MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *ASPERGILLUS* SPECIES ISOLATED FROM CORN FEED IN PENANG

by

NOOR ATIQAH ZULKIFLI

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LIST OF ABBREVIATIONS

µl	Microliter
µm	Micrometer
AFLP	Amplified Fragment Length Polymorphism
Bp	Base pair
Cm	Centimeter
CREA	Creatine Sucrose Agar
CYA	Czapek Yeast Extract Agar
CZ	Czapek-Dox
DG18	Dicholoran 18% Glycerol Agar
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleotide triphosphate
EtBr	Ethidium bromide
FAO	Food and Agricultural Organisation
FDA	Food and Drug Administration
G	Gram
H	Hour
ICBN	International Code of Botanical Nomenclature
ICPA	International Commissions of *Penicillium* and *Aspergillus.*
ITS	Internal Transcribe Spacer
Kb	Kilobase
Kg	Kilogram
L	Litre
LSU rDNA	Large subunit ribosomal deoxyribonucleic acid
M	Meter

M	Molar
mA	Miliampere
MAT	Mating type
MEA	Malt Extract Agar
MEB	Malt Extract Broth
Mm	Milimeter
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
Ml	Mililiter
MLP	microsatellite length polymorphisms
MLST	multilocus sequence typing
Min	Minutes
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
NNI	Nearest Neighbour Interchange
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Ppb	Parts per billion
RAPD	Random amplified polymorphic DNA
rRDNA	Ribosomal deoxyribonucleotide acid
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction Fragment Length polymorphism
Rpm	Revolution per minute
S	Second
SBS	Sick building syndrome

sp.	Species
spp.	Species
SSU rDNA	Small subunit ribosomal deoxyribonucleic acid
TBE	Tris-Boric acid-EDTA
TEF1α	Translation elongation factor 1α
UV	Ultraviolet light
WA	Water agar

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
®	Registered
±	Plus minus
™	Trade mark
a_w	Water activity

PENGECAMAN SECARA MORFOLOGI DAN MOLEKUL SPESIES *ASPERGILLUS* DARI MAKANAN TERNAKAN JAGUNG DI PULAU PINANG

ABSTRAK

Aspergillus adalah antara kulat yang paling lazim mencemar makanan ternakan dan bijirin simpanan termasuklah dedak jagung. Kajian mengenai *Aspergillus* dalam jagung adalah penting bagi memastikan keselamatan jagung sebagai makanan ternakan. Pengecaman *Aspergillus* dapat memberi petunjuk jenis mikotoksin yang hadir kerana spesies berbeza menghasil jenis mikotoksin berbeza. Dalam kajian ini, kaedah morfologi dan molekul telah digunakan untuk mengecam pencilan *Aspergillus* dari dedak jagung yang diperolehi dari kedai runcit di sekitar Pulau Pinang. Berdasarkan ciri morfologi, 202 pencilan telah dikenal pasti secara tentatif sebagai *A. niger* Kumpulan I dan Kumpulan II (116), *A. flavus* (32), *A. oryzae* (14), *A. fumigatus* (23), *A. clavatus* (2), dan *A. terreus* (15). Bagi pengecaman secara molekul, jujukan DNA bagi kawasan transkripsi dalaman (ITS) dan gen β-tubulin telah digunakan, dan lapan spesies telah dikenal pasti sebagai *A. niger* (48), *A. tubingensis* (68), *A. flavus* (12), *A. oryzae* (5), *A. fumigatus* (11), *A. clavatus* (2), dan *A. terreus* (7). Berdasarkan pengecaman secara molekul, wakil pencilan dari pengecaman secara morfologi telah disahkan dimana hampir semua pencilan Kumpulan I telah disahkan sebagai *A. niger* dan hampir semua pencilan Kumpulan II disahkan sebagai *A. tubingensis*. Analisis filogenetik berdasarkan kaedah hubungan jiran (NJ) dan kebolehjadian maksimum (ML) dengan 1000 replikasi set data individu dan gabungan data kawasan ITS dan gen β-tubulin menunjukkan pencilan daripada spesies yang sama dikelompokkan bersama dalam klad yang

tersendiri, kecuali pencilan *A. flavus* dan *A. oryzae* yang hanya dipisahkan dalam satu klad utama yang sama. Kajian ini menunjukkan dedak jagung dari kedai runcit di Pulau Pinang telah dicemari oleh beberapa jenis spesies *Aspergillus*, dan antaranya adalah spesies yang terkenal sebagai penghasil mikotoksin seperti *A. niger* dan *A. flavus.*

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *ASPERGILLUS* SPECIES ISOLATED FROM CORN FEED IN PENANG

ABSTRACT

Aspergillus is among the most prevalent fungus contaminating stored grains and feed including corn feed. The study of *Aspergillus* in corn feed is important to ensure the safety of corn feed used as livestock feed. Identification of *Aspergillus* can give indication of mycotoxin being produced, as different species produced different types of mycotoxin. In this study, morphological and molecular identification methods were used to identify *Aspergillus* from corn feed collected from sundry shops around Penang. Based on morphological characteristics, 202 isolates were tentatively identified as *A. niger* Groups I and II (116), *A. flavus* (32), *A. oryzae* (14), *A. fumigatus* (23), *A. clavatus* (2), and *A. terreus* (15). For molecular identification, DNA sequencing of ITS region and β-tubulin gene were used, and eight species were identified as *A. niger* (48), *A. tubingensis* (68), *A. flavus* (12), *A. oryzae* (5), *A. fumigatus* (11), *A. clavatus* (2), and *A. terreus* (7). Based on molecular identification, the representatives isolates of morphologically identified species were confirmed, with most isolates of Groups I were confirmed as *A. niger* and most isolates of Group II were confirmed as *A. tubingensis*. Phylogenetic analysis based on Neighbour Joining (NJ) and Maximum Likelihood (ML) methods with 1000 bootstrap values using individual and combined ITS region and β-tubulin sequences showed that the isolates from the same species were clustered in the same clade except *A. flavus* and *A. oryzae* isolates which were separated within the same main

