ASSESSMENT OF TOXIGENIC POTENTIAL AND GENETIC RELATEDNESS OF *ASPERGILLUS FLAVUS* POPULATIONS FROM DIFFERENT MAIZE GROWING REGIONS IN KENYA

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DECLARATION

This is my original work and has not been submitted for any award of degree in any other University or academic institution.

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DEDICATION

This work is dedicated to my parents, Mr. and Mrs. Oloo, my siblings and Matilda for their love, support and continuous encouragement.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABCF	African Biosciences Challenge Fund
AF	Aflatoxin
AFB	Aflatoxin Type B
AFG	Aflatoxin Type G
APA	Aflatoxin Producing Ability
AVF	Averufin
AVN	Averantin
BecA	Biosciences eastern and central Africa
BLAST	Basic Local Alignment Search Tool
CaM	Calmodulin
CDC	Centre for Disease Control and Prevention
CPA	Cyclopiazonic acid
DMDHST	Demethyldihydrosterigmatocystin
DMST	Demethylsterigmatocystin
DHOMST	Dihydro-O-methylsterigmatocystin
DHST	Dihydrosterigmatocystin
DNA	Deoxy Nucleic Acid
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
FB_1	Fumonisins B_1

FDA	Food and Drug Administration
GOK	Government of Kenya
Ha	Hectare
HAVN	5 -hydroxyaverantin
HPLC-FLD	High Performance Liquid Chromatography- Fluorescence Detection
ILRI	International Livestock Research Institute
ITS	Internally Transcribed Spacer region
Ksh.	Kenya shilling
$MgCl_2$	Magnesium Chloride
NCBI	The National Centre for Biotechnology Information
NCPB	National Cereal and Produce Board
NOR	Norsolorinic acid
OMST	O methylsterigmatocystin
PCR	Polymerase Chain Reaction
PPB	Parts per billion
PPM	Parts per Million
ST	Sterigmatocystin
TAE	Tris base, acetic acid and EDTA
VAL	Versiconal
VERA	Versicolorin A
VERB	Versicolorin B

VHA	Versiconal hemiacetal acetate

ABSTRACT

Maize (*Zea mays* L.*)* is a staple food in Kenya, being consumed at 98 Kg/person/year. Aflatoxin, a metabolite of *Aspergillus flavus* is a common maize contaminant, and Aflatoxin B_1 (AFB_1) is the most carcinogenic natural compound known. Highly toxigenic strains of *Aspergillus flavus* have been reported to frequently contaminate maize, causing fatal aflatoxin poisoning in Kenya. This study was carried out to assess genetic relatedness and toxigenic potential of *A. flavus* isolates (n=72) recovered from maize grains (n=120) sampled from Eastern, Coastal and Western regions of Kenya. Analyses of genetic sequences of internal transcribed spacer (ITS) regions of ribosomal DNA and partial calmodulin were used in assessing genetic relatedness. The isolates were further screened through PCR detection for presence of five aflatoxin structural genes (*afl*D, *afl*M, *afl*O, *afl*P and *afl*Q) and two regulatory genes (aflR and aflS). Profiling of aflatoxigenicity was performed through HPLC-FLD quantification of aflatoxin produced in growth medium. Levels of AFB_1 contamination of the maize samples were analyzed using ELISA and UPLC-FLD. The ITS gene sequence grouped all isolates as *A. flavus* whereas Calmodulin gene sequence data placed them in two clades; *A. flavus* and *A. minisclerotigenes*. *A. flavus* was widespread across all regions whereas *A. minisclerotigenes* was mostly found in Eastern region. The sequences of calmodulin gene exhibited wider genetic variation than those of ITS. All *A. minisclerotigenes* were highly toxigenic. *Aspergillus flavus* isolates varied widely in toxigenicity. *Aspergillus minisclerotigenes* isolates from Eastern region produced more of AFB_1 while those from Coastal and Western regions produced more of AFG_1. *Aspergillus minisclerotigenes* isolates produced more total aflatoxins than *A. flavu*s isolates, with an average total aflatoxin production of 41883.9μgkg^{-1} and 54.4μgkg^{-1}, respectively. Of seven aflatoxin genes tested, only two genes (*aflD* and *aflS*) were corelated to aflatoxin production. Maize samples from coastal region were most contaminated with AFB_1. Contamination levels of maize in Western and Eastern Kenya were not significantly different. This study lays foundation in formulating aflatoxin mitigation measures in Kenya and contributes to the understanding on molecular basis for the toxigenicity in *Aspergillus* species.

Key words: *Aspergillus flavus*, ITS, calmodulin, aflatoxigenicity potential, aflatoxin biosynthesis genes, maize

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Maize (*Zea mays* L.*)* ranks the third most cultivated cereal after wheat and rice in the whole world. Approximately 142 million hectares of land in the world is cultivated by maize producing about 637 million tons of this grain (FAO, 2002). Maize is consumed as food for humans and feed for animals and utilized as a raw material in many industries making it an important cereal crop in agricultural economy of the world. Ninety-six percent of Kenyans consume maize as a staple food (Mutiga *et al*., 2014) both in rural and urban. Maize is grown by both large- and small-scale farmers in Kenya with the latter producing 75% of the total maize. It has received major considerations in national food security because around 90% of households in rural areas of Kenya depend on this cereal (Ouma and De-Groote, 2011). Approximately 98 kilograms of maize is consumed in this country per person per annum translating to over thirty million 90-kilograms bags in a year with some families consuming it twice daily (Okoth, *et al.,* 2012).

There has been an increasing concern that Kenyan populations depending on maize are more likely to be exposed to toxic secondary metabolites of fungi called mycotoxins (Mutiga *et al*., 2014). Mycotoxins are produced by various genera of fungi and are poisonous to human beings and domestic animals (Mboya *et al.,* 2012). They often contaminate maize in the field when fungi grow as pathogens on the maize plant and during storage when the fungi grow

saprophytically on stored maize (Afsah-Hejri *et al.*, 2013; Smith *et al.*, 2012). This exposes humans and domestic animals depending on maize to a health risk. Aflatoxins, Fumonisins, Ochratoxin and Deoxynivalenol have been found to be the main mycotoxin groups associated with maize (Kimanya *et al.*, 2012). Of these, Aflatoxin, a metabolite of *Aspergillus flavus* and *A. parasiticus* is the most popular, widespread and of great economic importance in the agricultural sector (Magembe, 2016).

Aspergillus flavus being a fungus, is eukaryotic organism with filamentous growth. It is ubiquitous in nature and lives on living matter as a parasite and dead organic matter as a saprophyte. It is pathogenic to plants and animals and widely known for its capacity to produce aflatoxins (Probst *et al.,* 2012). The saprophytic feature of these fungi permits them to live on and generate nutrients from dead organic matter in the soil allowing them to infect other plants hence completing their life cycles even in absence of maize in the field (Scheidegger and Payne, 2003). The most common aflatoxin types produced by *A. flavus* are Aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2. Among these, AFB_1 is the most carcinogenic natural compound known to humans and is widely produced by the highly aflatoxigenic *A. flavus* strains in Kenyan maize growing areas (Okoth, *et al.,* 2012).

Acute exposure to aflatoxins is lethal and has killed over 470 people in Kenya in the last three decades (Mutiga *et al.*, 2014). Chronic exposure leads to liver cancer, and has been linked to immunosuppression, nutrient absorption impairment, stunted growth in children and increased morbidity (Zain, 2011).

Exposure is also thought to increase the risk of primary hepatocellular carcinoma and there exist positive relationship between prevalence of hepatitis infections and aflatoxin consumption (Williams, *et al.,* 2004). Aflatoxin contamination has resulted into loss of income because the contaminated maize is unhealthy for both human and livestock consumption. It also has an impact on trade because contaminated maize is unfit for the market creating the need to strictly regulate aflatoxin levels in food and feed in most markets of the world (Wagacha *et al*., 2008).

1.2 Statement of the Problem

The food security of Kenya relies on the availability of maize grown domestically hence the available maize in the stores of a household determines the food security of that household (World Bank, 2010). Despite several attempts to increase maize production, Kenya as a country does not produce enough maize to carter for her population leading to importation of large quantities of maize (Erastus, 2011). Unfortunately, the little available quantities of maize produced in Kenya has suffered severe attacks by *Aspergillus flavus* and aflatoxin leading to condemnation. The national Cereal and Produce Board (NCPB) in June 2018, held 984,102 90-kg bags of maize worth Ksh. 3.1 billion that were infested with fungi and damaged by insects hence rendered unfit for human consumption (Business Daily Newspaper, 2018). The impact of aflatoxin contamination of maize has affected large population of Kenya yearly without effective solution being concluded. Several attempts to eliminate the

aflatoxin contamination in this country have not fully succeeded in controlling the mycotoxins.

Highly toxigenic isolates of *A. flavus* with some genetic differences or mutations which existed only in Kenyan isolates have been shown (Probst et al, 2012). However, to obtain a full aflatoxigenicity potential report, it is important to determine how the isolates are genetically related. This study assessed toxigenic potential and the difference between toxigenic and atoxigenic isolates by comparing genetic variation among *A. flavus* isolates based on two universal gene markers. If stakeholders were aware of areas with the most toxigenic isolates, they could use these maps in creating awareness and alerts at different stages in the maize value chain. The study has mapped the hotspots of aflatoxigenic fungi. A study by Mutiga *et al*. (2014) found out that aflatoxin contamination mainly dominates Eastern part of Kenya. This study assessed whether the toxigenicity potential of *A. flavus* isolates is localized, and/or whether the observed high contamination levels are modulated by contemporary maize stress conditions. The purpose of the study was to assess toxigenic potential and genetic relatedness of *Aspergillus flavus* population from different maize growing regions of Kenya.

1.3 Justification of the Study

There has been lethal aflatoxicosis of humans for over three decades resulting from consumption of maize produced in this country especially in Eastern parts of Kenya. To manage and control aflatoxicosis, detailed knowledge about highly toxigenic strains of *A. flavus* and their mechanisms of toxigenicity

including their genetic variation need to be captured. Chronic exposure of aflatoxins has been reported in Kenya (Okoth *et al.*, 2012). Adequate information about the occurrence of highly toxigenic isolates across Kenyan maize growing areas is lacking creating a need to conduct this study. Aflatoxin contamination takes place in the whole production chain from cultivation to harvesting. Furthermore, aflatoxins are heat stable hence cannot be controlled through the cooking process. The use of biocontrol agents (BCA) and resistant maize cultivars to control toxigenic *Aspergillus flavus* has been implemented, however it is important to continuously monitor the fungus to be able to manage it. This study aimed at generating knowledge of the genetic relatedness of the isolates and their toxigenic potential which can facilitate better development of control methods.

1.4 Objectives of the study

1.4.1 General Objective

This study was carried out with the aim of assessing toxigenic potential and genetic relatedness of *Aspergillus flavus* population from different maize growing regions of Kenya.

1.4.2 Specific Objectives

1. To characterize and determine genetic relatedness of *Aspergillus flavus* strains from maize samples collected from different regions in Kenya using two genetic markers

2. To determine the correlation between presence of seven aflatoxin biosynthesis genes in *Aspergillus flavus* isolates and their aflatoxigenic potential
3. To compare aflatoxin B_1 contamination levels in maize sampled from different regions in Kenya

1.5 Hypothesis

The research was guided by the following hypotheses,

1. *Aspergillus flavus* populations from different maize growing regions in Kenya are genetically similar.
2. There is a correlation between the aflatoxigenicity potential and presence of aflatoxin biosynthesis genes.
3. There is no significant difference in contamination of maize samples with aflatoxin B_1 across the eight regions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Maize Production and Consumption in Kenya

Maize is a staple food for most Kenyan population and is cultivated under an area of approximately 1.6 million Ha producing around 26,000,000 bags of maize per year. It is produced by both large- and small-scale farmers in Kenya with the latter producing 75% of the total maize. Approximately 98 kilograms of maize is consumed in this country per person per annum translating to over thirty million 90-kilograms bags in a year (Okoth *et al.*, 2012). Maize is receiving major considerations in national food security because around 90% of rural households in Kenya depend on it (Maina *et al.*, 2016). Every Kenyan family grows maize either in a garden or farm sometimes for trade but mostly for consumption (Okoth *et al.*, 2012) portraying the usefulness of this crop in the country.

Maize production is closely linked to food security in Kenya for the fact that it is the country's most important staple crop. According to World Bank (2010), maize production in Kenya has been fluctuating over the last decade although the demand has been rising because of high population growth rate of approximately 2.9% per year. This has led to heightened maize shortages because the domestic consumption levels are higher than the total maize produced per year in this nation. This shortage is caused by increased urbanization, low per capita production and changing lifestyles and high dependence on corn-based diets as the staple food (Erastus, 2011). Even

though there are notable attempts to increase the production of maize in this country, the demand has sometimes exceeded the supply, necessitating the need to import large quantities of maize. To meet the large maize deficit, the Kenyan government usually import maize from other countries cope up with consumption patterns.

Maize has many uses in Kenya and world at large; it is utilized as a food; feed for domestic animals and as a raw material for industries (FAO, 2002). As food, maize is a basic component of the diet of millions of Kenyans because it is adapted to different agro-ecological zones, easy to cultivate, has high yields per hectare, good storage characteristics and versatile uses as food (Asiedu, 1989). A study by Kumar *et al.* (2007) indicated that around 50 to 55 % of the total maize produced in most developing countries is consumed as food. Compared to other grains as a feedstuff, maize is more efficiently converted to animal products such as eggs, milk and meat (Gatch and Munkvold, 1999). According to Lynch *et al.* (1999), maize is used industries worldwide as a primary raw material to produce fuels, ready-to-eat snack foods, starch, corn syrup, oil, dextrose, paper, protein, biodegradable chemicals, alcohol, textiles, breakfast cereals and cornmeal.

2.2 Mycotoxins Contamination of Maize

Maize is liable to contamination with toxic fungal metabolites known as mycotoxins. It can be infected by mycotoxin producing fungi before harvest, during harvest and during storage (Atanda *et al.*, 2011). Fungi that produce mycotoxins are prevalent in tropical and subtropical ecosystems. Shephard

(2008), reported that there is a high probability of exposure to mycotoxins for African populations depending on maize. Maize is a rich substrate for *Fusarium verticillioides* that produces fumonisin and two fungal species, *Aspergillus flavus* and *A. parasiticus* that produce Aflatoxins (Mutiga *et al.*, 2014). B_1 types of both Aflatoxins and Fumonisins are carcinogenic (Gelderblom *et al.,* 2002). Maize production in Kenya is carried out under agro-ecological conditions that aid fungal proliferation and accumulation of mycotoxins. Approximately three-quarters the total maize in Kenya is produced by small-scale farmers with poor resources under conditions that can predispose the maize to contamination with mycotoxin (Okoth *et al.*, 2012).

2.3 Aflatoxins in Maize

Aflatoxin is a carcinogenic and toxic secondary metabolite produced by several *Aspergillus* species when they infect maize and other crops. Because of its detrimental health effects, the levels of aflatoxin present in maize are strictly regulated by the FDA, and those with level higher than the limit set are condemned making maize producers to suffer an economic loss (Mylroie *et al.*, 2016). Despite such strict regulations, aflatoxin contamination remains to be a serious challenge in the maize production in Kenya. It detriments the health of human and animals as well as results in great economic losses in terms of crops production (Okoth *et al.,* 2012). In humans the exposure causes liver cancer and other negative health effects such as immunosuppression and stunted growth in children (Maina *et al.*, 2016). Aflatoxin contamination of animals' feeds have resulted into aflatoxicosis, immunosuppression, impaired growth,

kidney and liver tumours in rodents as well as reduction in the quality of milk and its products due to the presence of a derivative of AFB_1 called aflatoxin M1 (Lizárraga-Paulín *et al.*, 2011).

Aflatoxins are produced by cosmopolitan and ubiquitous fungi, *Aspergillus flavus* and *A. parasiticus* on various substrates that include cotton, wheat and maize. They are difuranocoumarin derivatives that consist of a coumarin ring and a double furan ring (Papp *et al*., 2002). Approximately twenty aflatoxins are known, with only Aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2 normally found in food (Espinosa-Calderón *et al*., 2011) (Figure 2-1). These abbreviations are indicative of the colours they exhibit/fluorescence under the ultraviolet light (385 nm) on thin layer chromatography plates where B represent blue and G present green-yellow (Bennett and Klich, 2003). AFB_1 is the most carcinogenic natural compound known to humans and is widely produced by the highly toxigenic strains of *A. flavus* in Kenyan maize growing areas. (Mutiga *et al.*, 2014).

Kenya has a high prevalence of aflatoxin contamination and several incidences of fatal aflatoxicosis have been reported for the last three decades resulting from consumption of *A. flavus* and aflatoxins contaminated maize (Mutiga *et al.*, 2014). The outbreak that occurred in 1981 resulted from drought that preceded torrential rainfall during harvesting period of maize grown domestically (Ngindu, *et al.*, 1982). The worst outbreak occurred mainly in Eastern regions of Kenya in 2004 and reported 125 deaths and 317 patient cases (Probst, *et al.*, 2007; CDC, 2004). It was due to early rains, inappropriate

harvesting time and poor storage of harvested maize in humid environments (Muthomi *et al.,* 2009). A total of 31,000 bags of maize in Mbeere in Embu County and 1,213 in Bura Irrigation Scheme in Tana River County were contaminated with aflatoxins in 2009 and condemned. In 2010, approximately 2.3 million bags of maize from Coastal and Eastern Kenya were contaminated with high levels of aflatoxins rendering it unhealthy for consumption by both human and animal (Kesley, 2010, FAO/UON, 2011).

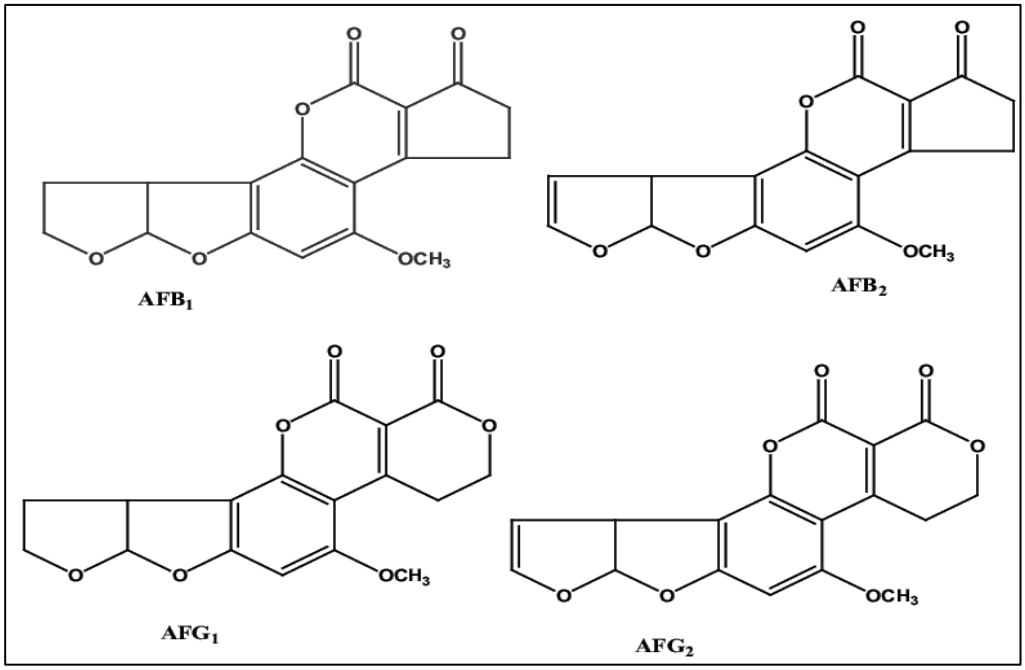

Figure 2-1: Ring structure of four common aflatoxin types that contaminate food. Sourced from (Oratile and Bareki, 2017)

2.4 *Aspergillus* section Flavi group

Aspergillus section *Flavi* is composed of closely related group of ubiquitous and cosmopolitan fungi (Klich, 2002), which seem to devote most of their lives growing saprophytically in the soil (Accinelli *et al.*, 2008).

2.4.1 Members of the *Aspergillus* section *Flavi*

Species belonging to this group grow as yellow to green molds in culture and they produce a unique conidiophore made up of a long stalk, which supports a swollen vesicle just like other *Aspergillus* species. The colonies are olive to lime green with a cream reverse on potato dextrose agar (PDA) at 25°C. Conidiogenous cells on the vesicle usually produce conidia with variable shapes and relatively thin and finely roughed walls. The optimal water activity (aw) range for ideal growth of *Aspergillus flavus* is 0.86 to 0.96 (Vujanovic *et al.,* 2001).

