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Distribution and molecular characterization of aflatoxin-producing and non-producing isolates of *Aspergillus* section Flavi for biological control of aflatoxin contamination in maize in Nigeria

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Distribution and molecular characterization of aflatoxin-producing and non-producing isolates of *Aspergillus* section Flavi for biological control of aflatoxin contamination in maize in Nigeria

Fungal communities in soils of Nigerian maize fields were examined to determine distributions of aflatoxin-producing fungi and to identify endemic atoxigenic strains of potential value as biological control agents for limiting aflatoxin contamination in West African crops. Over 1,000 isolates belonging to *Aspergillus* section Flavi were collected from soil of 55 Nigerian maize fields located in three agroecological zones by dilution plating onto modified Rose Bengal agar. The most common member of *Aspergillus* section Flavi (85% of isolates) was the *A. flavus* L-strain followed by the unnamed taxon known as strain S_{BG} (8%), *A. tamarii* (6%) and *A. parasiticus* (1%). The highest incidence of S_{BG} was in the Zaria district, and the lowest was in the Ogbomosho and Ado-Ekiti districts. Only 44% of 492 *A. flavus* isolates produced aflatoxins (limit of detection 5 ng g^{-1}). All S_{BG} and *A. parasiticus* isolates produced both B and G aflatoxins and greater than 300 μg g^{-1} total aflatoxins. Thirty two percent of the *A. flavus* isolates produced >1 μg g^{-1} total aflatoxins but no *A. flavus* isolate produced G aflatoxins. The most important aflatoxin producers varied by region. However, all regions had atoxigenic strains of potential value as biological control agents.

In addition, this study focused on 18 atoxigenic *A. flavus* Vegetative Compatibility Groups (VCGs) collected from naturally infected maize. Loci across the 68 kb aflatoxin biosynthesis gene cluster were compared among the 18 atoxigenic VCGs, an atoxigenic strain used commercially in North America to manage aflatoxins, and several aflatoxin producers. Five of the VCGs from Nigeria had large deletions (37kb to 65kb) extending from the telomeric side of the aflatoxin biosynthesis cluster. In one case (isolate AV0222), the deletion extended through the cluster to the adjacent sugar cluster. The remaining 12 atoxigenic VCGs, including the VCG used for aflatoxin management in North America contained all genes of the aflatoxin pathway. Comparison of pathway genes revealed more changes in atoxigenic than in aflatoxin-producing VCGs and several non-synonymous changes that are unique to atoxigenics. However, for some atoxigenic VCGs, additional sequencing and experimentation will be required to determine precise causes of atoxigenicity.

Finally, a set of atoxigenic and toxigenic *A. flavus* L-strains from each soil sample were analyzed to determine their morphological, physiological, and genetic characters. Isolates that produced high levels of toxin in liquid fermentation produced proportionally less spores and had a higher sclerotia mass on Czapeks agar (31°C, 25days), while isolates with high spore mass and less sclerotia mass produced less aflatoxin. Portions of the aflatoxin biosynthetic pathway genes, *aflR*, as well as genes from the *Aspergillus flavus* genome (*taka amylase* and *pecA*) were amplified by PCR and sequenced. Phylogenetic analysis of all three genes confirmed that the high B+G aflatoxin producing S_{BG} strain of West Africa did not belong to *A. minisclerotigenes* or Group II as previously assumed. *A. parasiticus* isolates of Nigeria were phylogenetically separated from those of other regions in the world. Within the *A. flavus* isolates, differences between toxigenic and atoxigenic L-strains were found in two of three analyzed genes.

Die Verteilung und molekulare Charaterisierung von aflatoxin produzierenden und nicht-produzierenden *Aspergillus* Isolaten für die Entwicklung einer biologischen Bekämpfungsmassnahme von aflatoxin kontaminierten Mais in Nigeria.

Die Verteilung von aflatoxinbildenden und nicht-aflatoxinbildende *Aspergillus* Stämmen aus nigerianischen Böden wurde bestimmt mit dem Ziel mögliche natürlich vorkommende nicht-aflatoxinbildenden *Aspergillus* Isolate als biologische Kontrolmaßnahme verwenden zu können. Aus insgesamt 55 Bodenproben nigerianischer Maisfelder, die über 3 agroecologische Zonen verteilt waren, wurden über 1000 *Aspergillus* Isolate auf Rose Bengal Agar isoliert. Der am häufigsten vorkommende Vertreter der Gattung *Aspergillus* war *A. flavus* L-Stamm (85%), gefolgt von dem namenlosen Taxon, bekannt als S_{BG}-Stamm (8%), *A. tamarii* (6%) und *A. parasiticus* (1%). Das höchste Aufkommen von S_{BG} Isolaten wurde im Bezirk Zaria ermittelt und das niedrigste in den Bezirken Ogobomosho und Ado-Ekiti. Nur 44% von 492 *A. flavus* Isolaten produzierten Aflatoxin bei einer Nachweisgrenze von 5 ng g^{-1}. Alle S_{BG} Isolate sowie *A. parasiticus* Isolate produzierten B- und G-Aflatoxine mit mehr als 300 µg g^{-1} total Aflatoxin. Von den *A. flavus* Isolaten produzierten 32% mehr als 1 µg g^{-1} total Aflatoxin und keiner der *A. flavus* Isolaten produzierte G-Aflatoxine. In allen Regionen wurden nicht-aflatoxinbildende *Aspergillus* Stämme gefunden. Diese *A. flavus* Isolate wurden auf ihre Tauglichkeit als biologische Kontrollmaßnahme weiter untersucht.

Desweiteren wurden 18 von Mais isolierte nicht-aflatoxinbildende *A. flavus* Vegetative Compatibility Groups (VCGs) untersucht. Verschiedene Genabschnitte von dem 68 kb grossen Gencluster des aflatoxin Biosyntheseweges wurden mit den 18 nicht-aflatoxinbildenden VCGs, einem gewerblich genutzten nicht-aflatoxinbildenden Stamm aus Nord Amerika und mehreren aflatoxinbildenden Stämmen verglichen. Von der telomerischen Seite ausgehend zeigten fünf VCGs fehlende Genabschnitte im Gencluster des aflatoxin Biosynthesewege von der Grösse von 37 bis 65kb. Dem Isolat AV0222 fehlte das komplette Gencluster des aflatoxin Biosyntheseweges bis zum angrenzenden Zuckergencluster. Für die restlichen nicht-aflatoxinbildenden VCGs, sowie das nicht-aflatoxinbildenden Isolat aus Nord Amerika wurden alle Gene des aflatoxin Biosyntheseweges nachgewiesen. Der direkte Vergleich der Genesequencen zeigte mehr Punktmutationen in nicht-aflatoxinbildenden Isolaten als in aflatoxinbildenden Isolaten, die zum Teil eine Änderung der Aminosäure zur Folge hatte. Mehr Forschungsarbeit ist notwendig, um zu ermitteln, warum einige *Aspergillus* Stämme kein Aflatoxin synthetisierten.

Letztendlich wurden nicht-aflatoxinbildende und aflatoxinbildende *A. flavus* L-Stämme auf deren morphologischen, physiologischen und genetischen Charateristika untersucht. Isolate, die in Flüssigfermentation einen hohen Anteil an Aflatoxin produzierten, zeigten proportional weniger Sporenwachstum und hatten ein höheres Sclerotiagewicht auf Czapeks Agar (31°C, 25 Tage). Hingegen produzierten Isolate mit hohem Sporenwachstum und niedrigem Sclerotiagewicht, weniger Aflatoxin. Genabschnitte des aflatoxin Biosyntheseweges *aflR*, sowie Gene des *Aspergillus* Genome (*Taka Amylase* und *PecA*) von verschiedenen *Aspergillus* Isolaten aus Nigeria und Weltweit wurden mittels PCR amplifiziert und sequenziert. Die phylogenetische Analyse von allen Genabschnitten bestätigte, dass anders als vorher angenommen, der B+G aflatoxinbildende S_{BG}-Stamm aus West Afrika weder der Art *A. minisclerotigenes* noch der Group II angehört. *A. parasiticus* Isolate Nigerias unterschieden sich phylogenetisch von denen aus anderen Regionen der Welt. Aflatoxinbildende und nicht-aflatoxinbildende *A. flavus* Isolate unterschieden sich phylogenetisch in zwei von drei analysierten Genen.

Table of contents

List of Chemicals

Chemical	Manufacturer
Agar Technical	Difco
Agar-Agar	EMD
Agar Bacteriological	Oxoid
Aflatoxin Mix	SupelCo.
Ammonium Iron (III) Citrate	Aldrich
Ammonium Molybdate	Fisher
Ammonium Sulfate	J.T. Baker
Bacto Agar	BD
Bacto Casamino Acids	BD
Bacto Malt Extract	BD
Bacto Peptone	BD
Bacto Tryptone	BD
Bacto Yeast Extract	BD
Calcium Chloride	J.T. Baker
Capsicum	Kalsec
Chloramphenicol	EMD
Citric Acid	Sigma
Cupric Sulfate, Pentahydrate	Sigma
Czapek-Dox Broth	Difco
Dextrose, Anhydrous	J.T. Baker
Difco Agar	BD
Difco Soluble Starch	BD
Ethyl Alcohol USP	Aaper
Ferric Sulfate, n-hydrate	J.T. Baker
Ferrous Sulfate, Heptahydrate	Sigma
Folic Acid	Sigma
Glycerol	EMD
Glycine	Sigma
Hypoxanthine	Sigma
L-Glutamine	Sigma
Magnesium Sulfate Heptahydrate	EMD
Manganous Sulfate, Monohydrate	Fisher
MES	Research Organics
MES, sodium salt	Research Organics

List of chemicals

myo-Inositol	Sigma
Nicotinic Acid	Sigma
p-(Dimethylamino)benzaldehyde	J.T. Baker
pH 10.00 blue buffer	Fisher
pH 4.00 red buffer	VWR
pH 7.00 yellow buffer	VWR
Phenolphthalein	Acros Organics
PIPPS	Calbiochem
Polyoxyethylene-	Sigma
Potassium Chlorate	EMD
Potassium Chloride	J.T. Baker
Potassium Hydroxide	Fisher
Potassium Phosphate, Dibasic	J.T. Baker
Potassium Phosphate, Monobasic	J.T. Baker
Potato Dextrose Broth	EM Science
Pyridoxine Hydrochloride	Sigma
Rose Bengal	Fisher
Rose Bengal sodium salt	Sigma
Silica Gel Dessicant	J.T. Baker
Skim Milk	Difco
Sodium Borate Decahydrate	EMD
Sodium Borate, 10-Hydrate	J.T. Baker
Sodium Chloride	J.T. Baker
Sodium Citrate Dihydrate	J.T. Baker
Sodium Hydroxide	J.T. Baker
Sodium Nitrate	J.T. Baker
Sodium Nitrite	J.T. Baker
Sodium Phosphate	J.T. Baker
Sodium Sulfate	EM Science
Sodium Sulfate, Anhydrous	EMD
Streptomycin Sulfate	ICN Biomedicals
Sucrose	J.T. Baker
Thiamine Hydrochloride	Sigma
Urea	J.T. Baker
Zinc Sulfate, Heptahydrate	Sigma
2,6-Dichloro-4-nitroaniline	Aldrich

CHAPTER ONE

GENERAL INTRODUCTION

1 BACKROUND OF THE RESEARCH TOPIC

Maize has been in the diet of West Africans for centuries (Iken et al., 2004). In Nigeria it has become the staple cereal due to its taste, cooking properties and potential to produce high yields. The rapid population growth over the past years in developing countries in West Africa has led to an ever increasing demand for food. In turn, the production of maize has steadily increased. Alone in Nigeria, the production of maize has risen nearly 10-fold in the last 25 years from 465,000 ha in 1980 to 4,466,000 ha in 2004 (FAOSTAT, data 2006). In West Africa the consumption frequency of maize is stable across socioeconomic lines and is consumed at a comparable frequency by rich and poor, educated and illiterate (Cardwell et al., 2006). Subsistence farmers in the savanna of Africa consume locally grown maize on average virtually everyday of the week (Cardwell et al., 2006).

Maize in West Africa is frequently contaminated with aflatoxins. Aflatoxins are toxic metabolites produced by fungi, mainly *Aspergillus flavus* and *A. parasiticus*. One of the most potent carcinogens produced in nature is aflatoxin B_1 and is therefore listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1993). The aflatoxin exposure in West Africa is mainly associated with the consumption of maize products (Egal et al., 2005; Bandyopadhyay, 2007). Therefore it is not surprising that human exposure to aflatoxin in West Africa is extremely high. Over 99% of the population in many regions have had long term exposure (Gong et al., 2002). As a result of this, many West Africans suffer from tremendous health problems such as immune system deficiency, liver cancer, impaired child development and other illnesses (Wild et al., 1992b; Wild et al., 1992a; Gong et al., 2004; Williams et al., 2004).

Over the years countries with known specific aflatoxin regulations have increased from 33 in 1981 (Schuller et al., 1983) to 77 in 1995 (F.A.O., 1997) and 100 in 2003 (F.A.O., 2004). However, more regulations regarding the amount of allowed aflatoxin in grain are still necessary. In the European Union, regulations limit the total amount of aflatoxin to 4 ng g^{-1}, whereas the regulations in some developing countries and the US limit the total aflatoxins to more than 20 ng g^{-1} in

foodstuffs intended for human consumption (F.A.O., 2004). In Nigeria regulations do exist and were set at 20 ng g^{-1} as the maximum permissible limit for total aflatoxin in foodstuff by the National Agency for Food, Drug Administration and Control (F.A.O., 2004). Nevertheless, these regulations are difficult to enforce. In Nigeria agricultural products are mostly produced from field to mouth by small-scale farmers or products are sold through local markets where "caveat emptor" remains the basic rule (Bandyopadhyay, 2007). Since these products rarely enter official channels, practical monitoring of aflatoxin seldom happens. Therefore, much of the food that is produced and consumed in Nigeria is often contaminated with high levels of aflatoxin.

Biological control strategies directed at utilizing non-aflatoxin (atoxigenic), strains to limit aflatoxin contamination on crops have been pursued for almost two decades (Cole et al., 1990; Dorner, 2004). These strategies seek to give atoxigenic strains a competitive edge and exclude their aflatoxin producing relatives, therefore decreasing the potential for contamination in crops and the environment. Successful strategies have been accomplished by using native non-aflatoxin producing strains such as *A. flavus*. (Cotty et al., 2006). In a joint project with the IITA, Africa, the University of Bonn, and the University of Arizona, research is being conducted on the potential for using native atoxigenic *Aspergillus flavus* strains to suppress aflatoxin producing fungi that infect maize in Nigeria. Initial efforts focused on identifying the ecology of the aflatoxin producing strains and potential biological control isolates of *A. flavus* in Nigeria.

2 THE GENUS *ASPERGILLUS*

The genus *Aspergillus*, a member of the phylum Ascomycota, includes over 185 known species. Several members of *Aspergillus* section Flavi produce aflatoxin. These includes *Aspergillus flavus* and *Aspergillus parasiticus*, as well as several less common taxa including *Aspergillus nomius, A. tamarii, A. pseudotamarii, A. minisclerotigenes* and *A. bombycis* (Klich et al., 1988; Cotty et al., 1994b). *Aspergillus* species classified outside of section Flavi can also produce aflatoxins. For example, *Aspergillus ochraceoroseus* from section *Ochraceorosei*, SCRR 1468, morphological resembling members of section *Circumdati*, and the ascomycete *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section Nidulantes, Cary et al., 2005) also produce aflatoxin. The group of aflatoxin producing species is more complex than previously thought. One

example of this is a West Africa strain of an unnamed taxon which may soon be described as a new important aflatoxin producer (Cotty et al., 1999).

3 POPULATION DYNAMICS

3.1 Life cycle

The fungi of *Aspergillus* section Flavi are one of the most abundant and widely distributed soil-borne molds and can be found anywhere on earth (Yu et al., 2005). *A. flavus* is a saprophytic fungus that is capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder, cotton, compost piles, dead insects and animal carcasses, outdoor and indoor air environments, stored grains, and even on live humans and animals (Klich, 1998).

The life cycle in agriculture fields can be divided into two stages: (1) colonization of plant debris in soil and (2) invasion of seeds and grain in actively growing crop plants (Figure 1) (Horn, 2007). Soil serves as a reservoir for primary inoculum of *A. flavus* and *A. parasiticus* (Horn et al., 1995; Payne, 1998). *A. parasiticus* appears to be more adapted to a soil environment, being prominent in peanuts, whereas *A. flavus* seems adapted to the aerial and foliar environment, being dominant in corn, cottonseed, and treenuts (Diener et al., 1987).

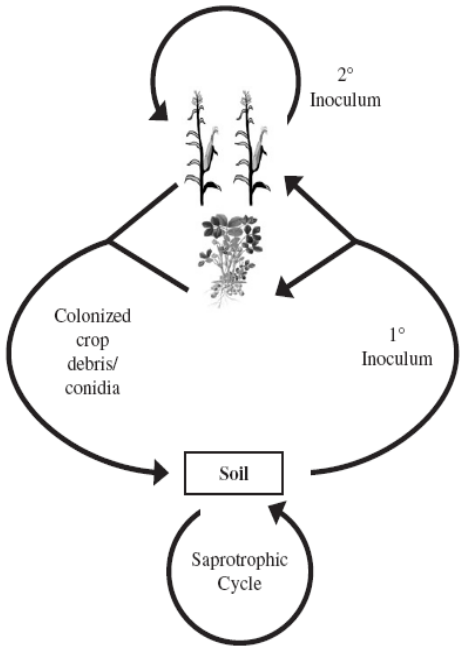


Figure 1 Life cycle of *Aspergillus flavus* and *A. parasiticus* in agricultural ecosystems (Horn, 2007)

Under adverse conditions such as dry and poor nutrition, the mycelium congregates to form resistant structures called sclerotia (Yu et al., 2005). Sclerotia are pigmented, compacted aggregates of hyphae, which resist unfavorable environmental conditions and are capable of remaining dormant for long periods (Wicklow et al., 1983; Cotty, 1988; Rollins et al., 1998). The fungus overwinters either as mycelium in plant debris and litter on the soil, on insects or as sclerotia in the soil (Diener et al., 1987). When the growth conditions are favorable the sclerotia either germinate to produce additional hyphae or they produce conidia (asexual spores), which can be further dispersed in the soil and air (Bennett et al., 1986; Cotty, 1988). The fungus mostly exists in the form of mycelium or asexual conidia spores.

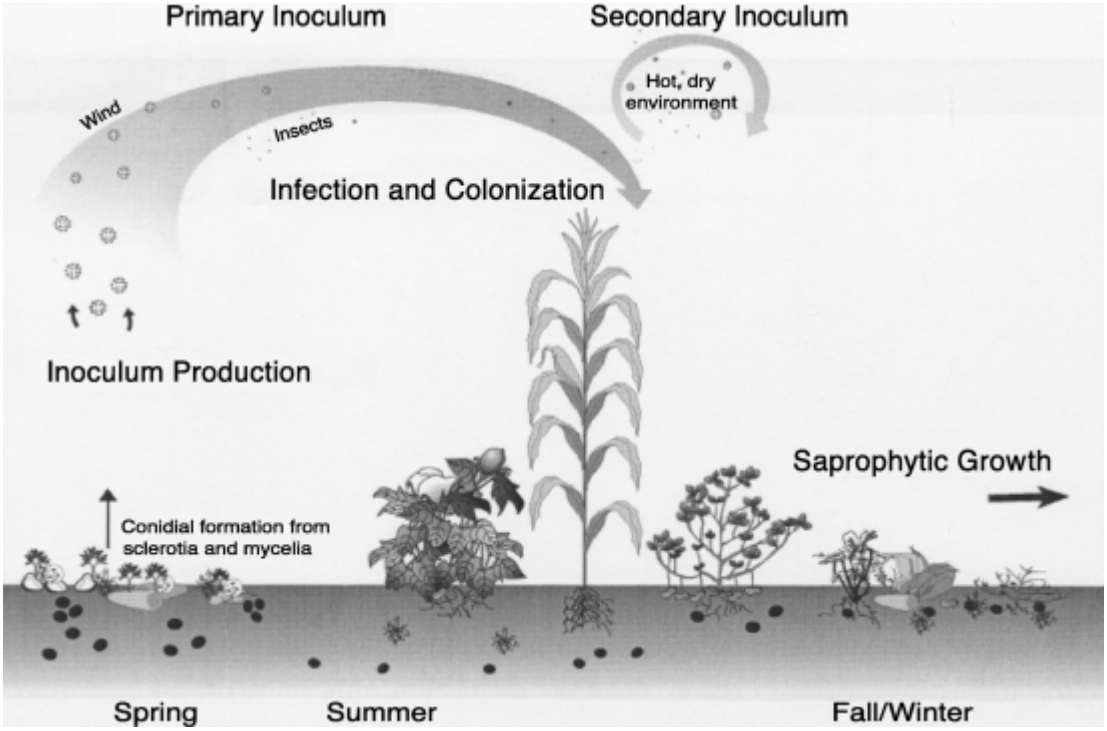


Figure 2 Diagram of the pre-harvest infection of cotton, corn, and peanuts by *Aspergillus flavus*. Sclerotia and conidia produced by *A. flavus* growing on crop debris and in the soil serve as primary inoculum for young plants in the spring. Later in the growing season, conidia produced on crop debris or on infected plants provide high levels of secondary inoculum when environmental conditions are conducive for disease development. (Copyright Marcel Dekker Inc.) (Scheidegger et al., 2003)

3.2 Phases of infection

Aflatoxin contamination can be divided into two distinct phases with the infection of the developing crop in the first phase and increase in contamination after maturation in the second phase (Cotty, 2001). Both phases contribute to many contamination events (Cotty et al., 2007). Weather

influences the two phases of contamination differently. During the first phase of contamination infections by *A. flavus* and *A. parasiticus* of susceptible crops are promoted due to wounding of developing crops by birds, mammals, insects, mechanically (e.g. hail) or drought stress and elevated temperatures (Figure 2) (Dowd, 1998; Payne, 1998; Guo et al., 2002). Its ability to attack seeds of both monocots and dicots, and to infect seeds produced both above and below the ground, demonstrates that this fungus has evolved a battery of mechanisms to breach the host's resistance (Yu et al., 2005). Conidia of plant, insect, and human derived strains of *A. flavus* rapidly colonize leaves, kernels, and insects injured during inoculation but do not affect uninjured plant or insect material (St. Leger et al., 2000).

3.3 Infection of crops

A. flavus causes diseases of various agricultural crops such as maize, cotton, groundnuts, peanuts, as well as tree nuts such as Brazil nuts, pecans, pistachios, and walnuts. In West Africa the most vulnerable crops are maize (*Zea mays*), groundnut (*Arachid hypogaea*), and tree nuts (Cardwell et al., 2006).

Maize has been studied most intensively with respect to infection by primary inoculum in soil (Horn, 2007). Aflatoxin contamination of corn occurs worldwide (Payne, 1992). The occurrence of *A. flavus* in field maize was first reported in 1920 (Taubenhaus, 1920). Aflatoxins can be produced in preharvest as well as in stored maize (Marsh et al., 1984; Hell et al., 2003). Infections of maize by *A. flavus* are complex and include colonization of silks as well as wounding of kernels by insects (Marsh et al., 1984; Brown et al., 1993). In nature, *A. flavus* can directly infect maize kernels under drought stress and high temperatures (32 to 36°C) known to compromise the host's physiological defense systems as well as cause cracks in the seed (Marsh et al., 1984; Payne et al., 1988; Smart et al., 1990). Nitidulids beetles (Nitidulidae) are known as major vectors that carry *A. flavus* from colonized crop debris in soil to developing maize ears (Lussenhop et al., 1990). Colonized waste maize kernels and cobs that overwinter following harvest also serve as important sources of maize infections due to wind dispersed conidia (Olanya et al., 1997; Jaime-Garcia et al., 2004). The effectiveness of primary inoculum in infecting crops has been most convincingly demonstrated in peanuts, maize and cotton seed with biological control in which strains of *A. flavus* and *A. parasiticus* are applied to the soil surface in form of inoculated grain (Dorner et al., 2007; Atehnkeng et al., 2008a; Cotty et al., 2008).