clade. The present study indicated that corn feed from sundry shops in Penang were contaminated with different types of *Aspergillus* species, and among the species were well known mycotoxin producers such as *A. niger* and *A. flavus.*

CHAPTER ONE

INTRODUCTION

Corn grain is globally used as a raw ingredient for livestock feed and in Malaysia it is mainly used in pig and poultry feed. Malaysian annual requirement for animal feed is approximately 7 million tonnes, in which more than 50 feed mills produce 4.9 million tonnes of the feed and home mixer produce 350 000 tonnes (Veterinary Health Malaysia, 2010). However, the raw ingredients for feed production including corn grain are imported from Argentina, India, and Brazil (Ghani, 2013). Malaysia's total corn grain import has been significantly increased from 2.8 million tonnes in 2012 to 3.1 million tonnes in 2013 as a respond to high demand from animal feed industry (International Grains Council, 2013).

Corn used for animal feed or corn feed is prone to fungal infection under favourable environmental conditions for fungal growth, especially during storage either in silo, shipment or in the shops and market. Fungal contamination including *Aspergillus* spp. is a worldwide problem, especially corn used for corn feed is imported and exported across the world. Contamination by *Aspergillus* spp. does not only reduce the quality and nutritional value of the corn but can also lead to mycotoxin contamination. Mycotoxin contamination by *Aspergillus* spp. can occur due to improper storage such as high moisture content and also present of insects and mites. Mycotoxin can affect human health and animal productivity, as the mycotoxin produced by toxigenic *Aspergillus* can persist in the food chain (Patron, 2006).

Therefore, identification and characterization of *Aspergillus* spp. contaminated corn feed are important as it will give clues or indication of mycotoxin produced. Several *Aspergillus* spp. such as *A. niger*, *A. flavus*, *A. fumigatus*, and *A. terreus* produced different types of mycotoxin. For example, *A. flavus* produced aflatoxins which are major problem globally. Contamination by aflatoxin caused losses and reduced corn quality, and may lead to severe aflatoxicosis among livestock (Sabran et al., 2012). Mycotoxin poisoning in humans and animals can occur through ingestion of contaminated feed or food, as mycotoxins can remain in the food chain due to its resistant being broken down either by digestion or food processing like freezing, heating, steaming or pasteurization (Patron, 2006). Consequently the mycotoxin can cause various health impacts such as liver and kidney toxicity, haemorrhage, cancer and even death.

The most common method used for identification of *Aspergillus* isolates is based on morphological characteristics. The genus *Aspergillus* is easily distinguished based on the spore bearing structures or the conidiophores, an erect hyphal with a swollen tip called vesicle, which bear conidia (Klich, 2002). The vesicle shape, and also the conidia colour, arrangement and size are among the important morphological characteristics for identification of *Aspergillus* to species level (Bennett, 2010). However, identification solely based on morphological characteristics is not sufficient, particularly to differentiate species within the same section or closely related species, which may showed phenotypic variation and overlapping features. The main purpose of morphological characterization is to sort the isolates into their respective groups or sections. Due to this limitation, molecular characterization using DNA sequencing is incorporated for confirmation of species identity. For DNA

sequencing, internal transcribed spacer (ITS) regions and β-tubulin gene are commonly used (Samson et al., 2010).

For identification of *Aspergillus,* sequencing of ITS region is recommended for subgenus or section level identification. This region is part of ribosomal gene which exists in two segments, ITSI and ITS2, separated between structural ribosomal RNAs (rRNA) 18S and 28S. The locus is easily amplified and is recommended as the prime fungal barcode (Samson et al., 2010). However, the region may show lack of variation within several closely related *Aspergillus* species. Thus, β–tubulin which is globular proteins is recommended for species level identification of *Aspergillus*, in which this protein coding gene contains introns which is highly variable especially at intron-rich 5′end, and more conserved at intron-poor 3′ end. Based on several studies, β–tubulin gene has resolved most of *Aspergillus* closely related species identity and phylogenetic relationship (Hong et al., 2005; Samson et al., 2006; Balajee et al., 2007; Varga et al, 2007a; Hong et al., 2010).

The occurrences and contamination of *Aspergillus* in corn feed might pose health risk to the livestock and also to human consuming the livestock products. Thus, correct species identification of *Aspergillus* spp. is important as the species identified will give indication of mycotoxin being produced. Therefore, the objectives of the present study were:

1. To isolate *Aspergillus* associated with corn feed from sundry shops around Penang and to identify the isolates using morphological characteristics and DNA sequencing of ITS region and β-tubulin gene.

