CHARACTERISATION OF *Fusarium oxysporum* ISOLATED FROM VARIOUS PLANTS AND NON-AGRICULTURAL SOILS IN MALAYSIA

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
µl	Microlitre
°C	Degree celcius
®	Registered identity assigned to a product
AFLP	Amplified Fragment Length Polymorphism
AIC	Akaike Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CLA	Carnation leaf-piece agar
cm	Centimetre
cm^3	Cubic centimetre
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DS	Disease severity
FOC	*Fusarium oxysporum* f. sp. *cubense*
FOSC	*Fusarium oxysporum* species complex
f. sp.	Formae speciales
g	Gram
h	Hour
IGS	Intergenic spacer
ITS	Internal transcribed spacer
kb	Kilobase
kg	Kilogram
kg/cm^2	Kilogram per centimetre square
L	Litre
mA	Milliampere
MEGA	Molecular Evolutionary Genetic Analysis
$MgCI_2$	Magnesium chloride
min	Minutes

ml	Millilitre
ML	Maximum Likelihood
mm	Millimetre
mtSSU	Mitochondrial small subunit
NaOCl	Sodium hypochlorite
NCBI	National Center for Biotechnology Information
NDM	Non-dermatophyte molds
ng	Nanogram
NJ	Neighbor-joining
NNI	Nearest-Neighbor-Interchange
NRRL	Northern Regional Research Laboratory
PPA	Peptone Pentacloronitrobenzene Agar
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Potential hydrogen
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomised complete block design
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
s	Second
SMC	Simple matching coefficient
sp.	Species
TBE	Tris-Borate-EDTA
TEF1-α	Translation elongation factor 1-alpha
™	Trademark
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetical Averages
UV	Ultraviolet light
V	Volt
var	Variety
WA	Water agar

PENCIRIAN *Fusarium oxysporum* YANG DIPENCILKAN DARIPADA PELBAGAI TANAMAN DAN TANAH BUKAN PERTANIAN DI MALAYSIA

ABSTRAK

Fusarium oxysporum ialah kulat kosmopolitan, terdiri daripada ahli patogen dan bukan patogen dan terkenal sebagai agen penyebab bagi beberapa penyakit termasuk penyakit layu dan reput pada pelbagai tanaman. Di Malaysia, kebanyakan kajian memberi tumpuan kepada pencilan *F. oxysporum* yang patogenik kerana implikasinya terhadap pengeluaran pertanian tetapi kurang perhatian diberikan terhadap pencilan yang tidak patogenik. Kajian ini cuba untuk menghuraikan isu berkenaan evolusi kepatogenan, kekhususan perumah dan kewujudan spesies krip dalam *F. oxysporum* dari populasi setempat. Objektif kajian ini adalah untuk memencil, mengenal pasti dan mencirikan pencilan *F. oxysporum* daripada pelbagai tanaman dan tanah bukan pertanian di Malaysia menggunakan ciri morfologi dan molekul, kepatogenan dan julat perumah serta analisis penjarak intergen-polimorfisme kepanjangan serpihan pembatasan (IGS-RFLP). Sejumlah 133 pencilan *Fusarium* sp. telah diperoleh daripada pelbagai tanaman berpenyakit (*Abelmoschus esculentus*, *Solanum melongena*, *Solanum tuberosum*, *Cucumis sativus*, *Solanum lycopersicum*, *Cucumis melo*, *Musa paradisiaca* var. *awak*, *Hymenocallis littoralis*, *Asparagus officinalis*, *Sansevieria trifasciata* dan *Hylocereus polyrhizus*) dan tanah bukan pertanian dari 12 negeri (Johor, Kedah, Kelantan, Melaka, Negeri Sembilan, Pahang, Pulau Pinang, Perak, Sabah, Sarawak, Selangor dan Terengganu) di Malaysia. Berdasarkan ciri morfologi, pencilan *Fusarium* tersebut telah dikenalpasti secara tentatif sebagai *F. oxysporum*. Perbandingan jujukan DNA penterjemahan pemanjangan faktor 1-alfa (TEF1-α) dan subunit kecil mitokondria (mtSSU)

menunjukkan pencilan tersebut adalah 98-100% sama dengan *F. oxysporum* dari GenBank, dengan itu mengesahkan identiti kulat tersebut. Pohon filogenetik kebolehjadian maksimum (ML) dan penyambungan bersebelahan (NJ) daripada set data gabungan TEF1-α dan mtSSU disimpulkan bahawa pencilan tersebut dikelompokkan mengikut perumah masing-masing kecuali untuk pencilan daripada *H. polyrhizus*, *S. trifasciata* dan tanah (Johor, Kelantan, Melaka, Pulau Pinang dan Sarawak). Keputusan ujian kepatogenan menunjukkan kesemua pencilan yang telah diuji adalah patogenik terhadap perumah masing-masing dengan mempamerkan simptom reput, layu dan bintik daun dan memcatatkan min keparahan penyakit (DS) yang berbeza. Kesemua pencilan yang diuji mempunyai julat perumah yang luas dengan min DS yang berbeza. Pencilan tersebut dikategorikan sebagai sangat virulen terhadap perumah asal tetapi menunjukkan kevirulenan yang sederhana hingga rendah terhadap perumah lain yang diuji. Analisis IGS-RFLP menggunakan enzim pembatasan AluI, BsuRI, HhaI, MspI dan RsaI telah menghasilkan sebanyak enam corak pembatasan (A-F). Tujuh belas haplotip IGS telah diperuntukkan untuk 133 pencilan *F. oxysporum,* menunjukkan variasi intraspesies antara pencilan. Analisis gugusan UPGMA menunjukkan majoriti pencilan *F. oxysporum* dikelompokkan mengikut keutamaan perumah dan lokasi geografi. Kesimpulannya, 133 pencilan *F. oxysporum* yang dipencilkan daripada pelbagai tanaman dan tanah bukan pertanian di Malaysia telah dikenalpasti menggunakan ciri morfologi dan molekul; patogenik dengan julat perumah yang luas dan pelbagai dari segi genetik dengan menunjukkan variasi intraspesies. Penemuan dalam kajian ini akan memberi manfaat kepada tujuan kuarantin, pemantauan dan pengurusan penyakit.