3.4 *Aspergillus* section Flavi species

Populations of section Flavi species are diverse and comprise individuals that differ greatly in phenotype, including characters such as conidial color, sclerotium production, presence of diffusible pigments and growth rate (Raper et al., 1965; Christensen, 1981; Horn et al., 1996). On the basis of physiological and morphological criteria, *A. flavus* can be divided into two types of strains (Cotty, 1989). The S-type isolates of *A. flavus* produce numerous small sclerotia (average diameter <400 µm) and fewer conidia than other *A. flavus* isolates (Figure 3) (Saito et al., 1986; Cotty, 1989). The S strain was originally described as *A. flavus* var. *parvisclerotigenus*, based on a type strain that produced on average much greater quantities of only B aflatoxins (Cotty, 1989; Saito et al., 1993). The L-type isolates of *A. flavus* produce larger and fewer sclerotia and is designated as "typical" isolates of *A. flavus* (Figure 4) (Saito et al., 1986). Strains resembling the S-type but having different physiological criteria have been reported in different regions of the world. These strains can also produce aflatoxin G and were found in Argentina, Thailand, Australia and West Africa (Saito et al., 1993; Geiser et al., 1998; Cotty et al., 1999; Fernandez Pinto et al., 2001). Recent studies designated most of these isolates to the *A. minisclerotigenes* (Pildain et al., 2008), however, the exact taxonomic affiliation of S_{BG} commonly found in West Africa remains unclear (Cotty et al., 1999; Atehnkeng et al., 2008b).

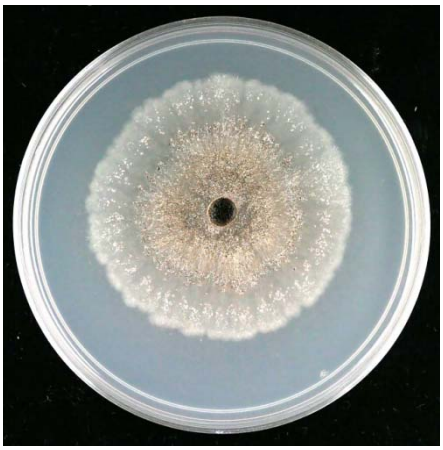


Figure 3 Macroscopic features of *Aspergillus flavus* S_{BG}-strain on Czapek's agar.

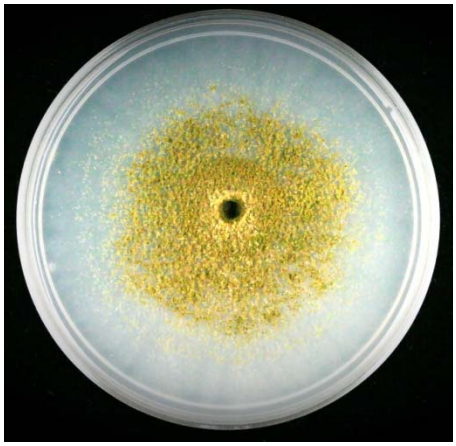


Figure 4 Macroscopic features of *Aspergillus flavus* L-strain on Czapek's agar.

4 AFLATOXINS

Aflatoxins are a group of structurally related toxic secondary metabolites produced mainly by certain strains of *A. flavus* and *A. parasiticus*. *A. flavus,* in particular, is a common contaminate in agriculture (Bhatnagar et al., 2001; Bennett et al., 2003). *Aspergillus bombysis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius, A. minisclerotigenes*, *Aspergillus pseudotamari* and the strain S_{BG} are also aflatoxin-producing species but occur less frequently (Goto et al., 1996; Cotty et al., 1999; Klich et al., 2000; Peterson et al., 2001; Pildain et al., 2008). The four major aflatoxins are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography (Bennett et al., 2003).

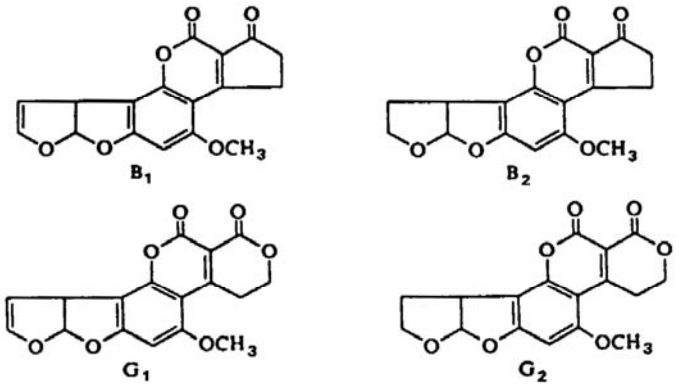


Figure 5 Chemical stuctures of aflatoxins B_1, B_2, G_1, G_2. (Bhatnagar et al., 1993)

Aflatoxins were first identified in the early 1960s and compose a family of toxic compounds (Wild et al., 2002). Aflatoxin B_1 is predominant and the most toxic and potent hepato-carcinogenic natural compound ever characterized (Squire, 1981; Bhatnagar et al., 2001). The conditions favoring formation of the aflatoxins have been described, as has their metabolism, toxicity, DNA adduct formation, mutagenic, and carcinogenic activity (Eaton et al., 1994). The immuno-suppressive properties of aflatoxin B_1, particularly on cell-mediated immunity, have been demonstrated in various animal models (Ali et al., 1994; Neiger et al., 1994; Pestka et al., 1994). A major metabolic of aflatoxin B_1 is aflatoxin M_1 which is usually excreted in the milk and urine of dairy cattle and other mammalian species that have consumed aflatoxin contaminated food or feed (Gourama et al., 1995).

4.1 Aflatoxin producing species within *Aspergillus* Section Flavi

Variability in production of aflatoxins, especially among *A. flavus* isolates, has often been reported (Cotty et al., 1994a; Mahoney et al., 1996; Orum et al., 1997). For example, only half of *A. flavus* strains produce aflatoxins, however, many of these strains produce more than 10^6 μg/kg (Klich et al., 1988; Cotty et al., 1994b). *A. flavus* only produces aflatoxins B_1 and B_2, whereas *A. parasiticus* and the West African S_{BG} strains in addition produce aflatoxins G_1 and G_2 (Cotty et al., 1994b; Cotty et al., 1999; Bennett et al., 2003). The S-type *A. flavus* produces high levels of B-aflatoxins, whereas the L-type produces on average less B-aflatoxins (Cotty, 1989; Cotty, 1997). *A. parasiticus* and the S_{BG} generally produce high levels of aflatoxins and populations are considerably less diverse in aflatoxin production than the *A. flavus* L strain (Horn et al., 1996; Cotty et al., 1999). Therefore, *A. parasiticus* isolates are typically toxigenic. The percent of naturally occurring *A. parasiticus* isolates not producing aflatoxins has been reported to range from 3 to 6% (Horn et al., 1996; Vaamonde et al., 2003; Barros et al., 2006).

4.2 Aflatoxin biosynthetic pathway

The aflatoxin pathway (Figure 6) represents one of the best-studied pathways of fungal secondary metabolism (Cleveland et al., 1990; Minto et al., 1997; Payne et al., 1998; Ehrlich et al., 2005). Aflatoxins are polyketides with characteristic dihydro- (B1 and G1) or tetrahydro- (B2 and G2) bisfuran rings (Bhatnagar et al., 1992; Minto et al., 1997) (Figure 5). The production of aflatoxin involves a complex biosynthetic pathway consisting of at least 25 genes (Yabe et al., 1999; Bhatnagar et al., 2003). All of the identified genes related to the biosynthesis are located within a 75kb DNA region in both *A. parasiticus* and *A. flavus*, and their relative positions in the cluster of both fungal species are similar (Yu et al., 2000b; Ehrlich et al., 2005).

Most of genes within the biosynthetic pathway are regulated by a single Zn_2Cys_6-type transcription factor, *aflR*, which is encoded by one of the genes in the cluster (Yu et al., 1997). The gene *aflJ*, adjacent to the *aflR*, is also involved in the regulation of transcription (Meyers et al., 1998; Chang, 2003). Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path: acetate → polyketide → anthraquinones→ xanthones → aflatoxins (Yu et al., 2004b). The steps of the aflatoxin pathway are summarized in Figure 6.

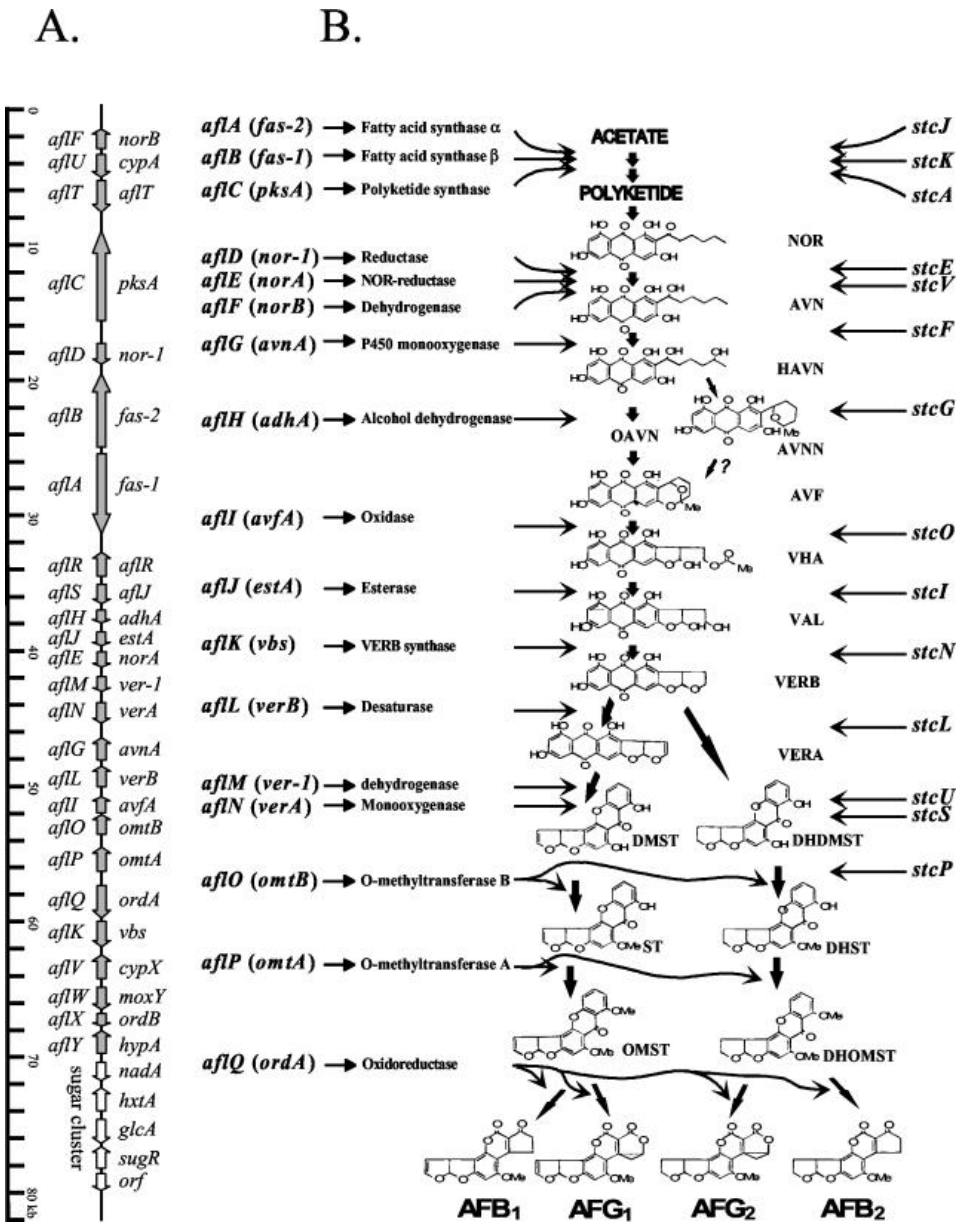


Figure 6 Clustered genes (left) and the aflatoxin biosynthetic pathway (right). The generally accepted pathway for aflatoxin biosynthesis is presented. The clustered genes with their new and old names are shown on the left. The vertical line represents the 82 kb aflatoxin biosynthetic pathway gene cluster plus sugar utilization gene cluster in A. parasiticus and A. flavus. The new gene names are given on the left of the vertical line and the old gene names are given on the right. Arrows along the vertical line indicate the direction of gene transcription. The ruler on the far left indicates the relative sizes of these genes in kilobase pairs. Arrows indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxy-averantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; and AFG2, aflatoxin G2. (Yabe et al., 1999)

CHAPTER TWO

GENERAL MATERIAL AND METHODS

1 CULTURE MEDIA AND REAGENTS

5/2 Agar

Ingredients	*Amounts*
V-8Juice	50 ml
Agar-Agar	15 g
Deionized distille water	950 ml
pH 5.2	

Modified Rose Bengal - Media[a]

Ingredients	*Amounts*
Sucrose	3g
$NaNO_3$	3 g
KH_2PO_4	0.75g
K_2HPO_4	0.25g
$MgSO_4*7H_2O$	0.5g
KCL	0.5g
NaCL	10g
Micronutrients	1ml
Rose Bengal	5m
Bacto Agar	10g
Deionized distille water	1000ml
pH 6.5	

[a]5ml Chloramphenicol stock solution added before autoclaving, 10ml Dichloran stock solution and 5ml Streptomycin stock solution added after autoclaving.

AFPA-Media

Ingredients	*Amounts*
Yeast Extract	20g
Bacto Peptone	10g
Ferric Ammonium Citrate	0.5g
Bacto Agar	20g
Deionized distille water	1000ml
pH 5.0	

CZ-Media (Czapeks)

Ingredients	*Amounts*
Sucrose	30g
$NaNO_3$	3g
KH_2PO_4	0.5g
K_2HPO_4	0.5g
$MgSO_4*7H_2O$	0.5g
KCL	0.5g
Micronutrients	1ml
Bacto Agar	20g
Deionized distille water	1000ml
pH 6.0	

Stock solutions

Dichloran

250mg Dichloran dissolved in 50ml Aceton

QS to 250ml with 100% Ethanol

Stored at room temperature

Streptomycin

1g Streptomycin Sulfate

QS to 100ml with deionized distilled water and filter sterilized into sterile culture tubes.

Stored in freezer

Rose Bengal

500mg Rose Bengal

30ml Ethanol

QS to 100ml with deionized distilled water

Stored at room temperature

Chloramphenicol

5g Chloramphenicol

QS to 500ml with 100% Ethanol

Stored at room temperature

A&M Micronutrients

Ingredients	*Amounts*
$Na_2B_4O_7*10\ H_2O$	0.7g
$(NH_4)_6Mo_7O_{24}*4H_2$	0.5g
$Fe_2(SO_4)_3*6\ H_2O$	10g
$CuSO_4*5H_2O$	0.3g
$MNSO_4*H_2O$	0.11g
$ZnSO_4*7H_2O$	17.6g

QS to 1000ml with deionized distilled water

2 SILICA GEL STORAGE

The isolates were inoculated on 5/2 agar and incubated for 5 to 10 days at room temperature. Deionized water was used to prepare 3% skim milk (Difco dehydrated skim milk). The milk was autoclaved for 12 min. and was cooled immediately after autoclaving. Vials (Wheaton, 12ml) were filled with silica gel (Silica gel, EM Science Grade H, Type II 6-12 mesh) to approximately 1/3 of the vial volume. The caps were autoclaved for 20 minutes and the vials were sterilized in a preheated oven at 180°C for 105 minutes. Aseptically, 700µl sterile skim milk was piped into 7ml sterile polystyrene tubes. Vials containing silica gel and tubes containing milk were placed on ice for approximately 5 minutes. The spores were swabbed off the plate by using sterile cotton applicators and transferred to the 3% skim milk. The tubes were capped, shaken, vortexed vigorously and cooled on ice. The spore suspensions were transferred to silica vials, vortexed, and cooled on ice. The isolates on silica were stored in a refrigerator at 12°C.

CHAPTER THREE

DISTRIBUTION OF *ASPERGILLUS* SECTION FLAVI IN SOILS OF MAIZE FIELDS IN THREE AGROECOLOGICAL ZONES OF NIGERIA

1 INTRODUCTION

Crops often become contaminated by aflatoxins, toxic fungal metabolites, in warm production areas throughout the world (WHO, 1979). Causal agents of these contamination events belong to *Aspergillus* section Flavi (Cotty et al., 1994b) and the species most frequently implicated in contamination are *A. flavus* and *A. parasiticus* (Cotty et al., 1994b). Fungi in *Aspergillus* section Flavi exist in complex communities composed of individuals that vary widely in aflatoxin-producing ability (Cotty, 2006). Individuals that do not produce aflatoxins, called atoxigenic, are common in *A. flavus* (Joffe et al., 1969; Schroeder et al., 1973; Lisker et al., 1993; Cotty, 1997). Based on morphological, genetic and physiological criteria, *A. flavus* can be divided into two morphotypes, commonly called strains (Cotty, 1994b). The most common strains are the S and L strains. The S-strain produces numerous, small sclerotia (average diameter <400 μm) and high levels of B-aflatoxins, while the L-type strain produces fewer, larger sclerotia, and on average, less B-aflatoxins (Garber et al., 1997). All *A. flavus* isolates produce only B-aflatoxins as a result of a 0.8 to 1.5kb deletion in the aflatoxin biosynthesis gene cluster (Ehrlich et al. 2004). Two common aflatoxin-producers, *A. parasiticus* and *A. nomius,* produce both B- and G-aflatoxins (Ehrlich et al., 2003). In the West African country of Benin, another less frequently identified producer of B- and G-aflatoxins is common (Saito et al., 1986; Cotty et al., 1999). This unnamed taxon (Egel, et. al. 1994) has been known as strain S_{BG}. S_{BG} has sclerotial morphology similar to the S-strain of *A. flavus*. However, S_{BG} is phylogenetically ancestral to both *A. flavus* and *A. parasiticus* (Egel et al., 1994; Ehrlich et al., 2003). Isolates that share traits with S_{BG} have been reported from Thailand, Argentina, and Australia (Saito et al., 1993; Geiser et al., 1998; Cotty et al., 1999; Fernandez Pinto et al., 2001) and several species have recently been described with characteristics similar to S_{BG} (Pildain et al., 2008). However, the exact taxonomic affiliation of S_{BG} remains unclear. Two other common aflatoxin-producing species, *A. parasiticus* and *A. nomius,* produce both B- and G-aflatoxins (Ehrlich et al., 2003).

Maize is an important staple food throughout most of Africa. In West Africa, many children rely exclusively on maize for nutrition after being weaned off mother's milk (Nwokolo et al., 1978; Adebajo et al., 1994). Maize is especially vulnerable to infection by mycotoxin-producing fungi in tropical and subtropical countries (Klich, 2002). In Nigeria official monitoring of the mycotoxins is sparse to non-existent, nevertheless, high concentrations of aflatoxin have been found in pre- and postharvest maize (Udoh et al., 2000; Bankole et al., 2003a; Kpodo et al., 2008). In West Africa, aflatoxin contaminated food is consumed daily by populations unaware that associated health risks include liver cancer and impaired child development (Jiang et al., 2005; Cardwell et al., 2006).

Interest in the distribution of species within *Aspergillus* section Flavi across Nigeria has increased because of recent attempts to utilize isolates of *A. flavus* that do not produce aflatoxins (atoxigenic strains) to reduce aflatoxin contamination (Atehnkeng et al., 2008b). Several atoxigenic strains of *A. flavus* are used commercially to reduce aflatoxin contamination in the USA (Antilla et al., 2002; Cleveland et al., 2003). To minimize human exposure to aflatoxins, aflatoxigenic strains may be displaced from crop environments by applying atoxigenic strains of *A. flavus* to soil of developing crops. Fungal communities resident in various locations differ widely in aflatoxin-producing potential (Schroeder et al., 1973; Lisker et al., 1993; Cotty, 1997). The structure and aflatoxin-producing potential of communities of *Aspergillus* section Flavi in Nigerian soil is previously unexplored. Knowledge of variability among fungal communities and the impact of agroecological zones on average aflatoxin-producing potential could be critical to selecting native, safe, and efficacious atoxigenic strains of *A. flavus* for use in biological control of aflatoxins in Nigeria.

The current study sought to assess distributions of species and strains within *Aspergillus* section Flavi across the three agroecological zones where most maize is produced in Nigeria. The results provide a clear picture of both how the average aflatoxin-producing potential of fungal communities varies across regions and which fungi have the greatest potential to contaminate crops in Nigeria. During the process, a large resource of atoxigenic *Aspergillus flavus* isolates of potential value as biocontrol agents was compiled from throughout the major maize producing regions of Nigeria.

2 MATERIAL AND METHODS

2.1 Survey sites

Soil samples were collected from fields where maize was planted in three agroecological zones (Figure 7). Nigeria is located in the tropical zone between latitude 4° and 14° N, and longitude 2° and 14° E. A vast portion of Nigeria has savanna vegetation which is classified into 3 agroecological zones: the Derived Savannah (DS); the Southern Guinea Savannah (SGS); and the Northern Guinea Savannah (NGS) (Cardwell et al., 2006). Over 2 million ha of maize is produced in Nigeria almost entirely within these zones. Growing periods vary among the zones from 151-180 days for NGS to 181-210 days for the SGS, and 211-270 days for the DS. A bimodal rainfall distribution occurs in both the DS (130 to 150 cm $year^{-1}$) and SGS (100 to 130 cm $year^{-1}$). Maximum temperatures range from 25 to 35°C in the DS and from 26 to 39°C in the SGS. The NGS has a unimodal rainfall distribution (90 to 100 cm $year^{-1}$) with maximum temperatures varying from 28 to 40°C. In general, temperature increases and rainfall decreases with increased latitude in this region.

2.2 Survey methods

A total of 11 districts in the three agroecological zones were selected for sampling: Five districts from the SGS (Mokwa, Bida, Minna, Abuja, and Akwanga); Five districts from the DS (Ogbomosho, Lafia, Markurdi, Lokoja, and Ado-Ekiti) and one district in the NGS (Zaria) (Figure 7). In each district soils were collected from five maize fields, 0.2 to 0.3 ha in size. Sampled fields were separated by at least 20 km. A single composite sample (50 to 60g) was collected from each field by collecting multiple sub-samples at three random locations to a depth of 4 cm.

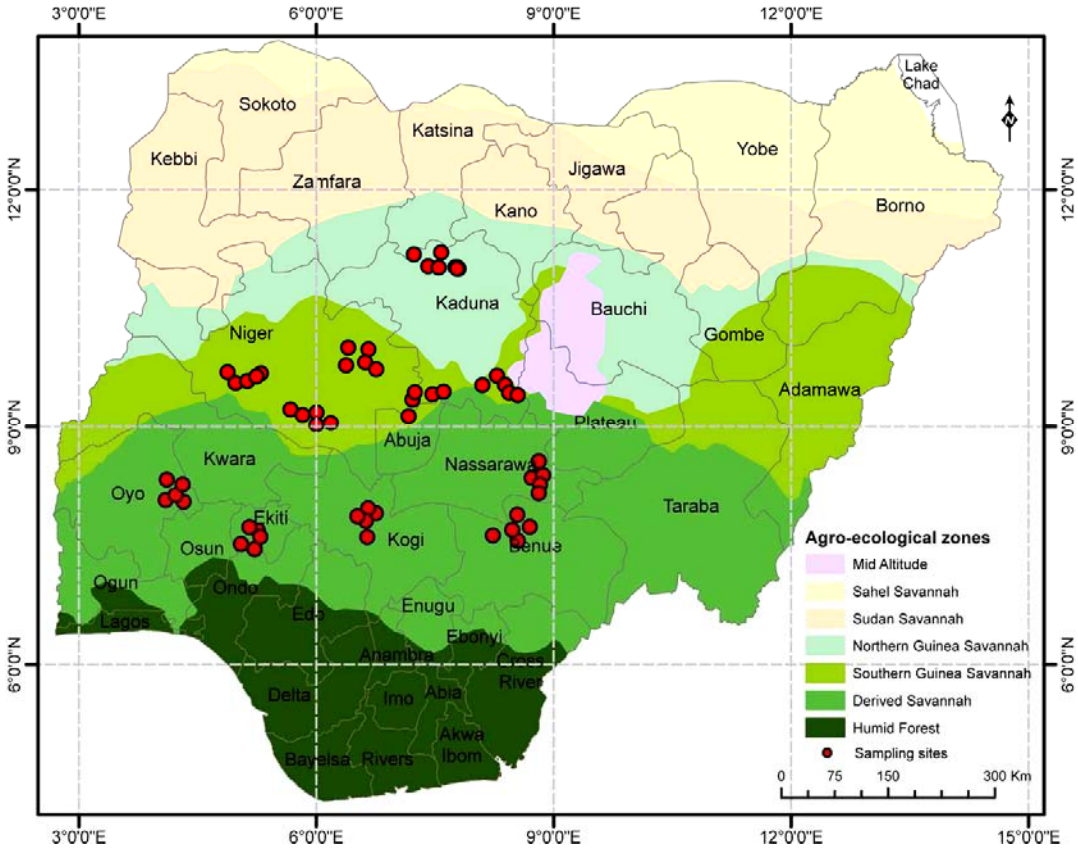


Figure 7 Map of Nigeria showing districts in three agro-ecological zones from which soil samples were collected to determine distribution of *Aspergillus* section Flavi strains and other fungal species.

