IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM HIGHLANDS IN MALAYSIA

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IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM HIGHLANDS IN MALAYSIA

by

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TABLES OF CONTENTS

CHAPTER 3 –METHODOLOGY

CHAPTER 4 - RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree of Celsius
~	Approximately
>	Bigger than
®	Registered
°	Degree of Latitude
°F	Degree of Fahrenheit
µg/kg	Microgram per kilogram/ ppm
µl	Microliter
µm	Micrometer
ACN	Acetonitrile
AFM1	Aflatoxins M1
aq	Aqueous
BEA	Beauvericin
BLAST	Basic Local Alignment Search Tool
bp	Basepair
CLA	Carnation Leaf Agar
cm	Centimeter
ddH_2O	Deionized Distilled Water
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EF-1α	α-Elongation factor
ELEM	Equine leukoencephalomalacia
ELISA	Enzyme-linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detection
EtBr	Etidium bromide
FAO	Food and Agricultural Organization
FHB	Fusarium Head Blight
FLR	Fluorescence
ft	Feet
FUM	Fumonisins
$FUMB_1$	Fumonisins B_1
h	Hour
H3	Histone
Ha	Hectare
HPLC	High Performance Liquid Chromatography
IGS	Intergenic Spacer
ITS	Internal transcribed spacer
kb	Kilobase

KCL	Potassium Chloride
kg	Kilogram
km	Kilometer
km^2	Square kilometer
KOH	Potassium hydroxide
L	Litre
m	Meter
M	Molar
mA	Miliampere
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
mL	Millilitre
ML	Maximum Likelihood
mm	Millimeter
mM	Millimol
MON	Moniliformin
MP	Maximum Parsimony
N	Normality
NCBI	National Center of Biotechnology Information
ng	Nanogram
NJ	Neighbor-joining
nm	Nanometer
NTSYS	Numerical Taxonomy System of Multivariable programme
OPA	O-phthaldialdehyde
OTA	Ochratoxin A
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction and Restriction Fragment Polymorphism
PDA	Potato Dextrose Agar
PDA	Photodiode Array
PPA	Pentachloronitrobenzene Agar
ppm	Part Per Million
rpm	Revolutions per minute
SBL	Sum of branch length
sp.	Species
SPE	Solid Phase Extraction
TBAHS	Tetrabutylammonium Hydrogen Sulphate
TBE	Tris-Borate EDTA
TEF-1α	Translation Elongation Factor alpha gene
TLC	Thin Layer Chromatography
TRI	Trichothecenes
U	Unit
UPGMA	Unweighted pair group method with arithmetical mean
UPLC	Ultra Performance Liquid Chromatography

USA	United State of America
UV	Ultraviolet
V	Volt
v	Volume
WA	Water Agar
ZEN	Zearalenone
β	Beta
µM	Micromol

PENGENALPASTIAN DAN PENCIRIAN SPESIES *Fusarium* DARIPADA KAWASAN TANAH TINGGI DI MALAYSIA

ABSTRAK

Fusarium merupakan salah satu genus kulat heterogenous yang merangkumi sebagai saprofit, patogenik dan toksigenik yang mudah di dapati di seluruh dunia. Akan tetapi, disebabkan kepelbagaiannya yang meluas ia sukar untuk dikenalpasti dan dikelaskan. Baru-baru ini, sebahagian spesies *Fusarium* yang sering dijumpai di kawasan beriklim sejuk dan kawasan separuh tropika telah dijumpai di kawasan tanah tinggi tropika termasuk di Malaysia. Kajian ini dijalankan untuk mengenalpasti spesies *Fusarium* tersebut dalam konteks kewujudan, kepelbagaian serta tahap toksigeniknya di Malaysia. Persampelan berperingkat dilakukan secara rawak daripada pelbagai perumah dan substrat di tiga kawasan tanah tinggi di Malaysia, iaitu Cameron Highlands dan Bukit Fraser di Pahang, dan Taman Kinabalu. Pemencilan *Fusarium* dilakukan menggunakan media agar-agar pentakloronitrobenzena (PPA) dan dieram dalam keadaan biasa (27$\pm$1°C). Sebanyak 123 pencilan *Fusarium* telah berjaya diperolehi dikenalpasti menggunakan kaedah morfologi. Empat spesies telah dikenalpasti iaitu *F. graminearum* (86.2%), diikuti *F. venenatum* (12.2%), *F. avenaceum* (0.8%) dan *F. sporotrichioides* (0.8%). Selain itu, pencirian menggunakan kaedah molekular dan profil mikotoksin juga digunakan untuk menyokong data morfologi. Pencirian molekular menggunakan analisis RFLP di kawasan IGS rDNA dan jujukan DNA menggunakan gen TEF-1α. Persamaan jujukan di antara spesies adalah sebanyak 88%- 99% melalui keputusan BLAST mengunakan pangkalan data GenBank manakala melalui pangkalan data Fusarium-ID adalah sebanyak 89% -99%. Empat mikotoksin telah dianalisis menggunakan Ultra-Performance Liquid Chromatography (UPLC) iaitu fumonisins ($FUMB_1$),

beauvericin (BEA), zearalenone (ZEN) dan moniliformin (MON) ke atas 40 pencilan. Majoriti *F. graminearum* telah menghasilkan ZEN pada kepekatan di antara 0.002 – 0.437 µg/kg dan *F. venenatum* di antara 0.006 – 0.014 µg/kg. Ini adalah kajian pertama yang dilakukan ke atas *F. avenaceum*, *F. graminearum*, *F. sporotrichioides* dan *F. venenatum* dari kawasan tanah tinggi di Malaysia.

IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM HIGHLANDS IN MALAYSIA

ABSTRACT

Fusarium is one of the heterogeneous genera of saprophytic, pathogenic, and toxigenic fungi distributed worldwide. However, such an important genus is considered to be one of the most highly diversified, thus difficult to identify and classify. Recently, several *Fusarium* species normally found in the temperate and subtropical regions were also reported to be in the highlands of tropical areas, including Malaysia. This research was to identify *Fusarium* species in context of occurrence, diversity, and toxigenicity. Phase sampling was done randomly from variable hosts and substrate in three locations, Cameron Highlands, Fraser's Hill and Kinabalu Park. The samples were plated on a semi-selective medium (pentachloronitrobenzene agar) and incubated in a standard incubation conditions (27±1°C). One-hundred twenty three (123) *Fusarium* isolates were single-spored and identified based on the morphological characteristics. Four species were identified i.e. *F. graminearum* (86.2%), followed by *F. venenatum* (12.2%), *F. avenaceum* (0.8%) and *F. sporotrichioides* (0.8%). Molecular characterization and mycotoxin profiles were used to support-the morphological data. The molecular characterization were carried out by using PCR-RFLP of the rDNA intergenic spacer (IGS) region and DNA sequences using Translation α-Elongation Factor (TEF) gene. BLAST search for sequence similarity by using GenBank database which demonstrated 88% - 99% whereas for Fusarium Database was between 89% - 99%. Four mycotoxins; fumonisins ($FUMB_1$), beauvericin (BEA), zearalenone (ZEN), and moniliformin (MON) produced by 40 isolates were analyzed by using Ultra-Performance Liquid

Chromatography (UPLC). Majority of *F. graminearum* were able to produce ZEN in concentrations between 0.002 – 0.437 µg/kg and *F. venenatum* between 0.006 – 0.014 µg/kg. This is the first record on the occurrence of *F. avenaceum*, *F. graminearum*, *F. sporotrichioides* and *F. venenatum* from highland areas in Malaysia.

