CHARACTERIZATION AND MYCOTOXIN PRODUCTION OF Aspergillus spp. ISOLATED FROM GROUNDNUTS (Arachis hypogaea) IN MALAYSIA

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by

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LIST OF ABREVIATIONS

µl	Microlitre
µg/g	Microgram per gram
µm	Micrometre
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Tool
Bp	Base pairs
β-tubulin	Beta tubulin
CH_3CN	Acetonitrile
C-Met	C-methyltransferase
CREA	Creatine sucrose agar
CYA	Czapek Yeast Extract Agar
CZ	Czapek Dox
DG18	Dichloran 18 % Glycerol agar
DNA	Deoxuribonucleic acid
dNTP	Deoxunucleotide triphosphate
FLR	Fluorescent detector
g	Gram
h	Hour
ICBN	International Code of Botanical Nomenclature
ICPA	International Commissions of *Penicillium* and *Aspergillus*

ITS	Internal Transcribe Spacer
KS	β-ketoacyl synthase
L	Litre
LSU rDNA	Large subunit ribosomal deoxyribonucleic acid
M	Molar
mA	Miliampere
MEA	Malt Extract Agar
MEB	Malt Extract Broth
Mm	Milimetre
ml	Mililitre
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
Min	Minute
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
NNI	Nearest Neighbor Interchange
OTA	Ochratoxin A
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PKS	Polyketide synthase
p.s.i	Per square inch
RAPD	Random amplified polymorphism DNA
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction Fragment Length polymorphism
r.p.m	Revolution per minute

S	Second
SBS	Sick building syndrome
spp.	Species
TBE	Tris-Borate-EDTA
UHPLC	Ultra-high performance liquid chromatography
WA	Water agar

LIST OF SYMBOLS

%	Percentage
°C	Degree celcius
™	Trade mark
±	Plus minus

PENCIRIAN DAN PENGHASILAN MIKOTOKSIN OLEH *Aspergillus* spp. YANG DIPENCIL DARIPADA KACANG TANAH (*Arachis hypogaea*) DI MALAYSIA

ABSTRAK

Kacang tanah (*Arachis hypogaea*) merupakan tanaman makanan yang penting di seluruh dunia, ditanam di negara-negara tropika, sub-tropika dan beriklim sederhana. Kacang tanah mengandungi kandungan nutrien yang tinggi dan ia mudah terdedah kepada pelbagai kulat pencemar, terutamanya *Aspergillus* spp. yang merupakan kulat penyimpanan yang terkenal. Kajian ini dijalankan untuk memencil dan mengenal pasti *Aspergillus* spp. dari kacang tanah dan juga untuk mengesan penghasilan mikotoksin oleh spesies toksigen iaitu *A. niger*, *A. flavus* dan *A. chevalieri*. Berdasarkan ciri-ciri morfologi, 118 pencilan telah dikenal pasti sebagai *A. niger* (54 pencilan), *A. flavus* (20 pencilan), *A. chevalieri* (18 pencilan), *A. restrictus* (16 pencilan), *A. aculeatus* (empat pencilan), *A. sydowii* (empat pencilan) and *A. fumigatus* (dua pencilan). Pengenalpastian secara molekul menggunakan jujukan nukleotida kawasan transkripsi dalaman (ITS), gen β-tubulin dan kalmodulin telah mengenalpasti 81 wakil pencilan yang juga telah dikenalpasti secara morfologi sebagai *A. niger* (35 pencilan), *A. tubingensis* (10 pencilan), *A. flavus* (sembilan pencilan), *A. chevalieri* (10 pencilan), *A. amstelodami* (10 pencilan), *A. aculeatus* (tiga pencilan), *A. sydowii* (dua pencilan) and *A. fumigatus* (dua pencilan). Pencilan dari pengenalpastian morfologi *A. restrictus* telah dikenal pasti semula sebagai *A. amstelodami* berdasarkan jujukan gen β-tubulin dan kalmodulin. Pohon filogenetik berdasarkan kaedah hubungan jiran (NJ) dan kebolehjadian maksimum (ML) dengan 1000 nilai butstrap menggunakan gabungan jujukan nukleotida ITS, β-tubulin dan

