CHARACTERISATION OF *Aspergillus* SECTION *Flavi* AND DEVELOPMENT OF AFLATOXINS LEVEL DETECTION METHODS IN FOOD GRAINS AND POULTRY FEEDS FROM MALAYSIA AND NIGERIA

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by

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LIST OF SYMBOLS

<	Less than
>	Greater than
±	Plus-minus sign
µ	Micro
Bw	Body weight
cm	Centimeter
g	gram
Kg	Kilogram
mg	Milligram
mL	Milliliter
ng	Nanogram
β	Beta

LIST OF ABBREVIATIONS

°C	Degree Celsius
AA	*Aspergilli*c acid
ABGs	Aflatoxin biosynthesis genes
ADM	*Aspergillus* differential medium
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
aflD	Aflatoxin biosynthesis gene D
aflM	Aflatoxin biosynthesis gene M
aflP	Aflatoxin biosynthesis gene P
aflR	Aflatoxin regulator gene R
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFPA	*Aspergillus flavus-Aspergillus parasiticus* agar
AIDS	Acquired immunodeficiency syndrome
amdS	Acetamidase
ANOVA	Analysia of Variance
AOAC	*Association of Official Analytical Chemists*
ASF	*Aspergillus* section *Flavi*
ATF	Aflatoxigenic fungi
ATR-FTIR	Attenuated total reflectance – Fourier transformed infrared spectroscopy
ATR-FTIR-PLS	Attenuated total reflectance – Fourier transformed infrared spectroscopy integrated with partial least squares regression.
BenA	Beta-tubulin gene A
CA	Coconut agar
CAM	Coconut agar medium
CaM	Calmodulin gene
CFU/g	Colony forming-units per gram
CPA	Cyclopiazonic acid
CZA	Cyzepak's agar
DERA	Dietary exposure risks to aflatoxins

DMST	Demethylsterigmatocystin
DNA	Deoxyribonucleic acid
e.g.	Example
EAC	East African Community
FAO	Food and Agriculture Organisation of the United Nations
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography – diode array detector
HPLC-PDAD	High performance liquid chromatography – prominence photodiode array detector
HPLC-PFLD	High performance liquid chromatography – prominence fluorescence detector
IARC	International Agency for Research on Cancer
IBM	International Business Machines Corporation
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICMSF	International Commission on Microbiological Specifications for Foods
ITS	Nuclear ribosomal internal transcribe spacer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
Km	Kilometer
Km/h	Kilometer per hour
LGA	Local Government Area
LPCB	Lactophenol cotton blue
L-strain	Strain producing large size sclerotia
mcm7	Minichromosome maintenance protein
MEA	Malt extract agar
MGA	Malt glucose agar
MIR	Mid-Infrared
ML	Maximum likelihood
nor1	Norsolorinic reductase regulator gene 1
NS-isolates	Non-sclerotia producing isolates
OECD	Organisation for Economic Co-operation and Development
omt	O-methyl transferase gene

P	p-value
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PLS	Partial least squares regression
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
pTLC	Preparative thin layer chromatography
qTLC	Quantitative thin layer chromatography
RAPD	Random Amplified Polymorphic *DNA*
rpb1	Ribonucleic acid polymerase II, largest subunit
RP-HPLC-PFLD-PDAD	Reverse phase – High performance liquid chromatography – prominence fluorescence detector – prominence photodiode array detector
RT	Retention time
RTC	Rural Transformation Centre
SD	Standard deviation
SDA	Sabroud dextrose agar
SPSS	Statistical software for social sciences
S-strain	Strain producing small size sclerotia
TAFs	Total aflatoxins
TLC	Thin layer chromatography
US	United State
USA	United State of America
USDA	United State Department of Agriculture
UV	Ultra violet
v/v	Volume by volume ratio
VCGs	Vegetative compatibility groups
ver1	Vesicolorin dehydrogenase regulator gene 1
WHO	World Health Organisation
YES	Yeast extract sucrose agar

LIST OF APPENDICES

KARAKTER BAGI SEKSYEN *ASPERGILLUS FLAVI* SERTA PEMBANGUNAN KAEDAH PENGESANAN PARAS AFLATOKSIN DALAM BIJIAN MAKANAN DAN MAKANAN HAIWAN TERNAKAN DARI MALAYSIA DAN NIGERIA

ABSTRAK

Seksyen *Aspergillus Flavi* (ASF) dan aflatoksin adalah antara bahan pencemar dalam makanan dan makanan haiwan ternakan yang paling kritikal, yang memberikan kesan buruk kepada ekonomi dan kesihatan awam. Ia menyumbang kepada sekitar 20 peratus kadar kematian akibat barah di dunia setiap tahun. Namun terdapat kekurangan data penyelidikan dari kebanyakan negeri di Malaysia dan Nigeria, di mana paras pencemaran ASF biasanya tinggi. Kajian ini dijalankan untuk menentukan tahap *bioburden* dan penyebaran mikoflora dalam 660 bijirin dan makanan haiwan ternakan dari Malaysia dan Nigeria dengan menggunakan teknik mikrobiologi; mengenal pasti, mengklasifikasi dan menyaring aflatoksigenisiti bagi ASF berdasarkan kaedah fenotipik, biokimia, molekul, dan filogenetik. Kaedah kuantifikasi aflatoksin secara kromatografi (*Thin Layer Chromatography* (TLC) dan *High Performance Liquid Chromatography* (HPLC)) dan spektroskopi (*Attenuated Total Reflectance – Fourier Transformed Infrared Spectroscopy* (ATR-FTIR)) telah dibangunkan dan disahkan; seterusnya digunakan untuk menentukan paras aflatoksin dalam sampel. Purata risiko pendedahan diet terhadap aflatoksin (DERA) dan risiko barah hati (HCC) turut ditentukan. Sebanyak 142 dan 185 kulat berfilamen dengan purata *bioburden* masing-masing antara $8.9 \times 10^4 \pm 1.6 \times 10^5$ hingga $1.0 \times 10^6 \pm 2.5 \times 10^5$ CFU/g dan $1.2 \times 10^5 \pm 1.7 \times 10^5$ hingga $4.0 \times 10^5 \pm 5.8 \times 10^4$ CFU/g telah diisolasi dari bijirin dan makanan haiwan ternakan, dari Malaysia dan Nigeria. Berdasarkan fenotip, ekstrolit dan data

urutan gen (*β – tubulin gene* dan *nuclear ribosomal internal transcribed spacer* (ITS) *gene*) sebanyak 74 isolat (Malaysia = 27, Nigeria = 47) telah dikenal pasti sebagai ASF (60 *A. flavus* dan 14 *A. oryzae*), yang mana 47 (Malaysia = 13, Nigeria = 34) menghasilkan aflatoksin pada media pepejal dan mempunyai gen biosintesis aflatoksin (aflR, aflP, aflD dan aflM). Sebaliknya, kaedah kromatografi dan spektroskopi yang dibangunkan menunjukkan ketepatan dan kepekaan yang tinggi dalam mengukur aflatoksin dalam urutan HPLC (R^2 > 99.9%) > ATR-FTIR (R^2 = 99.59%) > TLC (R^2 > 99%). HPLC menunjukkan bahawa paras aflatoksin (8.68 hingga 77.40 ng/g) dalam sampel dari Malaysia menandakan DERA yang rendah (3.27 hingga 35.88 ng aflatoksin/KgBw/hari) dan risiko barah hati (1.67 hingga 18.31% kejadian HCC/100,000 orang/tahun) membandingkan tahap aflatoksin (0.21 hingga 114.41 ng/g) dalam sampel dari Nigeria (purata DERA = 23.04 hingga 50.08 ng/KgBw/hari, risiko HCC = 26.6 hingga 57.94% kejadian HCC/100,000 orang/tahun). Keputusan menunjukkan bahawa (i) kaedah pengekstrakan dan kuantifikasi aflatoksin yang lebih baik (FTIR, TLC dan HPLC) dalam bijirin dan makanan haiwan telah dibangunkan dan disahkan dalam kajian ini dengan nilai kejituan 90% hingga 103%; (ii) *A. flavus* dan *A. oryzae* ialah spesies ASF utama yang dikenal pasti dalam sampel, dengan kemotip aflatoksigenik adalah lebih tinggi secara ketara daripada kumpulan bukan aflatoksigenik dan (iii) sebilangan besar sampel yang dianalisis mempunyai *bioburden* kulat dan aflatoksin melebihi had piawaian antarabangsa yang boleh membawa kepada lebih 10% kes HCC di kawasan kajian. Oleh itu, strategi kawalan dan pencegahan kulat/aflatoksin harus diperkukuh di kawasan kajian.

