OCCURRENCE AND CHARACTERIZATION OF *Fusarium* SPP. ISOLATED FROM MANGROVE SOILS

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by

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LIST OF SYMBOLS AND ABBREVIATION

AFLP	Amplified Fragment Length Polymorphism
BEA	Beauvericin
bp	Base pair
CA	Carrot agar
CLA	Carnation leaf –piece agar
CM	Complement medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FCSC	*Fusarium chlamydosporum* species complex
FDSC	*Fusarium dimerum* species complex
FFSC	*Fusarium fujikuroi* species complex.
FSSC	*Fusarium solani* species complex
FUM	Fumonisin
IGS	Intergenic spacer
ITS	Internal transcribed spacer
MAT	Mating type
MEGA	Molecular Evolution Genetic Analysis
ML	Maximum Likelihood
MON	Moniliformin
MP	Mating type
ND	Not detected
NJ	Neighbour Joining
NNI	nearest- Neighbour-interchange
OPA	O-phthaldialdehyde

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PCNB	Peptone PCNB Agar medium
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
SEM	Scanning Electron Microscopic
SPE	Solid-phase extraction
TBE	Tris-Boric acid -EDTA
TEF-1α	Translation elongation factor 1-α
UPLC	Ultra Pressure Liquid Chromatography
USDA	United states Department of Agriculture
var	Variety
WA	Water agar

KEBERLAKUAN DAN PENCIRIAN *Fusarium* SPP. DIPENCILKAN DARIPADA TANAH BAKAU

ABSTRAK

Genus *Fusarium* adalah antara kulat fitopatogen dan toksigen penting yang tersebar secara meluas dalam alam semula jadi. Kajian ini bertujuan menentukan keberlakuan dan ciri-ciri pencilan *Fusarium* daripada tanah bakau, yang terkenal dengan flora dan fauna, sebaliknya tidak begitu diketahui sebagai substrat bagi *Fusarium* spp.. Sejumlah 136 pencilan *Fusarium* telah dipencilkan daripada sampel tanah bakau yang diambil dari pada beberapa kawasan bakau di Pulau Pinang dan Kedah. Kajian ini menunjukkan 11 spesies *Fusarium* spp. telah dikenal pasti berdasarkan ciri-ciri morfologi, iaitu *F. solani* (n = 77), *F. verticillioides* (n = 20), *F. semitectum* (n = 17), *F. proliferatum* (n = 7), *F. oxysporum* (n = 3), *F. chlamydosporum* (n = 1), *F. camptoceras* (n = 1), *F. longipes* (n = 3), *F. merismoides* (n = 1), *F. lateritium* (n = 4) dan *F. decemcellulare* (n = 2). Pengenalpastian secara morfologi *Fusarium* disahkaa semula semula menggunakan gen TEF-1α dan 11 spesies *Fusarium* telah dikenal pasti secara molekul iaitu *F. solani* (n = 77), *F. verticillioides* (n = 20), *F. incarnatum* (n = 17), *F. proliferatum* (n = 7), *F. oxysporum* (n = 3), *F. chlamydosporum* (n = 1), *F. camptoceras* (n = 1), *F. longipes* (n = 3), *F. merismoides* (n = 1), *F. lateritium* (n = 4) and *F. rigidiuscula* (syn. *F. decemcellulare*) (n = 2). Analisis filogenetik jujukan individu dan gabungan TEF-1α, dan ITS mengesahkan identiti *F. solani*, di mana klad yang belum dijelaskan diperoleh, yang mencadangkan kumpulan atau spesies filogenetik baru. Analisis filogenetik ITS mengesahkan identiti morfologi *F. longipes, F. merismoides* dan *F. chlamydosporum.* Identiti morfologi *F. verticillioides, F. incarnatum, F.*

proliferatum, F. oxysporum, F. camptoceras, F. lateritium dan *F. decemcellulare* disahkan melalui jujukan TEF-1α dan analisis filogenetik jujukan secara individu dan gabungan daripada jujukan TEF-1α, dan β-tubulin. Kajian kacuk subur dijalankan bagi spesies ahli komplek spesies *F. fujikuroi* dan keputusan menunjukkan bahawa semua pencilan *F. proliferatum* membawa alel MAT-1, sedangkan semua pencilan *F. verticillioides* membawa alel MAT-2. Pencilan *F. proliferatum* berjaya mengacuk subur dengan strain penguji populasi mengawan D dan pencilan *F. verticillioides* dengan strain penguji populasi mengawan A, mengesahkan pengenalpastian kedua-dua spesies. Analisis mikotoksin dijalankan pada 11 pencilan *F. verticillioides* dan enam pencilan *F. proliferatum* kerana kedua-duanya terkenal sebagai penghasil mikotoksin. Pencilan kedua-dua spesies menghasilkan $FUMB_1$ dan MON di mana *F. verticillioides* menghasilkan $FUMB_1$ 5.77 -172.4 µg/g, *F. proliferatum* menghasilkan $FUMB_1$ 1.17-74.6 µg/g. *Fusarium verticillioides* menghasilkan MON 2.45 -14.0 µg/g dan *F. proliferatum* menghasilkan MON, 4.7-67.0 µg/g. sementara itu, tiada pencilan yang menghasilkan BEA. Berdasarkan analisis tanah, terdapat beberapa faktor yang boleh mempengaruhi jumlah pencilan, antaranya kelembapan, tekstur, pH, saliniti, kandungan karbon, dan kandungan nitrogen. Berdasarkan analisis tanah, kandungan karbon, pH, kelembapan dan saliniti bervariasi antara lokasi tetapi kandungan nitrogen tidak signifikan antara lokasi. Kajian ini menunjukkan bahawa spesies *Fusarium* spp. boleh bertolernsi dengan persekitaran bakau yang ekstrem.