The section *Flavi* group members that are known to produce aflatoxin include *Aspergillus flavus, A. parasiticus, A. nomius, A. bombycis* and *A. pseudotamarii* fungal species (Peterson *et al.,* 2001). Among these, *A. parasiticus* and *A. flavus* are the most relevant in agriculture as far as the production of aflatoxin is concerned. They are of great concern because of ability to produce potent carcinogenic aflatoxins (Cotty *et al.*, 2008; Klich, 2007) on important food crops before harvesting, during harvesting, storage, and eventually processing (Yu *et al.*, 2008). However, they have wide variations in the aflatoxin production capability.

The considerable diversity in morphology and aflatoxin production is a distinctive feature of *A. parasiticus* populations. It appears to be adapted favourably to soil environments and is common in peanuts. It is characterized by a woolly/cottony to granular texture and grows rapidly at both 25ºC and 37ºC. *A. parasiticus* usually produce conidia with more noticeable

ornamentation and dark green heads (Klich and Pitt, 1988). This feature is applied in distinguishing *A. parasiticus* from *A. flavus.* Their sclerotia if present, are dark brown in colour. Their infective propagules are conidia and sclerotia and can contaminate many crops they come in contact with (Bayman and Cotty, 1993). Phenotypically, *A. parasiticus* produces AFB_1, AFG_1, AFB_2, and AFG_2 (Bennett and Klich, 2003).

A. nominus, a third species in the category, has a comparable mycotoxin profile to *A. parasiticus.* However, it resembles *Aspergillus flavus* morphologically (Peterson *et al.*, 2001). The species was initially considered uncommon, but a study by Ehrlich *et al.,* (2007) have shown it is widely distributed and might be economically viable. Other fungi that produce aflatoxins but with less occurrence in nature are *Aspergillus pseudotamarii*, *A. ochraceoroseus* and *A. bombycis* (Peterson *et al.*, 2001; Klich *et al.*, 2000).

2.4.2 *Aspergillus flavus*

Aspergillus flavus is a fungus with a filamentous growth. It produces a thread like branching filaments termed hyphae. A network of hyphae known as the mycelium exudes extracellular enzymes which helps in breaking down complex food molecules into fine particles, which are then absorbed to supply energy and nutrients for the growth of the fungus (Klich and Pitt, 1988). It is impossible to see individual hyphae with the unaided eye, but one can easily see a mass of mycelium with conidia (asexual spores). At the tender stages of *A. flavus,* the conidia are yellow-green in color; however, spores become darker green as it matures. *Aspergillus flavus* can grow on numerous substrates

in nature; it primarily grows saprophytically on dead animal and plant tissue and in the soil hence very vital in the recycling of nutrients. *Aspergillus flavus* thrives well in hot and dry conditions with optimal growth temperature range of 25-42ºC but can still grow at 12-48ºC. Such a high optimum temperature plays a major role to its pathogenicity on humans. Proliferation of the fungi and production of aflatoxins are favored by high temperatures and moistures (Davari *et al*., 2015).

A. flavus is also pathogenic to many plant and animal species, with human beings and domesticated animals included. It infects seeds of cotton, peanuts, nut trees and corn. In most cases, sporulation of the fungus can be observed on injured seeds like maize kernels but oftenly, only a few kernels are visibly infected (Richard and Payne, 2003). It produces toxic aflatoxins on food sources which pose negative effects on the health of animals and humans. It attacks crops in the field, during harvest, transportation or during storage. *A. flavus* is ranked as the second leading cause of aspergillosis in humans after *A. fumigatus* (Okoth *et al.,* 2012). People infected *A. flavus* usually have weakened immunity. The epidemiology of *A. flavus* varies greatly with the host species. The fungus hibernates as either mycelium or resistant structures called sclerotia. The sclerotia either propagate to produce more hyphae or conidia (asexual spores) that are normally disseminated in the air and soil. The spores are dispersed to their hosts by wind or insects where they grow to cause infection (Richard and Payne, 2003).

2.4.3 Characterization of *Aspergillus flavus*

Characterization of fungi from *Aspergillus* section *Flav*i based on cultural and morphological features is difficult due to interspecific similarities and intraspecific variability (Rodrigues *et al*., 2009). Moreover, it is time consuming and often not straight-forward (Okoth *et al*., 2018). In *A. flavus*, phenotypic disparity and genetic diversity have been reported (Horn, 2007). Although internal transcribed spacer (ITS) region of ribosomal DNA sequence analysis has been used to identify fungal species, it may not be able to differentiate some closely related members of certain genus. Thus, specific genes such as β-tubulin, calmodulin and actin are used to accurately differentiate the members in a PCR-sequencing technique (Samson *et al*., 2007; Balajee *et al*., 2005). For example, recent studies in Portugal applied sequencing of calmodulin and ubiquitin genes to resolve genetic differences and to accurately identify *Aspergillus* species and other cryptic fungal species (Raquel *et al*., 2014). Thus, there is need to combine cultural, biochemical and robust molecular techniques, including analysis of specific genes to enhance accurate characterization of toxigenic fungal species.

The sclerotia morphology of *A. flavus* has been used to distinguish between S and L strains (Cotty and Cardwell, 1999). The S-strain that produces considerable quantities of small dark sclerotia, is the most toxigenic as it produces high levels of B1 and B2 type aflatoxins. On the other hand, the L-strain produces yellow to bright green colonies with small amounts of sclerotia and inconsiderable amounts of B1 and B2 type aflatoxins (Maina *et al*., 2016). The S strains, originally identified as *A. flavus* were associated with human

aflatoxicosis outbreak of 2004 in Kenya (Claudia Probst *et al*., 2007). They were later identified to be distinct from *A. flavus* and closely related to *A. minisclerotigenes* (Probst *et al*., 2012). *Aspergillus minisclerotigenes* which was first described in 2008 (Pildain *et al*., 2008), have typically small sclerotia but is genetically different from other S-strain isolates (Probst *et al*., 2012). Some of these isolates were previously described as *A. flavus* (Geiser *et al*., 1998; 2000). Isolates of *A. flavus* are known to produce only aflatoxin type B whereas *A. minisclerotigenes* isolates produce both aflatoxins B and G (Klich, 2007). Analysis of aflatoxigenicity potential of *A. flavus* and *A. minisclerotigenes* isolates from wider maize growing areas of Kenya would be essential to develop an effective aflatoxin mitigation strategy for different maize production environments.

2.5 Aflatoxigenicity Potential of *Aspergillus flavus*

This is the ability of *Aspergillus flavus* to produce aflatoxin. Those that produce aflatoxins are known to be aflatoxigenic while those that do not have been classified to be non-aflatoxigenic. Aflatoxigenic potential of *A. flavus* extremely varies. This has led to the strains in this species being grouped into five classes based on their aflatoxigenic profile (Giorni *et al.*, 2007). Five proposed classes include; chemotype I which produce Cyclopiazonic acid (CPA) and AFBs only; chemotype II which produce CPA, AFBs and AFGs; chemotype III that produce only AFBs; chemotype IV which produce only CPA; and chemotype V which do not produce aflatoxins at all. (Vaamonde *et al.*, 2003).

The failure of *Aspergillus flavus* to produce aflatoxins is brought about by occurrence of single nucleotide polymorphisms (SNPs) and gene deletions and/or insertions in aflatoxin biosynthesis genes (Okoth, *et al.*, 2018). Mutations do occur among the aflatoxigenic isolates of A. *flavus* that results into the loss of aflatoxin producing potential as reported by Solorzano *et al.*, (2014). The occurrence of atoxigenic *Aspergillus flavus* strains has been also found to be dependent on substrate (Vaamonde *et al.*, 2003) and geographic origin (Atehnkeng *et al.*, 2008). However, highly toxigenic *A. flavus* isolates with some genetic differences or mutations which existed only in Kenyan isolates have been shown (Probst *et al.*, 2012).

2.6 Aflatoxin Pathway Gene Cluster

Aflatoxins are secondary metabolites derived from polyketide that are produced through a complex conversion pathway outlined; Acetate – polyketide – Anthraquinones – Xanthones – aflatoxins (Yabe, 2003). The pathway of production of aflatoxins consists of 25 genes with some having their roles unclear in the biosynthesis of aflatoxins. To achieve uniformity and consistency in the naming of aflatoxin genes with their functions in the aflatoxin biosynthetic pathway in *Aspergillus*, a standard rule for naming genes was instituted (Bennett and Lasure, 1985). The genes whose roles in the aflatoxin biosynthesis pathway are clear have been assigned *aflA* to *aflQ* starting from the transition of acetate, through the various intermediates to aflatoxins. The *aflR* and *aflS* genes regulate the transcription of aflatoxin biosynthesis pathway genes. Those genes whose involvements in pathway are

unclear or ambiguous are named; *aflT, aflU, aflV, aflW, aflX* and *aflY* (Jiujiang *et al.*, 2004).

2.6.1 Aflatoxin Biosynthesis Structural Genes

The conversion of the primary metabolite, acetate, to the first stable AF biosynthesis intermediate, norsolorinic acid (NOR), is carried out by the action of three genes; aflA, aflB, and aflC. NOR is then converted to averantin (AVN) through the action of an enzyme encoded by aflD gene (Papa, 1982). aflF is also utilized in the conversion of norsolorinic acid to averantin. aflG gene encodes a cytochrome P450 monooxygenase which converts AVN to 5 - hydroxyaverantin (HAVN). The aflH the converts HAVN to averufin (AVF) which is converted by an oxidase encoded by aflI to versiconal hemiacetal acetate (VHA).

The conversion of VHA to versiconal (VAL) is ensured through the action of aflJ. The aflK then converts VAL to versicolorin B (VERB) that is converted to versicolorin A (VERA) by aflL. Two aflatoxin genes; aflM and aflN help to convert VERA to demethylsterigmatocystin (DMST). Another gene, aflO converts DMST and Demethyldihydrosterigmatocystin (DMDHST) to Sterigmatocystin (ST) and dihydrosterigmatocystin (DHST) respectively. The aflP converts ST and DMST to O methylsterigmatocystin (OMST) and dihydro-O-methylsterigmatocystin (DHOMST) respectively. The aflQ gene has two roles: it converts OMST to aflatoxin B_1 and G_1 and DMDHST to aflatoxin B_2 and G_2 (Yu et al., 1998). Some of the structural genes in the pathway play same roles (Figure 2-2).

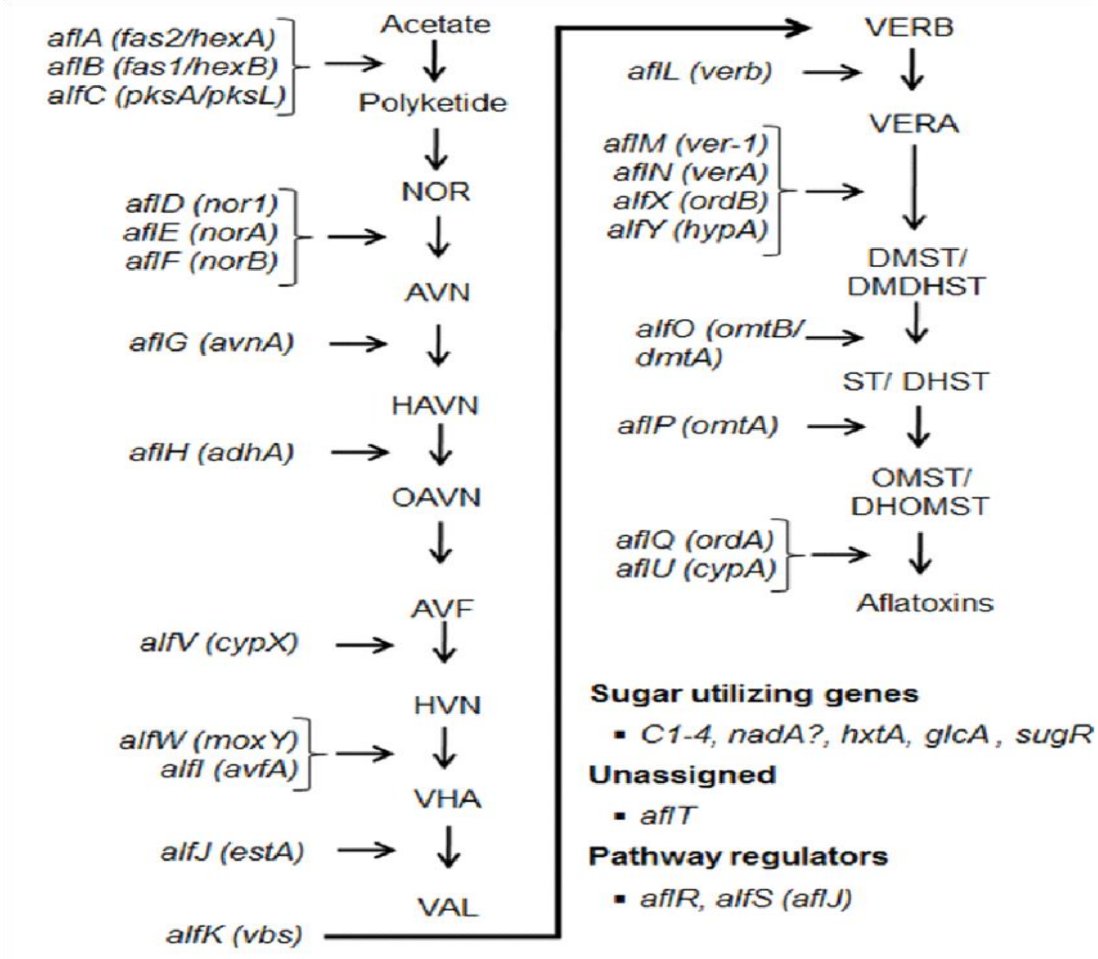

Figure 2-2: Schematic diagram of aflatoxin biosynthetic pathway with the genes involved and the roles they play. Sourced from (Dodia *et al*., 2014)

2.6.2 Aflatoxin Biosynthesis Regulation Genes

The *aflR (afl-2)* is a positive regulatory gene that activates the transcription of the structural genes of aflatoxin pathway. The transcription of genes involved in aflatoxin pathway are triggered once the AflR protein attaches to the AflR-binding motif (5_-TCGN5CGA-3_) in the promoter region of the structural genes. This has been shown in *A. flavus* (Ehrlich, *et al.*, 1999a) and in *A. parasiticus* (Ehrlich, *et al.*, 1999b). A study by Matsushima *et al.*, (2001)

showed that nontoxigenic strain, *Aspergillus sojae*, usually utilized in industrial fermentations, has a defective non-functional aflR gene which prevents the production of aflatoxin. Another important aflatoxin biosynthesis regulation gene is *aflS (*originally termed *aflJ*). It is located in the aflatoxin gene cluster next to the *aflR* gene and is implicated in the regulation of transcription (Meyers *et al.*, 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of study sites

The study was conducted across eight regions in Kenya namely; Western Kenya (Rachuonyo, Bungoma North, Kitale, Nandi), Eastern Kenya (Machakos, Meru, Makueni) and Coastal Kenya (Kilifi) regions (Figure 3-1). Western Kenya produces more than three-quarters of the total maize consumed in this country and have not reported serious aflatoxicosis outbreaks, (Okoth *et al.,* 2012). Nevertheless, the absence of aflatoxin surveillance and fatalities could mean that the effects of exposure to these toxins have gone unnoticed. Eastern Kenya is prone to drought and known as a hotspot for aflatoxins (Mutiga *et al.,* 2014). None of aflatoxin surveillance study has been done in Coastal Kenya.

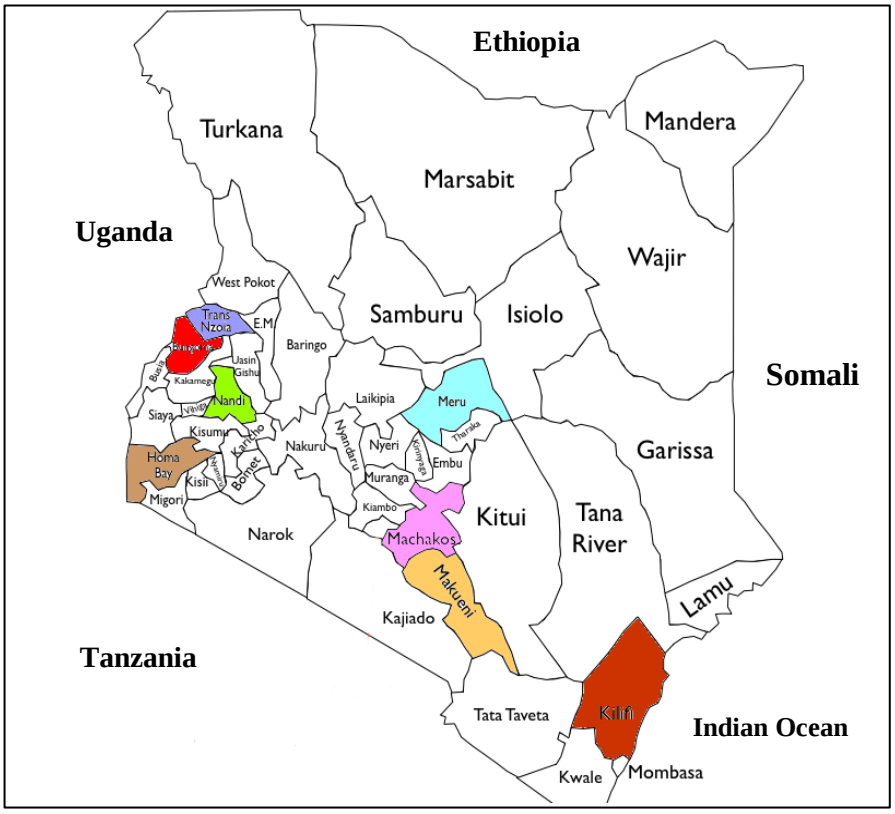

Figure 3-1: Map of Kenya with the colored regions showing counties where maize was sampled for analysis.

3.2 Research Design

This study was part of a larger project, Safe Food Safe Dairy (2010 – 2018), that assessed the presence of AFM_1, a derivative of AFB_1 from milk and urine from breast-feeding mothers and fresh cow milk. A cross sectional design was used to sample. Household selection criteria was purposively and was based on - those keeping cattle and have breast-feeding mothers with a child of less than five years of age. Village elders, chiefs and divisional teams comprising of staff members from departments of animal production and agriculture were incorporated in the project to ensure sustainability of the project, selection of

participating farmers and acceptance of the project by the community (Nyongesa *et al.*, 2015).

3.2.1 Sample size

Study sample size was determined using the area population formula by Cochran, (1977) to obtain a representative sample size.

$$n = \frac{\frac{z^2 \times p(1-p)}{e^2}}{1+\left(\frac{z^2 \times p(1-p)}{e^2 N}\right)}$$

Where; n = representative sample size

N= Population size

z = confidence level (1.96)

e = margin of error (0.05)

p = percentage value in decimal

Total qualifying house in all regions were = 1294

Total number of households in all regions were = 1429

$$P = \frac{\text{Total qualifying households}}{\text{Total number of households}}$$

1294/1429 = 0.906

1-p = 0.094

$$n = \frac{\left[\frac{1.96^2 \text{ X } 0.906 \text{ X } 0.094)}{0.05^2}\right]}{1+\left[\frac{1.96^2 \text{ X } 0.906 \text{ X } 0.094}{0.05^2 \text{ X}1429}\right]}$$

$$= \quad 120 \text{ households}$$

From the above calculation the sample size obtained was one hundred and twenty (120). To get the number of samples per region, the total number of samples was divided by eight regions. Fifteen households from each location were randomly selected using purposive sampling technique described above.

3.2.2 Maize Sample Collection

This was done between 15th August to 17th October 2017 as described in section 3.2 above. Two kilograms of shelled maize kernels from the household storage facilities within the sub-locations were randomly collected in sterile paper bags sealed and stored at 4ºC. The maize stored in sacks were sampled from different parts by driving a closed spear through the top and sides of each sack to collect a total of 2 kg of progressive samples. All sacks were sampled in stores that were having less than 10 sacks but for the stores with more than ten to one hundred, ten sacks were sampled randomly (Okoth, *et al.*, 2012). The samples were later taken to BecA-ILRI Hub for analysis.