2. To determine phylogenetic relationship among the *Aspergillus* spp. recovered from the corn feed.

CHAPTER TWO

LITERATURE REVIEW

2.1 Corn and Corn Feed

Corn (*Zea mays*) is also known as cereal grass, is an annual plant belonging to the Poaceae family (grass family) and is characterized by a hollow stem, parallel leaf venation, and alternating leaves off a central sheath. The ear of corn consists of hundreds of kernels (fruits/ cryopsis) fully covered the cob, and the cob is wrapped by a husk leaves and a mass of silky threads.

Corn is the third most important crop worldwide after rice and wheat (National Service for Agrifood Health and Quality, 2008), and is a very important food and fodder crop, serving as an energy source in both human and animal diets (Niaz and Dawar, 2009; Kranjaja et al., 2013). Annually, worldwide corn production is more than 814 million tonnes, in which more than 345 million tonnes are used for food, seed and industrial use. Corn is also primarily used as a staple food for more than 1.2 billion people including in sub-Saharan Africa (SSA) and Latin America. Globally more than 439 million tonnes are used for animal feed and residual use, where 60% of world total livestock feed are from corn, along with other main grains like wheat, barley, sorghum and oats (FAO, 2011; O'Brien, 2011; Nithya et al., 2012).

Corn used as livestock feed consumed 40% of worldwide corn production, used as a feed ingredient for beef and dairy cattle, lamb and sheep, pig, and mostly in

poultry. Corn is largely trade internationally as a raw material to the global feed industry, where the feeds are formulated and milled locally. The local feed manufacturers are highly relying on availability of imported corn (FAO, 2004).Globally, corn feed is popular as a livestock feed because it promotes faster weight gain and also cheaper than any other available feeds. Corn feed provides high energy but low in protein, and like any other cereal grains, contains high phosphorus but low calcium, thus require mixing with other supplements for good dietary animal feed (Lardy, 2013).

In Malaysia, the cost of producing corn is much higher than the cost of imported corn grain, and as 60 - 70% of total cost of livestock production is for feed, encourages the industry to choose imported corn (Mahmud, 2004). Malaysia, efficiently produce animal feed locally but for ingredients depend on imported raw materials, in which RM 2.5 billion was spent annually for importation and 50% of it is for corn (Zahari and Wong, 2009). The corn grain imports amounting to more than 3 million tonnes with 10% growing rate annually (Zahari and Wong, 2009; International Grains Council, 2013). Animal feed industry uses 70 - 80% of imported ingredients and only 20-30% local ingredients. Two major imported ingredients are corn and soy bean meal, others including vegetable, fish, meat and bone meal, also other mineral and vitamin sources, while the local feedstuffs include rice bran, palm kernel cake (PKC), tapioca, crop residue, and forage (Mahmud, 2004).

The poultry and pig industries are highly dependent on imported corn grain, in which most compound feed for non-ruminant (poultry and pig) are based on corn feed mixed with other additives such as amino acids, vitamins and minerals, while

the ruminants depend on locally available feedstuffs (Loh, 2002). Ghani (2013) reported in Global Agricultural Information Network (GAIN) that the demands for pork and broiler meat in Malaysia keep growing, thus forecasting the corn consumption to increase marginally in 2013-2014.

Livestock feed particularly stored grains are vulnerable to contamination of a variety of storage fungi (Muthomi et al., 2012). Fungal contamination of corn diminishes grain quality and germination, while increasing fatty acids, mustiness and spoilage. Eventually, the grain is unfit for human and livestock consumption due to discolouration, depleted nutritional value and potential contamination by mycotoxins (Niaz and Dawar, 2009; Muthomi et al., 2012). Corn grain is one of the crop commonly contaminated with mycotoxins, although the contaminated corn grain often appears without any visible sign of fungal infection (Reddy and Salleh, 2011; Muthomi et al., 2012).

2.1.1 Mycoflora and Mycotoxins associated with Corn Feed

Due to health risks associated with contaminated corn feed, studies of mycoflora and mycotoxins associated with stored grains and feed have been performed. There are two types of mycoflora, field and storage fungi. Field fungi invade on pre-harvest crops in the field such as *Alternaria*, *Fusarium*, *Clasdosporium*, *Aspergillus*, *Penicillium*, *Curvulari*, *Diplodia*, and *Gibberella*, whereas storage fungi invade post-harvest crops on seeds or grains during storage such as *Aspergillus*, *Penicillium* and *Rhizopus* (FAO, 2012). Corn feed is dried grain but vulnerable to fungal infection in favourable conditions, particularly *Aspergillus* which can grow in low water activity and at higher temperature. *Penicillium* has

variable requirements for temperature and moisture but preferred cooler climate, wet condition and lower pH and *Fusarium* is associated with dry condition followed by wet weather, whereas other and most fungi preferred adequate moisture, temperature and good aeration (Whitlow and Hagler, 2003).

In Malaysia, a study by Reddy and Salleh (2011) found 100% of corn feed samples were contaminated with *Aspergillus, Fusarium* and *Penicillium*. In Pakistan, 70% of the corn grain samples were infested by *Aspergillus* and *Penicillium*, and other 30% by 21 genera of other fungi such as *Fusarium, Dreschlera, Cladosporium, Curvularia, Arthrinium, Bipolaris*, and *Rhizopus* (Niaz and Dawar, 2009). Askun (2006) reported that in Turkey, 100% of corn grain was infested by *Aspergillus, Rhizopus, Fusarium* and *Penicillium*. Milicevic et al (2010) found *Penicillium, Fusarium, Paecilomyces, Aspergillus, Mucor*, and *Alternaria* in pig feed including corn feed in Serbia. Other studies in Spain, Iran, Nigeria, and Kenya on stored grains and corn feed also reported *Aspergillus, Penicillium* and *Fusarium* as the most common species (Ibiam et al., 2008; Khosravi et al., 2008; Sanchez-Hervas et al., 2008; Muthomi et al, 2012).