kalmodulin menunjukkan bahawa pencilan dari spesies yang sama telah dikelompokkan dalam klad yang sama. Topologi dan perkelompokan pencilan-pencilan *Aspergillus* dari spesies yang berbeza dalam pohon NJ adalah serupa dengan topologi dan perkelompokan pencilan-pencilan dalam pohon ML. Keputusan kajian ini menunjukkan bahawa ciri-ciri morfologi tidak mencukupi untuk mengenal pasti beberapa spesies *Aspergillus*, terutamanya *A. tubingensis* dan *A. amstelodami* daripada kacang tanah dan pengenalpastian secara molekul perlu dijalankan untuk mengenal pasti identiti spesies dengan tepat. Untuk analisis mikotoksin, pengesanan gen biosintesis mikotoksin boleh memberi petunjuk kepada penghasilan mikotoksin. Dalam kajian ini, dua gen okratoksin A, PKS15KS and PKS15C-MeT telah dikesan pada tujuh pencilan *A. niger* tetapi tiada satu pun pencilan tersebut menghasilkan okratoksin A apabila analisis secara kuantifikasi menggunakan kromatografi cecair berprestasi ultra tinggi. Bagi aflatoksin B1, gen Nor-1 (asid norsoloronik) dan Ver-1 (Versicolorin) dikesan pada semua pencilan *A. flavus* tetapi hanya dua pencilan *A. flavus*, KDH 7 dan KL 27b, menghasilkan aflatoksin B1 dengan kepekatan 1.0 μg/g dan 1.1 μg/g, masing-masing. Gen biosintesis okratoksin A serta gen Nor-1 dan Ver-1 yang tidak dapat dikesan pada pencilan *A. chevalieri* mungkin menunjukkan bahawa spesies ini tidak menghasilkan okratoksin A dan aflatoksin B1. Pelbagai spesies *Aspergillus* telah ditemui pada kacang tanah yang boleh membawa kepada pencemaran mikotoksin dimana spesies toksigen juga telah ditemui. Kehadiran *Aspergillus* spp. pada kacang tanah boleh mengurangkan kualiti kekacang serta mengurangkan hayat simpanannya.

CHARACTERIZATION AND MYCOTOXIN PRODUCTION OF *Aspergillus* spp. ISOLATED FROM GROUNDNUTS (*Arachis hypogaea)* IN MALAYSIA

ABSTRACT

Groundnut (*Arachis hypogaea*) is an important food crop worldwide, cultivated in tropical, sub-tropical and temperate countries. Groundnut contains high nutrients and it is susceptible to various fungal contaminants, especially *Aspergillus* spp. which is a well known storage fungi. The present study was conducted to isolate and identify *Aspergillus* species from groundnuts as well as to detect mycotoxin production by toxigenic species, namely *A. niger, A. flavus* and *A. chevalieri*. Based on morphological characteristics, 118 isolates were identified as *A. niger* (54 isolates), *A. flavus* (20 isolates), *A. chevalieri* (18 isolates), *A. restrictus* (16 isolates), *A. aculeatus* (four isolates), *A. sydowii* (four isolates) and *A. fumigatus* (two isolates). Molecular identification using nucleotide sequences of Internal Transcribed Spacer (ITS) region, β-tubulin and calmodulin genes identified 81 representative isolates that were also morphologically identified species of *A. niger* (35 isolates), *A. tubingensis* (10 isolates), *A. flavus* (nine isolates), *A. chevalieri* (10 isolates), *A. amstelodami* (10 isolates), *A. aculeatus* (three isolates), *A. sydowii* (two isolates) and *A.fumigatus* (two isolates). The isolates which were morphologically identified as *A. restrictus* were re-identified as *A. amstelodami* based on β-tubulin and calmodulin gene sequences. Phylogenetic tree based on Neighbour Joining (NJ) and Maximum Likelihood (ML) methods with 1000 bootstrap values using combined nucleotide sequences of the ITS region, β-tubulin and calmodulin showed that the isolates from the same species were grouped in the same clade. The topology and grouping of the *Aspergillus* isolates from different species in the NJ tree are similar with the topology

and grouping of isolates in the ML tree. The results of the present study showed that morphological characteristics are not sufficient for identification of some *Aspergillus* spp., particularly *A. tubingensis* and *A. amstelodami* from groundnuts, and molecular identification needs to be carried out for accurate species identity. For mycotoxin analysis, detection of mycotoxin biosynthesis genes can give an indication of mycotoxin production. In the present study, two ochratoxin A genes, PKS15KS and PKS15C-MeT were detected in seven *A. niger* isolates but none of the isolates produced ochratoxin A when quantification analysis was done using Ultra-High Performance Liquid Chromatography. As for aflatoxin B1, Nor-1 (norsolorinic acid) and Ver-1 (Versicolorin) genes were detected in all *A. flavus* isolates but only two *A. flavus* isolates, KDH 7 and KL 27b produced aflatoxin B1 with 1.0 μg/g and 1.1 μg/g concentrations, respectively. Ochratoxin A biosynthesis genes as well as Nor-1 and Ver-1 genes that were not detected from *A. chevalieri* isolates may indicate that the species did not produce ochratoxin A and aflatoxin B1. Various species of *Aspergillus* were found in groundnuts which may lead to potential mycotoxin contamination as toxigenic species were also recovered. The occurrence of *Aspergillus* spp. on groundnuts can reduce the quality of the legumes as well as reducing their shelf life.

CHAPTER ONE

INTRODUCTION

Groundnut (*Arachis hypogaea*) or peanut is a legume belonging to the family Fabaceae. In Malaysia, groundnut is often used as an ingredient in cooking. Groundnuts are commercialised as raw or roasted, salted and consumed as a snack. Raw shelled groundnuts are available in almost all supermarkets and sundry shops in Malaysia.