CHARACTERISATION OF *ASPERGILLUS* SECTION *FLAVI* AND DEVELOPMENT OF AFLATOXINS LEVEL DETECTION METHODS IN FOOD GRAINS AND POULTRY FEED SAMPLES FROM MALAYSIA AND NIGERIA

ABSTRACT

Aspergillus section *Flavi* (ASF) and their aflatoxins are among the most critical food and feed contaminants with deleterious economic and public health impacts. It contributes to about 20% of global cancer-related deaths annually. However, research data is lacking from most states in Malaysia and Nigeria, where ASF contamination is usually high. This study determines the bioburden and distribution of mycoflora in 660 Malaysian and Nigerian food grains and poultry feeds by utilising microbiological dilution plating techniques; in identifying, classifying, and screening the isolated ASF for aflatoxigenicity by phenotypic, biochemical, molecular, and phylogenetics methods. In addition, simplified chromatographic (Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC)) and spectroscopic (Attenuated Total Reflectance – Fourier Transformed Infrared Spectroscopy (ATR-FTIR)) aflatoxin quantification methods were developed and validated; hence applied to determine the aflatoxins level in the samples. The average dietary exposure risks to aflatoxins (DERA) and attributable liver cancer (HCC) risks were also determined. A total of 142 and 185 filamentous fungal isolates with average bioburden of $8.9 \times 10^4 \pm 1.6 \times 10^5$ to $1.0 \times 10^6 \pm 2.5 \times 10^5$ CFU/g and $1.2 \times 10^5 \pm 1.7 \times 10^5$ to $4.0 \times 10^5 \pm 5.8 \times 10^4$ CFU/g were obtained from the food grains and poultry feeds from Malaysia and Nigeria, respectively. Based on the phenotype, extrolites and gene sequence data (β – tubulin gene and nuclear ribosomal internal transcribed spacer (ITS) gene), 74 isolates

(Malaysia = 27, Nigeria = 47) were identified as ASF (60 *A. flavus* and 14 *A. oryzae*), of which 47 (Malaysia = 13, Nigeria=34) produced aflatoxins on solid media and possessed the aflatoxin biosynthesis genes (aflR, aflP, aflD and aflM). On the other hand, the developed chromatographic and spectroscopic methods showed high accuracy and sensitivity in quantifying aflatoxins in the order HPLC (R^2 > 99.9%) > FTIR (R^2 = 99.59%) > TLC (R^2 > 99%). The HPLC showed that the levels of aflatoxins (8.68 to 77.40 ng/g) in samples from Malaysia signified low DERA (3.27 to 35.88 ng of aflatoxins/KgBw/day) and HCC risks (1.67 to 18.31% incidence of HCC/100,000 peoples/year) compared the levels of aflatoxins (0.21 to 114.41 ng/g) in samples from Nigeria (mean DERA = 23.04 to 50.08 ng/KgBw/day, HCC risk = 26.61 to 57.94% incidence of HCC/100,000 peoples/year). Results showed that (i) improved aflatoxin extraction and quantification methods (FTIR, TLC and HPLC) in food grains and poultry feeds were developed and validated in this study with accuracy values of 90% to 103%; (ii) *A. flavus* and *A. oryzae* are the main ASF species identified in the samples, with aflatoxigenic chemotypes being significantly higher than the non-aflatoxigenic groups and (iii) significant number of the samples analysed have fungal bioburden and aflatoxins above the international regulatory limit which could lead to above than 10% HCC cases in the study regions. Hence, fungal/aflatoxin control and prevention strategies should be strengthened in the study regions.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Food security and safety are among the serious public health issues in the current atmosphere of expanding populations worldwide. Saprophytic toxigenic fungi have been identified among the most critically important biological agents that cause severe food insecurity and malnutrition (Udomkun *et al.*, 2017; Mahato *et al.*, 2019) through food intoxication and osmiotrophic hydrolytic spoilage processes of foods and feeds along the food chain. Fungal food intoxication and spoilage processes have resulted in humans, animal and plants' diseases and fatalities, as well as spoilage of a large percentage of the worlds' food products leading to a considerable reduction in plants and animal productivities and overall economic loss in many countries (Wartu *et al.*, 2015; Mansour *et al.*, 2015; Piacentini *et al.*, 2015; Adeyeye, 2016; Gacem and Ould, 2016; Pierron, 2016; Ji *et al.*, 2016; Momodu *et al.*, 2016; Nortaa and Sowley, 2016; Wolde, 2017; Schmale and Munkvold, 2018; Luo *et al.*, 2018; Negash, 2018; Patial *et al.*, 2018; Singh and Cotty, 2019). Thus, the need for rapid, sensitive, and reliable techniques to detect mycotoxin-producing fungi and mycotoxins in foods and feeds is of paramount importance.

Fungi responsible for food intoxication (mycotoxigenicity) are generally the *Aspergillus*, Fusarium and Penicillium species (Reddy et al., 2010; Salisu et al., 2019) that are capable of contaminating various agricultural products both at pre and post-harvesting stages. These fungi produce a plethora of spores (Ogunleye and Olaiya, 2015; Stanić, 2016) that ensure their dissemination, propagation and survival in various environments leading to their persistent contamination of foods and feeds at

various trophic levels. This adaptive feature makes their ubiquitous presence in both fresh and stored agricultural produce unavoidable.

Aspergillus section *Flavi* (ASF), subgenus *Circumdati,* has been described as the most economically important fungal contaminant in various stored food products due to their deleterious impacts on humans and animals (Singh and Cotty, 2019). *Aspergillus flavus,* a prominent member of the section, has been reported as the most common causative agent of superficial mycosis and the second most common aetiologic agent of inversive aspergillosis in humans (Frisvad *et al.,* 2019). ASF contained several toxigenic species that commonly contaminate various stored foods and feeds, and subsequently produce a wide array of toxic and carcinogenic metabolites (mycotoxins), including but not limited to aflatoxins, ochratoxin A, tenuazonic acid, 3- nitropropionic acid, Kojic acid, and cyclopiazonic acid (Varga *et al.,* 2015; Frisvad *et al.,* 2019).

Mycotoxin contamination of stored food products is mostly due to aflatoxins, the most toxic and carcinogenic mycotoxins produced by *Aspergillus* spp, especially *Aspergillus flavus, A. parasiticus,* and *A. nomius* when they grow in various stored foods (Dangora *et al.,* 2017; Medina *et al.,* 2017). Aflatoxin contamination of stored products is more common in the tropical and subtropical countries where the weather is constantly warm, and high relative humidity that favours the proliferation of the aflatoxigenic fungi and aflatoxin production, given that aflatoxin production is directly proportional to moisture (humidity), temperature, (Bbosa *et al.,* 2013; Medina *et al.,* 2017) and length of storage period (Jonathan and Esho, 2010). Many products with no detectable aflatoxin levels during exportation or importation are contaminated with high levels of aflatoxins at retailer and consumer levels (Villers *et al.,* 2014;

Gamuchirai *et al.*, 2019). Thus, the need for continuous monitoring and screening of stored foods and feeds for aflatoxins, especially at the consumer and retailer level, can never be overemphasised.

