distilled water. Results were documented by photocoping.

Table 6: Dilution series of the labeled probe and control DNA

Tube	**DNA (µl)**	**From tube #**	**DNA Dilution buffer (µl)**	**Dilution**
1		original		1:100
2	5	1	495	1:3.3
3	15	2	35	1:10
4	5	2	45	1:10
5	5	3	45	1:10
6	5	4	45	1:10
7	5	5	45	1:10
8	5	6	45	1:10
9	0	-	50	-

2.4.6.4 Hybridization

Hybridization was done in duplicate. A volume of 20 ml/100 cm^2 membrane/filter of the DIG Easy Hybridization buffer (Roche Molecular Biochemicals, South Africa) was pre-heated to hybridization temperature (45°C). The membrane was then pre-

hybridized (with the buffer – 50ml) for 30 min with gentle agitation in temperature-resistant plastic bags. The membranes were allowed to move freely within the bags.

Approximately 25ng/ml of DIG-labelled DNA probe was denatured by boiling for 5 min and rapidly cooled in ice. The denatured DIG-labelled DNA probe was added to the pre-heated DIG Easy hybridization buffer (with membrane). This was mixed thoroughly (foaming was avoided because it leads to background). The mixture was then incubated overnight (approximately 20 hours) with gentle agitation at 45°C.

After hybridization overnight, stringency washes were performed. The membrane was washed twice for 5 min in 50ml 2x SSC, 0.1% SDS at room temperature (25°C) under constant agitation. The membranes were then washed in 50ml 0.5x SSC, 0.1% SDS (pre-warmed to wash temperature) at 45°C under constant agitation.

All reactions that were carried were done under constant agitation. After hybridization and stringency washes, the membranes were rinsed briefly (1-5 min) in 50 ml washing buffer. After washing, the membranes were incubated for 30 min in 50ml blocking solution then incubated for another 30 min in 50ml antibody solution. The membranes were then washed twice for 15 min in 50ml washing buffer. The membranes were then equilibrated for 2-5 min in 50ml detection buffer. The membranes were incubated in 50 ml freshly prepared colour substrate solution in a dark coloured container with a lid in dark cabinet/room. During colour development the container was not shaken.

The membranes were exposed to light for short time periods for colour development monitoring. The reaction was stopped, when the desired band intensities were achieved, by washing the membrane for 5 min with distilled water. The membranes were not allowed to dry at any time (for re-probing purposes). They were stored in 50ml TE buffer.

2.4.6.5 Stripping and re-probing of DNA blots

Approximately 100ml of dimethylformamide (DMF) in a glass beaker was heated to 55ºC in a water bath under a fume hood. The membranes were then incubated in the

heated DMF until the blue colour precipitate was removed from the membranes. The membranes were then rinsed briefly in 100ml double distilled water. Then the membranes were washed for 2 x 20 min in 50 ml 0.2N NaOH, 0.1% SDS, at 37ºC under constant agitation, then equilibrated briefly in 2 x SSC (50ml). After these steps, the membranes were used directly for hybridization (as described in section 2.4.6.4).

CHAPTER 3: RESULTS

3.1 Aflatoxin Analysis

Aflatoxins were analysed using HPLC and this was done after two to three day of incubation (based on a study by Fente *et al*., 2001). All 12 *Aspergillus* isolates were analyzed (in duplicate) for aflatoxin production (AFB_1, AFB_2, AFG_1 and AFG_2). The mean (average) of the two results obtained was then used in the analysis as shown in Table 7 (readings/levels from each sample are shown in appendix 4b). Mean aflatoxin concentration levels obtained were divided into four categories (based on a previous study by Geiser *et al*., 2000), i.e. not detectable, barely detectable, moderately detectable and detectable by HPLC. The aflatoxin concentrations detected are also shown graphically in Figure 8b to figure 8e (for AFB_1, AFB_2, AFG_1 and AFG_2).

Table 7: Aflatoxin concentration levels detected

FUNGAL ISOLATES	**MEAN AFLATOXIN CONCENTRATIONS (µg/ml)±SD**				***MEAN TOTAL AFLATOXIN**
	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	
A. niger MLST	0	0	0	0	0
A. oryzae ATCC 3151	0	0	0	0	0
A. tamarii MLST1	0	0	0	0	0
A. flavus ATCC 32592	0	5.1±0.13	0	0	5.1
A. flavus PPRI 5065	0	0	0	0	0
A. flavus PPRI 7052	0	0	0	0	0
A. parasiticus ATCC 11970	0	23.7±0.60	0	0.010±0.01	23.71
A. parasiticus PPRI 2885	5.5±0.0004	36.7±0.32	0	28.3±0.13	70.5
A. parasiticus PPRI 3641	0.021±0.03	13.2±0.36	6.5±0.12	0.049±0.03	19.77
A. parasiticus PPRI 5063	6.4±0.16	25.6±0.25	0	0	32.0
A. parasiticus PPRI 5183	0.012±0.03	4±0.15	0	0	4.012
A. parasiticus PPRI 5990	0	22.7±0.06	0	0	22.7

*** Mean – average of two readings**

3.1.1 Aflatoxin B_1 (AFB_1)

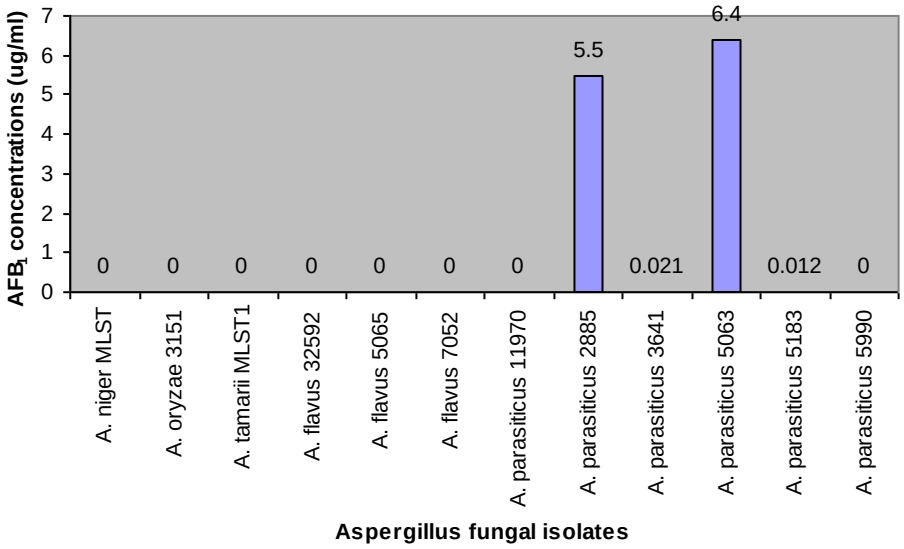


Figure 8b: AFB_1 concentrations detected by HPLC from12 *Aspergillus* isolates.

Four of the 12 isolates analyzed produced AFB_1 (as shown in Table 7 and Figure 8b). None of the three *A. flavus* isolates analyzed (i.e. *A. flavus* 32592, 5065 and 7052) produced any detectable AFB_1. On the other hand, four of the six *A. parasiticus* isolates produced AFB_1 and the levels were from barely detectable (0.012 ±0.03µg/ml) to moderately detectable (6.4±0.16 µg/ml).

3.1.2 Aflatoxin B_2 (AFB_2)

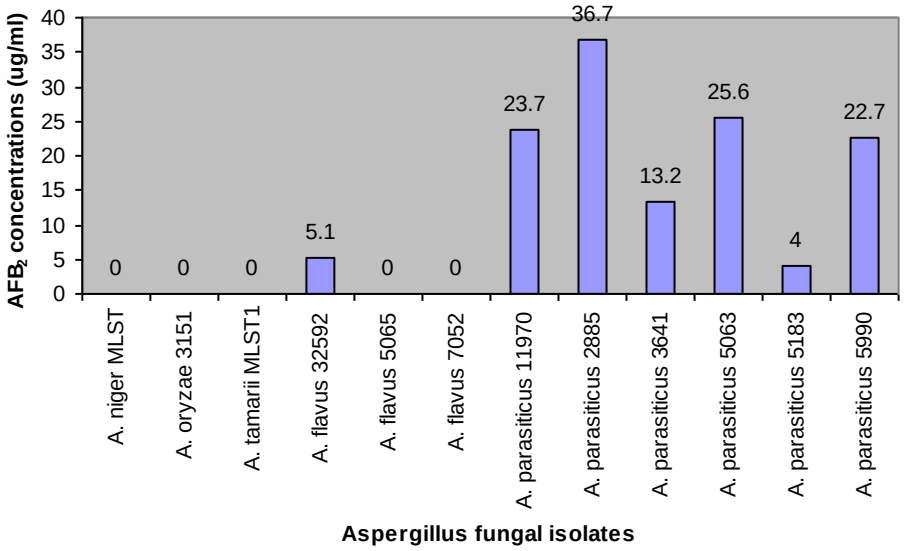


Figure 8c: AFB_2 concentrations detected by HPLC from12 *Aspergillus* isolates.

AFB_2 was produced by seven of the 12 isolates analyzed (as shown in Table 7 and Figure 8c). *A. flavus* 32592 was the only *A. flavus* isolate that produced AFB_2 and the levels were moderately detectable (5.1±0.13 µg/ml). All six *A. parasiticus* isolates analyzed produced AFB_2 and the levels were from moderately detectable (4±0.15 µg/ml) to detectable (36.7±0.32 µg/ml).

3.1.3 Aflatoxin G_1 (AFG_1)

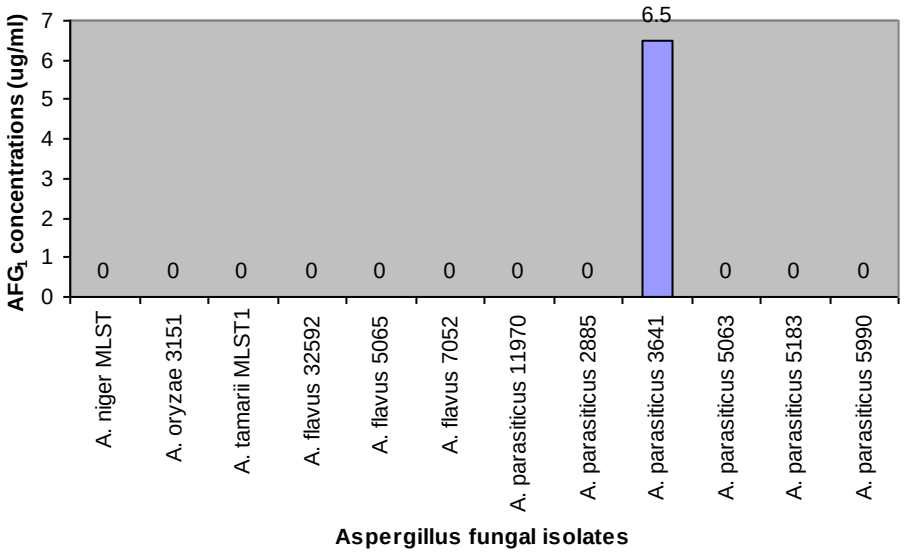


Figure 8d: AFG_1 concentrations detected by HPLC from12 *Aspergillus* isolates.

AFG_1 was produced by only one (*A. parasiticus* 3641) of the 12 isolates analyzed (as shown in Table 7 and Figure 8d) and the levels were moderately detectable (i.e. 6.5±0.12 µg/ml).

3.1.4 Aflatoxin G_2 (AFG_2)

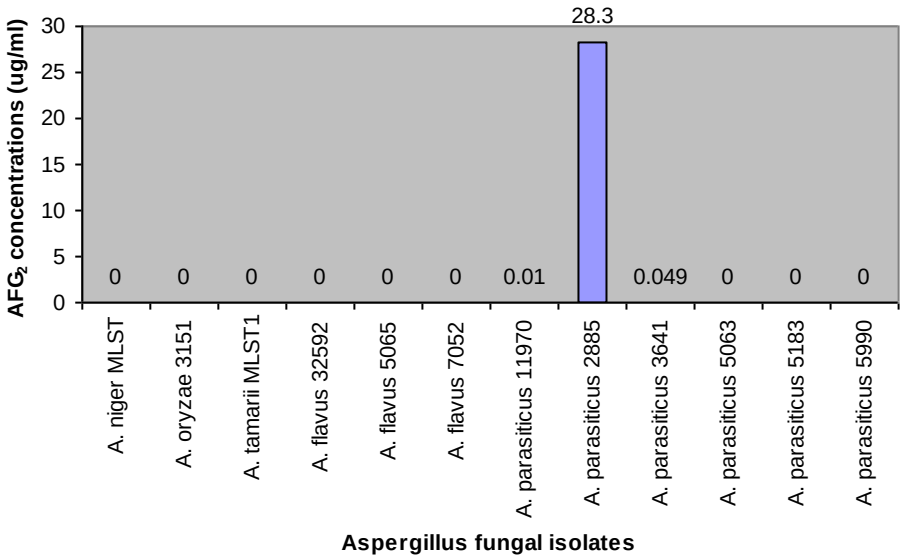


Figure 8e: AFG_2 concentrations detected by HPLC from12 *Aspergillus* isolates.

AFG_2 was produced by three of the 12 isolates analyzed (as shown in Table 7 and Figure 8e). None of the three *A. flavus* isolates produced any detectable levels of AFG_2. Three of the six *A. parasiticus* isolates (i.e. *A. parasiticus* 2885, 11970 and 3641) did produce AFG_2 and the levels were from barely detectable (0.010±0.01 µg/ml to detectable (28.3±0.13 µg/ml).

3.1.5 Mean total aflatoxin concentration

Mean total aflatoxin concentrations of all aflatoxigenic isolates analyzed is shown in Table 7. *A. parasiticus* 2885 produced the overall highest total amount of aflatoxin (70.5 µg/ml). *A. parasiticus* 5183 produced the overall least amount of total aflatoxin (4.012 µg/ml).

3.2 RAPD Analysis

All 12 *Aspergillus* isolates were subjected to RAPD analysis using 14 UBC primers. Six of the 14 gel profiles produced were thoroughly analyzed (see appendix 20 to 27 for the other eight gel profiles).

3.2.1 RAPD gel profile with primer UBC 204

Primer UBC 204 produced an intense band approximately 800bp (Figure 9, lane 2 and lanes 4-12) for 10 of the 12 isolates. This band was also present but less intense for *A. tamarii* MLST1 and it was lacking for non-aflatoxigenic *A. niger* MLST. Another intense band, approximately 1500bp, was shared by four of the twelve isolates (Figure 9, lane 2, 5, 6 and 10) i.e. non-aflatoxigenic *A. oryzae* 3151, *A. flavus* 7052, *A. flavus* 5065 and aflatoxigenic *A. parasiticus* 5063.

The three non-aflatoxigenic *Aspergillus* isolates i.e. *A. niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 produced different overall banding patterns to each other. However, *A. oryzae* 3151 and *A. tamarii* MLST1 shared two bands (approx. 1200bp and 800bp). The *A. niger* MLST banding pattern was completely different from the rest of the isolates analyzed and it also produce the largest band, approx. 2176bp (Figure 9, lane 1).

Non-aflatoxigenic isolate *A. oryzae* 3151 produced a banding pattern that was similar to that of non-aflatoxigenic *A. flavus* 5065 and *A. parasiticus* 5063**.** The producer of only AFB_2 (i.e. *A. flavus* 32592) shared only one band (800bp) with the other two *A. flavus* isolates (i.e. *A. flavus* 5065 and 7052). All six *A. parasiticus* isolates shared one intense band (800bp). *A. parasiticus* 5990 and *A. flavus* 32592 had similar banding patterns. The fragment/band number among all isolates analyzed ranged between one and three.

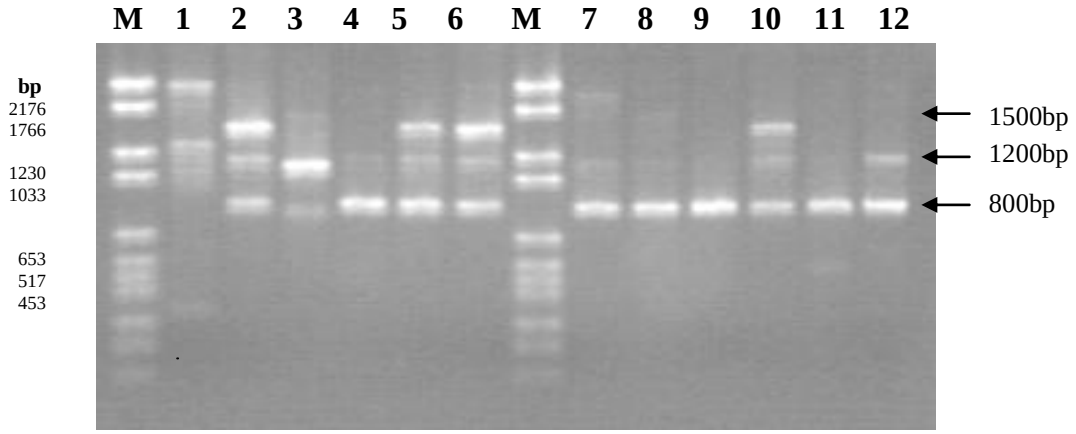


Figure 9: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 204. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.2.2 RAPD gel profile with primer UBC 229

With primer UBC 229 there were common bands produced. A band, approximately 1500bp (Figure 10, lane 2, 5, 6, 10 and 12), was shared by five of the 12 isolates i.e. non-aflatoxigenic *A. oryzae* 3151, *A. flavus* 7052, *A. flavus* 5065, aflatoxigenic *A. parasiticus* 5063 and 5990. A band, approximately 800bp (Figure 10, lane 1, 4-6 and lane 7-11), was shared by nine of the twelve isolates; *A. oryzae* 3151, *A. tamarii* MLST1 and *A. parasiticus* 5990 were the only three isolates that didn't produce this band. Three isolates: *A. niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 had unique banding patterns, no bands were shared.

