MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF *Aspergillus* spp. ISOLATED FROM ENCLOSED BUILDINGS IN PENINSULAR MALAYSIA

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by

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LIST OF ABBREVIATIONS

18S	Small Ribosomal Subunit
28S	Large Ribosomal Subunit
ABPA	Allergic Bronchopulmonary Aspergillosis
ACGIH	American Conference of Governmental Industrial Hygienists
AFLP	Amplified Fragment Length Polymorphism
AFs	Aflatoxins
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CBS	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre
CDC	Centers for Disease Control and Prevention
CEC	Commission of European Committees
CFU	Colony Forming Unit
CI	Consistency Index
CIT	Citrinin
CNI	Close Neighbour-Interchange
CYA	Czapek Yeast Extract Agar
CZ	Czapek Dox Agar
CZ37	Czapek Dox Agar at 37^0C
DDBJ	DNA Data Bank of Japan
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide triphosphates
EMBL	European Molecular Biology Laboratory
ERIC	Enterobacterial Repetitive Intergenic Consensus
EtBr	Ethidium bromide
ETS	External Transcribed Spacers
HVAC	Heating, Ventilation and Air Conditioning
IAQ	Indoor Air Quality

IARC	International Agency for Research on Cancer
IBT	Instituttet for Bioteknologi Culture Collection of Fungi
ICBN	International Code of Botanical Nomenclature
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer
ITS1	Internal Transcribed Spacer Region 1
ITS2	Internal Transcribed Spacer Region 2
LPCB	Lactophenol Cotton Blue
MEA	Malt Extract Agar
MEGA	Molecular Evolution and Genetic Analysis
$MgCl_2$	Magnesium chloride
MP	Maximum Parsimony
M-RB	Modified Rose Bengal Agar
NIOSH	National Institute for Occupational Safety and Health
NJ	Neighbour Joining
OSHA	U. S. Department of Labor, Occupational Safety and Health Administration
OTA	Ochratoxins A
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAMS	Random Amplified Microsatellites
RAPD	Random Amplification of Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RI	Retention Index
SBS	Sick Building Syndrome
SEM	Scanning Electron Microscope
sp.	Species
TBE	Tris Borate EDTA
TBS	Tight Building Syndrome
UV	Ultraviolet

VOCs	Volatile Organic Compounds
WA	Water Agar
WHO	World Health Organization

PENGECAMAN MORFOLOGI DAN MOLEKUL PENCILAN SPESIES *Aspergillus* DARIPADA BANGUNAN TERTUTUP DI SEMENANJUNG MALAYSIA

ABSTRAK

Aspergillus sering ditemui dan mudah tersebar di hampir semua jenis persekitaran. *Aspergillus* yang berpotensi bertindak secara patogenik, alergenik dan toksigenik pernah dilaporkan sebelum ini. Memandangkan kebanyakan individu menghabiskan lebih dari 80% masa mereka di dalam persekitaran tertutup, kehadiran *Aspergillus* dalam persekitaran sedemikian sangat penting untuk kesihatan manusia. Di samping itu, bioaerosol yang turut terdiri daripada spora *Aspergillus* juga bertindak sebagai salah satu faktor utama dalam mengawal kualiti udara dalaman yang baik. Di Malaysia, laporan tentang kehadiran *Aspergillus* dalam persekitaran dalaman masih terhad dan jarang dilaporkan. Dalam kajian ini, spesies *Aspergillus* ditemui secara konsisten pada semua persekitaran udara dalaman dengan 117 pencilan diperolehi dari sembilan negeri di Semenanjung Malaysia. Melalui pengenalpastian secara morfologi, *Aspergillus niger* (45 pencilan, 38%) adalah spesies yang paling sering dipencilkan daripada persekitaran dalaman diikuti oleh *Aspergillus japonicus* (32 pencilan, 27%), *Aspergillus flavus* (21 pencilan, 18%), *Aspergillus fumigatus* (14 pencilan; 12%), *Aspergillus sclerotiorum* (3 pencilan; 3%) dan *Aspergillus ochraceus* (2 pencilan; 2%). Setiap spesies telah dikenalpasti dan dicirikan berdasarkan ciri-ciri primer atau mikro-morfologi (seriat, saiz dan

bentuk vesikel, konidia dan konidiofor) dan ciri-ciri sekunder atau makro-morfologi (warna konidia dan miselia, diameter koloni dan kehadiran eksudat dan sklerotia). Dalam kajian molekul, jujukan DNA pada kawasan bukan pengkodan pada DNA ribosom (rDNA) iaitu kawasan penjarak transkripsi dalaman (ITS1 dan ITS2) dianalisis secara berasingan. Pencarian BLAST untuk mencari kesamaan jujukan menggunakan pengkalan data GenBank menunjukkan kesamaan jujukan antara 81% hingga 100% di kawasan ITS1, dan 82% hingga 100% di kawasan ITS2. Pada kedua-dua kawasan, pohon filogenetik yang dibina dengan menggunakan kaedah bumbungan jiran (NJ) dan parsimoni maksimum (MP) menunjukkan variasi antara spesies lebih tinggi dengan sebahagian besar pencilan telah berjaya dikumpulkan mengikut spesies. Kajian ini membuktikan bahawa kedua-dua kawasan ITS1 dan ITS2 perlu digunakan untuk pengenalpastian dan pencirian spesies *Aspergillus* yang lebih tepat. Ini merupakan catatan menyeluruh pertama berkenaan spesies *Aspergillus* dari bangunan tertutup di Semenanjung Malaysia.

MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF *Aspergillus* spp. ISOLATED FROM ENCLOSED BUILDINGS IN PENINSULAR MALAYSIA

ABSTRACT

Aspergillus is frequently found and easily spread in almost any type of environments. The potentially pathogenic, allergenic and toxigenic of *Aspergillus* have been reported previously. As most individuals spent more than 80% of their time indoors, the occurrence of *Aspergillus* in indoor environment is crucial to human health. Moreover, bioaerosols which also consists of *Aspergillus* spores could be one of the main factors in controlling a good Indoor Air Quality (IAQ). In Malaysia, reports on the occurrence of *Aspergillus* in indoor environments are still limited and rarely reported. In the present study, *Aspergillus* species were consistently found in all indoor air environments with a total of 117 isolates were obtained from nine states in Peninsular Malaysia. Through morphological identification, *Aspergillus niger* (45 isolates; 38%) were the most common species isolated from the indoor environment followed by *Aspergillus japonicus* (32 isolates; 27%), *Aspergillus flavus* (21 isolates; 18%), *Aspergillus fumigatus* (14 isolates; 12%), *Aspergillus sclerotiorum* (3 isolates; 3%) and *Aspergillus ochraceus* (2 isolates; 2%). Each species were identified and characterized based on primary characteristics or micromorphology (seriation, sizes and shapes of vesicles, conidia and conidiophores) and secondary characteristics or macromorphology (conidial and mycelia colour,

colony diameters and the presence of exudates and sclerotia). In molecular study, DNA sequences of non-coding regions of ribosomal DNA (rDNA), namely Internal Transcribed Spacer (ITS1 and ITS2) were analyzed separately. BLAST search for sequence similarity by using the GenBank database demonstrated 81% to 100% sequence similarity in ITS1 region, and 82% to 100% in ITS2 region. In both regions, phylogenetic trees which were constructed by using Neighbour Joining (NJ) and Maximum-Parsimony (MP) methods showed higher interspecies variation with most of the isolates were successfully clustered according to the species. The present study proved that both ITS1 and ITS2 regions should be used for a more accurate identification and characterization of *Aspergillus* species. This is the first comprehensive record of *Aspergillus* species from enclosed buildings in Peninsular Malaysia.

CHAPTER ONE

INTRODUCTION

Studies on aerobiology and air quality assessment had been carried out extensively in many types of indoor settings such as industrial (Simsekli *et al.,* 1999; Awad*,* 2007; Awad *et al.,* 2010), and occupied (Anderson *et al.,* 1996; Obbard and Fang, 2003) indoor environments including hospitals that are considered to be the most hygienic place. In addition, several studies were also conducted on microbial sampling in indoor and outdoor environment to interprete indoor sampling data which is based on total spore counts (Ren *et al*., 1999; Lee and Chang, 2000; Shelton *et al*., 2002; Zhu *et al.,* 2003).

The samplings that were occasionally conducted with measurements of chemical composition and/or physical aspects of buildings were carried out throughout seasonal or non-seasonal time by scientists for over a century to find correlation between environmental conditions in buildings with bioaerosol exposures (Morey, 2007). At the same time, the procedure of the air sampling was quite complicated and lack of consistent guideline. The procedure used vary between studies, depending on the goal of the researchers as some may start with building investigations while the others may directly conducting microbial sampling.

Since human exposure to these air contaminants may lead to health risk, several recommendations and guidelines have been created which involved remediation actions along with setting up maximum limits of bioaerosols particles in an indoor environment by government agencies around the world. For instance, 500 colony forming units (CFU)/m^3 in Singapore (Ministry of the Environment, 1996) and Hong Kong (The Government of the Hong Kong Special Administrative Region, 2007), 200 CFU/m^3 in United States of America (ACGIH, 1989) and 500 CFU/m^3 by World Health Organization (WHO, 2002) were proposed as the maximum limits. In Canada, indoor fungal quantities measured through CFU/m^3 should be lower than outside (Public Works and Government Services Canada, 2005) while other maximum limit also been introduced by individual authors (Gorny and Dutkiewicz, 2002) such as 1000 CFU/m^3 by Morey *et al.* (1984). These actions showed that contamination of indoor air environment by bioaerosols is an important area of concern especially when one considers that an average person spend most of their time indoors whether at home or work place.

Generally, airborne fungi that may contain allergens, toxins and irritants implicated in indoor air quality (IAQ) problems are listed as one of the air contaminating agents subsequently referred as 'bioaerosols contaminants' (Daisey *et al.,* 2003). In order to systematically evaluate the relationship between airborne fungi and the health effects they may cause, the fungal species or genera need to be known (Shelton *et al.,* 2002). There were

numerous air quality studies documenting the existence and examination of wide range of fungi in buildings (Table 1.1).