2.3 Strain isolation

Soil samples were dried in a forced air oven at 48 to 50°C for 48 hours, placed inside plastic bags, hammered to remove clods, and homogenized by hand-mixing. Isolates belonging to *Aspergillus* section Flavi were isolated by dilution plate technique on Modified Rose Bengal Agar (MRBA, Cotty, 1994a). In 7-ml sterile polystyrene tubes 1g of soil was suspended in 3 ml sterile water, mixed for 20 min on a Roto-Shake Genie (Scientific Industries, Bohemia, NY) and plated on MRBA at appropriate dilutions to allow collection of isolates from plates with fewer than 10 colonies. Plates were incubated in the dark for 3 days at 31°C. Colonies of *Aspergillus* section Flavi were identified by colony morphology. No more than 8 isolates were collected from each isolation and 17-20 isolates per sample were transferred to 5/2 agar (5% V-8 juice, and 2% agar, pH 5.2) for further characterization. After 5 days unilluminated at 31°C, isolates were classified on the basis of colony characteristics and conidial morphology (400X). Isolates with abundant small sclerotia (average diameter <400 µm) were initially classified as strain S_{BG} (Cotty

et al., 1999). Isolates with smooth conidia and large sclerotia (average diameter over 400 μm) were classified as the L strain of *A. flavus* (Cotty, 1989). *A. tamarii* and *A. parasiticus* were initially identified by colony and spore morphology (Klich et al., 1988) and identifications were confirmed by color reaction on AFPA (*A. flavus* and *A. parasiticus* agar, Pitt et al., 1983). Quantities of *Aspergillus* section Flavi in soils were calculated as colony forming units (CFU) per gram. A total of 1,089 total cultures were maintained as agar plugs in 4 ml vials containing 2 ml sterile distilled water at 4°C.

2.4 Aflatoxin production by isolated fungi

Aflatoxin-producing ability was quantified for *Aspergillus* section Flavi isolated strains randomly selected from each of the collected soil samples in order to determine both the fungi that produce the greatest quantities of aflatoxins and the frequency of occurrence of non-aflatoxin producers across Nigeria. Isolates belonged to *Aspergillus flavus* L-strain (492), *A. parasiticus* (7), *A. tamarii* (38) and to the strain S_{BG} (65). Isolates were fermented in Adye and Matales medium (A&M, Mateles et al., 1965) with 22.4 mM urea as the sole nitrogen source and adjusted to pH 4.7 prior to autoclaving (Cotty et al., 1999). Vials (15 ml containing 5 ml A&M) were seeded with approximately 2×10^3 conidia suspended in 100 μl water. After incubation (32°C, dark, 5 days) medium pH was measured, 3 ml acetone was added, and the contents were mixed by inverting. Vials were allowed to set for 1 h to allow lyses of fungal cells and extraction of aflatoxins from mycelia and conidia. Subsequently, the mycelia was collected on Whatman No. 4 filter paper, dried in a forced air oven (48°C, 3 days), and weighed to quantify fungal biomass. The filtrate was diluted as appropriate, spotted along side standards of aflatoxin B_1, B_2, G_1 and G_2 (Supelco, Bellefonte, PA, USA), and separated on thin-layer chromatography plates (silica gel 60, 20 mm) with the development solvent diethyl ether-methanol-water (96:3:1) (Cotty, 1997). Aflatoxin was quantified directly on TLC plates with a scanning densitometer (Camag TLC Scanner 3 with winCATS 1.4.2 software). In order to concentrate the aflatoxins potentially in extracts initially showing no detectable aflatoxin, these extracts were diluted with an equal volume of water and extracted with 3 ml methylene chloride. Aflatoxins partitioned into the methylene chloride fractions which were dried and the residues dissolved in 100 μl methylene chloride and subjected to thin-layer chromatography according to the above procedure.

2.5 Data analysis

Analyses were preformed with SAS (version 9.1.3, SAS Institute Inc., Cary, NC). Analysis of variance was performed on all data with the general linear model (GLM), suitable for unbalanced data. The GLM of SAS uses the least squares method to fit data to a general linear model. Tukey`s honestly significant difference (HSD) test was performed to compare treatment means at the 5% level. Analyses for percentage values, CFU g^{-1}, and aflatoxin concentrations were preformed with data transformed, using the arcsine of the square root, the natural logarithm (log), and the log (count +1), respectively. Districts and the agroecological zones were treated as class variables. Pearson`s correlations coefficients were generated to assess relationships between ecological and biological variables.

3 RESULTS

3.1 Distribution of *Aspergillus* section Flavi across Nigeria

In all 55 soil samples collected in Nigeria, *Aspergillus* section Flavi was detected. In total, 1,089 isolates belonging to the *Aspergillus* section Flavi were collected with 100 isolates from each district except for district Abuja with 89 (Table 1). The *A. flavus* L-strain was the most commonly isolated member of section Flavi (85%) across the three examined agroecological zones with L strain incidence exceeding 57% in all districts and reaching 99% in Ogbomosho. *A. tamarii*, with an average incidence of 6%, was found in 8 districts and in all three agroecological zones. *A. parasiticus* made up only 1% of section Flavi isolates collected and only occurred in 5 fields dispersed across the DS and SGS zones. Within *A. flavus* only L-strain isolates were detected. All isolates with sclerotial morphology similar to the S-strain produced both B- and G-aflatoxin and, as a result, were classified as the S_{BG} previously described from Benin, West Africa (Cotty et al., 1999; Ehrlich et al., 2003). S_{BG} was the second most commonly isolated member of section Flavi (8%) and was found in 10 districts and in all three agroecological zones studied (Table 1). The highest incidence of this strain was found in Zaria district (31%).

The S_{BG} isolates were significantly ($P>0.05$) more frequent in northern latitudes, while *A. flavus* was significantly more common in southern latitudes (Table 2 and 3). Incidences of the S_{BG} strain had a significant positive correlation with the longitude ($r = 0.29$, $P = 0.03$) and the

latitude (r = 0.36, P = 0.007), whereas incidences of the *A. flavus* L-strain had a significant negative correlation with longitude (r = -0.38, P = 0.004) and latitude (r = -0.43, P = 0.0009). There were significant negative correlations between the incidences of the S_{BG} and the L-strain (r= -0.84, $P < 0.0001$) (Table 3). Isolates of *A. tamarii* were significantly more frequent in the NGS and SGS than in the DS (Table 2) and incidence of *A. tamarii* was positively correlated with longitude (r = 0.33, P = 0.02), and latitude (r = 0.39, P = 0.004) but negatively correlated with the L-strain (r = -0.49, P = 0.0002) (Table 3). *A. parasiticus* was not significantly associated with any particular zone (Table 2).

The mean CFU of *Aspergillus* colonies per gram soil was extremely variable among the districts, ranging from 55 to 3,736. CFU counts were significantly different between the districts, however, not between the zones (Table 1 and 2). Only the incidences of *A. tamarii* had a significant negative correlation with the CFU g^{-1} (r = -0.38, P = 0.004) (Table 3).

Although soil pH varied significantly among districts, ranging from an average of 5.9 in Abuja to 7.3 in Akwanga, between the zones the soil pH was not significantly different.

Table 1 Soil pH, proportion of *Aspergillus* section Flavi composed of major taxa, and colony forming units (CFU) in maize field soil from districts across three agroecological zones (AEZ) in Nigeria[a]

		A. flavus	S_{BG}	*A. tamarii*	*A. parasiticus*		Soil pH		CFU g^{-1} soil	
AEZ[b]	District	(%)	(%)	(%)	(%)	Number isolated	Range	mean	range	mean
DS	Ogbomosho	99 a	0 b	1 b	0 a	100	6.04 - 6.99	6.4 ab	502 -5,761	2,661 a
	Ado-Ekiti	92 ab	1 ab	2 ab	5 a	100	5.99 - 7.3	6.5 ab	24 -668	178 abc
	Lafia	78 ab	19 ab	3 ab	0 a	100	5.85 - 7.1	6.3 ab	40 - 3,512	900 abc
	Makurdi	91 ab	3 ab	4 ab	2 a	100	5.13 - 7.01	6.1 ab	2 - 145	79 bc
	Lokoja	91 ab	9 ab	0 b	0 a	100	6.1 - 6.56	6.3 ab	22 - 4,781	1,946 ab
SGS	Mokwa	94 ab	5 ab	0 b	1 a	100	5.99 - 7.71	7.0 ab	50 -16,661	3,736 ab
	Bida	90 ab	3 ab	7 ab	0 a	100	6.06 - 7.91	6.8 ab	49 - 1,238	381 abc
	Minna	87 ab	2 ab	11 a	0 a	100	5.52 - 6.59	5.9 ab	80 - 956	531 abc
	Abuja	70 ab	12 ab	17 a	1 a	89	5.40 - 6.34	5.9 b	2 - 160	55 c
	Akwanga	89 ab	3 ab	7 ab	1 a	100	5.66 - 8.7	7.3 a	242 - 2,411	1,131 ab
NGS	Zaria	57 b	31 a	12 ab	0 a	100	5.75 - 6.95	6.5 ab	92 - 958	454 abc

[a] Percent data were arcsine square root and CFU data were log transformed prior to the analysis. Averages with a common letter in a column do not differ significantly by Tukey`s HSD test ($\alpha = 0.05$).

[b] NGS = Northern Guinea Savannah, SGS = Southern Guinea Savannah, and DS =Derived Savannah

Table 2 Variation among agroecological zones of Nigeria for soil pH, colony forming units (CFU) of *Aspergillus* section Flavi in soils, and total aflatoxin concentration[a]

AEZ[b]	No. of fields	Soil pH	CFU/g soil	% S_{BG}[c]	% Af[c]	% At[c]	%Ap[c]	No. of tested isolates	Aflatoxin B_1 (ng g^{-1})[d]
DS	25	6.58 a	1,153 a	6.4 b	90.4 a	1.8 b	1.4 a	288	278,976 b
SGS	25	6.33 a	1,167 a	4.7 b	86.0 a	8.7 a	0.6 a	266	133,568 c
NGS	5	6.53 a	396 a	31.0 a	57.0 b	12.0 a	0 a	50	335,517 a

[a] CFU g^{-1} and the total aflatoxin concentration were log (value +1) transformed for the analysis and percent data were arcsine square root transformed prior to statistical analysis. Means within a column followed by a different letter are significantly different (Tukey's HSD, $\alpha = 0.05$).

[b] AEZ = agroecological zone; DS = Derived Savannah, SGS = Southern Guinea Savannah, and NGS = Northern Guinea Savannah.

[c] Proportion of *Aspergillus* section Flavi belonging to various taxa. $\%S_{BG}$ = unnamed Taxon; %Af = *Aspergillus flavus*; %At = *A. tamarii*; %Ap = *A. parasiticus.*

[d] Mean aflatoxin of all aflatoxin producing taxa.

Table 3 Pearson`s correlation[a] coefficients of relationships among the quantity of *Aspergillus* section Flavi in soil (CFU g^{-1})[b], soil pH, longitude (LON), latitude (LAT), the proportions of isolates that are either the unnamed taxon S_{BG}, *A. flavus* (Af), *A. tamarii* (At), *A. parasiticus* (Ap), aflatoxin producing (Tox), or atoxigenic (Atox) and the average aflatoxin-producing ability[c]

	CFU	pH	LON	LAT	%S_{BG}	%Af	%At	%Ap	%Tox	%Atox	Toxin
CFU	1.00										
pH	0.24	1.00									
Lon	-0.24	-0.08	1.00								
Lat	0.06	0.10	0.13	1.00							
%S_{BG}	0.05	-0.10	**0.29***	**0.36****	1.00						
%Af	0.18	0.15	**-0.38****	**-0.43****	**-0.84*****	1.00					
%At	**-0.38****	-0.13	**0.33***	**0.39****	0.07	**-0.49****	1.00				
%Ap	-0.26	0.02	0.00	-0.17	-0.11	-0.12	-0.07	1.00			
%Tox	0.04	0.00	0.11	-0.17	0.13	-0.04	-0.18	0.13	1.00		
%Atox	-0.05	-0.06	-0.14	-0.01	-0.08	0.02	0.15	-0.11	**-0.84*****	1.00	
Toxin	-0.04	0.04	**0.30***	-0.01	**0.34****	**-0.27***	-0.04	0.21	**0.45****	**-0.54*****	1.00

[a] Correlation significance $P < 0.0001$ = ***, $0.0001 \geq P < 0.01$ = **, $0.01 \geq P < 0.05$ = *; n = 55

[b] CFU and the aflatoxin concentration were log (value +1) transformed prior to analyses.

[c] Percent data were arcsine square root transformed prior to analyses.

3.2 Distribution of aflatoxin-producing and atoxigenic *A. flavus* L-strains

Frequencies of aflatoxin production within *A. flavus* L-strain isolates varied among the districts (Figure 11) and agroecological zones of Nigeria. Overall, 56% of the tested isolates showed no detectable aflatoxin and were classified as atoxigenic. Significantly ($P<0.05$) greater proportions of *A. flavus* produced aflatoxins in NGS (61%) than in SGS (31%) (Figure 8). Incidences of atoxigenic and toxigenic *A. flavus* isolates were nearly balanced in the DS zone. Atoxigenic isolates made up significantly ($P<0.05$) greater proportions of the *A. flavus* communities than toxigenic in the districts Bida, Minna, Abuja, and Ado-Ekiti. In Lafia, Makurdi, and Zaria aflatoxin producers were significantly ($P<0.05$) more common than atoxigenics (Figure 9). In all the remaining districts, no significant differences were observed between incidences of toxigenic and atoxigenic strains. Across districts, the lowest and highest incidences of aflatoxin producers were observed in Bida (21%) and Lafia (65%), respectively (Figure 9).

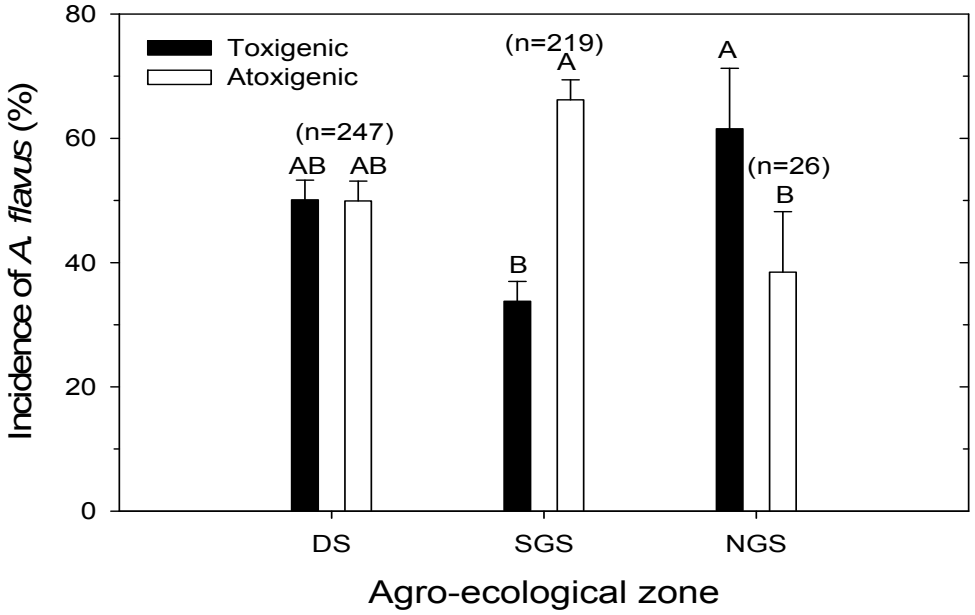


Figure 8 Distribution of aflatoxin-producing and atoxigenic isolates of the *Aspergillus flavus* L-strain among three agroecological zones in Nigeria. For each bar, vertical lines represent the standard error of the mean. DS = Derived Savannah, SGS = Southern Guinea Savannah, and NGS = Northern Guinea Savannah. Means not sharing a common letter are significantly different according to Tukey's HSD test ($\alpha = 0.05$)

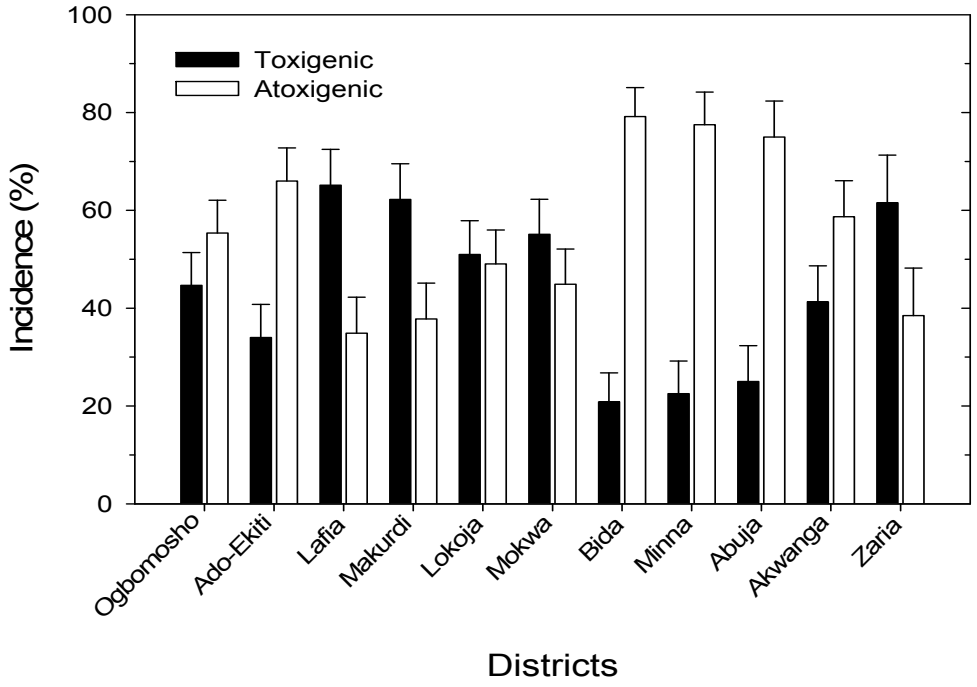


Figure 9 Incidence of aflatoxin-producing and atoxigenic isolates of the *Aspergillus flavus* L-strain isolated from soil samples of maize growing locations in Nigeria. Values shown are based on a mean of five locations within a district. Incidence values are based on the following numbers of isolates for each district Ogbomosho (56), Ado-Ekiti (50), Lafia (43), Makurdi (45), Lokoja (53), Mokwa (49), Bida (48), Minna (40), Abuja (36), Akwanga (46), and Zaria (26). For each bar, vertical lines represent the standard errors of the mean.

3.3 Aflatoxin Quantification

Aflatoxin-producing potential varied among isolates, species, districts, and agroecological zones (Table 4). All tested *A. tamarii* isolates produced no detectable aflatoxins. *A. flavus* isolates produced only B-aflatoxins and averaged 4.25×10^4 ng g^{-1} total aflatoxins (ranged = 0 to 2.46×10^6 ng g^{-1}). The strain S_{BG} averaged 1.56×10^6 ng g^{-1} total aflatoxins (ranged = 1.69×10^3 ng g^{-1} to 6.07×10^6 ng g^{-1}). All isolates of both S_{BG} and *A. parasiticus* produced both B- and G-aflatoxins. Isolates of *A. parasiticus* averaged 1.18×10^6 ng g^{-1} total aflatoxin (ranged = 9.04×10^4 ng g^{-1} to 2.72×10^6 ng g^{-1}).

S_{BG} made the greatest contribution to the aflatoxin-producing potential of fungal communities within seven districts (Figure 10). *A. flavus* contributed the most to the average aflatoxin-producing potential in fungal communities resident in soils in the districts Ogbomosho, Makurdi, and Akwanga (Figure 10). *A. parasiticus* was the greatest contributor to the average aflatoxin-producing potential only in Ado-Ekiti (Figure 10). According to Pearson`s correlation analysis, there was a significant positive correlation between the average aflatoxin-producing potential of fungal communities and percentage S_{BG} (r = 0.34, P = 0.01) while there was a

negative significant correlation with *A. flavus* (r = -0.26, P = 0.05) (Table 3). There was also a weak significant correlation between the district average aflatoxin-producing potential and latitude (r = 0.3, P = 0.02).

The average aflatoxin-producing potential varied widely among the districts. The highest average aflatoxin concentration was in the district of Lafia (6.94×10^5 ng g^{-1}), while the least was in the district Ogbomosho (9.00×10^3 ng g^{-1}) (Table 4). Average aflatoxin-producing potential of *Aspergillus* section Flavi isolates resident in the NGS (3.36×10^5 ng g^{-1}) was significantly greater than for isolates resident in the SGS (1.33×10^5 ng g^{-1}), which was significantly lower than those in DS (2.79×10^5 ng g^{-1}).

Although *A. flavus* isolates in the NGS (average = 6.20×10^4 ng g^{-1}) produced significantly more aflatoxin than isolates in the SGS (average = 1.90×10^4 ng g^{-1}), the S_{BG} isolates produced significantly less aflatoxin in the NGS (average = 8.88×10^5 ng g^{-1}) than in the DS (average = 1.92×10^6 ng g^{-1}) (Table 4). Of all aflatoxin producing *A. flavus* isolates, 62% produced more than 1,000 ng g^{-1} aflatoxin B_1 (Figure 11).

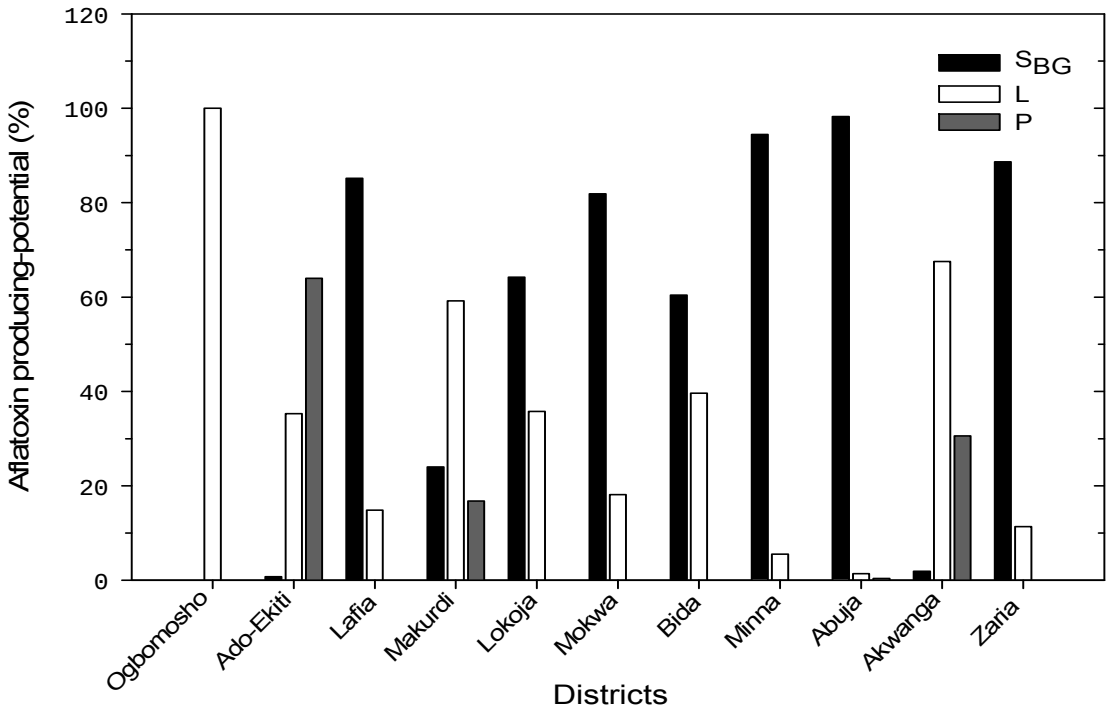


Figure 10 Contribution of *Aspergillus* species and strains to the average aflatoxin-producing potential of *Aspergillus* section Flavi communities resident in the 11 sampled Nigerian districts. Proportion of average aflatoxin-producing potential attributed to the species and strains = (Sum aflatoxin $B_1^{\text{species or strain}}$) / (Sum aflatoxin $B_1^{\text{all isolates}}$) * 100

Table 4 Mean aflatoxin quantities produced by three aflatoxin-producing taxa across three agroecological zones of Nigeria in liquid fermentation

				Aflatoxin (μg g^{-1})[c]							
				A. flavus	unnamed taxon S_{BG}			*A. parasiticus*			average toxin[d]
AEZ[a]	**Districts**	**N[b]**		**B_1**	**N[b]**	**B_1**	**G_1**	**N[b]**	**B_1**	**G_1**	**B_1**
DS	**Ogbomosho**	**56**	**mean**	**9 abcd**	**0**	--	--	--	--	--	**9 dc**
			range	0 - 175		--	--		--	--	0 - 175
	Ado-Ekiti	**50**	**mean**	**16 bcd**	**1**	**31 c**	**21 b**	**3**	**532**	**559 ab**	**43 dc**
			range	0 - 236		--	--		405 - 704	393 – 805	0 - 704
	Lafia	**43**	**mean**	**92 a**	**18**	**2,169 a**	**2,261 a**	**0**	--	--	**694 a**
			range	0 - 1,086		1,043 - 6,071	352 - 5,084		--	--	0 - 6,071
	Makurdi	**44**	**mean**	**26 ab**	**3**	**1,997 a**	**2,736 a**	**2**	**2,092**	**3,450 a**	**336 abc**
			range	0 - 614		1,637 - 2,676	1,919 - 3,944		1,467 - 2,717	1,942 - 4,957	0 - 6131
	Lokoja	**53**	**mean**	**85 abcd**	**8**	**1,551 a**	**2,158 a**	**0**	--	--	**278 abc**
			range	0 - 2,339		782 - 2,051	1,263 - 2,952		--	--	0 - 2,339
	total	**246**	**mean**	**45 ab**	**30**	**1,915 a**	**2,102 a**	**5**	**1,156**	**1,715 a**	**279 b**
			range	0 - 2,339		30 - 6,071	21 - 5,084		405 - 2,717	393 - 4,957	0 - 6,071
SGS	**Mokwa**	**49**	**mean**	**21 abc**	**4**	**1,751 a**	**1,751 ab**	**0**	--	--	**151 abc**
			range	0 - 335		381 - 3,768	198 - 3,258		--	--	0 - 3768
	Bida	**48**	**mean**	**4 d**	**2**	**178 abc**	**230 ab**	**0**	--	--	**10 d**
			range	0 - 65		27 - 329	58 -403		--	--	0 - 329
	Minna	**40**	**mean**	**1 d**	**1**	**817 ab**	**1,746 a**	**0**	--	--	**17 d**
			range	0 - 20		--	--		--	--	0 – 817
	Abuja	**36**	**mean**	**6 dc**	**9**	**2,293 a**	**2,434 a**	**1**	**90**	**99 b**	**403 bcd**
			range	0 - 152		1,144 - 3,425	494 - 4,108		--	--	0 - 3,425
	Akwanga	**46**	**mean**	**60 abcd**	**2**	**49 c**	**99 b**	**1**	**2,369**	**1,152 ab**	**96 dc**
			range	0 – 2,456		2 -96	2 - 197		--	--	0 - 2,456
	total	**219**	**mean**	**19 b**	**18**	**1,606 ab**	**1,669 ab**	**2**	**1,229**	**626 a**	**133 c**
			range	0 - 2,456		2 - 3,768	2 - 4,108		90 - 2,369	99 - 1,152	0 - 3,3768
NGS	**Zaria**	**27**	**mean**	**62 abc**	**17**	**888 abc**	**682 ab**	**0**	--	--	**336 ab**
			range	0 - 1,343		2 - 1,880	1 - 1,916		--	--	0 - 1,880
	total	**27**	**mean**	**62 a**	**17**	**888 b**	**682 b**	--	--	--	**336 a**
			range	0 - 1,343		2 - 1,880	1 - 1,916		--	--	0 - 1,880

[a] AEZ = agroecological zone; DS = Derived Savannah, SGS = Southern Guinea Savannah, and NGS = Northern Guinea Savannah.