OCCURRENCE AND CHARACTERIZATION OF *Fusarium* SPP. ISOLATED FROM MANGROVE SOILS

ABSTRACT

The genus *Fusarium* is among the most important phytopathogenic and toxigenic fungi, widely spread in nature. The aim of this study was to determine the occurrence and characterize *Fusarium* isolates from mangrove soils which are well known for its flora and fauna, but poorly known as substrate of *Fusarium* species. One hundred thirty six (136) isolates of *Fusarium* were isolated from mangrove soil samples collected from several mangroves areas in Penang and Kedah, Peninsular Malaysia. The present study morphologically identified 11 *Fusarium* species namely *F. solani* (n = 77), *F. verticillioides* (n = 12), *F. semitectum* (n = 16), *F. proliferatum* (n = 15), *F. oxysporum* (n = 3), *F. chlamydosporum* (n = 1), *F. camptoceras* (n = 2), *F. longipes* (n = 3), *F. merismoides* (n = 1), *F. lateritium* (n = 4), and *F. decemcellulare* (n = 2). The identification of morphologically identified *Fusarium* species was re-identified using TEF-1α gene and 11 species of *Fusarium* species were also molecularly identified as *F. solani* (n = 77), *F. verticillioides* (n = 20), *F. incarnatum* (syn. *F. semitectum*) (n = 17), *F. proliferatum* (n = 7), *F. oxysporum* (n = 3), *F. chlamydosporum* (n = 1), *F. camptoceras* (n = 1), *F. longipes* (n = 3), *F. merismoides* (n = 1), *F. lateritium* (n = 4) and *F. rigidiuscula* (syn. *F. decemcellulare*) (n = 2). Phylogenetic analysis of individual and combined sequences of TEF-1α and ITS confirmed the identity of *F. solani* of which undescribed clades were revealed which suggested new phylogenetic clade or species. Phylogenetic analysis of ITS confirmed the identity of *F. longipes*, *F. merismoides* and *F.*

chlamydosporum. The identity of *F. verticillioides*, *F. incarnatum, F. proliferatum, F. oxysporum, F. camptoceras, F. lateritium* and *F. rigidiuscula* were confirmed using TEF-1α and phylogenetic analysis of individual and combined sequences of TEF-1α, and β-tubulin. Mating study was conducted on *Fusarium* species belonging to *F. fujikuroi* species complex and the results showed all *F. proliferatum* isolates carried MAT-1 allele, whereas all *F. verticillioides* carried MAT-2 allele. Isolates of *F. proliferatum* successfully crossed fertile with tester strain of mating population D and isolates of *F. verticillioides* with tester strain of mating population A which confirmed the identification of both species. Mycotoxin analysis was done on 11 isolates of *F. verticillioides* and six isolates of *F. proliferatum* as both species are well-known mycotoxin producers. Isolates of both species produced fumonisin B_1 ($FUMB_{1)}$ and moniliformin (MON)of which *F. verticillioides* produced 5.77 -172.4 µg/g $FUMB_1$, *F. proliferatum* produced 1.17-74.6 µg/g $FUMB_1$, *F. verticillioides* produced 2.45 -14.0 µg/g MON and *F. proliferatum* 4.7-67.0 µg/g MON. None of the isolates produced beauvericin**.** Based on soil analysis, carbon content, pH, soil moisture and salinity varied between locations, but nitrogen content was not significant between the locations. The present study showed that *Fusarium* species can tolerate the extreme environment of mangrove ecosystem.

CHAPTER 1

GENERAL INTRODUCTION

Mangrove ecosystems are important as ecological and economic resources, which provide a wide variety of services such as source of wood, accumulation sites for sediment, carbon and nutrients. Traditionally, some mangrove plants are used for food such as the tender leaves of *Acrostichum* are the staple food of some Papua New Guineans, leaves of *Osbornia octodonata* are used as flavouring agents and fruits of *Avicennia marina* are used as vegetables (Australian Institute of Marine Science, 2014) (http://www.aims.gov.au/docs/projectnet/mangroves-uses.html). Mangroves are used as timber, fuel and medicine, however, during the last century, approximately one-third of the world's mangrove forests have been lost due to pollution, overharvesting and human activity such as clearing to make room for agricultural land and human settlements (Alongi, 2002, McLeod and Salam, 2006). In Myanmar, Malaysia and Indonesia, land expansion to plant rice and oil palm had contributed to major mangrove losses (Richards and Friess, 2015).

Mangrove is regarded as an extreme environment where the soils have high levels of salinity, high temperature, exposed to extreme tides and muddy anaerobic conditions (Kathiresan and Bingham, 2001; Araujo et al., 2010). However, mangrove ecosystems are habitat for halophytes plants, commercial fishes and crustaceans as well as animals such as birds and monkeys.

Microbes also play an important role in mangrove ecosystem, as they play an important role in recycling and conserving nutrients in the ecosystem. The mangrove

microbial community especially fungi transforms organic matter such as residual dead mangrove plants and animal remains into nutrients such as nitrogen and phosphorus. Therefore, microbial community contribute to plant fitness, survival and overall mangrove ecosystem resilience (Gomes et al., 2011)

Despite a few documentations on the occurrence of microorganisms in the mangrove soils, the knowledge on the diversity of fungi is not sufficient and many of the microorganisms remain uncharacterized. Fungi in mangrove environment play an important ecological role in decomposition of organic matter by production of a variety of extracellular degradative enzymes such as cellulase, xylanase, pectinase and amylase (Thatoi et al., 2013).