3.3 Isolation and Identification of *Aspergillus flavus*

Maize kernels (n=12) from each sample were assessed for internal colonization by fungi using direct plating technique (Pitt and Hocking, 1997). Four kernels

were surface sterilized by dipping in diluted commercial bleach (2.5% sodium hypochlorite) for 1 minute prior to rinsing thrice in sterile distilled water. Kernels were plated in three replicates on ¼ strength potato dextrose agar (PDA) amended with 2 millilitres of lactic acid to suppress bacterial contamination by creating acidic environment. Plated kernels were incubated 3 days at 31ºC. Fungal colonies from the plated kernels were sub-cultured in PDA to obtain pure cultures. Only *Aspergillus* species were selected for further analysis using cultural characteristics e.g. appearance of aerial mycelia, reverse surface, colony colour and growth rate. The cultural characteristics described in taxonomic keys by Klich (2002) were used to identify *Aspergillus* section *Flavi*. The isolates were sub-cultured and transferred to Aspergillus Flavus Parasiticus Agar (AFPA) as described by Muthomi, *et al*., (2009), then incubated in the dark at 28°C for 72 hours to identify putative *Aspergillus flavus colonies based on* orange reverse colour in the media.

3.4 DNA Extraction

The putative *Aspergillus flavus* isolates were sub-cultured in Malt Extract Agar (MEA) medium at 25°C for three days. Mycelia were harvest from the 3-day old cultures into 1.2 ml microtubes containing six sterile beads. Microtubes containing the mycelia were capped and stored at -80°C overnight prior to lyophilization (Christ Martin Alpha 2-4 LSCplus Lyophilizer). Microtubes with lyophilized mycelia were dipped in liquid nitrogen for 60 seconds and then ground into a fine powder using a Geno Grinder (SPEX SamplePrep, NJ, USA). Total genomic DNA was extracted using MagAttract 96 DNA Plant

Core Extraction Kit (QIAGEN Inc., Mississauga, Ontario), following the manufacturer's protocol. DNA quality and quantity were analyzed using 0.8% agarose gel electrophoresis and Nanodrop 2000c (Thermo Scientific, Wilmington, USA) Spectrophotometry, respectively.

3.5 PCR Amplification and Sequencing

The regions encoding these conserved genes; internal transcribed spacer (ITS1–5.8S-ITS2 cluster) region and the partial calmodulin gene were amplified through polymerase chain reaction (PCR) analysis and sequenced to determine genetic relatedness of the isolates. The DNA samples of the isolates (*n*=218) from section 3.4 (above) were amplified and sequenced using fungal universal primer sets of ITS1F and ITS4. A subset of 72 isolates which represented the genetic clusters of the entire collection (based on ITS sequence data) and the geographical regions were sequenced for partial calmodulin (CaM) gene using primers indicated in Table 3-1below. A 50 µL volume of a reaction mixture containing AccuPower® Taq PCR MasterMix ($MgCl_2$-free reaction buffer, 1.5 mM $MgCl_2$, 1 U of Taq polymerase, 250 µM of each dNTP), 0.2 µM of each primer and template DNA at a final concentration of 1 ng/µL was prepared.

PCR was carried out as follows - first step of denaturation at 94°C for 4min, 35 cycles of the following three steps - 45sec at 94°C, 45sec at specific annealing temperature (49.4°C for ITS and 56°C for CaM), 45sec at 72°C, and one final extension step of 10-minutes at 72°C. The PCR amplicons were assessed on 1.5% agarose gel at 100V for 45min. PCR products were purified using

QIAquick PCR Purification Kit (QIAGEN Inc., Mississauga, Ontario). Purified PCR products were shipped for sequencing at Bioneer Corporation Republic of South Korea. The ITS and partial Calmodulin sequences are available at NCBI data base with accession numbers details presented in respective results section.

3.6 Selection of Subset *Aspergillus flavus* for Further Analyses

Based on ITS sequence clusters and geographical location (region) of the isolates, 72 representative isolates were selected for further analysis to reduce number of duplicate sequences. For the clusters that had only one isolate, the isolate was selected regardless of its location. The clusters with more than one sample had their selection done on basis of the region and initial maize sample from which the *A. flavus* was isolated. In a case where more than one *A. flavus* in the same cluster were isolated from the same maize sample, only one isolate was picked randomly for further analyses.

3.7 Joining of ITS and Calmodulin Sequences

Using R software, the trimmed, assembled, aligned and edited sequences of ITS and Calmodulin were joined to have a longer sequence composed of the two regions. Phylogenies were performed on these data to obtain genetic diversity among the isolates and the number of unique alleles / sequences per region. The data was also related to toxigenic potential of the isolates, Calmodulin sequences and ITS sequences.

3.8 Screening for Aflatoxin Genes in *Aspergillus* Isolates

The subset of the putative *A. flavus* isolates (n=72) were screened for the seven aflatoxin genes; *aflD, aflM, aflO aflP, aflQ, aflR* and *aflS* by PCR analysis using the primers in Table 3-1 below. The *aflD* and *aflQ* genes were amplified using two different sets of primers to test consistency of the results (Table 5). A 20µL volume reaction mixture containing $MgCl_2$-free reaction buffer, 1.5 mM $MgCl_2$, 1 U of Taq polymerase, 250 µM of each dNTP, 0.2 µM of each primer and template DNA at a final concentration of 1 ng/µL was prepared. PCR was performed using the following steps: initial denaturation at 94°C for 3 minutes; 30 cycles of the following three steps: denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute and extension for 1 minute at 72°C; and one final extension step at 72°C for 10 minutes. The PCR amplicons were assessed on 1.5% agarose gel electrophoresis at 100V for 30 mins and were viewed under UV light. Presence of an amplified band was considered to indicate the presence of the gene.

Table 3-1: Sequences of the nucleotide primers used in the study

Target gene	Primer code	Primer sequence	Size (bp)	Reference
AflD	AflD-1	5'-CACTTAGCCATCACGGTCA-3'	852	Fakruddin *et al.*, 2015
	AflD-2rev	5'-GAGTTGAGATCCATCCGTG-3'		
	Nor1-F	5′-ACCGCTACGCCGGCACTCTCGGCAC-3′	400	Rodrigues *et al.*, 2009
	Nor1-R	5′-GTTGGCCGCCAGCTTCGACACTCCG -3′		
AflM	AflM-1	5'-AAGTTAATGGCGGAGACG-3'	470	Fakruddin *et al.*, 2015
	AflM-2rev	5'-TCTACCTGCTCATCGGTGA-3'		
AflO	AflO-1	5'-TCCAGAACAGACGATGTGG-3'	790	Fakruddin *et al.*, 2015
	AflO-2rev	5'-CGTTGGCTAGAGTTTGAGG-3'		
AflP	AflP-1	5'-AGCCCCGAAGACCATAAAC-3'	870	Fakruddin *et al.*, 2015
	AflP-2rev	5'-CCGAATGTCATGCTCCATC-3'		
AflQ	AflQ-1	5'-TCGTCCTTCCATCCTCTTG-3'	757	Fakruddin *et al.*, 2015
	AflQ-2rev	5'-ATGTGAGTAGCATCGGCATTC-3'		
	Ord1-gF	5′-TTA AGG CAG CGG AAT ACA AG-3′	719	Sweeney *et al.*, 2000
	Ord1-gR	5′-GAC GCC CAA AGC CGA ACA CAA A-3′		
AflR	AflR-1	5'-AAGCTCCGGGATAGCTGTA-3'	1079	Fakruddin *et al.*, 2015
	AflR-2rev	5'-AGGCCACTAAACCCGAGTA-3'		
AflS	AflS-1	5'-TGAATCCGTACCCTTTGAGG-3'	684	Fakruddin *et al.*, 2015
	AflS-2rev	5'-GGAATGGGATGGAGATGAGA-3'		

3.9 Aflatoxigenicity of the *Aspergillus* Isolates

An assay was set up to quantify the amount of aflatoxin production by individual isolates (n=72) on Yeast Extract Sucrose Agar (YESA) medium. Twenty-five millilitres of autoclaved YESA was dispensed in 9cm diameter Petri-plates and allowed to cool. A loopful mycelia of *A. flavus* isolates scraped from 9mm PDA plugs were sub-cultured on YESA plates and incubated in the dark at 28°C for 7 days to induce aflatoxin production as per the conditions established by Davis *et al*., 1966.

Five grams of fungal plug from each sample were transferred to sterile 50ml Falcon tube and cut into small pieces using a sterile blade. Twenty-five millilitres of 70% methanol was added and the slurry shaken in a mechanical shaker at 350rpm for 1 hour. The methanolic extract was centrifuged at

3500RPM for 10 minutes. Extract supernatant of volume 0.5ml was transferred into a 2 ml centrifuge tube and diluted with 0.5 ml of 1% acetic acid prior to centrifugation at 13000 RPM for 5 minutes. A total of 0.7ml of the diluent was then transferred into HPLC vial for analysis using Ultra High-Performance Liquid Chromatography.

Chromatographic separation was performed using a Shimadzu Nexera UHPLC system (Kyoto, Japan) fitted with a SIL-30AC Auto sampler, LC-20AD Prominence pumps and RF-20AXS Prominence Fluorescence detector. A Phenomenex Synergi Hydro – RP 2.5u, 100mm x 3.00mm column was used for separation of aflatoxins using methanol (40%) and 1% acetic acid (60 %) at a flow rate of 0.4ml/min with the column oven temperature set at 50°C. Liquid chromatography program was set at 8 minutes per run and 60% methanol used as flushing solution of column.

The LabSolutions data analysis software was used to establish a standard calibration curve from a plot of peak areas against the known concentration of the injected series of standards. The aflatoxin of interest was identified by comparing the retention time of the chromatographic peak of the target aflatoxin in the test sample and that of the corresponding standard chromatographic peak. The samples whose values lied above the linear range of the standard curve were diluted further and retested.

The content of aflatoxins in the test sample was calculated according to the formula shown below:

$$X\ (ng/g)\ = \frac{c\ x\ V\ x\ f}{W}$$

where, X—The content of aflatoxin in the test sample in ng/g;

c—The concentration of aflatoxin in the test sample in ng/mL;

V—Constant volume in mL; f—Dilution factor of the test solution;

W—The mass of the test sample in g

Total aflatoxin was estimated as the sum of the concentrations of individual aflatoxin types in each sample. For quality control, blank solvents (1:1 Mixture of 70% Methanol and 1%Acetic Acid), HPLC standard samples, repetition of one sample and blank YESA plate incubated at the same conditions as the cultured plates were included in the analysis procedure. Accuracy of the reported aflatoxin were acceptable if the determined values of the reference materials were within the 3±SD in the control chart of each analytical run.

3.10 Analysis of maize samples for Aflatoxin B_1

Subsamples of maize were taken from main samples and milled into flour using a laboratory miller (ROMER SERIES II™ MILL). Five grams of maize flour was weighed into 50ml falcon tube and labeled for each sample. To

extract aflatoxins, 25ml of 70% Methanol was added into falcon tube, vortexed for 1 minute and shaken in a shaker for 30mins. They were then centrifuged at 3500rpm for 10 mins and supernatant carefully transferred into 2ml Eppendorf tubes for aflatoxin contamination level analysis. Contamination levels was assessed by both enzyme-linked immunosorbent assay (ELISA) and Ultra High-Performance Liquid Chromatography – Fluorescence Detection (UPLC-FD) method.

3.10.1 ELISA Method

ELISA analysis was done using a commercially available Helica Aflatoxin B_1 ELISA kit following the manufacturer's protocol. Based on information offered by the manufacturer, the quantification limits of the ELISA kits were 1 and 20µg/kg or parts per billion (ppb). Accuracy test for ELISA Kits was conducted by determining the value of R^2 (coefficient of determination) for the regression of the optical density readings of the reaction standards which should be 0.995 to 1.000. Two reference samples with known aflatoxin levels, 5ppb (3-7ppb) and 21ppb (17-25ppb) and blank solution (70% Methanol) were used to validate the results obtained. Only results that passed the accuracy test and could be validated using reference and blank samples were accepted as true. Samples whose aflatoxin values fell below these ranges were considered lacking any detectable Aflatoxin B_1. Maize samples with contamination levels above the quantification limit of the ELISA Kit (20ppb) were diluted further. Those that could not yield any results using ELISA after dilution were assessed using UPLC-FLD.

3.10.2 UPLC-FLD Method

For Ultra High-Performance Liquid Chromatography (UPLC) analysis, 0.5ml methanolic extract supernatant was transferred into a 2 ml centrifuge tube and diluted with 0.5 ml of 1% acetic acid prior to centrifugation at 13000 RPM for 5 minutes. A total of 0.7ml of the diluent was then transferred into HPLC vial for analysis using Ultra High-Performance Liquid Chromatography as described in Section 3.9 above. For quality control, certified reference maize samples (17, 25 and 273ppb) and blank solvents (1:1 Mixture of 70% Methanol and 1%Acetic Acid) were included in the analysis procedure. Reference maize samples were obtained from Office of the Texas State Chemist -Aflatoxin Proficiency Testing in Eastern and Central Africa program (APTECA), Chiromo Campus, Nairobi, Kenya Accuracy of the reported aflatoxin were acceptable if the determined values of the reference materials were within the 3±SD in the control chart of each analytical run.

3.11 Data Analysis

All statistical evaluations were done using R software package version 3.4.4. Aflatoxin B_1 contamination analysis was done using one-way ANOVA and the difference of means analysed using Turkey T-test. The sequences were trimmed, assembled and edited using CLC Main Workbench 8.0.3 and then compared with the sequence database in the GenBank (http://www.ncbi.nlm.nih.gov/). Sequence alignment and corrections were done using MEGA 7. Redundant sequences were removed using Jalview software to get unique sequences. Phylogenetic trees based on Maximum

Likelihood were constructed based on the best model using both MEGA 7 and R software package.

CHAPTER FOUR

4.0 RESULTS

4.1 Characterization of *Aspergillus flavus* Isolates

4.1.1 Cultural Characterization

Aspergillus section *Flavi* isolates (*n*=258) were identified in the maize samples (n=120) from the three regions of Kenya. These isolates had yellow to green colonies with white mycelia at the edges and cream reverse on PDA (Figure 4-1).

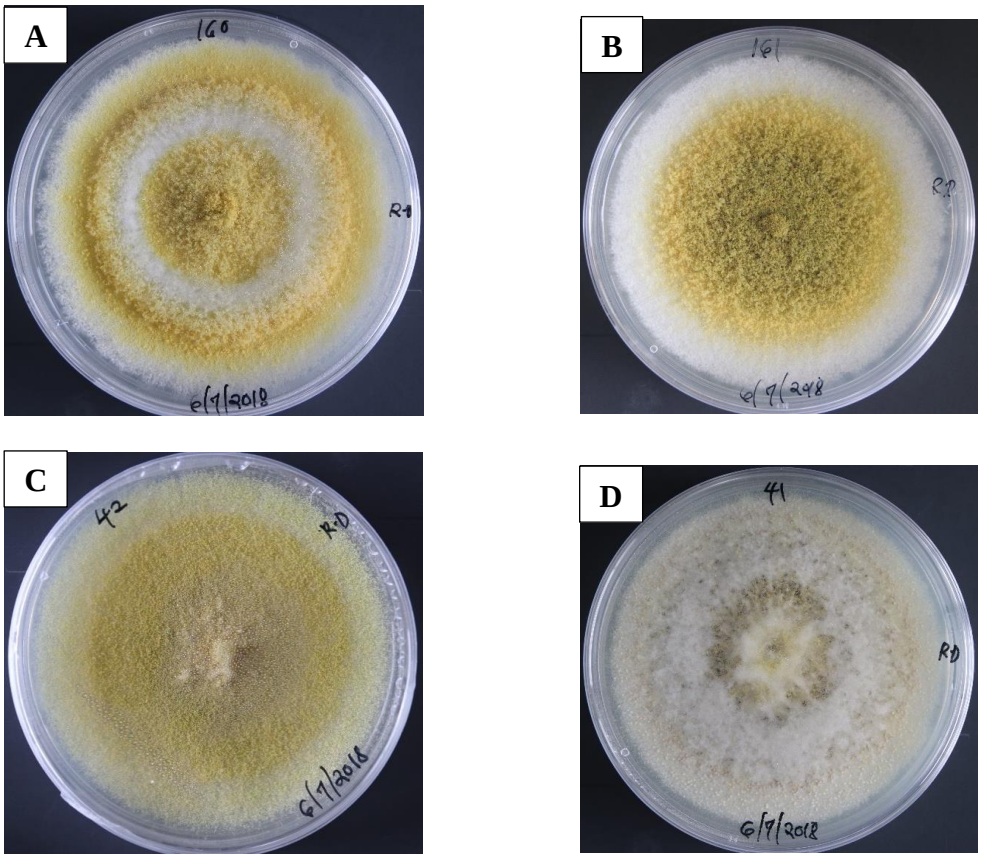

Figure 4-1: *Aspergillus flavus* isolates grown on Potato Dextrose Agar for 5 days at 28ºC showing mycelia and spores.

Based on an orange appearance of the colonies in the reverse of a petri-place containing the selective Aspergillus Flavus Parasiticus Agar (AFPA) medium, the majority (218/258, 84%) of the isolates were putatively identified as *A. flavus* (Figure 4-2). Owing to the scope of this study, isolates that were not culturally identified as *A. flavus* were dropped from the subsequent analyses. Out of these, 22, 6, 12, 59, 20, 33, 50 and 26 were recovered from the maize samples from Rachuonyo, Machakos, Makueni, Kitale, Meru, Bungoma, Kilifi, and Nandi respectively. The distribution of the putative *A. flavus* isolates, in increasing order, across the three major regions of sample collection in Kenya were as follows: Eastern (35/218, 16%), Coast (48/218, 22%) and western (135/218, 62%).

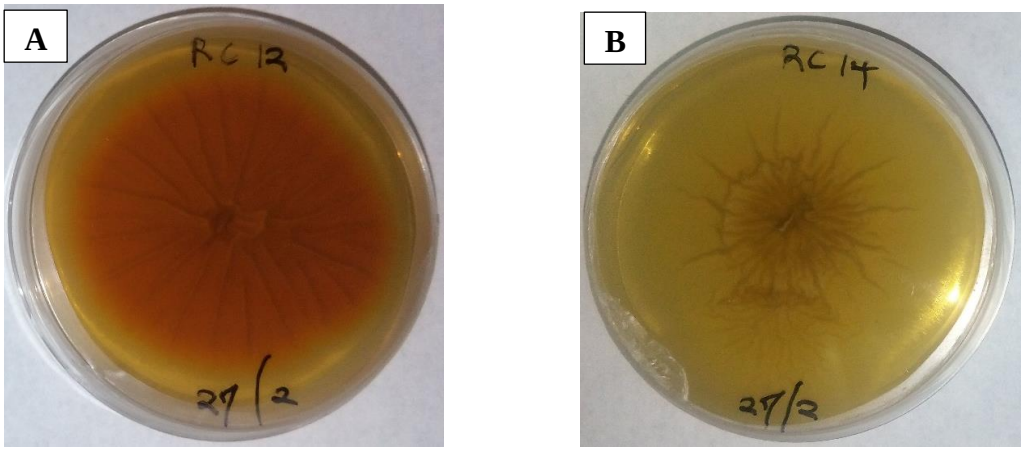

Figure 4-2: Reverse appearance of *Aspergillus* section *Flavi* isolates grown on Aspergillus Flavus Parasiticus Agar (AFPA). A: positive isolate (Orange reverse color); B: negative isolate (yellow reverse color)

4.1.2 Molecular Characterization

4.1.2.1 DNA Quality and Concentration

The DNA extracted from 221 *Aspergillus flavus* isolates were intact with good concentration as shown in figure 4-3. The concentrations ranged from 26-221ng/µl. The 230/260 ratio ranged from 1.6-2.1 and 260/280 ratio from 1.6 to 1.9.