[b] Number of isolates.

[c] Aflatoxin concentration values are in parts per million and were log (value +1) transformed prior to statistical analysis. Averages followed by the same letter in a column are not significantly different by Tukey`s HSD test ($\alpha = 0.05$).

[d] Mean aflatoxin of all three taxa.

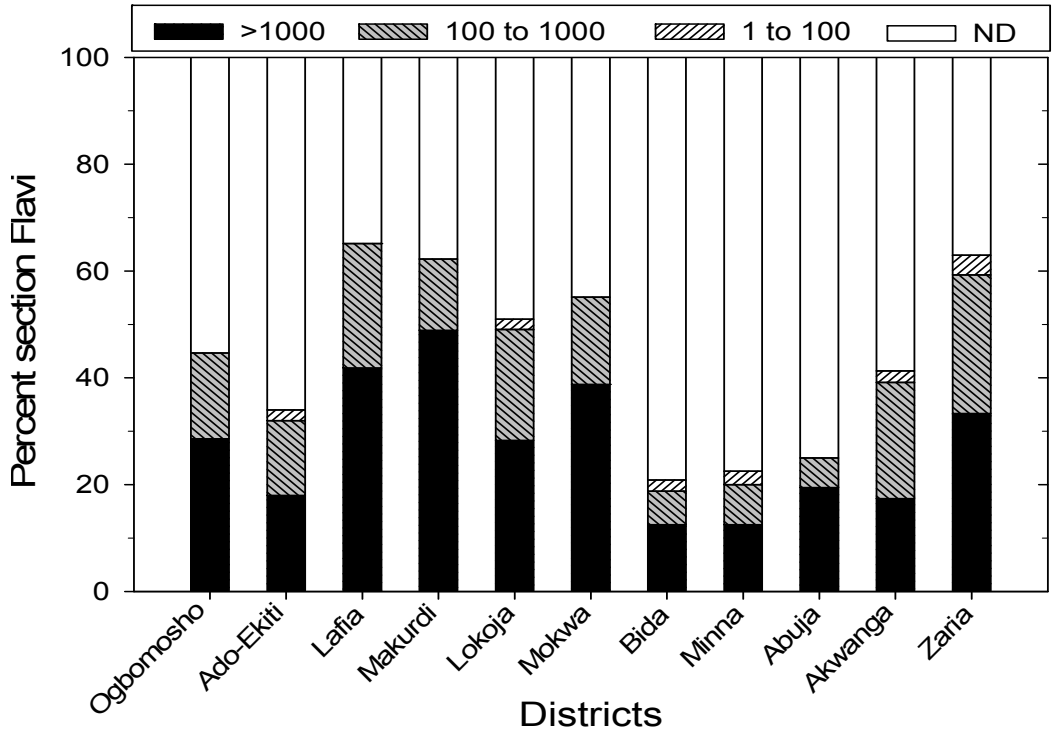


Figure 11 Variation among districts in the percent of *Aspergillus flavus* isolates that produce various quantities of aflatoxin B_1 (ng g^{-1} fungal biomass) in culture. ND = none detected.

4 DISCUSSION

A. flavus is the predominant member of *Aspergillus* section Flavi in cultivated maize fields across the three agroecological zones where most maize is produced in Nigeria (Table 1). *A. flavus* has similar dominance in other important maize producing regions of both West and East Africa (Cotty, 1997; Cardwell et al., 2002). Two species that produce G aflatoxins, *A. parasiticus* and the S_{BG}, were relatively common in some districts (Table 1). Isolates from Nigeria of both species produced high concentrations of both B- and G-aflatoxins (Table 4) and, as a result, in fields where these species were detected, they contribute substantially to the average aflatoxin-producing potential of resident fungal communities. *A. parasiticus* was restricted to a much smaller proportion of fields than S_{BG}. Therefore, it is more likely that contamination events in Nigeria involving G-aflatoxins are caused by S_{BG}, a genetically distinct West African species (Ehrlich, et al. 2003, 2005), than by *A. parasiticus*. As in other portions of the world (Cotty, 1997; Ehrlich, et. al. 2007), in Nigeria *A. tamarii* produces no aflatoxins, and is widely distributed.

The average aflatoxin-producing potential of *Aspergillus* section Flavi communities varies greatly across regions. For example, in both Argentina (Vaamonde et al., 2003) and Iran (Razzaghi-Abyaneh et al., 2006) less than 30% of the *A. flavus* produce aflatoxins, while in the southern USA, the majority of *A. flavus* isolates are aflatoxin producers (Cotty, 1997; Horn et al., 1999). The average aflatoxin-producing potential of section *Flavi* communities appears to be change with latitude. Cotty (1997) reported a negative correlation between latitude and *A. flavus* toxigenicity and Horn and Dorner (1999) observed greater proportions of L-strain isolates producing aflatoxins in southern than in northern peanut growing regions. In Nigeria, the percent *A. flavus* L-strain isolates that produced aflatoxins varied with geography and climate (Figure 8 and 9). Incidences of atoxigenic *A. flavus* varied widely among districts and agroecological zones with most *A. flavus* making aflatoxins in the warm, dry NGS zone and only 33% producing aflatoxins the SGS (Figure 8). However, in previous studies unacceptable aflatoxin concentrations were found in SGS maize (Sétamou et al., 1997; Hell et al., 2003; Atehnkeng et al., 2008b). Taken together, these observations demonstrate how aflatoxin levels unacceptable for human consumption may occur even in areas with relatively low frequencies of aflatoxin

producers. In the current study, 62% of aflatoxin-producing L-strain isolates produced more than 1,000 μg kg^{-1} aflatoxin B_1. This combined with high incidences allows the L-strain to be the largest contributor to the average aflatoxin-producing ability of fungal communities in three districts (Figure 10) and a potentially important causal agent of contamination in Nigeria.

Atoxigenic strains of *A. flavus* are common in crop environments (Joffe, 1969; Lisker et al., 1993; Cotty, 1997). Atoxigenic strains of *A. flavus* and/or *A. parasiticus* are used as biopesticides directed at minimizing crop contamination with aflatoxins (Cotty, 1994b; Dorner et al., 1998). Effective biological control necessitates high ratios of atoxigenic to toxigenic strains (Dorner et al., 2007). In the present study high incidences of native atoxigenic *A. flavus* strains were found in the districts Ogbomosho, Ado-Ekiti in the DS zone, and Bida, Minna, Abuja, and Akwanga in the SGS zone. These native atoxigenic strains are adapted to maize production areas in Nigeria and, as such, may have greater value than exotic strains as biocontrol agents for Nigeria.

Aspergillus section Flavi was resident in all sampled maize fields and quantities of section Flavi were higher on average in Nigeria than in neighbouring Benin (Cardwell et al., 2002). Densities of section Flavi in soil reflect fungal growth on crop associated organic matter. Maize cobs and other crop debris harbour section Flavi for at least 3 years after harvest (Jaime-Garcia et al., 2004). Following the aflatoxin epidemic year of 1988 in Iowa, high soil densities (1,231 CFU g^{-1}) of *A. flavus* were observed in harvested maize fields (Shearer et al., 1992). In the present study, similar densities (1,150 CFU g^{-1}) occurred in the SGS and DS. These high concentrations of propagules of aflatoxin-producing fungi may reflect frequent and wide spread aflatoxin contamination of susceptible crops in Nigeria.

The West African strain S_{BG} produces numerous small sclerotia similar to the S-strain of *A. flavus.* The S-strain is resident in several regions including North America (Cotty, 1997), Thailand (Ehrlich et al., 2007b), Argentina (Nesci et al., 2002), Italy (Giorni et al., 2007), and Kenya (Probst et al., 2007). However, S_{BG} produces both B- and G-aflatoxins whereas the S-strain produces only B-aflatoxins and molecular phylogenetics suggest that S_{BG} isolates represent a species distinct from both *A. flavus* and *A. parasiticus* (Egel et al., 1994; Ehrlich et al., 2003). During the current study, over 200 section Flavi isolates that produced numerous small sclerotia

were examined for aflatoxin production. All of these isolates produced both B and G aflatoxins indicating an absence of the *A. flavus* S-strain. Other studies in West Africa also failed to detect the *A. flavus* S-strain in either maize or soil (Cotty et al., 1999; Atehnkeng et al., 2008b). Therefore, in contrast to Kenya where the S-strain is the primary cause of maize aflatoxin contamination (Probst et al., 2007), the S-strain is either absent from West Africa or occurs at a very low frequency. Factors causing the S-strain of *A. flavus* to be dominant in portions of East Africa but undetectable in West Africa are not known. As in Benin (Cotty et al., 1999; Cardwell et al., 2002), S_{BG} isolates from Nigeria consistently produced greater quantities of aflatoxins than sympatric *A. flavus* L-strain isolates. This high aflatoxin-producing ability makes the S_{BG} a potentially important cause of contamination, even where it composes only a small proportion of section Flavi communities. Practices that might facilitate establishment of either the S_{BG} in other portions of Africa or the S-strain in West Africa should be discouraged.

Overall, S_{BG} isolates were less common than *A. flavus*. Nevertheless, S_{BG} distribution in Nigeria was similar to that in Benin (Cardwell et al., 2002). S_{BG} was less common in the south (SGS and DS) than in the north (NGS). Warm dry climates favoured S_{BG} over other members of section Flavi. S_{BG} isolates were most prevalent in the agroecological zones bordering the Sahara desert, where mean temperatures are high (Cardwell et al., 2002). In North America, the similarly adapted S-strain of *A. flavus* is also most common in dry, hot regions (Cotty, 1989; Cotty, 1997; Jaime-Garcia et al., 2006). The fungi causing most crop contamination are not necessarily the best adapted to infection (Mellon et al., 2004). Isolates that infect at relatively low frequencies but produce large quantities of aflatoxin may cause more contamination than more frequent isolates that produce little aflatoxin. Aflatoxin-producing potential and plant virulence are not correlated and isolates that produce high levels of aflatoxins may vary widely in virulence (Cotty, 1989). In the current study the S_{BG} isolates made the greatest contribution to the average aflatoxin-producing potential of fungal communities resident in certain soils. Relative virulence of S_{BG} isolates on maize, peanut, and other susceptible crops needs to be examined in order to fully evaluate the risk posed by this potent aflatoxin producer.

S_{BG} isolates produced very high levels of both B- and G-aflatoxins and incidences of the S_{BG} were correlated with the average toxigenicity of fungal communities. A similar relationship exists between incidences of the S-strain of *A. flavus* and average toxigenicity in North America

(Cotty, 1997). Even low frequencies of crop infection by the S_{BG} may adversely impact crop aflatoxin content. Therefore the S_{BG} is an important target for current efforts to control aflatoxin in West Africa (Atehnkeng, 2008).

Crop production practices vary across agroecological zones (Hell et al., 2000; Cardwell et al., 2006) and crop rotations influence the composition of fungal communities (Jaime-Garcia et al., 2006). S_{BG} incidence varies across agroecological zones (Table 2) and this variation may be caused in part by influences of crop rotation. Studies are needed to investigate which West African crops favour increased incidences of S_{BG}.

Atoxigenic strains of *A. flavus* and *A. parasiticus* have been used to minimize aflatoxin contamination in peanuts (Dorner et al., 1992), maize (Brown et al., 1991) and cotton (Cotty, 1994b). Results of the current study combined with a recently published study on Nigerian atoxigenic strains (Atehnkeng et al. 2008) suggest that atoxigenic strains of *A. flavus* could be useful in reducing aflatoxin contamination in the three most important maize production districts of Nigeria.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF ATOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS* FOR BIOLOGICAL CONTROL OF AFLATOXINS IN NIGERIA

1 INTRODUCTION

Aflatoxins are a group of toxic secondary metabolites produced by several species in *Aspergillus* section Flavi (Payne et al., 1998). *Aspergillus flavus* isolates produce B-aflatoxins, while *A. parasiticus*, *A. nomius,* and the unnamed taxon S_{BG} from West Africa produce both B- and G- aflatoxins (Diener et al., 1987; Kurtzman et al., 1987; Cotty et al., 1999). The more than twenty genes involved in aflatoxin biosynthesis are clustered in a 65 to 70 kb DNA region (Yu et al., 1995; Yu et al., 2004a; Yu et al., 2004b).

A. flavus is the most common causal agent of aflatoxin contamination in nature (Klich, 2007). Aflatoxins are highly carcinogenic and can contaminate food and feeds resulting in serious human and domestic animal health problems (Williams et al., 2004). Regulations limiting the concentrations of aflatoxins allowed in foods and feeds exist in most countries (van Egmond et al., 2007). Nevertheless, there are many regions, especially in Africa, where the products of small-scale farms move from field to mouth without any opportunity for practical monitoring of aflatoxin content. This can result in severe effects on human populations as with the recent outbreak of acute aflatoxicosis associated with aflatoxin contaminated maize in Kenya that resulted in hundreds of human mortalities (Lewis et al., 2005). In Nigeria, official monitoring of mycotoxin levels is rare, but high concentrations of aflatoxin in pre and postharvest maize have been reported (Adebajo et al., 1994; Udoh et al., 2000; Bankole et al., 2003b; Atehnkeng et al., 2008b).

A. flavus is very widely distributed with greater quantities of the fungus occurring in warm climates (Cotty et al., 1994b; Boyd et al., 1998). Gene flow within *A. flavus* is limited by a vegetative compatibility system (Papa, 1986; Bayman et al., 1991) that delineates the species into numerous genetic groups called Vegetative Compatibility Groups (VCGs). *A. flavus* VCGs are

clonal lineages (Ehrlich and Cotty, 2004) that exist in complex communities composed of many (VCGs). VCGs vary in many characteristics including aflatoxin producing ability.

Populations of *Aspergillus flavus* in an individual agricultural field contain isolates of many VCGs (Cotty et al., 1994b; Ehrlich et al., 2007a). VCGs with aflatoxin-producing potential are known to vary less among isolates within a VCG than among isolates from different VCGs (Kenneth et al., 2004). Communities resident in different fields, areas, and regions may vary widely in average aflatoxin-producing ability (Schroeder et al., 1973; Lisker et al., 1993; Cotty et al., 1997). Isolates and VCGs that do not produces aflatoxin, called atoxigenic, are common within *A. flavus* communities (Joffe et al., 1969; Schroeder et al., 1973; Lisker et al., 1993; Cotty et al., 1997). Surveys of *A. flavus* isolates from various geographic regions have revealed differences in the proportions of isolates that produce low, medium, and high amounts of aflatoxins (Cotty, 1997; Cotty et al., 1999; Horn et al., 1999). In Argentina (Vaamonde et al., 2003) and Iran (Razzaghi-Abyaneh et al., 2006) less than 30% of the *A. flavus* isolates were capable of producing aflatoxin whereas in Nigeria the number was much higher, exceeding 50% (Donner et al., 2008). In the southern USA most *A. flavus* isolates produce aflatoxin (Cotty, 1997; Horn et al., 1999).

In the USA, there are currently several atoxigenic strains of *A. flavus* used to reduce crop aflatoxin contamination through competitive exclusion of aflatoxin producers (Dorner, 2004; Cotty, 2006; Cotty et al., 2008). One of these strains, AF36, has successfully suppressed toxigenic strains of *A. flavus* on cottonseed in Arizona and Texas since 1999 (Antilla et al., 2002). Recently, efforts began to develop similar technology for use in Africa (Atehnkeng et al., 2008a; Hell et al., 2008).

The loss of aflatoxin production by members of *Aspergillus* section Flavi is still not well understood. For example, neither *A. sojae* nor *A. oryzae* produce aflatoxins (Wei et al., 1986), even though homologues of several aflatoxin biosynthetic genes have been found in them (Chang et al., 1995; Klich et al., 1995; Yu et al., 2000a). Both species have been used for centuries in the food fermentation industry and are generally considered safe (Machida et al., 2005; Chang et al., 2007). Although *A. oryzae* strains have the aflatoxin biosynthetic cluster, it is not functional. *A. oryzae* apparently is a domesticated form of *A. flavus* originating from an ancestral atoxigenic *A. flavus* (Wicklow, 1984; Kurtzman et al., 1986; Chang et al., 2006). The aflatoxin biosynthesis genes in *A. oryzae* contain deletions, frameshift mutations, and base pair substitutions that explain the lack of

aflatoxin production (Tominaga et al., 2006). Deletion of portions of the aflatoxin biosynthetic gene cluster within atoxigenic *A flavus* isolates is not rare (Chang et al. 2005) and strains of *A. flavus* with large deletions in the aflatoxin gene cluster have been used to study the genetics of aflatoxin biosynthesis for over a decade (Prieto et al., 1996). A single nucleotide polymorphism (SNP) in a polyketide synthase gene results in atoxigenicity in the biocontrol strain AF36 (Ehrlich et al., 2004; Ehrlich et al., 2007a). Nevertheless, molecular mechanisms responsible for loss of aflatoxin production are diverse and for most atoxigenic *A. flavus* specific genetic lesions resulting in atoxigenicity are not known.

In the current study, in order to provide a basis for understanding atoxigenicity in Nigeria, aflatoxin biosynthetic gene clusters of 21 atoxigenic *A. flavus* isolates belonging to 18 VCGs were compared with several aflatoxin producing *A. flavus* and with atoxigenic strain AF36, currently used to manage aflatoxins in North America. To further assess the diversity of atoxigenic strains available for biocontrol in Nigeria, relationships among the examined isolates were assessed with phylogenetic analysis. During this process, molecular characteristics were discovered that are useful both for monitoring the stability of the atoxigenic phenotype and for specifically identifying each of these candidate's biocontrol strains.

2 MATERIAL AND METHODS

2.1 Isolates, Vegetative Compatibility, and Aflatoxin Production

Aspergillus flavus isolates from Nigeria used in this study are listed in Table 5. Isolates originate from maize samples collected in several districts of Nigeria by the International Institute of Tropical Agriculture (IITA) (Atehnkeng et al., 2008b). Fungi were isolated from the maize by dilution plate technique on modified rose Bengal Agar (MRBA, Cotty, 1994a). All isolates belong to the L-strain morphotype of *A. flavus*, which produces sclerotia that are >400 µm in diameter (Cotty, 1989). The commercial biocontrol agent from the United States, *Aspergillus flavus* AF36 (ATCC96045) was used as a reference culture throughout the study. Methods for collection, strain identification, and vegetative compatibility group (VCG) characterization have been described (Bayman et al., 1991; Bayman et al., 1993; Cotty, 1994a; Cotty, 1997).

Aflatoxin was also quantified as previously described by Cotty (1997). 250 ml flasks containing 70 ml of a chemically defined medium with ammonium as the sole nitrogen source were inoculated with isolates belonging to *Aspergillus* section Flavi. After 5 days incubation, 50 ml aceton was added to lyse the mycelium and solubilize the aflatoxins. The cultures were filtered and the aflatoxins were partitioned into methylene chloride. Extracts negative for aflatoxin content were evaporated to dryness, dissolved in 60% methanol and loaded onto a column with immunoaffinity to aflatoxins (Aflatest P column, VICAM, Watertown, MA, USA). Aflatoxins were eluted from the column with methanol and the eluate was concentrated and spotted onto TLC plates and developed and quantified as previously described by Cotty and Cardwell (1999). The column clean up allowed detection of 0.5 ppb aflatoxin B1/gram mycelium.

Table 5 *Aspergillus flavus* strains used in the study

Isolate	VCG	Geographic origin	Aflatoxin Production
AV3279[a]	B	Lafia	No
AV3304[a]	D	Lafia	No
AV2216[a]	E	Abuja	No
AV0106	F	Ogbomosho	Yes
AV0222[a]	G	Ogbomosho	No
AV0173	H	Ogbomosho	No
AV0165	I	Ogbomosho	No
AV0452	L	Ogbomosho	No
AV3108[a]	M	Lafia	No
AV3150	N	Lafia	No
AV3228[a]	O	Lafia	Yes
AV3224	Q	Lafia	No
AV3303[a]	R	Lafia	No
AV3306	S	Lafia	No
AV0205	T	Ogbomosho	No
AV2757[a]	U	Akwanga	No
AV3058[a]	V	Akwanga	No
AV4216[a]	W	Lokoja	No
AV16127[a]	X	Kaduna	No
AV3020[a]	Y	Akwanga	No
AV0216	NA[c]	Ogbomosho	No
AV0230	NA[c]	Ogbomosho	No
AV3193	NA[c]	Lafia	No
AF36[b]	YV36	Arizona	No

[a] Isolates from Nigeria used in a previous study by Atehnkeng et. al. (2008)

[b] AF36 (ATCC 96045) a strain that produces no aflatoxin.

[c] NA = Not available. They could not be assigned to any VCG because of incompatibility.

2.2 DNA isolation

Fungi were cultured in 70 ml Czapek-Dox Broth (Difco) in 250ml flasks agitated 150 rpm at 32°C for 48-72 h. Mycelia were collected by vacuum filtration, ground to fine powder in liquid nitrogen and stored at -80°C. In 1.5 ml tubes 200 mg of mycelial powder was resuspended in 750 µl spermidine-SDS buffer (4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 0.5% SDS, 10 mM β-mercaptoethanol, 40 mM Tris-HCl pH 8.0). After adding an equal volume of Phenol, the suspension was centrifuged at 14,000 rpm for 15 min and the mycelial lysates were recovered and mixed with an equal volume of phenol/chloroform (1:1) and recentrifuged for 15 min at 14,000 rpm. The supernatant was mixed with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged. DNA was recovered from the aqueous phase by mixing a 0.1 volume of 3M sodium acetate pH 5.5 and adding 2 volumes of 100% ethanol. The DNA was pelleted by centrifugation, washed with 70% ethanol, air-dried and redissolved in TE buffer (20 mM Tris-HCl pH 7.5, 0.1 mM EDTA). Purity and average fragment size were visualized with SYBR Gold after 1.2% agarose gel electrophoresis. DNA concentration was measured with a spectrophotometer (model ND-1000, NanoDrop). From some of the samples, DNA was isolated using the FastDNA SPIN Kit and the FastPrep Instrument according to manufacturer`s instructions (Qbiogene, Inc., CA).