Groundnut cultivation and production are not extensive in Malaysia. The crop is more suitable to be used as crop rotation which is locally grown in rotation with other crops. Saleh and Masiron (1994) reported that Malaysia is popular with groundnuts which are grown as intercrop in small-holdings. Groundnuts are grown in the riverine and in rainfed rice areas in Kelantan, Terengganu, Kedah and Pahang (Halim and Ramli, 1980).

Groundnuts are mainly imported from Vietnam, USA, China, Thailand and Hongkong. A total of 44 871 tonnes of groundnuts were imported, mainly in the form of shelled nuts as a response to high demands (Halimah and Lum, 1992).

Groundnuts are imported across the world and thus contamination can easily occur. Under favourable conditions during storage either in the shops and markets or shipments in long journeys, groundnuts are prone to spoilage and contamination by

diverse groups of microorganisms particularly storage fungi. One of the storage fungi which is widely distributed is the *Aspergillus* spp. which can cause contamination in storage products including groundnuts. In addition to *Aspergillus*, *Rhizopus*, *Penicillium*, *Curvularia*, *Fusarium* and *Mucor* can also cause contamination in groundnuts under suitable storage conditions (Aliyu and Kutama, 2007). Hedayati et al. (2010) reported that *Aspergillus* was the most predominant species (70%) isolated from peanut in Sari, Iran.

Various species of *Aspergillus* have been reported to contaminate groundnuts in storage. Guchi (2015a) reported that groundnuts in storage are usually infected by *A. flavus, A. niger, A. nomius* and *A. tamari*. Some of the *Aspergillus* species contaminated groundnuts in storage produce mycotoxin. Common toxigenic *Aspergillus* spp. associated with groundnuts and other storage products are *A. niger*, *A. flavus*, *A. fumigatus* and *A. terreus*. Different *Aspergillus* spp. produced different types of mycotoxin such as aflatoxin, ochratoxin and gliotoxin. Based on a study by Bankole et al. (2005), groundnuts were contaminated by 38 isolates of *A. flavus* which produced aflatoxins. Reddy et al. (2010) detected aflatoxins B1, B2, G1, and G2, citrinin, fumagillin, diacetoxyscirpenol, T-2 toxin, satratoxin H and zearalenone in groundnuts. *Aspergillus flavus* mycotoxins have harmful effects on humans, animals, and crops that result in illnesses and economic losses (Giray et al., 2007).

The first step to detect fungal contamination by *Aspergillus* on groundnuts is by isolation of the fungus. After isolation, the common method used for identification of *Aspergillus* spp. is based on morphological and molecular

characterization. Morphological characteristics commonly used for identification are characteristics of conidiophores, arrangement of metulae or phialides on the vesicle, conidia and colony colour. However, morphological characteristics of some *Aspergillus* spp. are not sufficient for identification as the characters are similar or there are overlapping characters. Geiser (2007) reported that morphological characters of *Aspergillus* spp. may vary as sclerotia of some *Aspergillus* spp. are not present in all isolates. *Aspergillus* spp. growth may vary on different media and under different temperatures due to adaptations towards the surrounding condition. Thus, molecular identification need to be carried out for accurate species determination.

For molecular identification of *Aspergillus* spp., internal transcribed spacer region of the ribosomal DNA (rDNA) is commonly used as the ITS region can differentiate and distinguish most species of *Aspergillus*. However, sometimes the region may lack variation within several closely related *Aspergillus* species. Thus, secondary identification markers, namely β-tubulin and calmodulin genes are more suitable for *Aspergillus* identification and phylogenetic analysis. The β-tubulin gene has been used to resolve closely related *Aspergillus* species (Hong et al., 2005; Samson et al.,2006; Hong et al., 2010). Based on a study by Geiser et al. (1998), this protein coding gene shows a high rate of nucleotide substitutions and was phylogenetically informative to study the taxonomic relationships between closely related species. Calmodulin gene is also suitable as a secondary identification marker because this gene has a high degree of specificity for the identification of *Aspergillus* at the species level especially within the black *Aspergillus* (Samson et al., 2014).

Phylogenetic relationship for individual and combined nucleotide sequences of ITS region, β-tubulin and calmodulin were conducted for species confirmation by grouping the isolates of the *Aspergillus* species into distinct clades. The combination of multigene sequences of (ITS region, β-tubulin and calmodulin) can resolve species identity and determine the genetic relationships among species and sections in *Aspergillus* (Peterson, 2008).