Many studies of mycoflora in corn feed demonstrated *Aspergillus* as the most prevalent genus. Pitt and Hocking (2009) reported heavy invasion of Southeast Asian corn by *Aspergillus,* in which 85% to 100% of corn grain samples in Thailand were infested by *A. flavus*. Similar findings were observed in Indonesia and in the Philippines. In addition to *Aspergillus, Penicillium* and *Fusarium* are also commonly found contaminating stored grains used for animal feed including corn feed. Pitt and Hocking (2009) reported that *Aspergillus* species always compete with *Penicillium*

and *Fusarium*. Although *Aspergillus* is less diverse than *Penicillium*, it is able to grow at extreme temperatures and dry conditions, and its more rapid growth, favours *Aspergillus* as dominant spoilage organism in the tropics, whereas *Penicillium* is more dominant in temperate zones. Fungal invasion of toxigenic fungi like *Aspergillus*, *Penicillium* and *Fusarium* are usually associated with several types of mycotoxins (Pitt and Hocking, 2009).

Mycotoxins are secondary metabolites produce by certain fungal species, which are toxic and have harmful effect on animals and humans which consume or expose to the products. *Aspergillus*, *Penicillium* and *Fusarium* are the major-mycotoxin producing fungal genera in corn feed and other stored feedstuffs (Whitlow and Hagler, 1998). As the fungal grow, mycotoxins are produced either during pre-harvest, post-harvest, storage, transportation, processing, or feeding. Favourable conditions such as adequate moisture content and temperature, as well as insect activity and damage grain can highly influence mycotoxin contamination of feed grains. Other factor is storage period as prolonged fungal invasion give time for mycotoxin accumulation and increasing the concentration (Coulumbe, 1993). Mycotoxins have been detected in nearly all agricultural crops and products including corn, wheat, sorghum, oats, barley, rough rice, and soybean meal (Afsah-Birck et al., 2006: Hejri et al., 2013). Mycotoxins can persist in the food chain, from feed to livestock, and eventually humans.

Several types of mycotoxins have been reported to be associated with corn feed. In a study by Reddy and Salleh (2011) detected aflatoxins and fumonisins in corn feed in Malaysia. Whitlow and Hagler (1998) detected aflatoxins,

deoxynivalenol, zearalenone, T-2 toxin and fumonisins in corn feed, corn silage and in variety of other feedstuffs in North Carolina. Milicevic et al. (2010) detected ochratoxins, deoxynivalenol and zearalenone in mixed corn feed in Serbian. Rodrigues and Naehrer (2012) reported on mycotoxin contamination in worldwide feedstuffs including corn feed, detected aflatoxins, zearalenone, deoxynivalenol, fumonisins, T-2 toxin and ochratoxins, where fumonisins are reported to be highest in corn feed. Few studies reported aflatoxins as the most common mycotoxin in corn grain (Cassel et al., 2001; Muthomi et al, 2012).

Aspergillus is among the most prevalent toxigenic fungus with several common species produced significant mycotoxins in stored grains and corn feed such as aflatoxins, ochratoxin A (OTA), and cyclopiazonic acid (Whitlow et al., 2010). Aflatoxins are frequently detected in stored feed, corn grain and peanuts, which are produced by *A. flavus*, *A. parasiticus* and *A. sojae*. They are highly toxic to livestock and humans, which may cause acute aflatoxicosis, hepatic necrosis, haemorrhage and often death (Klich, 2002; Bennett, 2010). Cyclopiazonic acid is produced by *A. flavus*, *A. oryzae* or *A. tammarri* and is commonly co-produced with aflatoxins, causes hepatic necrosis, and resulting in death at high doses (Klich, 2002; Sanchez-Hervas et al., 2008). Ochratoxin A is reported in stored feed, corn grain, barley, and wheat, which is produced by *A. niger*, *A. carbonarious*, and *A. melleus*, (Klich, 2002). Ochratoxin A is teratogenic and carcinogenic, primarily targets the kidneys. In poultry, OTA may reduce antibody production, growth rate and egg production (Klich, 2002,) Other mycotoxins produced are fumigclavine and fumitremorgens by *A. fumigatus* which are found in silage and ingestion may cause malnutrition, diarrhoea, and death (Whitlow et al., 2010).

Penicillium is also commonly found in stored grains and corn feed, in which toxigenic species produce citrinin, panitrem, ochratoxins, patulin, cyclochlorotine, and erythroskyrin. Citrinin produced by *P. citrinium* has been detected in almost every kind of food including feed and grains like wheat, rice, and corn. Citrinin is renal toxin for pig, poultry, bird and dog, causes symptoms like watery diarrhoea, reduced weight gain and kidney degeneration in chickens, ducklings and turkeys (Pitt and Hocking, 1985). Panitrem A is produced by *P. canescens*, *P. crustosum*, *P. glandicola* and *P. janozewskii*, has been detected in majority of cereal and animal feed and has tremorgenic effect to domestic animal like dog, cat, sheep and cow (Pitt and Hocking, 1985).