CHARACTERISATION OF *Fusarium oxysporum* ISOLATED FROM VARIOUS PLANTS AND NON-AGRICULTURAL SOILS IN MALAYSIA

ABSTRACT

Fusarium oxysporum is a cosmopolitan fungus, consists of both pathogenic and non-pathogenic members and well-known as causal agent of several diseases including wilt and rot on various plants. In Malaysia, most studies are focusing on pathogenic isolates of *F. oxysporum* due to their implications on agricultural production, but less attention was given towards the non-pathogenic isolates. This study attempted to delineate issues of pathogenicity evolution, host specificity and the existence of cryptic species within *F. oxysporum* from local population. The objectives of the present study were to isolate, identify and characterise isolates of *F. oxysporum* from various plants and non-agricultural soils in Malaysia using morphological and molecular characteristics, pathogenicity and host range as well as intergenic spacer-restriction fragment length polymorphisms (IGS-RFLP) analysis. A total of 133 isolates of *Fusarium* sp. were recovered from various diseased plants (*Abelmoschus esculentus*, *Solanum melongena*, *Solanum tuberosum*, *Cucumis sativus*, *Solanum lycopersicum*, *Cucumis melo*, *Musa paradisiaca* var. *awak*, *Hymenocallis littoralis*, *Asparagus officinalis*, *Sansevieria trifasciata* and *Hylocereus polyrhizus*) and non-agricultural soils from 12 states (Johor, Kedah, Kelantan, Melaka, Negeri Sembilan, Pahang, Penang, Perak, Sabah, Sarawak, Selangor and Terengganu) in Malaysia. Based on morphological characteristics, the *Fusarium* isolates were tentatively identified as *F. oxysporum*. Comparison of DNA sequences of translation elongation factor 1-alpha (TEF1-α) and mitochondrial small subunit (mtSSU) showed that the isolates were 98-100% similar to *F. oxysporum* from GenBank, thus, confirming the

fungal identity. Phylogenetic trees of maximum likelihood (ML) and neighbor joining (NJ) of combined dataset of TEF1-α and mtSSU inferred that the isolates were clustered according to their respective hosts except for isolates from *H. polyrhizus*, *S. trifasciata* and soils (Johor, Kelantan, Melaka, Penang and Sarawak). The results of pathogenicity test indicated that all the tested isolates were pathogenic toward their respective hosts by exhibiting rot, wilt and leaf spot symptoms and recorded varied means disease severity (DS). All the tested isolates had wide host range with varied means DS. They were categorised as highly virulent toward their original hosts but demonstrated moderate to low virulence toward other tested hosts. The IGS-RFLP analysis using AluI, BsuRI, HhaI, MspI and RsaI resulted a total of six restriction patterns (A-F). Seventeen IGS haplotypes were assigned for 133 isolates of *F. oxysporum*, showing intraspecific variation among the isolates. The UPGMA cluster analysis showed that majority isolates of *F. oxysporum* were grouped according to host and geographical location preferences. As a conclusion, 133 isolates of *F. oxysporum* isolated from various plants and non-agricultural soils in Malaysia were identified using morphological and molecular characteristics; pathogenic with wide host range and genetically diverse by showing intraspecific variation. Findings in the present study will be beneficial for purposes of quarantine, disease monitoring and management.

CHAPTER 1

INTRODUCTION

The *Fusarium* (teleomorphs: *Gibberella* and *Nectria*) is a well-known fungal genus causing several economically important diseases on plants (Desjardins, 2003; Di Pietro et al., 2003). One of the notable species is *Fusarium oxysporum* which is cosmopolitan, anamorphic species that comprises both pathogenic and non-pathogenic isolates. The species has drawn much attention and well-studied because of its implications toward plants and humans. Pathogenic isolates of *F. oxysporum* causing destructive diseases on a wide host range such as rot and wilt and being considered among the world's most vital soilborne phytopathogens (Leslie and Summerell, 2006). In Malaysia, the *F. oxysporum* infects a number of plants such as banana, maize, oil palm, roselle, pineapple, tomato, cucumber and okra (Dita et al., 2010; Izzati et al., 2011; Bakar et al., 2013; Hafizi et al., 2013; Nurul Huda and Latiffah, 2014; Ibrahim et al., 2015). The specificity of the *F. oxysporum* in infecting plants, makes it categorised into formae speciales (f. sp.) (Baayen et al., 2000; O'Donnell et al., 2009) but some are not (Zhou and Everts, 2007; Webb et al., 2013). To date, it had more than 150 formae speciales and races based on plant species and cultivars that it can infect (Bertoldo et al., 2015; Rana et al., 2017).

Identification of *F. oxysporum* is primarily depends on its anamorph as its teleomorphic stage is unknown. Morphological identification based on macroscopic and microscopic characteristics is widely used to characterise *F. oxysporum*. The criteria include of size and shape of macroconidia and microconidia, the presence or absence of chlamydospores, colony appearance and conidiophore structure (Leslie and Summerell, 2006). However, the existence of colony variations and overlapping

morphological characteristics leads to difficulty in *Fusarium* identification (Gerlach and Nirenberg, 1982; Nelson et al., 1983). Hence, to overcome the limitation of classical taxonomic method in identifying *Fusarium* species, molecular tools are basically applied to assist taxonomical studies.

DNA sequences of translation elongation factor 1-alpha (TEF1-α) and mitochondrial small subunit (mtSSU) ribosomal DNA are two common genes that have been proven their effectiveness in distinguishing species within *Fusarium* especially for *F. oxysporum* (Baayen et al., 2000; Skovgaard et al., 2001). The TEF1-α gene consists of informative sequences and non-orthologous copies which can delimit isolates of *Fusarium* until species level (Geiser et al., 2004). Moreover, mtSSU gene has been widely used as an alternative to accurately delineate the identity of the *Fusarium* species (Bruns and Szaro, 1992). Phylogenetic analysis based on multigene is evidenced to assist in determining relationships among the isolates as well as to resolve the fungal identity (Fravel et al., 2003; Raja et al., 2017). From this approach, O'Donnell et al. (1998) revealed that isolates of *F. oxysporum* within a forma specialis are not necessarily monophyletic.

