

**SEGREGATION OF MICROSATELLITE
MARKERS, FERTILITY AND
PATHOGENICITY ON *Hylocereus undatus*
(DRAGON FRUITS) IN THE
INTERSPECIFIC HYBRID BETWEEN
Fusarium fujikuroi AND *Fusarium
proliferatum***

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UNIVERSITI SAINS MALAYSIA

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by

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for the degree of
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TABLE OF CONTENTS

| | |
|--|-------------|
| ACKNOWLEDGEMENT | ii |
| TABLE OF CONTENTS | iii |
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| LIST OF ABBREVIATIONS AND SYMBOLS | viii |
| ABSTRAK | xi |
| ABSTRACT | xiii |
| CHAPTER 1 GENERAL INTRODUCTION | 1 |
| CHAPTER 2 LITERATURE REVIEW | 4 |
| 2.1 History of Genus <i>Fusarium</i> taxonomy and classification | 4 |
| 2.2 Species concept in <i>Fusarium</i> | 6 |
| 2.2.1 Morphological species concept | 7 |
| 2.2.2 Biological species concept | 9 |
| 2.2.2(a) Mating population and mating fertility of Ascomycota | 9 |
| 2.2.3 Phylogenetic species concept | 11 |
| 2.3 <i>Fusarium fujikuroi</i> | 12 |
| 2.4 <i>Fusarium proliferatum</i> | 13 |
| 2.5 Interspecific hybrids between <i>F. fujikuroi</i> and <i>F. proliferatum</i> | 14 |
| 2.6 Host of research (Dragon fruit) | 14 |
| 2.6.1 Origin, distribution and ecology | 15 |
| 2.6.2 Botanical classification and <i>Hylocereus undatus</i> | 16 |

| | | |
|--|--|-----------|
| 2.6.3 | Nutrition and important of dragon fruit | 17 |
| 2.6.4 | Diseases of dragon fruit | 18 |
| 2.6.5 | Disease management..... | 21 |
| 2.7 | Pathogenicity test..... | 22 |
| 2.8 | Molecular characterization of <i>Fusarium</i> species..... | 24 |
| CHAPTER 3 FERTILITY AND PATHOGENICITY OF INTERSPECIFIC HYBRID BETWEEN <i>Fusarium fujikuroi</i> AND <i>Fusarium proliferatum</i> ON <i>Hylocereus undatus</i> IN MALAYSIA | | 26 |
| 3.1 | Introduction | 26 |
| 3.2 | Material and Methods..... | 28 |
| 3.2.1 | Pathogenicity test..... | 28 |
| 3.2.1(a) | Fungal isolates | 28 |
| 3.2.1(b) | Preparation of <i>H. undatus</i> seedlings..... | 29 |
| 3.2.1(c) | Preparation of inoculum, growth and symptom development..... | 29 |
| 3.2.1(d) | Disease severity (DS) and data analysis | 30 |
| 3.2.2 | Fertility test..... | 31 |
| 3.2.2(a) | Amplification of <i>MAT</i> alleles | 31 |
| 3.2.2(b) | Sexual crosses..... | 33 |
| 3.2.2(c) | Perithecia and ascospore examination..... | 34 |
| 3.3 | Results | 34 |
| 3.3.1 | Pathogenicity test..... | 34 |
| 3.3.1(a) | Symptoms and sign development | 34 |
| 3.3.1(b) | Disease severity (DS) | 37 |
| 3.3.2 | Fertility test..... | 45 |

| | |
|---|-----------|
| 3.3.2(a) Amplification of <i>MAT</i> alleles and sexual cross fertility | 45 |
| 3.4 Discussion and conclusion..... | 46 |
| CHAPTER 4 SEGREGATION OF MICROSATELLITE LOCI IN AN INTERSPECIFIC HYBRID BETWEEN <i>Fusarium fujikuroi</i> AND <i>F. proliferatum</i> | 51 |
| 4.1 Introduction | 51 |
| 4.2 Material and Method..... | 52 |
| 4.2.1 Selected strains..... | 52 |
| 4.2.2 DNA extraction..... | 55 |
| 4.2.3 DNA quantification..... | 56 |
| 4.2.4 Primer screening..... | 56 |
| 4.2.5 Microsatellite amplification..... | 56 |
| 4.2.6 Segregation microsatellite loci analysis..... | 57 |
| 4.3 Result..... | 58 |
| 4.3.1 Segregation of microsatellite loci | 58 |
| 4.4 Discussion and conclusion..... | 61 |
| CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION..... | 64 |
| REFERENCES | 67 |
| APPENDICES | |