Groundnut contamination by *Aspergillus* might cause health risk to human and livestock as groundnuts are commonly consumed directly. Moreover, mycotoxins that may be present in peanuts are also toxic and have harmful effects on animals and humans. Mycotoxins cannot be destroyed by heating because they are very heat stable. Heat treatments such as boiling water, roasting or even autoclaving cannot adequately destroy the mycotoxins. Thus, the present study was conducted to identify *Aspergillus* species and the species that are capable of producing aflatoxin and ochratoxin. Identification of *Aspergillus* spp. and mycotoxin detection are important for implementing suitable control strategies for groundnuts storage and this will lead to improving quality control of groundnuts for consumer safety. The specific objectives of the present study were:

1. To isolate and identify *Aspergillus* spp. from groundnuts using morphological and molecular characteristics.
2. To determine phylogenetic relationship among the *Aspergillus* spp. isolated from the groundnuts.
3. To determine and detect the mycotoxin produced by toxigenic *Aspergillus* spp.

CHAPTER TWO

LITERATURE REVIEW

2.1 Groundnuts

Groundnut or peanut (*Arachis hypogaea*) is a legume in the family Fabaceae, originated from South America. Groundnut is one of the most popular legume crops widely cultivated in tropical, sub-tropical and temperate countries (Aliyu and Kutama, 2007). Countries that are known as the leading world producers of groundnuts are China, India, Nigeria, the USA and Senegal (Shiyam, 2010). Groundnut is currently grown over 22.2 million hectares worldwide and the production is over 35 million tonnes (Payman et al., 2011).

About 75% of the groundnut production is used as food in Europe, North and South America while only 35% in Asia (Birthal et al., 2010). As a food based product, groundnuts are commercialised in raw or roasted, salted forms, consumed as a snack and used as cooking oil to make bread, biscuits and other bakery products. Groundnut is also used to make peanut butter, which is a famous groundnut product in the USA, Canada and Australia (Janilla et al., 2013). The oil produced from groundnut is also used to manufacture margarine, shortening and soap.

Groundnut is the 13th most important food crops in the world, 4th most important source of edible oil and 3rd most important source of vegetable protein as it is rich in nutrient contents. The legume is rich in vitamins, essential minerals and high in antioxidants (Halvorsen et al., 2002; Wu et al., 2004). Nutrients in peanuts

also play important roles for growth and energy as well as provide cardiovascular and metabolic benefits to humans which can be used as part of healthy diets (Ros, 2010). Other nutritional contents of groundnuts including phosphorus, folate, fiber, vitamin E, magnesium, trianin and niacin maintain and repair body tissues.

Nutrient content in groundnuts is very high in starch while the protein content ranged from 10-30%. The protein portion of the oil is used in the manufacturing of some textile fibres while protein cake which is oil cake residues is used as animal feed and also as soil fertilizer (Godio, 2013).

Since groundnut is high in nutrient content, it can get contaminated and damaged by various groups of fungi**.** Groundnut contamination can occur during pre-harvest, harvesting and post-harvest. Pre-harvest contamination by fungi is affected by soil moisture and soil temperature especially in hot, dry and high soil temperature. Pre-harvest contamination of groundnuts can also occur when there is extreme and prolonged drought stress more than 20 days at 29 – 31°C (Craufurd et al., 2006). *Aspergillus flavus* is a common fungal species that can cause groundnut contamination during pre-harvest (Craufurd et al., 2006; Guo et al., 2009).

Infestation of groundnuts by soil fungi can occur during growth and harvesting (Khodavaisy, 2012). Harvesting groundnut crops at optimum maturity is important as overmature or immature pods at harvest can lead to contamination (Torres et al., 2014) and late harvest can cause contamination of aflatoxin produced

by *Aspergillus* spp. (Singh and Oswalt, 1995). Groundnut contamination by fungi also occur due to mechanical damage during digging and threshing as well as harvesting using hand and hoe (Okello et al., 2013).

Post-harvest contamination can occur during storage, transportation and marketing. If groundnuts are damaged by fungal infestation or mechanical damage during pre-harvest, it will result in decrease of the legume quality during storage. Infestation by storage fungi and insects during storage can be avoided by storing the peanuts at low moisture content and low temperature (Atanda et al., 2011).

2.2 Mycoflora of Groundnuts and Storage Conditions

Groundnuts are very susceptible to contamination by various types of storage fungi and soil fungi. These fungi are common contaminants of groundnut seeds in the field and storage which can cause damage and yield loses. Thus, quality of groundnut seed is affected when infestation occurs.

Peanut seeds are highly susceptible to various types of storage fungi such as *Rhizoctonia* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Cladosporium* spp. and *Penicillium* spp. (Elwakil and El-Metwally, 2001). Several genera of fungi have been reported to contaminate groundnuts such as *A. flavus* (39.1%), *Penicillium* (9.2%), *Rhizopus* (7.2%), *Mucor* (2.5%), *Alternaria* (1.03%) and *Nigrospora* (0.5%) (Rostami et al., 2009). In a study by Hedayati et al. (2010), several types of storage fungi including *A. flavus*, *A. niger*, *Penicillium*, *Cladosporium* and *Rhodotorula* were

frequently isolated from dry roasted (63.7%) and raw (48.9%) forms of peanut kernels.