CHAPTER 1

INTRODUCTION

Highlands in the tropical region are characterized by high humidity, rainfall and less sunshine. As a result, these areas have cooler and wetter conditions than the lowland areas (Sanchez, 2000). The tropical rain forest occupies 59.4% of Malaysia's land area and these are found mainly in the hills and mountains with more than 2,000 m above the sea level (Mohd Hasmadi *et al.,* 2008). Tropical highlands temperature ranged between 64.4ºF (18ºC) to 73.4ºF (23ºC) whereas the temperature in coldest month ranged between 64.4ºF (18ºC) to -26.6ºF (-3ºC), and in warmest month are above 50ºF (10ºC) (Reynolds *et al.,* 2008). Therefore, the temperature of tropical highlands generally falls within the range of temperature in the temperate regions during summer months.

The vegetation at the highland areas is mainly oaks, laurels, conifers, myrtles and tea family (Andrews and Freestone, 1972). There are also a large proportion of temperate vegetables planted in highland tropical climates such as *Umbelliferae* (celery), *Solanaceae* (tomato, *Capsicum* sp.), *Cruciferae* (English cabbage, Chinese cabbage, mustard, and radish), *Leguminosae* (beans, peas) (Midmore *et al.,* 1996). The climate in highlands areas is moist, which is conducive for fungus includes Deuteromycetes or imperfect fungi to grow (Agrios, 2005).

Fusarium is the one of the most important genus of imperfect fungi tropics causing serious diseases on plants, humans and animals (Klahr *et al*., 2006). *Fusarium* is cosmopolitan, (Young *et al*., 1978; Nelson *et al.,* 1994; Arney *et al.,* 1997; Summerell *et al.,* 2010) can be found in most bioclimatic region of the world including tropical and temperate grasslands, shrublands, forests as well as harsh

desert and alpine environment, soils associated with plants, organic debris and any part of plants from deepest root to highest flowers (Leslie and Summerell, 2006).

Recently, several *Fusarium* species known to be present in the temperate region were found in tropical highlands of Malaysia such as Cameron Highland, Genting Highland and Fraser's Hill. Therefore, this finding could indicate that temperate *Fusarium* species exist in the highland areas of Malaysia. These species could be associated with the plants in the highland areas as these species are well known pathogen to cereals and small grains in temperate region. Thus, the main purpose of this study was to determine the diversity of *Fusarium* species in highland areas of Malaysia.

The identification and classification of *Fusarium* species is complex (Summerell *et al.,* 2003; Leslie and Summerell, 2006). The identification of *Fusarium* depends mostly on morphological characteristics such as the shapes and sizes of macroconidia, presence or absent of microconidia and chlamydospores, and shapes of basal foot cells on the macroconidia (Nelson *et al*., 1983; 1991; Leslie and Summerell, 2006). There are several identification manual based on morphological characteristics that can be used to identify *Fusarium* species such as descriptions by Wollenweber and Reinking (1935), Booth (1971), Joffe (1974), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), Burgess *et al.* (1994) and The *Fusarium* Laboratory Manual by Leslie and Summerell (2006).

When morphological characteristics are not sufficient to identify closely related *Fusarium* species, molecular characterization are commonly used. Among the technique used in molecular characterization is combination of polymerase chain reaction and restriction fragment polymorphism (PCR-RFLP). Polymerase chain

reaction and restriction fragment polymorphism (PCR-RFLP) is used because it can detect high level of sensitivity and specificity of fungi (Mirhendi *et al.,* 2001). After PCR, the PCR product will be digested using different types of restriction enzyme. For characterization of *Fusarium* species, intergenic spacer (IGS) region is commonly used in PCR-RFLP analysis as the region is most rapidly evolving spacer region (Hillis and Dixon, 1991), have high levels of sequence variability among species of the same genus (Bruns *et al.,* 1991) and shown variability which allow discrimination of closely related species (Appel *et al.,* 1995; Edel *et al.,* 1996; Llorens *et al.,* 2006a). The IGS-RFLP analysis has been used to characterize intraspecific variability in *Fusarium,* and it is simple, fast and routine determination and diagnosis technique (Edel *et al.,* 1996; Konstantinova and Yli-Mattila, 2004; Llorens *et al.,* 2006a; Patino *et al.,* 2006, Nitschke *et al.,* 2009; Fernandez-Ortuno *et al.,* 2011).

Another molecular technique that is commonly applied in taxonomic study of *Fusarium* is sequencing of alpha elongation factor gen (TEF-1α). This is because TEF-1α gene sequences were show high level of polymorphism among closely related species, it can provide useful information at species level and it became marker of choice as a single-locus identification tool in *Fusarium* (Geiser *et al.,* 2004).

Another aspect that should be the focus of any researcher working on *Fusarium* is the ability of the fungus to produce mycotoxins (fusariotoxin) which are the secondary metabolites produce by several species of *Fusarium*. There are different types and classes of mycotoxins produced by *Fusarium* and only 25 *Fusarium* species have been reported to produce mycotoxins (Salleh, 1989). These mycotoxins can enter the food chain in the field and during storage. As a result, the

mycotoxins will contaminate and give negative effect to animal and human health (Naicker *et al.,* 2007).

Among the mycotoxins, fumonisins B_1 ($FUMB_1$) is the most toxic and classified as Class II carcinogenic by International Agency for Research on Cancer (IARC, 1993). Other well-known mycotoxins are zearalenone (ZEN), trichothecenes (TRI), moniliformin (MON), and beauvericin (BEA). Each *Fusarium* species produces its own mycotoxins profiles. For example, most of the species in the section Discolor produce zearalenone (ZEN) and trichothecenes (TRI). Therefore, mycotoxin profiles will be used as one of the supporting criteria for identification of *Fusarium* species from tropical highlands areas.