Aspergillus spp and aflatoxin contamination of foods and feeds are generally of great concern today due to their deleterious impacts on public health, food safety, and the national economy of several countries (Hymery *et al.*, 2014; JECFA, 2018b). It is estimated that 25% of the global food supply is contaminated with aflatoxins (EAC Policy, 2018b), resulting in an estimated exposure of about 4.5 billion of the world's population to aflatoxin, which represents one sixth of deaths worldwide each year, much of which comes from less developed countries (Savage, 2018), where the majority of people use the most susceptible crops (grains and peanuts) as their staple diet (Udomkun *et al.*, 2017). In fact, research showed that no amount of aflatoxin might be considered safe for consumption as it can lead to acute or chronic aflatoxicoses. Globally, several human deaths have been recorded due to outbreaks of acute aflatoxicosis from many countries such as Thailand (Shank *et al.*, 1971), Nigeria (Oyelami *et al.*, 1997), Malaysia (Chao *et al.*, 1991), India (Reddy and Raghavender, 2007), Kenya (Yard *et al.*, 2013), Tanzania (Musewa *et al.*, 2016) and Uganda (Kamala *et al.*, 2018). On the other hand, chronic exposure to aflatoxins (prolong exposure to smaller doses of aflatoxins) have been associated with severe liver damage, liver cancer, gastrointestinal malabsorption, and immune deficiency (Gurav and Medhe, 2018). It can also lead to congenital disabilities, kwashiorkor, genotoxicity, and stunting in children (EAC Policy, 2018a; JECFA, 2018b).

Research has shown that aflatoxins decompose at 200 to 300^{o}C (Rustom, 1997), and hence they are not denatured by pasteurisation and most industrial or home

food processing processes. Humans become exposed to aflatoxins directly through oral ingestion of contaminated food (Wangia, 2017; Knutsen *et al.*, 2018; Ozluoymak and Guzel, 2018) or indirectly through consuming contaminated products from animals that fed on aflatoxin-contaminated feeds. Occupational exposure also occurs via the dermal route or inhalation of dust generated during the handling or processing of aflatoxin-contaminated grains and feeds (IARC, 2012; Susana *et al.*, 2014). Thus, food grains and feeds represent a significant source of human exposure to aflatoxins.

Furthermore, several studies showed that aflatoxins remained persistent upon transfer along the food chain from contaminated crops to herbivores animals via contaminated feeds, and subsequently to carnivores animals and humans via products such as meat (Magnussen and Parsi, 2013), milk (Tozzi *et al.*, 2016; Naeimipour *et al.*, 2018) and eggs (Greco *et al.*, 2014; Alshannaq and Yu, 2017) from the exposed animals. Poultry feeds are especially prone to high contamination rates by aflatoxins and other mycotoxins due to multicomponent raw materials from various crops from different environments with different fungal loads (Xu *et al.*, 2018). Many studies have reported the occurrence of aflatoxins in animal feeds above the minimum acceptable limits (Greco *et al.*, 2014). This high rate of contamination of feeds is most common in countries such as Nigeria and Malaysia, with hot and humid climatic conditions (Mgbeahuruike, 2016).

1.2 Statement of problems and rationale of the study

The unavoidable persistent, ubiquitous presence of *Aspergillus* section *Flavi* (ASF) in foods and feeds is a worldwide phenomenon of significant concern because of their potential detrimental economic and public health impacts. (Ibrahim *et al.*, 2018). Despite several studies on ASF from various research fields, the occurrence,

toxigenicity, genotypes and taxonomy of ASF are still inadequately resolved or not fully elucidated, as most of the efficient mycotoxin producers have yet to be identified (Frisvad *et al.*, 2019). Many toxigenic and atoxigenic new species belonging to the ASF are continuously isolated from various sources, such as *Aspergillus. novoparasiticus* from nuts (Juliana *et al.*, 2012), *A. bertholletius* from nuts (Taniwaki *et al.*, 2012), *A. sergii, A. mottae, A. transmontanensis,* from almonds and maize (Soares *et al.*, 2012), *A. korhogoensis* from peanuts (Carvajal-Campos *et al.*, 2017), *A. hancockii* from Australian soil (Pitt *et al.*, 2017), and *A. austwickii, A. aflatoxiformans, A. vandermerwei, A. neoalliaceus, A. subflavus , A. cerealis, A. aspearensis,* and *A. pipericola* from Nigerian soil (Frisvad *et al.*, 2019). Hence, knowledge of the toxigenicity and taxonomy of autochthonous ASF in various regions is very imperative, especially in countries with tropical locations that favour the proliferation of mycotoxigenic fungi, like Malaysia in Asia (Hejri, *et al.*, 2013a; Norlia *et al.*, 2018b, 2019) and Nigeria in Africa (Ibrahim *et al.*, 2018; Stepman, 2018).

Despite the fungi-friendly weather in Malaysia, there are generally inadequate studies on fungal and mycotoxin contamination of foods and feed products at consumer levels (Sabran *et al.*, 2013). Although most of the food items are screened for fungi and aflatoxins during importation, studies on commercially marketed samples showed that most of the food items become highly contaminated by fungi and mycotoxins at consumer and retailer levels in Malaysia in excess of the international regulatory limits in most of the samples (Chin *et al.*, 2012; Jalili and Jinap, 2012; Samsudin and Abdullah, 2013; Ali *et al.*, 2015; Nadira *et al.*, 2017). A recent study in Malaysia showed that peanut samples collected from importers had zero levels of aflatoxin. In contrast both toxigenic and atoxigenic ASF highly contaminated the peanut samples at retailer and consumer levels, in addition to high aflatoxin content

(above 120 ng/g) (Norlia *et al.*, 2018b, 2019). A study involving adults from five districts in Penang, Malaysia, reported that Malaysians are being exposed to high doses of aflatoxins but not up to the levels that can elicit acute or apparent toxicity, with men above 30 years of age being 3.08 times more exposed to AFB1 ($p = 0.026$) than those between 18 to 30 years in the study (Leong *et al.*, 2012). It has been estimated that about 13 HCC cases per 100,000 healthy people occur each year in Malaysia through aflatoxin-contaminated maize only (Vaghela and Afshari, 2017). According to Raihan and coworkers, yearly life lost due to HCC in Malaysia has increased by 31.5% from 1990 to 2018 (Raihan *et al.*, 2018).

However, based on the available literature from 2008 to 2020 (shown in chapter 2, section 2.4), most of the fungal and mycotoxin studies in Malaysia were conducted in few states, such as Selangor and Penang, only; little or no studies were conducted in most of the granary states, such as Perak, Perlis, and Kelantan where local farm products that have not been subjected to fungal and mycotoxin screening are in abundance (Salisu *et al.*, 2021). These issues call for more studies on fungi, especially the ASF, which produce the most toxic and carcinogenic mycotoxin (aflatoxins) in Malaysia. Hence this would help to generate adequate information on the incidence or prevalence of mycotoxigenic fungi and mycotoxins in foods and feeds at consumer levels, especially in the rural states that lacked previous studies data. This will eventually help evaluate the safety of the foods and feeds, generate and predict the degree of populations' exposure to mycotoxins for effective control.

On the other hand, Nigeria is one of the largest producers and consumers of grain foods among the African countries (USDA, 2020). Majorities of grain farmers in Nigeria reside in the northern part of the country where the storage facilities used

are traditional types or locked up stores without proper ventilation, leading to contamination of various farm products by fungi and subsequently mycotoxins (Ibrahim *et al.*, 2018). Foods sold in Nigerian markets are either from local retailers or directly from farmers; hence they are not subjected to fungal and mycotoxin screening before releasing to consumers. These issues have led to a high rate of fungal and mycotoxin contamination of most marketed foods in Nigeria. A recent review on dietary aflatoxin exposure in Nigeria showed that consumption of maize, rice, peanut, and wheat could be attributed to a very high risk of dietary aflatoxin exposure ranging from 1.7×10^{-4} to 9.9×10^{3} ng/KgBw/day, which could account for an estimated percentage incidence of HCC/100,000 population/year of 84.03 to 1,052.50% between 2009 to 2018 in the country (Salisu *et al.*, 2020b). These have led to several cases of aflatoxin related liver cancer deaths in the country (Atanda *et al.*, 2013), resulting in the loss of about five thousand lives and 155 million US dollars each year due to aflatoxins in Nigeria (Olayinka, 2018). However, just like in Malaysia, the reports on the dreadful ASF (taxonomy, distribution, aflatoxigenicity) are lacking in many Nigerian granary states; hence, studies addressing these issues are needed for effective mycotoxin control measures.