Groundnuts can easily get contaminated during storage especially at high temperature and high relative humidity. According to Guchi (2015a), the optimum temperature for storage fungi to grow on groundnuts is at 25 - 42°C, however, a particular fungal species has its own climatic requirement for growth. For example some species of *Penicillium* and *Cladosporium* which are cold-tolerant fungi can grow at low temperature below 20°C (Atanda et al., 2011) while *Aspergillus* spp. can grow at higher storage temperature, from 25 - 35°C (Lacey and Megan, 1991). Most of *Aspergillus* spp. can grow at low and relatively high storage temperature and thus, stored products including peanuts are susceptible to *Aspergillus* spp. compared to the other fungi. To prevent infestation of storage fungi, the temperature should be maintained between 0 - 10°C (Chang et al., 2013).

Suitable relative humidity is also important for storage fungi to grow. Relative humidity is closely related to the moisture content. For grains in storage, relative humidity of at least 65% which is equivalent to an equilibrium moisture content of 13% are required for growth of storage fungi (Atanda et al., 2011). The most prevalent storage fungi, *Aspergillus* spp. and *Penicillium* spp. can contaminate seeds at relative humidity from 65% to 90% (Chelkowski, 1991). These conditions are probably similar for infestation of storage fungi on groundnuts.

Other than storage fungi, soil fungi also has the ability to contaminate and damage peanut crop in the field. Soil fungi play a significant role in spoilage of the crop during preharvest in the field. Common soil fungi that have been found to contaminate peanuts are *Aspergillus*, *Penicillium*, *Rhizopus* and *Fusarium* species (Gachomo et al., 2004). Soil serves as reservoir of various fungi including toxigenic fungi. For example *A. flavus* and *A. parasiticus* can invade peanut seeds during maturation (Horn, 2005) and may produce aflatoxin, a carcinogenic mycotoxin which can reduce the quality of peanuts and give health implications to humans and animals (Guchi, 2015b).

2.3 The Importance of the Genus *Aspergillus*

Aspergillus spp. are ubiquitous filamentous fungi and among the most economically important fungal genera worldwide. Many species of *Aspergillus* are used in food industry, acid production and pharmaceutical products.

Many Asian food and beverages such as sake and soy sauce are produced from enzymes produced by *A. oryzae*, *A. sojae* and *A. awamori*, which are known as koji mold (Klich, 2002). *Aspergillus oryzae* was also used for enzyme production to produce traditional Japanese foods such as sauce, sake, bean curd with seasoning and vinegar (Machida et al., 2008).

Aspergillus spp. are involved in acid production, including citric and gluconic acids which are produced by *A. niger*. Citric acid is used in food and beverage

industries and act as an acidifier or antioxidant to preserve or enhance the flavors and aromas of food (Max et al., 2010). In the chemical industry, citric acid which is also produced by *A. niger* is used as a foaming agent for softening and treatment of textiles (Max et al., 2010). Gluconic acid produced by *A. niger* is used to impart refreshing sour taste in many food items such as wine and fruit juice (Ramachandran et al., 2006).

Aspergillus is also used in pharmaceutical and medicinal industries. Cholesterol-lowering drugs, Lovastatin was produced by a wild strain of *A. terreus* (Goswami et al., 2012) which also produced the metabolite terrecyclic acid A, used for anticancer and antibacterial activities (Bok et al., 2006). A chemical modification of echinocandin B produced by *Emericella nidulans/ rugulosa* is used as an anti-candida drug and Cilofungin which is a semi-synthetic drug (Huang et al., 1990).

Although there are many uses of *Aspergillus* in producing food products, many *Aspergillus* spp. are common contaminants of food such as cereal, nuts, vegetables, fruits and feed for livestock. Many *Aspergillus* spp. are heat tolerant and xerophilic which have the ability to grow on low water activity substrates and cause spoilage of stored food and animal feeds (Klich, 2002). *Aspergillus chevalieri* and *A. amstelodami* are examples of xerophilic *Aspergillus* which can contaminate maize, peanuts, soybeans and rice grains (Pitt and Hocking, 2009).

Many *Aspergillus* spp. have been reported on different types of food and feed. *Aspergillus flavus* and *A. niger* have been recovered from maize, wheat, rice and peanut (El-Shanshoury et al., 2014) while *A. carbonarius* was found in grapes (Pitt and Hocking, 2009). In a study by Reddy et al. (2009), *A. niger*, *A. ochraceus* and *A. parasiticus* are major contaminants of rice grains. When infestation occurs, nutritional value, texture, flavour and appearance of the food changes making it become unsuitable to consume as it may contain harmful effect to humans and animals.