Although, *Fusarium* species from highland areas have been reported in Malaysia, detail studies on the characterization and mycotoxin profiles have not been done, thus the present study was carried with the following objectives:

1. To isolate and identify *Fusarium* species from highland areas of Malaysia by using morphological approach,
2. To characterize the *Fusarium* isolates by using PCR-RFLP of IGS region and TEF-1α sequences, and
3. To determine the production of major mycotoxins (BEA, $FUMB_1$, MON and ZEN) by the *Fusarium* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Climatic condition of tropical region

Climate zone was first classified in the 19th century by a Russian climatologist, Wladimir Koppen. He recognized crucial link between climate and natural vegetation to set the boundaries of the climate zones. Koppen classification scheme were divided into five groups, A, B, C, D and E according to the distribution of vegetation types. The five groups were defined by distribution of natural vegetation as well as seasonal amount of precipitation and temperature in a particular zone. There are also certain factors which can influence the distribution of climatic zone namely latitude, elevation and the distance of the ocean (Reynolds *et al.,* 2008). Tropical or megathermal climate is define as group A which is characterized by constant high temperature (at sea level and low elevations) with 12 months of the year have average temperatures of 18°C or higher. Group A zone are divided into three areas, tropical rainforest, tropical monsoon and tropical wet and dry or savannah climate.

The most humid climate is tropical climate which has a very high annual rainfall and average monthly temperature of at least 18°C. These areas of tropical climates are from Amazonia, across equatorial Africa to Malaysia and Indonesia (Reynolds *et al.,* 2008). Malaysia lies near the Equator between latitude 1° and 7° North and between longitude 100°and 119° East (Lim and Azizan, 2004). The country is subjected to maritime influence and the interplay of wind systems, which originate in the Indian Ocean and South China Sea.

Malaysia is located in the tropical region which has hot wet equatorial climate with mean daily temperatures in lowlands throughout the year ranging from 21ºC to 32ºC, but cooler temperatures prevail at the higher altitudes about 16ºC to 26ºC. Therefore, the temperature of highlands tropic is slightly the same as temperature in the temperate region.

2.2 Highland areas of Malaysia

Malaysia covers an area about 329,758 km^2 comprising the Malay Peninsula and the states of Sabah and Sarawak in the northwestern coastal of Borneo Island. These two regions are separated by about 531 km of South China Sea. The Peninsular Malaysia consists of steep hills and mountain ranges (about 1, 200 m), and it cover about one-third of the plain surface of the Peninsula and run more or less parallel to the long axis of the country. Meanwhile, Sabah has physical pattern consists of narrow alluvial coastal plains backed by hilly, forested areas. This mountain acts as barriers to inland penetration. Sarawak consists of a flat and swampy coastal area and steepy undulating hills in the interior (about 1219 to 1828 m) (Andrews and Freestone, 1972; Ooi, 1976).

The highest highland in Malaysia is Mount Kinabalu with 4,095.2 metres (13,455 ft) (Harrisson, 1978). The vegetation zone in Mount Kinabalu can be divided by altitude. There are four vegetation zones which are the lowland Dipterocarp zone, the montane zone, the ultra basic zone and the summit zone. The lowland Dipterocarp zone is from 0- 3000 ft from lowland and among the vegetations are the family Sapindaceae, Leguminosae and *Parashorea* (Dipterocarpaceae). Montane zone is at 914 – 2590 m and consist of family from Ericaceae, Myrtaceae, Lauraceae, Guttiferae, Orchidaceae, Magnolia, mosses and fern are always found at this

elevation. *Rafflesia* was also found at the lowest part of this zone. The third zone is ultra basic zone at 2590 – 3200 m and plant from woody genera was limited. Only dwarf shrubs such as *Dacrydium gibbsiae* and *Leptospermum recurvum* are found. At elevation of 3200 – 4101 m majority of the soil almost disappeared and the only vegetation is *Rhododendron buxifolium* (Cockburn, 1978).

Cameron Highlands is also one of the most popular highlands in Malaysia located on the Main Range (Banjaran Titiwangsa) between 4°20'N-4°37'N and 101°20'-101°36'E consists of 71, 218 ha ranging from 300 m at the river valleys on the eastern boundary to 2110 m (Mount Irau) on western boundary. There are various types of forest in Cameron Highlands including lowland Dipterocarp forest (100-300 m), hill Dipterocarp forest (300-750m), upper Dipterocarp forest (750-1200 m), lower montane forest (1200-1500 m) and upper montane forest (>1500 m). The upper montane forest comprised Mount Brinchang, Mount Jasar, Mount Perdah and Mount Irau mountain peak. The cool and moist montane rain forest has a smaller and less diverse group of plants and animals. There are about 727 plant species which consists of dicots (53.4%), monocots (34.7%), fern and fern allies (11.8%). However, from 727 plant species in forest Cameron Highlands there are 445 (61.2%) plant species found in this area (Kumaran and Ainuddin, 2006). All the highland streams have lower temperature and have lower tree diversity dominated by oaks, chestnuts rather than dipterocarps (Yule *et al.,* 2009).

The temperature in the highlands is related to the altitude. If the altitude increased, the temperature drops at the rates of 0.549°C, 0.613°C and 0.711°C per 100 m increase in altitude. Mainly the temperature in the highlands of the tropics ranging from 17°C to 18°C every month for the whole year. Annual rainfall of the

highlands areas is from 3153 mm (highest) and 1969 mm (lowest) for 13-26 rain days/month.

Climate in highland areas affect the soil formations. Soils in highland areas are mainly derived from granite, with sandy to sandy clay loam textures, and are classified as paleudults. For example, in Cameron Highlands, the humus in virgin soil in the north reaches 80-100 cm in depth (Gunung Brinchang) and falling to 40 cm in the Central Tanah Rata and to a negligible layer of humus in the Ringlet of the south where the altitude is less and temperature is higher. The virgin soil 0.10 cm beneath the organic or humus layer comprised 40% sand, 16% silt, 44% clay, with pH 4.2 (Midmore *et al.,* 1996). In Mount Kinabalu, there are large areas of tertiary sedimentary substrate, interspersed with mosaics of ultramafic rock at below 3000 m (Jacobson, 1970). Meanwhile, at above 3000 m, the areas are dominated with granite (adamellite). The ultramafic rocks are remarkably high in magnesium and low in phosphorus compared with sedimentary and granitic rocks found at above 3000 m (Kitayama *et al.,* 2011)

2.3 The genus *Fusarium*

Fusarium is one of the most intensively studied fungi due to its role as plant pathogen, toxin producer and human pathogen (Leslie and Summerell, 2006). It is widely distributed in soil, plant debris, on subterranean and aerial plant parts and other organic substrates from tropical, temperate and as well as desert, alpine and Arctic environments. *Fusarium* is a group of soilborne fungi, abundant in the soil and frequently associated with plants as root parasites and as saprophytes. The fungus can also exist as colonizers of living plant parts or plant residues within the soil or adjacent to the soil surface (Nelson *et al.,* 1981; Leslie and Summerell, 2006).