2.3 PCR conditions

Oligonucleotide primer sets (Table 6) targeting PCR products of 0.3 to 1.2 kb were derived from aflatoxin biosynthetic pathway genes of *A. flavus* AF36 (AY 510455), AF13 (AY 510451) and AF70 (AY 510453). Some primers sets were based on Chang et al. (2005) and Ehrlich et al. (2005). Primer sets were also designed to amplify portions of the *pecA* and *taka amylase* genes:

pecA, 5'-GCTTAGCCTAGACTCAAG; 5'-AAGAGGAGTCCAGCTTGTG;

taka amylase, 5'-TATCCAGGGAATGGGCTT, 5'-TTAGAGGTCGTCCATGCTGCC

PCR used 5 ng genomic DNA, 50 pmol of forward and reverse oligonucleotides, and the HotMaster PCR kit (Eppendorf, Westbury, NY). Annealing temperatures were optimized for each primer set and varied from 48-60° C. The 50 µl PCR reactions were preformed with a MyCycler thermocycler (Bio-Rad Laboratories) with typical conditions 5 min at 95° C followed by 38 cycles at 95° C for 30 sec, 55° C for 20 sec, 72° C for 30 sec and 10 min at 72° C. Amplicons were visualized with SYBR Gold after 1.2% agarose gel electrophoresis. Sequencing was preformed by The Genomic Analysis and Technology Core Facility (GATC) at the University of Arizona, Tucson.

Table 6 PCR primer sets derived from aflatoxin biosynthetic genes and flanking regions[a]

Designation	Forward primer	Reverse primer
*C2**	TCGCCTTGTTCTCGCTATAC	ACACCTGATAGCGAGAGTTC
norB	GTGAGGGATAGCAATAAGTGT	TCCTGGATTTCCGCATAC
norB-cypA **	GTGCCCAGCARCTTGGTCCA	AGGACTTGATGATTCCTGCTC
aflT*	ATGACATGCTAATCGACGAG	AGGCGCATGCTACGGATC
pksA	GCTGGGATTCTGCATGGGTT	CCATCTGAGGCATCGCACA
fasA(hexA)*	TCCTATCCAGTCCACCTCGTA	CACATCTTTGTCTTGCCCGC
afIR	GGAAACAAGTCTTTTCTGG	CAGAGCGTGTGGTGGTTGAT
aflJ*	CTTCAACAACGACCCAAGGTT	AGATGAGATACACTGCCGCA
estA*	CGATGGGACTGACGGTGATT	ACCACGCCGCTGACTTTAT
norA	GGAGCACCTCAAGGAGAACA	GGAACCTTGCGTCGATTCTA
ver-1	AGCCAAAGTCGTGGTGAACT	CCATCCACCCCAATGATCT
omtA*	CAGGATATCATTGTGGACGG	CTCCTCTACCAGTGGCTTCG
verA*	CCGCAACACCACAAGTAGCA	AAACGCTCTCCAGGCACCTT
avnA*	GCGATAGAACTGACAAAGGCA	GAATGAGTCTCCAAAGGCGAG
verB	CCCAATACAGTTCCGCAGTC	AGTGAAGAGTGCCGACGATAA
avfA*	ATTCAAATCCTCGTTCGGTCG	TAGCCCGTTGGTTGTGTTCC
omtB	TTTACTCGGATTGGGATGTGGT	CGCAGTCCTTGTTAGAGGTGAT
vbs*	AACGAGCAGCGTAAGGGTCT	TCAGCCAGAGCATACACAGTG
*cypX**	GGAGCCTACCATTCGCAACA	GGCTTTGACGAACAGATTCCG
*ordB**	GCTGCTACTGGAATGAAGACC	ATGCGACGACAACCAAACG
hypA*	CGCAAGACGGCAGAGATACT	GCTCCTTCAGTTCCACACCA
glcA*	AGACACAGTCATCGCCTGTT	GGTGCGAATAGGTGCAGGTA

*Primer sets based on Chang et al. (2005)

**Primer set based on Ehrlich et al. (2004)

[a] Names in bold indicate sequenced genes.

2.4 Phylogenetic analysis

In addition to those sequences produced during the current study, phylogenetic analyses included sequences from Genbank for several aflatoxin-producing fungi including *A. flavus* L strain isolates AF13 (ATCC 96044) and NRRL3357, *A. flavus* S strain isolate AF70 (ATCC MYA384), and *A. parasiticus* isolate NRRL5862 (AY371490). DNA sequences were aligned with DNAMAN (Lynnon Biosoft, Vandereuil, Canada).

Aligned sequences of the combined gene datasets were subjected to phylogenetic analyses with PAUP* Version 4.0b10 for Microsoft Windows (Swofford, 2002). Parsimony trees were obtained using heuristic search methods with stepwise sequence addition and the tree-bisection-reconnection (TBR) branch-swapping algorithm. All sites were equally weighted and gaps treated as missing characters. Bootstrap analyses were based on 1,000 replicates.

2.5 Analysis of synonymous and non-synonymous substitutions

Sequences were aligned to codon-aligned nucleotide sequences of *A. flavus* AF13 from Genbank. Codon-aligned nucleotide sequences of *A. flavus* NRRL3357 were used to compute synonymous (silent) and non-synonymous (amino acid-altering) nucleotide substitutions. Minor manual modifications were made to the DNA alignments to optimize alignments and ensure indels did not erroneously split codons. Estimates of synonymous and non-synonymous substitution rates based on Nei and Gojobori 1986 and incorporating the statistic of Ota and Nei 1994 were made with SNAP (Korber, 2000).

The previously described pyrosequencing (Biotage, Uppsala, Sweden) method (Das, et al, 2008) was used to assess the distribution among the examined isolates of the single nucleotide polymorphisms in *pksA* responsible for atoxigenicity in *A. flavus* AF36 (Ehrlich et al., 2004).

2.6 Data analysis

Analyses were performed with SAS (version 9.1.3, SAS Institute Inc., Cary, NC). Analysis of variance was performed on all data with the general linear model (GLM) suitable for unbalanced data. The GLM of SAS uses the least squares method to fit data to a general linear model. Tukey`s honestly significant difference (HSD) test was performed to compare treatment means at the 5% level.

3 RESULTS

3.1 Aflatoxin production and vegetative compatibility groups

The 21 atoxigenic strains previously selected for use in biocontrol of aflatoxin contamination of maize (Atehnkeng et al., 2008b) were confirmed to be atoxigenic (limit of detection 0.5 aflatoxin B_1/gram mycelium) (Table 5). The two aflatoxin producing isolates from West Africa produced widely varying aflatoxin concentrations from 560.3 ng g^{-1} aflatoxin B for AV0106 to 23,068.5 ng g^{-1} for AV3228. These isolates belong to 18 atoxigenic and two toxigenic VCGs (Table 5). Complementary mutants could not be generated for three atoxigenic isolates and therefore were not assigned to a VCG.

3.2 Deletions in aflatoxin biosynthetic gene cluster of atoxigenic *A. flavus* isolates

PCR with the designed primer sets was successful at amplifying all 22 gene fragments attempted within the aflatoxin biosynthesis gene cluster for both aflatoxin producing isolates from West Africa. In addition all fragments were successfully amplified for *Aspergillus flavus* AF36, the atoxigenic strain used commercially for aflatoxin biological control in the USA, and for 13 of the 21 atoxigenic strains belonging to different VCGs from Nigeria. PCR results for seven of the atoxigenic *A. flavus* isolates from Nigeria were consistent and indicated large deletions in the aflatoxin biosynthetic pathway (Figure 12). Deletions extended from the entire cluster for AV0222 to deletion of all genes from *norB* through *norA*, over 35 kb. Five of these seven isolates belonged to a different VCG and all originated from the same district, Ogbomosho. The large deletions included the 5' end (proximal to the teleomere) of the aflatoxin gene cluster except for AV0173 and AV0452 which retained remnants of the *norB-cypA* region. For four VCGs, the PCR protocol failed to amplify the target 603 bp within the gene *cypX* (aflV) while protocols for the adjacent genes *vbs* (aflK) and *moxY* (aflW) produced the expected amplicons. These four VCGs originated from 3 districts (Figure 12 and Table 5). The protocols for the three genes not involved in aflatoxin biosynthesis, *pecA, taka amylase,* and *glcA,* produced the predicted amplicons for each of the 24 *Aspergillus flavus* isolates included in the current study.

All *A. flavus* isolates had deletions in the *norB–cypA* region when compared to the *norB-cypA* region of *A. parasiticus* (AY371490). In all isolates except AV3228, deletions included

coding regions for amino acids 1-280 of *norB* and 1-112 of *cypA*, and the *norB-cypA* intergenic region. In AV3228 coding regions for amino acids 1-181 and 300-310 of *norB* and 1-29 of *cypA* were deleted along with the *norB-cypA* intergenic region.

The pyrosequencing assay used to detect a SNP responsible for atoxigenicity in the biocontrol atoxigenic *A. flavus* AF36 (Ehrlich et al., 2004; Das et al., 2008) successfully identified the target SNP in AF36 but not in any of the other *A. flavus* isolates included in the current study.

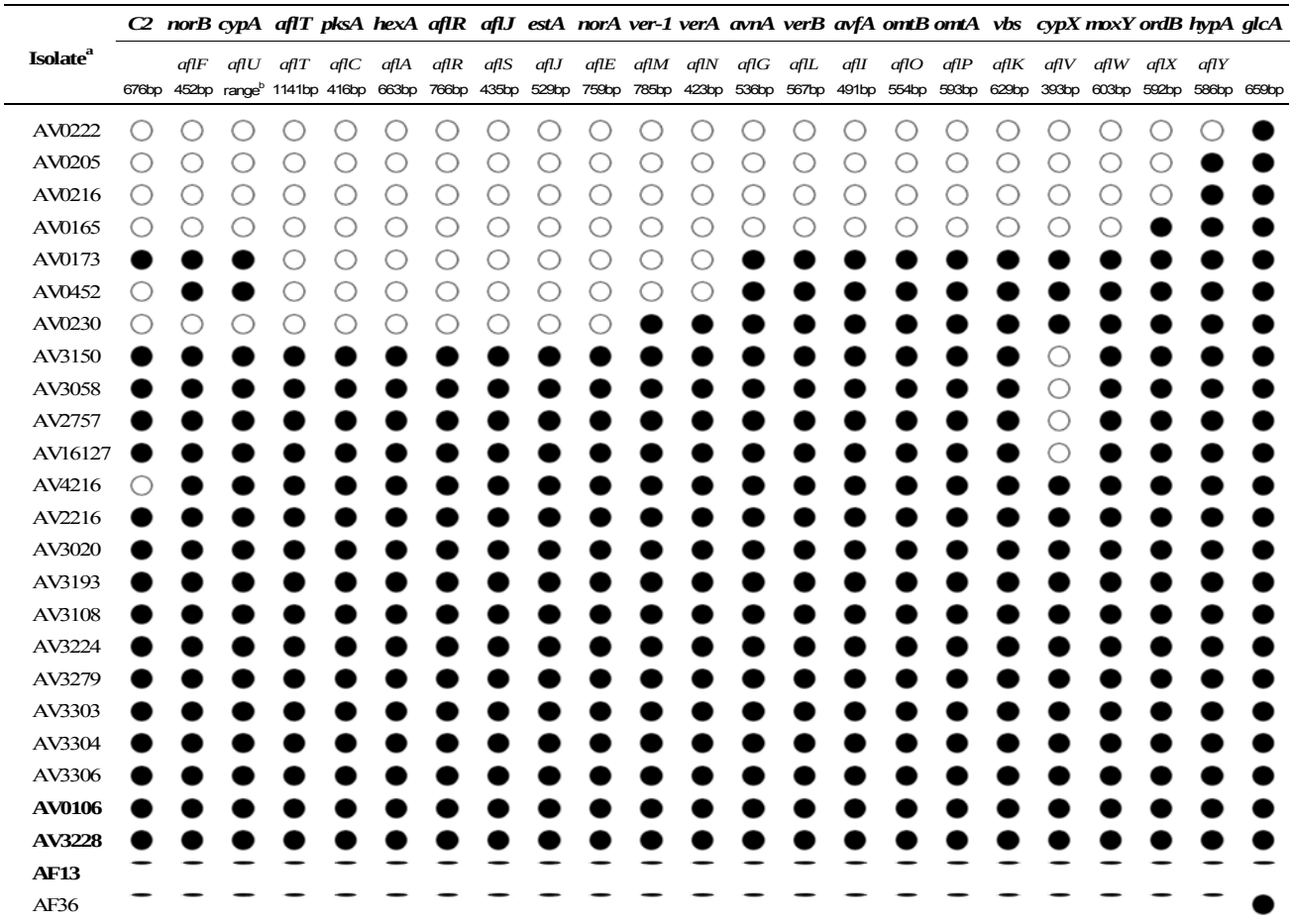


Figure 12 Segments of the *Aspergillus flavus* aflatoxin gene cluster present (filled circle) or absent (empty circle) in Nigerian A. flavus and United States isolates. Original gene names are above and new names below (Yu et al., 2004b). Amplicon sizes are indicated. The glcA is a gene in the sugar utilization cluster adjacent to the 3' end of the aflatoxin cluster; C2 is a gene - flanking region the aflatoxin gene cluster at the 5' end.

[a] Isolates names in bold indicate the aflatoxin producers

[b] Two amplicons sizes were detected. Amplicons of all isolates except AV3228 were 0.3 kb; the amplicons of AV3228 was 0.8 kb.

3.3 Distribution of polymorphisms

In *Aspergillus flavus* the aflatoxin biosynthetic gene cluster is 66.1 to 66.5 kb in length. In the current study, 0.4 kb to 8.1 kb of the cluster was sequenced for the 18 atoxigenic and two toxigenic VCGs not exhibiting large deletions in the cluster (Table 7). Of the sequenced cluster regions, 5 kb was coding sequence and 3.1 kb was non-coding. In addition, 3 gene pieces (2.2 kb) outside of the cluster were sequenced, including 649bp of *glcA*, a component of the sugar cluster adjacent to the aflatoxin-biosynthesis cluster. All polymorphisms were measured in relation to NRRL3357, the fully sequenced *A. flavus* and a producer of high concentrations of aflatoxins. Gene segments differed significantly (P = 0.05) in both total percentage polymorphism and non-synonymous substitutions with the greatest rates of polymorphism in *ver1* among atoxigenic VCGs and in *omtB* among all VCGs in both coding and non-coding segments. Among all VCGs, significantly (P = 0.05) more polymorphisms were detected in non-coding gene segments (3.4%) than in the coding (2.8%). This was true both when the toxigenic strains were included and when they were removed from the analysis.

Polymorphisms in the gene regions outside the aflatoxin cluster in atoxigenic VCGs had lower ranges of polymorphisms (total, 0.3 %, synonymous, 0.2%, non-synonymous, 0.1%) than genes within the aflatoxin cluster.

Polymorphisms in *hypA*, a gene present in all but one of the isolates from Nigeria, ranged from 0.0 % to 2.7 % in atoxigenics exhibiting a large cluster deletion (6 isolates) and from 0.0 % to 1.1% in atoxigenics with no deletion while the two aflatoxin producers had 0.4% to 0.9% polymorphism. For the isolates without large cluster deletion, there were significant (P = 0.05) differences in percent polymorphic sites between aflatoxin producers and atoxigenic strains in the coding gene segments of *aflT*, *hexA*, *aflR*, *aflJ*, *ver1*, and *omtB*. Only the gene segments of *aflT*, *ver1*, and *omtB* had significantly (P= 0.05) more non-synonymous substitutions in atoxigenic strains than in the toxigenic. Neither of the aflatoxin producers contained polymorphisms in the aflatoxin regulatory genes *aflR* and *aflJ*. All but one of the atoxigenics had polymorphisms in *aflR* and several atoxigenics had polymorphisms in the *aflJ* segment in which several changes were detected in protein sequences. Although the frequency of polymorphisms varied among atoxigenic VCGs for several genes (i.e. *avfA* and *omtA*) it was consistent among atoxigenics for many other genes. The frequency of polymorphisms in the coding and non-coding gene segments differed significantly

(P = 0.05) between the toxigenic and atoxigenic isolates, and in both cases atoxigenic strains had more polymorphism.

In total, the majority of the nucleotide changes in gene segments of the aflatoxin biosynthesis gene cluster resulted in synonymous substitutions, (Table 7). The ratio dn/ds, as calculated with SNAP (Korber, 2000) resulted in a ratio far less than one for most gene segments including genes with very high polymorphism rates (i.e. omtB and ver1). However, one gene had dn/ds values over 1. Twelve of 15 atoxigenics and one aflatoxin-producer had dn/ds greater than 1 for *avnA* gene segment. In that gene segment the highest ratio of synonymous to non-synonymous substitutions was also measured. In the *hypA* gene segment, 12 of 15 atoxigenics and one aflatoxin-producer contained 2 non-synonymous substitutions and no synonymous (Table 7). The ratio of non-synonymous to synonymous changes of all isolates was almost balanced in the *aflT* gene segment. Values for several genes could not be calculated because the values for d_S or Sd were only 0.

Although only 9.5% of the coding sequence of the aflatoxin biosynthesis pathway was sequenced, several DNA polymorphisms which lead to predicted amino acid changes were detected that did not occur in aflatoxin producing isolates (Table 8). Several of these cause changes in amino acid type (i.e. serine to proline or proloine to threonine) and reactivity (i.e. arginine to cysteine).

Table 7 Frequencies of Single Nucleotide Polymorphisms in sequenced segments of the aflatoxin biosynthesis gene cluster compared to the toxin producer NRRL3357 for isolates in the current study lacking a large deletion in the cluster

Genes[a]		Isolates																
		AV3193	AV3224	AV3108	AV3279	AV3304	AV3303	AV3150	AV3306	AF36	AV3058	AV2757	AV4216	AV3020	AV2216	AV16127	**AV3228**	**AV0106**
	Sd	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0
	Sn	1.0	1.0	2.0	1.0	1.0	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0
norB 120bp	**d_N/d_S**	0.284	0.284	NA	0.284	0.284	0.284	0.574	0.284	NA	0.284	0.284	NA	0.284	NA	0.284	NA	0.574
	P (%)	1.7	1.7	1.7	1.7	1.7	1.7	2.5	1.7	1.7	1.7	1.7	0.8	1.7	0.8	1.7	0.8	2.5
	Sn/Sd	1.0	1.0	NA	1.0	1.0	1.0	2.0	1.0	NA	1.0	1.0	NA	1.0	NA	1.0	NA	2.0
	Sd	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	2.0	3.0	2.0	0.0	2.0
	Sn	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	1.0	2.0	1.0	2.0	0.0	1.0
aflT 612bp	**d_N/d_S**	0.346	0.346	0.173	0.346	0.346	0.346	0.346	0.346	0.173	0.346	0.346	0.115	0.346	0.115	0.346	NA	0.173
	P (%)	0.7	0.7	0.5	0.7	0.7	0.7	0.7	0.7	0.5	0.7	0.7	0.7	0.7	0.7	0.7	0.0	0.5
	Sn/Sd	1.0	1.0	0.5	1.0	1.0	1.0	1.0	1.0	0.5	1.0	1.0	0.3	1.0	0.3	1.0	NA	0.5
	Sd	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	0.0	0.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
hexA 582bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0
	Sn/Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	NA
	Sd	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
aflR 354bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.339	NA	NA	NA
	P (%)	0.3	0.3	0.0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.6	0.3	0.0	0.0
	Sn/Sd	0.0	0.0	NA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	NA	NA
	Sd	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0
	Sn	0.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0
aflJ 190bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.5	0.5	0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0	0.0
	Sn/Sd	0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
estA 426bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn/Sd	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Sd	19.0	19.0	19.0	18.0	19.0	19.0	18.0	19.0	20.0	19.0	19.0	19.0	19.0	2.0	18.0	0.0	1.0
	Sn	1.0	2.0	0.0	1.0	2.0	2.0	1.0	2.0	0.0	2.0	2.0	1.0	2.0	0.0	1.0	0.0	0.0
ver1 483bp	**d_N/d_S**	0.015	0.030	NA	0.015	0.030	0.030	0.015	0.030	NA	0.030	0.030	0.015	0.030	NA	0.015	NA	NA
	P (%)	4.1	4.3	3.9	3.9	4.3	4.3	3.9	4.3	4.1	4.3	4.3	4.1	4.3	0.4	3.9	0.0	0.2
	Sn/Sd	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.1	NA	0.0
	Sd	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0
	Sn	4.0	4.0	3.0	4.0	4.0	4.0	4.0	4.0	3.0	4.0	4.0	4.0	4.0	2.0	4.0	2.0	4.0
avnA 387bp	**d_N/d_S**	1.290	1.290	0.963	1.290	1.290	1.290	1.290	1.290	0.477	1.290	1.290	1.290	1.290	0.319	1.290	0.645	1.290
	P (%)	1.3	1.3	1.0	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.0	1.3	0.8	1.3
	Sn/Sd	4.0	4.0	3.0	4.0	4.0	4.0	4.0	4.0	1.5	4.0	4.0	4.0	4.0	1.0	4.0	2.0	4.0
	Sd	5.0	5.0	18.0	5.0	5.0	5.0	5.0	5.0	18.0	5.0	5.0	5.0	5.0	19.0	5.0	1.0	5.0
	Sn	3.0	3.0	12.0	3.0	3.0	3.0	3.0	3.0	12.0	3.0	3.0	3.0	3.0	13.0	3.0	1.0	3.0
avfA 429bp	**d_N/d_S**	0.170	0.170	0.174	0.170	0.170	0.170	0.170	0.170	0.174	0.170	0.170	0.170	0.170	0.177	0.170	0.288	0.170
	P (%)	1.9	1.9	7.0	1.9	1.9	1.9	1.9	1.9	7.0	1.9	1.9	1.9	1.9	7.5	1.9	0.5	1.9
	Sn/Sd	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.7	0.6	1.0	0.6
	Sd	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	17.0	16.0	0.0	16.0
	Sn	5.0	5.0	3.0	5.0	5.0	5.0	5.0	5.0	4.0	5.0	5.0	5.0	5.0	4.0	5.0	0.0	5.0
omtB 249bp	**d_N/d_S**	0.094	0.094	0.056	0.094	0.094	0.094	0.094	0.094	0.075	0.094	0.094	0.094	0.094	0.069	0.094	NA	0.094
	P (%)	8.4	8.4	7.6	8.4	8.4	8.4	8.4	8.4	8.0	8.4	8.4	8.4	8.4	8.4	8.4	0.0	8.4
	Sn/Sd	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	NA	0.3
	Sd	2.0	2.0	2.0	7.0	2.0	2.0	7.0	2.0	7.0	2.0	2.0	7.0	2.0	6.0	2.0	0.0	7.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
omtA 122bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	1.6	1.6	1.6	5.7	1.6	1.6	5.7	1.6	5.7	1.6	1.6	5.7	1.6	4.9	1.6	0.0	5.7
	Sn/Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Na	0.0
	Sd	7.0	5.0	8.0	5.0	5.0	5.0	7.0	5.0	0.0	5.0	5.0	5.0	5.0	5.0	7.0	5.0	5.0
	Sn	3.0	2.0	0.0	1.0	2.0	2.0	2.0	2.0	0.0	2.0	2.0	1.0	2.0	1.0	3.0	1.0	1.0
vbs 540bp	**d_N/d_S**	0.123	0.116	NA	0.058	0.116	0.116	0.082	0.116	NA	0.116	0.116	0.058	0.116	0.058	0.123	0.058	0.058
	P (%)	1.9	1.3	1.5	1.1	1.3	1.3	1.7	1.3	0.0	1.3	1.3	1.1	1.3	1.1	1.9	1.1	1.1
	Sn/Sd	0.4	0.4	0.0	0.2	0.4	0.4	0.3	0.4	NA	0.4	0.4	0.2	0.4	0.2	0.4	0.2	0.2
	Sd	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn	2.0	2.0	0.0	0.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	0.0
hypA 525bp	**d_N/d_S**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.4	0.4	0.2	0.0	0.4	0.4	0.4	0.4	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.0
	Sn/Sd	NA	NA	0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Sd	1.0	1.0	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	1.0
	Sn	1.0	0.0	1.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	1.0
glcA 462bp	**d_N/d_S**	0.027	0.000	0.066	0.027	0.000	0.000	0.027	0.000	0.000	0.027	0.000	0.000	0.027	0.000	0.027	0.089	0.027
	P (%)	0.4	0.2	1.1	0.4	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.4	0.9	0.4
	Sn/Sd	1.0	0.0	0.3	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	0.3	1.0
total	**Sd**	58.0	55.0	73.0	59.0	55.0	55.0	61.0	55.0	69.0	55.0	55.0	61.0	55.0	59.0	57.0	10.0	39.0
	Sn	22.0	22.0	23.0	19.0	22.0	22.0	23.0	22.0	23.0	23.0	22.0	18.0	23.0	25.0	22.0	8.0	17.0