Aspergillus is known as an opportunistic pathogen of humans and animals (Pitt, 1994). Behnsen et al. (2008) reported that an opportunistic human pathogen, *A. fumigatus* causes severe systemic infections and is a major cause of fungal infections in immunocompromised patients. Aspergillosis is also another opportunistic fungal infection and is associated to a wide variety of diseases such as aspergilloma, allergic bronchopulmonary aspergillosis, tuberculosis, pneumothorax, bullous lung disease, nontuberculous mycobacterial pulmonary infection and chronic obstructive pulmonary disease (Denning et al., 2013). Among the species that often associate with aspergillosis are *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger*. *Aspergillus* is also associated with allergies such as asthma, allergic rhinitis, allergic sinusitis, broncho pulmonary mycoses and hypersensitivity pneumonitis (Pieckova and Wilkins, 2004).

Some of *Aspergillus* spp. can also grow and reproduce effectively in indoor air environments such as interior of offices, commercial buildings and residences

(Robbins et al., 2000). It has also been reported to be associated with sick building syndrome (SBS) (Pieckova and Wilkins, 2004). The syndrome is a condition whereby people experience symptoms of irritations of the skin, mucous membranes, headache and tiredness due to poor indoor air quality (Bakke et al., 2008; Terr, 2009). Several species have been found in indoor environments and contribute to the effect of SBS including *A. flavus*, *A. niger*, *A. oryzae*, *A. ochraceus*, *A. fumigatus* and *A. terreus* (Pavan and Manjunath, 2014).

2.4 Taxonomic History of *Aspergillus*

Aspergillus was first described by P. A. Micheli in 1729 and the name *Aspergillus* was based on the spore-bearing structure that resemble an aspergillum used to sprinkle holy water by the Catholic church (Raper and Fennell, 1965). Later, Michelli used *Aspergillus* and another species of microfungus, *Mucor* spp. to demonstrate asexual reproduction of fungi.

The first monograph describing species of *Aspergillus*, known as The Aspergilli was written by Thom and Church (1926), followed by A Manual of the Aspergilli by Thom and Raper (1945) which described 77 species and eight varieties. Later, Raper and Fennell (1965) published a monograph, The Genus *Aspergillus* that contained the description of 132 species which were divided into 18 varieties but in the manual there is no nomenclature status for classification into groups.

Gams et al. (1985) proposed the use of subgenera and sections of *Aspergillus*, and it was accepted by many *Aspergillus* taxonomists. Among them was Peterson (2000) who revised the taxa produced by Gams et al. (1985) and retained 12 of the 18 sections and eliminating three of subgenera based on sequences. In another taxonomic study, Pitt (2000) accepted 184 species of *Aspergillus* and 70 associated teleomorphic names based on morphological species concept. Samson (2000) listed another 36 taxa which had been published between 1992 and 1999. More than 40 species have been described since then and the total number of new species has increased to approximately 250 species (Geiser et al, 2007).

For delimitation of *Aspergillus* spp., polyphasic approach comprising molecular, morphological, physiological and in some cases ecological data is recommended (Samson et al., 2007a). Multiple sequences comprising three or more genes / regions namely, ITS, β-tubulin, calmodulin, actin and RNA polymerase are recommended for description of new taxa. Morphological descriptions based on macromorphological and micromorphological characteristics observed on recommended differential media also need to be provided.

The polyphasic approach was improved by Samson et al. (2014) with recommended standard working technique for identification and characterization of *Aspergillus.* In the publication, detail morphological observation is highlighted with the recommended method of observing microscopic and macroscopic characteristics as well as the recommended differential media used. For molecular identification and

phylogeny study, ITS and calmodulin genes are recommended for identification while β-tubulin and RPB2 genes are used for phylogeny.

The publication by Samson et al. (2014) also contain information on the list of accepted species which was 339 species, information on the ex-type strains with GenBank accession numbers for all recommended genes and regions used for identification and phylogeny of *Aspergillus* spp. The ex-type strains are also listed in the International Commision of *Penicillium* and *Aspergillus* (ICPA) website (http://www.aspergilluspenicillium.org/). The lists are regularly updated and contain information on the latest taxa of *Aspergillus*.

With the introduction of one fungus, one name concept which replaced dual nomenclature of naming fungal species, the nomenclature of *Aspergillus* is also affected. In the one fungus, one name concept or single system of naming a species, anamorph name is given priority over the teleomorph name. As for *Aspergillus*, the name *Aspergillus* (anamorph species) is synonymized with the teleomorph species such as *Eurotium*, *Emericella*, *Neosartorya* and *Petromyces* (Samson et al., 2014). Single name nomenclature of *Aspergillus* has been applied in a study by Hubka et al. (2013) on *Aspergillus* spp. section *Aspergillus* of which all the accepted *Eurotium* species are synonymized with *Aspergillus*.