Fusarium also produces significant mycotoxins in stored grains and corn feed such as zearalenon, deoxynivalenol, fumonisins, and T-2 toxin. Zearalenon is produced by *F. graminearum* and has been reported in corn feed causes enlarged or distorted prepubertal gilts, vaginal, uteri and shrunken ovaries in pig. Deoxynivalenol is produced by *F. culmorum* and *F. graminearum*, often associated with pig disorders like feed refusal, diarrhoea, reproductive failure and death. Fumonisins are produced by *F. verticilliodes* causes pulmonary edema in pig. T-2 toxin has low occurrence in corn feed sample, but reported to be associated with cow disorders such as gastrointestinal lesion, bloody diarrhoea, decreased milk production and even death (Whitlow and Hagler, 2005).

Fungal growth and mycotoxin production in corn feed are favoured by storage period and certain agricultural practices such as lack of hygiene equipments, insect and mite infestation, and high moisture content. According to Pitt and Hocking

(2009), deterioration of a particular crops and stored grains resulted from invasion of a specific fungus, and depend on climatic conditions. High ambient humidity even in sunny day in tropical countries causes high moisture content in stored grains and encourages rapid accumulation of toxigenic fungal growth particularly *Aspergillus*, *Penicillium* and *Fusarium* during processing or storage (Reddy et al., 2010).

Studies of naturally occurring mycotoxins in corn grain revealed that contamination may exceed regulatory limits. Reddy and Salleh (2011) reported 22.5% corn feed samples in Malaysia contained 20.6 - 135 µg/kg of aflatoxin B1 (AFB1) and Muthomi et al. (2012) reported over 4400 µg/kg of AFB1 in corn grain in Kenya. The US Food and Drug Administration (FDA) regulatory guidelines prohibit food and feed containing 20 ppb of mycotoxin for interstate commerce, of livestock feed including for young pig, poultry, and lactating animals, and also for human consumption (Cassel et al., 2001). In Malaysia, based on Malaysia Food Regulation 1985, through Food Act 1983, allowing maximum limit of 5 – 35 µg/kg for all mycological contaminants, as for groundnuts, 15 µg/kg need further processing and milk products should not exceed 0.5 µg/kg (Sabran et al., 2012). Even though, certain mycotoxin limits and regulations have been implemented, previous studies of mycoflora and mycotoxins on food and feedstuffs showed that mycotoxin contamination always exceed the limits.

2.2 The Genus *Aspergillus*

Aspergillus was first described in 1729 by P.A Michelli, after an aspergillum, a Catholic device used to sprinkle holy water, due to its resemblance to the spore-

bearing structure of the genus (Klich, 2002; Bennett, 2010). The genus is among the most abundant and widely distributed fungal species on earth, adapting to a variety of habitat and niches such as in air, water, soil, plant debris, decaying vegetation, sawdust litter, leaf litter, animal feed, organic compost piles and other similar environments (Patron, 2006; Varga et al., 2007a).

The genus *Aspergillus* is ubiquitous, most species can survive in any environment, but certain species are more prevalent at certain niches and environments depending on its ecology and adaptation. The genus is common soil inhabitant, has been found in a variety of substrates including desert, beach, grassland, rice fields, forest soils, and compost heaps (Balajee, 2009; Hong et al., 2010). In soil, *Aspergillus* species are mostly saprophyte usually associated with plant litter, and like any other fungi, plays a role as decomposer, nutrient cycler and could also cause post harvest diseases.

Several of *Aspergillus* species which disperse in air are clinically importance including the most prevalent species such as *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. These species have been isolated from indoor air environments including hospitals, schools, warehouses, flourmills, and poultry slaughterhouses, and often associated with sick building syndrome (SBS), a health symptoms like respiratory problems and headache due to poor indoor air quality (Kalogerakis et al., 2005). These species could also cause aspergillosis, referred to any disease or infection by *Aspergillus* (Klich, 2002).

Despite that, *Aspergillus* species are also widely exploited for industrial use, such as *A. oryzae* and *A. tammarii* are used in enzyme and food production, *A. niger* in acid organic production and *A. terreus* in medicinal compound production (Klich, 2002). On the other hand, several species are common contaminant of feed and foodstuffs like cereal grains, nuts, spices, vegetables, fruits, milk and eggs. *Aspergillus* has also been reported in products like jams and jellies, and also in acidic, salted and dried food, (Sanches-Hervas et al., 2008; Hocking 1994, Afsah-Hejri et al., 2013). Some common species like *A. niger*, *A. flavus*, *A. fumigatus* and *A. terreus* can be found in almost all environments but with different prevalence, such as *A. flavus* and *A. niger* are xerophilic and prevalent in dried grains (Pitt and Hocking, 2009). *Aspergillus clavatus* is alkaline-tolerant and commonly found in dung soil and manure (Varga et al., 2007a), *A. fumigatus* is prevalence in clinical settings which can thrive at high temperature up to 50°C (Hong et al., 2005) while *A. glaucus* group can be found in extreme niches such as acidic, dried, salted, and high sugar concentration foods (Afsah-Hejri et al., 2013).