The three *A. flavus* isolates (i.e. 5065, 7052 and 32592) had a common band of approximately 800bp. The second band (1500bp) was shared by *A. flavus* 5065 and 7052 and not by 32592. This band (1500bp) was very intense for 5065 and very faint for 7052. Five of the six *A. parasiticus* isolates shared one band (800bp). Other than sharing a common band, all of the six *A. parasiticus* isolates had unique overall banding patterns. *A. parasiticus* 5063 produced a banding pattern that was similar to that of *A. flavus* 7052. The fragment/band number ranged between one and three.

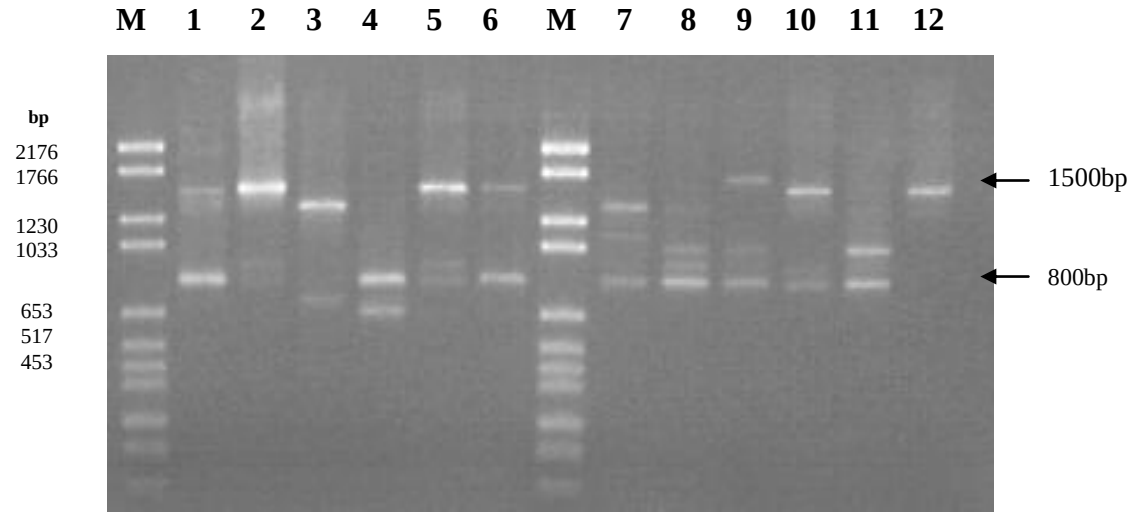


Figure 10: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 229. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.2.3 RAPD gel profile with primer UBC 246

The banding patterns produced by primer UBC 246 were characterized by one intense band, approximately 900bp that was shared by ten of the twelve isolates (Figure 11, lane 2, 4-6, and lane 7-12). Non-aflatoxigenic *A. niger* MLST and *A. tamarii* MLST1 were the only two isolates that didn't produce this 900bp band. Three non-aflatoxigenic isolates: *A. niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 shared no bands, each isolate had a unique banding pattern.

The three *A. flavus* isolates (i.e. 5065, 7052 and 32592) shared one band (900bp); non-aflatoxigenic 5065 and 7052 shared a second very faint band (approx. 1230bp, Figure 11, lane 5 and 6). *A. oryzae* 3151 also shared these two bands with the two *A. flavus* isolates. Aflatoxigenic *A. flavus* 32592 and *A. parasiticus* 3641 produced similar banding patterns. Also, aflatoxigenic *A. parasiticus* 2885 had a banding pattern similar to that of *A. flavus* 32592 and *A. parasiticus* 3641. *A. parasiticus* 5183 produced a unique band (approx. 653bp, Figure 11, lane 11). *A. parasiticus* 5063 produced a banding pattern that was similar to that of *A. oryzae* 3151.

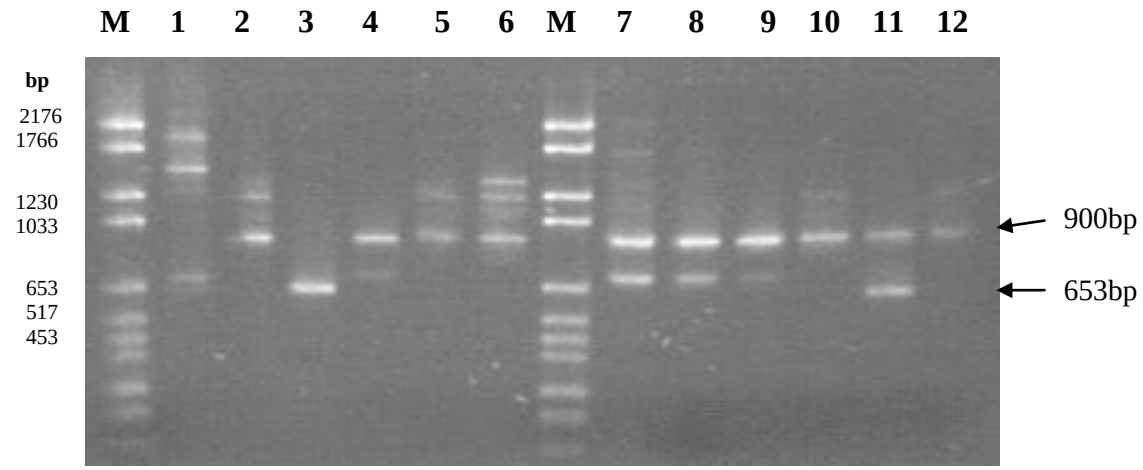


Figure 11: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 246. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamari* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.2.4 RAPD gel profile with primer UBC 284

Common bands were produced when primer UBC 284 was used. A band, approx. 980bp (Figure 12, lane 7, 8, 9, 11 and 12), was shared by five of the 12 isolates i.e. aflatoxigenic *A. parasiticus* 11970, 2885, 3641, 5183 and 5990. Another band, approx 800bp (Figure 12, lane 4 and lanes 7-10 and lane 12), was also shared by six of the 12 isolates i.e. aflatoxigenic *A. flavus* 32592, *A. parasiticus* 11970, 2885, 3641, 5063 and 5990. Another very faint band, approx 520bp (Figure 12, lane 2, 4, 6 and lanes 7-12), was shared by nine of the 12 isolates; *A. niger* MLST, *A. tamarii* MLST1 and *A. flavus* 5065 were the only three isolates that didn't produce this band.

The banding patterns of three non-aflatoxigenic isolates: *A. niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 were unique to each isolate. AFB2 producer, *A. flavus* 32592 shared one faint band (520bp) with non-aflatoxigenic *A. flavus* 7052. Non-aflatoxigenic *A. flavus* 5065 produced one intense band (approx 710bp - Figure 12, lane 2, 5 and 10) and this band was shared with *A. oryzae* 3151 and A. parasiticus 5063. *A. flavus* 7052 shared three bands (i.e. approximately 1300bp, 800bp and 520bp) with *A. oryzae* 3151.

Banding patterns produced by some *A. parasiticus* isolates appeared very close to each other. However, all six aflatoxigenic *A. parasiticus* isolates had the 520bp band in common. *A. parasiticus* 5063 was the only *A. parasiticus* isolate to produce the 710bp band. This isolate also shared some bands with *A. flavus* 7052 and *A. oryzae* 3151. This primer produced unique profiles for all twelve isolates.

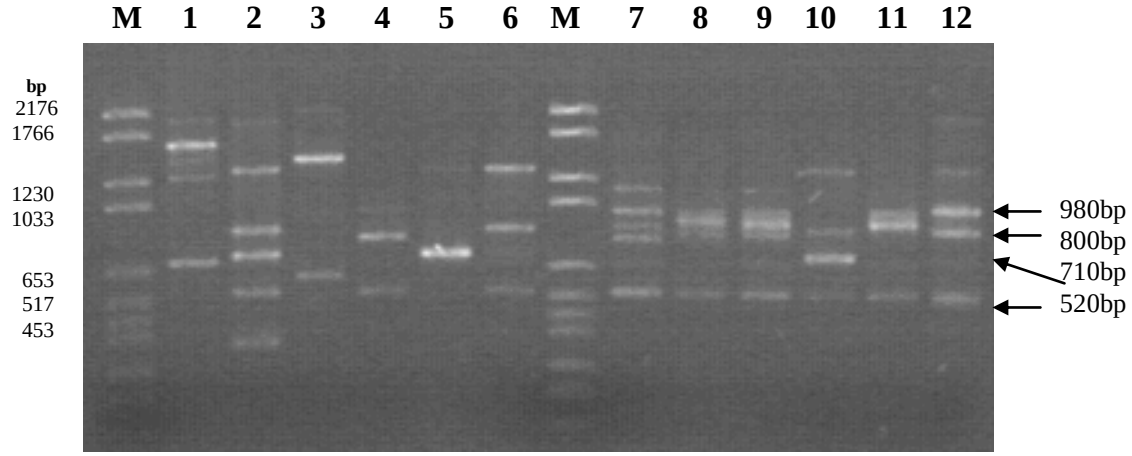


Figure 12: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 284. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.2.5 RAPD gel profile with primer UBC 285

Common bands were generated with primer UBC 285. Three intense bands were shared. A band, approx 1400bp (Figure 13, lane 1, 2, 5, 6 and 10) was shared by five of the 12 isolates i.e. non-aflatoxigenic *A. niger* MLST, *A. oryzae* 3151, *A. flavus* 7052, *A. flavus* 5065 and aflatoxigenic *A. parasiticus* 5063. A second intense band, approx 900bp (Figure 13, lane 2, 3, 5, 6, 10 and 12), was also shared by six of the 12 isolates i.e. *A. oryzae* 3151, *A tamarii* MLST1, *A. flavus* 5065, 7052, *A. parasiticus* 5063 and 5990. A third intense band, approx. 600bp (Figure 13, lane 2, 5 and 10), was shared by six of the 12 isolates (i.e. *A.flavus* 32592, *A. parasiticus* 11970, 2885, 3641, 5063 and 5183).

The banding pattern of *A. oryzae* 3151 was very similar to those of *A. flavus* 5065 and 7052. Aflatoxigenic *A. flavus* 32592 didn't share any bands with the other two *A.*

flavus isolates (i.e. 5065 and 7052), this isolate produced two small sized unique bands (approx. 653bp and 517bp). With the exception of aflatoxigenic *A. parasiticus* 5990, all aflatoxigenic *A. parasiticus* isolates produced visible banding patterns. Aflatoxigenic *A. parasiticus* 2885 and 3641 had similar banding patterns. Aflatoxigenic *A. parasiticus* 5063 shared two bands (1400bp & 900bp) with *A. flavus* 5065, 7052 and *A. oryzae* 3151.

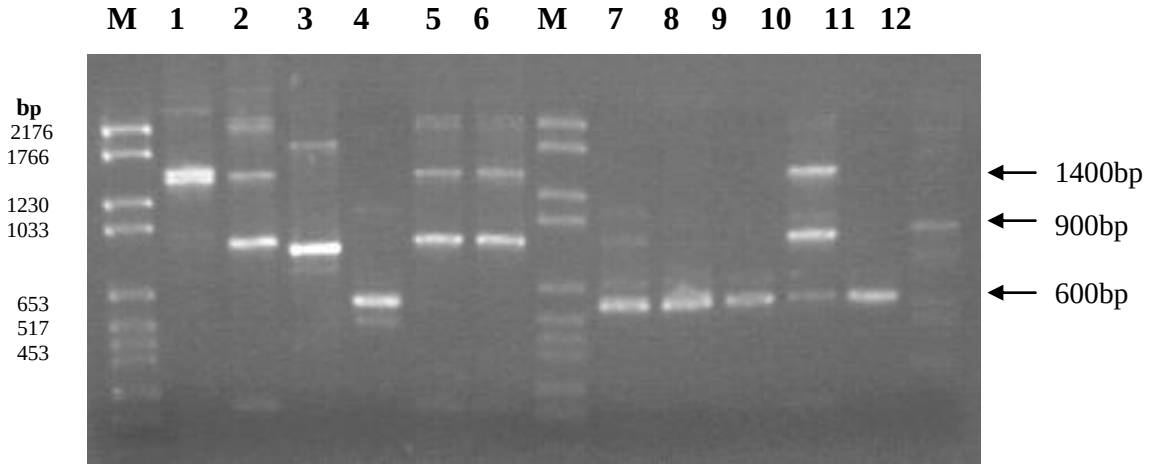


Figure 13: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 285. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamari* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.2.6 RAPD gel profile with primer UBC287

Primer UBC 287 also produced common bands. Two bands were shared by the isolates. One band, approx 1200bp (Figure 14, lane 2, 5 and 10), was shared by 10 of the 12 isolates; non-aflatoxigenic *A. oryzae* 3151 and *A. tamarii* MLST1 were the only two isolates that didn't produce a visible 1200bp band. The second band (approx. 900bp, Figure 14, lane 1, 4, 5, 6 and lanes 7-12) was also shared by 10 of the 12 isolates and *A. oryzae* 3151 and *A. tamarii* MLST1 were the two exceptions. Non-aflatoxigenic *A. niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 produced unique banding patterns and no bands were shared.

Aflatoxigenic *A. flavus* 32592 shared the two bands (1200bp and 900bp) with non-aflatoxigenic *A. flavus* 5065, 7052 and *A. niger* MLST. All six aflatoxigenic *A. parasiticus* isolates shared the two bands but the 900bp band was faint for some

isolates. *A. parasiticus* 3641, 11970 and 5990 produced similar banding patterns. Also, *A. parasiticus* 5063 and 5183 produced patterns that were almost similar. *A. parasiticus* 2885, *A. oryzae* 3151, *A. flavus* 32592, *A. flavus* 5065 and *A. flavus* 7052 also produced a faint band, approx. 453bp (Figure 14, lane 2, 4 and 8).

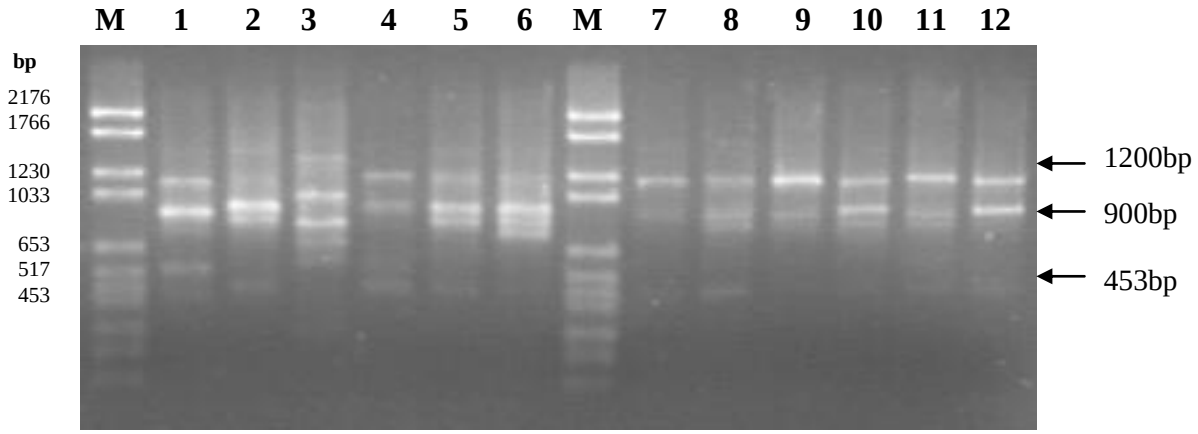


Figure 14: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 287. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

3.3 Dendograms

All 14 RAPD gel profile were subjected to dendogram analysis but only six of the 14 profiles were thoroughly analyzed (see appendix 28 to 35 for the other eight profiles). For dendogram analysis even the faintest bands were analyzed and the exact positions of each band were considered. Analysis of relatedness among isolates was done for similarities that were approximately between 100% and 60%; similarities approximately below 60% were not thoroughly analyzed.

3.3.1 Dendogram profile with primer UBC 204

Primer UBC 204 generated a dendogram with a high percentage of similarities among the 12 isolates analyzed (Figure 15). Four (two pairs) of the twelve isolates had 100% similar profiles. Non-aflatoxigenic *A. oryzae* 3151 and *A. flavus* 5065 were 100% similar to each other. Aflatoxigenic *A. flavus* 32592 and *A. parasiticus* 5990 were also 100% similar to each other. Three of the twelve isolates (i.e. *A. oryzae* 3151, *A. flavus* 5065 and 7052) generated profiles that were approximately 85% similar. Four of the

twelve isolates (i.e. *A. oryzae* 3151, *A. flavus* 5065, 7052 and *A. parasiticus* 5063) produced profiles that were approx 82% similar.

Overall, all six isolates (i.e. *A. oryzae* 3151, *A. flavus* 5065, 7072, 32592, *A parasiticus* 5063 and 5990) were approximately 65% similar. Aflatoxigenic *A. parasiticus* 3641 and 5183 were also 65% similar as was *A. parasiticus* 2885 to non-aflatoxigenic *A. tamarii* MLST1.