The present classification of *Aspergillus* based on National Centre for Biotechnology Information (NCBI) is as below (https://www.ncbi.nlm.nih.gov):

Kingdom : Fungi

Subkingdom: Dikarya

Phylum : Ascomycota

Subphylum : Pezizomycotina

Class : Eurotiomycetes

Order : Eurotiales

Family : Aspergillaceae

Genus : *Aspergillus*

2.5 Identification and Characterization of *Aspergillus* Species

2.5.1 Morphological Identification

Morphological identification is the most common method used to identify *Aspergillus* spp. and is based on similarity of the observable macroscopic and microscopic characteristics. In earlier taxonomic studies of *Aspergillus*, morphological identification and characterization is widely used (Raper and Fennell, 1965; Gam et al., 1985; Klich, 2002; McClenny, 2005).

For macroscopic characteristics, colony appearances which include colony colour, reverse colony colour, colony diameter, exudate, sclerotia and cleistothecia of the culture are used to group or to sort the isolates into sections. For example, isolates of *Aspergillus* in sections *Flavi*, *Fumigati* or *Nidulantes* commonly produced

yellow green, green or dark green colonies. Black and brown colonies are shown mainly by *Aspergillus* spp. from section *Nigri* while white colonies by species in section *Candidi*. *Aspergillus* spp. from section *Clavati* are identified by bluish grey colonies (Klich, 2002; Samson et al., 2010).

Distinctive features of microscopic characteristics used for identification are size and shape of conidia, seriation and conidiophore consisting of stipe, vesicle shape and size, phialides and metulae (Klich, 2002; Samson et al., 2010). *Aspergillus* spp. are classified into sections based on seriation either uniseriate, biseriate or both (Figure 2.1). Uniseriate form as refers to conidia-bearing phialides arising directly from the vesicle while biseriate refers to the phialide and metulae. *Aspergillus* spp. in section *Circumdati* and *Nidulantes* are examples of biseriate species and uniseriate species are shown by species in sections *Fumigati* and *Clavati.* Sections *Flavi*, *Nigri* and *Candidi* are both biseriate and uniseriate (Klich, 2002).

Phialide is a fertile area produced by a vesicle that produces long chains of conidia while metulae is a second layer between vesicle and phialide (Prakash and Jha, 2014). The apex of the stipe expands into the vesicle which has various shapes such as globose, spherical, pyriform, spathulate or clavate (Figure 2.2) which are important features to identify *Aspergillus* isolates to species level. The conidial head of *Aspergillus* can be a compact column, known as columnar while diverging, known as radiate (Samson et al., 2010) (Figure 2.3).

Other characteristics used to identify *Aspergillus* spp. are Hulle cells, cleistothecia and sclerotia. Hulle cells can be produced single or in chains, thick or smooth walled (Figure 2.3). Hulle cells of some *Aspergillus* spp. are associated with the cleistothecia which are produced in the teleomorph state. Cleistothecia are fruiting bodies with no natural openings, containing asci and ascospores. Sclerotia are masses of hyphae which does not contain spores (Klich, 2002).

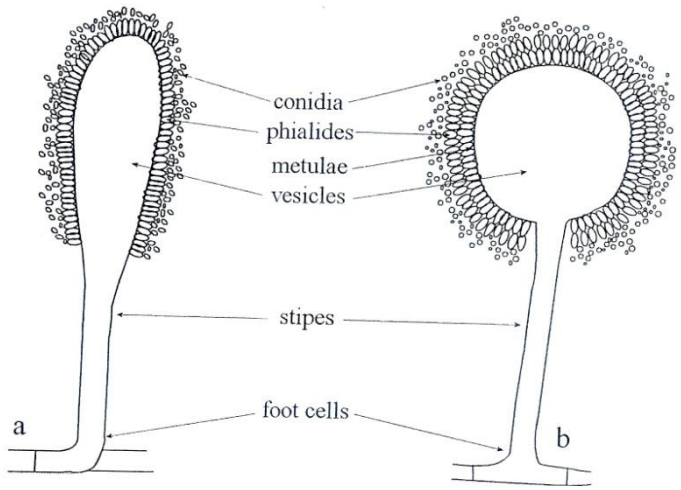

Figure 2.1: Conidiophore of (a) uniseriate (*A. clavatus*) and (b) biseriate (*A. flavus*) (Klich, 2002)

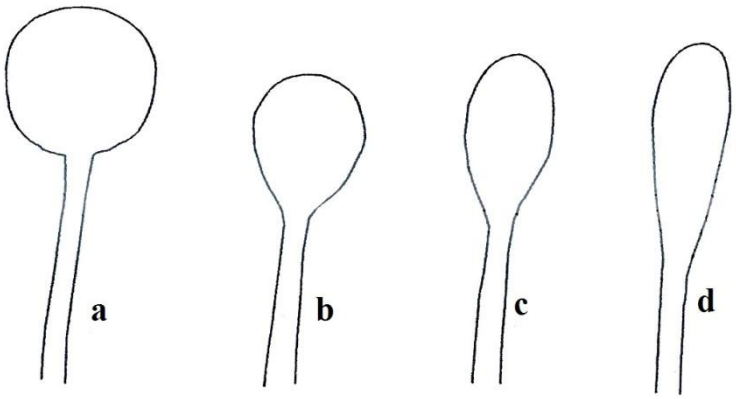

Figure 2.2: Vesicle shapes of *Aspergillus*. (a) Globose or spherical, (b) pyriform, (c) spathulate and (d) clavate (Klich, 2002).