LIST OF TABLES

| | Page |
|------------|---|
| Table 3. 1 | Rating of stem rot disease..... 30 |
| Table 3. 2 | Primer pairs of <i>MAT-1</i> and <i>MAT-2</i> used to detect <i>MAT</i> alleles of interspecific hybrid between <i>F. fujikuroi</i> and <i>F. proliferatum</i> 32 |
| Table 3. 3 | Disease severity of interspecific hybrids between <i>F. fujikuroi</i> and <i>F. proliferatum</i> on <i>H. undatus</i> 37 |
| Table 3. 4 | Frequency mating type allele (<i>MAT</i>) of interspecific hybrid between <i>F. fujikuroi</i> and <i>F. proliferatum</i> isolates 45 |
| Table 4. 1 | Interspecific hybrid strains and parental strains used in microsatellite analysis..... 53 |
| Table 4. 2 | Primers for microsatellite analysis..... 57 |
| Table 4. 3 | Segregation ratio of microsatellite markers among the progeny 60 |

LIST OF FIGURES

| | | Page |
|-------------|--|-------------|
| Figure 3. 1 | Structural organization of mating type (<i>MAT</i>) alleles with the priming sites of <i>Fusarium fujikuroi</i> species complex. Size of protein are given in amino acid and size of DNA in bp (Leslie and Summerell, 2006)..... | 32 |
| Figure 3. 2 | Result of pathogenicity test showing stem rot caused by parental strains. (A, B) <i>F. fujikuroi</i> ; (A) Brown-yellowish sunken lesion symptom appeared on 3rd day of inoculation; (B) Necrotic lesion after 10th days of inoculation; (C, D) <i>F. proliferatum</i> ; (C) Brown-yellowish sunken lesion symptom appeared on 3 rd day of inoculation; (D) Necrotic lesion after 10 th days of inoculation. Inoculation at bottom is control..... | 35 |
| Figure 3. 3 | Result of pathogenicity test showing stem rot caused by representative interspecific hybrid. (A, B) Strain CD170406; (A) Brown-yellowish sunken lesion symptom appeared on 3rd day of inoculation; (B) Necrotic lesion after 10th days of inoculation; (C,D) Strain CD144E-50; (C) No symptom on 3 rd day of inoculation; (D) No symptom appeared on 10 th day of inoculation. Inoculation at bottom is control..... | 36 |

LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---------------|---|
| μL | Microliter (10^{-3} ml) |
| μM | Micromolar |
| AFLP | Amplified fragment length polymorphism |
| ANOVA | Analysis of variance |
| bp | Base pair |
| CA | Carrot agar |
| CAPS | Cleaved amplified polymorphic sequence |
| cM | Centimorgan |
| cm | Centimetre |
| DNA | Deoxyribonucleic acid |
| DS | Disease severity |
| dNTPs | Deoxyribonucleotide triphosphate |
| FFSC | <i>Fusarium fujikuroi</i> species complex |
| GFSC | <i>Gibberella fujikuroi</i> species complex |
| g | Gram |
| h | Hour |

| | |
|-------------------|---------------------------------------|
| kg | Kilogram |
| LOD | Logarithm of the odds |
| MAT | Mating type |
| MgCl ₂ | Magnesium chloride |
| MP | Mating population |
| mA | Milliampere |
| min | Minutes |
| mg | Milligram |
| mL | Millilitre |
| mM | Millimolar |
| ng | Nanogram |
| nm | Nanometre |
| °C | Degree centigrade |
| PCR | Polymerase Chain Reaction |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| RAPD | Random amplified polymorphic DNA RFLP |
| rpm | revolution per min |

| | |
|------|---|
| SPSS | Statistical Package for social science SSRs |
| s | second |
| TBE | Tris-borate-EDTA |
| UV | Ultraviolet light |
| V | Voltage |