Although molecular tools can be used to identify pathogenic isolates of *F. oxysporum*, determining its pathogenicity still relies largely on bioassays or pathogenicity tests. Pathogenicity is referring to the potential capacity to cause disease on host plants while virulence is the degree of pathology caused by the fungal isolates which related to the ability of the pathogen to colonise and multiply within the host (Agrios, 2005; Casadevall, 2007). Different pathogenic isolates of *F. oxysporum* can display different levels of virulence toward their host plant which mainly influenced by environmental conditions such as temperature and humidity (Pasanen et al., 1991).

The growth of fungal hyphae and conidia production contributed to the fungal virulence. Deciphering pathogenesis of *F. oxysporum* not only allowing a better understanding on how the fungus attacks host plants, it also provides additional information to control plant diseases with new strategy to prevent, delay or restrict fungal development.

Some fungal pathogens able to infect a specific host, but some able to infect several hosts. In order to determine host specificity of a fungal pathogen, host range test needs to be conducted. Host specificity within formae speciales of *F. oxysporum* have been studied extensively (Lievens et al., 2008). Knowledge of pathogen host range will help in managing plant disease including use of resistant varieties to crop rotation, elimination of reservoirs, landscape planning, surveillance, quarantine, risk modeling and anticipation of disease emergences (Morris and Moury, 2019).

Besides, understanding the genetic diversity of *F. oxysporum* is essential to formulate effective disease control methods and important in the selection or breeding of resistant cultivars. Molecular markers have been used to identify systematic relationships and diversity between pathogens and diseases. Of the various technology that has been developed, restriction fragment length polymorphisms (RFLP) analysis of mitochondrial or nuclear DNA has been intensively used to resolve the genetic diversity within and between the formae speciales and among non-pathogenic isolates of *F. oxysporum* (Kachuei et al., 2015). A combination of polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) is commonly used to resolve genetic variation among the isolates (Mirete et al., 2003). There are several regions of ribosomal DNA (rDNA) that can be used in PCR-RFLP analysis such as 18S, 5.8S, 28S, ITS and intergenic spacer region (IGS). The region that regularly used in PCR-

RFLP analysis is IGS as it allows discrimination of closely related isolates and showed higher variability at intraspecific level, lack of selective constrains and suitable to estimate the genetic relationships among the isolates of *F. oxysporum* (Llorens et al., 2006).

In Malaysia, most studies on *F. oxysporum* have been focused on pathogenic isolates due to their impact on agricultural crop production. Although non-pathogenic isolates of *F. oxysporum* are widespread and genetically more diverse than pathogenic isolates, they are not well studied. Therefore, this study attempted to address several issues of *F. oxysporum* such as: the occurrence of pathogenicity evolution among the pathogenic and non-pathogenic isolates of *F. oxysporum* (Skovgaard et al., 2002); the occurrence of polyphyletic origin of host specificity in many formae speciales of *F. oxysporum* (van Dam et al., 2018); the discoveries of two new species namely *F. commune* (Skovgaard et al., 2003) and *F. foeten* (Schroers et al., 2004) within *F. oxysporum* species complex (FOSC) showed this species complex comprises multiple morphologically cryptic but genetically different species. To address the highlighted issues in local population, a study on pathogenicity and host range, phylogenetic relationships and genetic diversity of *F. oxysporum* isolates is highly importance. Therefore, objectives of the present study were:

1) to isolate, identify and characterise *F. oxysporum* from various plants and non-agricultural soils in Malaysia using morphological and molecular characterisation
2) to determine pathogenicity of *F. oxysporum* isolates towards their respective hosts and to evaluate host range towards the other tested hosts
3) to assess genetic diversity and intraspecific variation of *F. oxysporum* isolates using IGS-RFLP analysis

CHAPTER 2

LITERATURE REVIEW

2.1 Agricultural in Malaysia

Agriculture is defined as the production of food and goods through farming. Agricultural production includes food (cereals, vegetables, fruits and meat), feed (grains and fodder for the production of food for livestock), fiber (cotton, flax, hemp, silk and wool), furniture (rubber and rattan are grown for the wood by-products), ornamentals (cutflowers, cutfoliage, nursery plants and edge crops), flowers (grown for celebration, commemoration and felicity) and biofuel (methane from biomass, ethanol and biodiesel) for the nation (Campbell et al., 2014; Harris and Fuller, 2014; Velten et al., 2015). Agriculture plays an importance role in economic development of most countries including Malaysia (Saari et al., 2015).

Malaysia is a Southeast Asian country which located in the tropics consists of two regions, the Peninsular Malaysia and East Malaysia (Sabah and Sarawak). The area of the country has a total of 330,803 km^2 in which Peninsular with 138,000 km^2 and East Malaysia with 192,803 km^2 (Ab Rahman et al., 2013). Malaysia has an equatorial climate which experiences hot and humid weathers throughout the year. Malaysia has a total of 32.98 million hectares (ha) of lands with only 31% is arable land (Olaniyi et al., 2013). Agriculture is one of the main land uses in Malaysia with a total of 10.31 million ha of land, of which 6.19 million, 1.81 million and 2.31 million ha are estimated to be suitable for agriculture in Peninsular Malaysia, Sarawak and Sabah, respectively (Olaniyi et al., 2013). In Malaysia, oil palm, rice, rubber and cocoa are the major crops grown by public and private sectors.

In agricultural ecosystem, plants can be divided into crops and weeds (Norris, 2005; Reimer et al., 2019). A crop is a beneficial plant which is grown for certain purposes. These plants are purposely grown for numerous usages such as for food, ornamentation, fiber, organic farming, soil improvement, landscaping, medicines and many more. Contrary, a weed can be considered as undesirable plant which grows ubiquitously. Agricultural crops can be divided into two main categories namely agronomic and horticultural crops (Menges et al., 1985; Roberson, 2000). Agronomic crops or known as field crops are commonly grown in large-scale which mostly involved of herbaceous plants. The examples of agronomic crops are seed legumes, cereals, sugar crops, root and tuber crops, pasture and forage crops, latex and rubber and fiber crops (Blair et al., 2016; Liang et al., 2017).