In Malaysia, studies related to fungal communities of mangrove ecosystem have been conducted. However, the study was focused on marine fungi and phylloplane fungi (Kuthubutheen et al., 1984; Alias t al., 2000; Alias et al., 2010). Studies on the soil microfungi in mangrove ecosystem are lacking, although this type of fungi are vital as decomposer for ecological function.

Among the important soil microfungal inhabitant is *Fusarium* which is a cosmopolitan fungal genera, comprising both pathogenic and non-pathogenic strains associated with plants, animals and human. Many species of *Fusarium* are soil borne pathogen and facultative parasites. As soil borne pathogens, *Fusarium* species have been recovered from many agriculture or cultivated soils of which the presence of *Fusarium* is commonly associated with plant debris and plant roots. *Fusarium* species have also been isolated from non-agriculture soils such as peat soil, arid environment, Artic soil and mangrove soils (Latiffah et al., 2010b; Mandeel et al., 1995; Kommendahl et al., 1988; Latiffah et al., 2010a). These types of soil are regarded as extreme environment.

The occurrence of *Fusarium* species in mangrove soil is largely unexplored although *Fusarium* is among the important fungal genera causing diseases to plants, animal and human. *Fusarium* species residing in mangrove soil might be pathogenic as well as saprophyte as many species are facultative parasites. Therefore, mangrove soil could be reservoir for pathogenic species.

For identification and characterization of *Fusarium* species, three species concept are usually applied. Morphological species concept is based on morphological characteristics which group together isolates that show similar characteristics. For *Fusarium*, the main characteristics used for morphological identification are the shapes and size of macroconidia, microconidia, chlamydospores and culture pigmentation (Nelson et al., 1994).

The main purpose of morphological characterization is to group the isolates with similar features in the same group. However, for some species such as species in a species complex, the morphological characteristics are very similar. Therefore, misidentification can often occur if the identification is solely based on morphological characteristics. To overcome these weaknesses, molecular identification followed by analysis of phylogenetic are commonly applied.

Phylogenetic species concept or molecular identification is based on nucleotide sequence of a particular protein coding gene. For *Fusarium*, TEF-1-α gene is recommended for species identification as this gene occurs as single copy and has a high level of sequence polymorphism among closely related species (Geiser et al., 2004). In phylogenetic species concept, a species is differentiate based on the smallest phylogenetic clade of individual or population that show a diagnostic character (Nixon and Wheeler, 1990).

For *Fusarium* species, in addition to TEF-1-α, β-tubulin and ITS region are also used for phylogenetic analysis depending on the species. β-tubulin is among the most useful markers for studying closely related *Fusarium* species, and appears to be a more phylogenetically informative gene to characterize species in a species complex (Geiser et al., 2004)

Although ITS region is the barcode marker for fungi, for *Fusarium* species, there is significant overlap of sequences between many *Fusarium* species (Leslie and Summerell, 2006). ITS region works well for phylogenetic analysis of *F. solani* (O'Donnell, 2000; Chehri et al., 2015) as it is a very informative locus due to its highly repetitive characteristic region (Brasileiro et al., 2004) and its high degree of diversity (O'Donnell and Gray, 1994).

Other species concept use for *Fusarium* species identification is biological species concept or mating study. Mating study involves crosses between isolates from the same mating population (MP) and can only be applied for species that has sexual stage or teleomorphic stage such as species in *Fusarium fujikuroi* species complex (FFSC). Sexual crosses or mating study requires interaction between two mating type alleles, with successful production of perithecia. Most of the time, isolates belonging to the same biological species are sexually fertile, indicating that the isolates belong to the same MP. In FFSC, 12 mating populations or biological species have been identified, designated as MP-A to MP-L (Leslie and Summerell, 2006).

Some *Fusarium* species are also toxigenic, producing different types of mycotoxin such as beauvericin, fumonisins and zearalenone, all of which are found to contaminate animal feed and human foods (Rheeder et al., 2002, Kim et al., 2013).

Natural occurrences of mycotoxins such as fumonisins, zearalenone and trichothecenes have been reported in cereal crops such as corn and wheat (Yazar and Omurtag, 2008). Mycotoxin produced by *Fusarium* in the soil could be related to the survival of this fungus. The production of these mycotoxins can be used as supporting data for *Fusarium* species identification and characterization as species in the same species complex produce the same type of mycotoxin.

Due to the importance of the genus *Fusarium* as pathogen to plants, animals and humans, the present study was conducted to determine the occurrence of *Fusarium* species in mangrove soil. Thus, the specific objectives are as follows:

(1) To isolate and identify *Fusarium* species in mangrove soil by using morphological and molecular identification using TEF-1-α sequences.

(2) To determine phylogenetic relationships of *Fusarium* isolates based on TEF-1-α, β-tubulin and ITS sequences.

(3) To investigate the mating type alleles and mycotoxin production of *Fusarium* species members of FFSC.