Table 1.1: Predominant fungal genera isolated from various enclosed buildings

Genus	Type of buildings	References
Aspergillus	Hospital	Qudiesat *et al*. (2009), Fonseca *et al*. (2010)
Cladosporium, Aspergillus	Flourmill	Awad *et al.* (2010)
Penicillium, Alternaria	Food industry	
Scopulariopsis, Eurotium	Poultry house	
Aspergillus, Cladosporium	Textile industry	
Aspergillus, Penicillium	Flourmill	Awad (2007)
Cladosporium, Penicillium	Poultry slaughterhouse	Haas *et al*. (2005)
Cladosporium, Aspergillus	Renovated buildings	Awad *et al.* (2004)
Cladosporium, Penicillium	Various buildings	Shelton *et al.* (2002)
Cladosporium, Penicillium	Food production, warehouse	Simsekli *et al.* (1999)
Cladosporium, Penicillium	Dwellings	Ren *et al.* (1999)

Aspergillus and *Penicillium* have been reported as common airborne fungi with the ability to colonize different types of building substrates such as dry wall, conventional ceiling tile and gypsum wall board. At the same time, the two genera have the ability to produce some of the most important mycotoxins (Frisvad *et al.,* 2007) for example aflatoxins (AFs) and ochratoxins A (OTA) that have negative health effects such as acute liver damage, respiratory problems, irritation and infectious diseases (Grossi *et al*., 2000; Gent *et al*., 2002; Kuhn and Ghannoum, 2003; Yuchong *et al.,* 2010). The mycotoxins routes into the body can be in two ways; indirectly consumed contaminated food products or directly by inhalation of the fungal spores through respiratory system and dermal

contact. Out of 20 *Aspergillus* species that are known to cause human diseases, approximately 90% invasive aspergillosis (Denning, 1998) and most of pulmonary diseases are caused by *A. fumigatus,* whereas most of the sinus disease is caused by *A. niger* and *A. flavus* (Perfect *et al*., 2001).

In a study by Awad (2007), spores of *Aspergillus* are predominantly found in both indoor and outdoor environment while a ten years study by Falvey and Streifel (2007) reported that it is almost impossible that any environment is completely devoid of *Aspergillus* spores thus making the exposure to *Aspergillus* is difficult to avoid. Recently, a study conducted by Fonseca *et al.* (2010) had confirmed previous hypothesis of aerosolization of *Aspergillus* spores by Anaissie *et al.* (2002). Fonseca *et al.* (2010) also suggested that *Aspergillus* spores are smaller than other dematiaceous fungi and the abundance of *Aspergillus* colonies can oppress other dematiaceous colonies. According to Lacey (1996), the ubiquitous presence of *Aspergillus* in the indoor and outdoor environments is the result of its high sporulating capacity with 1-100 conidia/m^3. These factors may be the reasons why *Aspergillus* become naturally airborne and always found as the common fungal genus isolated from indoor environments. Hence, in particular protective sites such as operation theatre, most studies recommended a maximum of 5 CFU/m^3 and less than 0.1 CFU/m^3 of *Aspergillus* spores (Morris *et al*., 2000).

Accurate identification of *Aspergillus* remains difficult due to the complicated and overlapping morphological and biochemical characteristics

within the species. Okuda *et al.* (2000), for instance, had reported that variety of factors such as volume of media and air exchange followed by the size of inoculums and sporadic light affected the colony and microscopic characteristics. According to Klich (2006), standardized plating and incubation regimes are required in morphological approach while several steps e.g. adequate training of laboratory professionals and recognizing atypical variants of common aspergilli should be considered to improve the quality of this traditional approach (McClenny, 2005).

Filamentous fungi have mostly been characterized by using morphological method which involved observing cultural morphologies such as colony colour characteristics on specific culture media, the size, shape, and development of sexual and asexual spores and spore-forming structures, and/or physiological characteristics (Glass and Donaldson, 1995). Earlier studies on identification of *Aspergillus* species fully relied on morphological observation. Morphological observations are routinely used for identification because of their simplicity, least expensive and quite accurate (Klich, 2006). Hence, morphological approach is still an important method to be used in taxonomic work. Moreover, several monographs are available for references such as Raper and Fennell (1965), Klich and Pitt (1988) and Klich (2002).

In addition of using morphological methods, molecular methods have been widely applied for identification and characterization of *Aspergillus* species. Among the method used is sequencing of the ITS regions. ITS1 and ITS2

regions are located between the small (18S) and large-subunit (28S) rDNA genes with 5.8S become the separator of the two ITS regions (Henry et al., 2000). Hinrikson *et al.* (2005b) described ITS regions as the potential targets besides the 5' end of the large-subunit rRNA gene (D1-D2 region). Moreover, for amplification of ITS regions, universal primers are available (White *et al.,* 1990). The primers were derived from conserved regions of both 18S and 28S rDNA, and had been proved to be helpful in identification, characterization and determining relationships of *Aspergillus.*

Therefore, the objectives of this study were:

1. To isolate *Aspergillus* species from indoor buildings throughout Peninsular Malaysia by surface and air sampling methods. The places of sampling were randomly chosen from enclosed buildings where contamination of fungi were reported or suspected.