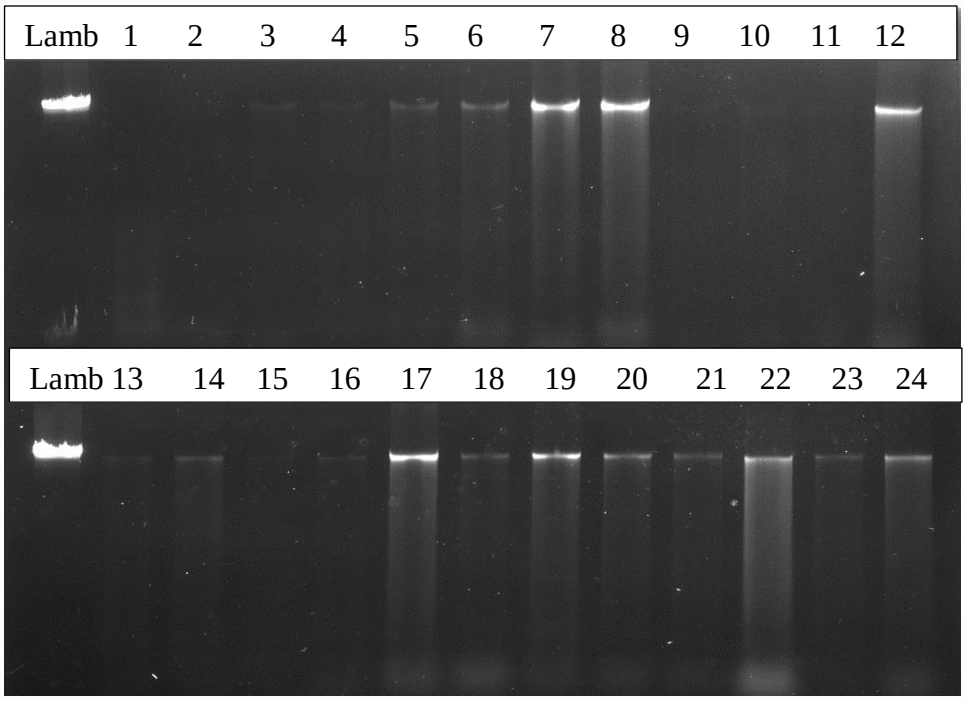

Figure 4-3: Gel image for genomic DNA of *Aspergillus flavus* isolates run in 0.8% Agarose Gel electrophoresis for 45 minutes and viewed under ultra violet light.

4.1.2.2 Identification by ITS Region Gene Marker

Polymerase Chain Reaction amplification of DNA of *Aspergillus flavus* isolates revealed single specific bands of 550 to 650pb when PCR product were run in 1.5% Gel electrophoresis (Figure 4-4). All ITS region sequence of 221

A. flavus isolates were good and assembled when analyzed. After sequence alignment, a fragment containing 593 nucleotides was used for analysis. The identities inferred from NCBI Database revealed 3 isolates as *Aspergillus parasiticus* (100%). Ninety-nine percent of the sequenced isolates (n=218) were identified as *A. flavus*. *A. parasiticus* isolates were eliminated since the study focused on *A. flavus* only. Only 10 unique sequences were obtained after removing redundant sequences and named as cluster 1 to 10. Seven clusters had only 1 isolate each. A total of 186 *A. flavus* isolates had similar ITS sequences. Two clusters had 11 and 14 isolates with similar sequences (Table 4-1).

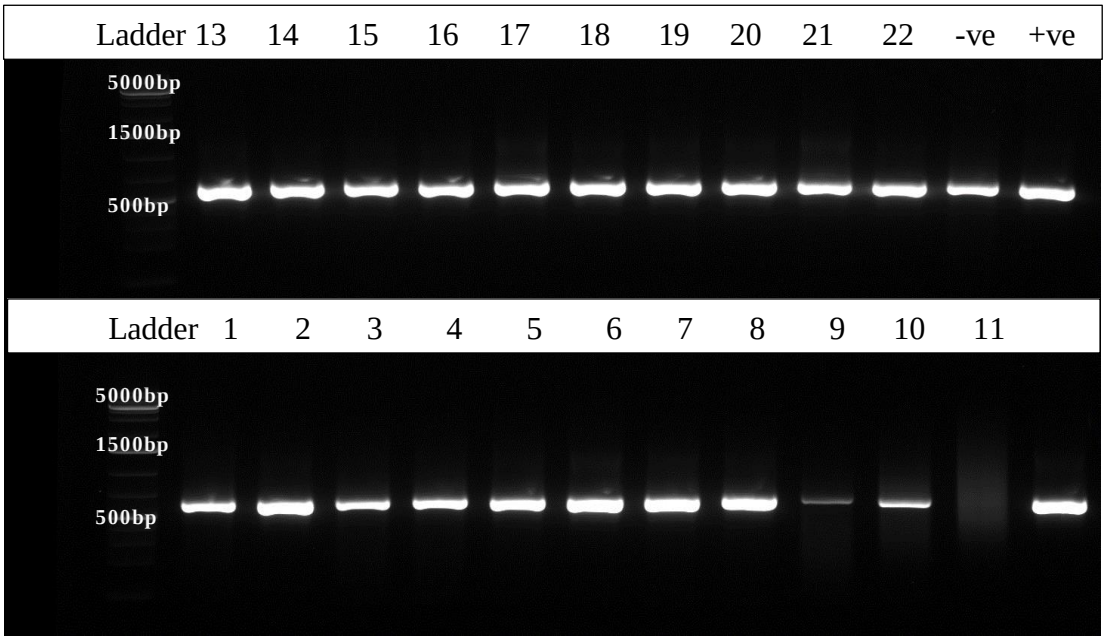

Figure 4-4: Gel image of ITS PCR products of *Aspergillus flavus isolates* in 1.5% Agarose Gel electrophoresis captured under ultra violet light.

Table 4-1: Clades of *Aspergillus flavus* isolates based on Internally Transcribed Spacer (ITS) region sequences.

Cluster	Isolate ID	Total Isolates	NCBI Identity
1	26	1	*A. flavus*
4	42	1	*A. flavus*
2	167	1	*A. flavus*
3	199	1	*A. flavus*
5	16, 160, 181, 21, 216, 219, 22, 224, 225, 25, 36, 39, 52, 64	14	*A. flavus*
6	161	1	*A. flavus*
7	104, 105, 106, 118, 120, 4, 5, 60, 62, 63, 72	11	*A. flavus*
8	1, 10, 100, 101, 102, 103, 107, 108, 109, 11, 110, 111, 112, 113, 114, 115, 116, 117, 119, 12, 121, 122, 123, 124, 125, 126, 127, 128, 129, 13, 130, 131, 132, 133, 134, 137, 138, 139, 14, 140, 141, 142, 145, 146, 147, 148, 149, 15, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 162, 163, 164, 165, 168, 17, 170, 171, 172, 173, 174, 179, 18, 182, 183, 184, 185, 186, 187, 188, 189, 19, 190, 191, 192, 193, 194, 195, 196, 197, 198, 2, 20, 200, 201, 202, 203, 204, 205, 207, 208, 209, 210, 211, 212, 213, 214, 215, 217, 218, 220, 221, 222, 223, 226, 227, 228, 229, 23, 230, 231, 232, 233, 234, 235, 24, 28, 29, 3, 30, 31, 32, 33, 34, 35, 37, 38, 40, 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 6, 61, 65, 66, 67, 68, 69, 7, 70, 71, 73, 74, 75, 76, 77, 78, 79, 8, 85, 86, 87, 88, 89, 9, 90, 91, 92, 93, 95, 96, 97, 98, 99	186	*A. flavus*
9	206	1	*A. flavus*
10	135	1	*A. flavus*

Out of 593 nucleotides (starting from sequence TTAAGTTCAGCGGGTATCCC) used in the analyses, only 30 nucleotide positions were informative. There was only one insertion/deletion observed, the rest were single nucleotide polymorphisms (SNPs). Most of the polymorphisms observed (n=13; 43.33%) were identified in isolate 135 (cluster 10) only. Isolate 206 (cluster 9) only had 3 polymorphisms and shared 5 SNPs

with isolate 135. The remaining 216 isolates in 8 clusters exhibited polymorphism in only 9 nucleotide positions. ITS region was found to be more conserved in most nucleotide positions of these isolates (Table 4-2). Phylogenic analyses of the 10 unique ITS sequences (clusters) in MEGA 7 using Maximum Likelihood based on Tamura 3-parameter model [Tamura, 1992] showed a variation among *A. flavus* isolates. Cluster 10 (Isolate 135) formed most different from other clusters as seen from its genetic distance. Cluster 1, 2, 3, 4, 5, 6, 7 and 8 were different but with close origin to each other. Cluster 9 is closer to cluster 10 than other clusters (Figure 4-5).

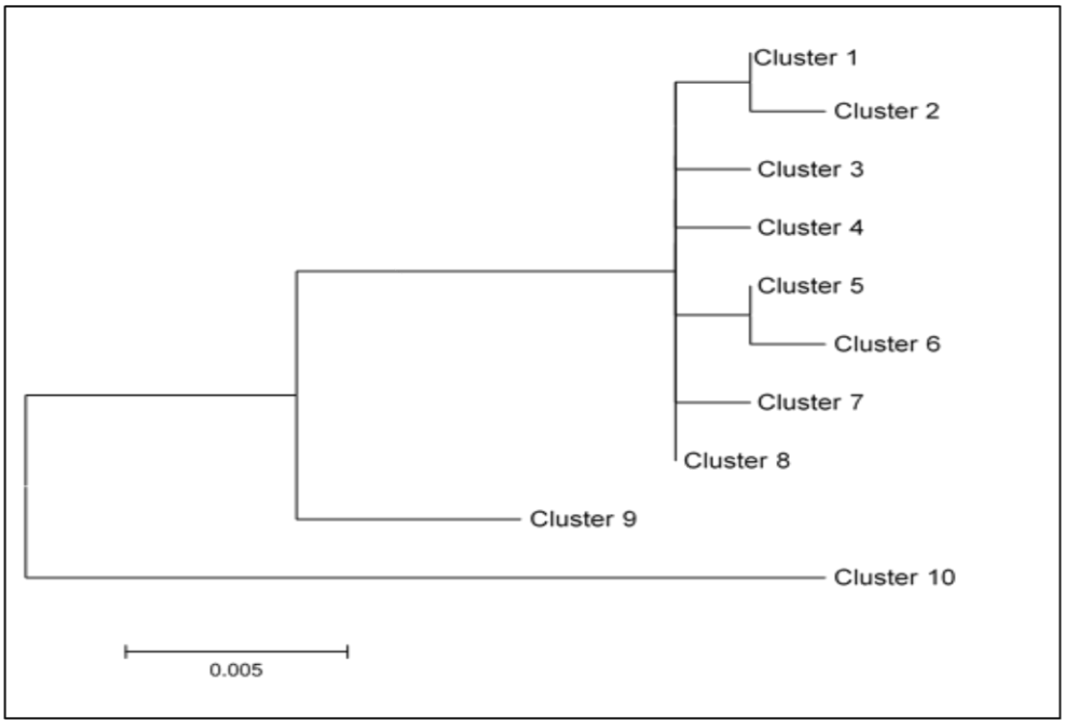

Figure 4-5: Genetic relationship of isolates of *Aspergillus flavus* from three regions of Kenya**,** as characterized using sequences of the internal transcribed spacer (ITS) region. Dendrogram was derived using Tamura 3-parameter model in MEGA 7

Table 4-2: *Aspergillus flavus* isolates showing nucleotide substitutions, deletions and insertions on ITS gene region

Isolate Cluster Groups	Position and type of SNP observed	Total No. of SNPS
Cluster 1	77: Substitution of C with A	1
Cluster 2	77: Substitution of C with A 87: Substitution of G with C	2
Cluster 3	38: Substitution of A with C 48: Insertion of A	2
Cluster 4	490: Substitution of C with A	1
Cluster 6	63: Substitution of C with T 466: Substitution of T with C	2
Cluster 7	29: Substitution of C with T 399: Substitution of T with C	2
Cluster 8	466: Substitution of T with C	1
Cluster 9	122, 164, 175, 391, 406, 450 and 587: Substitution of G with A 133: Substitution of G with C	8
Cluster 10	164, 172, 175, 266, 274, 344, 346, 376, 391, 401, 406, 410, 483, and 587: Substitution of G with A 189, 333, 452 and 486: Substitution of G with C	18

4.1.2.1 *Aspergillus flavus* Isolates Selected for Further Analyses

Seventy-two representative isolates were selected for further analysis to reduce number of duplicate sequences. For the 7 clusters that had only one isolate, the isolate was selected regardless of its location. The clusters with more than one sample had their selection done on basis of the region and initial maize sample from which the *A. flavus* was isolated. Only one isolate was picked randomly

for further analyses in maize samples that had more than one isolate belonging in the same cluster (Table 4-3).

Table 4-3: *Aspergillus flavus* isolates selected for further analysis based on ITS Clusters and geographical locations.

Cluster	Cluster total	Samples	Region	Regional total
1	1	26	Kilifi	1
2	1	167	Kitale	1
3	1	199	Kitale	1
4	1	42	Kilifi	1
5	10	22, 25, 36, 39	Kilifi	4
		52, 64	Rachuonyo	2
		160, 181	Kitale	2
		216, 225	Nandi	2
6	1	161	Kitale	1
7	6	4	Kilifi	1
		60, 62,	Rachuonyo	2
		72	Machakos	1
		104, 120	Bungoma	2
8	49	6, 8, 15, 28, 38, 41, 44	Kilifi	7
		51, 56, 59, 61, 65, 66, 71	Rachuonyo	7
		73, 74, 76	Machakos	3
		78, 85, 86, 88, 92, 96, 102	Meru	7
		103, 107, 109, 117, 122, 124, 128	Bungoma	7
		137, 138, 141, 145, 231	Makueni	5
		147, 152, 156, 157, 165, 179, 185	Kitale	7
		207, 210, 218, 223, 226, 228	Nandi	6
9	1	206	Nandi	1
10	1	135	Bungoma	1

4.1.2.2 Analyses of Variation using Partial Calmodulin gene marker

Polymerase Chain Reaction amplification of DNA of *Aspergillus flavus* isolates revealed single specific bands of 650 to 750pb when PCR product were run in 1.5% Gel electrophoresis (Figure 4-6).

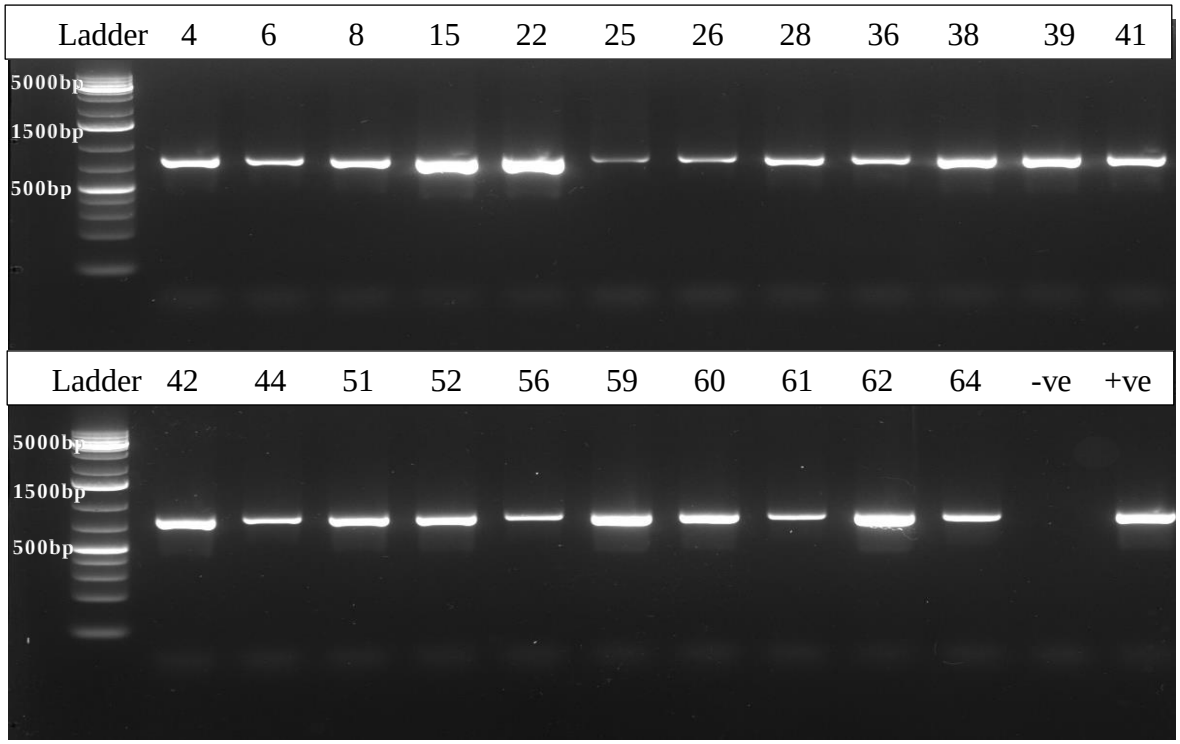

Figure 4-6: Gel image of Calmodulin PCR products in 1.5% Agarose Gel electrophoresis

Figure 4-7 and Table 4-4 and 4-5 present the genetic variation observed among the putative *Aspergillus flavus* isolates based on partial Calmodulin sequences. All Calmodulin gene region sequences of 72 *A. flavus* isolates were good and assembled when analyzed. After sequence alignment, a fragment containing 720 nucleotides (starting from the sequence; CACCATTTTTACAGCCGCAA) was used for analysis. Thirty characters were informative among the nucleotides used in analyses of Calmodulin sequences. Three nucleotides

positions showed indels, SNPs were observed in the rest (Table 4-4 and 4-5). Calmodulin gene sequences showed wider variation than ITS gene sequences. There was a total of 17 unique sequence as compared to 10 of ITS. Calmodulin gene sequence separated the isolates initially identified as *A. flavus* with ITS region into *A. flavus* and *A. minisclerotigenes*.

Generally, *A. minisclerotigenes* had more variants (SNPs) than *A. flavus* within the region. Overall, there were six SNPs that could potentially differentiate between *A. minisclerotigenes* from *A. flavus*. Based on these sequences, only two SNPs at positions 424 and 701 within the partial calmodulin gene, could discriminate clearly between the two species (Table4-6).

A total of 87.5% of the isolates (n=63) were identified as *Aspergillus flavus* using NCBI Database. Nine out of seventy-two isolates (12.5%) were identified as *Aspergillus minisclerotigenes*. The data showed two major clades, namely *A. flavus* formed one clade *A. minisclerotigenes* forming the second clade. Several subclades were observed in the clade of *A. flavus* indicating a wide variation among *Aspergillus flavus* species. *A. flavus* isolates were distributed diverse in all regions. Seven (7) *A. minisclerotigenes* isolates originated from Eastern Kenya (Meru and Makueni) with only two isolated from other regions (Kilifi and Rachuonyo). Phylogenetic tree with highest log likelihood (-1222.81) for 72 *A. flavus* isolates using Maximum Likelihood method has been shown (Figure 4-7).