2.2.1 Taxonomic history of *Aspergillus*

Aspergillus was first known to produce conidiospores or conidia by mitosis, classified as an asexual or anamorphic species. Later, the teleomorph state which produces ascospores through meiosis was discovered in 1854 and described. By then, *Aspergillus* was refered to the anamorph (sexual) state and up to this decade is linked to approximately ten different 'teleomorph' genera (Klich, 2002; Geiser et al., 2007). Approximately one-third of these species have a known teleomorph stage, and all but five of those are homothallic, in which a few homothallic species produce spores through both mitosis and meiosis (Geiser et al., 2007). Based on National Centre for

Biotechnology Information (NCBI) classification system*Aspergillus* is classes as below:

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Eurotiomycetes

Subclass: Eurotiomycetidae

Order: Eurotiales

Family: Aspergillaceae

Genus: *Aspergillus*

Aspergillus, like other members of subphylum Pezizomycotina produce both mitotic (asexual) and meiotic (sexual) spores, a condition known as pleomorphism (Bennett, 2010). According to the International Code of Botanical Nomenclature (ICBN) in article 59, 1910, *Aspergillus* species with a sexual phase can have two names, but the sexual or teleomorph phase has privileged over the asexual or anamorph phase (Cline, 2005; Mcneill et al., 2011). However, most taxonomists ignored this rule and instead followed the scheme devised by Raper and Fennel (1965), by naming the species according to the state of the observation (Geiser et al., 2007).

The first monograph of *Aspergillus* was first published by Thom and Church (1926) followed by Thom and Raper (1945) which described 77 species and eight

varieties. Later, Raper and Fennell (1965) described 132 species with 18 varieties which outlined the teleomorph name under the anamorph name. The monograph classified the species into 18 informal groups based on probable relationship deduced from the 'groups' define by previous monographs (Thom and Church, 1926; Thom and Raper, 1945). Gams et al. (1985) revised the informal grouping and divided the species into sections and changed some infrageneric taxa. Peterson (2000) further adapted the manual by Gams et al. (1985) conserving 12 of 18 sections, and modifying and deleting categories previously proposed by Gam et al. (1985). Pitt et al. (2000) have listed 182 taxa, and Samson (2000) listed 36 taxa that had been published between 1992 and 1999. Since then, over 40 new species have been described, with a total of approximately 250 species and is about to significantly increase (Geiser et al., 2007; Samson et al., 2010).

The traditional taxonomic system solely using morphology approach resulted in debatable taxonomic schemes. However, since molecular method was introduced a clear distinction between species was achieved, particularly based on nuclear DNA which allows analysis of fungal genetic and phylogenetic relationship (Samson et al., 2006). Over the last two decades many molecular methods have been used for rapid identification and accurate classification of *Aspergillus* species, including restriction fragment length polymorphisms (RFLP) analysis (Girardin et al., 1993; Varga et al., 1993; Kiziz et al., 2014) and also PCR based method such as microsatellite length polymorphisms (MLP) analysis (Bart et al., 1998), random amplified polymorphism DNA (RAPD) analysis (Yuan et al., 1995; Shin et al., 2001), amplified fragments length polymorphism (AFLP) analysis (Warris et al., 2003; Varga et al., 2007b), and multilocus sequence typing (MLST) analysis (Bain et al., 2007). These methods have

been reviewed by Varga (2006). Currently, the whole genomes of at least eight *Aspergillus* species are available in *Aspergillus* Genome Database (AspGD; http://www.aspgd.org), acts a comparative platform for exploring the diversity of *Aspergillus* species, also gives wider target sequences for researchers to compare with their own *Aspergillus* isolates (Arnaud et al., 2012). However for *Aspergillus* identification, sequencing analysis of ITS region and β-tubulin gene were proposed as target sequence, as these two loci particularly β-tubulin has resolved many closely related *Aspergillus* species (Balajee et al., 2007).

Due to inefficiency of older taxonomy, the species and varieties described since 1965 were critically reviewed. Later revisions of *Aspergillus* taxonomy involved great nomenclatural changes, with new taxa were added particularly in genera *Emericella* and *Neosartoya* (Samson et al., 2006). Peterson et al. (2008) listed 21 sections, in which each section comprised related *Aspergillus* and its teleomorph species.

Older taxonomy highly depends on morphological approach to separate each section and its members. For example, in terms of colony colour, section Nigri bear black colony, Flavi green to yellowish green colony, Terrei tan to brown colony, and Fumigati greyish-blue green colony (Klich, 2002). Up to this decade, morphological approach is still reliable for sections separation but not for species identification, DNA sequencing of ITS region is also considered reliable for section-level identification, but for species-level identification, other protein coding genes such as β-tubulin and calmodulin genes are included (Balajee et al., 2008).

The section comprised subgroups, consisted of more closely related and morphologically similar species, sibling species and cryptic species. For instance, the members of *A. niger* aggregate in section Nigri have morphologically similar characteristics (Abarca et al., 2004). In previous taxonomic system, the present of cryptic species among single morphospecies could not be detected but later it is proven to be distinct species based on molecular methods (Hong et al., 2005). Sibling species were previously separated based on similar morphological characters but later it was shown that the species are genetically similar (Rodrigues et al., 2007). These sub-groups are also referred to as complex species, which indicated more than one species can exist within the sub-group (Balajee et al., 2007). Due to the occurrence of cryptic species, *Aspergillus* taxonomy of particular sections have been intensively revised and re-investigated including the most common sections such as sections Nigri (Abarca et al., 2004; Samson et al., 2004; Samson et al., 2007b; Silva et al., 2011;Varga et al., 2011a), Flavi (Geiser et al., 2000, Varga et al., 2011b), Fumigati (Hong et al., 2005; Samson et al., 2007c; Hong et al., 2010), Clavati (Varga et al., 2007a), Terrei (Balajee et al., 2009; Samson et al, 2011), and Usti (Houbraken et al., 2007).