**SEGREGASI PENANDA MIKROSATELIT, TRAIT KESUBURAN DAN
KEPATOGENAN PADA *Hylocereus undatus* (BUAH NAGA) DALAM
HIBRID BERLAINAN SPESIES DI ANTARA *Fusarium fujikuroi* DAN
*Fusarium proliferatum***

ABSTRAK

Genus *Fusarium* adalah kumpulan yang paling biasa dan penting dalam patogen tumbuhan, mengandung banyak spesies yang terdapat diseluruh dunia dan menyerang banyak tumbuhan penting. Pengenalpastian spesies di dalam genus boleh dikenalpasti melalui morfologi, biologi dan teknik molekular. Walaubagaimana pun, peristiwa unik apabila dua spesies yang berkait rapat iaitu *Fusarium fujikuroi* dan *Fusarium proliferatum* mampu bersenyawa dan menghasilkan progeneri. Lebih kurang 500 progeneri hibrid berlainan spesies di antara *F. fujikuroi* dan *F. proliferatum* dengan menggunakan kondisi makmal telah dikumpulkan, disimpan dan telah dikenalpasti sebelum ini. Maka, objektif daripada kajian ini adalah mencirikan dengan lebih mendalam untuk hibrid berlainan spesies ini. Perlanjutan daripada kajian sebelum ini, beberapa progeneri telah dipilih dan dilanjutkan informasi mengenai hibrid tersebut seperti ujian kepatogenan, ujian kesuburan dan pendekatan molekular menggunakan penanda mikrosatelit. Ujian kepatogenan telah dijalankan pada buah naga sebagai perumah. Suntikan ampaian spora adalah teknik yang digunakan untuk menginokulasi progeneri pada batang buah naga. Daripada hasil kajian, 214 isolasi menunjukkan pelbagai darjah kevirulenan di mana 33 dikelaskan sebagai tidak virulen, 172 adalah virulen rendah, 7 adalah virulen sederhana dan 2 isolasi virulen tinggi. Ujian kesuburan telah dijalankan ke atas progeneri untuk menentukan daya biak mereka. Kajian kesuburan menunjukkan 137 progeneri membawa *MAT-1* alel dan 77 membawa *MAT-2* alel. 11 progeneri adalah subur

menyilang dengan strain penguji MP-C dan MP-D manakala satu progeneri subur silang dengan strain penguji MP-D sahaja. Akhir sekali, daripada 10 primer mikrosatelit yang telah diuji, hanya penanda (AG)₇C, (CTG)₅ dan (GTGC)₄ menghasilkan jumlah jalur polimorfisme yang tinggi. 13 penanda mikrosatelit yang polimorfisme telah terhasil daripada primer yang diuji, pengasingan penanda mikrosatelit menunjukkan progeneri terherot kepada alel *F. fujikuroi*. Walaubagaimanapun, trait dalam kalangan progeneri tidak berpisah secara bersama. Jadi, menunjukkan kepatogenan, kesuburan dan jenis pengawanan adalah fenotaip bebas. Oleh demikian, kajian ini menunjukkan keunikan fenotaip dan genotaip dalam hibrid berlainan spesies. Tambahan lagi, menunjukkan penanda mikrosatelit adalah penanda yang boleh diharap, boleh diulang dan alat yang mudah untuk pembelajaran kebolehubahan genetik dalam patogen kulat tumbuhan.

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ABSTRACT

Fusarium genus is the most common and important groups of plant pathogens that distributed worldwide and attack many economically important crops. Species identification within the genus was well distinguished based on morphology, biology, and molecular approach. However, a unique event where two closely related species which were *Fusarium fujikuroi* and *Fusarium proliferatum* was able to cross to produce viable progeny. At laboratory condition about 500 progenies of interspecific hybrid between *F. fujikuroi* and *F. proliferatum* have been collected, preserved, and initially characterized. Thus, the main objective of this research is to further characterize the interspecific hybrids. Continuing from the previous research, several progenies have been selected and extend the information regarding those hybrids such as pathogenicity test, fertility test and molecular approach using microsatellite marker. Pathogenicity test on dragon fruit plant as a host and conidia suspension injection technique were used. From the result, 214 isolates showed variable degree of virulence, 33 were categorized as non-pathogenic, 172 were classified as low virulence, 7 were classified as moderate virulence and 2 isolates were high virulence. Fertility test was conducted on the progeny to determine their fecundity. Fertility study shown 137 progeny carried *MAT-1* allele and 77 progeny carried *MAT-2* allele. 11 progenies are cross fertile with tester strain MP- C and MP-D and one progeny was cross fertile with MP-D tester strain only. Finally, off the 10 microsatellite primers tested, only primer