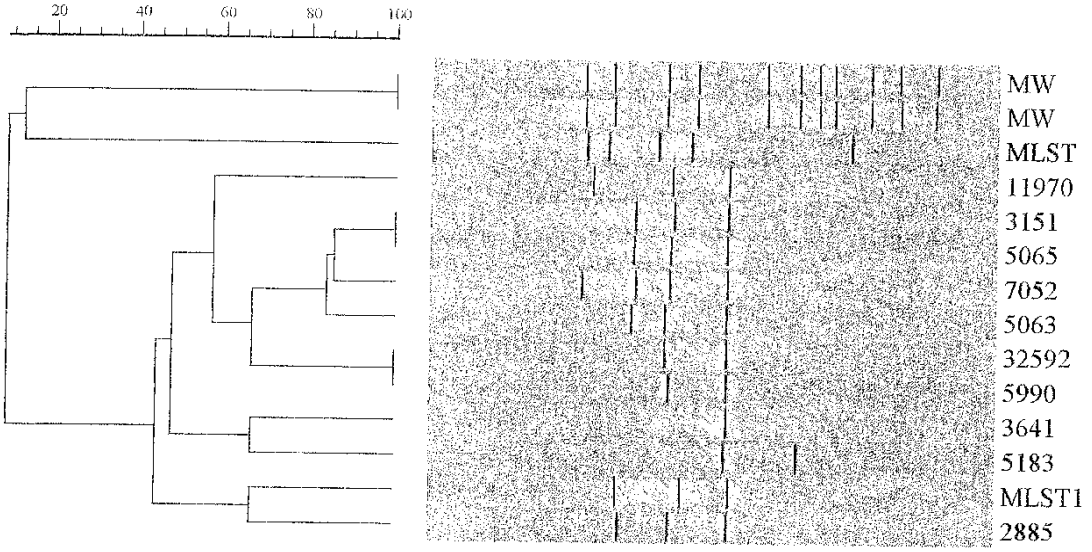


Figure 15: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 204. Keys/Lanes: MW - Molecular Marker VI; MLST - *A. niger* MLST; 11970 - *A. parasiticus* 11970; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 7052 - *A. flavus* 7052; 5063 - *A. parasiticus* 5063; 32592 - *A. flavus* 32592; 5990 - *A. parasiticus* 5990; 3641 – A. parasiticus 3641; 5183 - *A. parasiticus* 5183; MLST1 - *A. tamarii* MLST1; 2885 - *A. parasiticus* 2885.

3.3.2 Dendogram profile with primer UBC 229

Primer UBC 229 produced a number of profiles that were less than 100% similar, only a few profiles were more than 60% similar. The non-aflatoxigenic *A. niger* MLST and the *A. flavus* 5065 produced profiles that were approximately 78% similar (Figure 16). Aflatoxigenic *A. parasiticus* 5063 profile weas approximately 73% similar to both *A. niger* MLST and *A. flavus* 5065. The aflatoxigenic *A. parasiticus* 2885 and *A. parasiticus* 3641 produced profiles that were approximately 66% similar. Approximately 66% similarities were also observed between non-aflatoxigenic *A. oryzae* 3151 and aflatoxigenic *A. parasiticus* 5990.

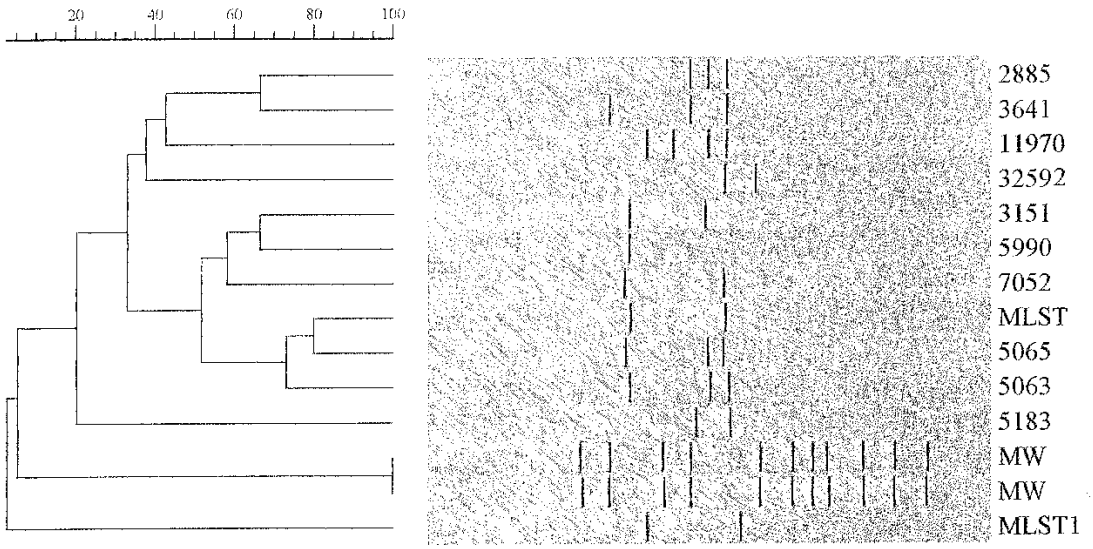


Figure 16: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 229. Keys/Lanes: 2885 - *A. parasiticus* 2885; 3641 – *A. parasiticus* 3641; 11970 - *A. parasiticus* 11970; 32592 - *A. flavus* 32592; 3151- *A. oryzae* 3151; 5990 - *A. parasiticus* 5990; 7052 - *A. flavus* 7052; MLST - *A. niger* MLST; 5065 - *A. flavus* 5065; 5063 - *A. parasiticus* 5063; 5183 - *A. parasiticus* 5183; MW - Molecular Marker VI; MLST1 - *A. tamarii* MLST1

3.3.3 Dendogram profile with primer UBC 246

With primer UBC 246, non-aflatoxigenic *A. oryzae* 3151 and aflatoxigenic *A. parasiticus* 5063 produced 100% similar profiles. Aflatoxigenic *A. flavus* 32592 and *A. parasiticus* 3641 also produced 100% similar profiles (Figure 17). The non-aflatoxigenic A. *flavus* 5065 produced a profile that was approximately 75% similar to *A. oryzae* 3151 and *A. parasiticus* 5063. The non-aflatoxigenic *A. tamarii* MLST1 and aflatoxigenic *A.parasiticus* 5183 produced profiles that were approximately 66% similar. Aflatoxigenic *A. parasiticus* 11970 and *A. parasiticus* 2885 also produced profiles that were also approximately 66% similar.

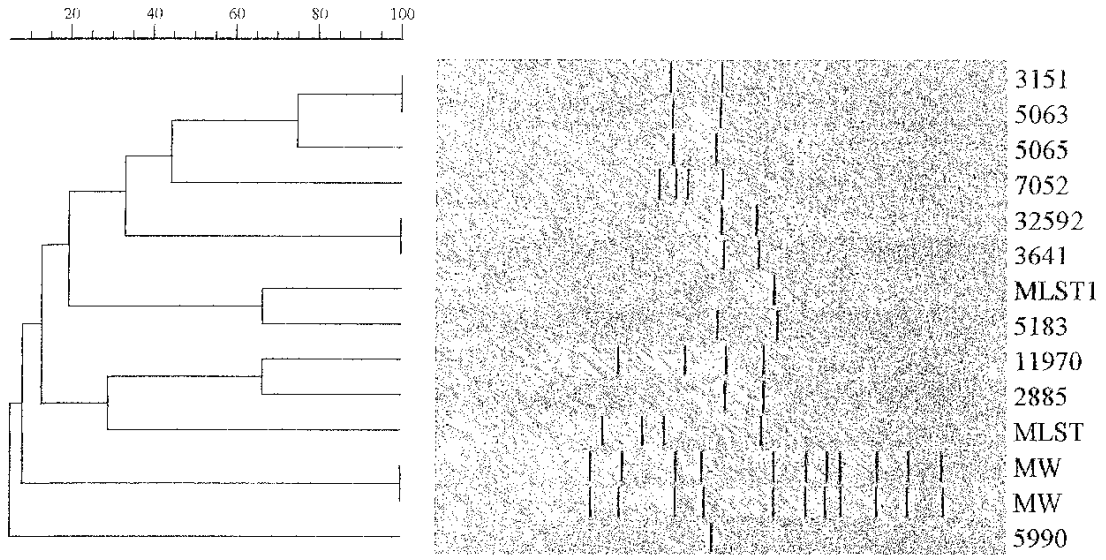


Figure 17: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 246. Keys/Lanes: 3151- *A. oryzae* 3151; 5063 - *A. parasiticus* 5063; 5065 - *A. flavus* 5065; 7052 - *A. flavus* 7052; 32592 - *A. flavus* 32592; 3641 – *A. parasiticus* 3641; MLST1 - *A. tamarii* MLST1; 5183 - *A. parasiticus* 5183; 11970 - *A. parasiticus* 11970; 2885 - *A. parasiticus* 2885; MLST - *A. niger* MLST; MW - Molecular Marker VI; 5990 - *A. parasiticus* 5990

3.3.4 Dendogram profile with primer UBC 284

Primer UBC 284 produced two profiles that were identical, i.e. aflatoxigenic *A. parasiticus* 11970 and *A. parasiticus* 3641 had profiles that were 100% similar (Figure 18). Aflatoxigenic A. *parasiticus* 2885 produced a profile that was approximately 77% similar to the two isolates (11970 and 3641). The non-aflatoxigenic *A. oryzae* 3151 and the aflatoxigenic *A. parasiticus* 5063 produced profiles that were 80% similar. Non-aflatoxigenic *A. flavus* 7052 produced profiles that were approximately 62% similar to both 3151 and 5063.

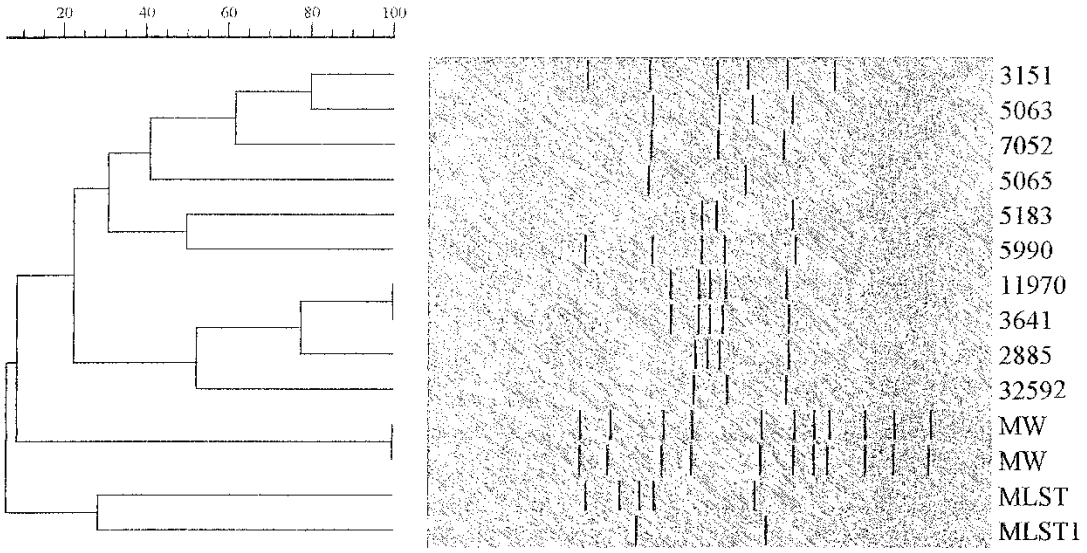


Figure 18: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 284. Keys/Lanes: 3151- *A. oryzae* 3151; 5063 - *A. parasiticus* 5063; 7052 - *A. flavus* 7052; 5065 - *A. flavus* 5065; 5183 - *A. parasiticus* 5183; 5990 - *A. parasiticus* 5990; 11970 - *A. parasiticus* 11970; 3641 – *A.parasiticus* 3641; 2885 - *A. parasiticus* 2885; 32592 - *A. flavus* 32592; MW - Molecular Marker VI; MLST - *A. niger* MLST; MLST1 - *A. tamarii* MLST1

3.3.5 Dendogram profile with primer UBC 285

The aflatoxigenic *A. parasiticus* 2885 and *A. parasiticus* 3641 produced 100% similar profiles with primer UBC 285 (Figure 19). Non-aflatoxigenic *A. oryzae* 3151 and *A. flavus* 7052 produced profiles that were approximately 85% similar. While non-aflatoxigenic *A. flavus* 5065 produced profiles that were approximately 85 % similar to both *A. oryzae* 3151 and *A. flavus* 7052. Other profiles generated were too low to analyze.

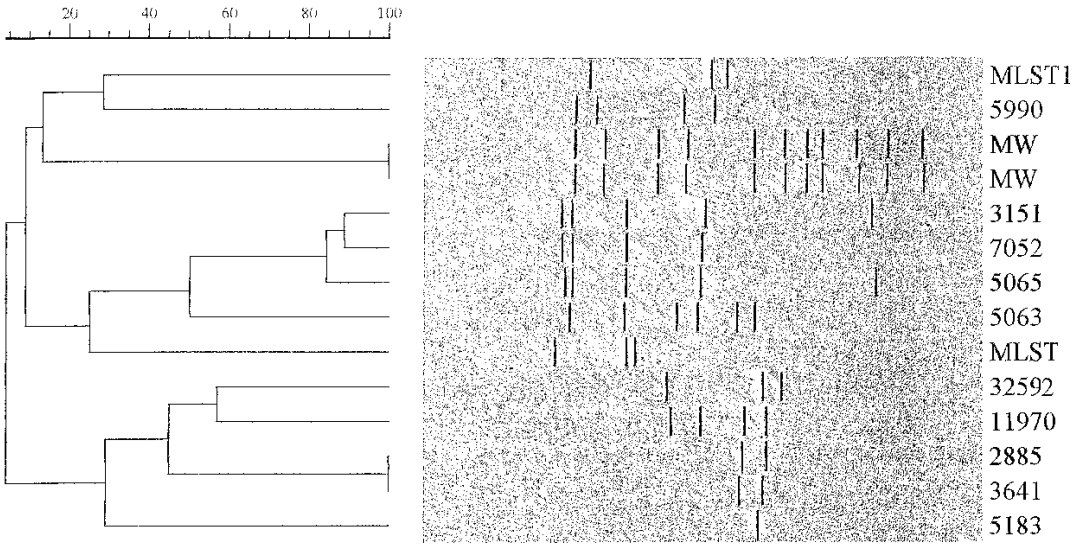


Figure 19: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 285. Keys/Lanes: MLST1 - *A. tamarii* MLST1; 5990 - *A. parasiticus* 5990; MW - Molecular Marker VI; 3151- *A. oryzae* 3151; 7052 - *A. flavus* 7052; 5065 - *A. flavus* 5065; 5063 - *A. parasiticus* 5063; MLST - *A. niger* MLST; 32592 - *A. flavus* 32592; 11970 - *A. parasiticus* 11970; 2885 - *A. parasiticus* 2885; 5183 - *A. parasiticus* 5183.

3.3.6 Dendogram profile with primer UBC 287

Primer UBC 287 produced a number of profiles that were similar (Figure 20). Three aflatoxigenic *A. parasiticus* isolates (i.e.11970, 3641 and 5990) produced profiles that were 100% similar. Aflatoxigenic *A. parasiticus* 5183 produced profiles that were 80% similar to *A. parasiticus* 11970, 3641 and 5990. Aflatoxigenic *A. parasiticus* 5063 had profiles that were 65% similar to all of the abovementioned isolates (i.e. *A. parasiticus* 11970, 3641, 5990 and 5183). *A. niger* MLST produced a profile that was approximately 62% similar to all of the abovementioned isolates (i.e. 11970, 3541, 5990, 5183 and 5063). Aflatoxigenic *A. flavus* 32592 and non-aflatoxigenic *A. flavus* 5065 produced profiles that were 85% similar. There was also approximately 60% similarity between *A. oryzae* 3151 and *A. flavus* 5065.

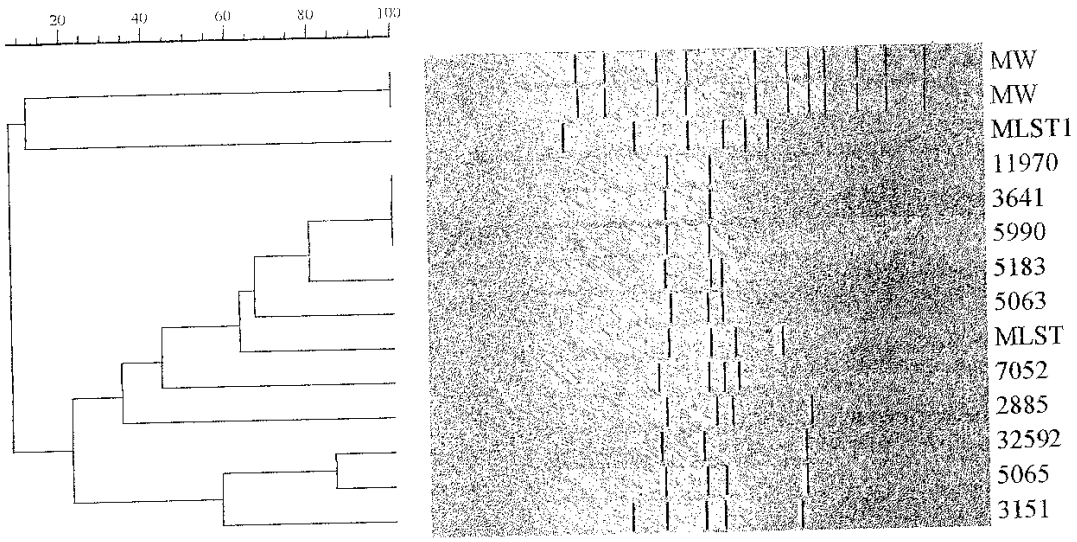


Figure 20: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 287. Keys/Lanes: MW – Molecular Marker VI; MLST1 – *A. tamarii* MLST1; 11970 – *A. parasiticus* 11970; 3641 – A. parasiticus 3641; 5990 – *A. parasiticus* 5990; 5183 – *A. parasiticus* 5183; 5063 - *A. parasiticus* 5063; MLST – *A. niger* MLST; 7052 – *A. flavus* 7052; 2885 – *A. parasiticus* 2885; 32592 – *A. flavus* 32592; 5065 – *A. flavus* 5065; 3151- *A. oryzae* 3151

All significant dendogram similarities from this study are summarizes in Table 8.