Fusarium can act as plant pathogenic fungus causing various types of diseases on a variety of tropical plants such as vascular wilt, root rots, rots of fruit, seed, and storage tissue, stem and stalk rots, canker, die-back and leaf diseases. Diseases caused by pathogenic *Fusarium* species can reduce yield and quality of food crops worldwide (Nelson *et al.,* 1981). In temperate regions, *Fusarium* species infected cereals such as wheat, barley, oats, sorghum and maize and caused diseases known as Fusarium head blight, Fusarium ear rot, foot rot and crown rot.

2.3.1 History of *Fusarium* systematic

The first *Fusarium* species, *F. roseum* was described by Link (1809) based on an asexual non-septa spore which was fusiform or canoe or banana-shaped, borne on a stroma (Booth, 1971). Following the description by Link, majority of the earlier research work on *Fusarium* was focused on the diagnosis, identification and enumeration of the taxa that caused plant diseases. However, there are still many of the *Fusarium* species in taxonomic systems are poorly defined as confusion and controversial issues regarding the characters used for identification. Therefore, after over a 100 years later, all the complicated system in *Fusarium* taxonomy was simplified by Wollenweber and Reinking (1935) in a monograph '*Die Fusarien'* with approximately 1000 names which was included into 16 sections, 65 species, 77 subspecific and 22 forms. In Wollenweber and Reinking (1935) system, the species with common similarities were grouped into a same section based on the characteristics of microconidia, chlamydospores and macroconidia. The sections were than divided into species, variety and forms based on the characters of the stroma, present or absent of sclerotia, the number of septation and the length and width of the macroconidia. There were a few weaknesses in Wollenweber and Reinking's species description. The characters used in the monograph are unstable,

which can easily be altered by environmental conditions. Mutation and culture variation was not recognized since the culture was not originated from single conidia (Nelson, 1991; Nelson *et al.,* 1994). However, their studies provided the first standard reference for *Fusarium* taxonomy.

In 1940s, Snyder and Hansen reduced the number of *Fusarium* species to nine and introduced the single spore technique which made the species identification more reliable. The nine species identified by Snyder and Hansen were *F. oxysporum*, *F. solani*, *F. moniliforme*, *F. roseum*, *F. lateritium*, *F. tricinctum*, *F. nivale*, *F. rigidiuscula*, and *F. episphaeria* which was based on morphology of macroconidia, environmental conditions and variability of the *Fusarium* species (Nelson, 1991). Snyder and Hansen species concept was popular because the systems were easy to use as every isolate could relatively be easily identified (Leslie and Summerell, 2006). They introduced the taxonomy system with *F. oxysporum* (section Elegans) and *F. solani* (section Martiella) as the basis for their system and these two species descriptions are still widely used until today.

The taxonomic system by Gordon (1952) was based on *Fusarium* species from cereal seeds, a variety of plants and soils from both temperate and tropical areas. The taxonomy concept by Gordon was similar with Wollenweber and Reinking's (1935) than Snyder and Hansen in 1940s. There were 26 species described in Gordon's system and in the system, teleomorph stage was taken into consideration for species description (Nelson, 1991).

Fusarium taxonomic system developed by Raillo (1950) used cultures from single conidia and recommended that the shape of the apical cell was the main character in species determination. Raillo (1950) also recommended that incurvature

of conidia, length of apical cell, number of septa, width of conidia were the characters to separate subspecies and varieties. Cultural characters such as pigmentation, present of sclerotia and mode of spore formation were the characters to separate the forms.

Taxonomic system by Bilai (1955) was focused on the importance of variability and mutation of *Fusarium* cultures, as well as physiological characters especially the effects of temperature, moisture, length of growth period and composition of the medium. This system was used in Russia although it was not accepted in other parts of the world as the system combined some of the Wollenweber and Reinking sections such as section Liseola with Elegans and Gibbosum with Discolor. Bilai (1955) and Raillo (1950) work together and published a book known as *Fusarii* which contained nine section, 26 species and 29 varieties.

Messiaen and Cassini's (1968), introduced aclassification system which was based on Snyder and Hansen's classification but subspecies identification of *F. roseum*were based on botanical variation than cultural variation as suggested by Snyder and Hansen (1940, 1941).

A significant development in taxonomy of *Fusarium* was made by Booth (1971) in a form of a monograph, 'The Genus *Fusarium*'. The monograph contains keys to the sections and species which were similar with the taxonomic system by Wollenweber and Reinking's. Booth introduced the use of the morphology of conidiogenous cell to separate section and species and microconidia as identification criteria. The length and shape of microconidiophores were also shown to be reliable characters to separate species such as to distinguish *F. oxysporum*, *F. solani* and *F. moniliforme*. The identification system by Booth (1971) made species identification

easier especially to the taxonomist, mycologist and plant pathologist to characterize isolates into sections and species.

Joffe's philosophy about classification system was identical to the system proposed by Wollenweber and Reinking (1935). The isolates examined were from soil, debris and seeds and included 13 section, 33 species and 14 varieties. The monograph by Joffe (1974) received little attention outside the mycotoxins research community as the monograph was only focusing on mycotoxin instead of taxonomy (Liddell, 1991).

Gerlach and Nirenberg (1982) system used the concept introduced by Wollenweber and Reinking in which they described 78 species that were arranged in sections with clear illustration and line drawings to enhance the original drawings by Wollenweber and Reinking. Gerlach and Nirenberg (1982) grew the cultures on the same eight different media as Wollenweber and Reinking's. Their system concentrated more on the differences than similarities of culture morphology and the basis for a new species and variety was defined by slight cultural differences. The new species established was based on a single culture and sometimes on a single mutant culture. The difficulty of understanding this taxonomic system persisted since the philosophy and techniques used followed Wollenweber and Reinking's system.

Nelson *et al.* (1983) species description is a combination of several classification systems from the United States and Anglo-European systems as well as their own descriptions, but the descriptions was not widely accepted outside the United States (Lindell, 1991). The number of species was reduced and varieties and forms were combined with appropriate species since many of the varieties and forms may have been cultural variants or mutants. Nelson *et al.* (1983) classification

manual are equipped with illustrated photos of macroconidia, microconidia, conidiophores and chlamydospores produce from carnation leaf agar (CLA) which are essential for identification. The manual is cross-indexes to the taxonomic systems of Wollenweber and Reinking (1935), Snyder and Hansen (1940s), Messiaen and Cassini (1968), Booth (1971), Joffe (1974), and Gerlach and Nirenberg (1982) which included the synoptic keys for identification of sections and species.