(AG)₇C, (CTG)₅ and (GTGC)₄ produce a high number of polymorphic band. 13 polymorphic microsatellite markers was generated, segregation of microsatellite marker shows that it distorted towards *F. fujikuroi* allele. However, the traits did not segregate together among the progenies. Thus, it shows pathogenicity, fertility and mating type are independent phenotype. Hence, the current research demonstrate the unique phenotype and genotype of interspecific hybrid. In addition, demonstrate that microsatellite marker is reliable, reproducible and technically simple tool for studying genetic variability in plant pathogenic fungi.

CHAPTER 1

GENERAL INTRODUCTION

The *Fusarium* genus comprises many important species that are pathogenic to plants. It is distributed worldwide and cause diseases to many economically agricultural and horticultural crops (Leslie *et al.*, 2006; Kvas *et al.*, 2009). Common diseases caused by *Fusarium* are Fusarium wilt of banana caused by *F. oxysporum* f.sp. *cubense* (Ploetz *et al.*, 2015), and vascular wilt of palm oil (Fusariose) caused by *F. oxysporum* f. sp. *elaedis* (Julie *et al.*, 2006). Some *Fusarium* species have a wide and narrow host range. Example of *Fusarium* species with narrow host range are *F. fujikuroi* cause bakanae disease of rice, *F. mangiferae* cause mango malformation, *F. graminearum* and *F. verticillioides* effect cereals (Mohd Zainudin *et al.*, 2008; Ma *et al.*, 2010; Mohamed Nor *et al.*, 2013). On the other hand, *Fusarium* species with a wide host range are *F. proliferatum* which affects dragon fruit, onion, garlic, asparagus and maize, while *F. oxysporum* infects both monocotyledonous and dicotyledonous (Moretti *et al.*, 2007; Ma *et al.*, 2010). One of the most important agricultural fruit in Malaysia is *Hylocereus* species which has become increasingly demand due to its high nutrient content and healing properties (Rita *et al.*, 2013). Dragon fruit is divided into three varieties, *H. undatus* that have white-fleshed with scarlet skin, *H. polyrhizus* which red-fleshed with scarlet skin and *Selenicereus megalanthus* that have white-fleshed with yellow skin (Mahani and Halimi 2007). However, only two varieties are commercially cultivated in Malaysia namely *H. undatus* and *H. polyrhizus* due to the suitability of tropical climate, rainfall requirement, soil type and light intensity. Dragon fruit is rich in vitamins which help a lot for the digestive system due to its fiber, prevent colon cancer and diabetes, neutralize toxic substances such as heavy metal and reduce cholesterol

level with high blood pressure (He *et al.*, 2012). Example *Fusarium* species that have been reported effect dragon fruit are *Fusarium semitectum*, *Fusarium oxysporum* and *Fusarium moniliforme* which can cause stem rot (Mohd *et al.*, 2010). Other causal agents that affect dragon fruit are *Bipolaris cactivora* which causes fruit rot (Tarnowski *et al.*, 2010; He *et al.*, 2012), *Botryodiplodia* species which causes brown spots and *Septogloeum* species causing stem lesion (Zheng *et al.*, 2009).

Taxonomy of the genus *Fusarium* has been studied since 1800s, since then the identification system has been improved with the application of new technology (Wollenweber and Reinking, 1935; Snyder and Hansen, 1945; Nelson *et al.*, 1983; Nirenberg, 1989; Leslie and Summerell, 2006). The combination of morphological species concept, biological species concept, and phylogenetic species concept was applied in distinguishing the species in genus *Fusarium*. The problem occurs when two closely related species are able to cross fertile and produce viable progeny, for example, *F. fujikuroi* and *F. proliferatum* in the FFSC group. Both species have similar morphological characteristics and are closely related regarding gene sequences (Leslie and Summerell, 2006). It has been reported that the interspecific hybrid between *F. fujikuroi* and *F. proliferatum* was isolated from native tall grass prairie of grassland in the United States (Leslie *et al.*, 2004) and dragon fruit (Masratul Hawa, 2014). Laboratory generated progeny from sexual crosses between *F. fujikuroi* and *F. proliferatum* was successfully isolated and preserved (Leslie *et al.*, 2004, Mohamed Nor, 2014). Initially, about 500 progenies were characterized based on amplified fragment length polymorphisms (AFLPs) fingerprint. The progeny of an interspecific cross could produce progeny with unique pathogenicity and secondary metabolite profile. The mixed genetic information from the parents