2. To identify and characterize *Aspergillus* species based on morphological characteristics. Micro- and macroscopic observations including primary characteristics (formation of conidial heads, conidiophores and sterigmata) and secondary characteristics (colony appearance, pigmentation and colony diameter) were carried out.

3. To characterize and examine the genetic relationship among *Aspergillus* isolates through sequence analysis of ITS region (ITS1

and ITS2 regions) of rDNA. Phylogenetic trees were constructed by neighbor-joining and maximum parsimony methods by using Molecular Evolution and Genetic Analysis (MEGA 4.0) package.

CHAPTER TWO

LITERATURE REVIEW

2.1 Indoor Air Quality (IAQ)

Indoor Air Quality (IAQ) refers to quality of air within enclosed buildings, including homes, offices and dwellings. During the last two decades, air quality in enclosed areas was not the primary focus compared to outdoor air but in recent years, the studies on IAQ had been increasing. According to Sundell (2004), since mid-1800's the topic on air quality has been a growing interest that started during the hygienic revolution, followed by an outdoor environmental issue and finally became one of the major concerns during 1960s, until today.

As IAQ is related to human health and may cause discomfort to the occupants, IAQ became an important subject starting from 1960s. In the mean time, air quality assessment has been conducted in different closed environment or indoor buildings such as hospitals (Kiertiburanakul *et al*., 2007; Fonseca *et al.*, 2010), homes (Ren *et al.*, 1999), schools (Cooley *et al.*,1998; Lee and Chang, 2000; Daisey *et al.*, 2003), offices (Wargocki *et al.*, 2000; Kalogerakis *et al*., 2005), dairies and bakeries (Palmas *et al.*, 1989), warehouses (Simsekli *et al.*, 1999), flourmills (Awad, 2007; Awad *et al*., 2010), poultry slaughterhouse (Hass *et al.*, 2005), and even in commercial aircrafts (Lee *at al*., 1999).

The interest in IAQ has lead to many books and publications with a wide scope covered. Guidelines and documents regarding indoor fungal contamination issues were published by several agencies such as World Health Organization (WHO, 2002), American Conference of Governmental Industrial Hygienists (ACGIH, 1989), U.S. Department of Labor, Occupational Safety and Health Administration (OSHA, 1999), Commission of European Committees (CEC, 1993), Health Canada (Health Canada, 1995), Public Works and Government Services Canada (Public Works and Government Services Canada, 2005), Government of the Hong Kong Special Administrative Region (The Government of the Hong Kong Special Administrative Region, 2007) and Ministry of the Environment, Singapore (Ministry of the Environment, 1996).

2.1.1 IAQ in Malaysia

Malaysian understanding regarding IAQ is still low and studies on IAQ that focused on bioaerosols in Malaysia are still rare compared to those in the developed countries. Developed nations such as the United States, have their own regulations or laws regarding IAQ, while Malaysia only has the IAQ Code of Practice issued by the Department of Occupational Safety and Health in 2005.

It is believe that only after the incident of ‘fungal attack’ in Sultan Ismail Hospital in Johor Bahru, the awareness had arised. The new-10 storey hospital which cost RM 557.8 million was opened to the public in July 2004 but within three months, it was closed and only re-opened after spending RM11.5 million for decontamination work (Salleh, 2005). This case was referred to as the main

testimony of the importance of early IAQ maintenance in order to prevent such a high-cost bill caused by the remediation and antimicrobial treatment. Moreover, the possible impacts of contaminated building to the occupants and patients health was also highlighted and seriously considered during this case. After this incident several other IAQ studies have been conducted in Malaysia (Lian *et al*. 2007; Kumar *et al.* 2005; Syazwan *et al.* 2009).

2.2 Sick Building Syndrome (SBS)

The fact that most individuals spent more than 85% of their time indoors with around 35 gallons of air breathe each day, make a good quality of indoor air very crucial and important to human's health. We are risking our health and facing a possibility of having sick building syndrome (SBS) if the indoor environments such as homes, offices and even cars contain particulate matters that can cause negative effects that are easily inhaled during breathing (Zelikoff *et al.,* 2003).

Sick Building Syndrome (SBS) is a common terminology that refers to a series of health symptoms caused by poor indoor air quality. Stolwijk (1991) described SBS or 'tight-building syndrome' (TBS) as the occurrence of an excessive number of subjective complaints by the occupants of a building while according to Burge (2004), SBS is one of the health problems related to working in office type buildings and the symptoms can be varied between the times spent in a particular building. The first study of SBS was carried out by Finnegan

et al. (1984) and since then, it was believed that SBS normally occurred in ventilated environment. Burge *et al.* (1987) came out with a report on SBS that included a wide range of buildings including those with natural ventilations.

2.2.1 Causes of SBS

Spengler *et al.* (2000) divided air pollutants into three main categories i.e. biological origin including bacteria, fungi, virus, molds, pollen and animal hairs; chemical origin such as cleansers, solvents, fuels and combustion products and lastly, particles or aerosols that are light enough to be suspended in the air. In addition to these categories, internal building environment plays a major role in evaluation of IAQ level. Lee and Chang (2000) proposed carbon dioxide, temperature, relative humidity, formaldehyde, respirable particulate matters, total bacteria and primary air pollutants as the parameters or the source of pollution in such buildings. Stolwijk (1991) had listed physical problems such as leakage of water, insufficient maintenance of heating, ventilation and air conditioning (HVAC) systems and low ventilation effectiveness as causes contributing to SBS in buildings.