Burgess *et al.* (1994) worked on *Fusarium* species primarily in Australia and published a laboratory manual which is similar with Nelson *et al.* (1983) species descriptions which contains a useful synoptic key. The used of section within the genus has been avoided, pending determination of the affinities of several newly-described species and a revaluation of the validity of the section defined by Wollenweber and Reinking and other taxonomist working on *Fusarium*. Over than 40 000 cultures of *Fusarium* were examined to give an updated description for *Fusarium* species.

Leslie and Summerell (2006) published the *Fusarium* Laboratory Manual which contained a compilation of species description by several researchers. The manual contains methods for identification of *Fusarium* species based on morphological, biological, and phylogenetic species concepts.

The taxonomy of *Fusarium* species is still evolving. There are many species which are not-well described especially species from tropical and subtropical plants. According to Summerell *et al.* (2003), there are still lack of information on described symptoms and undescribed species based on morphological features of tropical and subtropical origins. Over the years, taxonomy of *Fusarium* changes after numerous

studies have been carried out. The latest classification of the genus *Fusarium* is as follows (http://www.uniprot.org/taxonomy/942786):

Superkingdom: Eukaryota

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Subclass: Hypocreomycetidae

Order: Hypocreales

Genus: *Fusarium*

2.3.2 Morphological Characteristics of *Fusarium* species

Generally, the morphological species concepts are based on similarity of the observable morphological characters. Morphological characteristics are the most common used criteria for identification of *Fusarium* species, which can be divided into two groups, primary and secondary characteristics. Primary characters usually used to separate species are based on the morphology of macroconidia, microconidia, presence or absence of chlamydospores and conidiophores. Secondary characteristics are the pigmentation, colony morphology and growth rates (Burgess *et al.,* 1994; Nelson *et al.,* 1994; Leslie and Summerell, 2006)

For identification of *Fusarium* species, macroconidia is the first structure examined. The most important character for identification is the distinctly shape macroconidia and mode of formation which are remarkably consistent characters (Burgess *et al.,* 1994). On Carnation Leaf Agar (CLA), the macroconidia characters

observe are the shape and size (width and length), the number of septa and the shape of apical and basal cells (Summerell *et al.,* 2003; Leslie and Summerell, 2006). There are three basic shaped of macroconidia, namely slender, straight and almost needle-like macroconidia (**Figures 2.1** and **2.2**). Usually the cells in the middle of macroconidia are wider than the cells at the ends. Both ends of macroconidia are the important character in morphological determination. The left part (**Figure 2.1A1**) is the apical cell whereas the right end (**Figure 2.1A2**) is the basal cell. The four general forms of apical cells are blunt, papillate, hooked and tapering. Furthermore, the common basal cells shape has four general forms which are foot-shaped, elongated foot shaped, distinctly notched and barely notched (**Figures 2.1** and **2.2**) (Burgess *et al.,* 1994; Leslie and Summerell, 2006).

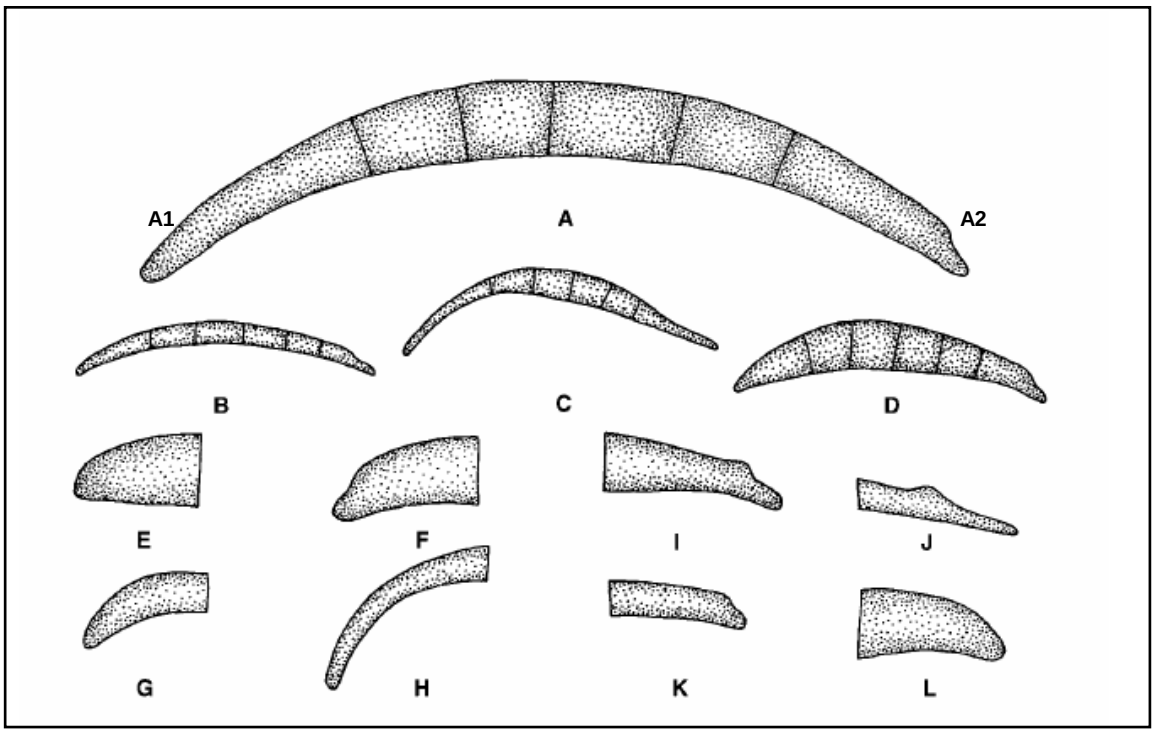

Figure 2.1: Different types of apical and basal cell of macroconidia of *Fusarium* species. (**A1)**Apical cell, (**A2**) Basal cell. (**B-D**) Shape of macroconidia. (**E-H**) Macroconidia apical cell shapes (**I-L**) Macroconidia basal cell shapes (Leslie and Summerell, 2006).