(4) To analyse the mangrove soil samples and to detect the occurrence of the *Fusarium* species

Chapter 2

LITERATURE REVIEW

2.1 Mangrove Ecosystem

The mangrove ecosystem is commonly referral to as the mangrove forest, tide forest or mangle. Mangrove forest is defined as a type of forest on tidal mudflats along the sea coast extending along the streams where the water is brackish (Faridah et al., 2014). Mangroves are an ecosystem that comprise salt-tolerant trees, grow mainly in the intertidal areas and estuary mouths between land and sea. The mangroves have specially adapted aerial and salt-filtering roots, as well as salt excreting leaves that enable them to occupy the saline wetlands, where other plant life usually cannot survive in this condition. Mangrove is also home to many molluscs, crustaceans, insects, fish, reptiles, amphibians, birds, mammals and microscopic organisms (Hotel, 1995).

As ecological and environmental resources, mangrove forests protect coastlines against erosive wave action and strong coastal winds, and serve as natural barriers against tsunamis and torrential storms. The mangrove forest also prevent salt water from intruding into rivers, and retain, concentrate and recycle nutrients and remove toxins through a natural filtering process. Furthermore, it is an important breeding ground for many fishes, crabs, prawns and other marine animals, which are essential for sustaining a viable fishing industry (Jusoff, 2013).

In Malaysia, the mangrove forests are important natural resources for coastal communities and provide resources such as fishes, shells and other gastropods for fishermen as well as provide fuel wood, poles and other building materials for local constructions.

2.2 Mangrove Soil Physical and Chemical Properties

The physical and chemical characteristics of the mangrove soils are determined by their muddy, anaerobic nature, and plant debris. These characteristics affect the potential and productivity of the soil (Eden and Ndon, 2001). The properties of mangrove soils are also influenced by species of mangrove tree which can be used as indicators of soil type (Effiong and Ayolagha, 2010). Red mangrove including *Bruquiera, Ceriops, Kandelia* and *Rhizophora* trees are adapted to mud and sandy soil with low oxygen while Black mangrove including *Avicennia* usually found further inland adapted to sandy, silty clay and loam soils (Bulow and Ferdinand, 2013)

Soil organic carbon is the main component of soil organic matter, which is extremely important in all soil processes. Organic material in the soil is mainly derived from plant and animal material residues, which are decomposed by microbes under the influence of temperature, moisture and soil conditions. High carbon content of mangrove soil was documented by several studies. A comparative study between two sites of mangrove soil was carried out in the Maldives and demonstrated that very dark soil is rich in organic matter (Shazra et al., 2008). Bouillon et al. (2003) reported organic carbon content of three different mangrove ecosystems in the Godavari Delta, India and south-west Sri Lanka ranged from 0.6 –

31.7%. Significant variation in organic carbon of mangrove and non-mangrove soils was reported by Uyub et al. (2011), but no significant differences were observed for other parameters such as pH or sulphate content, which determine the distribution of sulphate-reducing bacteria (SRB).

Nitrogen in the soil exists in many forms, and can transform easily from one form to another and flows into and out of the soil ecosystem. Sreeja et al. (2009) reported significant differences in nitrogen content of mangrove and non-mangrove soils in wetlands of the Kannur District while Lacerda (1995) reported nitrogen contents in mangrove soils differed between soils under different trees which showed higher nitrogen soil content under *Avicennia schaueriana* (0·35% at 1-5 cm) than under *Rhizophora mangle* (0·16% at 1-5 cm) in mangrove forests from south-eastern Brazil.

Soil texture refers to the relative proportions of the soil components, which include sand, silt and clay. The texture of mangrove soil located in coastal areas at Red Sea of Nabq and Ras Mohammed Protectorates in Egypt (Khalil et al., 2013) was clay, sandy to sandy loam. The nature of soil texture of sediment and soil texture in arid zone mangroves of Kachchh Gujarat India was characterized by the abundance of silty loam, silty clay and silty clay loam (Saravanakumar et al., 2008). In a study by Plante et al. (2006) showed there was no association between soil texture and soil organic matter comprising coarse- and fine-particulate organic matter.

Soil pH refers to the degree of soil acidity or alkalinity. The pH is an important chemical factor for solubility and production of dissolved organic carbon, because of the influence of humic compounds on charge density, or due to a positive or negative stimulation of microbial activity (Andersson and Nilsson, 2001). Mangrove soil pH ranged from strongly to moderately acid, slightly acid to slightly alkaline in nature (Effiong and Ayolagha, 2010). Madavasamy and Panneerselvam (2013) showed the pH values of mangrove soil from Karangadu, Ramanathapuram District, India were alkaline varying from 7.02 to 7.69. Soil pH was regarded as best predictor of bacterial population composition, whereas the composition of the fungal community was most strongly associated with changes in soil nutrient status (Lauber et al., 2008).

Soil moisture content is dependent on the soil type. Saturated heavy clay soil can hold more water than saturated sandy soil, from which most of the water drains due to gravity (Waugh, 2000). Mangrove soils are regularly water-logged and loaded with salt. The moisture content of mangrove soils reported by Faizuddin and Islam (2003) ranged from 28.6 - 43.3% by dry weight. Soil moisture influences the microbial community and activity in the soil. Nandy et al. (2004) stated that soil moisture content with other factors such as organic matter, pH, affects the density and diversity of microbes in mangrove soil. Soil moisture can be useful for microorganisms in two ways, serves as supply of hydrogen/oxygen and acts as a solvent and carrier of nutrients such as nitrogen, phosphorus and sulphur. Moisture content is also one of the factors correlated with root fungal population structure (Burke et al., 2009).