According to Warrington and Janick (2014), horticultural crops are referred as garden crops. There are several crops have been classified as horticulture crops such as fruits (banana, mango, dragon fruit and pineapple), vegetables (crucifers, cucurbits, legume vegetables, lilies and solanaceous crop) and ornamentals (Indian tree, orchids and ferns) as well as spices (black pepper, garlic and ginger) and medicinal plants (*Melicope ptelefolia*, tenggek burung; *Portulaca oleracea*, helang pasir; *Curcuma aeruginosa*, temu hitam; and *Annona muricate*, durian belanda).

2.2 Non-agricultural soils

Non-agricultural soil can be defined as a soil type which does not use for any agricultural activities or development and thus, no agricultural products are yielded (Melišková, 2018). Soil is a mixture of liquid, minerals, organic matter and gasses. Besides that, some microorganisms used soil as their habitat as well as for their growth, multiplication, survival and dissemination (Chuankun et al., 2004). Soils also serve as

media for growth of all types of plants and as a water holding reservoir for moisture. Several environmental factors such as biological activities, biomass carbon and nitrogen, climate, organic matter content, season, soil moisture, tillage systems and the physicochemical properties of soil may significantly affect the diversity of microorganism's community in the soil environment (Liang et al., 2011).

Like other microorganisms, fungi can be found mostly in every environment especially in soil. They have high capacity and plasticity to adapt with different forms in response to unfavourable conditions (Sun et al., 2005). The activity and diversity of fungi in soils are controlled by various abiotic (moisture, salinity, soil pH, structure and temperature) and biotic (plants and other organisms) factors (López-Bucio et al., 2015; Rouphael et al., 2015). For example, *Fusarium* species can survive in plant debris, live close to the soil surface and more interestingly, some species consist of a resistant structure known as chlamydospore that enable them to survive longer in the soil (Nagao et al., 1990; Leslie and Summerell, 2006).

2.3 *Fusarium oxysporum* species complex (FOSC)

Fusarium oxysporum is a widely distributed fungus which primarily can be found in the soils (Leslie and Summerell, 2006). This species is described as a species complex which consists of multiple morphologically cryptic species with high genetic diversity among the isolates (Ellis et al., 2014). Its complexity can be delineated with multiple phylogenetic origins with majority of their formae speciales are polyphyletic (O'Donnell et al., 1998; Baayen et al., 2000; Skovgaard et al., 2001). The polyphyletic origin of *F. oxysporum* was first observed in isolates of *F. oxysporum* f. sp. *cubense* that formed three separated clades that potentially represented several morphologically cryptic species (O'Donnell et al., 1998). The discovery of polyphyletic origins of *F.*

oxysporum f. sp. *cubense* revealed that isolates within this forma specialis are more closely related to isolates in other formae speciales compared to among themselves (Groenewald et al., 2006; Fourie et al., 2009).

Fusarium oxysporum species complex (FOSC) formed a sister group to the *F. fujikuroi* species complex (FFSC) that harbours sexual species with *Gibberella* teleomorphs, such as *F. verticillioides* and *F. fujikuroi* (Skovgaard et al., 2002). Two new *Fusarium* species namely *F. commune* (Skovgaard et al., 2003) and *F. foetens* (Schroers et al., 2004) are sister taxon of the FOSC. Recognising species boundaries in the FOSC represents a challenge because of the lack of taxonomic characters, the diverse biology of the component isolates, its broad distribution and the anthropogenic influence on its evolutionary dynamics through agricultural production.

Like other fungi, *F. oxysporum* has the ability to adapt in response to any changes or new environments due to exerts selection pressure (McDonald, 1997). The dynamics of the evolution of fungi are determined by five evolutionary forces which are mutation, natural selection, genetic drift, gene flow and mating or reproduction systems (McDonald and Linde, 2002).

2.3.1 Taxonomical classification

The taxonomical classification of the genus *Fusarium* is still evolving and becoming complicated over the years after numerous studies have been carried out. Based on National Center for Biotechnology Information (NCBI), *F. oxysporum* can be classified as:

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Subclass: Hypocreomycetidae

Order: Hypocreales

Genus: *Fusarium*

Species: *Fusarium oxysporum*

2.3.2 Formae speciales and races

The pathogenic isolates of *F. oxysporum* is commonly related to high level of host specificity and they were assigned into formae speciales according to which host plants that they can infect (Baayen et al., 2000). Some of the formae speciales are further subdivided into races based on pathogenicity to a set of differential cultivars within the same plant species.

According to Michielse and Rep (2009), formae speciales are used to characterise intraspecific relationship. Each forma specialis of *F. oxysporum* consists of one or several clonal lineages (Arie, 2010; Nirmaladevi et al., 2016). Isolates of *F. oxysporum* which attack the same crop are considered belong to the same forma specialis. For example, isolates of *F. oxysporum* that are pathogenic toward bananas and plantains are called *F. oxysporum* f. sp. *cubense* (FOC) (Ploetz, 2006).

Mostly pathogenic isolates of *F. oxysporum* attack or cause disease to only a single crop such as *F. oxysporum* f. sp. *dianthi* infects carnations and *F. oxysporum* f. sp. *vasinfectum* infects cotton. However, several studies showed that pathogenic isolates of *F. oxysporum* from one forma specialis also can cause disease on other hosts or formae speciales (Cafri et al., 2005; Webb et al., 2012; López-Orona et al., 2019). Roncero et al. (2003) stated that formae speciales are differ in epidemiology, symptomology and cultivar susceptibility. The genetic basis of host specificity (forma specialis) and cultivar specificity (pathogenic race) of *F. oxysporum* is unknown (Baayen et al., 2000). These pathogenic fungi are morphologically indistinguishable from each other, as well as from non-pathogenic members of *F. oxysporum*.

Races in *F. oxysporum* are recognised by their pathogenicity to distinct set of cultivars. Although most formae speciales of *F. oxysporum* are grouped into different races, some exceptions occur where different races have not yet been reported such as within *F. oxysporum* f. sp. *radicis-lycopersici* (Primo et al., 2001).

In FOC, there are four identified races namely race 1 individuals attack Gros Michel, 'Silk' (AAB), 'Pome' (AAB), 'Pisang Awak' (ABB), 'Maqueno' (AAB) and Latundan cultivars; race 2 attacks 'Bluggoe' and other plantains; race 3 attacks *Heliconia* species (race 3 does not cause disease to banana and therefore not considered part of the FOC race structure anymore); race 4 is pathogenic to Cavendish bananas and all cultivars susceptible to races 1 and 2 (Ploetz, 2006; Buddenhagen, 2007).