Other factors that can be the starting-point or increasing the widespread of SBS are volatile organic compounds (VOCs) and age of the building (Norback *et al.*, 1990), building hygiene, ventilation and air filtration (Anderson *et al*., 1996), HVAC systems (Mendell and Smith, 1990; Bourbeau *et al.*, 1996; Seppanen *et al.*, 1999; Niven *et al*., 2000; Zhu *et al*., 2003), environmental factors (particulates, air temperature, humidity, velocity, ions and carbon

dioxide) (Burge *et al*., 1987; Jaakkola *et al*., 1989; Hedge *et al*., 1993; Nordstrom *et al.*, 1994; Niven *et al.*, 2000), water damage (Wessen and Schoeps, 1996), dust (Gyntelberg *et al.*, 1994; Niven *et al*., 2000; Mendell *et al*., 2002), smokes from cigarettes (Robertson *et al.*, 1991; Raynal *et al.*, 1995; Mizoue *et al.*, 2001), air-conditioned appliances (Harrison *et al*., 1987; Tecelescu *et al*., 1998; Costa and Brickus, 2000) and other bioaerosols (Cooley *et al*., 1998; Daisey *et al*., 2003).

In addition to IAQ assessment in contaminated area, a series of questionnaire were made and used in several studies in order to detect or measure the appearance of SBS faced by the building occupants. Through the answers, air quality can be described by the health status and comfort of the occupants. NIOSH (1991) and other publications such as Raw *et al.* (1995) have built a basic guidance questionnaire for the purpose of studying SBS. According to Ooi *et al.* (1998), the building occupants are considered as having SBS if they had at least one symptom of SBS that appear at least once in a week. Besides that, the symptoms must show a recovery progress when the patients are away from the building and he or she must report the symptom occurrence of at least 1-3 days per week during 4 weeks. A significant result between health surveys using questionnaire distributed to the building occupants with IAQ problem was reported by Cooley *et al.* (1998).

2.2.2 Health Effects due to SBS

General health complaints or non-specific symptoms related to SBS are headache, running nose, rhinitis, breathing problem, eyes, throat and skin irritation, lethargy, sneezing, dizziness, chest tightness, inability to concentrate and objectionable odors. People with weakened immune system, old people and individuals that expose long enough to contaminated air are facing the high possibility to get these symptoms or show similar health problems to cold or flu-like syndromes (Cooley *et al.,* 1998; Tsai and Gershwin, 2002; Crameri *et al.,* 2006). Michael *et al.* (2000) divided SBS symptoms into two groups i.e. upper respiratory (eye, nose, throat and sinus) and lower respiratory (tight chest, difficulty in breathing, and coughing).

In serious cases, fungal infection such as pneumonia or aspergillosis might occur to those who have health problems that involved respiratory system. The condition of patients that already suffered with asthma, chronic bronchitis, uncontrolled diabetes and cancer can be worsen if they are not treated with a proper treatment and breathe in good quality of air. Some studies showed that there are several diseases that have a direct correlation with microbial contaminants in air such as asthma and bronchial hyper-reactivity (Ross *et al*., 2000).

Construction workers also face the same problems whenever they are involved with renovation or construction of a building that is heavily contaminated with fungal spores. They are risking their life by breathing in

contaminated air and the dispersal of the fungal spores to the environment may increased due to their actions.

2.3 Bioaerosol

Kalogerakis *et al.* (2005) considered airborne particles from biological origin such as bacteria, viruses, fungi and pollen with the fragments as bioaerosols. There are three respirable ranged sized for microorganism or bioaerosols (Burrell, 1991). Particle sizes in a range of 30-60 µm comprised dirts or fibers that can be filtered out in the nasal cavity while 10 - 20 µm particles are the size of hyphal fragments, pollens and smaller inert particles, which may adhere to the primary and secondary bronchi or also known as the major airways. The particles size that can be considered as respirable particles and may penetrate the lung are those in the range between 1 to 5 µm, mostly consist of bacteria and fungal spores.

To emphasize the public to be aware and take action on their health status was shown by World Health Organization (WHO) in collaboration with Health Department Alliance in 2009 to produce an information brochure in response to WHO Indoor Air Guidelines on Dampness and Mould (Anon., 2009). This brochure contained the methods on how to prevent the occurrence of bioaerosols or reducing the exposure to dampness in indoor environment. Besides being made available to the public, the brochure also represent WHO policy on against indoor air pollutants caused by biological agents.

2.4 *Aspergillus* as Human Pathogen, Allergen and Mycotoxin Producer

Aspergillus is a spore-forming fungi that may lead to aerosol dispersal and respiratory problems. This genus can act as a pathogen as it infect human through food, inhalation of smell or touch (Salleh, 1998). The arrangement of *Aspergillus* spores make it easy to disperse and among the most common species trapped during air sampling.