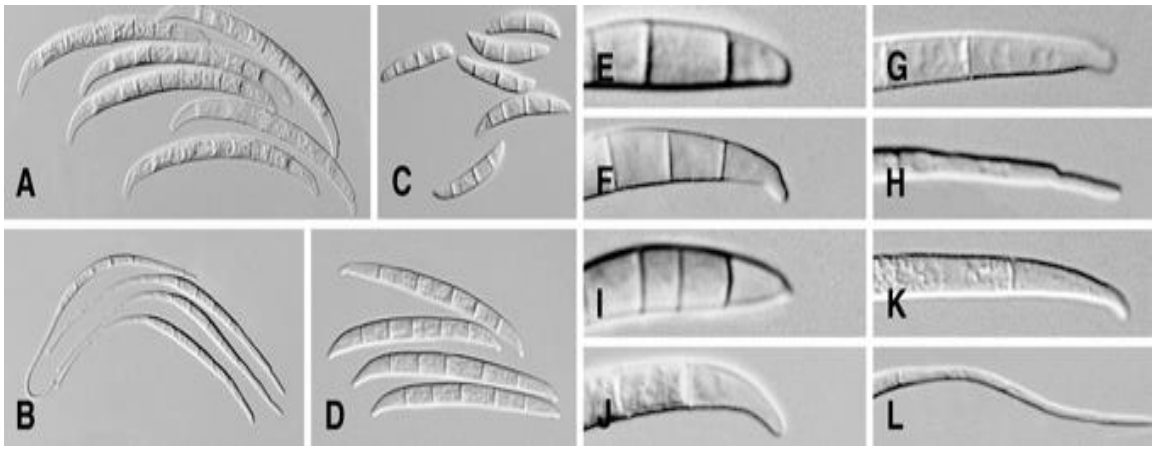

Figure 2.2: Variation of basal and apical cells of macroconidia in *Fusarium* species (Summerell *et al*., 2003).

Other important characters used in identification of *Fusarium* species is the presence or absence of microconidia as well as the size, shape and mode of formation of the microconidia. Microconidia is the smaller conidia produced in the aerial mycelia which vary in shapes and sizes. The most frequent shapes of microconidia are oval, reniform, obovoid, pyriform, napiform and globose (**Figure 2.3**) (Burgess *et al.,* 1994; Leslie and Summerell, 2006). In addition, microconidia may be found on the false heads (**Figure 2.4B**) and in chain forms (**Figures 2.4E** and **2.4F).** Macroconidia and microconidia can be found on the conidiogenous cell as monophialides or polyphialides in the aerial mycelium. Monophialides have only single opening per cell while polyphialides has more than one opening per cell. The length for each monophialides and polyphialides can also be important characters to differentiate between the *Fusarium* species (Nelson *et al.,* 1994; Leslie and Summerell, 2006)

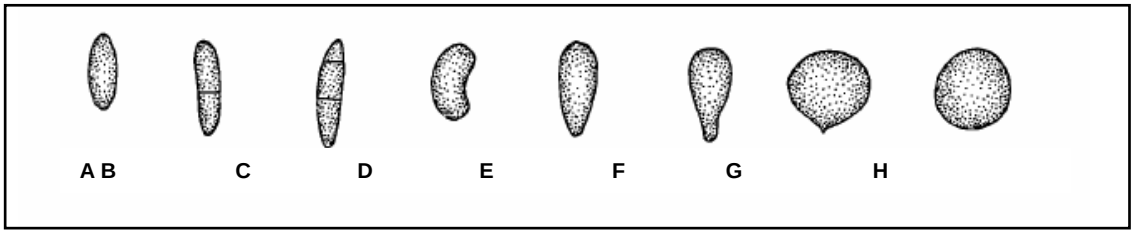

Figure 2.3: Various shapes of *Fusarium*microconidia (Leslie and Summerell, 2006).

Chlamydospores are one of the important characters for many *Fusarium* species description. Chlamydospore is an asexual spore, with thick wall and function as survival structure (Schippers and van Eck, 1981). These structures usually are more common on carnation leaf pieces than on the agar surface but more specialised in soil agar than CLA and take a long time (~6 weeks) to form (Summerell *et al.,* 2003). If chlamydospores are present, the structure can be formed singly, doubly, in clumps or in chains (**Figure 2.5**). Chlamydospore can easily be confused with pseudochlamydospores and swollen cells (Burgess *et al.,* 1994; Summerell *et al.,* 2003; Leslie and Summerell, 2006). However, true chlamydospores can easily be distinguish by the formation of thick wall, a warty appearance (verrucose) and light coloration (usually, yellow-brown).

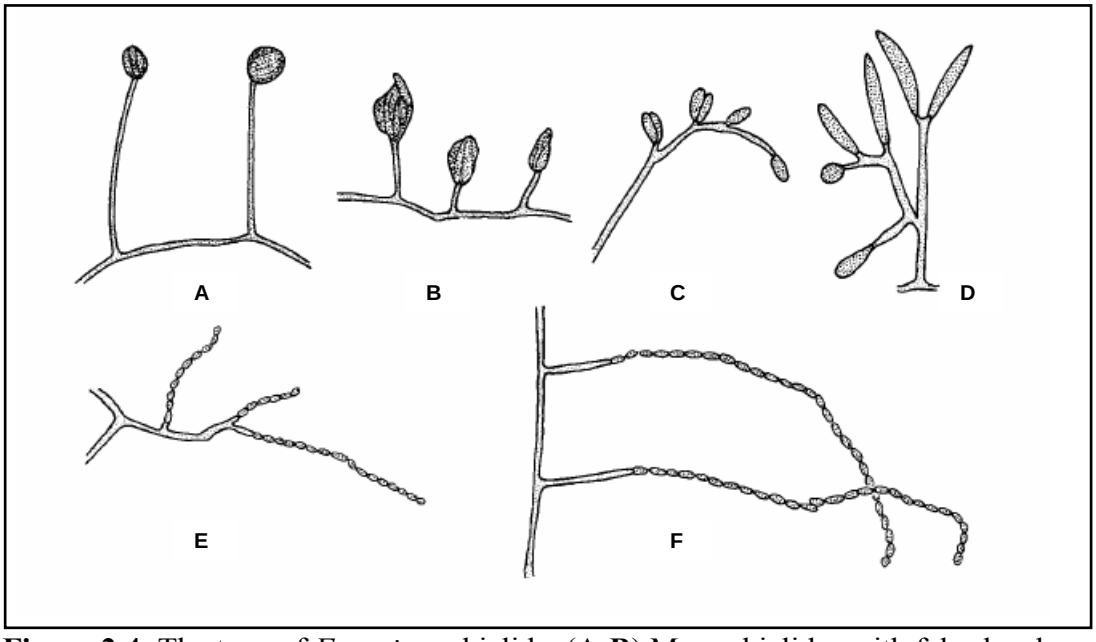

Figure 2.4: The type of *Fusarium* phialide. (**A-B**) Monophialides with false head. (**C-D**) Polyphialides. (**E-F**) Microconidia in chain (Leslie and Summerell, 2006).