2.3.3 Role as saprophyte and endophyte

Fusarium oxysporum associated with plants can exist as a saprophyte which feeds on dead or decaying organic matters or colonises diseased roots and stems of the plants (Leslie and Summerell, 2006). Plant debris in soils play a significant role as nutrient reservoir for *F. oxysporum* to survive in soils as a saprotroph (Fravel et al., 2003). This fungus also able to survive for long periods on organic matter in soil and in the rhizosphere of many plant species (Trouvelot et al., 2002; Leslie and Summerell, 2006). As a saprophyte, *F. oxysporum* has the ability to degrade lignin and complex carbohydrates associated with soil organic materials (Promputtha et al., 2010; Karim et al., 2016). This *Fusarium* species also helps in the carbon cycle and interacts with plants through exchanging of organic and inorganic compounds (Tiwari et al., 2008). *Fusarium oxysporum* acts as a decomposer which degrades simple polymers such as pectin and cellulose from plant debris (Karim et al., 2016). It secretes the extracellular enzymes such as amylases, cellulases, pectinases and polyphenol oxidases which degrade these polymers and resulted the released of nutrients into the ecosystems. Lignin and lignocellulose which considered as complex structural polymers have the ability to promote the growth of *F. oxysporum* which in turn this fungus will decompose the material associated (Deacon, 1997).

Apart of being as saprophyte, *F. oxysporum* also can act as an endophyte by colonising inside living plants but do not cause any noticeable disease symptoms toward its host (Leslie and Summerell, 2006). Endophytic *F. oxysporum* is commonly involves in the plant's physiological activities such as storage, secretion of sugars and may help the plant host in adapting to its habitat, promoting plant growth and protecting plant from abiotic and biotic stress (Sieber, 2002; Schulz and Boyle, 2005;

Rodriguez and Redman, 2008). Furthermore, the role of endophytic *F. oxysporum* has been proposed as a biological way to control several diseases by inducing resistance in the host (Alabouvette et al., 2009).

Vu et al. (2006) reported that *F. oxysporum* endophytes have the ability to induce systemic resistance in banana to against burrowing nematode, *Radopholus similis* in glasshouse experiments. *Fusarium oxysporum* also has been identified as the predominant species establishing endophytic relationships with banana plants. The ability of endophytic *F. oxysporum* isolates to protect banana plants against pests and diseases has been demonstrated in laboratory and plant house experiments (Gold and Dubois, 2005; Nel et al., 2006).

2.3.4 Role as a plant pathogen

Besides being as saprophyte and endophyte, *F. oxysporum* also plays an important role in causing several plant diseases in tropical and temperate regions (Baayen et al., 2000; Flood, 2006; Latiffah et al., 2010). Among the important diseases caused by *F. oxysporum* are wilt, rot and damping-off diseases. *Fusarium oxysporum* is known to cause wilt diseases in a wide variety of economically important crops such as fruits, vegetables, ornamental and cucurbits (Leslie and Summerell, 2006; Michielse and Rep, 2009). *Fusarium oxysporum* can survive prolonged in the soils by producing chlamydospores and when the environment is favourable for infection, the conidia will be dispersed and initiate infection to the new plants.

Panama disease is a devastating disease infecting banana plant worldwide caused by *F. oxysporum* f. sp. *cubense* which recorded significant yield losses each year (Groenewald et al., 2006). The first external symptom of Panama disease is yellowing of lower leaves which later turn brown and dry out. Leaf yellowing begins

along the margin and advances toward the midribs. Yellowing and buckling progress from older to younger leaves, and lead to entire plant dies. Internally, the discoloration of the inner tissue occurs in the corm and pseudostem (Ploetz, 2006). The discoloration is usually seen as a reddish-brown of the xylem, develops in feeder roots, the initial sites of infection (Ploetz and Pegg, 2000). There are various reports regarding *F. oxysporum* f. sp. *cubense* that affected banana in Malaysia (Liew, 1997; Wong et al., 2019).

Fusarium wilt of tobacco which caused by *F. oxysporum* f. sp. *nicotianae* is widespread in tobacco growing regions of the world and the infection causes major losses to the growers (LaMondia, 2015). The symptoms can be characterised by the rapid wilting and browning of the older leaves followed by the younger leaves and shoots (Ramakrishnan and Sreenivas, 2012). Dying of the leaves eventually leads to plant death. A brown discoloration formed internally in the vicinity of the vascular tissue (Ramakrishnan and Sreenivas, 2012). Several studies were conducted on Fusarium wilt of tobacco. Shenoi et al. (2004) and Berruezo et al. (2018) reported the incidence of tobacco wilt caused by *F. oxysporum* in Karnataka, India and Argentina, respectively.

Besides, Fusarium wilt is also considered as one of the most important diseases that affects tomato (*Solanum lycopersicum*) cultivation which caused by *F. oxysporum* f. sp. *lycopersici* (Srinivas et al., 2019). *Fusarium oxysporum* f. sp. *lycopersici* has been described over 100 years ago in the UK (Massee, 1895) which causes tomato wilting (Inami et al., 2014), resulting in low yields and high economic losses (Arie et al., 2007; Panthee and Chen, 2010), exceeding 50% in production systems in Mexico (Apodaca et al., 2004). The typical symptoms of Fusarium wilt of tomato are the leaves

become yellowing, flaccidity and wilting. On the roots and stems, necrosis and brownish discoloration were observed followed by reddish coloration of the vascular tissue. Wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* has been observed worldwide including Malaysia (Rozlianah and Sariah, 2010).

The other agricultural crops that affected by wilt disease are cabbage (*Brassica* sp.) caused by *F. oxysporum* f. sp. *conglutinans*, onion (*Allium* sp.) by *F. oxysporum* f. sp. *cepae*, watermelon (*Citrullus* sp.) by *F. oxysporum* f. sp. *niveum* and cucumber (*Cucumis sativus*) by *F. oxysporum* f. sp. *cucumerinum* (Chen et al., 2013; Borrego-Benjumea et al., 2014; Liu et al., 2017).