In recent years, identification manual of *Aspergillus* is based on a polyphasic approach, which is a combination of several methods such as morphological characteristics with colour photos, extrolite profiles and molecular methods (Samson et al., 2011; Silva et al., 2011; Varga et al., 2011a). Manuals written by Klich (2002) and Samson et al. (2010) are focusing on common *Aspergillus* in food products and indoor environments. The manuals by Klich (2002) and Samson et al. (2010) described morphological appearances of *Aspergillus* spp on differential media such

as Malt Extract Agar (MEA) and Czapex Yeast Agar (CYA). In Samson et al. (2010) manual also includes information on genes and regions used for DNA sequencing as well as secondary metabolites produced by *Aspergillus* species.

2.3 Identification of *Aspergillus* Species

2.3.1 Morphological Identification

The most common method used to identify fungal species is by using morphological characteristics which is based on similarity of the observable morphological features such as colony appearances and shape of conidia and vesicle. Most *Aspergillus* species have been described by using morphological features to distinguish species especially in earlier studies (Raper and Fennel., 1965; Gam et al., 1985). Thus, important monographs on *Aspergillus* taxonomies are strictly based on morphological characteristics.

The defining morphological character of *Aspergillus* is the spore-bearing structure called conidiophores, which is the erect hyphal branch enlarges at its tips, forming vesicles **(Figure 2.1).** The vesicles produce a fertile area called phialides that produce long chains of conidia or conidiospores. The shape and size of the vesicles, and the arrangement, colour and the size of the conidia are among the most important characteristics for identification. For instance, the vesicle of *A. clavatus* is clavate where most species have globose, sub-globose to pyrifom vesicles (**Figure 2.2)**. *Aspergillus fumigatus* and *A. terreus* have columnar arrangement, whereas *A. niger* and *A. clavatus* have radial arrangement (**Figure 2.3**). Another characteristic is seriation, either uniseriate or biseriate (**Figure 2.1**). Vesicles with two cell layers,

phialide and metulae are biseriate such as *A. flavus* and *A. terreus*, while vesicles that produce only phialide layers are uniseriate such as *A. fumigatus* and *A. clavatus* (Klich, 2002).

Other features used for identification include cleistothecia, Hűlle cells and sclerotia. Cleistothecia are generally produced in the teleomorph state, and act as sexual reproductive structures that contain ascospores borne within asci. Hűlle cells are usually found to be associated with cleistothecia, in which the cells are thick, and globose (**Figure 2.3**). Sclerotia are rounded masses of mycelium that resemble cleistothecia but the structure does not have sexual spores. Sclerotia may serve as resting structures to allow the species to survive in harsh conditions. Both cleistothecia and sclerotia generally have a rounded shape and may scattered abundantly (Klich, 2002).

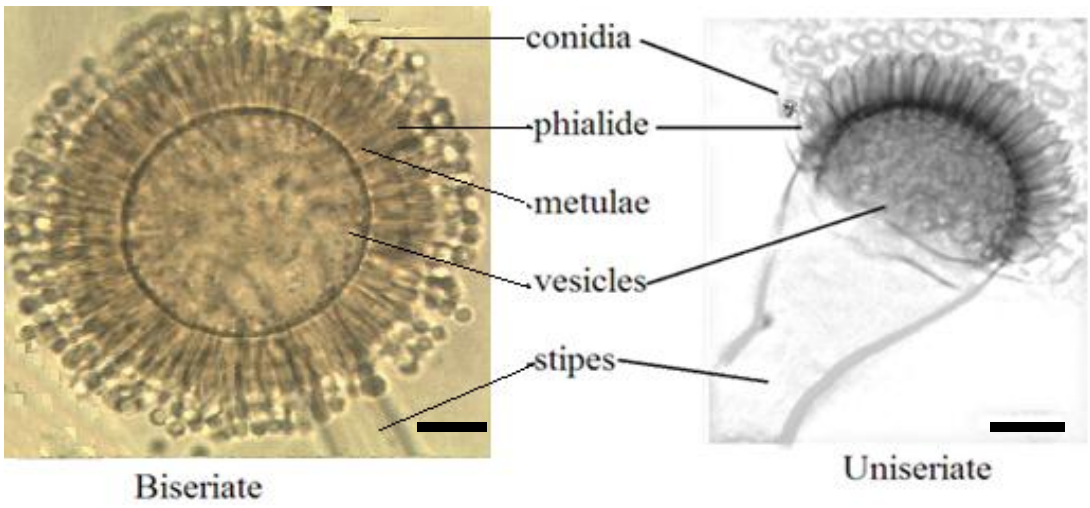

Figure 2.1: Conidiophore *of A. niger* (biseriate) and *A. fumigatus* (uniseriate)

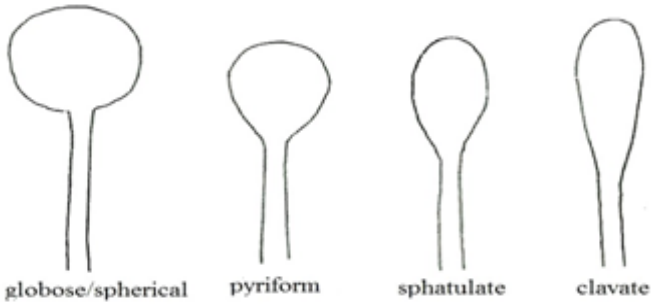

Figure 2.2: Some common vesicle shapes (Klich, 2002)