One of the well-known fungal infection caused by *Aspergillus* is aspergillosis caused by inhalation of the spores. According to Stevens *et al*. (2000), aspergillosis can be divided into three main categories; i) invasive aspergillosis which involves several organ systems; ii) pulmonary aspergilloma; and iii) allergic bronchopulmonary aspergillosis (ABPA). The first description of ABPA in a child was made by Slavin *et al.* (1970). Later, Rhame *et al.* (1984) reported the incidence of aspergillosis among immunosupressed patients in a hospital. Most of the findings showed that *A. fumigatus* is the most common human pathogen causing aspergillosis followed by *A. flavus* as the second most common isolated pathogen. Fungal allergen was also caused by *A. fumigatus* with over 25 allergens have been reported (Mari and Riccioli, 2004).

Some of the species in the genus *Aspergillus* are also known to produce secondary metabolites called mycotoxins which have been reported to be carcinogenic, teratogenic, tremorgenic, haemorrhagic, nephrogenic, hepatogenic and dermatitis to both humans and animals (Santos *et al.,* 2001;

Altuntas *et al*., 2003; Speijers, 2004). Nielsen (2003) reported that six species of *Aspergillus* namely, *A. flavus, A. fumigatus, A. niger, A. ochraceus, A. ustus* and *A. versicolor* are indoor fungi that have the ability to produce mycotoxins.

Mycotoxins produced by *Aspergillus* such as aflatoxins (AFs), ochratoxins A (OTA), citrinin (CIT) and sterigmatocystin can contaminate food commodities and subsequently causing diseases to human. The presence of these mycotoxins has also been reported in stored products like rice (Gonzalez-Salgado *et al.,* 2005, Nguyen *et al.,* 2007, Reddy *et al.*, 2009), cereals (Zaied *et al.,* 2009), nuts (Iqbal *et al.,* 2006), cereals (Villa and Markaki, 2008), flour (Vega *et al.,* 2009) and maize (Youssef, 2009).

Currently more than 400 types of mycotoxins have been identified worldwide. Among the mycotoxins, AFs and OTA have been classified as group 1 and group 2B human carcinogens by the International Agency for Research on Cancer (IARC, 1993). Fumonisin B_2 which commonly produced by *Fusarium* sp., is also produced by *A. niger* (Frisvad *et al.*, 2007). Major mycotoxins produced with several related illness caused by *Aspergillus* species are listed in Table 2.1.

Table 2.1: Mycotoxins produced by *Aspergillus* and the related illness effecting humans

Toxins	Fungal species	Disease
Aflatoxins	*A. flavus, A. parasiticus*	Aflatoxicosis, hepatitis, liver cancer, childhood cirrhosis, Reye's syndrome, Kwashiorkor
Ochratoxin	*A. ochraceus*	Urinary tract tumors
Sterigmatocystin	*A. versicolor*	Cancer
Cyclopiazonic acid	*Aspergillus* sp.	Kodua poisoning

2.5 Taxonomy of the Genus *Aspergillus*

Micheli was the first to give the name *Aspergillus* in 1729, when he noted that the spore columns radiated from a central structure produce a pattern that resembled an aspergillum, a device used by priests to sprinkle holy water (Raper and Fennell, 1965). Later, Micheli used *Aspergillus* and *Mucor* sp. as the example of asexual reproducing fungi in the formation of spores. During the early 1850s, deBary found that the fungus he examined, *Eurotium herbariorum* by Link was part of the same mycelium as *Aspergillus glaucus* which was previously identified by Link in 1809 (Ainsworth, 1976). Thus, the confusion on the *Aspergillus* taxonomy started.

According to the rules of International Code of Botanical Nomenclature (ICBN), it is required for *Aspergillus* species that have a sexual phase to have two names and the sexual phase (teleomorph) have more privileged over the

asexual name (anamorph) (Baker and Bennett, 2007). Even GeneBank followed this rule, for example *A. nidulans* is found listed as *Emericella nidulans*. Nevertheless, the rules had been applied differently as many taxonomist and scientist following the lead of Raper and Fennell (1965) prefer to use teleomorph names for ascosporic species.

In 1926, Charles Thom and Mable Church studied *Aspergillus* cultures in the laboratory under a controlled condition. Together with a large taxonomic literature, they managed to publish 'The Aspergilli', the first monograph of *Aspergillus* (Thom and Church, 1926) followed by 'A Manual of the Aspergilli' in 1945 (Thom and Raper, 1945). In 1965, Raper and Fennell published another monograph of the genus *Aspergillus* that contained 132 species which divided into 18 varieties or groups. Earlier, Thom and Raper (1945) published a monograph which contained 77 species, 8 varieties and 4 mutations (Raper and Fennell, 1965). In these monographs, the *Aspergillus* species was commonly identified through the production of aspergillum, the asexual spores. Even though 'The Genus *Aspergillus*' by Raper and Fennell was believed to be the last completed monograph, it was still complex to be used due to several issues described by Geiser *et al.* (2007).