In addition, *F. oxysporum* also responsible in causing fruit, root and crown rots on many agricultural crops. For fruit rots, the infected tissue basically will turn leathery, beige to light or dark brown in colour and sunken. Under humid conditions, white surface of mycelium will be observed on the infected fruits. This disease occurrence has been reported in Italy, Colombia (Bayona et al., 2011) and Korea (Aktaruzzaman et al., 2014). A study by Chehri et al. (2011) reported that the same pathogen was responsible to cause dry rot disease of potato tubers in Malaysia. The occurrences of *F. oxysporum* associated with eggplant's fruit have also been reported in Turkey (Altinok, 2005) and Iran (Safikhani et al., 2013). Fruit rot of *F. oxysporum* was also reported to be occurred on other hosts namely cucumber and melon (Morsy et al., 2009; Seo and Kim, 2017).

Crown rot or root rot causes deterioration and rotting of the tissues at the crown or root of the plant causing the leaves to turn yellow, collapse and die. As the disease progressed, the infected stem developed brownish water soaked lesions near the soil line. Besides, brown-black discoloration can be observed in the cortex of the tap or

main lateral roots and taproot. When diseased plants are sectioned lengthwise, extensive brown discoloration and rot can be observed in the cortex of the crown and roots (Ozbay and Newman, 2004). This disease was reported to affect pepper (Pérez-Hernández et al., 2014), asparagus (Borrego-Benjumea et al., 2014) and marijuana (Punja and Rodriguez, 2018). Occurrence of crown rot of oil palm in Malaysia has been studied by Hafizi et al. (2013). The common symptoms of crown rot disease on oil palm are appearance of small, brown necrotic lesions on spear leaf leaflets. With age, the lesions expanded and extensive rotting of leaflets occurred (Chinchilla, 2008; Akino and Kondo, 2012).

Apart of wilt and rot diseases, *F. oxysporum* also synonym to cause damping-off disease. *Fusarium oxysporum* can attack seedlings of many plant species, including *Eucalyptus viminalis* (Salerno et al., 2004), *Pinus pinea* (Machón et al., 2009), *P. merkusii* (Achmad et al., 2012) and *Acacia mangium* (Widyastuti et al., 2013). The early symptom of damping-off disease was indicated by wilted seedling, and the rot began from base up to whole seedling stem at the age of 6 days old until fourth to sixth week post-sowing (Horst, 2013; Widyastuti et al., 2013).

2.3.5 Role as a human pathogen

Fusarium oxysporum is a versatile fungus in which it is not only plays roles as saprophyte and endophyte, but it also can be pathogenic towards plants and humans. About 35% of cases involving human infections caused by *F. oxysporum* (Hennequin et al., 1997; Jain et al., 2011). It was reported to cause fusariosis including onychomycosis, keratomycosis and infected immunocompromised patients (Nucci and Anaissie, 2007).

Fusariosis is a fungal infection of the genus *Fusarium* which an emerging infectious disease in immunocompromised patients that may present as a localised skin infection, mycetoma or pneumonia (Nucci and Anaissie, 2007). *Fusarium oxysporum* was reported as one of the *Fusarium* species that responsible for invasive fusariosis in humans (Jain et al., 2011).

Onychmycosis is a type of nail plate infection which caused by yeasts or molds (Carvalho et al., 2014). *Fusarium oxysporum* is one of the causal agents of this fungal infection and the incidences have been reported in adults and immunosuppressed individuals (Tosti et al., 2000; Guilhermetti et al., 2007; Ranawaka et al., 2012). A study by Carvalho et al. (2014) has reported the first case of congenital onychomycosis in a 60-day-old child caused by *F. oxysporum*. Similarly, other studies also have stated that *F. oxysporum* accounts for most cases of onychomycosis (Godoy et al., 2004; Brilhante et al., 2005; Ninet et al., 2005). This pathogen can infect and invades the healthy human nail by penetrating of nail layers unassisted and causes onychomycosis (Veiga et al., 2018).

Fungal keratitis, also known as keratomycosis, is an important disease caused by microbial keratitis in the general population. Keratomycosis is defined as invasive infection of corneal stroma caused by variety of fungi (Kulkarni et al., 2017). *Apergillus* and *Fusarium* are two major causal agents of fungal keratitis. Several species of *Fusarium* including *F. oxysporum* can cause this disease, and more than 50% of all fungal keratitis are caused by this genus. Besides *F. oxysporum*, other *Fusarium* species that also can cause keratitis are *F. avenascus*, *F. dimerum*, *F.verticillioides*, *F. poae* and *F. solani* (Wang et al., 2009).

Besides, *F. oxysporum* also can cause disseminated disease in severely immunocompromised patients. Several studies documented that *F. oxysporum* has infected patients with stem cell transplant, allogeneic bone marrow or infected recipients of solid organ (Sampathkumar and Paya, 2001; Marr et al., 2002). According to Nucci and Anaissie (2007), *F. oxysporum* was ranked as the second highest *Fusarium* species after *F. solani* which causes invasive infections in immunosuppressed individuals. Besides, *F. oxysporum* also was reported to cause pneumonia in an immunocompetent host in USA (Gorman et al., 2006).

2.4 Identification of *F. oxysporum*

2.4.1 Morphological identification

Morphological characteristics are the most common criteria used by researchers in identification of *Fusarium* species particularly *F. oxysporum* (Leslie and Summerell, 2006). This classical taxonomic method is useful to understand the evolution of morphological characters (Raja et al., 2017). It is basically involving examination of similarities and differences in observable features of the fungal isolates. To observe all the phenotypic features of the genus *Fusarium*, potato dextrose agar (PDA) and carnation leaf agar (CLA) were commonly used (Leslie and Summerell, 2006). Primary and secondary characters are the main characters adopted for identification of *F. oxysporum*.

The primary characteristics include observation of shape and size of macroconidia, microconidia and chlamydospores; and the structure of conidiogenous cells (Leslie and Summerell, 2006). *Fusarium oxysporum* produces three types of asexual spores namely macroconidia, microconidia and chlamydospores. The macroconidia are sickle-shaped, straight to slightly curved, relatively slender and thin

walled with three to four septa. Macroconidia can be produced from sporodochia or aerial mycelia and they have a foot shaped to pointed basal cell and tapered and curved, sometimes pointed apical cell. Meanwhile, the microconidia are usually round, oval or reniform in shape and non-septate. Microconidia are produced in false heads on short monophialides of the hyphae. Chlamydospores can be presented singly or in pairs on the intercalary or the terminal regions of hyphae. The chlamydospores are formed by the modification of the hyphal and conidial cells through the condensation of their contents (Ohara and Tsuge, 2004; Leslie and Summerell, 2006).