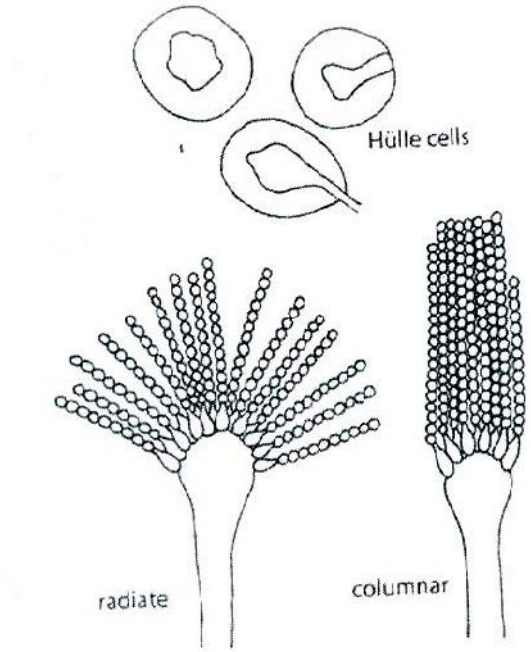

Figure 2.3: Conidial arrangement and Hulle cells of *Aspergillus* (Samson et al., 2010)

2.5.2 Differential Media

Differential media are used for morphological identification and characterization of *Aspergillus,* as well as to distinguish several species. In earlier taxonomic study, Czapek Dox agar (CZ) was used and this medium contain sucrose and nitrate, and is still widely used until today (Raper and Fennel, 1965). Several differential media are also recommended by Klich (2002) including Malt Extract Agar (MEA) incubated at 25°C, Czapek Yeast Agar (CYA) incubated at 25°C and 37°C, and CYA with 20% sucrose and these media are incubated at different temperatures, particularly to observe colony appearances (Klich, 2002). Samson et at. (2010) added two additional media, Creatine Sucrose Agar (CREA) and Dichloran 18 % Glycerol agar (DG18) for characterization of xerophilic *Aspergillus*. Samson et al. (2014) recommended to use CZ, 20 % sucrose CYA (CY20S), 20 % sucrose MEA (MEA20S), Yeast Extract Sucrose agar (YES), DG18, Oatmeal Agar (OA) and CREA for *Aspergillus* morphological identification and characterization.

Each medium has specific purposes and various macroscopic and microscopic features of *Aspergillus* can be observed. Differential media incubated at certain temperatures are used to distinguish several species of *Aspergillus* as it depends on physiological adaptation of a particular species. Xerophilic *Aspergillus* are commonly grown on CYA20S, MEA20S and DG18 while for studying extrolite profiling, isolates are grown on YES and CYA. Most *Aspergillus* spp. section *Nigri* and *Flavi* grow well on DG18 which indicated good growth of xerophilic species. The use of OA media is to observe the formation of sexual reproductive structures of *Aspergillus* spp. (Samson et al., 2010).

Several differential media, CZ, CYA, MEA and CYA20S have been used by Diba et al. (2007) for identification of *Aspergillus* spp. from clinical specimens and found that *A. flavus, A.niger* and *A. fumigatus* were the most common species recovered. Eight species, namely *A. ficcum, A. flavus, A. flavus* var. *columnaris, A. terreus* var. *aureus, A. fumigatus, Emericella nidulans, Em. rugulosa* and *A. terricola* var. *americana* isolated from soils were identified using only two differential media, CZA and MEA (Afzal et al., 2013).

Some *Aspergillus* spp. could not be identified or distinguished macroscopically when cultured on differential media. For example, *Aspergillus* spp. aggregate comprising *A. niger, A. tubingensis* and *A. foetidus* as well as *A. japonicus* and *A. aculeatus* could not be distinguished based on macroscopic characters observed on CYA25 and MEA. On CYA25 and MEA, the three species aggregate produced dark brown to black colonies while *A. aculeatus* and *A. japonicus* have dark brown to grey colonies (Silva et al., 2011)

For identification of some *Aspergillus* spp., misidentification can occur due to variability and overlapping characters, particularly for species within the same section. Most *Aspergillus* spp. in section *Nigri* produced black colony and similar globose to sub-globose vesicle and globose conidia (Klich, 2002) which can lead to misidentification. Misidentification can also occur among *Aspergillus* spp. section *Flavi*, particularly to distinguish *A. nomius* and *A. tamari* from *A. flavus* as the three species produced yellow colony (Tam et al., 2014). In addition, some clinical isolates showed slow sporulation and aberrant conidiophores such as species from section *Fumigati* (Balajee et al., 2007). Therefore, to assist in identification and for