2.3 The Genus *Fusarium*

Fusarium is a filamentous fungus from the class Sordariomycetes, order Hypocreales and family Nectriaceae. The genus comprises more than 200 species, which can act as saprophyte, endophyte and pathogen to plants, animals and humans (Leslie and Summerell, 2006). Therefore, the genus *Fusarium* is one of the fungal genera distributed worldwide and can be found in the tropical, temperate, extreme environment such as the Artic, alpine as well as arid and desert areas. Species of *Fusarium* are encountered in many substrates such as in different types of soils including agricultural soils planted with different crops and forest soils such as peat forest, hardwood forest, rain forest, grassland and pasture soils. Species of *Fusarium* can also be found in infected and healthy plants, infected animal and human as well as causing contamination in indoor environment.

In Malaysia, a few studies have been conducted on the occurrence of mangrove fungi**.** Alias et al. (1995) reported more than 100 fungal isolates on mangrove wood. Alias et al. (2010) also documented 139 marine ascomycetes collected from senescent and decaying substrata in selected Malaysian mangroves. A total of 173 samples collected from mangrove Sulu Sea included Jambongan, Mandidarah and Malawali Islands, yielded 78 marine manglicolous taxa of which 66 Ascomycota, 17 anamorphic morphs and one Basidiomycota were identified (Awaluddin et al, 2015). Latiffah et al. (2010) isolated and identified three *Fusarium* species, *F. solani*, *F. oxysporum* and *F. verticillioides* from mangrove soil in Kampung Pantai Acheh, Balik Pulau, Pulau Pinang, Malaysia.

2.3.1 *Fusarium* as Soil Inhabitant

Many *Fusarium* species are saprophytic soil inhabitants which allows growth and colonization of debris in the soil. Saprophytic *Fusarium* plays an important role in soil biology and obtains their nourishment by breaking down dead and decaying organic matter into simple substances (Whalen and Sampedro, 2010).

Fusarium is widely distributed in soils in different geographic regions (Burgess et al., 1994; Sangalang et al., 1995). *Fusarium* species have been reported from agriculture soil such as in a study by Bogale et al. (2009) where *F. solani* was mainly isolated from the agricultural soil previously planted with different cereals and pulses. Different species of *Fusarium* have also been isolated from several agricultural soils planted with potato, pea, bean, wheat, corn and rice in northwest of Iran (Saremi and Okhovvat, 2008). Maina et al. (2009) recovered 1,865 *Fusarium* isolates from soil planted with corn, nappier grass, planted forests of pines and cypress in Taita Taveta, Kenya which resulted in identification of 26 *Fusarium* species, and *F. oxysporum* and *F. solani* being the dominant species recovered in this area.

Fusarium has also been reported from non-agricultural soils. In a study by Saremi and Saremi (2013), 17 *Fusarium* species were identified from 12 soil samples collected from four different climatic zones including humid, semi-arid, extra-arid and arid zones. Behera et al. (2012) investigated the diversity of soil fungi from mangroves areas in the Mahanadi Delta, Orissa, India and successfully identified 22 species of *Fusarium*.

2.3.2 *Fusarium* as Plant Pathogen

Fusarium is among one of the economically important plant pathogens infecting various types of agricultural crops. The plant diseases caused by *Fusarium* species are not restricted to any particular region or crop and cause problems both in temperate and tropical agricultural crops (Brown and Proctor, 2013). *Fusarium* species can cause various types of diseases including vascular wilt, stem rots, root and crown rots, head and seed blights, and canker. There are several wilt diseases caused by *Fusarium* species such as wilt of banana, wilt of melon and wilt of cucumber. Several *Fusarium* species are capable causing multiple diseases on the same plant depending on the host and environment (Brown and Proctor, 2013).

Stem and root rot are also common diseases caused by *Fusarium*, mainly *F. solani* and *F. oxysporum.* Among the crops, *F. solani* was the causal pathogen of root and fruit rot of *Cucurbita* spp., pumpkin and root and stem rot of pea (Horst, 2001). *Fusarium oxysporum* has been reported causing root rot of soybean, apple, sage (Horst, 2001) and tomato (Szczechura et al., 2013). Other than *F. oxysporum* and *F. solani, F. verticillioides* and *F. proliferatum* has also been associated with root rot as well as wilt of asparagus (Waskiewicz et al., 2013).

Other well known diseases associated with *Fusarium* is ear rot of corn caused by several *Fusarium* species including *F. verticillioides, F. proliferatum* and *F. subglutinans.* These species can also infect corn seedling and developing kernels (Dragich and Nelson, 2014). *Fusarium* species are responsible for many postharvest diseases such as tomato fruit rot which commonly caused by *F. solani* (Abu Bakar et al., 2013), pumpkin rot caused by *F. solani* (Wyenandt et al., 2010) and peppers and

eggplants rot which are frequently caused by *F. equiseti*, *F. verticillioides F. solani* and *F. oxysporum* (Barkai-Golan, 2001).

2.3.3 *Fusarium* as Animal and Human Pathogen

Fusarium species can cause various types of infection to humans including superficial infection such as keratitis and onychomycosis, locally invasive, or disseminated infections and may also cause allergic such as sinusitis. *Fusarium* that causes human mycoses usually exists as saprophyte in the environment (Short et al., 2011).

Approximately 15 species of *Fusarium* have been reported to be associated with human and animal diseases including *F. solani, F. oxysporum, F. verticillioides, F. proliferatum* and *F. anthophilum* (Jain et al., 2011). Zhang et al. (2006) indicated that *F. falciforme*, a phylogenetic species in the *F. solani* species complex (FSSC) was the most common species associated with fungal keratitis which is found in soil and plant debris, and act as the main source of infection.