Table 8: Summary of the significant percentage similarities

PRIMERS	% SIMILARITIES
UBC 204	100% - *A. flavus* 5065 and *A.oryzae* 3151 85% - *A. flavus* 5065 and *A.flavus* 7052 82% - *A. parasiticus* 5063 and *A.oryzae* 3151 82% - *A. flavus* 5065 and *A. parasiticus* 5063
UBC 229	73% - *A. flavus* 5065 and *A. parasiticus* 5063
UBC 246	100% - *A. oryzae* 3151 and *A. parasiticus* 5063 75% - *A. flavus* 5065 and *A.oryzae* 3151 75% - *A. flavus* 5065 and *A. parasiticus* 5063
UBC 284	100% - *A. parasiticus* 11970 and 3641 80% - *A. parasiticus* 5063 and *A. oryzae* 3151
UBC 285	81% - *A. flavus* 5065 and *A. oryzae* 3151
UBC 287	100% - *A. parasiticus* 11970 and 3641 & 5990 85% - *A. flavus* 5065 and 32592 60% *A. flavus* 5065 and *A. oryzae* 3151

3.4 Presence of the *afl*R gene

Based on a study by Chang *et al.* (1995) all 12 *Aspergillus* isolates were subjected to PCR amplification of genomic DNA using primers that generate a fragment of approximately 450bp of the *afl*R gene.

Nine of the 12 isolates analyzed produced this 450bp PCR *afl*R gene product as the major PCR product (Figure 21). The non-aflatoxigenic, *A. oryzae* 3151, *A. niger* MLST and *A. tamarii* MLST1 were the only three isolates that did not produce the 450bp *afl*R gene product but did produce a faint minor product of approximately 900bp (Figure 21, lanes 1-3). All three *A. flavus* isolates (i.e. 32592, 5065 and 7052) did produce the 450bp product (Figure 21, lanes 4-6). *A. flavus* (5065 and 7052) also produced the minor 800bp PCR product while *A. flavus* 32592 produced a minor product of approximately 700bp. Also, all six aflatoxigenic *A. parasiticus* isolates (i.e. *A. parasiticus* 11970, 2885, 3641, 5063, 5183 and 5990) produced the 450bp *afl*R gene product. This *afl*R gene product for *A. parasiticus* 11970 was faint when compared to the rest of the *afl*R gene producers.

All nine potentially aflatoxigenic isolates (*A. flavus* and *A. parasiticus*) seemed to have what looked like 'extra bands or minor PCR prducts' (approximately 900bp and 800bp) which could be another copy of the *afl*R gene or fragmented DNA.

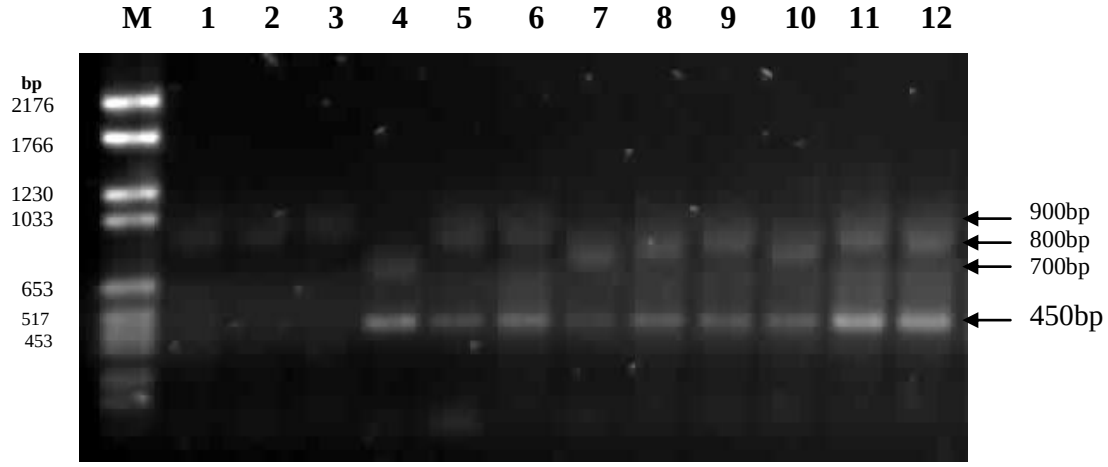


Figure 21: Gel profile showing the *afl*R gene. Key/Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990.

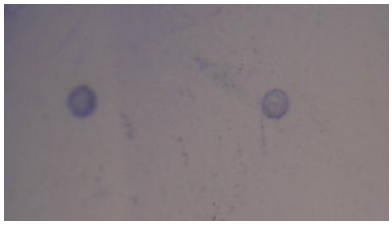
Correlation of aflatoxin production and the presence of the *afl*R gene is shown in Table 9.

Table 9: Correlation between aflatoxin production and the presence of the *afl*R gene

FUNGAL ISOLATE	AFLATOXIN PRODUCTION	PRESENCE OF *afl*R GENE
A. niger MLST	none	-
A. oryzae 3151	none	-
A. tamarii MLST1	none	-
A. flavus ATCC 32592	AFB_2 (5.1±0.13 μg/ml)	+
A. flavus PPRI 5065	none	+
A. flavus PPRI 7052	none	+
A. parasiticus ATCC 11970	AFB_2 (23.7±0.60μg/ml) AFG_2 (0.10 ±0.01μg/ml)	+
A. parasiticus PPRI 2885	AFB_1 (5.5±0.0004 μg/ml) AFB_2 (36.7±0.32 μg/ml) AFG_2 (28.3±0.13 μg/ml)	+
A. parasiticus PPRI 3641	AFB_1 (0.021±0.03μg/ml) AFB_2 (13.2±0.36 μg/ml) AFG_1 (6.5±0.12 μg/ml) AFG_2 (0.049±0.003 μg/ml)	+
A. parasiticus PPRI 5063	AFB_1 (6.4±0.16 μg/ml) AFB_2 (25.6 μg/ml)	+
A. parasiticus PPRI 5183	AFB_1 (0.012±0.03 μg/ml) AFB_2 (4.0±0.15 μg/ml)	+
A. parasiticus PPRI 5990	AFB_2 (22.7±0.06 μg/ml	+

3.5 Probe labelling and quantification

The 450bp PCR product that was produced by *A. parasiticus* 5990 was used as a probe. After purification, the probe and control DNA were labelled, successfully. Successful labelling was determined by the appearance of a coloured precipitate on the membrane (shown in Figure 22a).



A **B**

Figure 22a: Labelled probes profiles. Keys: **A –** labelling from the 450bp DNA probe; **B –** labelling from the control DNA.

Quantification of labelling efficiency was achieved, successfully, through a dilution series of both the labelled probe and the labelled control DNA. The results from this dilution series (Table 6) are shown in Figure 22b. As previously described in section 3.5, successful labeling was determined by the appearance of a coloured precipitate on the membrane. Even though the dilution series was carried out in nine tubes, the coloured precipitates were observed in seven of the nine tubes (tube 8-9 were too diute to show a reaction). A negative control (dilution buffer with no DNA) did not produce any coloured precipitate.

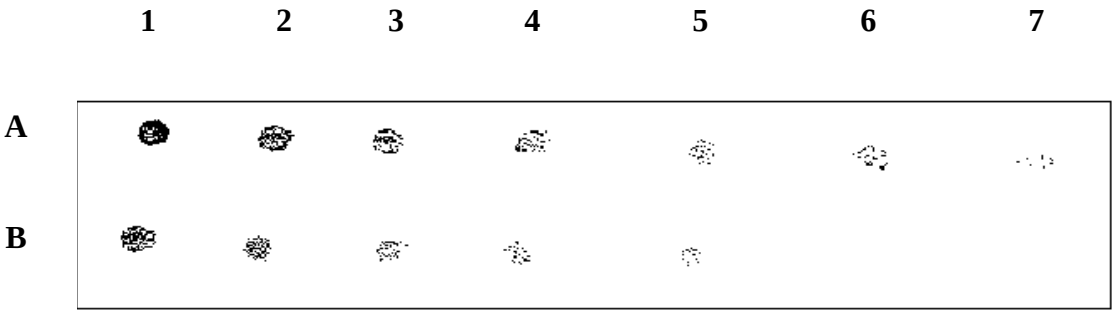


Figure 22b: Quantification (labelling efficiency) profile. Keys: **1-7** represents the tube dilution series; **A –** labelled control DNA dilution series; **B –** labelled probe dilution series.

3.6 Restriction analysis and hybridization

Digestion of genomic DNA by the five restriction enzymes (i.e. *Hind*III, *Eco*RV, *Eco*RI, *Dra*I and *Bgl*I) was characterized by the presence of a smear after electrophoresis (see appendix 36 to 40). These gels were immediately used for Southern blotting then subjected to hybridization using the labeled DNA probe.

The hybridization results that were obtained were negative for all the membranes that were subjected to this process i.e. the desired spots were not achieved. This was thought to be due to a number of reasons which are explained in the discussion section of this analysis. The membranes were then subjected to stripping/re-probing where hybridization conditions were optimized but still the desired spots were not achieved.

CHAPTER 4: DISCUSSION

4.1 Aflatoxin analysis

Although aflatoxins are primarily produced by species of the genus *Aspergillus*, not all isolates (of a particular species) are capable of producing aflatoxins. Also, other *Aspergillus* species have been proven to be totally incapable of producing aflatoxins (Matsushima *et al.,* 2001a). Therefore, one of the objectives of this study was to analyze aflatoxin production (AFB_1, AFB_2, AFG_1 and AFG_2) from 12 *Aspergillus* isolates.

Parameters such as culture media, temperature, pH and the time of incubation are some of the critical factors that were optimized in this study so as to promote aflatoxin production in all 12 isolates used in this study. SMKY broth (pH 6.0) was used in this study as liquid medium for aflatoxin production. SMKY was chosen because in a study by Roy *et al*. (2003) it was shown to promote aflatoxin production. Incubation time and temperature in this study were kept at 2-3 days at 25ºC. These two parameters were chosen because two previous studies (Fente *et al*., 2001; Matsushima *et al*., 2001b) had used parameters in this range, successfully, to analyze aflatoxin production.

The isolates *Aspergillus niger* MLST, *A. oryzae* 3151 and *A. tamarii* MLST1 did not produce any detectable amounts of the four aflatoxins analyzed (Table 7). This was expected because all three isolates belong to the species that are known and have been shown (in a previous study) to be incapable of producing aflatoxins (Matsushima *et al.,* 2001a).

Of the three isolates, *A. oryzae* has been the most extensively studied because of its use for preparation of food products (Watson *et al.,* 1999). Goto *et al*. (1996) found an unrecognized form of *A. tamarii* which produced AFB_1 and AFB_2. However, they couldn't prove beyond a reasonable doubt that *A. tamarii* species are potentially aflatoxigenic. Therefore, non-production of aflatoxin by *A. tamarii* MLST was not unexpected. Also, *A. niger* has not been found to be capable of producing any aflatoxins. Since HPLC is the most reliable method for detecting aflatoxins at very

low levels (Neue, 1997), the results that were obtained for these three isolates are regarded as conclusive.

A. flavus species are known to produce two of the four aflatoxins that were analyzed (Reddy and Waliyar, 2005) i.e. AFB_1 and AFB_2. However, all three *A. flavus* isolates used in this study (i.e. *A. flavus* 32592, 5065 and 7052) did not produce any detectable levels of AFB_1. The non-production of AFB_1 by these three isolates was unexpected because *A. flavus* was one of the first species (discovered decades ago) found to produce AFB_1 (Trail *et al.*, 1995).

In the case of *A. flavus* 5065 (isolated from a facial cream), the non-production of AFB_1 was due to two factors:- the composition of the substrate (facial cream) and the composition of the culture medium (SMKY). The composition (i.e. nutrients) of the facial cream were favourable for fungal growth but unfavourable for aflatoxin production. Furtado *et al*. (2005) showed that in some cases (in *Aspergillus* species) optimal conditions for fungal growth are not the same for secondary metabolites formation. In contrast to this fact, some *Aspergillus* isolates are known to strive under unfavourable conditions to produce aflatoxins (Fente *et al.*, 2001), however, this was not the case for *A. flavus* 5065.

Secondly, after the isolation from the facial cream, the isolate was cultured under laboratory conditions that were favourable for aflatoxin production. The components/nutrients of the culture medium that was used in this study (SMKY broth, pH 6.0) still did not induce aflatoxin production from this isolate. SMKY broth therefore, did not contain appropriate nutrients to induce aflatoxin production for *A. flavus* 5065 (and *A. flavus* 7052) or the levels of aflatoxin produced were too low to be detected by the HPLC. Roy *et al.* (2003) found that 122 of 199 *A. flavus* isolates produced aflatoxins when they were cultured in SMKY broth. Therefore, the choice of a liquid medium is crucial as it is possible that if these two isolates are cultured in a different liquid medium they will produce aflatoxins. Al-Hilli and Smith (1992) showed that some culture media will stimulate aflatoxin production (in *A. flavus* isolates) only in the presence of additional nutrients (such as propionic acid).

The pH of the SMKY broth (6.0) was expected to be favourable for aflatoxin production because *A. flavus* is known to produce aflatoxins only at acidic pH. However, this did not happen with *A. flavus* 5065 and *A. flavus* 7052. Erhlich *et al*. (2005) found atypical strain of *A. flavus* from West Africa that produced less aflatoxin at acidic pH and this could have been the case with the two *A. flavus* isolates in our study.

On the other hand, *A. flavus* 32592 isolate was aflatoxigenic in the fact that it did produce AFB_2 (even though it lacked AFB_1 production) (Table 7). The non-production of AFB_1 was due to facts that have been explained in the previous section with the other two isolates (i.e. *A. flavus* 5065 and 7052). The incubation time (2-3 days) as well, was not optimal for these isolates to produce AFB_1. Even though incubation time was 2-3 days, all 12 cultures were extracted for aflatoxin on the third day of incubation. In a study by Fente *et al.,* (2001) it was shown that aflatoxins were detected by HPLC on the third day of incubation and for some *Aspegillus* cultures it took two days for them to poduce aflatoxins. However, they also showed that for some *Aspergillus* isolates it took longer than three days for them to produce aflatoxins. Therefore, three days of incubation were probably not long enough for AFB_1 production by *A. flavus* 32595 and also not long enough for *A. flavus* 5065 and 7052 to produce AFB_1 and AFB_2.

AFB_2 was produced by only one of the three *A. flavus* isolates analyzed i.e. *A. flavus* 32592 (Table 7). This isolate produced moderately detectable levels of 5.1±0.13 μg/ml. The fact that this isolate did produce AFB_2 even though it did not produce AFB_1 was not unexpected at all because both aflatoxins are produced separately in the biosynthetic pathway. The conditions/parameters were, therefore, favourable for AFB_2 production.

As it was expected, AFG_1 and AFG_2 were not produced by any of the three *A. flavus* isolates (Table 7). AFG_1 and AFG_2. are (currently) exclusively produced by *A. parasiticus* isolates (Reddy and Waliyar, 2005).

All six *A. parasiticus* isolates produced AFB_2 whereas four of the six *A. parasiticus* isolates used in this study (i.e. *A. parasiticus* 2885, 3641, 5063 and 5183) produced AFB_1 (Table 7) This was expected because it corroborates the hypothesis that the production of AFB_2 is not dependent on AFB_1 production (Yabe *et al.*, 1999). One pathway leads to the production of AFB_1 and the other leads to AFB_2. Also, conditions such incubation temperature and culture media were optimum for enzymes that catalyzed the AFB_2 biosynthetic pathway than the AFB_1 one (Furtado *et al.*, 2005).

AFG_1 was the least produced of the four aflatoxins, only *A. parasiticus* 3641 produced AFG_1 whereas AFG_2 was produced by three of the six *A. parasiticus* isolates (i.e. *A. parasiticus* 11970, 2885 and 3641) (Table 7). Even though most of the *A. parasiticus* isolates had produced the B-group aflatoxins, that did not necessarily mean they will automatically produce the G-group aflatoxins. Yabe *et al*. (1999) obtained data that supported the hypothesis that the G- and B-group aflatoxins are produced independent of each other from the same substrate. That is, the B-group aflatoxins are not the precursors of the G-group aflatoxins.

A. parasiticus 5990 – isolated from peanuts, only produced AFB_2 (22.7±0.06 μg/ml) and no AFB_1. This was unexpected because AFB_1 was 'originally' isolated from groundnuts (Reddy and Waliyar, 2005). However, even those isolates with the ability to produce aflatoxins may not produce them all the time. The non-production of the other three aflatoxins is due an inhibition by one (or all) of the parameters which on the other hand were favourable for the production of AFB_2. Also, most fungi have a broad pH optima (from 5.5 to 7.5) but many enzymes involved in aflatoxin biosynthesis often function over a narrow pH range (Furtado *et al.*, 2005) which was probably unfavourable for this isolate.

A. parasiticus 3641 is the only isolate used in this study that produced all four aflatoxins (Table 7). This basically means that the temperature, pH, incubation time, composition of culture media and moisture levels were all optimum for the production of all four aflatoxins by this isolate.

AFB_2 was the overall predominantly produced aflatoxin in this study (Table 7). This finding was unexpected because AFB_1 is normally the most predominantly produced aflatoxin in previous studies (Reddy and Waliyar, 2000). None of the PPRI isolates (analyzed in this study) were initially tested (by PPRI) for aflatoxin production (*personal communication).*

Horn and Dorner (2001) aimed to test the hypothesis that strains of *A. flavus* (aflatoxigenic) often degenerate with repeated sub-culturing resulting in loss of aflatoxin production. They found that loss of aflatoxin production varied within strains and generation lines within a strain often showed different rates of loss of aflatoxin production. For example, the isolate NRRL 29474 lost the ability to produce AFB_1 by the second generation. Therefore, the lack of AFB_1 production by most isolates in this study is due to repeated sub-culturing which resulted in genetic mutations in DNA coding for regulating aflatoxin biosynthesis.