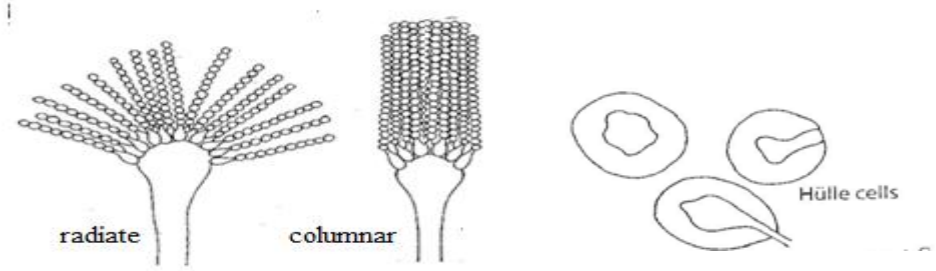

Figure 2.3: Conidial arrangement and Hulle cells (Samson et al., 2010)

For morphological identification, generally fungal cultures are grown on known media. In early taxonomic work, a medium containing sucrose and nitrate was widely used known as 'Czapex-Dox (CZ) medium' (Raper and Fennel, 1965). In current method of morphological identification, different media incubated at several temperatures were applied, and several criteria are observed such as macro- and micromorphology, colony diameter and production of coloured metabolites (Klich, 2002; Geiser, et al., 2007). Klich (2002) used Malt Extract Agar (MEA) at 25 °C; Czapex Yeast Agar (CYA) at 25°C and 37°C, and CYA with 20% sugar. Samson et al. (2010) also used the same media as Klich (2002) but added two more media, Dicholoran Glycerol at 18% (DG-18) and Creatine sucrose Agar (CREA). Different morphological appearances such as colony colour and diameter as well as microscopic features shown by each species are used to distinguish the species. Different media and temperatures are used to distinguish species based on different physiological adaptation. For example, section Nigri and Flavi showed good growth on DG-18 which indicated good xerophilic species (Samson et al., 2010), while *A. fumigatus* can grow at 50°C but not at 10°C, whereas other related species such as *A. lentulus*, *A. fumigatiaffinis*, and *A. novofumigatus* grow at 10°C but not at 50°C (Hong et al., 2005).

Due to variability and overlapping morphological characteristics, misidentification can occur. Species within section tend to have overlapping morphological characters. *Aspergillus* species within section Nigri produced black colony and similar globose to sub-globose vesicles (Klich, 2002). *Aspergillus terreus* was reported to have very variable colony appearances from poor to heavy and fluffy sporulation (Balajee et al., 2009). Sclerotia which considered as a characteristic of

some species can be absent in some isolates, for example only certain isolates of *A. tubingensis* and *A. flavus* produced sclerotia (Klich, 2002; Geiser et al., 2007). Balajee et al. (2007) reported that some clinical isolates showed irregular cultural characteristics that did not match the accepted species description such as reduced sporulation and aberrant conidiophore formation. Domestic culture of *A. oryzae* strains were also reported to show reduce sporulation compared to environmental isolates, which due to loss of certain characteristics for adaptation in nature (Kurtzman et al., 1986)

Morphological identification has been reported to be reliable for section level identification, which is very important to sort the isolates for further identification by other methods. In a study by Balajee et al. (2009) using clinical isolates showed a good correlation between morphological and sequencing analysis of ITS region to identify *Aspergillus* species at section level. The study concluded that clinical laboratories can continue using morphological methods but the species identified must be labelled as complex species indicated the possibility of existence of more than one species within the single morphospecies identified. For instance *A. fumigatus* species complex indicated present of *A. fumigatus* and possibly other related species.

Morphological identification is still widely used, even though the characteristics observed could not be used to accurately identify species to species level, but enough to give indication or clues of sections or species complex (Balajee et al., 2008). The survey by American Society for Microbiology (ASM, 2005) reported that 89% of laboratories in the United States are still using morphological

methods as the isolation and cultural identification are easy, quick and more affordable compared to molecular methods. In a few studies, identification solely based on morphological characteristics has been applied such as for identification of *Aspergillus* species from corn (Niaz and Dawar, 2009), cornmeal (Nithya et al., 2012) and rice grain (Reddy et al., 2010). For accurate identification these studies should be confirmed by molecular methods.

2.3.2 Molecular Identification

Molecular identification and characterization using DNA sequencing is widely used. DNA sequencing used for identification very much depends on the target locus. The locus should be ortologous, have a high level of interspecies variation with low intraspecific variation, easy to amplify and a standardized 'universal' primer set, and the locus should not undergo recombination (Balajee et al., 2007).

The internal transcribed spacer (ITS) region has been proposed as the prime fungal barcode as it satisfies the requirement of a 'universal' marker including highly variable within taxonomically distinct fungal species and also easy to be amplified (Samson et al., 2010). The region is part of nuclear ribosomal with two segments; ITS1 and ITS2 divided by 5.8S rDNA, and located between nuclear small- and large-subunit rRNA genes. This region can be used as the initial step of identification including in unknown fungal isolates to be categorized into appropriate genus, subgenus and section up to species level (Balajee et al., 2009). For some species, ITS region showed a high variability among subgroups of the species such as *Rhizoctonia*