Diseases caused by *Fusarium* in animal are parallel to diseases that infect human. *Fusarium solani* was reported causing immunocompetent diseases of mice and causing degradation of exoskeleton of shrimps and turtles (Brown and Proctor, 2013). Other *Fusarium* species such as *F. moniliforme, F. oxysporum* as well as *F. solani* have been reported causing black gill disease of kuruma prawn (*Penaeus japonicas*) in Japan (Hatai, 2012).

Table 2.1 summarises common *Fusarium* species associated with diseases on plants, humans and animals.

Table 2.1: Common *Fusarium* species associated with diseases on plants, humans and animals

Fusarium species	Type of Diseases
F. solani	- Crown and foot rot of cucurbits, sudden death syndrome in soybean, damping-off of seedlings and root rot of plants. - Fusarial infections on body and bloodstream, causing keratitis, endophtalmitis, onychomycosis, cutaneous and subcutaneous infections, arthritis and mycetoma and sinusitis
F. oxysporum	- Panama wilt (wilt of banana), wilt of cotton, sweet potato, tomato, palms, melon and cucumber, rotting of the bulbs (basal rot) and yellowing of the leaves of daffodils. - Infections in immunocompromised individual and, skin lesions
F. verticillioides	- Rotting of all corn plant parts, seedling decay rot, stalk rot, ear rot and kernel rot of corn and bakanae disease of rice
F. graminearum	- Root rot and seedling blight of wheat, barley, root rot and Gibberella ear rot in corn, head blight or 'scab' on rice and oats
F. sambucinum	- Ears of corn diseases, dry rot of potato and dry rot of stored tubers
F. semitectum	- Associate with canker on walnuts, pod and seed rot on beans and storage rot of mushroom
F. proliferatum	- Damping-off and rots of young corn, rot of garlic bulbs and malformation of mango - Soft tissue infection on finger and infections in immunocompromised individual
F. sporotrichioides	- Ear rot in corn, foliar spots on forage corn and head blight in wheat - Alimentary toxic aleukia and hemorrhagic syndrome
F. poae	- Ear blight of wheat, silvertop disease of grasses (white head or white ear) and diseases of small-grain cereals. - Alimentary toxic aleukia in human and hemorrhagic syndrome of animal

F. equiseti	- Damping-off on Aleppo pine, crown roots of wheat and dry rot and rhizomes rot of ginger
F. avenaceum	- Seedling blight, brown food rot and ear blight, attacks all cereals and damping-off, wilt of *Fagus sylvatica* and several legumes and carnations. - Burn spot disease syndrome in noble crayfish
F. dimerum	- Diseases of potato plant - Keratomycoses stem cell infection of human, eye infections of human and vesicles in disseminated disease.
F. subglutinans	- Short and leaf blight, shoot wilting and seedling stunting of coleoptiles and nod of corn, damping-off in *Pinus merkusii* seedlings, late-season mortality in longleaf pine nurseries and associated with stalk rot and cob rot of corn - Mycetoma diseases in human
F. culmorum	- Foot rot, root rot and head blight on wheat, barley, oats, rye, corn, sorghum
F. acuminatum	- Associated with roots and crowns diseases of a variety of hosts such as soybean and sunflowers
F. andiyazi	- Stalk rot in sorghum millets and rice - Fatal breakthrough infection in immunocompromised patients and causes keratitis
F. chlamydosporum	- Damping-off on Aleppo Pine - Human mycoses, particularly in immunocompromised patients and invasive disease
F. circinatum	- Damping-off of seedlings, canker and dieback of pine
F. crookwellense	- Tip blight on cones of hop, head blight disease of small grains and maize, mild root rot and foot rot of wheat and associated with seedling death
F. decemcellulare	- Canker on various l fruit trees, such as., avocado, cacao, guarana and mango, wilt and vascular and flower necrosis of rambutan and dieback of mango
F. fujikuroi	- Bakanae or foolish seedling disease of rice, crown and root of rice seedlings, ear and root rot of corn
F. longipes	- Diseases of guava, crown rot of wheat

F. mangiferae	- Vegetative and inflorescence malformation on mango
F. pseudograminearum	- Crown rot on cereals including wheat, barley, rye and triticale and grassy weeds
F. sacchari	- Pokkah boeng of sorghum, wilt of sugar cane and can reduce the quality of harvested crop

2.3.4 History of *Fusarium* Classification System

The genus *Fusarium* was first reported by Link in 1809, which was identified based on banana-shaped conidia (Leslie and Summerell, 2006). Intensive studies of *Fusarium* taxonomy and classification system began with Wollenweber and Reinking (1935) of which they developed simplified classification system of *Fusarium* and 1000 species was identified. However, Wollenweber and Reinking (1935) developed the identification system based on differences of the morphological features which was complex and difficult to use.

Another comprehensive *Fusarium* taxonomy and classification was done by Snyder and Hansen (1954) in which they used cultures derived from single spore for species identifications, and focused on the morphological similarities. In their classification system, only nine species was recognized. Snyder and Hansen (1954) descriptions of *F. solani* and *F. oxysporum* are still used until today.

Booth (1971) introduced an identification system of *Fusarium* based on the characteristics of macroconidia and the conidiogenous cells. In Booth (1971) identification system, the information of sexual state and keys for species identification were also included.

Gerlach and Nirenberg (1982) adapted the classification and identification system by Wollenweber and Reinking (1935), and as a result they described 78 species. The species were arranged in sections based on the differences of morphological features.