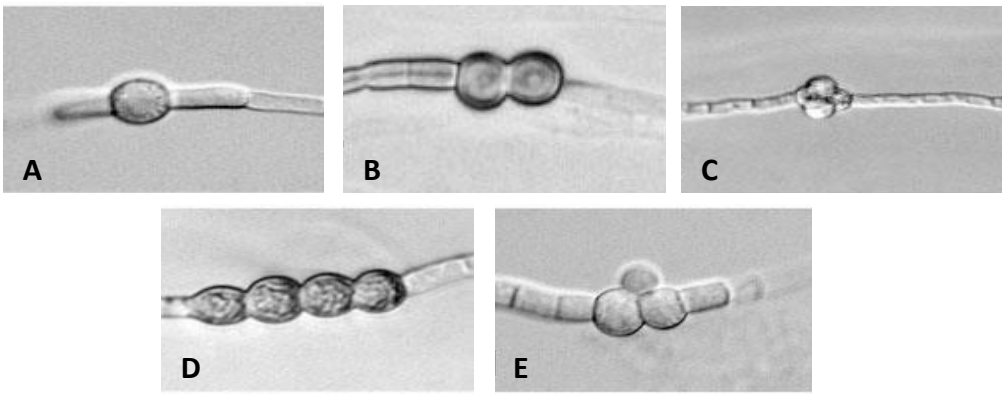

Figure 2.5: Various form of chlamydospores**. (A)** Single **(B)** In-pairs **(C)** Clustered.**(D)** Chains with verrucose appearance **(E)** Smooth-walled chlamydospores (Leslie and Summerell, 2006).

Pigmentation, colony morphology and growth rates can be useful in describing *Fusarium* species (Nelson *et al*., 1994). Pigmentation can be use to describe the *Fusarium* species according to their section by using Potato Dextrose Agar (PDA). For example red pigmentation is usually representing section Discolor and purple pigmentation for section Elegans or Liseola. Growth rate is also useful character to differentiate *Fusarium* species. There are species that are grow slower and others are fast grower. Measurement of growth rate on PDA usually originated from single spores and grown for three days at 25°C and 30°C. Accurate measurement is made by measuring the colony diameter (Leslie and Summerell, 2006).

2.4 Biogeography of *Fusarium* species

The centre of origin for *Fusarium* is difficult to determine as the genus have been reported in various environments and are dispersed globally. Therefore, the combinations of morphological and phylogenetic traits are used to split and create 'cosmopolitan' cultures and also to obtain information on the early spreading of *Fusarium* species in different types of ecosystems on global basis. Two main factors

which can gives information on the origin of various *Fusarium* species are climate and host, which are related to geographical location.

Fusarium species are diverse in both tropical and temperate regions (Gordon, 1960) which indicated that climate is an important factor which can determine the potential for occurrence of *Fusarium* species on a broad, regional scale. The climate and even a local variations in weather can limit the range of species observed and also can influence their relative frequency. For example, crown disease from wheat in cooler temperature caused by *F. culmorum* while *F. pseudograminearum* and *F. graminearum* are more common in warmer subtropical and arid regions (Backhouse and Burgess, 2002). The distribution of individual species can also depending on changes in temperature and rainfall patterns. For instance, *F. longipes* and *F. beomiforme* require warmer temperatures and higher moisture levels typical of those found in tropical regions. These two species are commonly found in the tropics and subtropics (Sangalang *et al.,* 1995).

Host is also the other main factors which can determine the origin of *Fusarium* species. For example, host for Fusarium Head Blight (FHB) usually found in temperate region, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) (Ward *et al,* 2008), oat (*Avena sativa*), maize (*Zea mays*) and even rice (*Oryza sativa*) (Pineiro *et al.,* 1996; Carter *et al,* 2002). Moreover, stem and root diseases in legumes were also caused by *F. avenaceum* which is common in temperate regions throughout the world (Kellock *et al.,* 1978; Lamprecht *et al.,* 1988).

In temperate area of Australia, *F. avenaceum, F. culmorum*, *F. sambucinum*, and *F. acuminatum* were recovered in temperate area of Australia, whereas *F. longipes*, *F. beomiforme*, *F. moniliforme*, *F. proliferatum*, *F. anthophilum* and *F.*

semitectum were recovered from tropical area of Australia (Burgess *et al.,* 1988; Saremi *et al.,* 1999).

2.5 Molecular characterization

In fungal taxonomy, PCR-based technique has been used for identification and characterization of *Fusarium* species. (Leslie and Summerell, 2006). The techniques have been applied to examine the variation among species which can provide the information on genetic relationship, taxonomy, population, gene cloning, molecular markers, as well as identify and distinguish species, formae specials, races and strains (Donaldson *et al.,* 1995; Walsh *et al.,* 2010). Molecular techniques are also useful in the fields of mycotoxicology, fungal diversity, fungal systematic, as well as its facilitating the prevention, control or treatment of infectious diseases.

Various molecular techniques have been developed based on PCR which included random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990), restriction fragment length polymorphism (RFLP) (Saiki *et al.*, 1985), amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995), arbitrarily-primed polymerase chain reaction (AP-PCR), inter simple sequence repeat (ISSR), and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) . One of the most common methods used for *Fusarium* detection and identification is species-specific PCR assays combined with DNA sequencing and phylogenetic analysis (Geiser*et al.,* 2004). For example, Demeke *et al*. (2005) used the species-specific PCR-based assays for the detection of nine *Fusarium* species, *F. avenaceum, F. acuminatum, F. crookwellense, F. culmorum, F. equiseti, F. graminearum, F. poae, F. pseudograminearum* and *F. sporotrichioides* from wheat, barley, oat, corn and rye.

2.5.1 Restriction Fragment Length Polymorphism of intergenic spacer region (RFLP-IGS)

Ribosomal DNA (rDNA) is a region to encode ribosomal RNA and important in protein synthesis. There are three regions for coding the ribosomal unit which two are ITS regions (ITS1 and ITS2) and IGS (Schlotterer, 1998). rDNA sequences contain both conserved and variable regions that could be used in phylogenetic and taxonomic studies to distinguish species at different taxonomy levels species or genus levels as well as at intraspecific level (Edel *et al.,* 1995; Llorens *et al.,* 2006a). There are three conserved regions in the rDNA namely 18S, 5.8S and 28S of rDNA which are not suitable for identification of species (Hillis and Dixon, 1991; Appel and Gordon, 1995). Intraspecies evaluations and variability within species can be shown by variable regions such as the spacer region, of the ITS and IGS regions. The intergenic spacer region (IGS) separates units of rDNA and appears to be the most rapidly evolving spacer regions. Closely related species may show considerable divergence in IGS, often reflecting both length and sequence variation (Hillis and Dixon, 1991). **Figure 2.6** shows the location of intergenic spacer region in rDNA.