Based on the HPLC results, the total mean aflatoxin levels ranged between 4 μg/ml and 32 μg/ml. Reproducibility of results is very good with HPLC. It's very important to know how much aflatoxin an isolate produces for monitoring purposes. In the US and other European countries, acceptable aflatoxin levels have been set. These guidelines are used to monitor 'safe' and 'unsafe' products. However, in South Africa no such guidelines exist, therefore, making it difficult to monitor aflatoxin levels in products.

4.2 Random Amplified Polymorphic DNA (RAPD) and dendogram analysis

Baleiras-Couto *et al*. (1995) indicated that in RAPD, the level of differentiation, either interspecies or intraspecies, depends highly on the primers used. Yuan *et al*. (1995) used RAPD assays to differentiate *A. parasiticus* from *A. sojae* with three decamer primers, but the same primers could not separate *A. flavus* from *A. oryzae*.

Throughout this analysis there were a few significant common banding patterns produced with different primers. The non-aflatoxigenic *A. flavus* 5065 and *A. oryzae* 3151 isolates produced a number of similar banding patterns with different primers. Four of the six primers used produced very similar banding patterns between these

two isolates. The results of this study are supported by findings by Geiser *et al.*, 1998. They showed that both species are genetically similar to each other.

When primer UBC 204 was used, both *A. flavus* 5065 and *A. oryzae* 3151 produced banding patterns that were 100% identical (Figure 15, Table 8). With primer UBC 246 the similarities were approximately 75% (Figure 17, Table 8) and 81% with primer UBC 285 (Figure 19, Table 8). Primer UBC 287 (Figure 20, Table 8) produced banding patterns that were approximately 60% similar. These results indicate a very high genetic similarity between these two isolates. Geiser *et al*. (1998) failed to find evidence that *A. flavus* and *A. oryzae* are independent species. They suggested that *A. oryzae* have evolved by domestication from *A. flavus*. In this study, it was found that the *A. flavus* 5065 isolate was more similar to *A. oryzae* 3151 than to the other two *A. flavus* isolates (i.e. *A. flavus* 32592 and 7052). However, this finding is highly dependent on the primer used.

The second significant banding pattern was between *A. flavus* 5065 and aflatoxigenic *A. parasiticus* 5063. Three of the six primers (UBC 204, 229 and 246) (Figure 15-17, Table 8) showed high similarities between these two isolates. The similarities between the two isolates were 73% to 82%. Both species' molecular biology has been studied actively over the years. These species share sufficient DNA sequence similarities that it has been proposed that they should be reduced to varietal status (Takahashi *et al.*, 2002).

Another significant banding pattern observed was between the *A. oryzae* 3151 and *A. parasiticus* 5063 isolates. Three of the six primers produced banding patterns that were very similar between these two isolates. The primers UBC 204, 246 and 284 (Figure 15, 17 and 18, Table 8) produced similarities that were approximately 82%, 100% and 80% respectively.

In summary: *A. flavus* 5065, *A. oryzae* 3151 and *A. parasiticus* 5063 are more genetically similar to each other than to the rest of the isolates. Both *A. flavus* 5065 and *A. oryzae* 3151 were found to be non-aflatoxigenic whereas *A. parasiticus* 5063 did produce AFB_1 and AFB_2 (G-grp was not produced). This finding was very interesting because normally *A. parasiticus* is more genetically similar to *A. sojae*

than to *A. oryzae* (Geiser *et al.,* 1998). This *A. parasiticus* isolate is most likely a different 'strain' of *A. parasiticus* isolate because it was less related to the other *A. parasiticus* isolates. It is important to also note that although *A. flavus* and *A. parasiticus* are easily grown under lab conditions, they are notorious for their genetic instability when repeatedly transferred in culture (Horn and Dorner, 2001).

Some of the isolates were expected to form similar banding patterns because of their known genetic similarity as previously shown by Matsushima *et al.* (2001a) did not produce the same in the present study. The three *A. flavus* isolates did not produce any 100% similarities among themselves with any of the primers used. The banding pattern of *A. flavus* 32592 was found to be completely different from that of the other two *A. flavus* isolates (i.e. *A. flavus* 5065 and 7052). *A. flavus* 32592 was also the only aflatoxigenic *A. flavus* isolate. Based on this observation, some 'partial' differentiation between aflatoxigenic and non-aflatoxigenic isolates was achieved in this study. With primer UBC 204 and 284 (Figure 15 and 18, Table 8) the isolates *A. flavus* 5065 and 7052 were found to be approximately 85% and 81% similar respectively. Although both species did share many common bands with the different primers used, these are the highest similarities that were observed. As previously mentioned this was probably due to genetic differences that can occurred between isolates.

Significant banding patterns among *A. parasiticus* isolates (all aflatoxigenic) was not as high as it was with the other isolates that were analyzed, with the exception of *A. parasiticus* 5063. With primer UBC 284 and 287, *A. parasiticus* 11970 and 3641 were 100% similar to each other (Figure 18 and 20, Table 8). This means that these two isolates were more genetically similar to each other than to the rest of the *A. parasiticus* isolates. However, since this was observed with two primers only, it cannot be considered as conclusive evidence.

Overall, primer UBC 204 produced the highest number of similarities between isolates (Table 8, section 3.3.6). The similarities were between both aflatoxigenic and non-aflatoxigenic isolates, therefore making it difficult differentiate between the two. No high levels of differentiation were observed. This corroborates the hypothesis that RAPD assays depend primarily on the primer used (Baleiras-Couto *et al*., 1995).

4.3 *Presence of the aflR gene*

The *afl*R gene is involved in the regulation of transcription of aflatoxin biosynthesis genes. Therefore, the isolates that had produced aflatoxins or those that are known to be capable of producing them were expected to produce this *afl*R gene. Nine of the 12 isolates produced the expected 450 bp *afl*R gene PCR product (Figure 21).

Two *A. tamarii* isolates tested in a study by Chang *et al*. (1995) using the same primers did not produce this PCR product. Our isolate, *A. tamarii* MLST1 also did not produce the PCR product for the *afl*R gene (Figure 21). This isolate is non-aflatoxigenic, therefore, the presence of this gene was not expected. Non-aflatoxigenic *A. niger* MLST did not produce this *afl*R gene, as was expected. None of the previous studies have conclusively shown the presence of the *afl*R gene in these two species.

Chang *et al*. (1995) did an experiment using the same primers that were used in this analysis and they found that one of the three *A. oryzae* isolates (i.e. *A. oryzae* SRRC 2353) they tested did produce the *afl*R gene PCR product. In our analysis, non-aflatoxigenic *A. oryzae* 3151 did not produce the expected *afl*R gene PCR product. Absence of this gene product can mean either the isolate does not possess this gene or the primer binding sites on the gene were significantly different from that of the other isolates. In the absence of the *afl*R gene isolates produce very low levels of aflatoxin under inducing conditions.

A study by Lee *et al*. (2006) aimed to find out why *A. oryzae* does not produce aflatoxins and this study focused mainly on the *afl*R gene sequence. This study found that there are base variations within the sequence at certain positions. These base variations often lead pre-mature stop codons, which may lead to pre-termination of aflatoxin production.

The three *A. flavus* isolates i.e., 32592, 5065 and 7052 did produce the expected *alf*R gene PCR product (Figure 21). This finding was consistent with the findings of Lee *et al*. (2006) as the *afl*R gene was ‘initially’ discovered in *A. flavus* and *A. parasiticus* species. It is almost always expected that the *A. flavus* isolates produce this gene.

Even though *A. flavus* 7052 and 5065 were found to be non-aflatoxigenic, the *afl*R gene PCR product was present. Presence of this *afl*R gene in these two isolates indicates that there was a high degree of similarity between their target DNA and the primers used and it also indicates that these isolates have the ability/potential to produce aflatoxins (Chang *et al*., 1995). *A. flavus* isolates i.e., 32592, 5065 and 7052 also produced what looked like an extra copy of the *afl*R gene. However, this was not analysed further due to the fact that there was a possibility that it was not an extra copy of *afl*R but some fragmented DNA. Also, the objective of the study was to analyze the 450bp product.

All six aflatoxigenic *A. parasiticus* isolates (i.e. 11970, 2885, 3641, 5063, 5183 and 5990) did produce the *afl*R genes (Figure 21). However, the band for isolate *A. parasiticus*11970 was faint in comparison. The faintness of this PCR product was due to poor PCR amplification process and/or low degree of similarity between the target DNA and the primers. This finding is support by a study by Chang *et al.* (1995) where they showed that poor amplification process could lead to the absence and/or weak signal of a PCR product.

Overall, the isolates that were expected to produce this *afl*R gene did produce it irrespective of whether or not they were aflatoxigenic or non-aflatoxigenic. Also, the production of this gene shows that the PCR amplification conditions were optimal.

The correlation of aflatoxin production and the presence of *afl*R gene is shown in Table 9, section 3.4. These results support the fact that both *A. flavus* and *A. parasiticus* species are potential aflatoxin producers, which means they are expected to produce the *afl*R gene. All *A. flavus* and *A. parasiticus* isolates used in this study did produce the *afl*R gene. Athough two *A. flavus* isolates were not aflatoxigenic, the presence of the aflR gene shows that they have the potential to produce the toxins. As for *A. niger, A. tamarii* and *A. oryzae* it was expected of them to lack the *afl*R and be non-aflatoxigenic.

4.4 Hybridiation

For this analysis, hybridization was not successful even after stripping and reprobing of the membranes. The ‘no signal’ results were probably due to a number of reasons and/or hybridization shortfalls. During the first hybridization attempt, it was discovered (after overnight incubation) that the temperature of the hybridization oven was unstable and climbed to temperatures (up to 70ºC) that were above the hybridization temperature (which was 45ºC). Due to this problem, positive results were not expected and ‘no signal’ results were obtained. Correct/optimum temperatures (hybridization and washing) play a very crucial role in the hybridization process as they are one of the major steps that lead to obtaining positive hybridization results. That is, if the temperature is too high probes cannot stay bound to target DNA and if too low non-specific binding of probe will occur (Vernier *et al.,* 1996). Both scenarios lead to negative results. The positive control did not yield the expected results.

The efficiency of hybridization between the nucleic acid on the membrane and the nucleic acid in the hybridization solution is one of the other hybridization limitations (Alberts *et al.,* 1998). In this analysis some of the smears (DNA restrictions) that were obtained were very faint i.e. the DNA concentrations were too low for Southern blotting. This fact could have limited the amount of DNA transferred to the membrane during blotting. Research indicates that under typical blot hybridization conditions, only 0.5-5% of the target molecules on the membrane are actually bound to available probe (Brown, 1998a; Brown; 1998b).

During the first analysis the probe concentration was kept to a minimum and it had also labeled very efficiently. Research has shown that low probe concentration can also lead to low sensitivity in the hybridization process (Brown, 1998a; Brown; 1998b). On the other hand using probe concentration higher than 25ng/ml is totally discouraged as it leads to non-specific binding of the probe. Therefore, a slight increase (not more than 10ng/ml) in probe concentration was a good potential for positive results. However, this did not improve the results.

Stripping and re-probing was done (three times) for all the membranes. A shaker (with a stable temperature) was used, all conditions were optimized and the probe

concentration was slightly increased. However, the desired results were still not obtained. Studies have shown that blots/membranes can be used multiple times by stripping previously bound probe. However, removing hybridized probe is not always a simple task though (Vernier *et al.*, 1996; Alcamo, 1999; Tait, 1997). Although some stripping protocols can be effective at removing hybridized DNA probes, they often adversely affect the nucleic acid on the blot. A significant portion of the target DNA bound to the blot is typically rendered non-functional. This happens in two ways: either the DNA is removed from the blot altogether, or by being chemically altered so that it cannot be hybridized to probe (Vernier *et al.*, 1996; Alcamo, 1999; Tait, 1997). Positive hybridization results were very crucial for this analysis as they were going to be combined with the other results obtained so as to form an overall conclusive finding.

CONCLUSION

Finding a rapid detection method for aflatoxigenic *Aspergillus* isolates proved more challenging/complex than envisaged. Four methods were investigated in this analysis: HPLC for detection of aflatoxins; RAPD, RFLP and PCR methods were used for the differentiation of aflatoxigenic and non-aflatoxigenic isolates.

Of the techniques used HPLC proved to be a very reliable and good method of detecting aflatoxins even at very low concentrations. However, it was also a very time-consuming technique i.e., aflatoxin standards and sample preparations; aflatoxin detection process and calculating concentrations. HPLC is also a vey expensive technique that requires expertise in order to operate properly. Also, use of incorrect equipment/reagents such as columns and mobile phases can results in the detection of incorret compounds. For aflatoxin detection and quantification, HPLC is still the method of choice. In this study it proved reliable in detecting and quantifying all four aflatoxins. Correct detecting of aflatoxin production from the 12 isolates was critical because the rest of the analyses in the study were depended on whether the isolates were aflatoxigenic or non-aflatoxigenic. Reproducibility of results is also very good with HPLC, provided the conditions are optimized all the time.

RAPD and dendogram analysis also proved to be very simple and fast techniques and the fact that very little foreknowledge of the genetics of the isolates was required made it easier. However, both techniques also required expertise and background knowledge of PCR and interpretation of dendograms. PCR is also an expensive technique. The dendogram software package was also very expensive (and was not purchased) and the analysis was then done at another institution. This was challenging and time-consuming. One advantage of dendogram analysis was that the results were obtained very quickly.

The RAPD and dendogram genetic profiles of the non-aflatoxigenic isolates did not prove to be conclusively similar to each other. Also, there were no conclusive results that showed differentiation between aflatoxigenic and non-aflatoxigenic. Genetic profiles mostly depended on the primer that was used and this is one of the shortfalls of RAPD analysis. Choosing primers that highly differentiated aflatoxigenic from

non-aflatoxigenic isolates proved to be very important for future studies. However, reproducibility was not consistent. RAPD analysis is very sensitive to reaction conditions. Also, since dendograms were dependant on RAPD analysis, it was crucial to optimize the condition for PCR-RAPD. Good quality genomic DNA was used and PCR conditions were kept optimum and this assisted in the improvement of reproducibility.

RFLP analysis was an easy technique but it required large amounts of DNA (when compared to RAPD analysis) and longer incubation time. The non-aflatoxigenic isolates were sufficiently digested by the REs, that is, the smears were more visible (to the naked eye). For some aflatoxigenic isolates used in the study the digestions were poor and one of the major shortfalls of this technique is that DNA methylation inhibits cutting by some REs. This technique was time consuming mainly because of the Southern blotting and hybridization that followed. Southern blotting proved to be an easy to understand/follow technique which, however, could result in negative results if its not followed properly or optimized. Hybridization proved to be 'technically challenging' and led to 'no signal' results. However, the technique is easy to master, provided conditions are optimum, in order to produce positive results.

PCR for detection of the *aflR* gene was an easy and automated technique, however, the number of amplification cycles were longer, which made this process time-consuming. PCR the conditions were optimum especially the annealing temperature (which is very crucial). If the annealing temperature is incorrect or above the melting temperature of the enzyme, the 'undesired results' will not be obtained. All *A. flavus* and *A. parasiticus* isolates analysed produced a band corresponding to the *afl*R gene. Both species are known to be potentially aflatoxigenic, even though two *A. flavus* isolates were found to be non-aflatoxigenic in this analysis. The three non-aflatoxigenic isolates didn't produce this gene. Therefore, the presence of an *afl*R gene can be used as one of the key methods of detecting aflatoxigenic isolates.

Overall, all methods/techniques that were used in this analysis proved useful in the process of finding a rapid detection method for characterizing aflatoxigenic and non-aflatoxigenic *Aspergillus* isolates.

Some recommendation for this type of analysis include optimizing RFLP conditions, especially DNA concentration in order to improve the Southern blotting/hybridization results. Also, the hybridization conditions should be optimized. This will assist to avoid stripping and re-probing since stripping blots does more damage than good. All equipment's working conditions (e.g. PCR machine, hybridization ovens etc.) should be checked prior to use so as to avoid getting false/negative results or delays.

Future work should focus more on the study and analysis of the *afl*R gene as a tool of differentiating aflatoxigenic isolates from the non-aflatoxienic ones. This should be used in conjuction with Southern blotting/hybridization analysis.

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92. **Wellinder, B. S., Kornfelf, T. and Sorensen, H. H.** 1995. HPLC. *Analytical Chemistry* **67**:39-43.
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APPENDICES

APPENDIX 1

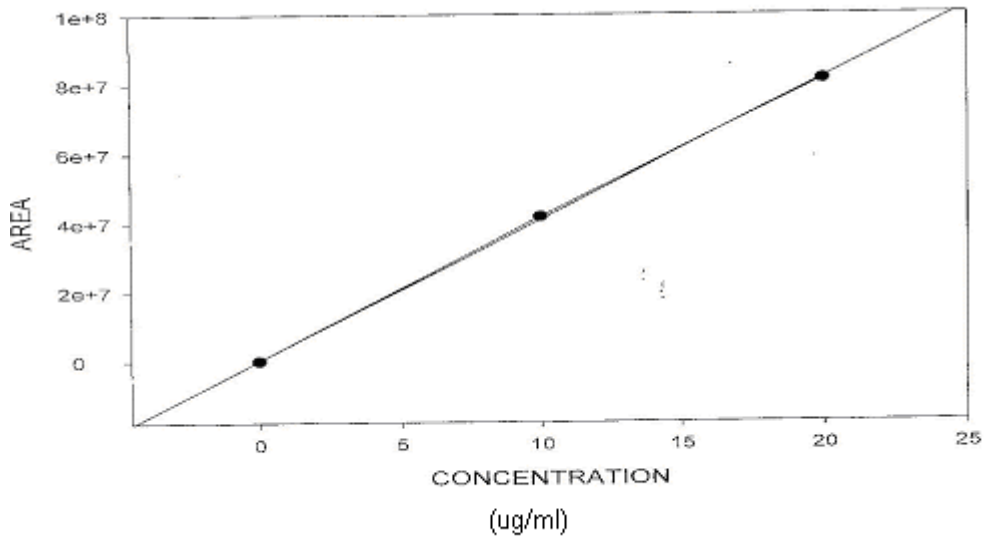

Figure 23: AFB_1 Standard curve.