Furthermore, the substantial detrimental impacts aflatoxins have on public health and the worlds' economy has led to the continuous demand for simple, sensitive, and reliable aflatoxins detection and quantification techniques to properly assessing both the relevant risk of exposure and the relevant toxicological risk for humans and animals, as well as ensuring that regulatory levels set by the European Union (EU) or other international organisations are met (Miklós *et al.*, 2020). Although various techniques have been developed to determine aflatoxins, most of them are only applicable to certain food or feed categories. At the same time, other methods are

complex, requiring special sample clean-up processes, which in turn require sufficient expertise from the analyst (Wacoo *et al.*, 2014). Thus, the need for the development of simple, rapid, sensitive, and robust techniques for accurate determination of even lower levels of aflatoxins in foods and feeds without requiring the complex stages of sample clean-up is very imperative. The present study aims to address the preceded highlighted problems, hence its significance and contributions to knowledge.

1.3 Hypotheses of the study

H_1: A significant difference will exist in the levels of fungal and aflatoxin contamination of food grains and poultry feeds from Malaysia and Nigeria.

H_2: Differences will be observed between the chemotypes of *Aspergillus* section *Flavi* contaminating food grains and poultry feeds in Malaysia and Nigeria.

H_3 Differences will be observed between the nucleotide sequences (genotype) and aflatoxigenicities of *Aspergillus* section *Flavi* contaminating food grains and poultry feeds in Malaysia and Nigeria.

H_4: Chromatographic techniques such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and spectroscopy (attenuated total reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR)) can be simplified and improved for efficient detection and quantification of aflatoxins from the complex matrices of food grains and poultry feeds without involving the carcinogenic chloroform-extraction or the complex sample clean-up stages.

H_5: A significant difference will exist in the HCC risks attributable to levels of aflatoxins in food grains and poultry feeds from Malaysia and Nigeria.

1.4 Objectives of the study

1.4.1 General objective

The present study aims to characterise *Aspergillus* section *Flavi* and develop aflatoxins detection methods in food grains and poultry feed samples from Malaysian and Nigerian open markets.

1.4.2 Specific objectives

I. To determine the bioburden, phenotypes, and distribution of filamentous fungal flora contaminating common food grains (maize, rice, wheat, peanut) and poultry feeds in Kelantan state (Malaysia) and Katsina state (Nigeria).

II. To determine the aflatoxigenicity of the *Aspergillus* section *Flavi* isolated from the samples and characterise them into chemotypes based on their extrolites.

III. To molecularly identify the *Aspergillus* section *Flavi* isolates based on the nucleotide sequences in the β-tubulin and ITS genes in their genomic DNA; and further characterise them into aflatoxigenic and non-aflatoxigenic strains based on the presence or absence of aflatoxin biosynthesis genes (*aflD*, *aflM*, *aflR*, and *aflP*) by polymerase chain reaction.

IV. To develop and validate chromatographic (TLC and HPLC) and spectroscopic (ATR-FTIR) techniques for efficient detection and quantification of aflatoxins in food grains and poultry feed samples.

V. To apply the developed methods (TLC, HPLC, ATR-FTIR) to determine the levels of aflatoxins in the Malaysian and Nigerian food grains and poultry feed samples, and estimate the populations' liver cancer risks attributable to dietary aflatoxin exposure in both Malaysia and Nigeria.

CHAPTER 2

LITERATURE REVIEW

2.1 A compendium of the genus *Aspergillus*

The genus *Aspergillus* (plural: *Aspergilli*) is one of the largest fungal genera comprising a group of ascomycetous, filamentous, hyaline, septate fungi that commonly reproduce asexually using spores (ascospores) borne on aseptate conidium. *Aspergilli* were first catalogued by an Italian biologist and priest, Pier Antonio Micheli, who coined the name "*Aspergillus*" in 1972, as a group of moulds with a common anamorphic (asexual) spore-bearing structure (conidium) that resemble an *Aspergillum* (Latin *Asperges* - device used by Roman Catholic clergy to sprinkle holy water) (Bennett, 2016).

Aspergillus species are generally fast-growing, appearing phenotypically as white, yellow, green-yellow, bright green, yellowish-brown to black with a dense mass of erect conidia (Kidd *et al.*, 2016). On routine mycological media, *Aspergillus* colonies are velvety and powdery, often with a white periphery. The reverse of the colonies is usually colourless, milk or brownish-yellow (Gordon and Julie, 2018). When viewed microscopically, *Aspergilli* have hyaline septate hyphae and several foot cells that elongate into erect, aseptate, smooth or rough-walled conidiophores (aerial hyphae or conidia) that culminate in a multinucleate globose to flask-shaped vesicle which can be uniseriate (if it produces a palisade-like layer of multinucleated, radially-arranged, tubular outgrowths called phialides/sterigmata that fully or partly covers the vesicle) or biseriate (if it produces a double layer of sterigmata composed of a layer of primary phialides called metulae covered by a secondary layer of phialides/sterigmata) (Kidd *et al.*, 2016). The conidial head comprises the vesicle, phialides, metulae (if present), and conidia (Figure 2.1). Conidia (conidiospores) are one-celled, hyaline or

pigmented, with a thick wall composed of a rough spiny outer layer called epispore and a smooth inner layer called an endospore. They are generated in long dry chains that can be divergent (radiate) or aggregated in compact columns (columnar). In addition, some members may produce Hülle cells or sclerotia (a hard dark resting body of the fungi, containing a mass of hyphal threads, capable of remaining dormant for long periods) (Kidd *et al.*, 2016).