could give rise to strains that are more pathogenic than either of the parents. Further characterization of the interspecific hybrids is important to understand the biology of this hybrids if they are found in nature. In this research, the progeny of interspecific hybrids is characterized based on the segregation of microsatellite markers, pathogenicity on dragon fruit, and fertility. Microsatellite (SSRs) was performed in the characterization of *Fusarium* species based on genotype where it can form phylogenies, has high level of polymorphism and good for studying genetic variation.

Despite the importance of dragon fruit as a food grain and reported with many diseases (Mercado-Silva, 2018), there are reports of interspecific hybrid occurring between *F. fujikuroi* and *F. proliferatum* in Malaysia by Mohd *et al.*, (2017). In this study, dragon fruit was selected as the host for pathogenicity test. It is expected that the progeny would be pathogenic or less pathogenic than their parents based on observed inheritance of the pathogenic phenotype. From the segregation of microsatellite loci, we could establish the optimum and polymorphic microsatellite primer for this hybrid and determine the progeny's preferable allele which would either follow the Mendelian segregation ratio or not. Microsatellite is one of the finger printing techniques that will be used in this experiment due to its consistency and reproducibility. Because of the above reasons, these studies focused on the biological species concept which sexual cross-fertility test, virulence of the hybrid using pathogenicity test and molecular technique by using microsatellite (SSRs). Thus, the research objectives of this study are; (i) to determine the interspecific hybrid between *F. fujikuroi* and *F. proliferatum* is pathogenic to dragon fruit; (ii) to investigate the fertility and fecundity of an interspecific hybrid; and (iii) to study the segregation of microsatellite loci in an interspecific hybrid.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Genus *Fusarium* taxonomy and classification

Taxonomy is the science of systematic classification of living or fossilized organism while classification is the arrangement of the categories based on related groups that will be divided or combined (Booth, 1975). In the beginning, there was confusion and inconclusive disorder in *Fusarium*, with more than 1000 species, varieties and forms named on the basis of superficial observation (Toussoun & Nelson, 1975). The genus *Fusarium* for the first, was separate with Sections and the pioneer idea in grouping them is based on similar morphological characteristics.

Pioneer recognizing of genus *Fusarium* by Link 1809 with the presence of canoe- or banana-shaped conidia. About more than 1000 species have been described but most of the species were no longer survive, poorly defined and make species diagnosis very complicated. The complicated system was reconstructed by Wollenweber and Reinking (1935) which establishing basis for all modern taxonomic systems in *Fusarium*, based on the morphological characteristic such as macroconidia, presence and shape microconidia and pigmentation. Corresponding to that, 16 sections, 65 species and 77 varieties and forms have been diagnosed. However, the process also have a weakness which cultural media and incubation time were not standardized and the culture was not from the single spore. In the middle 1930s, Raillo managed to study *Fusarium* classification base on variability of the morphological characters from single spore and another character for separate sub-species and varieties was length of apical cell, length and width conidia, number of conidia septa, while presence and absence formation of sclerotia, colony

pigmentation and mode of sporulation were used to separate special form. As a result, Raillo managed to recognize 55 species, 10 sub-species, 55 varieties and 61 forms grouped in 17 sections and 12 sub sections.

Next, Snyder and Hansen on 1940s and 1950s study on taxonomy by using a single technique in an identification, with their philosophy which focusing on similarities between isolates make up species rather than differences characteristic between isolates. As a result number of species was reduced to 9 which *F. episphaeria*, *F. lateritium*, *F. moniliforme*, *F. nivale*, *F. oxysporum*, *F. rigidiuscula*, *F. roseum*, *F. solani* and *F. tricinctum*. 2 species from Snyder and Hansen's studied which *F. oxysporum* and *F. solani* are still in widely used as reference for identification until today.