APPENDIX 2

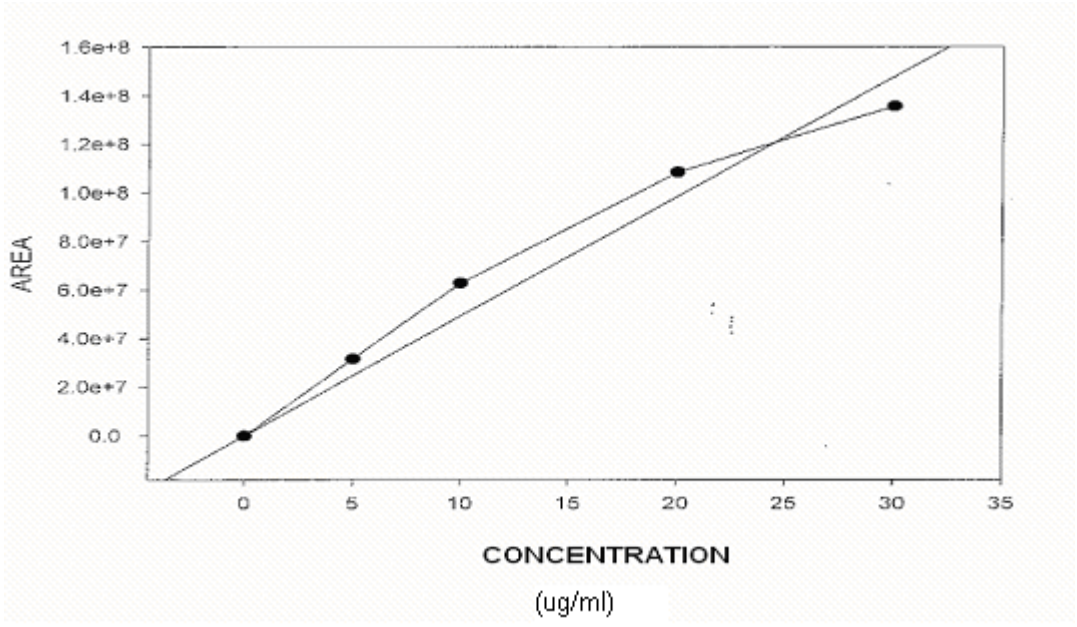

Figure 24: AFB_2 Standard curve.

APPENDIX 3

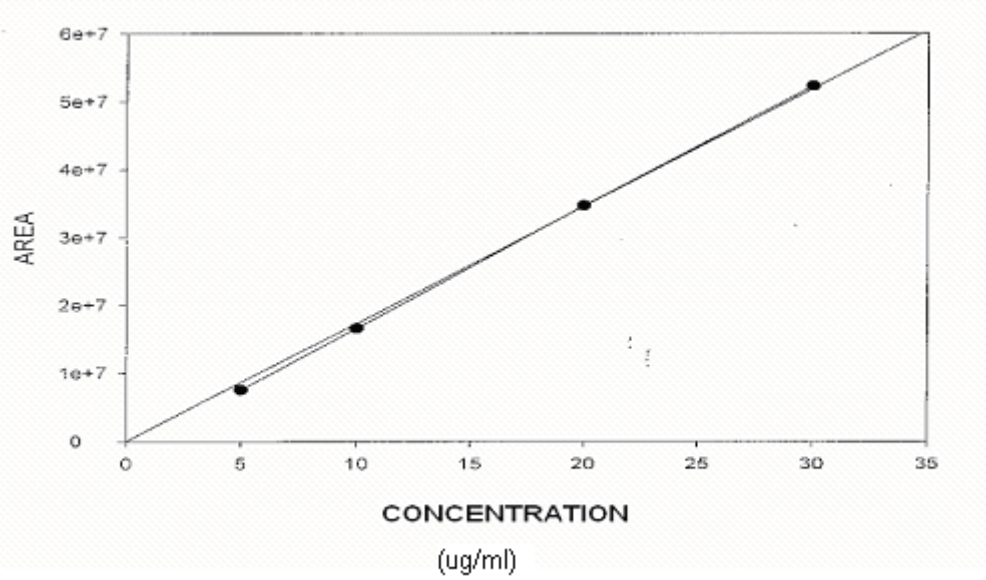

Figure 25: AFG_1 Standard curve for aflatoxin.

APPENDIX 4

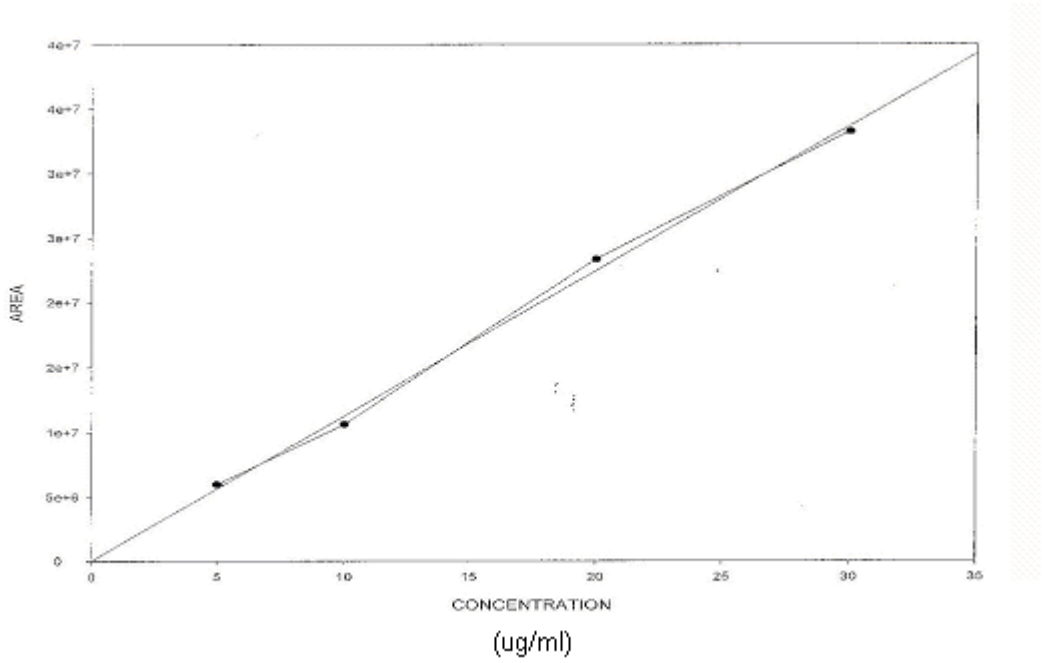

Figure 26: AFG_2 Standard curve.

APPENDIX 4B

Formula for calculating Standard error of mean:

SE = Standard Deviation /square root of mean of sample population

AFLATOXIN CONCENTRATIONS RESULTS

FUNGAL ISOLATE	TYPE OF AFLATOXIN	SAMPLE 1	SAMPLE 2	MEAN/AVERAGE	STANDARD DEVIATION	STANDARD OF ERROR
32592	AFB_2	4.9	5.3	5.1	0.3	0.13
11970	AFB_2	25.8	21.6	23.7	3.0	0.60
	AFG_2	0.009	0.011	0.010	0.001	0.01
2885	AFB_1	5.8	5.2	5.5	0.001	0.0004
	AFB_2	35.3	38.1	36.7	2.0	0.32
	AFG_2	28.8	27.8	28.3	0.7	0.13
3641	AFB_1	0.019	0.023	0.021	0.003	0.03
	AFB_2	14.1	12.3	13.2	1.3	0.36
	AFG_1	6.3	6.7	6.5	0.3	0.12
	AFG_2	0.045	0.053	0.049	0.005	0.03
5063	AFB_1	6.7	6.1	6.4	0.4	0.16
	AFB_2	26.5	24.7	25.6	1.3	0.25
5183	AFB_1	0.010	0.014	0.012	0.003	0.03
	AFB_2	4.2	3.8	4	0.3	0.15
5990	AFB_2	22.5	22.9	22.7	0.3	0.06

APPENDIX 5

SMKY Broth (pH 6.0)

Sucrose	20g
Magnesium sulphate	0.5g
Potassium nitrate	3.0g
Yeast extract	7g
Water	100ml

APPENDIX 6

Genomic DNA extraction buffer

2M Tris-HCl (pH 8.5)	10ml
1M NaCl	25ml
0.5M EDTA (pH 8.0)	5ml
10% SDS	5ml
Water	55ml

APPENDIX 7

RNaseA solution

RNaseA powder	200mg
TE buffer	10ml

APPENDIX 8

TE buffer

10mM Tris-HCl (pH 8.0)

1mM EDTA (pH 8.0)

Water (to make up to 10ml)

APPENDIX 9

50X TAE buffer

2M Tris-base	242.2g
0.5M EDTA (pH 8.0)	100ml
Acetic acid	57.1ml
Water	1000ml

APPENDIX 10

1X TAE buffer

50X TAE buffer	1ml
Water	49ml

APPENDIX 11

0.8% Agarose gel

Agarose gel	0.8g
1X TAE buffer	100ml

APPENDIX 12

2% Agarose gel

Agarose gel	2g
1X TAE buffer	100ml

APPENDIX 13

Gel loading buffer

Sucrose	8g
Bromophenol blue	50mg
Water	20ml

APPENDIX 14

Ethidium bromide solution

Ethidium bromide stock (10mg/ml)	10µl
Water	200ml

APPENDIX 15

2X SSC solution

0.3M NaCl	17.532g
0.03M Sodium citrate	8.823g
Water	1000ml

APPENDIX 16

5X SSC solution

0.7M NaCl	43.83g
0.075M Sodium citrate	22.0575g
Water	1000ml

APPENDIX 17

20X SSC solution (pH 7.0)

3M NaCl	175.32g
0.3M Sodium citrate	88.23g
Water	1000ml

APPENDIX 18

Table 10: Composition/preparation of reagents/solutions for hybridization and determination of labeling efficiency.

Solution	Composition/Preparation	Storage and Stability	Use
Washing Buffer	0.1M Maleic acid, 0.15M NaCl; pH 7.5 (20ºC); 0.3% (v/v) Tween 20	15-25ºC stable	Removal of unbound antibody
Maleic acid buffer	0.1M Maleic acid, 0.15M NaCl; adjust with NaOH (solid) to pH 7.5 (20ºC)	15-25ºC stable	Dilution of Blocking solution
Detection buffer	0.1M Tris-HCl, 0.1M NaCl, pH 9.5 (20ºC)	15-25ºC stable	Adjustment of pH 9.5
TE-buffer	10mM Tris-HCl, 1mM EDTA, pH 8.0	15-25ºC stable	Stopping colour reaction

APPENDIX 19

Table 11: Composition/preparation of kit working solutions for hybridization and determination of labeling efficiency.

Solution	Composition/Preparation	Storage and Stability	Use
Blocking stock solution (10 x conc)	Dissolve blocking reagent 10% (w/v) in Maleic acid buffer under constant stirring on a heating block (65ºC) or heat in a microwave, autoclave. Solution remains opaque.	2-8ºC	Preparation of Blocking solution
Blocking solution	Prepare a 1X working solution by diluting the 10X Blocking solution 1:10 in Maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites on the membrane
Antibody solution	Centrifuge Anti-Digoxigenin-AP for 5 min @ 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:5 000 in Blocking solution	12 hrs @ 2-8ºC	Binding to the DIG-labeled probe
Color substrate solution	Add 40µl of NBT/BCIP to 2ml of Detection buffer. **Note:** Store protected from light.	Always prepare fresh	Visualization of antibody-binding

APPENDIX 20

RAPD Gel profile with primer 211

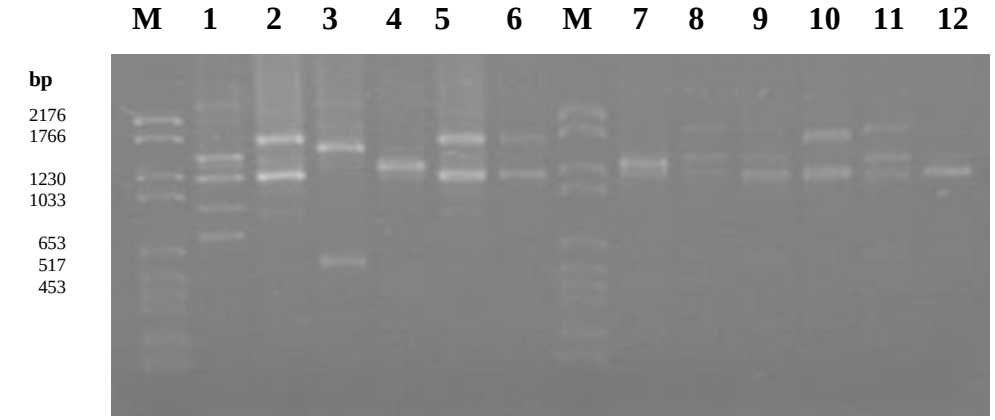

Figure 27: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 211. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 21

RAPD Gel profile with primer 213

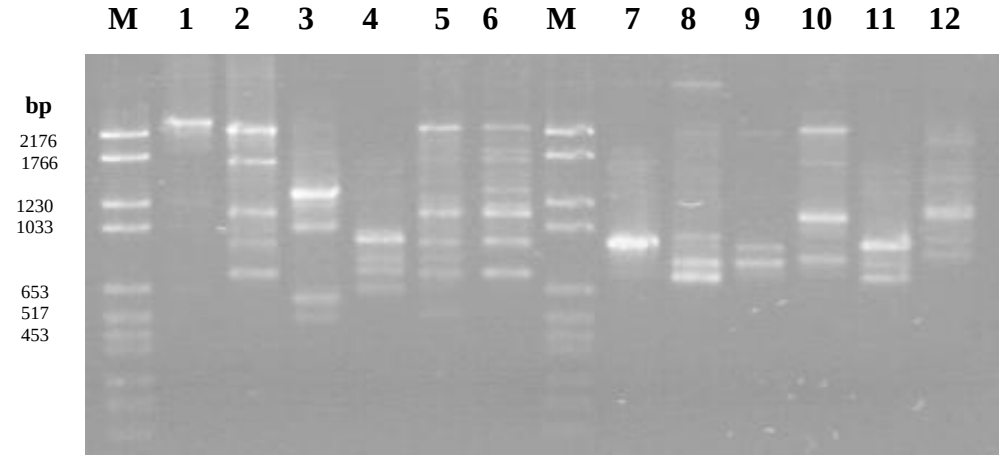

Figure 28: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 213. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 22

RAPD Gel profile with primer 220

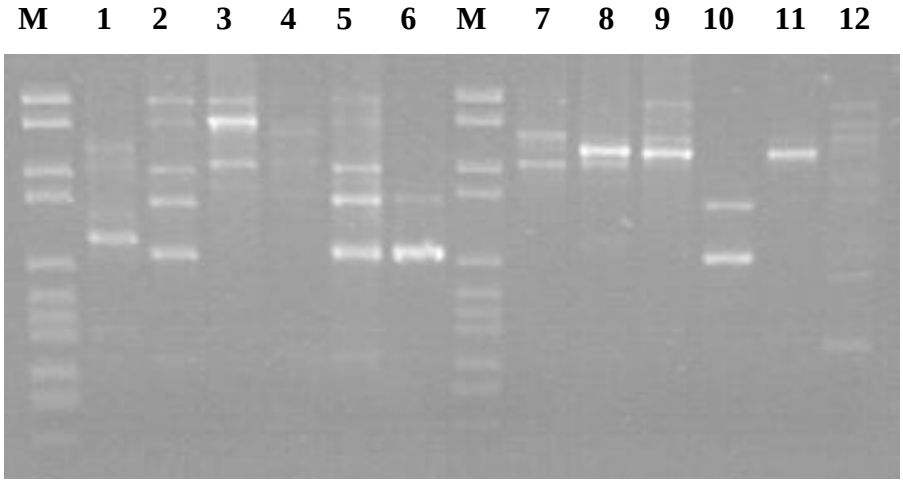

Figure 29: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 220. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 23

RAPD Gel profile with primer 230

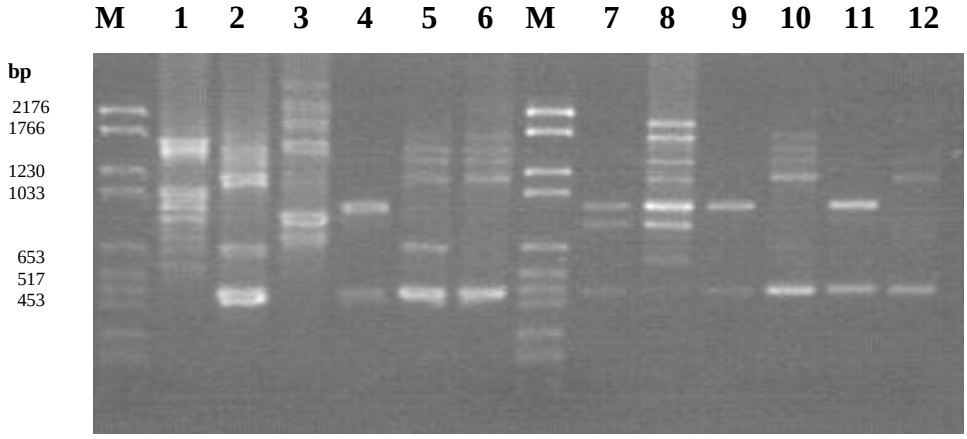

Figure 30: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 230. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 24