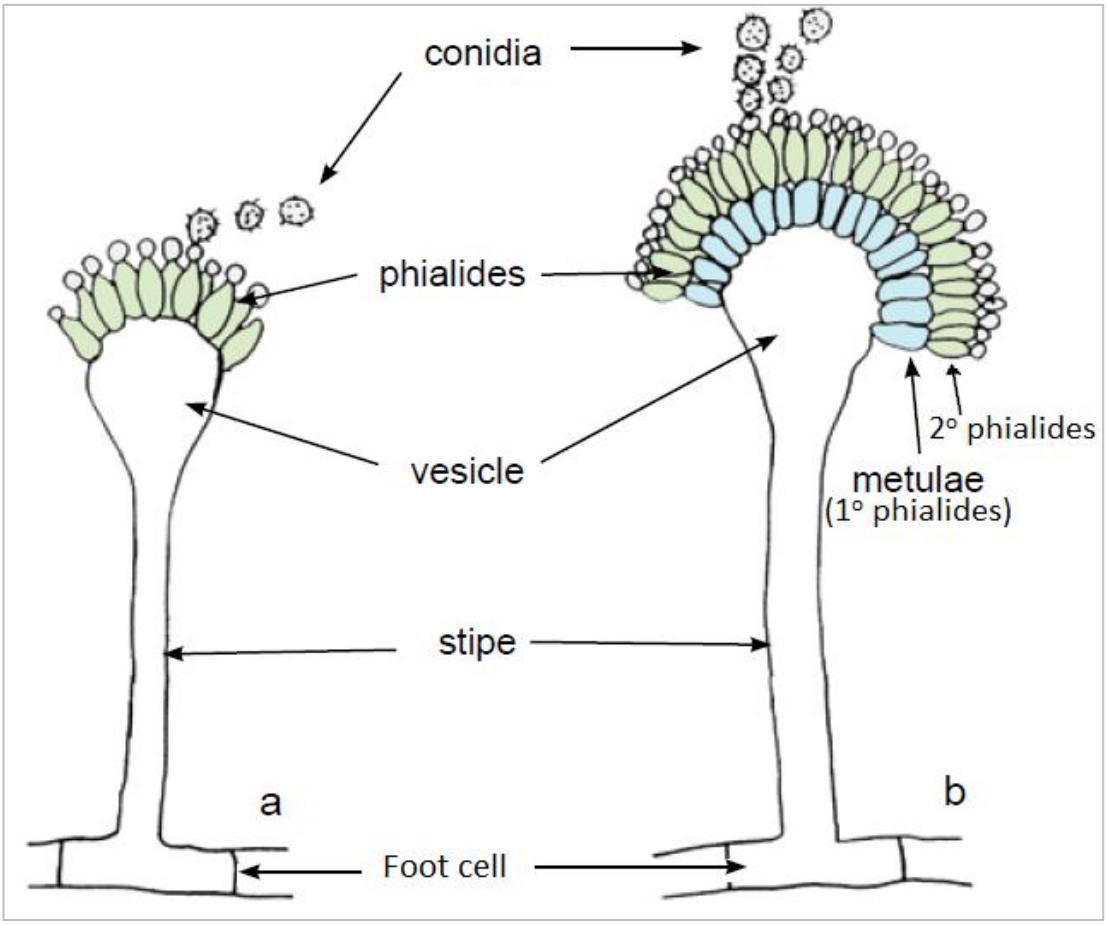

Figure 2.1: Typical conidia of *Aspergillus* (a) uniseriate (b) biseriate. Adopted from Kidd *et al.*, (2016)

2.1.1 Ecology of *Aspergilli*

Aspergilli produce a plethora of microscopic spores (Paulussen *et al.*, 2017; Ibrahim *et al.*, 2018) that aid in their dissemination and ubiquitous presence in various

habitats worldwide. The spores are airborne and can remain persistent and viable in the environment due to their stress resistance and hydrophobicity, making them remain viable even in water bodies (Stevenson *et al.*, 2015; Wyatt *et al.*, 2015). The spores commonly contaminate air, vegetation, foods, drinking water, rooms, and animal bodies making the *Aspergilli* cosmopolitan in both indoor environments (air, surfaces of buildings, food, and other household appliances) and outdoor habitats such as soil, air (bioaerosols), vegetation, in/on human/animal bodies, decaying organic matter, and water bodies (fresh water and marine habitats) (Samson *et al.*, 2014; Paulussen *et al.*, 2017; Tsang *et al.*, 2018), given that the presence of moisture greatly enhances their growth.

Many *Aspergillus* species are extremophiles, capable of growing in high salt and sugar solutions, high or low temperature and pH, and low oxygen (Houbraken *et al.*, 2014). These features, coupled with their achlorophyllous nature (growth that does not require sunlight energy) and other virulence characteristics, make them grow in deep tissues of the human/animal bodies, leading to mycosis.

2.1.2 Metabolism and economic importance of *Aspergilli*

Nutritionally, members of the genus *Aspergillus* are osmiotrophic heterotrophs (obtaining nutrients by absorption) with versatile metabolism that enable their proliferation in a wide range of substrates. In addition, they secrete a variety of extracellular hydrolytic enzymes (Frisvad and Larsen, 2015; Bennett, 2016; Gautier *et al.*, 2016; Campos, 2019; Houbraken *et al.*, 2020) to break down complex nutrients in the environment into simpler forms that the hyphae can absorb. For these degradative metabolic capabilities, *Aspergilli* play an important role in nutrient cycles in the

environment, where they serve as decomposers in biogeochemical cycles (Bennett, 2016).

Besides the decomposition of organic matter, these fungi secrete several exometabolites/extrolites (secondary metabolites) with high biotechnological, food, economic, health, and social impacts. *Aspergilli* has been reported to produce about two thousand various kinds of economically important extrolites (Frisvad, 2014). Several species such as *Aspergillus oryzae* and *A. sojae* are used in various food industries to produce fermented foods (e.g., miso and soy sauce) (Tsang *et al.*, 2018). In addition, species such as *A. niger* and *A. sojae* are used in the production of enzymes and organic acids (Houbraken *et al.*, 2014; Ichishima, 2016), while some other species such as *Aspergillus terreus* are used for the production of pharmaceutical products (Frisvad *et al.*, 2019; Houbraken *et al.*, 2020).

However, despite their tremendous beneficial roles, some *Aspergillus* species are associated with many detrimental effects through indoor air contamination (e.g., *A. versicolor*), food spoilage (e.g., *A. proliferans*), food intoxication (e.g., *A. flavus, A. parasiticus*, and *A. nomius*) and disease causation (e.g., *A. fumigatus, A. clavatus, A. flavus*), leading to deleterious impacts on public health, food safety, and the national economy of several countries (Monge *et al.*, 2013; Rashid *et al.*, 2013; Hymery *et al.*, 2014; JECFA, 2018b).

2.1.3 Taxonomy and nomenclature of the genus *Aspergillus*

The genus *Aspergillus* contained highly diverse species belonging to the family *Trichiocomaceae,* which is listed under the taxonomic order *Eurotiales* of the class *Eurotiomycetes* in the phylum Ascomycota. Since the introduction of the genus name "*Aspergillus"* in 1729 by Micheli and its subsequent validation (in 1768) and

sanctioning (in 1832) by von Haller and Fries, respectively, *Aspergilli* were being classified based on their morphological characteristics (Micheli, 1729; Haller, 1768; Fries, 1832). The foundation of the infrageneric classification began with the work of Thom and Church, (1926) and Blochwitz (Tsang *et al.*, 2018), who used phenotypic characteristics and divided the *Aspergilli* into seven and 18 sub-generic groups, respectively. Subsequently, the groups were accommodated into six subgenera and 18 sections by Gams and colleagues (Gams *et al.*, 1986), later revised and re-classified into eight subgenera containing 22 sections based on multilocus DNA sequence data and phylogenetic analysis (Peterson *et al.*, 2008). After that, several revisions were made as new species were being discovered and added to the groups until 2010 when the concept of one-fungus-one-name was introduced, leading to re-grouping and renaming of many species (Frisvad, 2014).

In the modern nomenclature, *Aspergilli* are named and classified using polyphasic approach (phenotypic, chemotypic and molecular/phylogenetic characteristics) (Tsang *et al.*, 2018), which leads to intensive reviews of the previously accepted species which were incorporated into the genus based on morphological characteristics. Thus, the previous taxonomic list was considered outdated because many new species were described, and most of the existing species that were considered synonymous based on morphology were phylogenetically characterised as distinct species (Samson *et al.*, 2014).

Based on the polyphasic approach, Houbraken *et al.*, (2014) and Hubka *et al.*, (2015) proposed grouping *Aspergilli* into four subgenera (*Aspergillus, Circumdati, Fumigati* and *Nidulantes*) containing 20 sections. Maintaining the four subgenera, three new sections were proposed by Samson and colleagues (Samson *et al.*, 2014),

making 23 sections with a total of 339 species in the four subgenera (Gautier *et al.*, 2016). Revisions continued as many species were identified and added to the genus. Recently, Houbraken *et al.*, (2020) used an advanced polyphasic approach (phylogenetic analysis of nine-gene data sets plus phenotypic, physiologic and extrolite features) to produce an updated list of *Aspergilli*, which now consists of six subgenera (*Aspergillus, Circumdati, Fumigati, Polypaecilum, Cremei,* and *Nidulantes*) containing 27 sections and 75 series with a total of 446 species (Figure 2.2).