A combination of several classification systems of *Fusarium* was the main criteria used by Nelson et al. (1983) in their identification system. Nelson et al. (1983) reduced the number of species to 30 species by combining the varieties and forms into appropriate species. For identification of species, Nelson et al. (1983) observed the microscopic features of macroconidia, microconidia, conidiophores and chlamydospores.

A laboratory manual for identification of *Fusarium* was published by Leslie and Summerell (2006) which consist of a compilation of species descriptions by several *Fusarium* taxonomists. The manual include detailed methods and descriptions on the media used for identification, isolation method as well as molecular identification method and mating studies.

2.4 *Fusarium* Species Identification

2.4.1 Morphological Identification

Morphological identification is widely used for identification of *Fusarium* species and it is based on similarity and differences of morphological characters. Early taxonomic work on *Fusarium* was based on morphological characteristics involving both primary characteristics of macroconidia, microconidia and

chlamydospores, and secondary characteristics including growth rate and pigmentation (Nelson et al., 1983; Leslie and Summerell, 2006).

Shape of macroconidia is an important feature to distinguish *Fusarium* species. The shapes of macroconidia can be slender, falcate, straight, fusiform or needle like and are assessed according to their size, septa and the shape of the basal and apical cells. Apical cell shape can be blunt, papillate, hooked or tapering, while basal cell can be foot-shaped, elongated foot-shaped or distinctly-elongated (Nelson et al., 1983; Leslie and Summerell, 2006). Based on the macroconidia shapes, several species of *Fusarium* can be distinguished such as *F. solani, F. equiseti* and *F. decemcellulare.*

Microconidia are smaller than macroconidia, and the shapes vary from oval, two or three-cell, oval, obovoid with a flattened base, reniform, pyriform or globose, commonly, have 0–3 septa (Nelson et al., 1983; Leslie and Summerell, 2006). *Fusarium* species produce microconidia on aerial mycelia which can be in chain or false heads. Microconidia in chain either long or short are produced by *Fusarium* member of FFSC. The microconidia are the most distinguishing feature of *F. anthophilum* which is globose or pyriform (Leslie and Summerell, 2006).

A phialide is aerial mycelium that consists of conidiogenous cells that produce meso and/or microconidia. A monophialide can either be branched or unbranched (Nelson et al., 1983). *Fusarium* species produce two types of phialides, i.e., monophialides and polyphialides. The types of phialides are mainly used to sort the isolates into groups although some species can be distinguish based on the

formation of the polyphialides such as to differentiate between *F. solani* and *F. oxysporum* while cross-shaped polyphialides are commonly used to identify *F. nelsonii*.

Chlamydospores are survival structures with thick-walled and rough structures. Chlamydospores can exist as single, in pairs, in short chains on somatic hyphae, and sometimes at the terminal (Nelson et al., 1983). Some *Fusarium* species produce chlamydospores such as *F. solani* and *F. oxysporum*. *Fusarium* species in FFSC including *F. verticillioides* and *F. proliferatum* do not produce chlamydospores.

Secondary characteristics of *Fusarium* species are also used to help in the identification process. The characteristics include growth rate, colony appearance and pigmentation observed on PDA. The growth rate varies among *Fusarium* species, from fast to very slow-growing, for example, *F. venenatum* and *F. incarnatum* are fast growers, whereas *F. merismoides* and *F. dimerium* are slow growers. Colony appearance and pigmentation also vary among *Fusarium* species. These characteristics are used to sort the isolates into groups or sections / species complex. Commonly dark purple pigmentations are produced by *Fusarium* species in FFSC and red pigmentations produced by *F. torulosum.*

2.4.2 Molecular Identification

Molecular identification is widely used in fungal systematics as the method can be applied to study the variation among fungal isolates, as well as provide

information on the genetic relationships, taxonomy and population structure of many groups of fungi (Cooley, 1992).

For *Fusarium* species, molecular identification is usually applied to identify *Fusarium* isolates that have similar morphological characteristics. For example, many species in a species complex such as FFSC and FSSC produce similar macroconidial features and colony appearance. Therefore, molecular identification is used to differentiate species in a species complex.

A few PCR based techniques have been used in identification, characterization and to observe genetic variation of *Fusarium* species. Among the techniques Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) have been used as tools to characterize and to study genetic relationships of *Fusarium* species. RAPD analysis has used to distinguish mating population of *Fusarium* isolates causing bakanae disease of rice (Voigt et al., 1995), to determine genetic variation of *F. oxysporum* f.sp. *psidii* from guava (Gupta, 2012) and to characterize *F. oxysporum* f.sp. *citri* causing wilt of citrus (Hannachi et al., 2015). Similar with RAPD, AFLP has been applied to differentiate *Fusarium* species associated with an endemic wild rice (*Oryza australiensis*) (Petrovic et al., 2013), to characterize *F. oxysporum* and *F. pseudocircinatum* causing dieback of koa (*Acacia koa*) (Shiraishi et al., 2012) and to distinguish high virulent isolates and less virulent isolates of *F. oxysporum* f. sp. *momordicae* causing wilt of bitter gourd (*Momordica charantia* L.) (Chen et al., 2015).

Nowadays, DNA sequencing of a particular gene or region is widely used for fungal identification as well as to determine genetic variation and phylogenetic analysis. DNA sequence data is now routinely used for identification of *Fusarium* as DNA sequence data can provide rapid and reliable species identity. By conducting phylogenetic analysis, DNA sequence can be used to distinguish between *Fusarium* species that show similar morphological characteristics as well as to distinguish isolates in a species complex. For molecular identification and phylogenetic analysis of *Fusarium* species, TEF1-α, β–tubulin genes and ITS region are commonly used as the genes and region are highly informative and recommended by many researchers (O'Donnell, 2000; Leslie et al., 2003; Geiser et al., 2004; Nalim et al., 2011; Castaño et al., 2014). For *Fusarium* species, in addition to GenBank a database known as Fusarium-ID is used for BLAST search (Geiser, et al., 2004).