The secondary morphological features include colony appearance of the culture, pigmentation produced by the colony and growth rate. Some *Fusarium* species produced sporodochia which are masses of macroconidia that formed on PDA or CLA with varied colours, depending on the species. Potato dextrose agar (PDA) is a nutrient rich medium used to examine colony features of the *Fusarium* species (Leslie and Summerell, 2006). Temperature and incubation conditions such as light regime (12 h light/ 12 h dark) are important factors which will affect the colony pigmentation (Burgess et al., 1994; Saremi et al., 2007). The measurement of growth rate is made after 3 days of incubation at 25°C to 30°C on PDA plate using single spore culture (Leslie and Summerell, 2006).

Unfortunately, morphological characteristics alone is sometimes insufficient to accurately identify genus *Fusarium* until species level due to similar features shared by closely related species. Moreover, morphological characters can regularly be misleading because of hybridization (Olson and Stenlid, 2002; Hughes et al., 2013), cryptic speciation (Kohn, 2005; Giraud et al., 2008; Foltz et al., 2013; Lücking et al., 2014) and convergent evolution (Brun and Silar, 2010). To classify *Fusarium* species

based on morphology solely can be tough, especially for the nonexperts as there are a limited number of key characteristics that can be used for identification. All these shortcomings subsequently will lead to confusion and misidentification in determining species of *Fusarium.*

2.4.2 Molecular identification

To solve the shortcomings of morphological identification, many researchers have turned to molecular approach such as DNA sequence-based methods for identifying species within *Fusarium.* DNA sequences basically can provide rapid, accurate and reliable species identity. One of the methods in sequence-based identification is DNA barcoding. In this method, the researcher will compare an unknown sequence against a sequence database such as from GenBank, NCBI (Raja et al., 2017).

Several genes are applied to accurately identify *F. oxysporum*. The most common regions used are internal transcribed spacer (ITS) regions, protein coding genes such as translation elongation factor 1-alpha (TEF1-α), mitochondrial small subunit (mtSSU) and beta-tubulin (β-tubulin) which appeared to be useful in *F. oxysporum* identification (Leslie and Summerell, 2006).

2.4.2(a) Internal transcribed spacer (ITS)

The ITS is a non-coding region comprised of two informative regions, ITS1 and ITS2 which are located between 18S and 28S ribosomal subunits and separated by the 5.8S ribosomal subunit (Michaelsen et al., 2006) (Figure 2.1). The ITS region can be amplified from a wide range of fungi including *F. oxysporum* using primers ITS1 and ITS4 (Zarrin et al., 2016). Many different universal primers have been designed

to amplify the ITS region and the most common are ITS1, ITS2, ITS3, ITS4 and ITS5 (Bellemain et al., 2010).

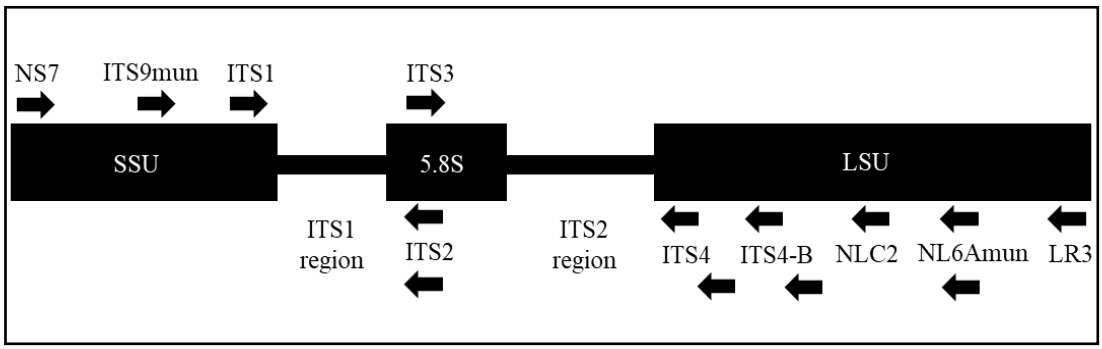

Figure 2.1: Schematic diagram of ITS gene with ITS1 and ITS2 primers location used for identification of fungal isolates (Toju et al., 2012).

ITS region has been widely used as a molecular marker in several studies of *Fusarium* (Mirete et al., 2013; Singha et al., 2016; Zarrin et al., 2016). Leyva-Mir et al. (2018) used ITS region to confirm the identify *F. oxysporum* as the causal agent of Fusarium wilt of stevia. Similarly, Campos et al. (2019) used ITS to verify the causal agent of Fusarium ear rot of maize in Portugal which suspected to be *F. oxysporum*.

However, there are some disadvantages associated with the use of ITS region, in which the region is insufficient of variability to distinguish various species from the genus *Fusarium* and lead to difficulty to resolve identity until species level (Mirhendi et al., 2010). Previous study showed that ITS sequence data failed to differentiate several species complexes within *Fusarium* (O'Donnell et al., 2007). Furthermore, ITS also unable to resolve identity of closely related species within *Fusarium* (O'Donnell et al., 2015). The low variability of the ITS region has led to the application of several other conserved genes such as TEF1-α, β-tubulin and mtSSU rDNA (Stewart et al., 2006; O'Donnell et al., 2013; Ramdial et al., 2016; Maryani et al., 2019).

2.4.2(b) Translation elongation factor 1-alpha (TEF1-α)

Besides ribosomal gene, protein coding gene was regularly applied for fungal identification. Translation elongation factor 1-alpha (TEF1-α) which encodes an essential part of the protein translation machinery is commonly used for identification of *F. oxysporum* (Geiser et al., 2004). This gene presents as single locus or multiple identical loci with a high level of sequence polymorphism makes it suitable as a molecular phylogenetic marker. The gene also provides non-orthologous copies in most of the fungal species and it is highly informative to differentiate species especially in the genus *Fusarium* (Geiser et al., 2004). The most common primer pair used in *Fusarium* identification particularly *F. oxysporum* is EF1/EF2 (Geiser et al., 2004) (Figure 2.2).