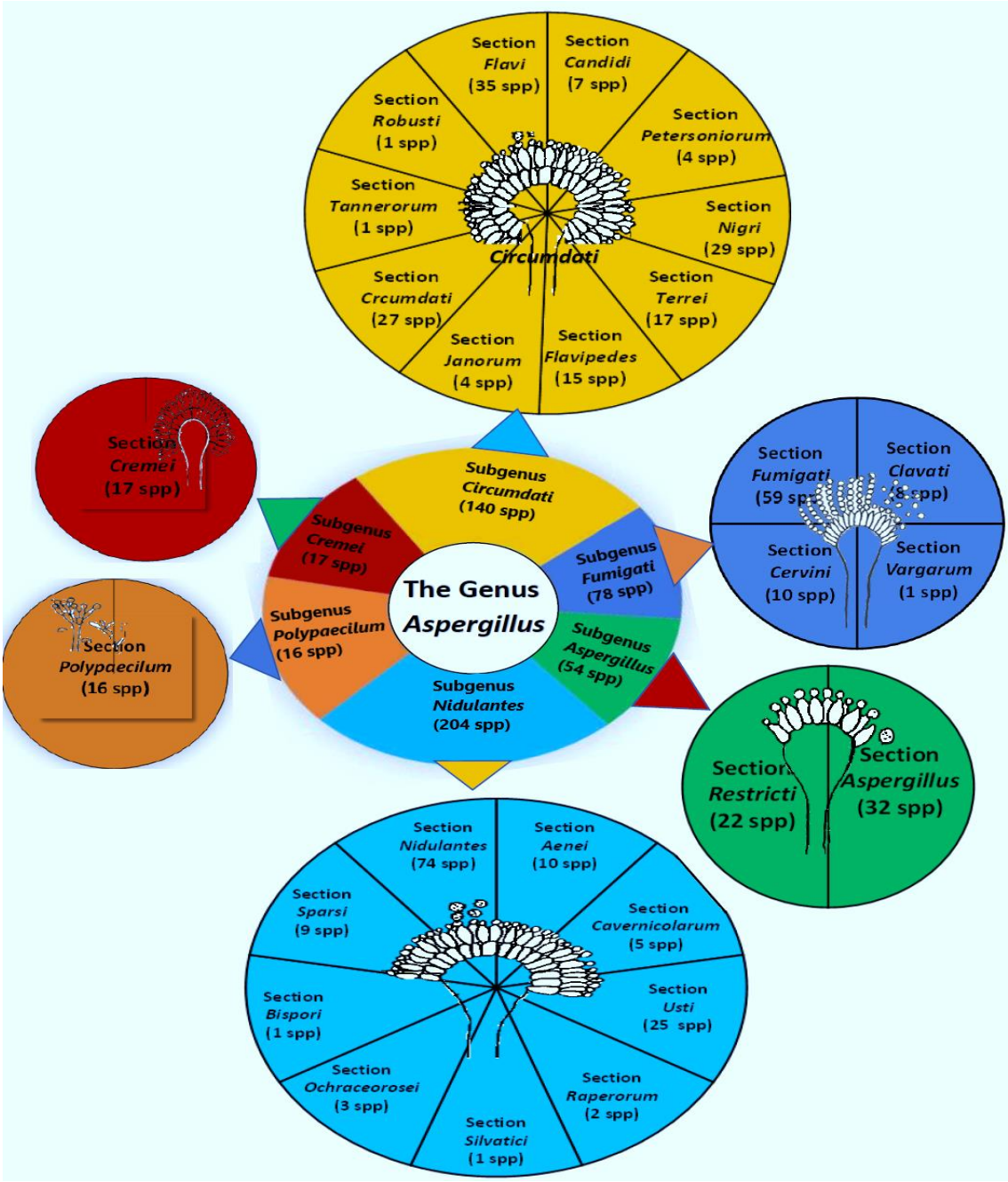

Figure 2.2: Current sub-genera and sections in the genus *Aspergillus*. Adapted from Houbraken *et al.*, (2020)

2.2 *Aspergillus* section *Flavi*

Aspergillus sub-genus *Circumdati* section *Flavi* (ASF) are the most economically important fungi with the highest biotechnological, health, and social impacts worldwide (Frisvad, 2014; Frisvad and Larsen, 2015; Campos, 2019; Frisvad

et al., 2019). ASF represent the most interesting group of mould fungi from risk assessment and analysis perspectives (Campos, 2019). This is due to their general worldwide distribution, rapid growth, and ability to interact with, colonise, and contaminate various crops and stored foods, leading to deterioration and intoxication which consequently lead to severe food insecurity and manifestations of both acute and chronic mycotoxicosis in addition to severe economic losses (Ji *et al.*, 2016; Momodu *et al.*, 2016; Nortaa and Sowley, 2016; Pierron, 2016; Wolde, 2017; Luo *et al.*, 2018; Negash, 2018; Patial *et al.*, 2018; Schmale and Munkvold, 2018; Singh and Cotty, 2019).

Macroscopically, ASF grow rapidly on most mycological media into radial-velvety colonies (Gordon and Julie, 2018) with bright green to yellow or brown surfaces composed of dense erect aerial hyphae with numerous conidia bearing dusty spores (Kidd *et al.*, 2016). Most species produce few to numerous dark brown sclerotia, which may be small (< 400 µm in s-strains) (Figure 2.3a) or large (> 400 µm in L-strains) (Figure 2.1b); while some species do not produce sclerotia (Figure 2.3c). A white border may surround the colonies, and just like other *Aspergilli*, the reverse side of the colonies is creamy, white, or colourless (Gordon and Julie, 2018). Microscopically, ASF are distinguished from other *Aspergilli* based on their characteristic globose vesicle bearing radially arranged uniseriate or biseriate phialides (Figure 2.3d) (Kidd *et al.*, 2016; Gordon and Julie, 2018; Houbraken *et al.*, 2020).