Messiaen and Cassini (1967), using Snyder and Hansen's model with modification, which the major modification was replacing botanical varieties in place of cultivar and diagnosed with some addition of morphological characteristics such as size and width macroconidia, abundance of chlamydospores, growth rate and pigmentation. In 1971, Booth come with the publication of the monograph, The Genus *Fusarium* which character in an identification was sporogenous cell morphological characteristic such as conidiophores, size and shape conidia, pigmentation, presence or absence and position of chlamydospores, growth rate and substrate compound to distinct between sections and species. After all, Booth reduced the 143 species and varieties to 44 species and 7 varieties.

Abraham Joffe (1986) published a monograph, *Fusarium* species, *Their Biology and Toxicology*, studied a large number of *Fusarium* isolates from soils, wilting or decaying plants and seeds. As consequence, Joffe recognized 13 sections,

33 species and 14 varieties. Gerlach and Nirenberg (1982), carried on with the philosophy of Wollenweber and Reinking published pictorial atlas with a well-illustrated work that uses astonishing photograph that enhance the Wollenweber's original line drawing. Another researcher, Paul E. Nelson, T. A. Toussoun and F. O. Marasas managed to publish an identification manual for *Fusarium* in 1983. As a result the number of species was reduced and some varieties and forms were combined with the appropriate species name. In 2006, Leslie and Summerell managed to produce a book of *Fusarium Laboratory Manual* which contain a compilation of species description by several researcher. Morphological, biological and phylogenetic species concept were included for a *Fusarium* species identification.

2.2 Species concept in *Fusarium*

The classification and identification systems of *Fusarium* that approached by different researcher have led to difficulties in species identification. Inconclusive *Fusarium* species which have same characteristic can be differentiated and identified with combination of three species concept, morphological, biological and phylogenetic species concepts. However, expectation result using these three species concept might be different or similar, because morphological species description can be wrong due to genetic mutation that change important morphology character, biological species principle are not possible if the strains are not sexually fertile and phylogenetic species definitions will be wrong if hybridization between species occurs frequently on an evolutionary time scale, it will somehow provide information on species identity, evolutionary or phylogeny of the species (Leslie *et al.*, 2001).

2.2.1 Morphological species concept

Morphological or another word is typological species concept are basically about a morphology type or individual characteristic that can represent the variation within an entire species. This species concept are the easiest and simplest method in an identification and taxonomy of *Fusarium* species, which very useful for preliminary classification. Based on Taylor *et al.* (2000), the greatest strength of morphological species concept for fungi at its general compatibility and qualities to any fungal taxon and its historical used. In general, to examine or determine in morphology species concept, focusing on general character is the most rational method rather than trying to pin down every variation in form and structure because it can lead to confusion, dissatisfaction and incorrigible identification.

Morphology characteristic can be divided into two main character, which primary (physical) and secondary (physiological) characteristic. Primary characters used to identify species in *Fusarium* were morphology of macroconidia, microconidia, conidiophores and chlamydospores. Morphology of macroconidia is the core for characteristic for characterization not only of the species but also of the genus *Fusarium*. Macroconidia of *Fusarium* species have three basic shapes, first is straight thin needle-like shape, next is dorsiventral curvature which have approximately same width along their entire length, finally shape is the dorsal (upper) side is clearly more curved than the ventral (lower) side and in the middle of spore body commonly more wider than the cell at the end. Apical and basal cell shape also were observed in differentiate the species. Apical cell have four general shapes, which blunt, papillate, hooked and tapering. The basal or foot cell also have four general shapes, which foot shaped, distinctly notched, elongated foot shape and barely notched (Leslie and Summerell, 2006).