[a] Only amplicons including coding regions with protein information included; gene names and length of the portion of coding region used;. co region; Sd, synonymous substitutions; Sn, non-synonymous substitutions; non-synonymous (d_N) and synonymous (d_S) as calculated by applying the Jukes-Cantor correction for back-mutations;

d_N/d_S, ratio of non-synonymous to synonymous substitutions; P, the percentage polymorphic sites for each gene/sequences; Sn/Sd is the simple ratio of non-synonymous to synonymous substitutions; NA, not applicable because uncalculated pairs of Sn/Sd or d_N/d_S; Isolates names in bold indicate the aflatoxin producers

Table 8 Non-synonymous nucleotide substitutions in gene sequences of atoxigenic *Aspergillus. flavus* isolates which were not found in the toxigenic isolates NRRL3357, AF13, AF70, AV3228, and AV0106

Genes	Nt change	Nt position with respect to the translation start site	Amino acid change	Amino acid position with respect to the translation start	Isolates
norB					
	G → A	1057	G → S	353	AV0452, AV0173
	T → C	1075	V → A	359	AF36, AV3108
aflT					
	G → T	710	M → I	237	AV3193, AV3303, AV3150, AV3303, AV3224, AV3279, AV3304, AV3058, AV16127, AV2757, AV3020
aflR					
	G → C	191	M → I	64	AV2216
aflJ					
	T → C	768	S → P	257	AV3303, AV3150, AV3306, AV3224, AV3279, AV3304, AV3058, AV2757, AV3020
ver1					
	G → A	198	D → N	67	AV3303, AV3306, AV3224, AV3304, AV3058, AV2757, AV3020
	G → A	213	E → K	72	AV3193, AV3303, AV3150, AV3306, AV3279, AV3304, AV3058, AV16127, AV2757, AV4216, AV3020
	C → T	390	R → C	131	AV0230
avnA					
	C → T	159	L → F	54	AV0230
	T → A	191	D → E	64	AF36
	C → A	294	P → T	99	AV3108
	A → C	415	D → G	139	AV0230
omtB					
	A → G	234	T → A	79	AF36
	A → G	297	T → A	100	AV0452, AV0173
vbs					
	G → A	1065	G → R	551	AV3193, AV16127
	G → A	1785	V → I	596	AV0230
	T → C	1791	S → P	598	AV3193, AV3150, AV16127, AV3279, AV2216
	G → T	1809	G → C	604	AV0452
hypA					
	G → C	975	P → A	326	AV0173, AV0216, AV0452
	A → G	1087	Y → C	363	AF36
	G → T	1118	K → N	373	AV3193, AV3224, AV3303, AV3306, AV3150, AV3058, AV3020, AV2757, AV2216, AV16127, AV0425, AV4216
	G → C	1149	E → Q	384	AV0173, AV0216, AV0452
	T → A	1220	N → K	407	AV0173, AV0216, AV0452
	T → C	1251	F → L	418	AV0173, AV0216, AV0452

3.4 Phylogenetic analysis

Phylogenetic analyses on the concatenated gene segments of the aflatoxin gene cluster (*norb-hypA*) were performed with reference aflatoxin producers on those atoxigenic strains without large deletions in the cluster (Figure 13). The analysis showed diverse clade structure of the atoxigenic strains. Twelve of 14 atoxigenic isolates developed a highly bootstrap supported structure with well supported subgroups, whereas the phylogenetic analysis of the other genes showed little structure. Those groups were separated from the toxigenic isolates of Nigeria and from the USA. Nevertheless, two atoxigenic isolates, AV3108 and AV2216 were closely related to *A. flavus* toxin producing isolates from the US. The toxigenic isolates AV3228 and AV0106 which showed previously the lowest polymorphisms of the analyzed gene segments, shared a separated group with the toxin producing isolates AF70 and NRRL3357.

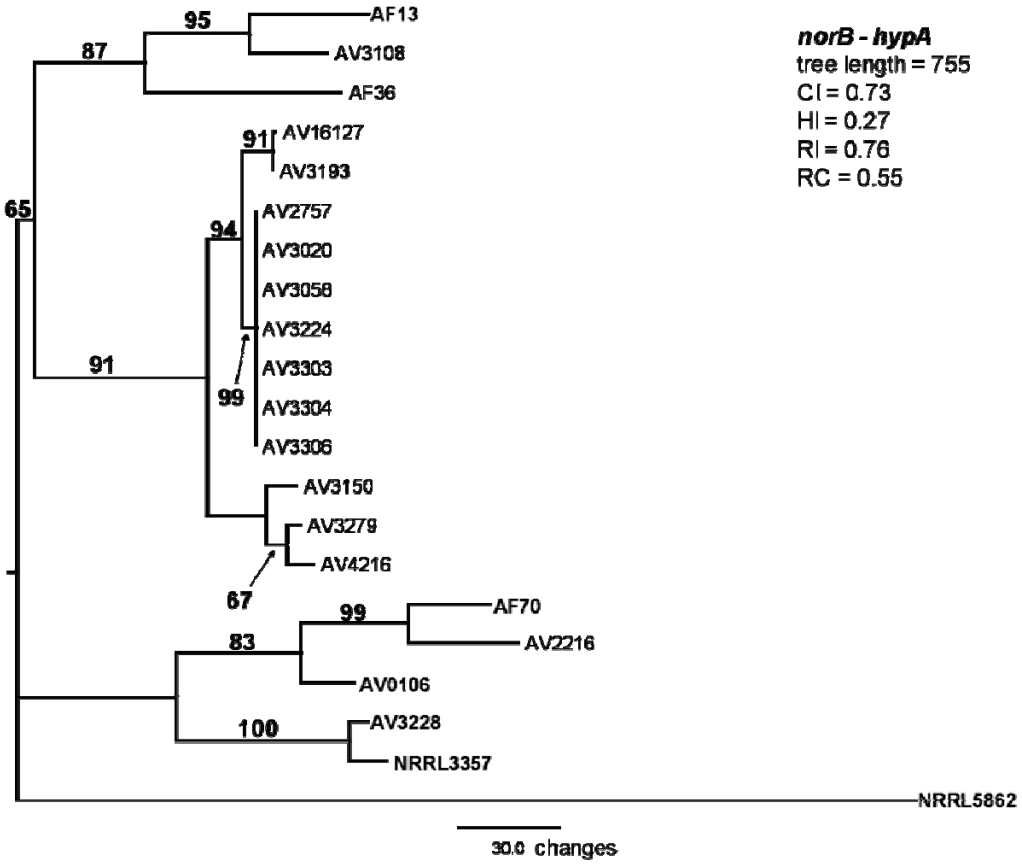


Figure 13 One of five most-parsimonious (MP) trees based on the combined aflatoxin biosynthesis gene dataset (*norB, aflT, hexA, aflR, aflJ, estA, ver1, avnA, avfA, omtB, omtA, vbs,* and *hypA*) with 7,825 total characters, 238 parsimony informative. Tree was rooted with *A. parasiticus* (NRRL5862). Bootstrap values based on 1000 replicates are shown above the line. Aflatoxins producers are bold. CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

The phylogenetic analysis from genes outside the cluster (*taka amylase-pecA-glcA*) showed a clear separation of five atoxigenic VCGs that contained large deletions in the aflatoxin gene cluster (Figure 14). Isolate AV0452 and AV0173, which show the same deletion pattern within the aflatoxin gene cluster, shared a well supported subgroup within the atoxigenic isolates. Similar groupings were also found by analyzing a combined sequencing data of gene segments from *avnA-hypA* (not shown). The majority of Nigerian atoxigenic isolates shared a clade separated from those atoxigenic and toxigenic isolates of the USA.

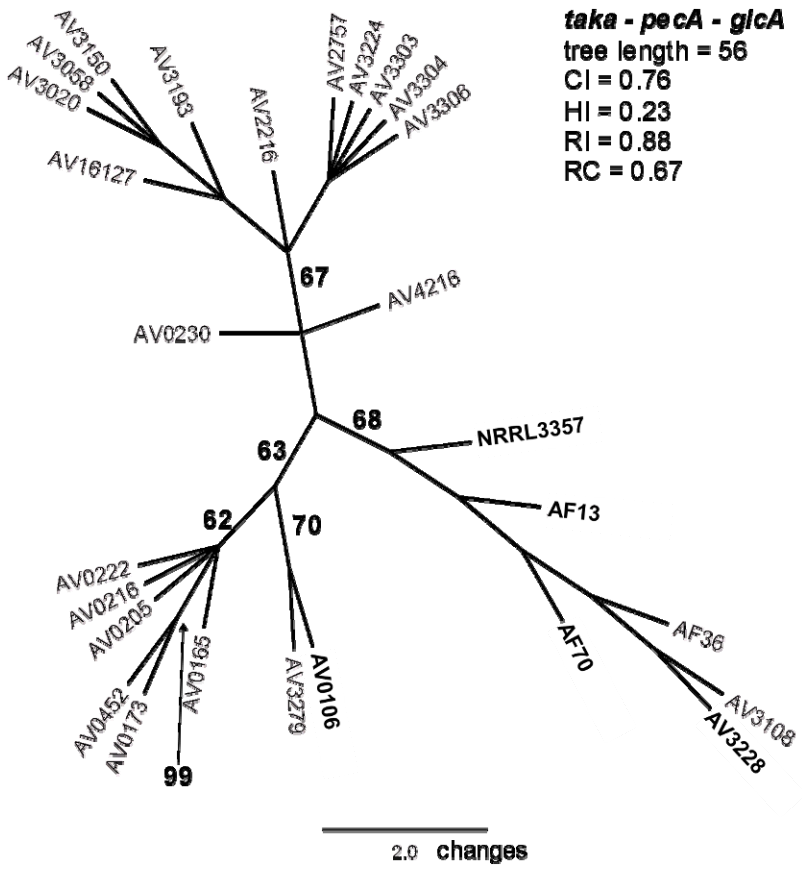


Figure 14 One of 86 most-parsimonious (MP) trees based on the combined gene dataset taka-amylase, *pecA*, and *glcA*. Of 2035 total characters, 24 were parsimony informative. Tree is unrooted. Bootstrap values based on 1,000 replicates are shown above the line. Aflatoxins producers are bold. CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

4 DISCUSSION

A. flavus isolates that do not produce aflatoxin have been found worldwide and are not rare (Cotty et al., 1994b). Some of them are used successfully to reduce aflatoxin contamination of susceptible crops in fields by competitively excluding native aflatoxin producing *A. flavus* strains (Antilla et al., 2002; Dorner, 2004). There is concern, however, in using *A. flavus* isolates that do not produce aflatoxin under laboratory conditions as a biological control agent, due to the fact that these isolates may be able to produce aflatoxin under certain conditions. Therefore, strong controls are necessary to prove the safety and stability of atoxigenic strains before their use as biological control agents. The atoxigenicity of *A. flavus* isolate AF36, a registered biopesticide, is the result of a single nucleotide polymorphism which inserts a stop codon near the beginning of the *pksA* coding sequence for the polyketide synthase (Ehrlich et al., 2004). In the current study, no single nucleotide polymorphism was found in the *pksA* sequences of the atoxigenic isolates of Nigeria. Nevertheless, different molecular lesions were observed in atoxigenic *A. flavus* isolates of various VCGs within the aflatoxin pathway genes.

In the present study, atoxigenic *A. flavus* isolates from Nigeria were found that did not generate any PCR products of several tested genes within the aflatoxin gene cluster. Deletions within the aflatoxin gene cluster were found previously in isolates from *A. flavus* and the closely related *A. oryzae* (Kusumoto et al., 2000; Chang et al., 2005). Kusumoto et al. (2000) classified strains of *A. oryzae* into 3 groups based on the deletion pattern. Strains belonging to group two and three contained large deletions, while group one had a nearly intact aflatoxin gene cluster. Lee et al. (2006) confirmed that *A. oryzae* isolates belonging to group 2 had deletions on the left side of the aflatoxin gene cluster extending to a chromosome breakpoint in the gene *ver-1*. The deletion type of group 2 is probably identical to that of isolate AV0230 of the current study. Interestingly, several deletion patterns observed by Chang et al. (2005) were found as well within Nigerian atoxigenic *A. flavus* VCGs. Nevertheless, in the current study new types of deletions were found. Isolates AV0205, AV0216, and AV0165 contained large lesions extending to *ordB* and *hypA*, at the distal of the aflatoxin gene cluster. No PCR products of four atoxigenic *A. flavus* isolates of the gene *cypX* were generated that could cause atoxigenicity. Wen et al. (2005) demonstrated that a *cypX*-deleted mutant of the *A. parasiticus* isolate NRRL2999 lost toxigenicity. The lesion of the

isolates AV0173 and AV0452 (Figure12) was similar to deletion pattern C identified by Chang et al. (2005). The phylogenetic analysis of the combined sequence data set of *taka-pecA-glcA* and other genes (not shown) revealed that those isolates shared one well supported subgroup within the isolates with large deletions patterns. The loss of the entire aflatoxin gene cluster of isolate AV0222 is comparable with the deletion pattern H by Chang et al. (2005). The putative absence of genes within the aflatoxin gene cluster offers a practical solution for selecting a completely safe atoxigenic strain for the use in a biological control management. Nevertheless, further studies are required to prove whether the strains have really lost genes in the cluster.

Interestingly, only atoxigenic isolates from the district Ogbomosho formed a unique group containing large deletions in the aflatoxin gene cluster, while isolates from other districts were only missing various genes. All the Ogbomosho strains belong to different VCGs and were found within a relatively small geographic area. Therefore, it could be concluded that these atoxigenic isolates all stem from within the district of Ogbomosho. The occurrence of VCGs with such large deletions patterns originating from only one district gives rise to the hypothesis that these isolates arose from a common ancestral source. However, it is not known which selective forces active in West Africa are responsible for such large deletion patterns. The combined phylogenetic tree *taka amylase–pecA-glcA* showed similar results of a well supported clade of those isolates adding strength to the above hypothesis.

In the present study, the deletions occurred mostly from the left side of the aflatoxin gene cluster toward the utilization sugar cluster. Chang et al. (2005) reported a similar incident. Genes controlling secondary metabolites are generally organized in clusters, many of which are species-specific (Nierman et al., 2005). As has been observed for mammals, nematodes and yeasts, repeats and subtelomeric sequences are associated with rearranged regions (Eichler et al., 2003; Galagan et al., 2005). The aflatoxin gene cluster of the toxigenic *A. flavus* isolate NRRL3357 genome is biased toward the telomere (Chang et al., 2007). Similarly, the left side of *A. oryzae* isolate RIB40 aflatoxin gene cluster and that of RIB62 is known to be close to the telomere (Lee et al., 2006). According to genome sequences of *A. oryzae* RIB40, the distance from the beginning of the aflatoxin gene cluster to the telomere is only 18kb. A comparison of genes within the biosynthesis gene cluster of main aflatoxin producing *Aspergillus* species showed that the right side of the aflatoxin gene cluster, toward the utilization sugar cluster is well conserved whereas the regions left

sided toward the telomere are highly variable (Ehrlich et al., 2005). Preliminary, genome analyses reveal large non-syntenous regions resulting from insertions or deletions in subtelomeric sequences, intra-moleculare recombination, and variation in the number of gene duplications (Nierman et al., 2005). For example, a deletion in the *cypA* gene is the reason why *A. flavus* is incapable of producing G aflatoxins. The *cypA* gene is located toward the telomeric region at the beginning of the aflatoxin gene cluster. Studies have suggested that the location of the aflatoxin gene cluster in the telomeric region of *A. nidulans, A. oryzae* and *A. flavus* would facilitate gene loss as well as recombination, DNA inversions, partial deletions, translocations and other genomic rearrangements (Kusumoto et al., 2000; Chang et al., 2005; Ehrlich et al., 2005; Wong et al., 2005; Carbone et al., 2007b). Thus, the large deletions found within the gene cluster are as a result of the instability of the subtelomeric region of the aflatoxin gene cluster.

Eleven atoxigenic VCGs were tested previously to evaluate *A. flavus* strains as potential biocontrol agents for maize in Nigeria (Atehnkeng et al., 2008a). Those isolates showed a significant impact in aflatoxin reduction in maize which ranged from 70.1% to 99.9%. AV3279 was the most effective atoxigenic isolate (>99.3%) followed by the isolates AV3303, AV0222, and AV4216 with an average aflatoxin reduction of greater than 92% (Atehnkeng et al., 2008a). In the present study 8 isolates of those previously tested VCGs had an intact aflatoxin biosynthesis gene cluster whereas isolate AV3058, AV2757, and AV16127 could not generate PCR products of gene *cypX* and the entire gene cluster was deleted for isolate AV0222. Only 9.5% of the entire coding sequence of the genes along the aflatoxin pathway was sequenced. A defective gene that resulted in atoxigenicity was not observed in isolates with intact aflatoxin gene clusters. Nevertheless, differences of aflatoxin pathway gene sequences were observed between atoxigenic and toxigenic strains. The phylogenetic analysis of gene segments within aflatoxin gene clusters and outside showed that the majority of atoxigenic isolates were well separated from those which produce aflatoxin. The comparison of polymorphism in aflatoxin coding gene segments of atoxigenic isolates to that of the toxin producing isolate NRRL3357 revealed that significantly more polymorphism occurred in the atoxigenic isolates. The percent polymorphism for gene ver1 averaged 4.0% for the atoxigenic strains while it was only 0.1% for toxigenic isolates. Interestingly, the majority of the nucleotide changes in portions of the genes *avnA* and *hypA* resulted in non-synonymous amino acid substitutions for most of the isolates. These observations suggest that there is a selection for non-synonymous substitutions in *avnA*, consistent with selective

pressure. Several isolates had polymorphisms that lead in predicted amino acid changes that may have influence on the aflatoxin productivity. Especially one amino acid change occurred in the *aflJ* gene in 9 atoxigenic isolates but not in the main toxigenic strains. Gene *aflJ* is known to be involved in regulation of the aflatoxin biosynthesis pathway (Meyers et al., 1998; Ehrlich et al., 1999; Chang et al., 2000). Similarly to the present study the majority of atoxigenic strains of *A. flavus* and *A. oryzae* had an intact aflatoxin gene clusters (Kusumoto et al., 2000; Chang et al., 2005; Lee et al., 2006). Atoxigenicity probably results from point mutations (Ehrlich et al., 2004) or small deletions in genes that regulate the function of the aflatoxin production (Calvo et al., 2004). Since aflatoxin production requires the normal function of the complete aflatoxin biosynthesis gene cluster, it is suspected that dysfunctional or abnormal genes in the aflatoxin gene cluster inhibit aflatoxin production. Nevertheless, the molecular mechanisms responsible for atoxigenicity in *A. flavus* appear to be diverse and are difficult to determine, especially for those isolates that seem to have an intact aflatoxin gene cluster. In conclusion, the mechanism of atoxigenicity on isolates with intact gene cluster is still not readily apparent, however, differences between toxigenic and atoxigenic isolates were observed. The sequence data of these strains is important for further studies to provide a safe biological control management. Further studies of the coding sequence in the aflatoxin pathway gene are required to find the gene defect which results in atoxigenicity.

The production of aflatoxins by the species *Aspergillus* involves approximately 25 genes within a complex biosynthetic pathway which requires a considerable expenditure of energy (Yu et al., 2004b). Ehrlich et. al. (2005) estimated that the aflatoxin gene cluster of *A. flavus* was maintained for at least 25 million years and that the loss of genes for production of G-aflatoxin must have occurred less than 17 million years ago. The high conservation of the cluster components needed for B-aflatoxins along certain *A. flavus* strains suggested important adaptative values for aflatoxins in character-shaping niches (Ehrlich et al., 2005). Around half of the *A. flavus* L-strains do not produce aflatoxin in Nigeria (Atehnkeng et al., 2008b; Donner et al., 2008). In contrast, *A. parasiticus*, *A. nomius,* and the unnamed taxon of West Africa are usually highly toxigenic (Kurtzman et al., 1987; Ehrlich et al., 2003; Donner et al., 2008). All these high toxin producing species within the section Flavi are phylogenetically different from the *A. flavus* isolates (Geiser et al., 2000a; Ehrlich et al., 2005; Ehrlich et al., 2007b). The function of aflatoxins is still under debate and much speculation about its role has been published. Some hypotheses are that aflatoxins may protect against microbes in the soil or competing insects (Matsumura et al., 1967; Drummond

et al., 1990; Dowd, 1992), that they facilitate growth of the fungus on carbon rich sources by the removal of excess acetate (Bu'lock, 1965) or that they promote conidial and sclerotial development (Cotty, 1988; Calvo et al., 2001; Chang et al., 2002; Wilkinson et al., 2004). Aflatoxins are to a large degree not phytotoxic (McLean et al., 1995; Hasan, 2001) and are not suspected to be involved in plant virulence any more than atoxigenic strains (Cotty, 1989). The ability of the fungus to produce aflatoxins is not essential to growth under certain conditions and is not required for successful competition with other aflatoxin-producing strains (Horn et al., 2000; Bhatnagar et al., 2003). It also has been argued that if aflatoxins are advantageous to the fungus, then aflatoxin producers should eventually replace atoxigenics over time (Carter et al., 2002). Nevertheless, in nature, atoxigenic and toxigenic *A. flavus* successfully coexist and atoxigenic *A. flavus* strains are equally capable of infecting susceptible crops (Cotty, 1989; Cotty, 1997). The aflatoxin-producing ability appears unnecessary when observing *A. flavus* success in occupying certain plant-associated niches (Cotty, 1989; Cotty, 1997). Ehrlich et al. (2005) suggested that over the last several million years the average adaptive value of aflatoxins may have decreased by the movement into new niches or loss of formative niches in the current environment. Therefore, since aflatoxins do not seem to be advantageous to *A. flavus* and if anything use unnecessary energy, the loss of this aflatoxin producing ability should only serve to make the fungus more energy efficient.

In the present study, isolates belonging to different VCGs had identical deletion patterns and were closely related. Chang et al. (2005) reported that isolates belonging to the same VCG also had identical deletion patterns. No genetic exchange was found among *A. flavus* atoxigenic VCG isolates and toxin-producing isolates collected from six geographically separated regions, suggesting that recombination among VCGs is rare (Ehrlich et al. 2007). Therefore, in the present study, isolates of the same VCG most likely have the same identical deletions patterns.

Sequencing of the aflatoxins biosynthesis gene cluster has shown that for many isolates belonging to different VCGs, genes could not be amplified and, therefore, are likely to be missing or highly abnormal. However, most of the atoxigenics had all the examined biosynthesis genes. Therefore, further studies are required to prove whether the strains with the occurrences of deletions have a total loss of function in aflatoxin production and which defect causes atoxigenicity in strains with intact aflatoxin gene clusters. This study shows that many atoxigenic *A. flavus* isolates of the district Ogbomosho are good candidates for a safe biological control management in Nigeria.

CHAPTER FIVE

CHARACTERIZATION OF AFLATOXIN-PRODUCING AND NON-PRODUCING STRAINS OF *ASPERGILLUS* SECTION FLAVI IN NIGERIA

1 INTRODUCTION

In agricultural fields of West Africa, *Aspergillus flavus* is the main producer of carcinogenic aflatoxins (Atehnkeng et al., 2008b; Donner et al., 2008). In addition to *A. flavus,* other aflatoxin-producing species like *A. parasiticus* and the unnamed taxon S_{BG} were also reported to be present, but in much lower amounts (Cardwell et al., 2002; Atehnkeng et al., 2008b). The usual aflatoxin regulatory limits that exist in most developed countries are also present in Nigeria but the necessitated regulatory controls are largely unenforceable or difficult to carry out (Bandyopadhyay, 2007). Thus, aflatoxin exposure in humans is high in many regions of West Africa and mainly associated with contaminated maize (Gong et al., 2002; Bandyopadhyay, 2007). Therefore, interest in the diversity within the *Aspergillus* sections Flavi has increased because of recent suggestions that atoxigenic strains might be applied to agricultural fields in Nigeria in order to decrease the aflatoxin contamination in maize (Atehnkeng et al., 2008a; Hell et al., 2008).