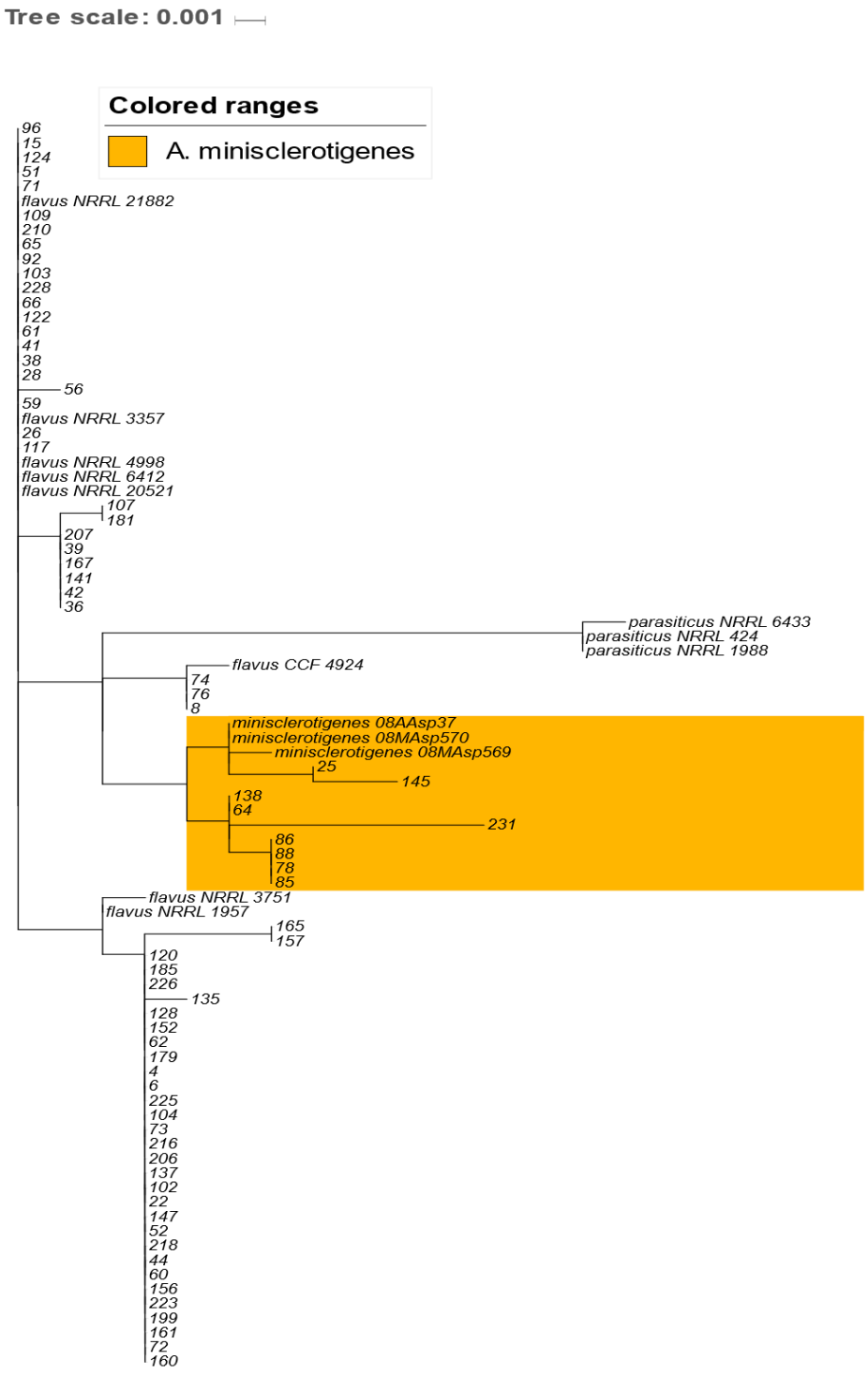

Figure 4-7: Phylogenetic tree of calmodulin sequences for *Aspergillus flavus* isolates drawn to scale with representative samples obtained from NCBI Database. Orange color represent the *Aspergillus minisclerotigenes* clade

Table 4-4: *Aspergillus flavus* isolates showing nucleotide positions with single nucleotide polymorphism and their total numbers on Calmodulin gene region

NCBI identity	Isolates	Nucleotide position and Polymorphism type	Total No of SNPs
A. flavus	8, 76, 74	105, 256 and 532: Substitution of G with A 144: Substitution of A with G	4
A. flavus	73, 4, 44, 52, 6, 60, 62, 72, 225, 223, 22, 218, 216, 206, 199, 185, 179, 161, 160, 156, 152, 147,137, 128, 120, 102, 104	222 and 533: Substitution of G with A 543: Substitution of A with T 557: Insertion of A	4
A. flavus	181, 107	16: Substitution of C with G 447: Substitution of G with A	2
A. flavus	165, 157	30, 33, 222 and 530: Substitution of G with A 34: Insertion of G 35: Substitution of C with T 541: Substitution of A with T 557: Insertion of A	8
A. flavus	42, 141, 167, 207, 36, 39	16: Substitution of C with G	1
A. flavus	135	222 and 531: Substitution of G with A 542: Substitution of A with T 557: Insertion of A 576: Substitution of A with C	5
A. flavus	56	508: Substitution of T with A 607: Insertion of A	2

Table 4-5: *Aspergillus minisclerotigenes* isolates showing nucleotide positions with single nucleotide polymorphism and their total numbers on Calmodulin gene region

Isolate ID	Nucleotide position and Polymorphism observed	Total No of SNPs
145	2: Substitution of A with C 39, 255, 303: Substitution of G with A 144 and 424: Substitution of A with G 701: Substitution of T with A 672: Substitution of T with C 15: Substitution of C with T	9
25	2: Substitution of A with C 39, 205 and 303: Substitution of G with A 144 and 424: Substitution of A with G 701: Substitution of T with A	7
231	39 and 255: Substitution of G with A 144 and 424: Substitution of A with G 701: Substitution of T with A 203 and 218: Substitution of C with T 243 and 466: Substitution of C with G 344 and 437: Substitution of G with T	11
138, 64	144 and 424: Substitution of A with G 255: Substitution of G with A 701: Substitution of T with A 466: Substitution of C with G	5
88, 86, 85, 78	144 and 424: Substitution of A with G 255 and 691: Substitution of G with A 701: Substitution of T with A 466: Substitution of C with G	6

Table 4-6: Positions of the calmodulin gene showing two SNPs that can discriminate between *Aspergillus minisclerotigenes* from *Aspergillus flavus* isolates from three regions of Kenya

Nucleotide position in Calmodulin sequence	39	144	424	466	691	701
A. minisclerotigenes	A(3)/G(6)	G	G	G(7)/C(2)	A(4)/G(5)	A
A. flavus	G	A(60)/G(3)	A	C	A	T

4.1.2.3 Analysis of ITS and Calmodulin encoding Regions Combined

When ITS region and partial Calmodulin sequences were aligned and merged, the resultant sequence was 1,313 nucleotides. To obtain a better resolution in the assessment of the relationships between the two species, phylogenetic analyses of the isolates (n=72) was conducted using the combined sequences. The phylogeny grouped the isolates into 27 genetic clades (herein after named alleles) compared to 10 and 13 obtained from ITS and Calmodulin sequences respectively. Based on this analysis, *A. minisclerotigenes* formed a unique clade together with three *A. flavus* isolates. Two of the isolates that grouped together with *A. minisclerotigenes* were from Eastern Kenya. The clade with *A. minisclerotigenes* had 78% (7 out of the 9) isolates that originated from Eastern region. Tracing back on the root of genetic divergence, *A. minisclerotigenes* formed a clade with three other *A. flavus* isolates (Sample 8, 74 and 76) but in different subclades. Two of these *A. flavus* isolates came from Eastern Kenya (Machakos). The remaining clades had only *A. flavus* isolates showing more variability and were distributed across all eight regions (Figure 4-8).

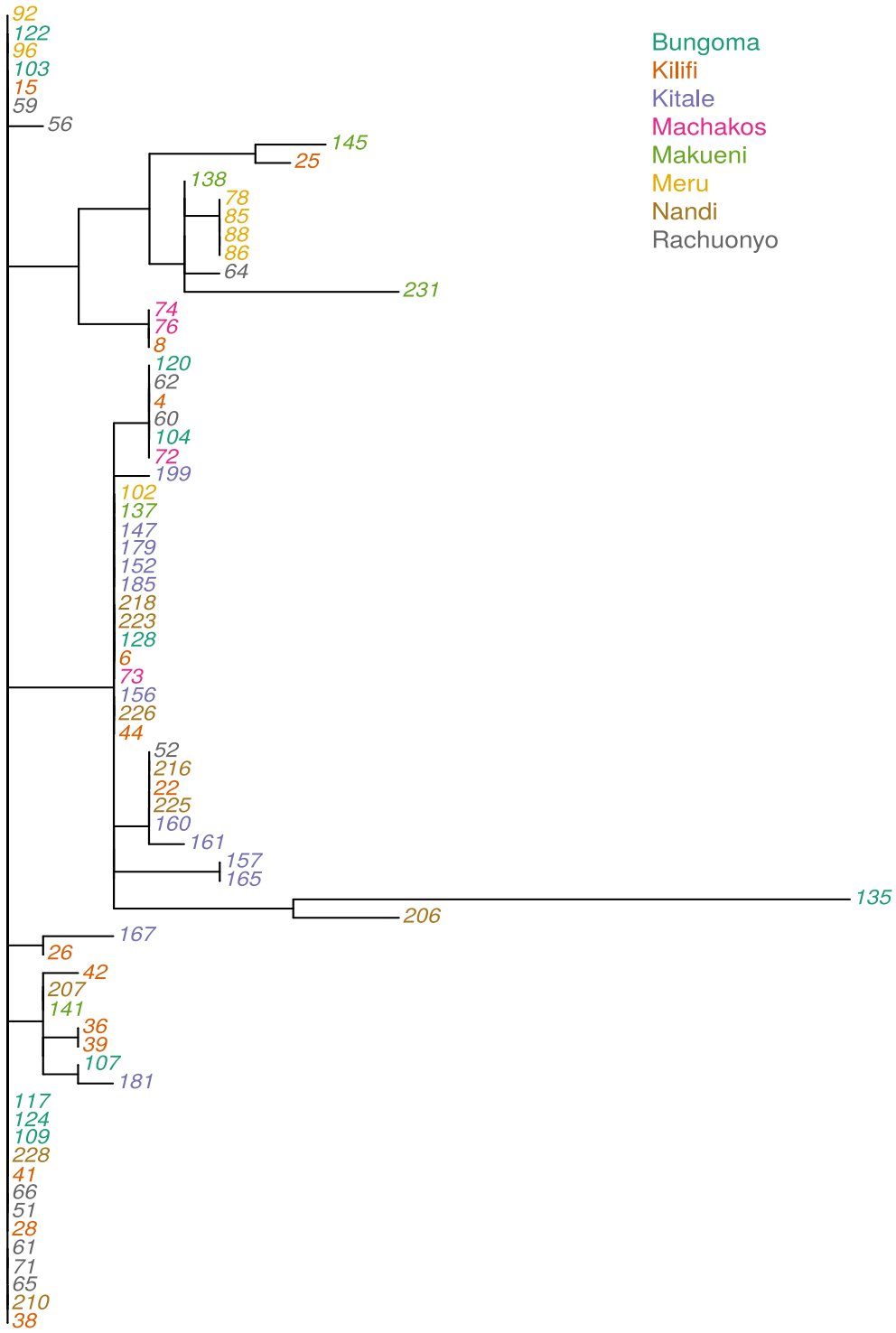

Figure 4-8: Phylogenetic tree of joined sequences of ITS and Calmodulin showing *Aspergillus flavus* isolates and the regions

The distinct alleles (named in a range from 1 to 27) derived from the combined sequences were called and used for further phylogenetic analyses (Figure 4-9). Most of the isolates that had clustered together in individual ITS and Partial Calmodulin gene phylogenetic analyses were separated using the combined sequences. Genetic distances varied for the isolates with Allele 27 having highest genetic divergence. Alleles 12, 20 and 21 contained isolates which were more similar than the rest of the groups. Number of isolates in each allele differed. Allele 20 had the most (17) *A. flavus* isolates, 17 out of 27 alleles had only one isolate (Table 4-7). Genetic distances varied for the isolates with Allele 27 have highest genetic divergence. Allele 12, 20 and 21 had the smallest genetic distances (Figure 4-9).

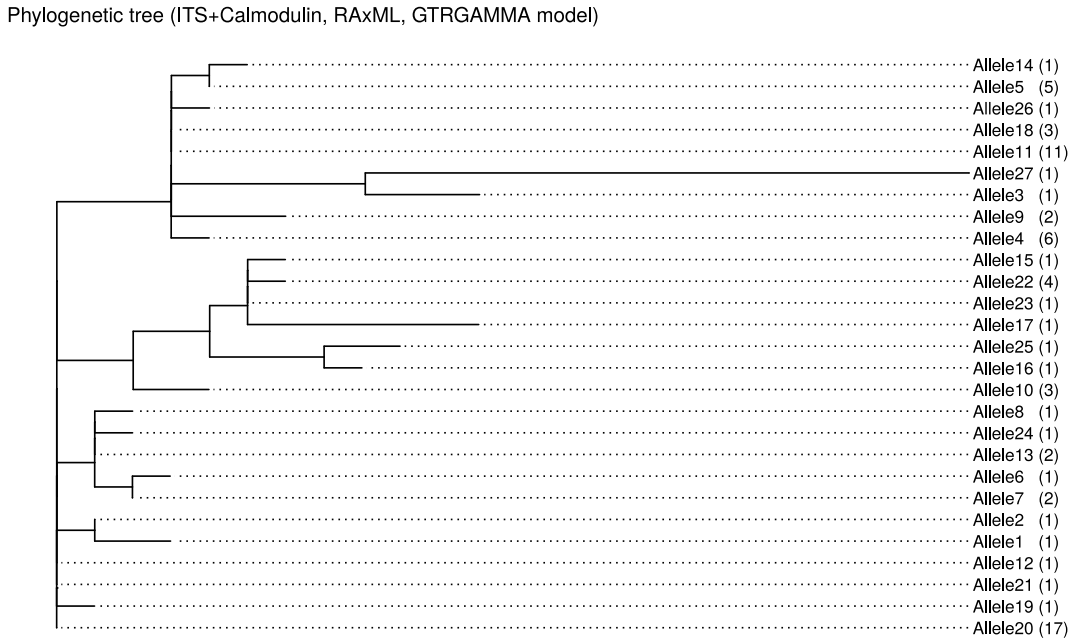

Figure 4-9: Phylogenetic tree of the different alleles observed using joined ITS and Calmodulin sequences with the total count of sequences bearing the allele indicated in brackets.

Table 4-7: *Aspergillus flavus* isolates belonging in each allele and their identities from NCBI GenBank Database using Calmodulin sequences

Allele	Samples	Identity
Allele1	167	*A. flavus*
Allele2	26	*A. flavus*
Allele3	206	*A. flavus*
Allele4	72, 62, 60, 4, 120, 104	*A. flavus*
Allele5	52, 225, 22, 216, 160	*A. flavus*
Allele6	181	*A. flavus*
Allele7	39, 36	*A. flavus*
Allele8	42	*A. flavus*
Allele9	165, 157	*A. flavus*
Allele10	8, 76,74, 73, 44, 226, 223, 218, 156, 152, 147, 137,	*A. flavus*
Allele11	128, 102	*A. flavus*
Allele12	59	*A. flavus*
Allele13	207, 141	*A. flavus*
Allele14	161	*A. flavus*
Allele15	64	*A. minisclerotigenes*
Allele16	25	*A. minisclerotigenes*
Allele17	231	*A. minisclerotigenes*
Allele18	6, 185, 179	*A. flavus*
Allele19	56	*A. flavus*
Allele20	96, 92, 71, 66, 65, 61, 51, 41, 38, 28, 228, 210, 15, 124, 122, 109, 103	*A. flavus*
Allele21	117	*A. flavus*
Allele22	88, 86, 85, 78,	*A. minisclerotigenes*
Allele23	138	*A. minisclerotigenes*
Allele24	107	*A. flavus*
Allele25	145	*A. minisclerotigenes*
Allele26	199	*A. flavus*
Allele27	135	*A. flavus*

4.2 Toxigenicity and Aflatoxin Genes in *Aspergillus flavus*

4.2.1 Analysis of Toxigenic Potential of *Aspergillus flavus* Isolates

Sample 4 was duplicated and produced similar results and the blank YESA plate did not show any value in all types of aflatoxin. The two controls

validated the accuracy of measurement. The production of each type of aflatoxin differed in all isolate. A higher correlation was observed between the production of aflatoxin B_1 and B_2 (0.9604) than aflatoxin G_1 and G_2 (0.924). Likewise, aflatoxin B_1 and total aflatoxin correlation (0.832) was higher than that of AFG_1 and Total aflatoxin (0.727). The correlation between the production of AFB_1 and AFG_1 was very low. (Table 4-8 and 4-9).

All the *Aspergillus flavus* isolates varied in the production of aflatoxins ranging from 2.5 to 110800.4ppb. A limit aflatoxin production ability (APA) of 100ppb was applied to separate highly aflatoxigenic from low aflatoxigenic isolates. Out of 72 isolates tested, 28 isolates (38.9%) were highly aflatoxigenic (APA above 100ppb). Seventeen of them produced only AFBs, 4 produced both AFBs and AFGs and 7 produced only AFGs. Seventeen (17) isolates (23.6%) had APA of above 1000ppb with 13 isolates producing only AFBs and 4 producing both AFBs and AFGs. Meru and Kilifi both had 4, Makueni and Rachuonyo had 3 each and Nandi, Bungoma and Kitale had one each. Machakos had only one highly toxigenic isolate with APA 245ppb. Thirteen isolates produced more than 10000ppb of total aflatoxin (Figure 4-10). All toxigenic isolates from Meru produced mostly AFBs and had APA above 40,000ppb. The most toxigenic strain was recovered from maize sample from Nandi (118800.4ppb) and it produced both AFBs and AFGs. A total of 14 isolates widespread in all eight regions, produced less than 20ppb of total aflatoxin (Figure 4-11).

Almost all highly toxigenic strains produced more of aflatoxin B_1 compared to other types (Figure 4-12). Contrarily, all low toxigenic strains produced higher levels of aflatoxin G_1 than the other aflatoxin types (Figure 4-13). However, some of isolates produced high amounts of both aflatoxin B_1 and G_1. All *A. minisclerotigenes* isolates produced high amounts of aflatoxins. *Aspergillus minisclerotigenes* strains recovered from Eastern Kenya (Meru and Makueni) samples produced more of Aflatoxin BI while those from other regions (Kilifi and Rachuonyo) produced more of Aflatoxin G_1 (Table 4-8and 4-9).

Table 4-8: Aflatoxin produced by *Aspergillus flavus* isolates in Yeast Extract Sucrose Agar after incubation at 28ºC for 7 days. Values reading zero indicate that the aflatoxin was not detected.

Sample ID	Isolate Identity	Region	AFB_1 (ppb)	AFB_2 (ppb)	AFG_1 (ppb)	AFG_2 (ppb)	Total Aflatoxin (ppb)	Aflatoxin Type produced
4	*A. flavus*	Kilifi	0	0	76.8	2.9	79.8	G
6	*A. flavus*	Kilifi	0.9	0	34.4	0	35.4	G
8	*A. flavus*	Kilifi	1	0.1	10.2	2.7	14.1	G
15	*A. flavus*	Kilifi	21	0.2	50.2	0	71.3	B and G
22	*A. flavus*	Kilifi	0.5	0	11.9	0	12.4	G
25	*A. minisclerotigenes*	Kilifi	8548.1	588.9	68332.5	3032.2	80501.7	G and B
26	*A. flavus*	Kilifi	15897.6	338.9	223.5	18.7	16478.7	B and G
28	*A. flavus*	Kilifi	21.9	0.1	12.3	0	34.4	G and B
36	*A. flavus*	Kilifi	298.1	10.2	0	0.1	308.5	B
38	*A. flavus*	Kilifi	2.1	0.3	101.4	0.5	104.3	G
39	*A. flavus*	Kilifi	949.1	5.4	52.4	2.4	1009.3	B and G
41	*A. flavus*	Kilifi	0	0.1	79.7	0.8	80.5	G
42	*A. flavus*	Kilifi	1002.4	8	162.8	0.8	1174	B and G
44	*A. flavus*	Kilifi	0	0	17.4	0	17.4	G
51	*A. flavus*	Rachuonyo	0.5	0.4	84.8	0.4	86	G
52	*A. flavus*	Rachuonyo	1.6	0	20.9	0	22.5	G
56	*A. flavus*	Rachuonyo	0.5	0	15.2	0	15.7	G
59	*A. flavus*	Rachuonyo	11491.6	279.4	126.5	18	11915.5	B and G
60	*A. flavus*	Rachuonyo	0	0	53.7	2.1	55.8	G
61	*A. flavus*	Rachuonyo	0.5	0.2	24.1	2	26.8	G
62	*A. flavus*	Rachuonyo	0	0	47.2	1.8	49.1	G
64	*A. minisclerotigenes*	Rachuonyo	4038.3	120.7	25651.1	606.3	30416.4	G and B
65	*A. flavus*	Rachuonyo	3155	27.2	64.3	6	3252.5	B and G
66	*A. flavus*	Rachuonyo	9.8	0.4	102.2	0.6	113	G and G
71	*A. flavus*	Rachuonyo	0.5	0	8.3	0	8.8	G
72	*A. flavus*	Machakos	0.7	0	242.1	2.1	244.9	G
73	*A. flavus*	Machakos	0	1.6	20.8	1.4	23.8	G
74	*A. flavus*	Machakos	0.6	0	89.2	3.8	93.6	G
76	*A. flavus*	Machakos	0	0	14	3.2	17.3	G
78	*A. minisclerotigenes*	Meru	40124	1706.5	0	53.4	41883.9	B
85	*A. minisclerotigenes*	Meru	77908.9	3928.6	0	144.4	81982	B and G
86	*A. minisclerotigenes*	Meru	50398.3	2496.6	5.3	85	52985.2	B
88	*A. minisclerotigenes*	Meru	49411.3	2648.8	23	63.1	52146.2	B
92	*A. flavus*	Meru	56.4	0.7	10.6	0.7	68.4	B and G
96	*A. flavus*	Meru	0	0.1	55.6	0	55.7	G
102	*A. flavus*	Meru	0	0.1	10.9	3.2	14.3	G
4repeat	*A. flavus*	Kilifi	0	0	76.8	2.9	79.7	G
Blank			0	0	0	0	0	None

Table 4-9: Aflatoxin produced by *Aspergillus flavus* isolates in Yeast Extract Sucrose Agar after incubation at 28ºC for 7 days. Values reading zero indicate that the aflatoxin was not detected.