A group of *Aspergillus* that was previously described in 1965 had been typified, revised, given formal taxonomic status as sections (18 sections) and six subgenera were added by Samson and Gams (1985) and Gams *et al.* (1985). Thirty-five years later, Peterson (2000) revised this genus through phylogenetic

analysis of the large subunit of rDNA and proposed to eliminate three of the six subgenera published by Gams *et al*. (1985), retaining 12 of the 18 sections, modifying three of the sections and deleting the other three. In the same year, Pitt *et al.* (2000) came out with 182 names of the latest recognized species of the genus and since that year, another 46 new species descriptions have been published, bringing the total number to approximately 250 species (Geiser *et al*., 2007). Summary of the important manuals and monographs of *Aspergillus* taxonomy are shown in Table 2.2.

Table 2.2: Important taxonomic treatments and identification manuals for the genus *Aspergillus* (Bennett, 2010)

Year	References
1926	Thom, C., and Church, M. The Aspergilli (Baltimore: Williams & Wilkins)
1954	Thom, C., and Raper, K. B. A Manual of the Aspergilli (Baltimore: Williams & Wilkins)
1965	Raper, K. B., and Fennell, D. I. The Genus *Aspergillus* (Baltimore: Williams & Wilkins)
1985	Samson, R. A., and Pitt, J. I. Advances in *Penicillium* and *Aspergillus* Systematics (New York : Plenum)
1988	Klich, M. A., and Pitt, J. I. A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs (North Ryde, Australia: Division of Food Processing)
1989	Kozakiewicz, A. *Aspergillus* Species on Stored Products (Wallingford: CAB International)
1990	Samson, R. A., and Pitt, J. I. eds. Modern Concepts in *Penicilliium* and *Aspergillus* Classification (New York: Plenum Press)
2000	Samson, R. A., and Pitt, J. I. eds. Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* classification (Amsterdam: Harwood Academic Publications)
2002	Klich, M. A. Identification of Common *Aspergillus* Species (Utrecht: Centraalbureau voor Schimmelcultures)
2008	Samson, R. A., and Varga, J. *Aspergillus* systematic in the genomic era (Urect: CBS Fungal Biodiversity Centre)

According to the present classification, Geiser *et al.* (2006) listed *Aspergillus* in the order Eurotilaes (Subclass : Eurotiomycetidae, Class : Eurotiomycetes, Subphylum : Pezizomycotina, Phylum : Ascomycota). The same classification also used by NCBI with the added Trichocomaceae as the Family of these filamentous fungi (http://www.ncbi.nlm.nih.gov/Taxonomy/Broser). The latest valuable general guide been used for identification of common species of *Aspergillus* had been described by Klich (2002) whereby he formulated a new key to the species along with illustrated references for *Aspergillus* identification as presented in Table 2.3.

Although *Aspergillus* can act as homothallic species, this is not the main feature of *Aspergillus* as only less than half of these species can produce a sexual stage. However, there is a hyphothesis that *Aspergillus* species with known sexual stages tend to be minor players in the clinical realm since reports on infections by the teleomorph stages are very rare (Geiser, 2008). *Emericella, Eurotium* and *Sclerocleista* are the only three sexual stages that have more than three species of *Aspergillus* compared to other five genera namely *Chaetosartorya, Fennellia, Hemicarpenteles, Neosartorya* and *Petromyces* (Pitt *et al.*, 2000). Recently, another four sexual stages were added to the group i.e. *Neopetromyces, Warcupiella, Neocarpenteles* and *Dichotomyces* (Table 2.4).

Table 2.3: Summary of *Aspergillus* current scheme for identification

Subgenus *Aspergillus* – uniseriate, xerophilic, growth on CY20S>CYA25, grey green conidia

Section *Aspergillus* – teleomorph *Eurotium* – yellow cleistothecia with wall made up of a single layer of polygonal pseudoparenchymatous cells, hyaline ascospores

Eurotium amstelodami
Eurotium chevalieri
Eurotium herbariorum

Section *Restricti* – strictly anamorphic, slow growth on all media

A. penicilliodes
A. restrictus

Subgenus *Fumigati* – uniseriate, vesicle predominantly pyriform, conidia grey green, blue green to orange

Section *Fumigati* – telemorph *Neosartorya* conidia grey green to blue green

Neosartorya fischeri
A. fumigatus

Section *Cervini* – conidia light orange to orange-grey

A. cervinus
A. kanagawaensis

Subgenus *Ornati* – uniseriate, conidia grey-green, yellow-green or olive brown

Section Ornati

Sclerocleista ornate
A. paradoxus

Subgenus *Clavati* – uniseriate, vesicles predominantly clavate, conidia grey-green

Section *Clavati* – as for subgenus

A. clavatus

Subgenus *Nidulantes* – biseriate, conidial colours variable

Section *Nidulantes* – stipes short often brown, conidia green, Hulle cells often present, most species with *Emericella* telemorph. Cleistothecia soft-walled, surrounded by Hulle cells, ascospores red to purple.