RAPD Gel profile with primer 270

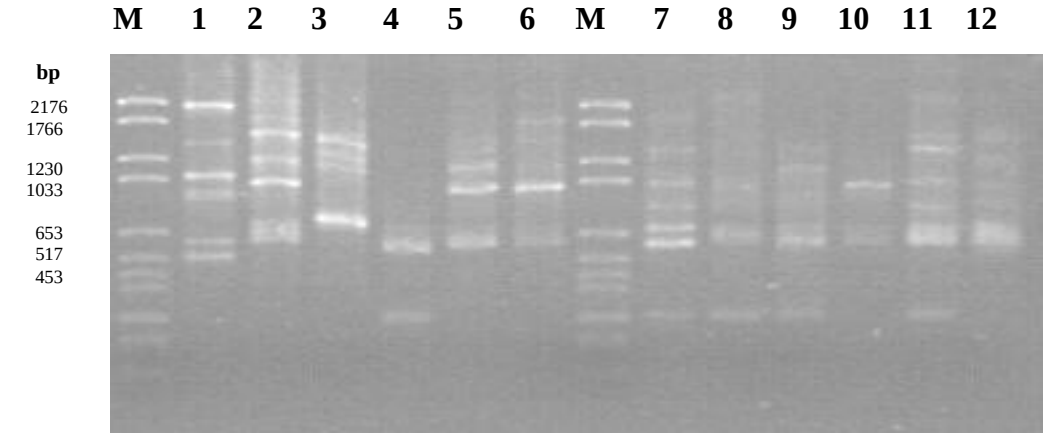

Figure 31: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 270. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 25

RAPD Gel profile with primer 286

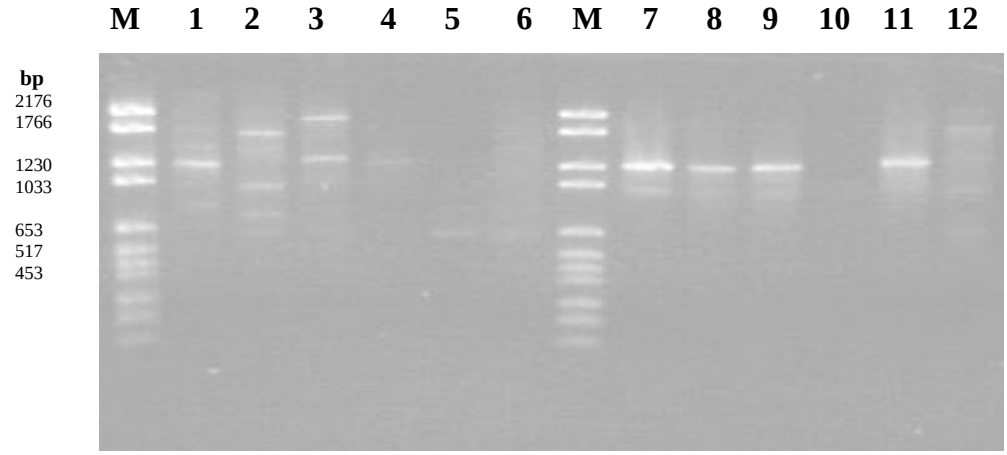

Figure 32: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 286. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 26

RAPD Gel profile with primer 295

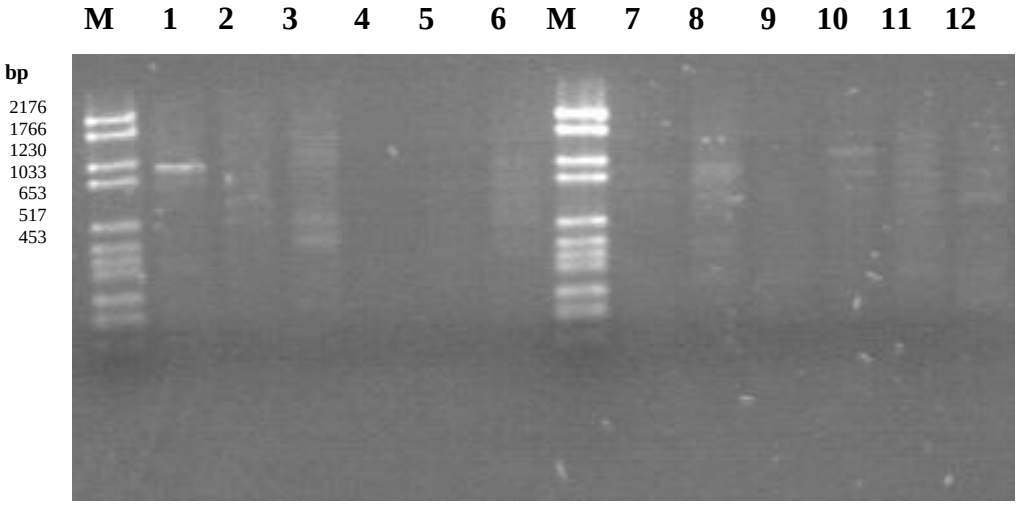

Figure 33: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 295. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 27

RAPD Gel profile with primer 300

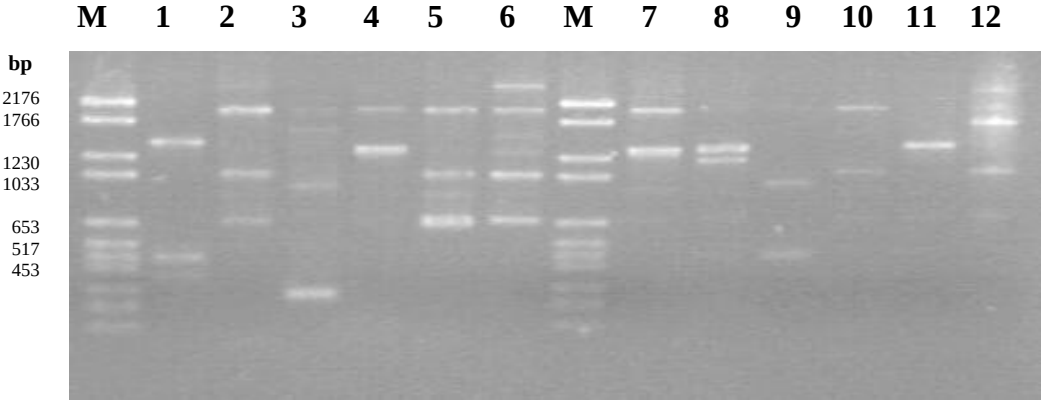

Figure 34: RAPD gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 300. Lanes: M - Molecular Marker VI; 1 - *A. niger* MLST; 2 - *A. oryzae* 3151; 3 - *A. tamarii* MLST1; 4 - *A. flavus* 32592; 5 - *A. flavus* 5065; 6 - *A. flavus* 7052; 7 - *A. parasiticus* 11970; 8 - *A. parasiticus* 2885; 9 - *A. parasiticus* 3641; 10 - *A. parasiticus* 5063; 11 - *A. parasiticus* 5183; 12 - *A. parasiticus* 5990

APPENDIX 28

Dendogram profile with primer 211

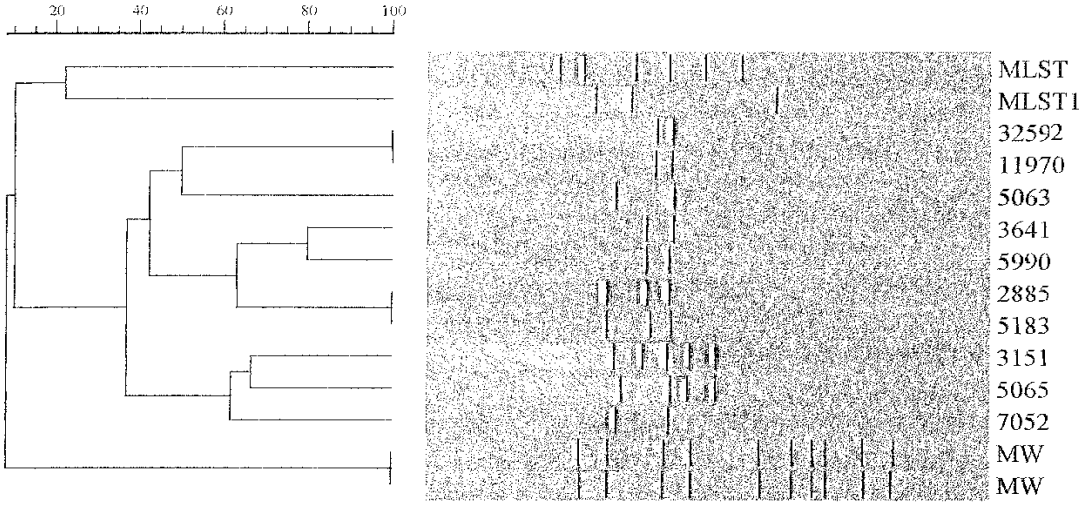

Figure 35: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 211. Keys/Lanes: MLST - *A. niger* MLST; MLST1 - *A. tamarii* MLST1; 32592 - *A. flavus* 32592; 11970 - *A. parasiticus* 11970; 5063 - *A. parasiticus* 5063; 3641 – *A. parasiticus* 3641; 5990 - *A. parasiticus* 5990; 2885 - *A. parasiticus* 2885; 5183 - *A. parasiticus* 5183; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 7052 - *A. flavus* 7052; MW - Molecular Marker VI

APPENDIX 29

Dendogram profile with primer 213

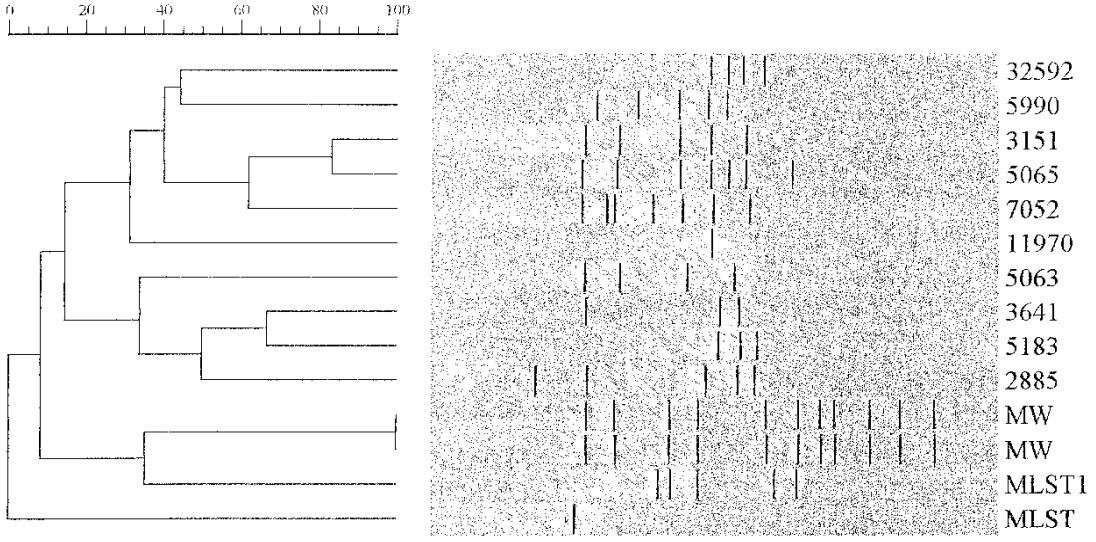

Figure 36: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 213. Keys/Lanes: 32592 - *A. flavus* 32592; 5990 - *A. parasiticus* 5990; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 7052 - *A. flavus* 7052; 11970 - *A. parasiticus* 11970; 5063 - *A. parasiticus* 5063; 3641 – *A. parasiticus* 3641; 5183 - *A. parasiticus* 5183; 2885 - *A. parasiticus* 2885; MW - Molecular Marker VI; MLST1 - *A. tamarii* MLST1; MLST - *A. niger* MLST

APPENDIX 30

Dendogram profile with primer 220

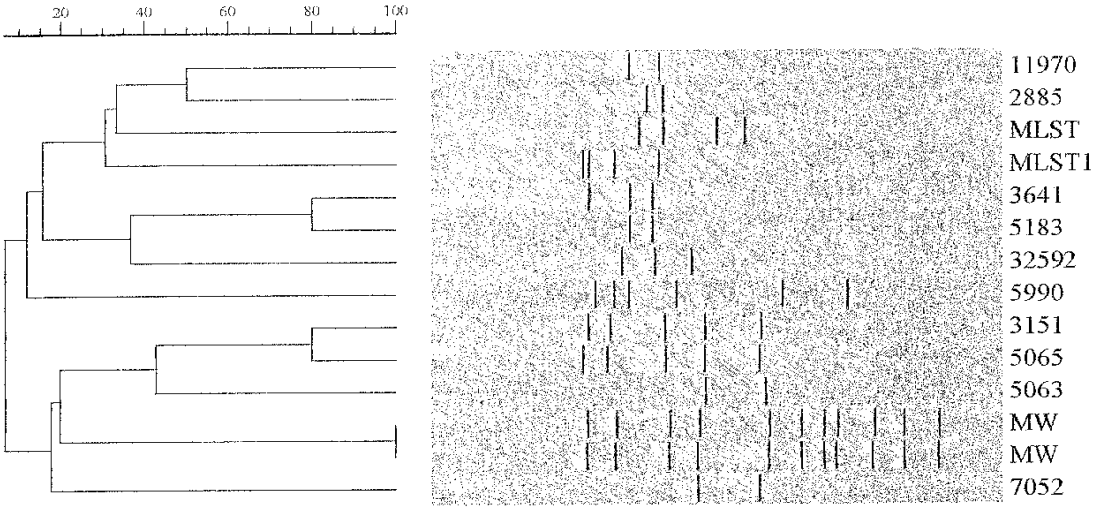

Figure 37: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 220. Keys/Lanes: 11970 - *A. parasiticus* 11970; 2885 - *A. parasiticus* 2885; MLST - *A. niger* MLST; MLST1 - *A. tamarii* MLST1; 3641 – *A. parasiticus* 3641; 5183 - *A. parasiticus* 5183; 32592 - *A. flavus* 32592; 5990 - *A. parasiticus* 5990; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 5063 - *A. parasiticus* 5063; MW - Molecular Marker VI; 7052 - *A. flavus* 7052

APPENDIX 31

Dendogram profile with primer 230

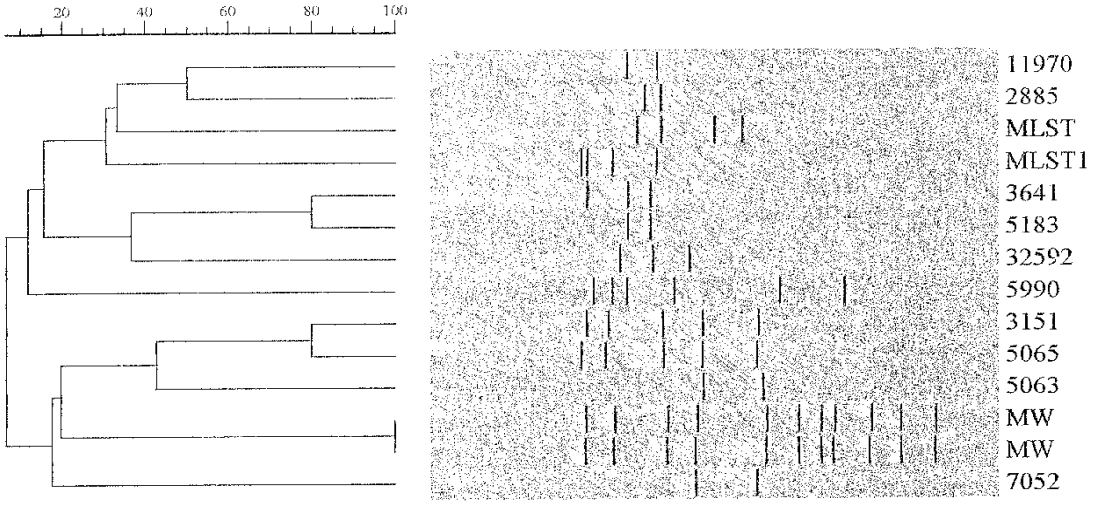

Figure 38: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 230. Keys/Lanes: 11970 - *A. parasiticus* 11970; 2885 - *A. parasiticus* 2885; MLST - *A. niger* MLST; MLST1 - *A. tamarii* MLST1; 3641 – *A. parasiticus* 3641; 5183 - *A. parasiticus* 5183; 32592 - *A. flavus* 32592; 5990 - *A. parasiticus* 5990; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 5063 - *A. parasiticus* 5063; MW - Molecular Marker VI; 7052 - *A. flavus* 7052

APPENDIX 32

Dendogram profile with primer 270

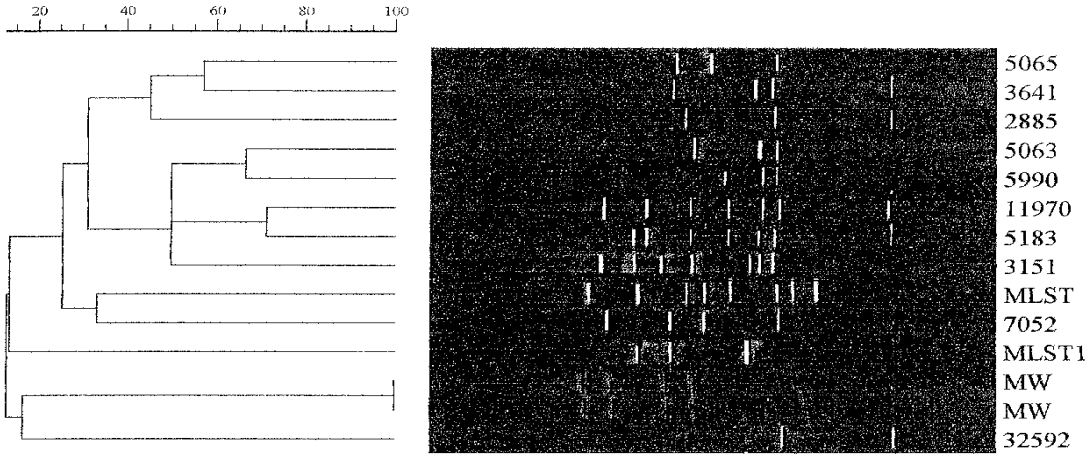

Figure 39: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 270. Keys/Lanes: 5065 - *A. flavus* 5065; 3641 – *A. parasiticus* 3641; 2885 - *A. parasiticus* 2885; 5063 - *A. parasiticus* 5063; 5990 - *A. parasiticus* 5990; 11970 - *A. parasiticus* 11970; 5183 - *A. parasiticus* 5183; 3151- *A. oryzae* 3151; MLST - *A. niger* MLST; 7052 - *A. flavus* 7052; MLST1 - *A. tamarii* MLST1; MW - Molecular Marker VI; 32592 - *A. flavus* 32592