Sample ID	Isolate Identity	Region	AFB_1 (ppb)	AFB_2 (ppb)	AFG_1 (ppb)	AFG_2 (ppb)	Total Aflatoxin (ppb)	Aflatoxin Type produced
103	*A. flavus*	Bungoma	497.6	10.8	20.1	0	528.5	B and G
104	*A. flavus*	Bungoma	0	0	97.2	2.9	100.1	G
107	*A. flavus*	Bungoma	0	0	112.7	0	112.7	G
109	*A. flavus*	Bungoma	17.4	12.6	33.8	0.8	64.6	B and G
117	*A. flavus*	Bungoma	29283.6	430.1	16.8	29.4	29759.9	B
120	*A. flavus*	Bungoma	0	0.5	60.5	2.7	63.7	G
122	*A. flavus*	Bungoma	0	0.1	4.3	0.1	4.6	G
124	*A. flavus*	Bungoma	0	0	42.3	0	42.3	G
128	*A. flavus*	Bungoma	8.8	0.8	52.2	2.9	64.7	B and G
135	*A. flavus*	Bungoma	0.8	0.3	124.9	0	126	G
137	*A. flavus*	Makueni	0.8	0.1	21.3	2.6	24.8	G
138	*A. minisclerotigenes*	Makueni	34924.5	1474.9	0	1.6	36401	B
141	*A. flavus*	Makueni	0	0	1.9	0.6	2.5	G
145	*A. minisclerotigenes*	Makueni	24216	665.4	34.2	29.7	24945.3	B
147	*A. flavus*	Kitale	2.6	0	65	0.5	68	G
152	*A. flavus*	Kitale	0.8	0	11.9	4	16.7	G
156	*A. flavus*	Kitale	0	0.3	23.4	0.6	24.3	G
157	*A. flavus*	Kitale	1	0.1	0	5.1	6.3	G and B
160	*A. flavus*	Kitale	0	0	15.1	1.1	16.2	G
161	*A. flavus*	Kitale	0.7	0	11.2	0.1	12.1	G
165	*A. flavus*	Kitale	0	0.2	19.4	2.1	21.6	G
167	*A. flavus*	Kitale	5453.6	87.2	78.3	5.2	5624.3	B
179	*A. flavus*	Kitale	4.1	0.3	30.4	0.2	35	G and B
181	*A. flavus*	Kitale	0	0.3	40.8	2.2	43.3	G
185	*A. flavus*	Kitale	5	0.7	39.5	5.6	50.8	G and B
199	*A. flavus*	Kitale	0	0.1	32.7	2.5	35.3	G
206	*A. flavus*	Nandi	325.3	11.8	22.2	0.2	359.6	B
207	*A. flavus*	Nandi	42309.3	958.8	74098.5	1433.9	118800.4	B and G
210	*A. flavus*	Nandi	235.3	3	16.7	0.1	255	B
216	*A. flavus*	Nandi	1.7	0.1	34.5	0.1	36.5	G and B
218	*A. flavus*	Nandi	0.5	0	11.9	2.2	14.6	G
223	*A. flavus*	Nandi	24.6	0.6	23.9	4.3	53.3	B and G
225	*A. flavus*	Nandi	0	0	54.4	0	54.4	G
226	*A. flavus*	Nandi	4.7	0.7	47.7	0.1	53.2	G and B
228	*A. flavus*	Nandi	0	0.3	118.7	1	120	G
231	*A. minisclerotigenes*	Makueni	10052.7	351.5	19	8.3	10431.5	B

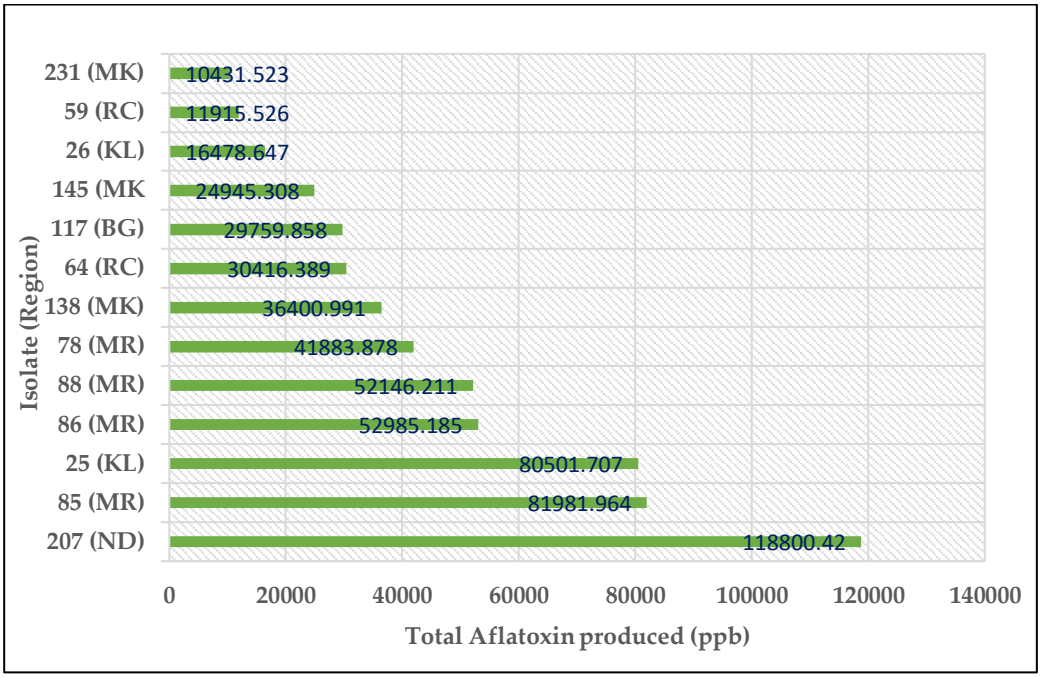

Figure 4-10: *Aspergillus flavus* isolates with toxigenic potential above 10000ppb in Yeast Extract Sucrose Agar. (The abbreviations after the isolate I.D represent the region from which the fungus was isolated. ND =Nandi, MR=Meru, KL=Kilifi, Mk=Makueni, RC=Rachuonyo).

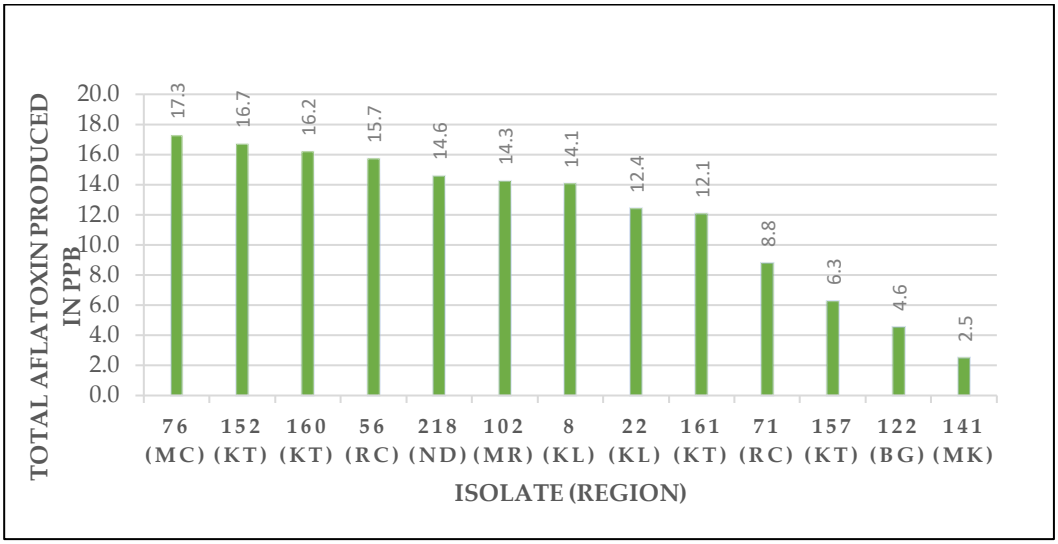

Figure 4-11: A graph of *Aspergillus flavus* isolates with lowest toxigenic potential (The abbreviations after the isolate identification number represent the region from which the fungus was isolated. MC=Machakos, KT=Kitale, RC=Rachuonyo, ND =Nandi, MR=Meru, KL=Kilifi, BG=Bungoma, MK=Makueni).

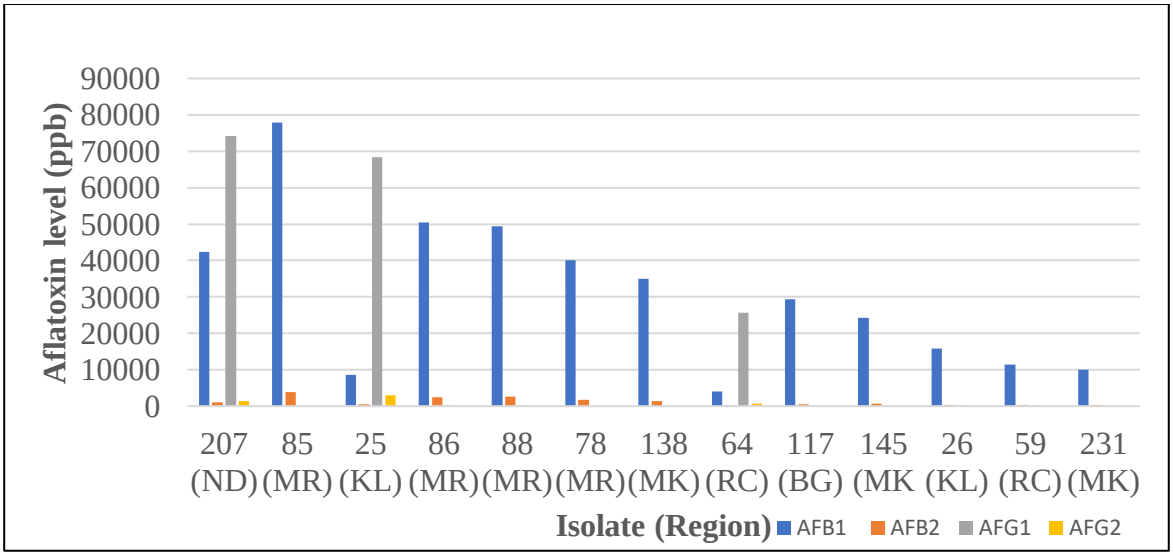

Figure 4-12: Aflatoxin types produced by high aflatoxigenic *Aspergillus flavus* isolates**.** (The abbreviations after the isolate identification number represent the

region from which the fungus was isolated. ND=Nandi, MR=Meru, KL=Kilifi, MK=Makueni, RC=Rachuonyo, BG=Bungoma).

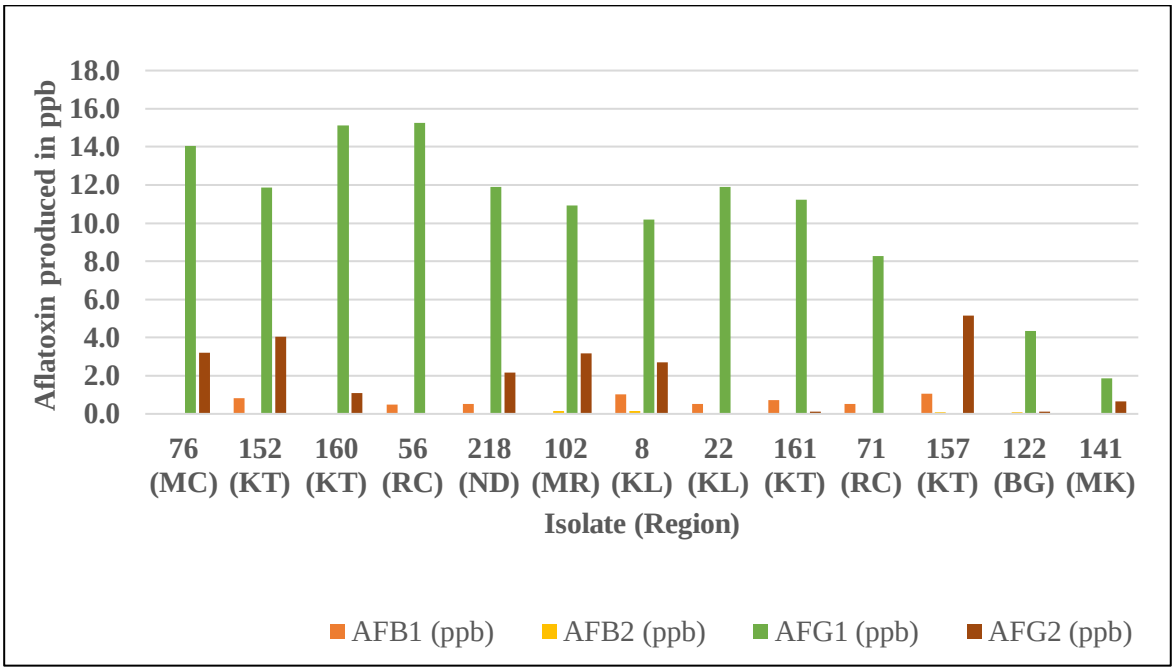

Figure 4-13: Aflatoxin types produced by low aflatoxigenic *Aspergillus flavus* isolates. (The abbreviations after the isolate identification number represent the region from which the fungus was isolated. MC=Machakos, KT=Kitale, RC=Rachuonyo, ND=Nandi, MR=Meru, KL=Kilifi, BG=Bungoma, MK=Makueni)

4.2.2 Toxigenicity and Sequence Data Relationship

When toxigenic potential was compared between species, a higher aflatoxin production potential was observed in *A. minisclerotigenes* (mean, 45743.7ppb; median, 41883.9ppb) compared to *A. flavus* (mean, 3048.9ppb; median, 54.4). All *A. minisclerotigenes* isolates produced high amounts of aflatoxins ranging from 10431.5 to 81982 ppb. *Aspergillus flavus* varied greatly in production of

the aflatoxin with most of them producing low quantities of aflatoxins. The production of total aflatoxin was significantly higher (P= 4.28e-06) in *A. minisclerotigenes* as compared to *A. flavus* (Figure 4-14)

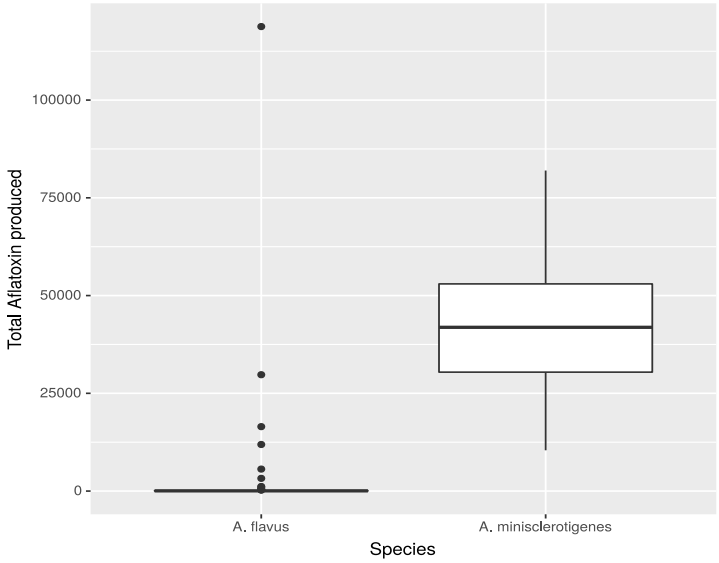

Figure 4-14: A boxplot showing the comparison of the total Aflatoxin production potential between *Aspergillus flavus* and *Aspergillus minisclerotigenes*

Even though most of the *A. minisclerotigenes* originated from Eastern, the region from which the *Aspergillus* isolate was recovered did not significantly contribute the production of aflatoxin (Figure 4-15).

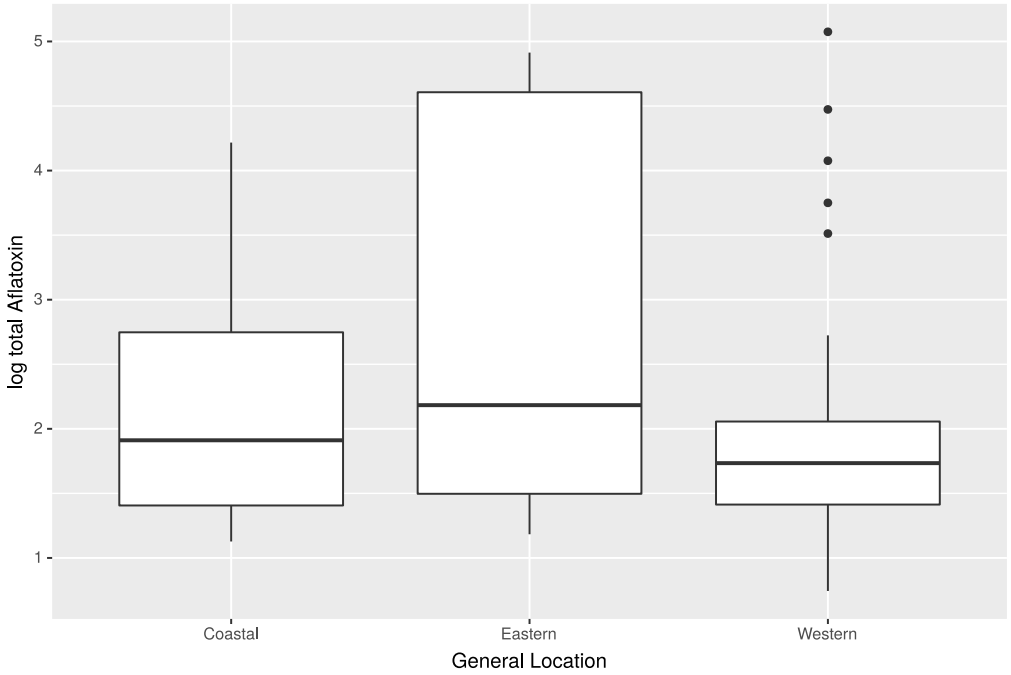

Figure 4-15: A boxplot presentation of the effect of the region from which the aspergillus isolate was recovered to the production of total aflatoxin. Total aflatoxin produced have been logarithmically transformed using $\log_{10}X$

The alleles obtained from combined ITS and partial calmodulin sequences were related with the geographical region and toxigenicity potential data. It was observed that some alleles had only isolates with high toxigenic potential whereas other had only low toxigenic isolates. Some alleles had isolates with both high and low toxigenicity. Allele 15, 22, 23, 17, 25 and 16 had only isolates with high toxigenicity whereas Allele 11 had 11 isolates with low toxigenicity. Allele 13 had 2 isolates, one with highest toxigenicity (118,800.4µg/Kg) and another one with lowest toxigenicity (2.5 µg/Kg). The comparison was done for total aflatoxin (Figure 4-16) and aflatoxin B_1 (Figure 4-17) that is known to be carcinogenic.