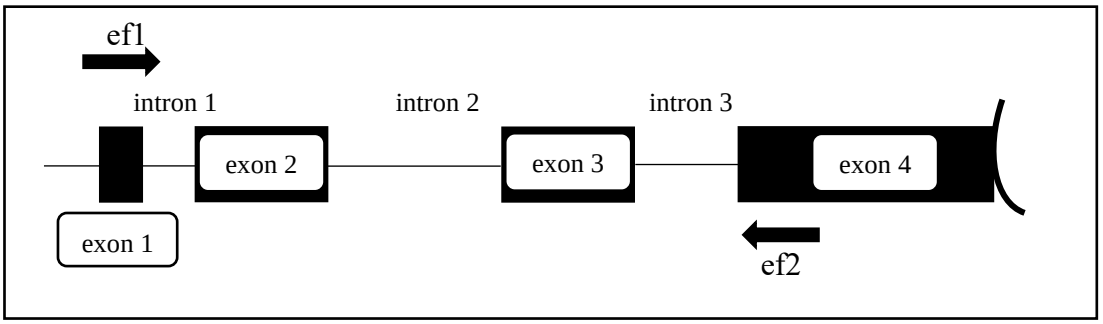

Figure 2.2: Schematic diagram of TEF1-α gene with EF1 and EF2 primers location used for identification of fungal isolates (Geiser et al., 2004).

Translation elongation factor 1-alpha (TEF1-α) gene was first used to study the lineage in FOSC showing 50% higher resolution level compared to mtSSU rDNA (O'Donnell et al., 1998). This gene also appears to be consistently single-copy in *Fusarium* species and has high level of sequence polymorphism among closely related species compared to other protein-coding genes such as calmodulin, β-tubulin and histone H3 (Geiser et al., 2004).

The role of TEF1-α gene in assisting identification of *F. oxysporum* has been proven by several studies (Geiser et al., 2004; Kristensen et al., 2005). A study by Rooney-Latham et al. (2011) had successfully identified *F. oxysporum* from wilt of passion fruit using TEF1-α gene. Mohammed et al. (2016) used TEF1-α gene to confirm the causal pathogen of crown and root rot disease of tomato. Other study conducted by Nitschke et al. (2009) found that sequences of TEF1-α merely managed to recognise different species of *Fusarium* namely *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. tricinctum* and *F. venenatum* isolated from infected sugar beet.

2.4.2(c) Mitochondrial small subunit (mtSSU)

In many organisms, mitochondrial DNA has a higher rate of evolution than nuclear DNA (Allio et al., 2017). The DNA sequence data of 18S, 26S, ITS and mitochondrial rDNA are the most frequently used in recent phylogenetic studies of eukaryotic cells due to ubiquitous occurrence and essential functions. Mitochondrial small subunit (mtSSU) rDNA gene was reported to evolve 16 times faster than 18S rDNA (Hong et al., 2002). The rDNA found in the nuclear genome of eukaryotes usually consists of tandem repeated units and it tends to be homogenised through concerted evolution (Richard et al., 2008). Therefore, phylogenies based on 18S or ITS rDNA should be verified by other sources of data in which sequence of mtSSU rDNA serve this purpose (Hong et al., 2002). Commonly, ms1 and ms2 primers are used for the amplification of the mtSSU ribosomal DNA gene (Ellis et al., 2014) (Figure 2.3).

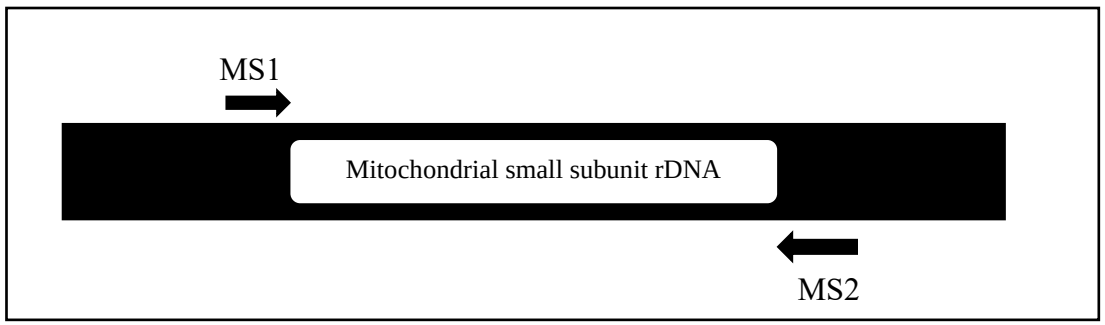

Figure 2.3: Schematic diagram of mtSSU gene with MS1 and MS2 primers location used for identification of fungal isolates (White et al., 1990).

As an effective molecular marker, mtSSU gene is widely used for identification purposes (Kristensen et al., 2005; Mbofung et al., 2007). For example, Fourie et al. (2009) used the gene to confirm identity of *F. oxysporum* isolated from infected banana. Similarly, Ellis et al. (2014) verified identity of *F. oxysporum* isolated from soybean roots using the same gene. Besides, the sequences of mtSSU have been utilised in *Fusarium* phylogenetic analysis (Li et al., 2000; Knutsen et al., 2004; Kristensen et al., 2005; Mbofung et al., 2007).

Apart from *F. oxysporum*, other fungal species also used mtSSU gene in molecular identification. A study by Kim et al. (2012) confirmed the identity of *F. commune* isolates based on mtSSU sequences. Hong et al. (2002) implied that mtSSU rDNA sequence contained considerable information to resolve phylogenetic relationships of both higher and lower ranks of taxa among several genera of Hymenomycetes and genus *Ganoderma*.

2.4.2(d) Beta tubulin (β-tubulin)

Tubulin can be classified into three members namely, α, β and γ tubulins and showed homology in the fungal genomes (Dutcher, 2001). The β-tubulin is a monomeric globular protein which it has been successfully used for species delineation in *Fusarium* species (Zhao et al., 2014a; Karim et al., 2016). This gene also useful in