Variation among the isolates within the *Aspergillus* section Flavi is evident in genetic, physiological, and morphological character (Cotty et al., 1994b). Populations within the *Aspergillus* section Flavi tend to be extremely diverse in terms of vegetative compatibility groups (Cotty et al., 1994b). Morphological and physiological divergence within *A. flavus* can vary, for instance, *A. flavus* was divided into "S" and "L" types based on the size of its sclerotia (Cotty, 1989). S-type *A. flavus* isolates produce numerous small sclerotia and fewer conidia than other *A. flavus* isolates and were originally described as *A. flavus parvisclerotigenus* (Saito et al., 1986; Cotty, 1989; Saito et al., 1993), whereas the "typical" *A. flavus* isolates, the so called L-strain, produce larger and fewer sclerotia (Saito et al., 1986; Cotty, 1989). Physiological characters vary as well, as in the case of the aflatoxin-producing L-strain isolates of *A. flavus* which produce anywhere from no detectable aflatoxin to great levels of only B-aflatoxin (Cotty et al., 1994b). In

contrast, the S-strain isolates produce high levels of aflatoxin and S-strain atoxigenic isolates are rarely found in natural environments. In addition, Geiser et. al. (1998, 2000) subdivided *A. flavus* into group I and II based on RFLPs of nuclear-coding genes and DNA sequences. Group I contains both L and S strains that produce aflatoxins B_1 and B_2, whereas Group II comprises only S-strains that often produce B- and G- aflatoxins and represents, at least in part, an unnamed taxon. These B- and G-aflatoxin producers are present in Australia, Thailand, Argentina, and West Africa (Saito et al., 1993; Cotty et al., 1999; Geiser et al., 2000a; Vaamonde et al., 2003). None have been reported from the United states (Cotty et al., 1999; Horn et al., 1999). In West Africa even though only the *A. flavus* strains resembling the S-type occur, they have the ability to produce great levels of both B and G aflatoxins (Cotty et al., 1999; Donner et al., 2008). In a recent study, Pildain et al. (2008) designated those B+G aflatoxin producers which produce typical small sclerotia and were found commonly in peanut plant and fields as *A. minisclerotigenes*. Some of those isolates have been described as *A. flavus* group II by Geiser et al. (1998, 2000). Nevertheless, it is not clear if the B+G producers resembling the S-strain isolated from Nigerian maize fields belong to this new taxon. The diversity of species within the *Aspergillus* sections *Flavi* appears to make it more difficult to identify species with only conventional morphological and physiological methods. Therefore, in the present study sequence data of three genes was used to determine phylogenetic relationships among *Aspergillus* section Flavi isolates that originate from Nigeria.

Aspergillus species like *A. flavus* and *A. parasiticus* are strictly mitotic and reproduce asexually (Geiser et al., 1996). These fungi mostly exist in the form of asexual spores called conidia, which are dispersed by wind, water and insects. Several generations of fungi can be produced in a single growing season (Chang et al., 2004). Under certain conditions, some isolates of *A. flavus* and *A. parasiticus* produce a specialized structure of mycelia, called sclerotia (Bennett et al., 1979; Wicklow et al., 1983). Sclerotia are pigmented, compacted aggregate hyphae, which are capable of resisting unfavorable environmental conditions and remaining dormant long periods of time (Cotty, 1988; Wicklow et al., 1993; Rollins et al., 1998). Isolates of *A. flavus* and *A. parasiticus* that produce aflatoxins do not always produce sclerotia (Bennett et al., 1979; Wang et al., 1993). Nevertheless, a positive correlation between aflatoxin biosynthesis and conidial production in *A. parasiticus* and an inverse relationship between aflatoxin biosynthesis and

sclerotial production has been observed (Guzman de Pena et al., 1997). No such relationship has been observed for *A. flavus* L-strain isolates.

In this study, the relationship of different soil populations of *A. flavus* L-strains according to aflatoxin biosynthesis along with sclerotia and condial production was examined. These relationships combined with the phylogenetic analysis of the main aflatoxin-producing species and atoxigenic isolates help support our understanding of the biology of the *Aspergillus* section Flavi strains resident in Nigeria.

2 MATERIAL AND METHODS

Soils samples were collected from maize fields in 11 districts across three agroecological zones within Nigeria. In each district samples were taken from five different maize fields. Fungal isolates belonging to *Aspergillus* section Flavi were isolated by dilution plate technique on Modified Rose Bengal Agar (MRBA, Cotty, 1994a). These isolates were screened for aflatoxin producing ability in liquid fermentation as previously described (Cotty, 1997; Atehnkeng et al., 2008b; Donner et al., 2008). Isolates were classified into different known species based on their production of only B-aflatoxins, or both B- and G-aflatoxins, and on their characteristic growth patterns, such as colony and sclerotial morphology, on various media Czapek-Dox Agar (BD Diagnostics, Sparks, MD), 5/2 agar (5% V8 juice and 2% agar, pH 5.2), and AFPA agar (*A. flavus* and *parasiticus* agar,Pitt et al., 1983) (Cotty, 1994a). Isolates which produced only B-aflatoxins or no aflatoxin had sclerotia on average over 400µm in diameter and were classified as L-type *A. flavus* isolates. Isolates which produced abundant small sclerotia similar to the S-type *A. flavus* but produced both B- and G-aflatoxins were initially classified as unnamed taxon S_{BG} (Cotty et al., 1999). Isolates that produced B- and G-aflatoxins, dark green colonies, rough conidia, and reacted on AFPA agar plates similar to typical *A. parasiticus* were assumed to be *A. parasiticus*.

2.1 Culture collections used in this study

In this study a set of 54 highly toxigenic and 56 atoxigenic *A. flavus* L-strain isolates were used which were taken from the soil of 56 Nigerian maize fields located in three agroecological zones (Donner et al., 2008). For comparison, isolates from various countries other than Nigeria were also used. The atoxigenic *A. flavus* L-strain isolate AF36, the toxigenic isolates AF13 as well as the S-strain isolate AF70 and AF12 from the USA were analyzed (Cotty, 1989). *A. parasiticus* isolate CP-461, which does not produce aflatoxin but the penultimate precursor, O-methylsterigmatocystin (Dorner et al., 1984; Bhatnagar et al., 1987) from the USA was also used. Additionally, the aflatoxin producing *A. parasiticus* isolate NRRL 2999, a wild-type isolate from Uganda (Rambo et al., 1974) and the toxigenic *A. parasiticus* isolate BN009E (Cotty et al., 1999) from Benin were analyzed. From Nigeria, 8 *A. parasiticus* isolates were used along with 18

isolates of unnamed taxon S_{BG} isolated from maize fields, one unnamed taxon isolate A-11612 (Hesseltine et al., 1970), and one *A. minisclerotigenes* NRRL A-11611 isolate (Hesseltine et al., 1970; Pildain et al., 2008). Isolate BN008R, BN040B, and BN038G of the unnamed taxon S_{BG} were also included from Benin (Cotty et al., 1999). *A. nomius* NRRL 13137 (Kurtzman et al., 1987) was used as an outgroup.

2.2 Sequencing and phylogenetic analysis

Centrifuge tubes with 10 ml liquid Czapeks medium were inoculated with spores which were incubated at 32°C for 48-72 h. After centrifuging at 10,000g for 15 min (Sorvall RC 5 B Plus, Newtown, CT, USA) mycelia masses were separated on Miracloth (Calbiochem, Corp. La Jolla, California). DNA was isolated with FastDNA SPIN Kit according to the manufacturer`s instruction (Qbiogene, Irvine, CA). DNA concentration was measured with a spectrophotometer (model ND-1000, NanoDrop).

Portions of the aflatoxin regulatory gene aflR, the polygalacturonase encoding gene pecA and the taka-amylase gene (*taka*) were amplified by using a HotMaster PCR kit (Eppendorf, Westbury, NY). The primer sequences used were as follows 5`-3`: *aflR*1: GGAA-ACAAGTCTTTTCTGG, *aflR*2: CAGAGCGTGTGGTGGTTGAT *pecA*1: GCTTAGC-CTAGACTCAAG, *pecA*2: AAGAGGAGTCCAGCTTGTG; *taka*1: TATCCAGGGAATGGGCTT, *taka*2: TTAGAGGTCGTCCATGCTGCC. The 50 µl reaction mixture included 3 µl DNA (5 ng/µl), 5 µl of 10× PCR buffer, 1 µl of 10 mM dNTPs, 1.2 µl (10 ng/µl) of each primer, 0.3 µl Taq polymerase, and 38.3 µl deionized water. The PCR reactions were preformed with a DNA thermo cycler (MyCycler, Bio-Rad Laboratories) using the following conditions: 5 min at 95° C followed by 38 cycles at 95° C for 30 sec, 55° C for 20 sec, 72° C for 30 sec. A final extension was conducted for 10 min at 72° C. The annealing temperature was optimized for each primer set with the result that the annealing temperature varied from 49-59°C. Amplicons were visualized with SYBR Gold after 1.2% agarose gel electrophoresis. Sequencing was preformed by The Genomic Analysis and Technology Core Facility (GATC) at the University of Arizona, Tucson.

DNA manipulations and alignments were made using DNAMAN (Lynnon Biosoft Vandreuil, Quebec, Canada). Phylogenetic analyses were preformed using PAUP* Version 4.0b10 for Microsoft Windows (Swofford, 2002) for parsimony and bootstrap analysis. Bootstrap values were generated by 1,000 replications of the bootstrap procedure. Gaps were treated as missing data and thus were excluded from the analysis. *A. nomius* NRRL 13137 was used as an outgroup.

2.3 Conidia and sclerotia production

The production of conidial and sclerotial was measured for toxigenic and atoxigenic *A. flavus* L-strain isolates originating from Nigeria. One atoxigenic and one toxigenic isolate were chosen from each of 55 locations. Nine-centimeter Petri dishes with 20 ml of Czapeks medium were inoculated in the center with 15 µl of distilled water containing about 1,000 spores. Cultures were grown unilluminated at 32°C.

After 25 days, the plates were washed three times with 100% ethanol, and then distilled water was added to the suspension up to 100 ml. Conidial concentrations were measured in 20 ml sample tubes using a turbidity meter (Orbeco-Hellige Digital Direct-Reading Tubidimeter, Orbeco Analysis Systems Inc., New York, USA). A linear nephelometric turbidity unit (NTU) vs colony forming unit (CFU) standard curve was developed to relate turbidity to conidial concentration. The spore concentration was extrapolated from the NTU /CFU standard curve.

The sclerotia were collected after having been rubbed and washed off the plate with water. After vacuum filtration on preweighed filter paper (Whatsman No. 4), the sclerotia were dried at 42°C for 48 hours, and weighed.

2.4 Data analysis

Pearson correlations coefficients were generated with SAS (version 9.1.3, SAS Institute Inc., Cary, NC) to assess relationships of sclerotia weight, conidial production in NTU, and aflatoxin-producing ability in liquid fermentation of Nigerian *A. flavus* L-strains. Linear regression analysis and nonlinear regression analysis were preformed with SigmaPlot 10 (Systat Software Inc.). For these analyses, the isolates were grouped based on their ability to produce spores (NTU) in 8 groups. The size of groups averaged 14 isolates (ranged= 8-19 isolates).

3 RESULTS

3.1 Phylogenetic analysis

The phylogenetic analysis was performed with three portions of the genes *aflR*, *pecA*, and *taka-amylase* as well as a combined dataset of all three genes. This analysis showed that the B+G producing *A. minisclerotigenes* isolate A11611 was placed bootstrap supported between the *A. flavus*, *A. parasiticus* and the unnamed taxon S_{BG} isolates. In all genes, the Nigerian S_{BG} isolates, including the *A. flavus* isolate A11612 and the S_{BG} isolates of Benin (Cotty et al., 1999) were clustered in one well bootstrap supported clade (Figure 15, 16, and 17). Within the *A. flavus* isolates, a separation of toxigenic and atoxigenic isolates appeared. These separations were found in the analysis performed with the sequences of *aflR* and *pecA* (Figure15 and 17). Nevertheless, these separations were rarely supported by high bootstrap values and the combined dataset showed a diverse structure (Figure 18). Interestingly, Nigerian isolates belonging to *A. parasiticus* were found to occur in a distinct phylogenetic group, whereas, the *A. parasiticus* isolate of the United States, Uganda and the one isolate of Benin were placed strongly bootstrap supported separately from those of Nigeria. The *A. parasiticus* isolate 2999 of Uganda lay between the isolates of Nigeria, Benin, and the one of the United States in only one of the three genes, the *aflR*.

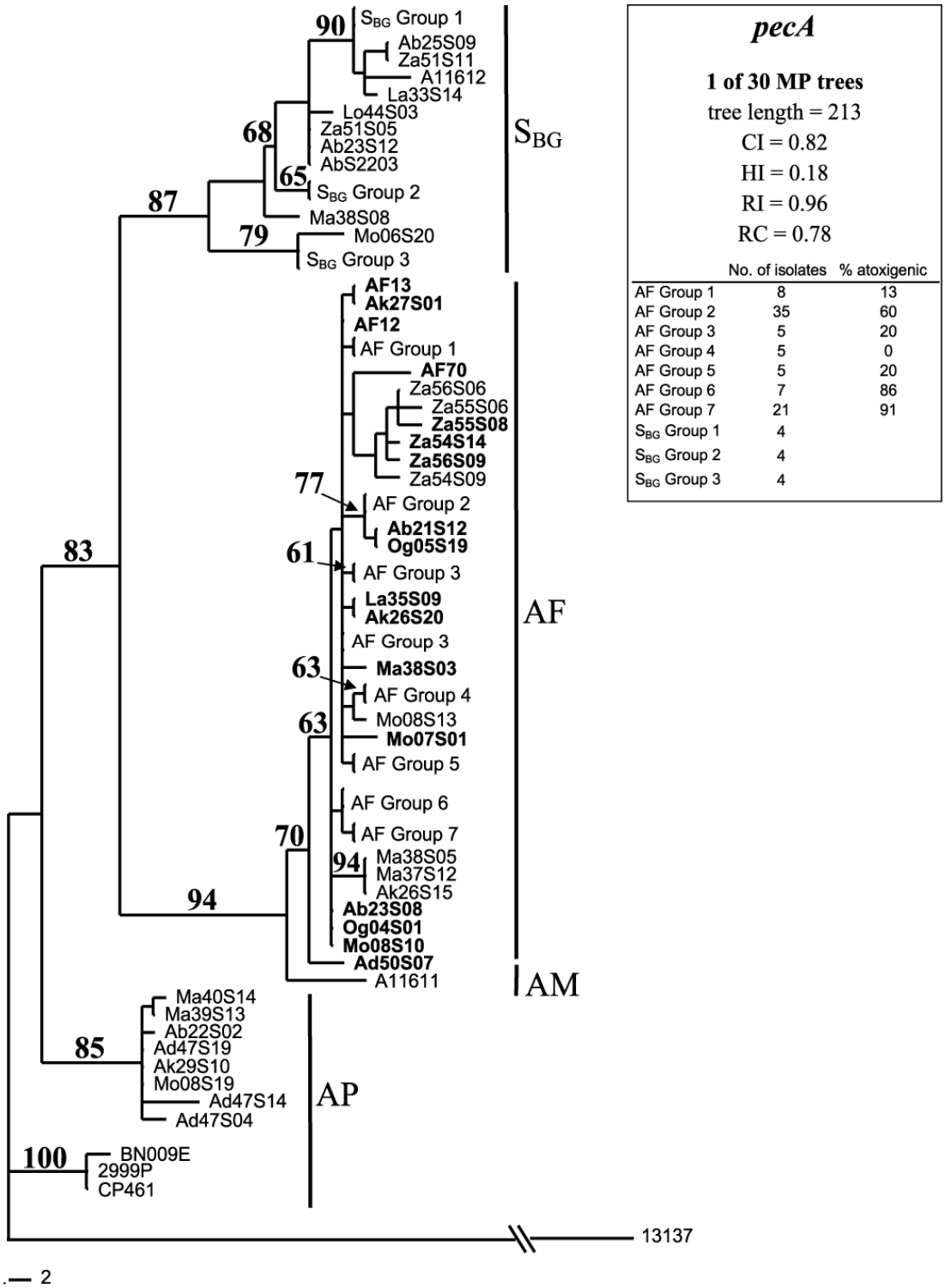


Figure 15 One of the most-parsimonious (MP) trees based on *pecA* gene sequence. Of 674 total characters, 64 were parsimony informative. Tree was rooted with *Aspergillus. nomius* (A13137). Bootstrap values based on 1,000 replicates are shown above the line. Toxigenic *A. flavus* isolates were written in bold. S_{BG}, unnamed taxon; AF, *A. flavus*; AM, *A. minisclerotigenes*; AP, *A. parasiticus*; CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index

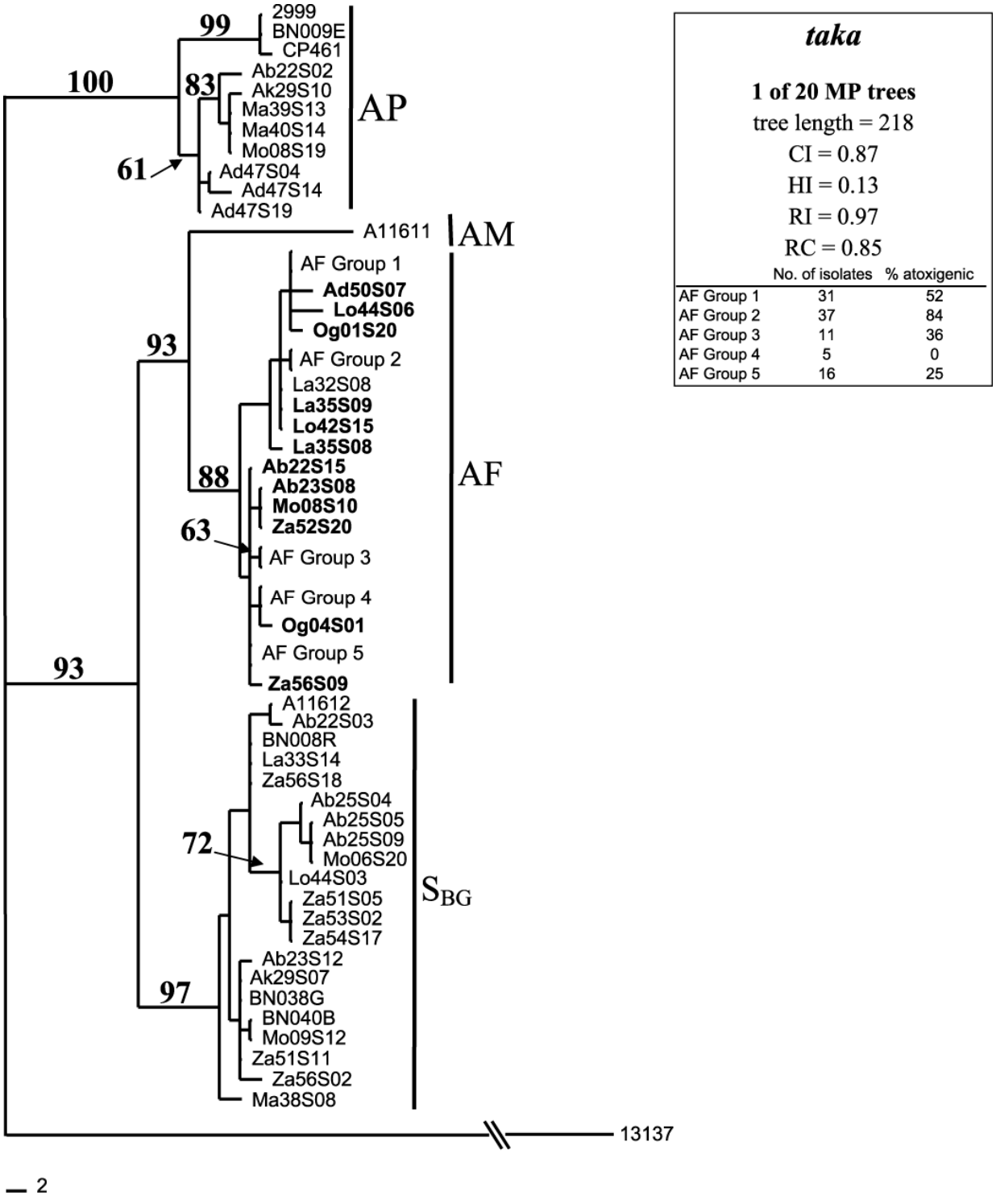


Figure 16 One of the most-parsimonious (MP) trees based on *taka-amylase* gene sequence. Of 811 total characters, 78 were parsimony informative. Tree was rooted with *Aspergillus. nomius* (A13137). Bootstrap values based on 1,000 replicates are shown above the line. Toxigenic *A. flavus* isolates were written in bold. S_{BG}, unnamed taxon; AF, *A. flavus*; AM, *A. minisclerotigenes*; AP, *A. parasiticus*;CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

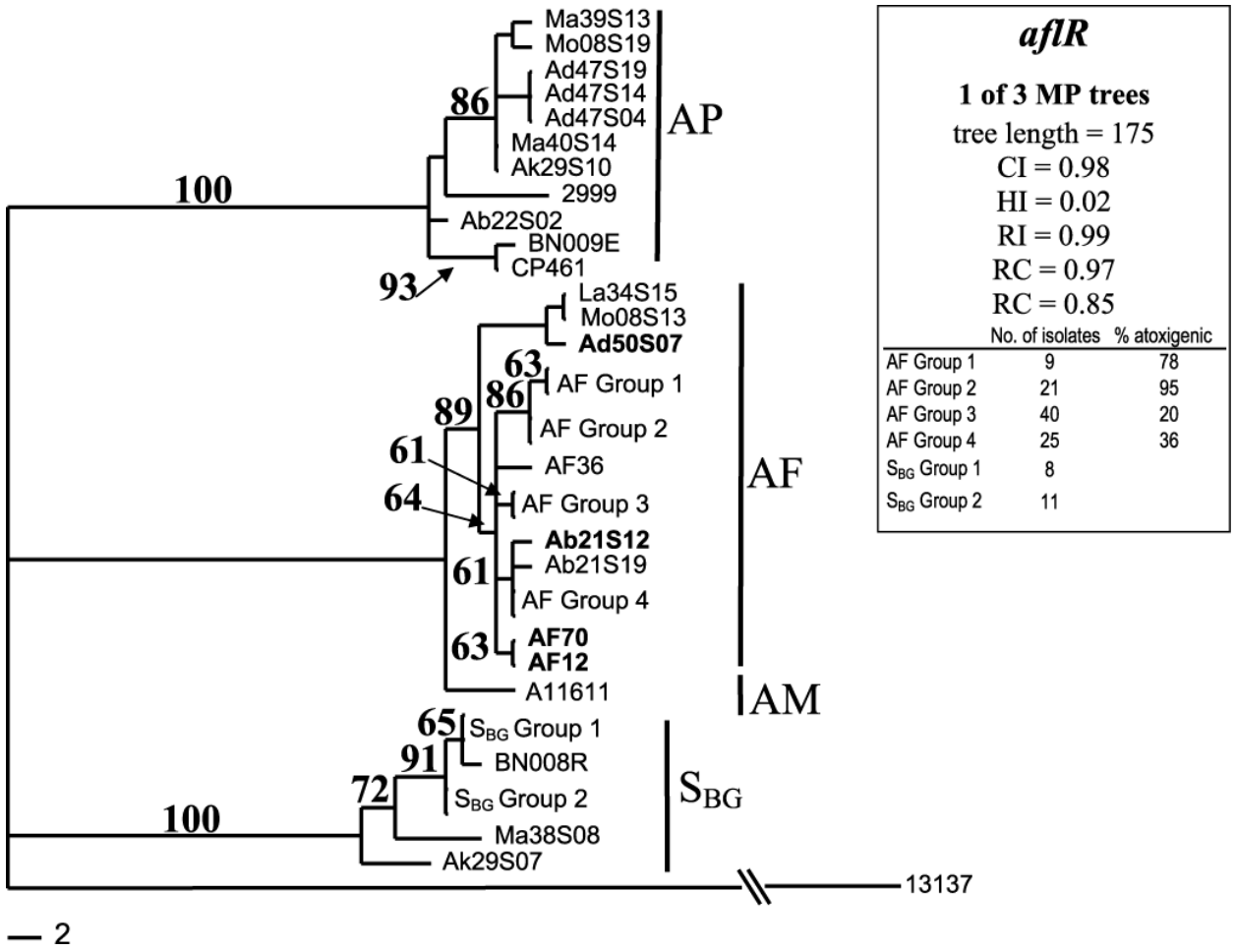


Figure 17. One of the most-parsimonious (MP) trees based on *aflR* gene sequence. Of 685 total characters, 69 were parsimony informative. Tree was rooted with *Aspergillus nomius* (A13137). Bootstrap values based on 1,000 replicates are shown above the line. Toxigenic *A. flavus* isolates were written in bold. S_{BG}, unnamed taxon; AF, *A. flavus*; AM, *A. minisclerotigenes*; AP, *A. parasiticus*;CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