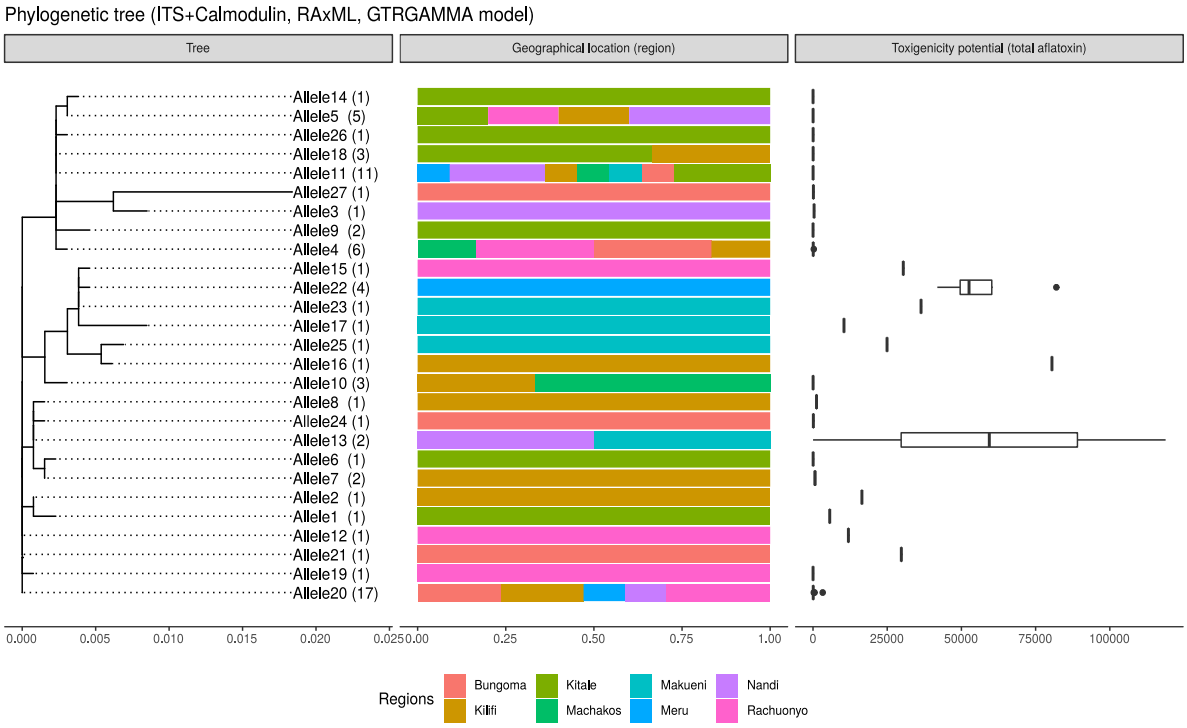

Figure 4-16: Diagrammatic presentation of the comparison between distinct alleles obtained from combined ITS and Calmodulin sequences of the *Aspergillus flavus* population, their geographical location and quantity of Total aflatoxin they produced

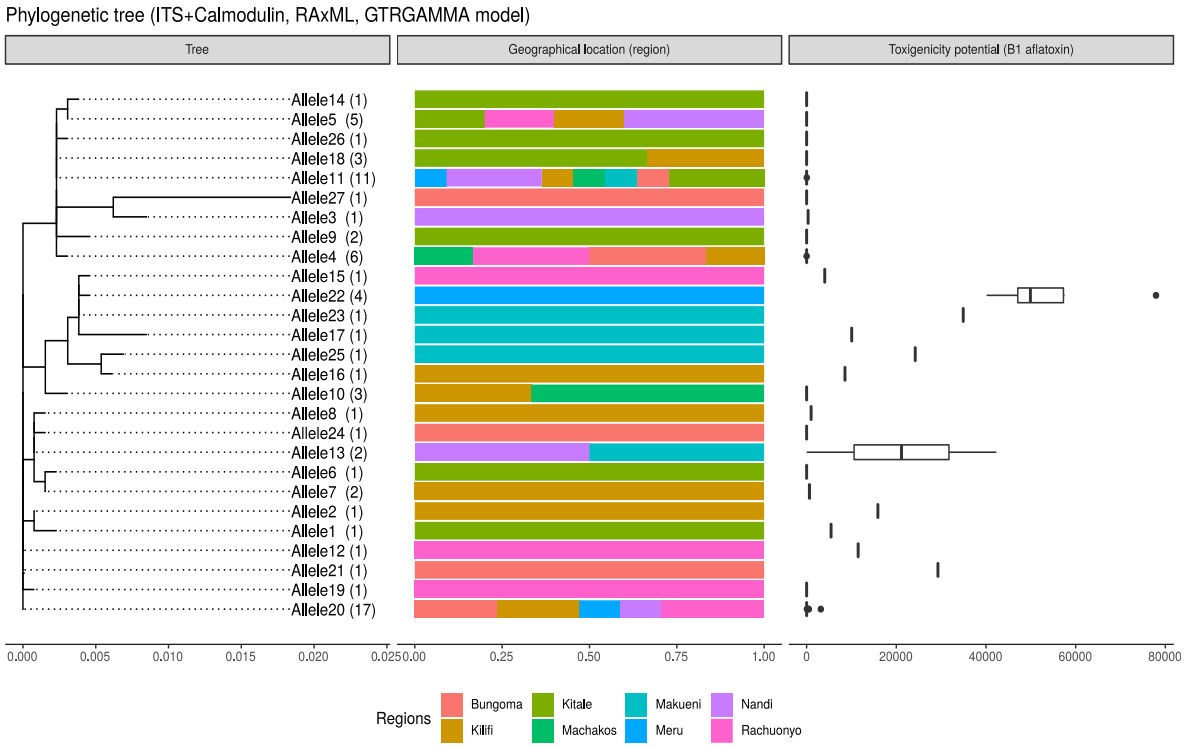

Figure 4-17: Diagrammatic presentation of the comparison between distinct alleles obtained from combined ITS and Calmodulin sequences of the *Aspergillus flavus* population, their geographical location and quantity of aflatoxin B_1 they produced

Similarities of isolates was assessed based on allelic diversity, inferred by counting the number of different alleles represented in each region. Diversity of the isolates was highest in Western (n=23) followed by Eastern (n=11) and least in Coastal (n=10). Observed allelic diversity ranged from 3 to 10 with Kilifi having 10 and Machakos and Meru having 3 out of 27 total alleles observed. Focusing on specific regions, the regions from Eastern Kenya generally had fewer alleles as compared to other. (Figure 4-18).

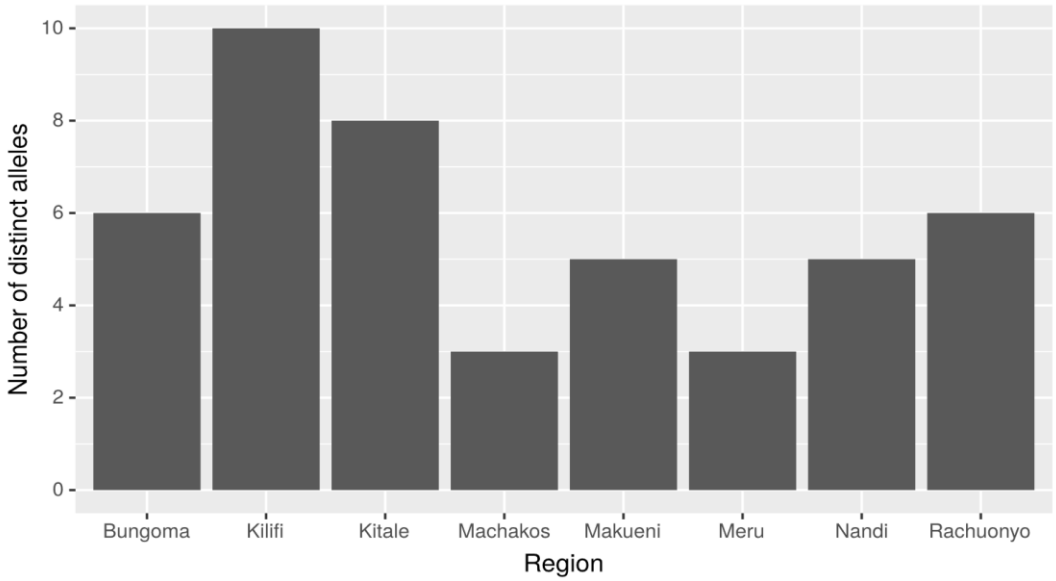

Figure 4-18: A comparison of number of distinct alleles observed from the combined ITS and Calmodulin sequences of *Aspergillus flavus* isolates by geographical region.

4.2.3 Screening for Aflatoxin Biosynthetic Pathway Genes

The tables 4-10 and 4-11 presents Aflatoxin biosynthesis genes profile of *A. flavus* and *A. minisclerotigenes* isolates as revealed by PCR detection method. The PCR data placed the isolates into different groups based on number of amplicons present. Among the seven genes screened, only *aflQ* gene based on Ord1-gF/ Ord1-gR primer set was present in all the 72 isolates. Because *aflD* and *aflQ* genes were amplified using two sets of primers each, the total number of amplicons per isolates were 9. Sixty-six percent of the isolates (n=48) showed presence of all 9 amplicons. Regardless of the primer set used, 75% (n=54) confirmed the presence of the seven aflatoxin genes. The *aflD* and *aflQ* amplifications differed when different sets of primers were used to amplify the same genes. Some isolates showed presence of these genes when one set of

primers was used and absence of the same gene using a different set of primers. The Nor1-F/Nor-R primer set showed presence of *aflD* in 60 isolates compared to 51 of AflD-1/AflD-2rev. Ord1-gF/ Ord1-gR primer set showed presence of *aflQ* gene in all 72 isolates while AflQ-1/AflQ-2rev primer set showed presence in only 63 isolates. Regulatory genes; *afl*R and *afl*S were present in 58 and 56 *A. flavus* isolates respectively (Table 4-10 and 4-11).

Table 4-10: Aflatoxin biosynthesis genes profile of *Aspergillus flavus* isolates. Presence of gene through PCR band detection denoted by positive (+) and absence by negative (-) sign. The abbreviations after the isolate identification number represent the region from which the fungus was isolated. KL=Kilifi, RC=Rachuonyo, MC=Machakos, MR=Meru)

Isolate Identity	aflD (aflD-1/aflD-2rev primers)	aflD (Nor1-F and Nor-R primers)	aflM	aflO	aflP	aflQ (AflQ-1/AflQ-2rev primers)	aflQ (Ord1-gF/ Ord1-gR primers)	aflR	aflS	Sum of amplicons
4(KL)	+	+	+	+	+	+	+	+	+	9
6(KL)	-	-	+	+	+	+	+	-	-	5
8(KL)	+	+	+	+	+	+	+	+	+	9
15(KL)	-	+	+	+	+	+	+	-	-	6
22(KL)	+	+	+	+	+	+	+	+	+	9
25(KL)	-	+	-	+	+	+	+	-	-	5
26(KL)	+	+	+	+	+	+	+	+	+	9
28(KL)	+	+	+	+	+	+	+	+	+	9
36(KL)	+	+	+	+	+	+	+	+	+	9
38(KL)	-	-	-	-	-	-	+	-	-	1
39(KL)	+	+	+	+	+	+	+	+	+	9
41(KL)	-	-	-	+	+	+	+	-	-	4
42(KL)	+	+	+	+	+	+	+	+	+	9
44(KL)	+	+	+	+	+	+	+	+	+	9
51(RC)	-	-	-	-	+	-	+	+	-	3
52(RC)	+	+	+	+	+	+	+	+	+	9
56(RC)	-	-	-	+	+	+	+	-	-	4
59(RC)	+	+	+	+	+	+	+	+	+	9
60(RC)	+	+	+	+	+	+	+	+	+	9
61(RC)	-	+	+	+	+	+	+	+	+	8
62(RC)	+	+	+	+	+	+	+	+	+	9
64(RC)	-	+	+	+	+	+	+	-	+	7
65(RC)	+	+	+	+	+	+	+	+	+	9
66(RC)	+	+	+	+	+	+	+	+	+	9
71(RC)	-	-	-	+	+	+	+	-	-	4
72(MC)	+	+	+	+	+	+	+	+	+	9
73(MC)	+	+	+	+	+	+	+	+	+	9
74(MC)	+	+	+	+	+	+	+	+	+	9
76(MC)	+	+	+	+	+	+	+	+	+	9
78(MR)	+	+	+	+	+	+	+	+	+	9
85(MR)	+	+	+	+	+	+	+	+	+	9
86(MR)	-	+	+	+	+	+	+	+	+	8
88(MR)	+	+	+	+	+	+	+	+	+	9
92(MR)	+	+	+	+	+	+	+	+	+	9
96(MR)	-	-	+	+	+	+	+	+	-	6
102(MR)	+	+	+	+	+	+	+	+	+	9

Table 4-11: Aflatoxin biosynthesis genes profile of *Aspergillus flavus* isolates. Presence of gene through PCR band detection denoted by positive (+) and absence by negative (-) sign. The abbreviations after the isolate identification number represent the region from which the fungus was isolated MK=Makueni, BG=Bungoma, KT=Kitale, ND=Nandi)

Isolate Identity	aflD (aflD-1/aflD-2rev primers)	aflD (Nor1-F and Nor-R primers)	aflM	aflO	aflP	aflQ (AflQ-1/AflQ-2rev primers)	aflQ (Ord1-gF/ Ord1-gR primers)	aflR	aflS	Sum of amplicons
103(BG)	+	+	+	+	+	+	+	+	+	9
104(BG)	+	+	+	+	+	+	+	+	+	9
107(BG)	+	+	+	+	+	+	+	+	+	9
109(BG)	+	+	+	+	+	+	+	+	+	9
117(BG)	+	+	+	+	+	+	+	+	+	9
120(BG)	+	+	+	+	+	+	+	+	+	9
122(BG)	-	-	-	-	-	-	+	-	-	1
124(BG)	-	-	+	+	+	+	+	+	-	6
128(BG)	+	+	+	+	+	+	+	+	+	9
135(BG)	+	+	+	+	+	+	+	+	+	9
137(MK)	+	+	+	+	+	+	+	+	+	9
138(MK)	+	+	+	+	+	+	+	+	+	9
141(MK)	-	+	+	+	+	-	+	+	-	6
145(MK)	+	+	+	+	+	+	+	-	+	8
147(KT)	+	+	+	+	+	+	+	+	+	9
152(KT)	+	+	+	+	+	+	+	+	+	9
156(KT)	+	+	+	+	+	+	+	+	+	9
157(KT)	-	-	+	+	+	-	+	-	-	4
160(KT)	+	+	+	+	+	+	+	+	+	9
161(KT)	+	+	+	+	+	-	+	+	+	8
165(KT)	-	-	+	+	+	-	+	-	-	4
167(KT)	+	+	+	+	+	+	+	+	+	9
179(KT)	+	+	+	+	+	+	+	+	+	9
181(KT)	+	+	+	+	+	+	+	+	+	9
185(KT)	-	+	+	+	+	+	+	+	+	8
199(KT)	+	+	+	+	+	+	+	+	+	9
206(ND)	+	+	+	+	+	-	+	-	-	6
207(ND)	-	+	+	+	+	+	+	+	+	8
210(ND)	+	+	+	+	+	+	+	+	+	9
216(ND)	+	+	+	+	+	+	+	+	+	9
218(ND)	+	+	+	+	+	+	+	+	+	9
223(ND)	+	+	+	+	+	+	+	+	+	9
225(ND)	+	+	+	+	+	+	+	+	+	9
226(ND)	+	+	+	+	+	+	+	+	+	9
228(ND)	-	-	-	-	-	-	+	-	-	1
231(ND)	-	+	+	+	+	+	+	+	+	8

There was no common relationship between the production of aflatoxin and presence of the 7 genes, different observations were made when relating aflatoxin genes and aflatoxin production potential (Table 4-12). Some isolates had all the seven genes and produced aflatoxins while other isolates lacked one or more genes still produced aflatoxins. Category A has isolates that showed presence of all 7 aflatoxin biosynthetic pathway genes but produced low concentrations of aflatoxins. Category B is composed of isolates that lack one or more of the AF genes but still produced high quantities of aflatoxins. Category C has the isolates that lacked one or several AF genes and produced low quantities of aflatoxins. Category D has the isolates that confirmed the presence of 7 aflatoxins genes through PCR detection and produced high quantities of aflatoxins.

Table 4-12: Categories of relationships between aflatoxin produced in Yeast Extract Sucrose Agar media and presence of aflatoxin biosynthesis genes in *Aspergillus flavus* population

Category	Sample Identity	Geographical Location	Total AF genes present	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total Aflatoxin
	22	Kilifi	7	0.51	0	11.90	0	12.412
	8	Kilifi	7	1.03	0.14	10.18	2.72	14.06
A	**102**	Meru	7	0	0.15	10.92	3.19	14.259
	218	Nandi	7	0.53	0	11.89	2.15	14.572
	160	Kitale	7	0	0	15.12	1.08	16.194
	25	Kilifi	4	8548.15	588.89	68332.48	3032.19	80501.71
	206	Nandi	5	325.33	11.77	22.21	0.25	359.56
B	**228**	Nandi	1	0	0.29	118.71	1.01	120.00
	38	Kilifi	1	2.14	0.31	101.36	0.50	104.31
	51	Rachuonyo	3	0.48	0.37	84.75	0.40	86.01
	165	Kitale	4	0	0.174	19.37	2.074	21.616
	56	Rachuonyo	3	0.47	0	15.24	0	15.718
C	**71**	Rachuonyo	3	0.51	0	8.29	0	8.803
	157	Kitale	4	1.04	0.089	0	5.14	6.272
	122	Bungoma	1	0	0.089	4.338	0.123	4.55
	85	Meru	7	77908.94	3928.64	0	144.39	81981.96
	88	Meru	7	49411.28	2648.80	22.98	63.15	52146.21
D	**78**	Meru	7	40124.01	1706.49	0	53.37	41883.88
	138	Makueni	7	34924.52	1434.88	0	41.59	36400.99
	117	Bungoma	7	29283.56	430.10	16.79	29.40	29759.86

The risk factor analysis of individual genes on the production of aflatoxin was assessed using stepwise regression. Since the individual aflatoxin produced by the *A. flavus* isolates ranged from 0 to 77908.937 μgkg^{-1}, the data was logarithmically transformed (Log10 (x+1). The best model for predictor genes that had influence on toxigenicity of the isolates selected by Akaike information criterion (AIC) regression has been shown (Table 4-13). It was observed that *aflD* based on Nor1-F/Nor-R set of primers (*afl*D") and *aflS* were always significant predictors whose presence increased aflatoxin Bs and total aflatoxin production levels (p value <0.05). The combined interaction of *afl*D"

and *afl*R in *A. flavus* isolate had a negative effect on the production of total aflatoxins (p=0.028). The other genes did not show significant influence the toxigenicity of the isolates. However, at a higher significant level, (p<0.1), the presence of *afl*D based on AflD-1/AflD-2rev primers decreased the production of total aflatoxins which was opposite of the effect of the same gene when amplified by Nor1-F/Nor-R set of primers.

Table 4-13: Association between the aflatoxin biosynthetic genes and the aflatoxin production potential in Kenyan *Aspergillus flavus* and *Aspergillus minisclerotigenes* isolates. Gene effects were compared in a stepwise regression model and the best model was selected based on Akaike information criterion

Risk Factor	Coefficients	Std Error	t-value	Significance P Value
*afl*D'	-0.8736	0.4439	-1.968	0.053
*afl*D''	3.4093	1.162	2.934	0.005
*afl*M	-0.239	0.6628	-0.361	0.720
*afl*R	0.4392	0.7546	0.582	0.563
*afl*S	2.4061	0.7943	3.029	0.004
*afl*D'' and *afl*M	-2.109	1.4374	-1.467	0.147
*afl*D'' and *afl*R	-2.2789	1.0133	-2.249	0.028
Intercept	1.4965	0.423	3.538	0.001

By coding the genes as presence (1) and absence (0) and concatenating the resultant numbers (in the order: aflD (aflD-1/aflD-2rev primers), aflD (Nor1-F and Nor-R primers), aflM, aflO, aflP, aflQ (AflQ-1/AflQ-2rev primers), aflQ (Ord1-gF/ Ord1-gR primers), aflR and aflS), to have one number for each isolate, 16 gene combinations were obtained. Ten combinations had one *Aspergillus* isolate each, while the remaining six combinations, which were analyzed further, had more than one isolate. The analysis of effects of gene

group combinations on the production of aflatoxin potential showed that isolates with gene combination of group E had highest capacity of aflatoxin production (10,470.3 μgkg^{-1}). Isolates in group C had least aflatoxin production potential of 11.9 μgkg^{-1} (Table 4-14, Figure 4-19).