2.4.2 (a) Translation Elongation Factor TEF1-α Gene

Translation elongation factor 1-α gene encodes an essential part of the protein translation mechanism, is the marker of choice for identification of *Fusarium* species. The gene appears to occur as non-orthologous copies in *Fusarium* and is highly informative, and shows a high level of sequence polymorphism among closely related species (Geiser et al., 2004). Plate 2.1 shows a part of TEF1-α gene with location of introns and exons and position of the primers used to amplify the gene.

Many studies have used TEF-1α for identification and characterization of *Fusarium* species. By using TEF-1α sequence, saprophytic, endophytic and pathogenic *Fusarium* species were grouped separately and formed distinct clades (Barik and Tayung, 2012). Latiffah et al. (2013) used TEF-1α sequences to

characterize *F. semitectum* isolates from fruit rot of vegetable fruits from different hosts. The results showed that the isolates were highly variable and the grouping of the isolates were not according to the host. By using TEF-1α sequences, 65 *Fusarium* isolates from sugar beet were identified as *F. avenaceum, F. cerealis, F. culmorum, F. equiseti, F. graminearum, F. oxysporum, F. proliferatum, F. redolens, F. solani, F. tricinctum,* and *F. venenatum* (Nitschke et al., 2009).

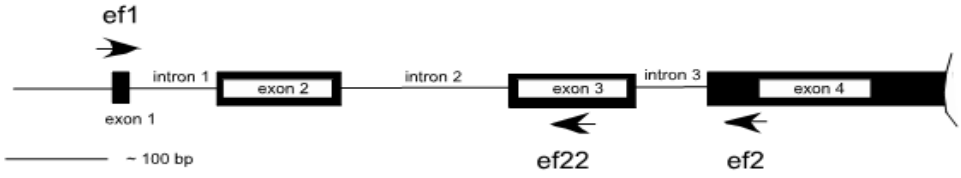

Plate 2.1: Map of TEF-1α primer positions. Positions of forward (right-pointing arrow) and reverse (left-pointing arrow) primers (Geiser et al., 2004).

2.4.2 (b) β –tubulin Gene

Tubulin family include three members which are α, β and γ tubulins and show homology in the fungal genomes (Dutcher, 2001). The β-tubulin encoding gene is among the most prominent diagnostic genes for *Fusarium* species detection (Esser et al., 2007). Plate 2.2 shows part of the β-tubulin gene and the position of several primers used to amplify the gene.

β-tubulin is commonly used for phylogenetic analysis of *Fusarium* species. Schroes et al. (2009) used β-tubulin to conduct phylogenetic analysis of *F. dimerum*

and based on combined sequences of β-tubulin, ITS, LSU rDNA and TEF-1α. The *F. dimerum* isolates were formed groups according to macroconidial features. Phylogenetic analysis of *F. oxysporum* f. sp. *vasinfectum* isolated from cotton in Uzbekistan using β-tubulin showed that the isolates of the formae speciales can be classified into three races (Egamberdiev et al., 2013).

Plate 2.2: Map of β-tubulin location and primers. Positions of forward (right-pointing arrow) and reverse (left-pointing arrow) primers (Glass and Donaldson,1995).

2.4.2 (c) ITS Region

The ribosomal DNA repeat unit contains genic and non-genic or spacer regions. Each repeat unit consists of a copy of 18S-5.8S and 28S-like rDNA and two spacers. Several primers were designed to amplify one repeat unit of ITS (Plate 2.3). The spacers regions namely ITS1 and ITS2 are more variable, which makes the ITS region useful in systematics and taxonomic studies of *Fusarium* at species-level (Park and Min, 2005). However, ITS region has limited uses for several *Fusarium* species such as species of FFSC because ITS sequences are not informative for these species (Leslie and Summerell, 2006) as well as it cannot resolve the closely related members of FFSC (Kebabc et al., 2014) due to exist area

of paralogous or xenologous segments in ITS2 sequences (O'Donnell and Cigelnik, 1997).

Internal transcribed spacer region has been used by Oechsler et al. (2009) to differentiate *Fusarium* isolates from ocular sources of 52 patients diagnosed with *Fusarium* keratitis. Sequence analysis of ITS region differentiate the isolates into *F. solani*, *F. oxysporum*, *F. incarnatum-equiseti*, *F. dimerum*, and *Fusarium* sp. Wilson et al. (2004) conducted phylogenetic analysis using ITS to differentiate *F. sporotrichioides* and *F. langsethiae* from grain samples. The phylogenetic analysis differentiated the two species into two groups as well as showed genetic evidence for their close genetic relationship.

Plate 2.3: Map of nuclear ribosomal RNA genes and their ITS regions. Positions of forward (right-pointing arrow) and reverse (left-pointing arrow) primers (Toju et al., 2012).

2.4.2(d) Phylogenetic Analysis

Phylogenetic analysis is used to study the evolutionary development between and among a group of organisms, to study the relationship from the ancestors to the descendents as well as used in taxonomic and systematic studies based on DNA sequence data. There are two major phylogenetic tree-building methods; phenetic methods which is based on distances and represented by neighbour joining method