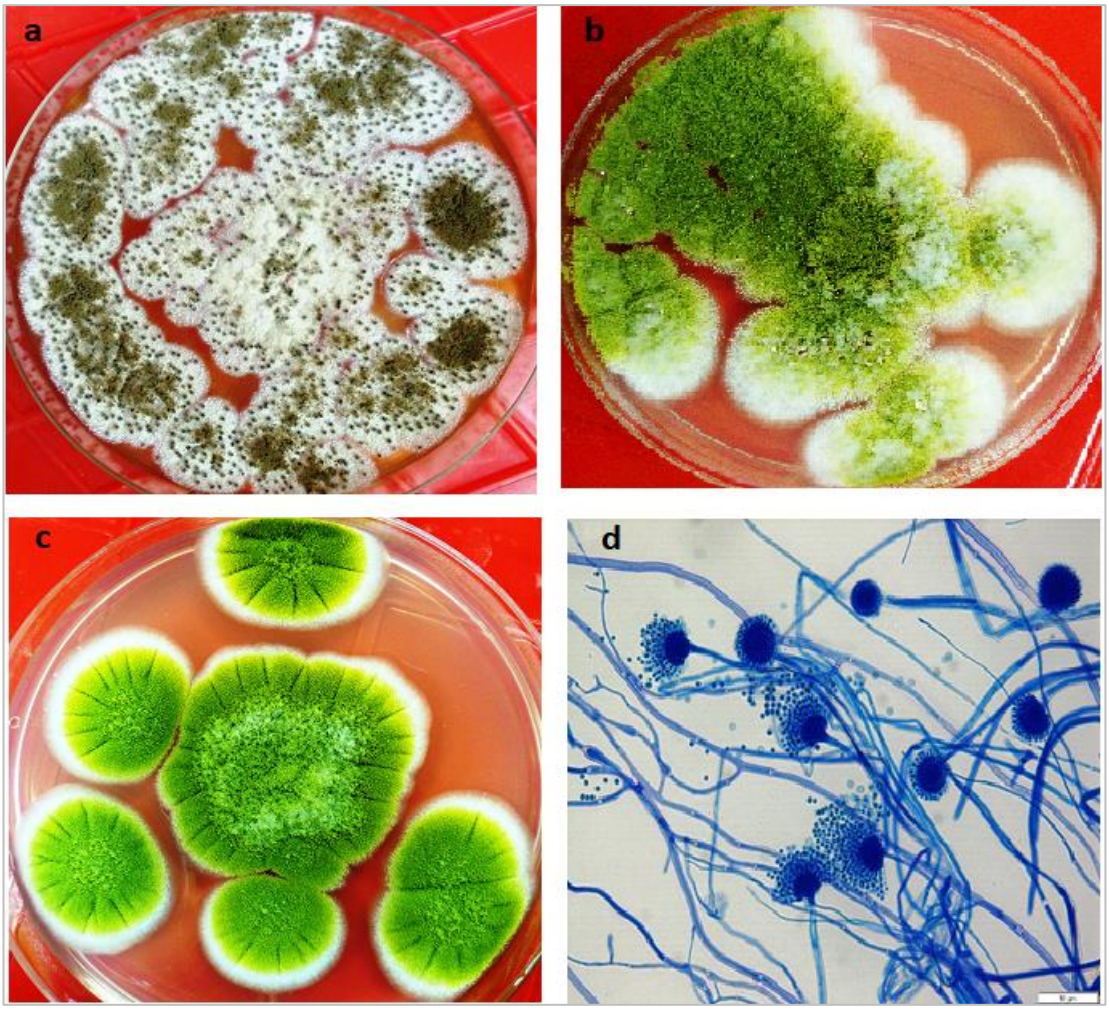

Figure 2.3: Phenotypic appearance of typical *Aspergillus* section *Flavi* cultured on potato dextrose agar (PDA). (a) colonies with few conidia and numerous small sclerotia, (b) dense conidia with a small number of large sclerotia, (c) dense conidia but no sclerotia, and (d) microscopy of the conidia stained with lactophenol cotton blue (LCB) stain and observed under x40 objective lens of a bright field microscope. Adopted from Salisu *et al.*, (2019)

2.2.1 Species in the *Aspergillus* section *Flavi*

The makeup of ASF has changed many times during the last two decades. Several species have been classified as *Aspergillus* section *Flavi* in the past, primarily using conventional techniques in which phenotypic macroscopic and microscopic characteristics were used to identify and assign species to specific taxon (Houbraken *et al.*, 2014). However, studies showed that the phenotype of filamentous fungi is

influenced by environmental and nutritional circumstances, resulting in overlapping phenotypical characteristics and extensive morphologic variability brought by genetic variations (Campos, 2019), making morphological studies to distinguish between isolates difficult. Hence, molecular approaches using real-time polymerase chain reaction, RAPD (Random Amplified Polymorphic DNA) analysis, etc., were employed for species identification (Godet and Munaut, 2010). The nucleotide sequences of the primary identification genes, such as nuclear ribosomal internal transcribed spacer (ITS), calmodulin, beta-tubulin, etc., are being used to identify species and determine their evolutionary phylogenetic relationships. However, because members of ASF shared numerous conserved characteristics, identifying changes at the molecular level can be difficult/challenging. For example, the genomes of *Aspergillus parasiticus* and *A. flavus* have about 97 to 99 per cent nucleotide similarity (Chang *et al.*, 2007).

Multivariate methods using phenotypic (macroscopic and microscopic morphology), metabolic/chemotypic (lipidomic, proteomic and metabolomic), physiological and genetic characteristics are presently used to classify ASF in the attempt to resolve the challenges. The section has been divided into eight distinct clades (*Aspergillus flavus* – clade, *A. alliaceus* – clade, *A. avenaceus* – clade, *A. coremiiformis* – clade, *A. bertholletius*- clade, *A. leporis* – clade, *A. tamarii* – clade, and *A. nomius*- clade) based on the similarities and differences in their DNA sequence, extrolites, and morphology in accordance to the current taxonomic protocol (Houbraken *et al.*, 2014, 2020; Frisvad *et al.*, 2019). According to the recent taxonomic updates, 37 species were identified as members of the *Aspergillus* section *Flavi.* In addition, the detailed phylogenetic relationship among the species has been published (Figure 2.4) (Frisvad *et al.*, 2019).

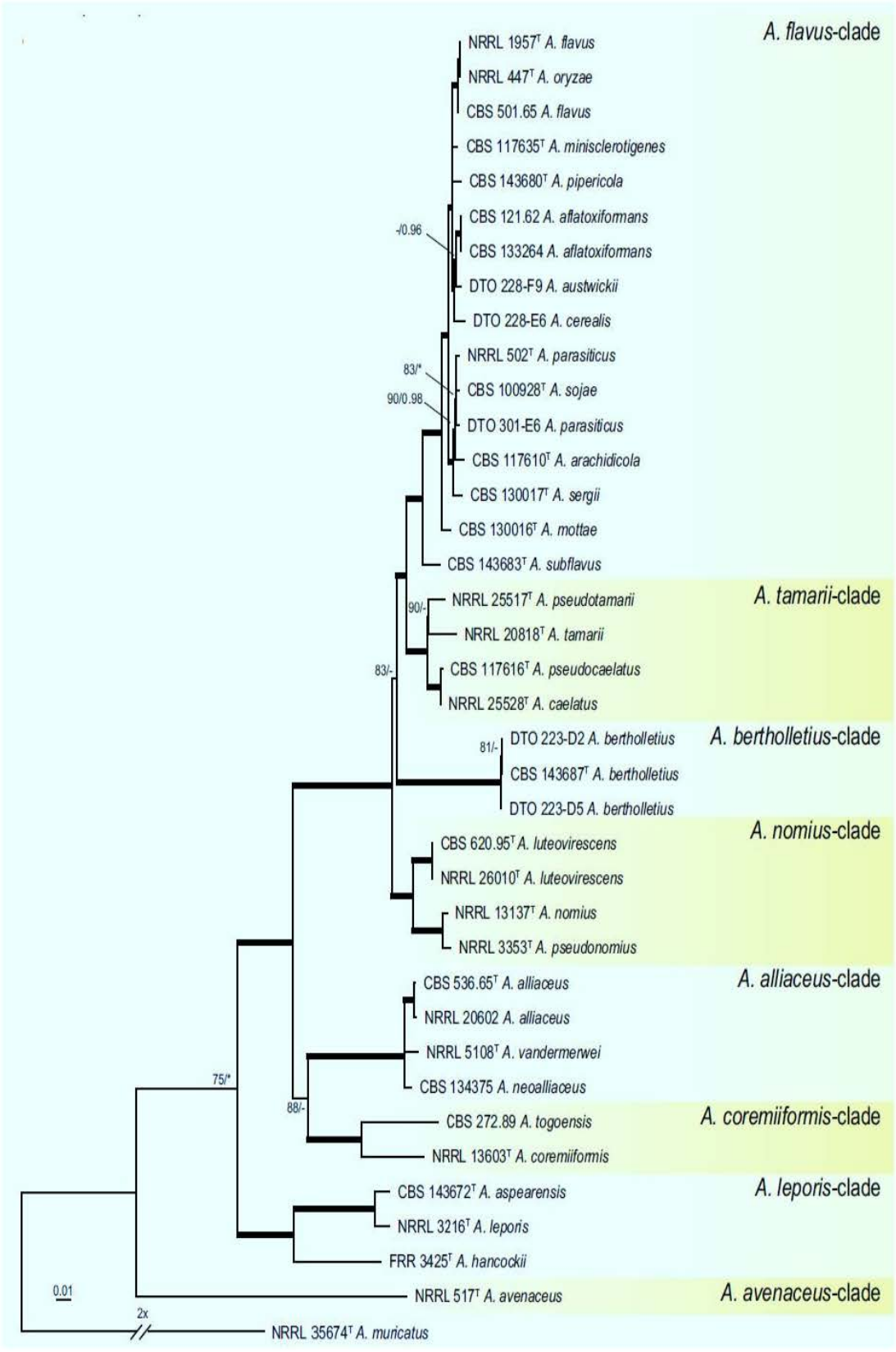

Figure 2.4: Phylogenetic tree showing the taxonomic relationships among the clades and species in the *Aspergillus* section *Flavi* based on the partial nucleotide sequences of their *BenA, CaM,* and *RPB2* genes (adopted from Frisvad *et al.*, (2019)).