Table 4-14: Aflatoxin biosynthesis genes combination having more than one *Aspergillus* isolate

Gene effect groups	Concatenation of genes	No. of *Aspergillus flavus* Isolates bearing the genes	Median Aflatoxin Production Log10(1+X)	Median Aflatoxin Production (μgkg^{-1})
A	000000001	3	2.02	103.713
B	000110011	3	1.22	15.596
C	001110001	2	1.11	11.882
D	001111011	2	1.69	47.978
E	011111111	5	4.02	10470.285
F	111111111	48	1.82	65.069

* genes ordered as follows: aflD (aflD-1/aflD-2rev primers), aflD (Nor1-F and Nor-R primers), aflM, aflO, aflP,aflQ (AflQ-1/AflQ-2rev primers), aflQ (Ord1-gF/ Ord1-gR primers), aflR and aflS). Appearance of codes 1 or 0 denotes the gene that is present or absent respectively in the concatenation

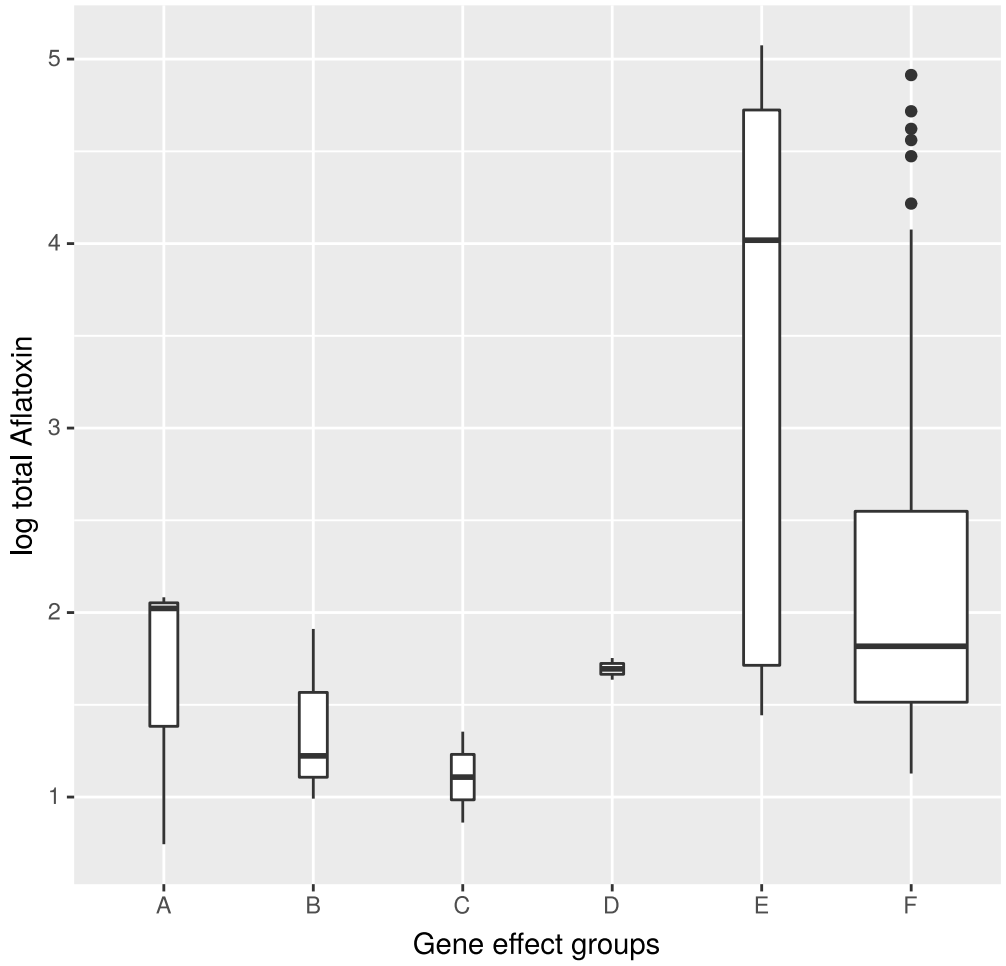

Figure 4-19: Effect of different groups of aflatoxin biosynthesis gene combination on the production of total aflatoxin in *Aspergillus flavus* and *Aspergillus minisclerotigenes* isolates

4.3 Maize Contamination Level of Aflatoxin B_1

Among 120 maize samples from eight regions of Kenya, 12% (n=14) were contaminated above the regulatory limit (> 10 ppb). Most (88%) of the samples (n=106) were not contaminated with Aflatoxin B_1 above the regulatory limit. The highest percentage of maize contaminated Aflatoxin B_1 was observed in Kilifi (n=7; 46.67%) (Figure 4-20). Highly contaminated samples came from

Meru with contamination levels of 9052 and 8902 parts per billion. All the samples from Nandi and Machakos were not contaminated above the regulatory limit. (Table 4-15).

Table 4-15: Aflatoxin B_1 contamination levels in maize samples assayed by Enzyme linked Immunosorbent Assay and Ultra High-Performance Liquid Chromatography-Fluorescence Detection

Region	Rachuonyo	Bungoma	Kitale	Nandi	Machakos	Makueni	Meru	Kilifi
Sample contamination in (ppb)	9.6	12.3	1.6	1.1	0.3	19.5	1.4	17.0
	1.2	6.9	0.9	1.5	0.7	0.7	1.6	16.0
	6.7	1.2	1.2	0.9	1.4	5.2	1.5	284.4
	2.1	1.2	0.6	1.0	3.9	0.7	9052.4	182.9
	0.9	2.3	1.7	1.8	1.9	0.9	1.5	1.4
	2.1	5.7	1.1	0.6	2.1	0.2	1.9	4.4
	1.2	1.0	1.9	1.9	1.2	0.6	1.4	713.2
	1.6	1.0	2.0	2.0	1.1	2.4	1.4	119.6
	7.9	2.0	2.1	1.6	1.3	1.2	2.3	7.2
	1.1	1.7	64.0	1.6	0.8	1.9	1.6	4.6
	1.3	1.5	17.1	0.7	3.0	1.6	8902.4	1.8
	18.5	1.2	1.0	1.4	1.2	1.6	1.9	1.9
	1.4	1.5	2.1	1.3	1.4	1.1	1.4	628.0
	2.2	2.0	0.8	1.7	1.8	0.9	0.8	1.9
	1.0	5.4	1.5	1.7	1.8	1.1	0.6	1.0
Range	0.9-18.5	1.0-12.6	0.6-64	0.6-2.0	0.3-3.9	0.2-19.5	0.6-9052.4	1.0-713.2
% <1ppb	6.67	0.00	20.00	20.00	20.00	40.00	13.33	0.00
% 1-10ppb	86.67	93.33	66.67	80.00	80.00	53.33	73.33	53.3
% >10ppb	6.67	6.67	13.33	0.00	0.00	6.67	13.33	46.67

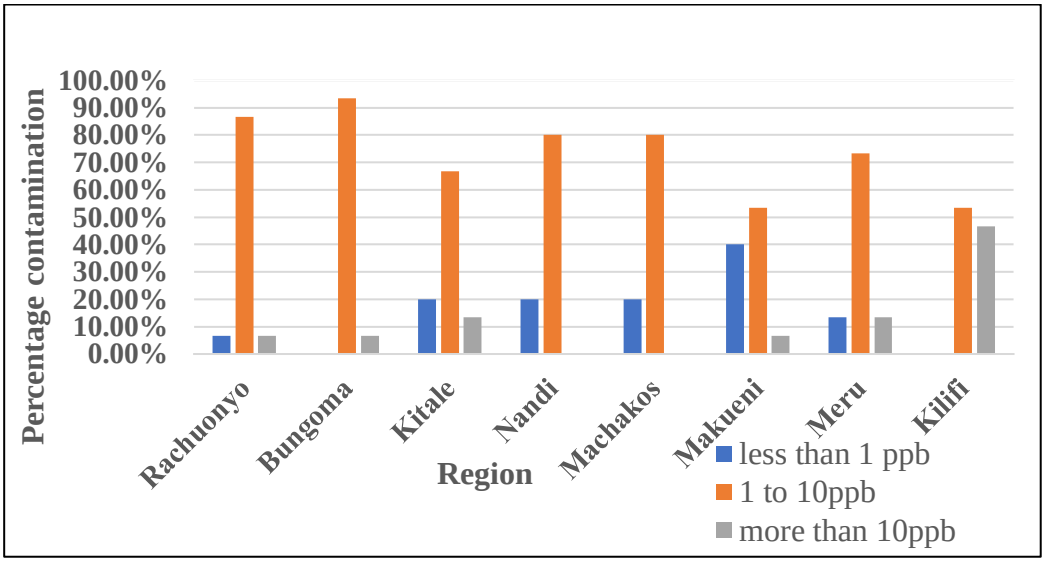

Figure 4-20: Comparison of percentage aflatoxin B_1 contamination of maize sampled from eight regions of Kenya showing those contaminated below 1 part per billion, between 1 to 10 parts per billion and above the accepted level (10ppb)

The overall range of contamination was 0.3 to 9052 ppb. The outliers came from Kilifi and Meru. The raw data was not continuous to allow for direct analysis and therefore was transformed using Log_{10} transformation. To avoid negative logarithm transformed data on contamination values that are less than one, we used $\log_{10}$ (1+X) transformation. There was no significant difference (p=0.000578) among aflatoxin contamination levels in regions of Western Kenya (Rachuonyo, Bungoma, Kitale and Nandi) and Eastern Kenya (Machakos and Makueni and Meru). Aflatoxin B_1 contamination levels in Kilifi was significantly different from all other regions except Meru. Meru contamination level was not significantly difference from all the regions (Table 4-16).

Table 4-16: Log_{10} (1+X) transformed means of Aflatoxin B_1 contamination of maize sampled from the eight regions under the study

Region	Mean of Aflatoxin B_1 (Log10)
Bungoma	0.53 ± 0.06a
Kilifi	1.29 ± 0.24b
Kitale	0.52 ± 0.11a
Machakos	0.39 ± 0.03a
Makueni	0.41 ± 0.07a
Meru	0.86 ± 0.32ab
Nandi	0.37 ± 0.02a
Rachuonyo	0.55 ± 0.08a
$F_{(7,112)}$	4.023
p-value	0.000578

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

A population identified as *Aspergillus flavus* at first using cultural AFPA characteristics was separated into *A. flavus* and *A. minisclerotigenes* using two molecular markers. Cultural features were important but not enough for characterization of the *Aspergillus flavus*. According to Rodrigues *et al.*, (2009), integration of cultural and morphological features, aflatoxin production profile and molecular characterization is necessary to achieve full characterization. All *A. minisclerotigenes* originated from Eastern Kenya except two that came from Western and Coastal Kenya. *A. flavus* was common across all the regions. These findings indicate that *A. flavus* and *A. minisclerotigenes* are spatially distributed. Okoth *et al* (2018) found similar results and attributed them to evolutionary origins differences between the two species.

The partial calmodulin gene marker revealed wider variation among *A. flavus* than ITS gene marker. It separated the isolates identified as *A. flavus* using ITS marker into *A. flavus* and a few *A. minisclerotigenes*. ITS region was found to be more conserved than Calmodulin gene encoding region. More genetically informative positions were found in Calmodulin sequence. Phylogeny of combined ITS and Calmodulin sequences showed wider variation than the two markers separate. It revealed 3 *Aspergillus flavus* isolates closely related to *A. minisclerotigenes* forming a clade distinct from other *A. flavus* strains. Two of

the 3 *A. flavus* isolates were from Eastern Kenya. Allele distribution per region showed that *A. flavus* was very diverse. Isolates from Eastern Kenya were less diverse compared to other regions hence low recombination and genetic variability in the aflatoxin gene cluster (Olarte *et al.*, 2012). This could support the existence of high aflatoxigenic strains in this region.

All the *Aspergillus flavus* isolates produced aflatoxins but in different levels that ranged between 2.5 to 118,800.4 µg/Kg. The aflatoxin production potential of *Aspergillus flavus* isolates was highly variable implying great possibility of genetic recombination among toxigenic and atoxigenic strains belonging to this species (Taylor *et al.*,1999). High aflatoxigenic strains of *Aspergillus flavus* were distributed across the regions. This can be attributed to the high genetic variability, aflatoxin heritability, and recombination in the aflatoxin gene cluster (Olarte *et al.*, 2012). The unit of quantification of aflatoxin produced by *A. flavus* isolates is very important in drawing the line between aflatoxigenic and non-aflatoxigenic isolates. In other studies (Fakruddin *et al.*, 2015; Youssuf *et al.*, 2016), aflatoxin producing ability of *Aspergillus* species have been quantified in µg/g. I have quantified aflatoxins in µg/Kg and found all the isolates to be aflatoxigenic.

It is normally known that not every *Aspergillus flavus* strain is capable of producing aflatoxins, and those that are normally produce aflatoxins B_1 and B_2 only (Klich, 2007). However, this study found some isolates of *Aspergillus flavus* that produced both AFBs and AFGs. For instance, sample 65 and 207 were confirmed to be *Aspergillus flavus* but they produced both AFBs and

AFGs contrary to the general rule. Okoth *et al.*, (2012) found similar results. Even though some isolates produced AFBs and AFGs, the production of AFBs was higher in general hence the higher correlation between the total AF and AFBs than Total AF and AFGs. Some *A. flavus* that produced only AFGs but in lower levels. On other hand, *A. minisclerotigenes* have been classified to produce both AFB's and AFG's (Pildain *et al.*, 2008). This was not the only case in this study, *A. minisclerotigenes* isolate from Makueni (sample 138) produced only AFB's. Okoth *et al.*, (2018) also found *A. minisclerotigenes* isolates that produced only AFB's. Furthermore, *A. minisclerotigenes* from Eastern Kenya produced more AFB's while those from Western and Coastal Kenya produced more AFG's.

Some *A. flavus* isolates clustered together based on both ITS and Calmodulin sequences but still varied greatly in aflatoxin producing ability. For instance, sample 207 and 141 which recorded highest and lowest aflatoxin producing ability respectively all belonged to allele 13. Several studies have found that the mycotoxin producing ability of *Aspergillus flavus* is highly variable and dependent on substrate (Vaamonde *et al.*, 2003) and geographical origin (Atehnkeng *et al.*, 2008; Giorni *et al.*, 2007; Razzaghi-Abyaneh *et al.*, 2006).

PCR detection of 5 structural AF biosynthesis (aflD, aflM, aflO, aflP and aflQ) and 2 AF pathway regulation (aflR and aflS) genes could not categorically distinguish aflatoxigenic and non-aflatoxigenic *A. flavus* strains. It was expected that since all the isolates produced aflatoxins, they would all confirm the presence of 7 aflatoxin genes. However, some *A. flavus* isolates lacked one

or more of the genes. Similar results were found by Okoth *et al.,* (2018), Gallo *et al.,* (2012) and Levin (2012) who explained this to the intraspecific and interspecific genetic mutations within the targeted binding site of the primers. Some isolates had all 7 genes but with low aflatoxin producing ability. This could be due to; other defects at various protein and molecular levels (Yin *et al*., 2008) and low copy number and/or expression of one or more genes of the pathway resulting to a slowed function. Some of the isolates bore all 7 genes and were highly aflatoxigenic while other isolates lacked one or more genes and had low toxigenicity. Only aflQ based on Nor1-F/Nor-R primer set was present in all isolates hence correlated with the aflatoxigenicity data. Rodrigues *et al.*, (2009) found a correlation between aflatoxigenicity and expression of aflQ using the same primer set. Individual gene effect on total aflatoxin production revealed that only the presence of *aflD* based on Nor1-F/Nor-R primer set and *aflS* were significant predictors whose presence increased the total aflatoxin levels. Analysis of combined effect of genes based on the presence or absence of respective gene' amplicon revealed that the absence of only *aflD* based AflD-1/AflD-2rev primer set significantly alleviate the ability of isolates to produce aflatoxin.

The specificity of two sets of primers for both aflD and aflQ were also tested. It was not always possible to get consistent results using different sets of primers. The presence of aflD gene in was revealed more frequently in Nor1-F/Nor-R (80.33% of *A. flavus* isolates) compared to AflD-1/AflD-2rev (70.83%). Furthermore, AflD-1/AflD-2rev primer set showed absence of aflD gene in *A.*

flavus isolates that recorded the same with the Nor1-F/Nor-R primers but the opposite was not true. This can be due to the smaller amplicon size of Nor1-F/Nor-R (400bp compared to 852bp of AflD-1/AflD-2rev) whose amplification is more efficient than larger fragments (Harris *et al.*, 1998). Ord1-gF/ Ord1-gR showed presence of aflQ gene in all *A. flavus* isolates while AflQ-1/AflQ-2rev showed presence in only 87.5%. This can be attributed to mutational differences in binding sites of the primers (Levin, 2012).

These findings have clearly indicated that the presence of the seven screened genes in *Aspergillus flavus* is not enough to characterize aflatoxigenic and non-aflatoxigenic strains. Apparently, the inability of *A. flavus* isolate to produce aflatoxin need not to be only related to an incomplete PCR-based detection pattern of AF genes. The inactivation of genes in AF biosynthetic pathway may result from different types of mutations in some A. *flavus* strains (Geisen, 1996). Other studies (Flaherty and Payne, 1997; Chang *et al.*,1999a, 1999b; Cary *et al.*, 2002; Ehrlich *et al.*, 2003; Chang *et al.*,2007) have proposed that aflatoxin biosynthesis regulations in *Aspergillus* species consist of a complicated pattern of positive and negative acting transcriptional regulatory factors influenced by nutritional and environmental characteristics.

A total of 85% of the maize samples had detectable aflatoxin quantities and 16% contaminated above the set limit for human consumption. Approximately half of Kilifi samples were contaminated above regulation limit. This is the first aflatoxin surveillance to be done in the region. There is limited information regarding status of aflatoxin occurrence in Coastal region. As a

result, the region has suffered lack of awareness that could give way to control and better management strategies. Most two contaminated samples came Meru at levels above 8900 µg/Kg. These observations could be explained by lack of the required facility for monitoring the maize cultivated and consumed at a local level in this country (Okoth, 2016). Samples from Machakos and Makueni showed a general low contamination level with aflatoxin B_1. This was contrary to the past documented reports of aflatoxin contamination in this region (Azziz-Baumgartner *et al.*, 2005, Muthomi *et al.*, 2009, Daniel *et al.*, 2011, Mutiga *et al.*, 2014). This could be attributed to good farming practices by the farmers, biocontrol agents introduced thriving and outcompeting the aflatoxin producing strains and inability of the small sample size used to represent the population. Despite few samples being contaminated above regulatory limit, all Western Kenya regions recorded a general low aflatoxin B_1 contamination levels. The same results were reported by Mutiga *et al* (2015). The region is leading in maize production in this country and has not reported any human aflatoxicoses case (Okoth *et al.*, 2012).

The contamination of staple food such as maize is common but remains unnoticed due to lack of surveillance. This disparity calls for continuous monitoring of mycotoxins, even in regions with typically low contamination. Oloo, (2010) argued that integration of higher learning institutions in monitoring and evaluating the status of quality control and food safety through research should be considered since they have the required knowledge. Aflatoxin limit standards set by regulatory board should be made accessible to

the citizens. Currently, in Kenya, standards are not only purchased but also hard to understand unlike in other nations, where they are widely distributed to the clients free of charge (Sirma *et al.*, 2018).

5.2 Conclusion

Use of cultural features is not enough to fully characterize *Aspergillus flavus*. There exist minor differences that cannot be expressed phenotypically hence complete classification requires integration of cultural, morphological and molecular characterization. Even so, having more genetic markers is essential since it gives more accurate findings. Partial Calmodulin gene marker showed more genetic diversity in form of deletion, insertion or replacement of nucleotides than internally transcribed spacer region which appeared to me more conserved. *A. flavus* strains were diverse across all the regions in this study whereas *A. minisclerotigenes* were mostly found in Eastern Kenya (Meru and Makueni) with only one in Kilifi and Rachuonyo.

There is no isolate in our study that did not produce any aflatoxin, however the production differed in concentrations. Despite that most of the isolates had low toxigenicity potential. Aflatoxigenicity of the *Aspergillus flavus* isolates varied greatly. All *A. minisclerotigenes* isolates had high toxigenic potential. Presence of aflatoxin biosynthesis genes could not reveal toxigenic and non-toxigenic strains. However, presence of *aflD* and *aflS* significantly increases the amount of total aflatoxin production. It was always impossible to get consistent results when two different sets of primers were used to amplify

similar gene. The aflQ based on Nor1-F/Nor-R primer set was present in all isolates used in this study.

Aflatoxin B_1 contamination continues to be a threat to the staple food of Kenyans from Meru and Kilifi as our findings indicated risk of high contamination in these regions. There was a general low contamination in other regions in this study. Machakos and Nandi region did not have any sample with contamination above regulatory limit. Aflatoxin B_1 contamination levels of maize samples from Rachuonyo, Bungoma, Kitale, Nandi, Machakos, Makueni and Meru were not significantly different. Contamination levels of Kilifi maize samples were significantly different from levels of all other regions' samples except Meru.

5.3 Recommendations

The following are recommended based on the findings of this study;

- Calmodulin gene marker discriminated the strains better than internal transcribed spacer region (ITS) marker. Molecular characterization based on use of more markers should be applied to achieve high accuracy levels of classification.
- *Aspergillus minisclerotigenes* proved to be highly toxigenic. Awareness should be created to all the stake holders concerned in the management of aflatoxin contamination.
- Isolates showed absence of some of the seven aflatoxin biosynthetic pathway genes tested in this study but still produce copious amounts of

aflatoxins. Genetic loci associated with aflatoxigenicity in *Aspergillus flavus* population need to be mapped.

- Screening of all the genes in aflatoxin biosynthetic pathway genes and correlating the data with aflatoxin production ability should be done to depict more about the combination of gene effect to the aflatoxigenicity.
- Awareness creation about aflatoxin contamination and management practices and strategies.
- Kilifi region samples had highest aflatoxin contamination levels. Large-sample based surveillance of aflatoxin should be done in Coastal region especially Kilifi.

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