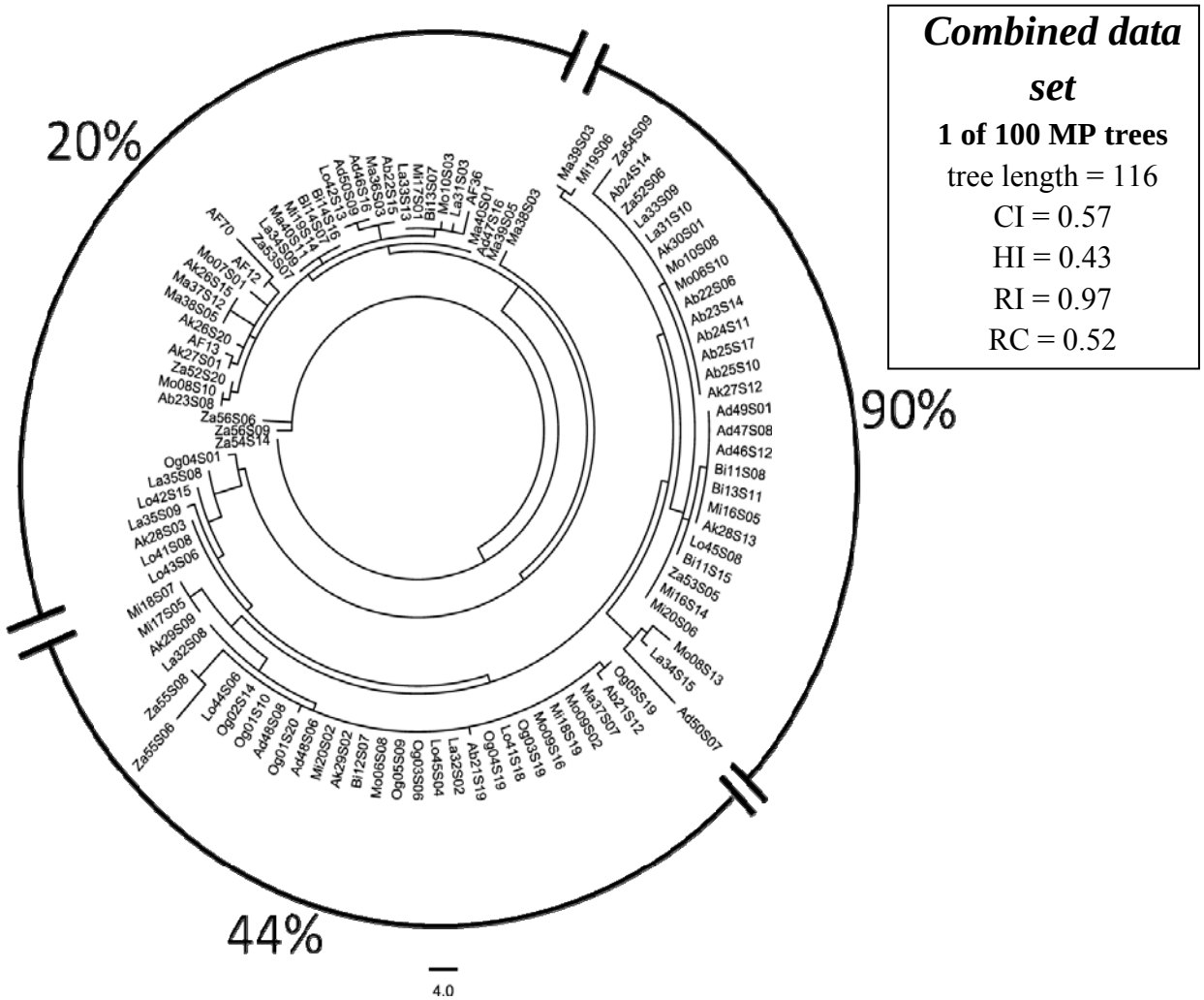


Figure 18 One of 100 most-parsimonious (MP) trees based on the combined dataset *taka-pecA-aflR* and calculated only with *Aspergillus flavus* isolates. Of 2,137 total characters, 44 were parsimony informative. Tree is unrooted. The outer ring divided the isolates in three groups the isolates. The proportion of atoxigenic *A. flavus* isolates in each group is shown in percent. CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

3.2 Conidial, sclerotial and aflatoxin production

The toxigenic *A. flavus* L-strain isolates produced only B-aflatoxins and averaged 20.62 * 10^4 ng g^{-1} total aflatoxins (ranged = 34.86 ng g^{-1} to 245.62 * 10^4 ng g^{-1}). Isolates of the Nigerian unnamed taxon strain S_{BG} averaged 1.56 * 10^6 ng g^{-1} aflatoxins B_1 (ranged = 1.69 * 10^3 ng g^{-1} to 6.07 * 10^6 ng g^{-1}) and averaged 1.65 * 10^6 ng g^{-1} aflatoxins G_1 (ranged = 1.10 * 10^3 ng g^{-1} to 5.08 * 10^6 ng g^{-1}). All isolates of both S_{BG} and *A. parasiticus* produced both B- and G-aflatoxins. Isolates

of *A. parasiticus* averaged 1.18 * 10^6 ng g^{-1} aflatoxin B_1 (ranged = 9.04 * 10^4 ng g^{-1} to 2.72 * 10^6 ng g^{-1}) and averaged 1.40 * 10^6 ng g^{-1} aflatoxin G_1 (ranged = 9.94 * 10^4 ng g^{-1} to 4.97 * 10^6 ng g^{-1}).

According to the Pearson's correlation analyses, there were significant negative correlations between both the production of conidia mass and the mass of sclerotia (r = -0.58, P < 0.0001) and aflatoxin-producing ability in liquid fermentation (r = -0.27, P = 0.0040) (Table 9). However, the weight of sclerotia was significant positive correlated with the aflatoxin-producing ability (r = 0.28, P = 0.0027).

Table 9 Pearson`s correlation coefficients of relationships among the quantity NTU of conidia, sclerotia weight, and of aflatoxin-producing ability of *Aspergillus flavus* L-strain isolates[a] N=110

	NTU	**sclerotia**	**aflatoxin**
NTU	1.00	-0.58 *<0.0001*	-0.27 *0.0040*
sclerotia	-0.58 *<0.0001*	1.00	0.28 *0.0027*
aflatoxin	-0.27 *0.0040*	0.28 *0.0027*	1.00

[a]Normal font represents coefficient of correlation and italics represents probability of significance

A. flavus L-strain isolates with an increasing ability to produce conidia on a Czapek's media, showed decreasing ability to produce aflatoxin in liquid fermentations (Figure 19). Inversely, isolates with an increasing ability to produce sclerotia, showed an increasing production of aflatoxin (Figure 20). *A. flavus* L-strain isolates with a decreasing ability to produce sclerotia, showed a proportional increasing production of conidia on the Czapeks plates (Figure 21).

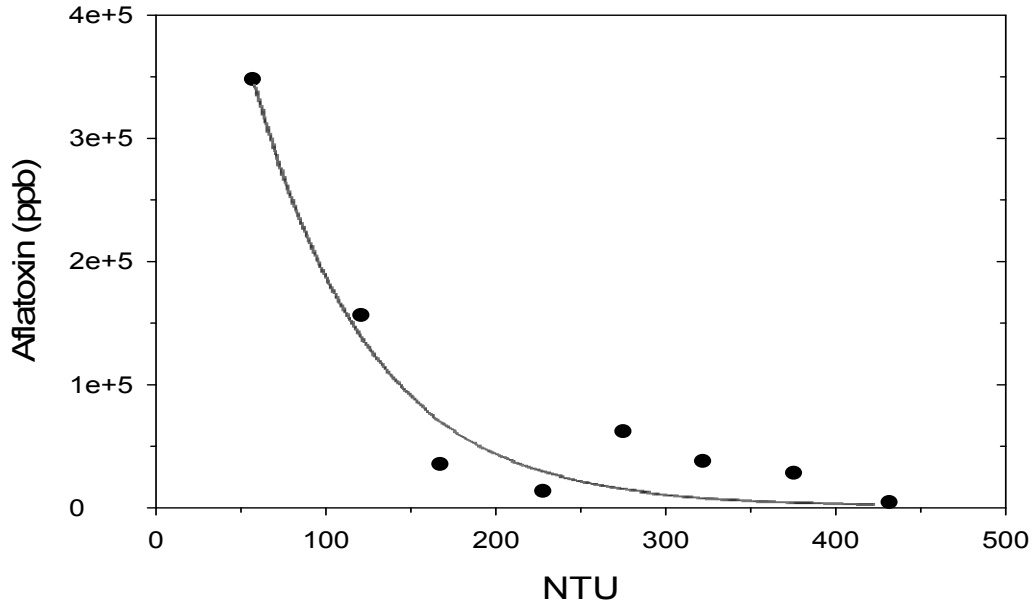
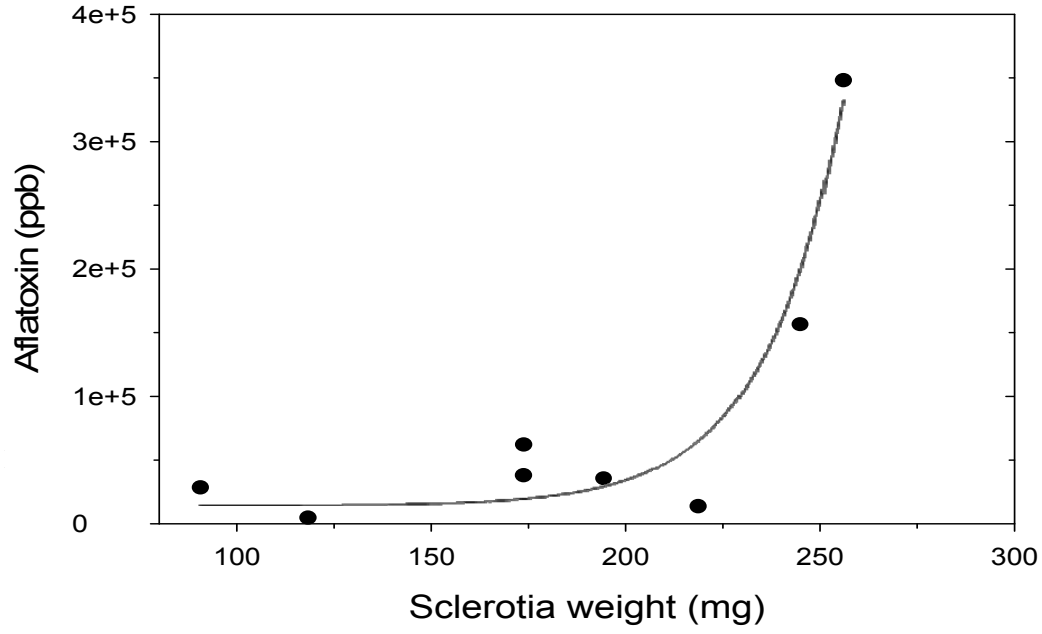


Figure 19 Aflatoxin concentration (ppb) in liquid fermentation vs. conidial production in NTU after 25 days on Czapeks medium. $Y = 784{,}192 * EXP^{(-0.0142 * X)}$; $R^2 = 0.942$



ays on Czapeks. Y =

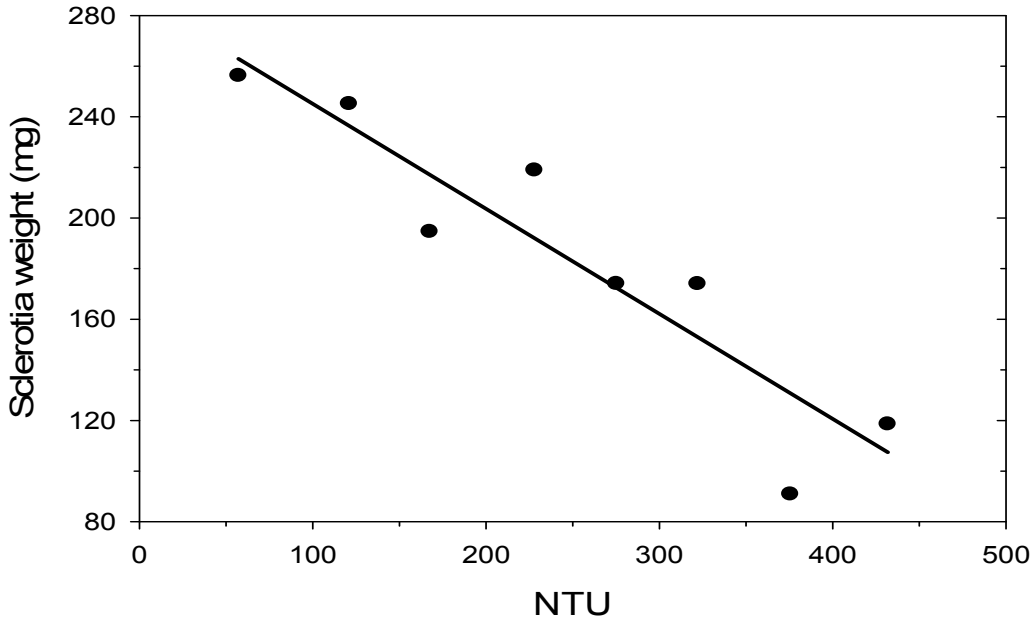


Figure 21 Sclerotia weight (mg) after 25 days on Czapeks medium vs. conidial production in NTU. The regression is linear. $Y = 286.6 - 0.415 X$; $R^2 = 0.85$

4 DISCUSSION

The most common aflatoxin-producing species, *Aspergillus flavus*, can be divided into two strains based on its morphological and physiological characters. The S-type produces numerous microsclerotia and high levels of aflatoxins, whereas the L-type produces fewer, large sclerotia and on average little to no aflatoxin (Saito et al., 1986; Cotty, 1989). The *A. flavus* S-strain, termed S_B, has been commonly found in North America and recently in East Africa (Cotty, 1989; Cotty, 1997; Jaime-Garcia et al., 2006; Probst et al., 2007). These isolates produce only B-aflatoxins. Molecular phylogenetics suggests that S_B isolates are closely related to the *A. flavus* type culture and other L strain isolates (Egel et al., 1994). Nevertheless, other strains morphologically similar to the S_B but with the ability to produce B- and G-aflatoxins, termed S_{BG}, were found in Argentina, Thailand, Australia, and West Africa. Recent studies revealed that the B- and G-aflatoxin-producing isolates in Thailand belong to *A. nomius* (Ehrlich et al., 2007b) and that most Group II isolates were classified as a new taxon, *A. minisclerotigenes* by Pildain et al. (2008). The delineation of S_{BG} type from West Africa has lead to some controversy and confusion for almost four decades. The current work contributes to the gradual development of an understanding of S_{BG} type isolates originating from West Africa.

In the current work, as reported in previous studies, only S_{BG} isolates were found. Neither S_B nor *A. minisclerotigenes* isolates were found in West Africa (Cotty et al., 1999; Atehnkeng et al., 2008b; Donner et al., 2008). The S_{BG} type was first reported by Hesseltine et. al. (1970), who combined three isolates into one new unnamed taxon. One isolate from North America produced only B-aflatoxin and was clearly assigned to S_B *A. flavus*. The other two isolates, A-11611 and A-11612, from Nigeria produced B- and G-aflatoxins and were later known as strain S_{BG} (Egel et al., 1994; Cotty et al., 1999). However, Saito et al. (1986) considered both the S_B and S_{BG} to be "atypical" variants of *A. flavus*, and applied the name *A. flavus* var. *parvisclerotigenus* (Saito et al., 1993). In the current study, the West African S_{BG} isolates were placed phylogenetically between *A. flavus* and *A. parasiticus* in agreement with previous studies (Egel et al., 1994; Ehrlich et al., 2003; Ehrlich et al., 2005; Ehrlich et al., 2007b). Therefore, it was suggested that the S_{BG} were ancestral to both *A. flavus* and *A. parasiticus*. In a recent study, Pildain et al. (2008) assigned *A. flavus*

Group II and S_{BG} isolates originating from Australia, Argentina, Texas, and Nigeria as the new taxon *A. minisclerotigenes*. Nevertheless, the S_{BG} strains found in West African maize fields were neither related to *A. minisclerotigenes* nor to Group II by Geiser et al. (2000). One of the *A. minisclerotigenes* isolates, A-11611, interfered clearly in all three gene regions with the S_{BG}s from Nigeria. *A. minisclerotigenes* was in all tested gene segments closely related to the *A. flavus* isolates, whereas the S_{BG} isolates showed a clear distance from *A. flavus* and *A. parasiticus*. In this study, another strain, A-11612, analyzed by Hesseltine et al. (1970) and Egel et al. (1994) was found strongly included within the West African S_{BG} isolates, using all three gene regions tested. The phylogenetic analysis by Egel et al. (1994) of A-11611 and A-11612 indicated that these isolates were in two distinct phylogenetic groups, which is in agreement with the present study.

Interestingly, most *A. minisclerotigenes* isolates and those belonging to Group II were found in peanuts and soil, while the West African S_{BG} isolates were commonly isolated from maize fields (Cardwell et al., 2002; Atehnkeng et al., 2008a; Donner et al., 2008; Pildain et al., 2008). Until now, neither *A. minisclerotigenes* isolates nor other S-type isolates were found in soil or from maize in West Africa. Adaptations of species within section Flavi is evident within diverse animal and plant-associated niches in geographically isolated environments (Cotty, 1989; Cotty et al., 1994a). The unique *Aspergillus* FP-1 found in Japan and Texas is associated with fields cultivated with sugarcane (Kumeda et al., 2003; Garber et al., 2006). Therefore, the special type of S-strain occurring in West Africa is probably well adapted to maize and its geographical environment. Nevertheless, it is not known which selective forces active in West Africa are responsible for these highly aflatoxin producing S_{BG} strains in maize.

It is still unclear why the unnamed taxon produces both aflatoxins. The most recent common ancestor to section *Flavi* most likely produced higher levels of G_1 aflatoxin relative to B_1 or O-methylsterigmatocystin (OMST) (Carbone et al., 2007a). Since no species is known to produce only G-aflatoxins, Carbone et. al. (2007) suggested that the most recent common ancestor of section Flavi was a B-and G-aflatoxin-producer and that the selection has been acting on the G_1/B_1 ratio. Isolates of the unnamed taxon and *A. parasiticus* produced on the average higher ratios of G_1 aflatoxins relative to B_1. These observations seem consistent with the hypothesis by Carbone

et al. (2007) that the unnamed taxons of West Africa and *A. parasiticus* are one of the most common recent ancestors of section Flavi.

Phylogenetic affiliations not described previously by current taxonomic schemes were also discovered. The *A. parasiticus* isolates formed two distinct clades in two of three trees. Especially the *A. parasiticus* isolates from Nigeria, which are morphologically and physiologically distinct from *A. parasiticus*, formed one separate clade from those of United States, Benin, and Uganda. Therefore, these isolates are only present in a relatively small geographic area. Such diversity has been found for *A. nomius* in Thailand and for *A. flavus* in the United States (Bayman et al., 1993; Ehrlich et al., 2007b).

It is evident that *A. flavus* S-strain isolates belong to clades in which the ability to produce aflatoxin is highly conserved, whereas L-type isolates belong to clades in which the aflatoxin-producing ability is highly variable (Geiser et al., 2000b; Ehrlich et al., 2003). The phylogenetic analyses of the aflR sequence presented here a separate *A. flavus* S-strain and L-strain set in distinct clades in agreement with previous studies (Cotty, 1997; Geiser et al., 2000b; Ehrlich et al., 2003). Similar to phylogenetic analyzes by Geiser et. al. (2000), atoxigenic *A. flavus* isolates were grouped into a separate clade from those which produce aflatoxin. The results of this study further demonstrate a tendency towards separation of atoxigenics and toxigenics isolates into groups with similar patterns. Some groups contained high proportions of atoxigenics up to 95% while other groups contained only 25% whereas in yet other groups, the proportions were equal. Only high bootstrap values were detected in the *aflR* data set.

It has been suggested that sclerotia may play an important role in survival and dispersal of *A. flavus*. This role may account for selective pressure to produce sclerotia, despite their metabolic cost. Aflatoxins may have a function in correlation to sclerotia and therefore, a relation between sclerotia morphogenesis (S-and L-strain) and the aflatoxins biosynthesis (Wicklow et al., 1983; Cotty, 1997; Guzman de Pena et al., 1997; Nesci et al., 2007). High aflatoxin production has been associated with phenotypes having increased sclerotial formation, particularly in the S morphotype species of section Flavi (Cotty, 1997; Geiser et al., 2000b). In the present study, a positive correlation between aflatoxin and the sclerotia mass within *A. flavus* L-strain isolates were

observed. Similar results were also found in Argentina (Novas et al., 2002; Pildain et al., 2004). It was suggested that the ability of the fungus to produce aflatoxin and sclerotia at the same time may contribute to its defense against natural predators, especially insects (Wright et al., 1982; Willetts et al., 1992). The present study showed that an increasing production of spore mass was proportionally combined with a decreasing productions of sclerotia within the *A flavus* L-strain isolates. Less aflatoxin producing L-strains produced a greater spore mass than highly toxigenics. Differences within the L-strains are reminiscent of the distinction between *r*- and *K*- selected species (Harper, 1977); L-strains that produce numerous conidia should be favored for colonization, whereas L-strains with a combined ability to produce sclerotia and aflatoxin devote more resources to resistant structures useful for survival.

Since efforts have begun to initiate a biological management with native atoxigenic L-strains to control the contamination in maize (Atehnkeng et al., 2008a; Hell et al., 2008), the combination of less aflatoxin producing isolates with the ability to produce high spore mass could help support a control management. A successful biological control of aflatoxin contamination necessitates very high ratios of atoxigenic to toxigenic strains in the field (Horn, 2007). Infection of aerial crops, like maize, occur primarily through wind and insect dispersal of conidia, and wounding greatly enhances colonization by *A. flavus* (Diener et al., 1987; Payne, 1998). The ability of native atoxigenic isolates to produce great amounts of conidia combined with other control management techniques could be a competitive advantage against toxigenic strains in the field. Nevertheless, the natural ratio of highly toxigenic to atoxigenic strains is almost equal on tested maize and in soil (Atehnkeng et al., 2008b; Donner et al., 2008). Studies in North America have shown that a relatively low proportion of highly toxigenic strains can cause the vast majority of aflatoxin contamination (Cotty, 1996). Thus, a biological control management with an atoxigenic strain is necessary to prevent aflatoxin contamination in Nigeria.

In West Africa, unique species of section Flavi occur which are highly diverse genetically, physiologically, and morphologically. My research has shown that the diversity among these isolates varies greatly in comparison to other geographical regions which are also home to Flavi isolates. Therefore, knowledge of the specific nature of the West African isolates is needed in order

to develop the most effective biological control management for reducing aflatoxin contamination of maize in Nigeria.

GENERAL CONCLUSION

The conclusions of the present thesis are:

1. The most common member of *Aspergillus* section Flavi from the more than 1,000 isolates collected from soil of 55 Nigerian maize fields located in three agroecological zones in Nigeria was the *A. flavus* L-strain (85%), followed by the strain S_{BG} (8%), *A. tamarii* (6%) and *A. parasiticus* (1%).
2. All S_{BG} and *A. parasiticus* isolates produced both B- and G-aflatoxins and greater than 300 µg g^{-1} total aflatoxins.
3. Only 44% of 492 *A. flavus* isolates produced aflatoxins (limit of detection 5 ng g^{-1}). Thirty two percent of the *A. flavus* isolates produced >1 µg g^{-1} total aflatoxins, but no *A. flavus* isolate produced G-aflatoxins.
4. Five of the 20 VCGs from Nigeria had large deletions (37kb to 65kb) extending from the teleomeric side of the aflatoxin biosynthesis cluster. In one case (isolate AV0222) the deletion extended through the cluster to the adjacent sugar cluster. The remaining twelve atoxigenic VCGs, including the VCG used for aflatoxin management in North America contained all genes of the aflatoxin pathway.
5. Comparison of pathway genes revealed more changes in atoxigenic than in aflatoxin-producing VCGs and several non-synonymous changes that are unique to atoxigenics. These observations support the existence of atoxigenicity for very long periods. However, for some atoxigenic VCGs, additional sequencing and experimentation will be required to determine precise causes of atoxigenicity.
6. The phylogenetic analysis revealed that the atoxigenic VCGs and isolates with large deletions in the aflatoxin biosynthetic gene cluster were closely related.
7. The phylogenetic analysis revealed that atoxigenic *A. flavus* isolates were grouped into a separate clade from those which produce aflatoxin.

8. The phylogenetically analyzed S_{BG} isolates of Nigeria revealed that they neither belong to the *A. minisclerotigenes* nor to the Group II isolates. Therefore, the S_{BG} isolates of West Africa are unique isolates resembling the S-strains but occurring in only a relatively small area.
9. *A. parasiticus* isolates of Nigeria differed phylogenetically from those originating from Benin, Uganda, and the United States.
10. *A. flavus* L-strain isolates with an increasing ability to produce conidia on a Czapek's media, showed decreasing ability to produce aflatoxin in liquid fermentations. Inversely, isolates with an increasing ability to produce sclerotia, showed an increasing production of aflatoxin. *A. flavus* L-strain isolates with a decreasing ability to produce sclerotia, showed a proportional increasing production of conidia on the Czapeks plates.

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