2.2.2 Reproductive cycles in *Aspergillus* section *Flavi*

Although *Aspergillus* species were previously thought to be anamorphic (reproduce asexually), several species have been shown to generate ascocarps (Tsang *et al.*, 2018). There is also evidence that sexual reproduction in some *Aspergilli* occurs recessively. Currently, four types of reproductive cycles were identified in ASF and some other *Aspergilli*: vegetative, asexual, sexual, and parasexual reproductive cycles (Varga *et al.*, 2014; Bennett, 2016; Tsang *et al.*, 2018; Campos, 2019).

The vegetative reproduction occurs among the multinucleate vegetative mycelia whereby the mycelium fragment into small pieces/fragments, each of which can grow independently (under favourable conditions) into a new thallus. Vegetative fragmentation is most common in mutant species that lacked the *SepA* gene, which regulates septation in filamentous fungi (McIntyre *et al.*, 2001).

Asexual reproduction is carried out by a group of aerial hyphae called conidiophores (conidia) which produce chains of unicellular, air-borne reproductive structures called spores by interstitial mitotic cell division of the phialides (Bennett, 2016). The asexual cycle (conidiogenesis) can be divided into three distinct phases; starting with the growth phase necessary for the sporogenous cells to acquire the ability to respond to the induced signals, progressing with the initiation of the developmental pathway, and ending in the development of regulatory events leading to conidiation (sporulation) (Adams *et al.*, 1998). These processes are regulated by several genes, of which *wetA*, *brlA*, and *abaA* genes are critical for the initiation of conidiation, developmental stages, and maturation of spores (Boylan *et al.*, 1987; Adams *et al.*, 1988, 1998; Mirabito *et al.*, 1989; Marshall and Timberlake, 1991; Ichinomiya *et al.*, 2005). Once released, the spores are dispersed in the environment by wind, animals, insects, or water. Then, under favourable environmental conditions (presence of

moisture, worm weather, and nutrient), the spores germinate into new fungi by producing germ tubes that subsequently produce hyphal branches and septation to form networks of branched hyphae (mycelium). Being stress-resistant, the spores can remain dormant in the environment for a long period until they found suitable germination conditions (Stevenson *et al.*, 2015; Wyatt *et al.*, 2015). The schematic illustration of the reproductive cycles in ASF is depicted in Figure 2.5.

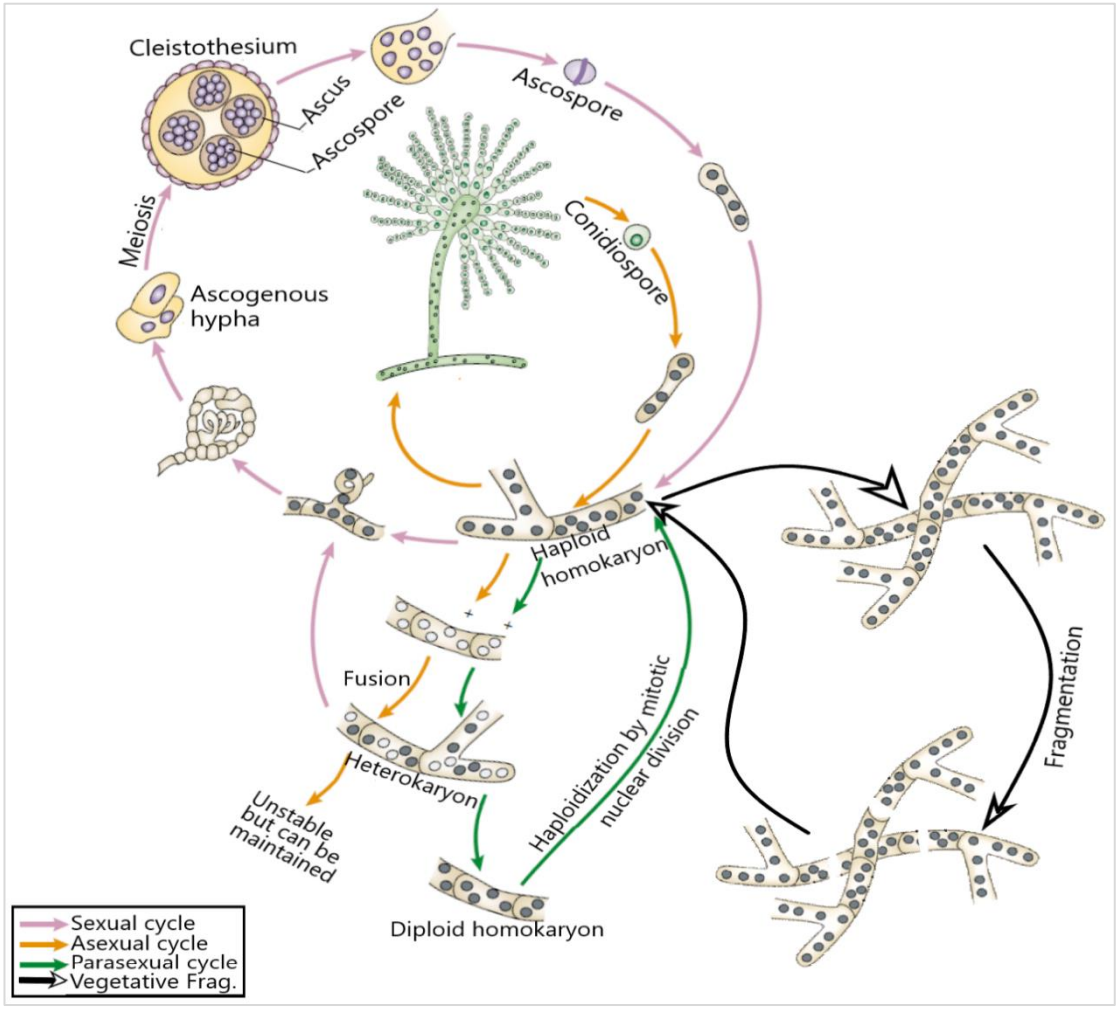

Figure 2.5: Reproductive cycles in *Aspergillus* section *Flavi* represented by *Aspergillus nidulans.* Adapted from Casselton and Zolan, (2002).

Similarly, the sexual reproductive cycle uses meiotically generated spores called ascospores, meiospores, or teliospores. Unlike asexual reproduction, which

occurs in all species of *Aspergillus*, sexual reproduction in ASF occurs in species that exhibit heterothallism, possession of vegetative hyphae capable of differentiating into compatible 'male hyphae' (antheridium) and 'female hyphae' (ascogonium) either on the same filament or different filament of the same mycelium. The sexual compatibility is mediated by two structurally unrelated but commonly occurring MAT1-1 and MAT1-2 mating genes (idiomophs), which are generally found in the MAT1 locus in the same fungus (homothallic species, e.g., *Petromyces alliaceus*) or different fungus (Heterothallic species, e.g., *A. flavus* and *A. parasiticus*) (Dyer and Kück, 2017; Horn *et al.*, 2017).

Crossing/mating of individuals with opposite mating types leads to karyogamy and subsequent development of a sexual structure (ascocarp containing ascospores) in *A. flavus* and *A. parasiticus* (Horn *et al.*, 2009), as follows. First, contact between antheridium and ascogonium leads to the fusion of intracellular materials from the two hyphae (plasmogamy) but not their haploid nuclei, producing dikaryotic hyphae (having two haploid nuclei in each cell, one from each mother cell) (Casselton and Zolan, 2002). Second, plasmogamy is followed by ascocarp formation (development of the fertilised ascogonium into a fruiting body, ascocarp, also called cleistothecium), where karyogamy occurs (fusion of the two haploid nuclei) to produce diploid ascogenous hyphae in the ascocarp, which are reduced to haploid by meiotic division to produce eight ascospores in each ascus (sac-like structure) of the ascogenous hyphae (Noble and Andrianopoulos, 2013). Finally, the ascospores are released by mature cleistothecium into the environment, where they germinate into new fungi under favourable conditions (Figure 2.5). The whole sexual cycle takes about seven to ten days (Adams *et al.*, 1988).