APPENDIX 33

Dendogram profile with primer 286

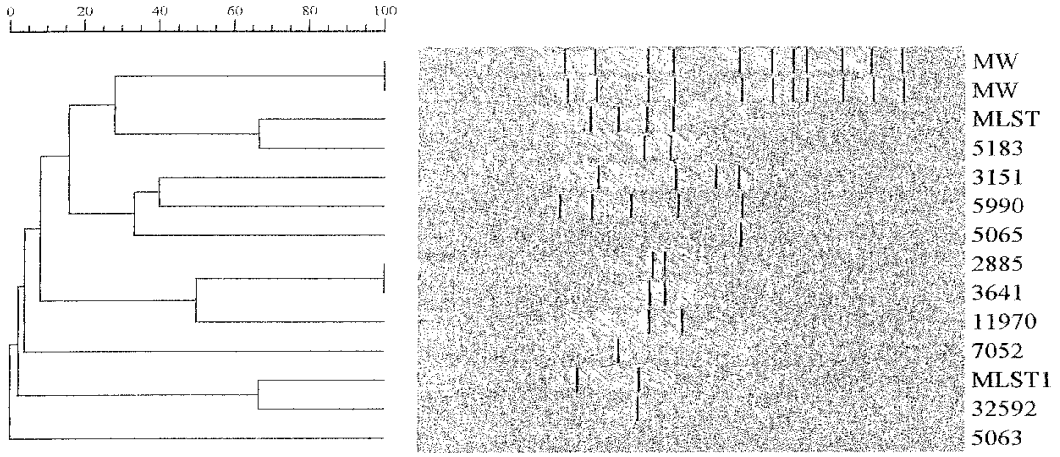

Figure 40: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 286. Keys/Lanes: MW - Molecular Marker VI; MLST - *A. niger* MLST; 5183 - *A. parasiticus* 5183; 3151- *A. oryzae* 3151; 5990 - *A. parasiticus* 5990; 5065 - *A. flavus* 5065; 2885 - *A. parasiticus* 2885; 3641 – *A. parasiticus* 3641; 11970 - *A. parasiticus* 11970; 7052 - *A. flavus* 7052; MLST1 - *A. tamarii* MLST1; 32592 - *A. flavus* 32592; 5063 - *A. parasiticus* 5063

APPENDIX 34

Dendogram profile with primer 295

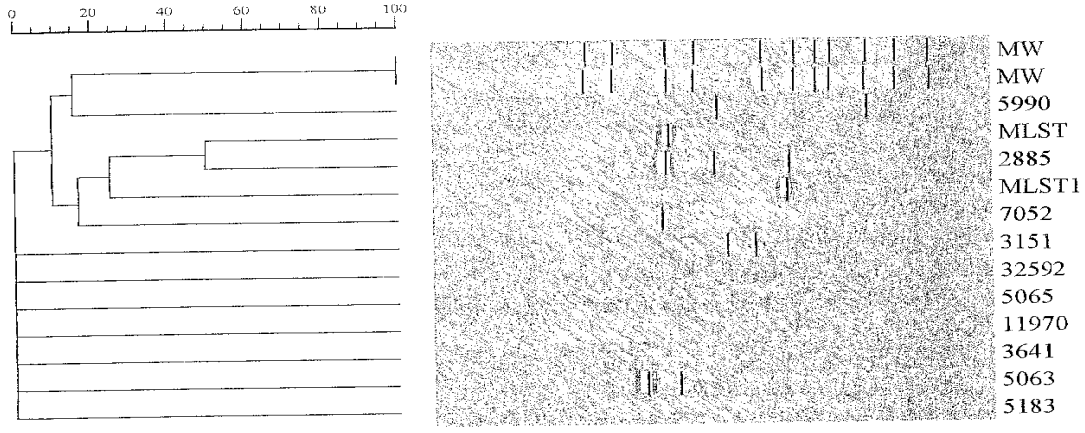

Figure 41: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 295. Keys/Lanes: MW - Molecular Marker VI; 5990 - *A. parasiticus* 5990; MLST - *A. niger* MLST; 2885 - *A. parasiticus* 2885; MLST1 - *A. tamarii* MLST1; 7052 - *A. flavus* 7052; 3151- *A. oryzae* 3151; 32592 - *A. flavus* 32592; 5065 - *A. flavus* 5065; 11970 - *A. parasiticus* 11970; 3641 – *A. parasiticus* 3641; 5063 - *A. parasiticus* 5063; 5183 - *A. parasiticus* 5183

APPENDIX 35

Dendogram profile with primer 300

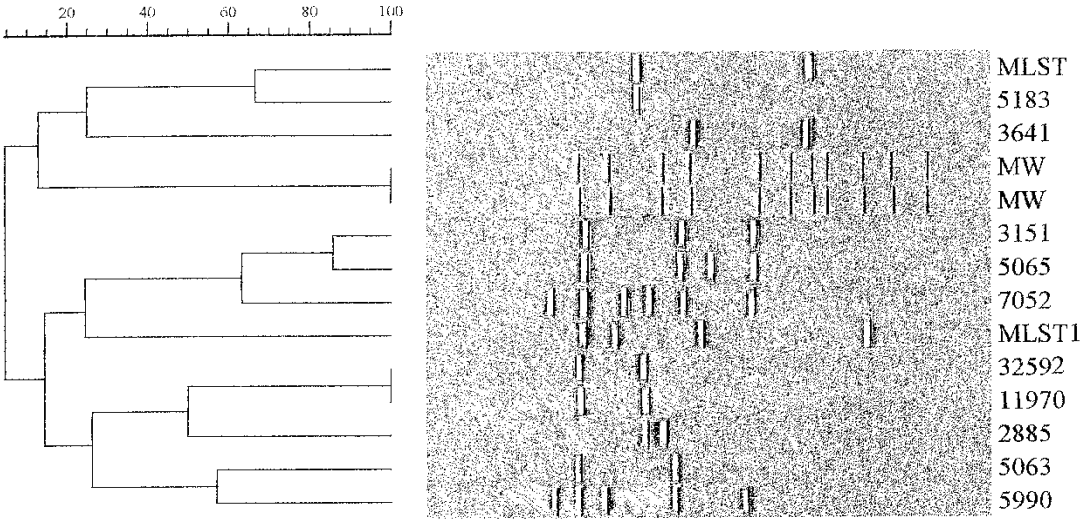

Figure 42: Dendogram gel pattern/profile of twelve *Aspergillus* isolates generated with primer UBC 300. Keys/Lanes: MLST - *A. niger* MLST; 5183 - *A. parasiticus* 5183; 3641 – *A. parasiticus* 3641; MW - Molecular Marker VI; 3151- *A. oryzae* 3151; 5065 - *A. flavus* 5065; 7052 - *A. flavus* 7052; MLST1 - *A. tamarii* MLST1; 32592 - *A. flavus* 32592; 11970 - *A. parasiticus* 11970; 2885 - *A. parasiticus* 2885; 5063 - *A. parasiticus* 5063; 5990 - *A. parasiticus* 5990

APPENDIX 36

Restriction analysis by *Hind*III

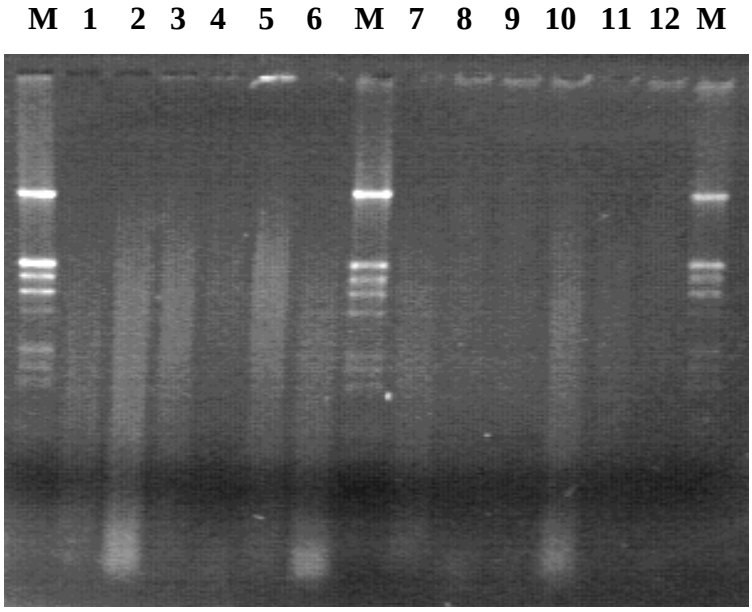

Figure 43: Restriction by *Hind*III enzyme.Key: M-Molecular Marker III, 1-*A. niger* MLST, 2-*A. oryzae* 3151, 3-*A. tamarii* MLST1, 4-*A. flavus* 32592, 5-*A. flavus* 5065, 6-*A. flavus* 7052, 7-*A. parasiticus* 11970, 8- *A. parasiticus* 2885, 9- *A. parasiticus* 3641, 10- *A. parasiticus* 5063, 11- *A. parasiticus* 5183, 12- *A. parasiticus* 5990.

APPENDIX 37

Restriction analysis by *EcoR*V

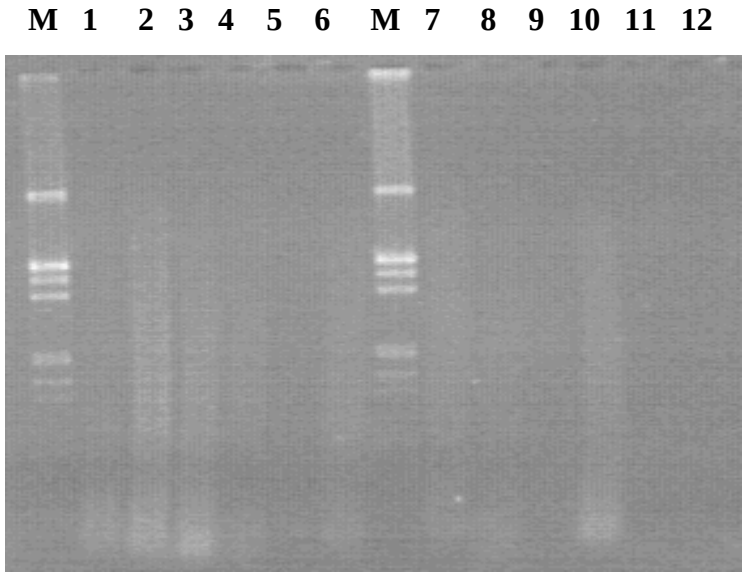

Figure 44: Restriction by *EcoR*V enzyme. Key: M-Molecular Marker III, 1-*A. niger* MLST, 2-*A. oryzae* 3151, 3-*A. tamarii* MLST1, 4-*A. flavus* 32592, 5-*A. flavus* 5065, 6-*A. flavus* 7052, 7-*A. parasiticus* 11970, 8- *A. parasiticus* 2885, 9- *A. parasiticus* 3641, 10- *A. parasiticus* 5063, 11- *A. parasiticus* 5183, 12- *A. parasiticus* 5990.

APPENDIX 38

Restriction analysis by *EcoR*I

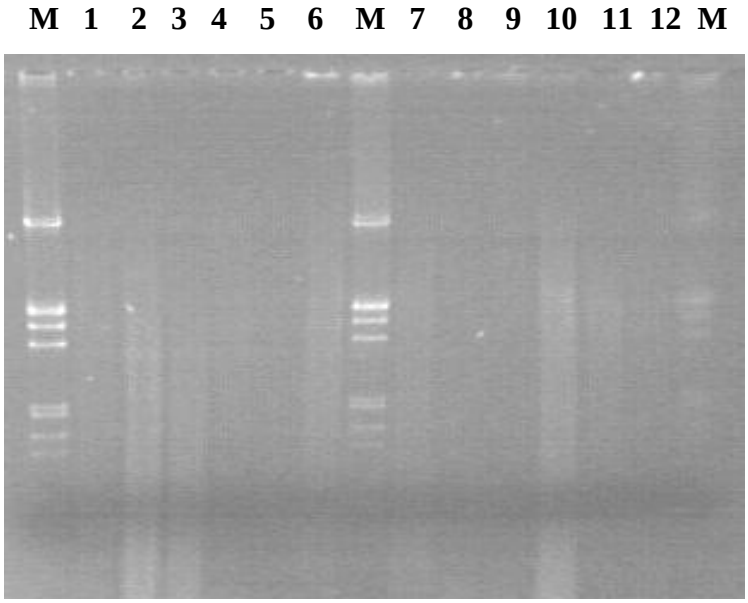

Figure 44: Restriction by *EcoR*I enzyme. Key: M-Molecular Marker III, 1-*A. niger* MLST, 2-*A. oryzae* 3151, 3-*A. tamarii* MLST1, 4-*A. flavus* 32592, 5-*A. flavus* 5065, 6-*A. flavus* 7052, 7-*A. parasiticus* 11970, 8- *A. parasiticus* 2885, 9- *A. parasiticus* 3641, 10- *A. parasiticus* 5063, 11- *A. parasiticus* 5183, 12- *A. parasiticus* 5990.

APPENDIX 39

Restriction analysis by *Dra*I

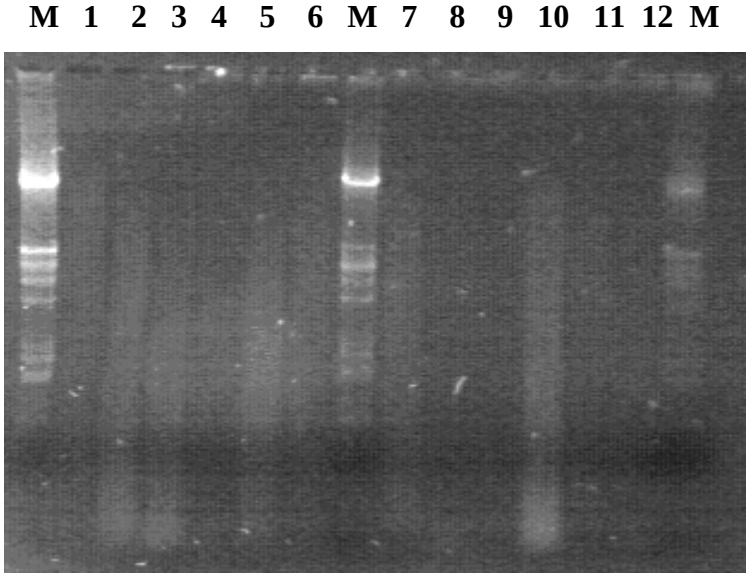

Figure 46: Restriction by *Dra*I enzyme. Key: M-Molecular Marker III, 1-*A. niger* MLST, 2-*A. oryzae* 3151, 3-*A. tamarii* MLST1, 4-*A. flavus* 32592, 5-*A. flavus* 5065, 6-*A. flavus* 7052, 7-*A. parasiticus* 11970, 8- *A. parasiticus* 2885, 9- *A. parasiticus* 3641, 10- *A. parasiticus* 5063, 11- *A. parasiticus* 5183, 12- *A. parasiticus* 5990.

APPENDIX 40

Restriction analysis by *Bgl*I

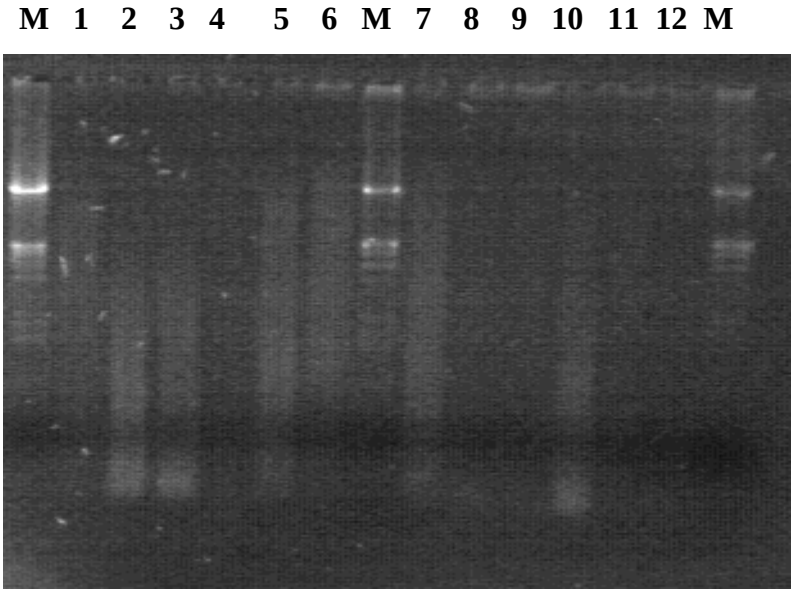

Figure 47: Restriction by *Bgl*I enzyme. Key: M-Molecular Marker III, 1-*A. niger* MLST, 2-*A. oryzae* 3151, 3-*A. tamarii* MLST1, 4-*A. flavus* 32592, 5-*A. flavus* 5065, 6-*A. flavus* 7052, 7-*A. parasiticus* 11970, 8- *A. parasiticus* 2885, 9- *A. parasiticus* 3641, 10- *A. parasiticus* 5063, 11- *A. parasiticus* 5183, 12- *A. parasiticus* 5990.