Emericella nidulans
Emericella quadrilineata
Emericella rugulosa
A. unguis

Section *Versicolores* – stipes hyaline to brown, conidia green, grey green or blue green

A. caespitosus
A. sydowii
A. versicolor

Section *Usti* – stipes brown, conidia dull red, brown or olive

A. puniceus
A. ustus

Section *Terrei* – stipes hyaline, conidia white to buff

A. terreus

Table 2.3: continued

Section *Flavipedes* – stipes hyaline to pale brown, conidia white to buff
- *A. carneus*
- *A. flavipes*
- *A. niveus*

Subgenus *Circumdati* – uniseriate or biseriate, vesicles spherical to pyriform

Section *Wentii* – conidia buff, yellow brown or olive brown
- *A. wentii*

Section *Flavi* – conidia yellow green to olive brown
- *A. alliaceus*
- *A. flavus*
- *A. oryzae*
- *A. parasiticus*
- *A. sojae*
- *A. tamarii*

Section *Nigri* – stipes smooth-walled, conidia black or near black
- *A. awamori*
- *A. carbonarius*
- *A. foetidus*
- *A. japonicus*
- *A. niger*

Section *Circumdati* – predominantly biseriate, conidia yellow, buff or ochraceus
- *A. auricomus*
- *A. melleus*
- *A. ochraceus*
- *A. ostianus*
- *A. sclerotiorum*

Section *Candidi* – conidia white or nearly white
- *A. candidus*

Section *Cremei* – conidia brown, yellow or blue-green
- *A. cremea*

Section *Sparsi* – conidia pale grey to olive buff
- *A. sparsus*

Subgenus *Stilbothamnium* – species forming synnemata

Source: Identification of Common *Aspergillus* species by Klich (2002) with slight modifications

Table 2.4: Sexual stages associated with the genus *Aspergillus* (Geiser, 2008) with slight modifications

Genus	Some phylogenetically associated species
Neosartorya	*A. fumigatus, N. fischeri, A. lentulus*
Emericella	*E. nidulans, E.echinulata, E. quadrilineata, E. rugulosa, A. ustus, A. calidoustus, A. versiclor*
Fennellia	*A. terreus, F. flavipes*
Petromyces	*P. alliaceus, A. flavus, A. parasiticus, A. niger*
Neopetromyces	*A. ochraceus, A. sclerotium*
Eurotium	*E. amstelodami, E. herbariorum, E. Glaucum*
Chaetosartorya	*C. chrysella, A. wentii, C. cremea*
Sclerocleista	*A. ornatus*
Warcupiella	*A. spinulosus*
Hemicarpenteles	*A. paradoxus*
Neocarpenteles	*A. clavatus*
Dichotomyces	Weak phylogenetic association with *A. clavatus* and *Neosartorya*

The number of species in *Aspergillus* keep on stretching and widening as new species were discovered and existing classification are refined. Samson *et al.* (2006) also suggested that misidentifications and incorrect names always occurred in *Aspergillus* nomenclature.

2.6 Identification and Classification of *Aspergillus* Species

Identification of *Aspergillus* relies mainly on morphological observations although this method tedious and time consuming. However, morphological observations could be used in identification for almost all species in the genus *Aspergillus* (Klich, 2006). Moreover, Raper and Fennell (1965), Samson *et al.*

(2000), and Klich (2002) basically used morphological techniques in their identifications.

Specific identification of *Aspergillus* usually involved a single-spored or pure culture along with examination on culture media of known composition. The important thing needed in handling *Aspergillus* through morphological observation is the researcher has to have a good understanding of the genus characteristics. Besides that, a proper handling of the working cultures and microscopes are also required in the identification process.

2.7 Morphological Characteristics

Raper and Fennell (1965) divided two different keys to classify *Aspergillus* into groups, primarily on morphology that involved the number of sterigmata series and on colour of the conidial heads. From these main keys, other morphological characteristics were added to support the classification system.

Secondary characteristics also play a significant role in describing the *Aspergillus* species. These macromorphology features are conidial and mycelia colour, reverse colour of the colony, colony diameter and the presence of exudates and sclerotia. Conidial colour is useful in subgeneric classification. Colony diameter is taken by measuring the maximum diameter of each three-point cultures of *Aspergillus* on the growth media.

2.8 Molecular Characteristics

Molecular methods could provide useful and convenient ways in clarifying the taxonomy and identification of *Aspergillus* species. Several molecular methods have been developed and used to assist in taxonomic studies of fungi which include Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Microsatellites (RAMS).

2.8.1 PCR-based Technique

PCR is commonly used to study and provide information on the variation, taxonomy, population structure, epidemiology and genetic relationship among fungal species (Cooley, 1992). Several PCR-based techniques have been successfully applied in studying genetics and relationships between *Aspergillus* isolates; intra-species and inter-species. Previously, ITS-RFLP technique was used for identification of *A. fumigatus* (Katz *et al.*, 1998; Semighini *et al.*, 2001), *A. oryzae* (Wicklow *et al.*, 2007) and *A. niger* (Someren *et al.*, 1991). Inter-species variation between *Aspergillus* species were also observed through the same technique (Moody and Tyler, 1990). In addition to RFLP, other molecular methods used in genetic characterization of *Aspergillus* were RAPD (Loudon *et al.,* 1993; Leenders *et al*., 1996 and 1999; Hamari *et al*., 1997; Brandt *et al.*, 1998; Rath, 2001; Fungaro *et al*., 2004; Heinemann *et al.*, 2004), microsatellite polymorphism analysis (Guarro *et al.*, 2005) and AFLP (Montiel *et al.,* 2003).