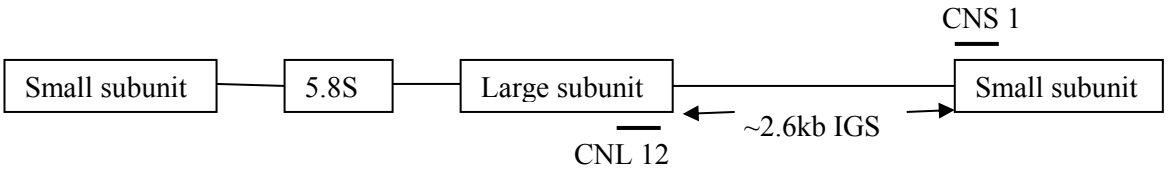

Figure 2.6: The approximate location of CNL12 and CNS1 primers and the location of intergenic spacer region in rDNA

PCR-RFLP analysis of IGS region is a suitable technique to characterize intraspecific variations and became a tool for delimiting specific or subspecific taxa where the morphological and physiological differences were unclear. The restriction analysis using IGS region proven an as appropriate method for taxonomic studies in

Fusarium (Mirete *et al.,* 2003; Llorens *et al.,* 2006a; Hsuan *et al.,* 2010; Jurado *et al.,* 2012; Srinivasan *et al.,* 2012), *Aspergillus* (Moody and Tyler, 1990), *Entomophaga* (Walsh *et al.,* 1990), *Metarhizium anisopliae* (Hughes *et al.,* 2004) and *Pyrenophora graminea* (Aminnejad *et al.,* 2009). This RFLP-IGS analysis also showed higher variability than ITS sequences (Donaldson *et al.,* 1995; Jurado *et al.,* 2005)

2.5.2 DNA Sequencing and Phylogenetic analysis

Phylogenetic is used to study evolutionary development or relationship of a group of organism (Leslie and Summerell, 2006) and is commonly used to clarify many taxonomic difficulties (Taylor *et al.,* 2000). Phylogenetic studies of *Fusarium* species can be carried out by using a combination of several genes and regions. Several genes and regions commonly used in phylogenetic studies of *Fusarium* species were elongation factor-1 alpha (EF-1α) (Aoki and O'Donnell,1999a; Knutsen *et al.,* 2004), histone (H3) (Steenkamp *et al.,* 1999), β-tubulin (O'Donnell *et al.,* 1998a; Seifert and Levesque,2004), calmodulin genes and ITS1 and ITS2 regions (Waalwijk *et al.,* 1996; O'Donnell and Cigelnik, 1997) and the intergenic spacer region (IGS) (Yli-Mattila and Gagkaeva, 2010).

Not all the genes and regions used in phylogenetic analysis work well for all *Fusarium* species in the section Liseola. For examples, *F. avenaceum*, *F. arthrosproioides/ F. tricinctum, F. sporotrichioides/ F. langsethiae* and lineages of *F. graminearum* do not work well with ITS region (O'Donnell *et al.,* 2000). β-tubulin gene did not work well for phylogenetic analysis within the *F. solani* species complex (Sampietro *et al.,* 2010). TEF-1α gene is widely used in identification and has been reported to have sufficient variability to differentiate *Fusarium* species. The gene is also useful for phylogenetic and taxonomy of *Fusarium* species with the set-up of FUSARIUM-ID v.1.0 database (Geiser*et al.,* 2004; Nitschke *et al.,* 2009).

O'Donnell *et al.* (1998a) and Kristensen *et al.* (2005) reported that phylogenetic analysis of TEF-1α sequences can be used very successfully to identify monophyletic species of *Fusarium* in term for species separation and toxigenic potential.

2.6 Mycotoxins produced by *Fusarium* species

Mycotoxins are secondary metabolites that are not directly involved in the normal growth, development and reproduction of fungi (Turner *et al.,* 2009). Mycotoxins contaminated food and feeds in the temperate and tropical regions, and the Food and Agricultural Organization of the United Nation (FAO) reported that about 25% of the world's food crops are significantly contaminated with mycotoxins. Examples of food and commodities affected by mycotoxins are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans, and fruit (Turner *et al.,* 2009). About 90% of mycotoxins are carcinogenic and International Agency for Research and Cancer (IARC) (1993) reported that well-known mycotoxins are classified according to their carcinogenic ability (**Table 2.1)**.

Table 2.1: Classification of mycotoxins based on their carcinogenic ability to mammals

Mycotoxins	Group/ Class of human carcinogen	Data
Aflatoxins B_1(AFB_1), Fumonisins (FUM)	1	Carcinogenic to human
Aflatoxins M1 (AFM1), Ochratoxin A (OTA)	2	Have ability carcinogenic to human
Patulin, Zearalenone (ZEN), Trichothecenes (TRI)	3	Not carcinogenic

*Source from Salleh (1998) and Reddy *et al.* (2010).

Fusarium species produced several types of mycotoxins (fusariotoxins) and can cause mycotoxicoses (fusariotoxicoses). The natural occurrence of the mycotoxins from *Fusarium* species are detected not only in temperate countries but

also in the tropics. Over the last 20 years, research on the genus *Fusarium* has intensified, especially with the Fusarium head blight (FHB) epidemics on wheat, barley, oats, maize and rice as well as deoxynivalenol (DON) contamination issue (Parry *et al.,* 1995). Placinta *et al.* (1999) reported that multiple major mycotoxins of fungi contaminated cereal grains and animal feed are TRI, ZEN and FUM in temperate region. In Asia, *Fusarium* mycotoxins, trichothecenes and zearalenone have been found in Korean polished rice (Park *et al.,* 2005). The mycotoxin also have been reported occurring in Vietnam, which was about 21.88% *Fusarium* species identified in rice samples tested containing zearalenone and DON (Trung *et al.,* 2001).

Food and feed contamination with *Fusarium* mycotoxins is the focus of interest of many researchers in mycology, agronomy, plant pathology, food technology, toxicology and animal health. There was no region in the world that escapes the problem of mycotoxins and there are about 300 harmful mycotoxins reported worldwide (Iheshiulor *et al.,* 2011). From these mycotoxins, TRI, ZEN, MON, and FUM are the most important mycotoxins which can give negative economic impact such as loss of human and animal life, reduced livestock production, disposal of contaminated foods and feeds, increased health care and veterinary care costs investment in research and application to reduce the severity of the mycotoxins problem (Bottalico, 1998; Placinta *et al.,* 1999; Logrieco *et al*., 2002; Zain, 2011).