The presence or absence of microconidia as well as the mode of formation is an important characters in *Fusarium* taxonomy and differentiation of species. Microconidia have several common shape which oval, reniform, obovoid, pyriform, napiform, globose and fusiform. Besides that, morphology of conidiogenous cell and presence or absence of chlamyospore are another crucial character for examined and separation of *Fusarium* species. There are two common type of conidiogenous cell which monophialide and polyphialide. Monophialide have only single opening while polyphialide have multiple opening. Certain *Fusarium* species only produce one type of conidiogenous cell but there are also *Fusarium* species can produce more than one type of conidiogenous cell. Arrangement microconidia on phialide also one of another character that can be highlighted in *Fusarium* classification, whether it be singly, in false head only or in false head and chain. If chlamydo-spores are present, they may be formed singly, in pairs, in clumps or in chains, with either rough or smooth walls (Leslie and Summerell 2006).

A number of secondary character (physiological) used in the identification of *Fusarium* species. There are pigmentation of colonies, growth rate, presence or absence of sporodochia and sclerotia. Pigmentation is one of the eminent character in secondary character. Sample condition and media used are the crucial for the production of pigmentation, preferably potato dextrose agar (PDA) is used with 12:12 hour fluorescent light:dark light at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Growth rate is another character that can be highlighted in secondary character. Basically, measurement are made on PDA media and incubate about three days at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Some species of *Fusarium* produced sporodochia character which a place production of macroconidia by grow on carnation leaf agar (CLA). The production and formation macroconidia in sporodochia are more uniform and consistent compared with macroconidia that

form on aerial mycelium.

2.2.2 Biological species concept

Based on (Mayr, 1963), the biological species concept is defined as groups populations that manage or have a potential to interbreed with each other. These species concept have change the focus from individual to populations and define species by the population interaction between them rather than by compare with static standard. This experiment is an inexpensive in term of reagent preparation but expensive in term of the time consumable for a result and also need a quality incubator. For *Fusarium* species, biological species concept, are group of species that shared characters several genetic information that accessible within a sexual reproductive population and involved interbreeding or sexual crosses within a same group of species. Fertile progeny that produced from sexual crosses, will assigned to mating population (MP). Instead of assigning isolate, biological species concept also can help in term of infertility and fertility of the species. As a result, a biological species classification has been developed for species within the *Fusarium fujikuroi* species complex (FFSC) and *Fusarium solani* species complex (Leslie, 1995).

2.2.2(a) Mating population and mating fertility of Ascomycota

Filamentous ascomycete fungi are usually found with haploid mycelia that grow via conidial spore and hyphal elongation. Within the ascomycetes, there are three mode of sexual reproductive strategies homothallic, pseudohomothallic and haploid heterothallic and each species limited to a single reproductive strategy (Nelson 1996). Individual strains of homothallic fungi and individual haploid, strain of pseudohomothallic fungi might be self-fertile or cross with other their species to complete the sexual life cycle. In this particular research, heterothallic mode strategy

is the main focus which require involvement from two different genetically parents for a complete sexual cross. Crosses in this mode, conducted by the mating type locus and one of the two parents are female reproductive functions. In heterothallic species, the crossed parents must be different mating type. Mating type are molecular mechanism that regulate compatibility in sexually reproducing eukaryotes and these fungi is controlled by a single locus with two alleles termed varyingly “*A*”/”*a*”, “*a*”/”*α*”, “+”/”-“, or “*mat1-1*”/”*mat1-2*” depending on the organism (Nelson 1996). For population purpose, mating type in ascomycete can considered to be determined by one locus with two functional alleles.

In addition to mating type, an idealized member of heterothallic species of ascomycete is a self-fertile hermaphrodite which can produce male gametes and formation of female reproductive structure. When the male gamete is fertilized with female structure, then the structure matures to form ascospores that derived from meiotic products of a diploid, biparental nucleus. The male gamete originated is termed the male parent which produce from an asexually, a sexual or a mycelial fragment, while the female reproductive structure develops is termed the female parent which more elaborate which requiring a high degree of cellular specialization and development of highly organized structure called carotenoid.

2.2.3 Phylogenetic species concept

Implementation of phylogenetic species concept to *Fusarium* genus is a new development that resolve the unclear and unresolved taxonomic problem. However, if an improper applied or misinterpreted the data, the outcome will be a confusion and inconsistency result. The purpose of phylogenetic species concept is to classify taxonomic diversity into logical unit that allow descriptive sorting of diversity and species recognition. These phylogenetic analyses, sorted the taxonomic based on their similarities evolutionary history. Refer to Harrington and Rizzo (1999) which have same early definition of Nixon and Wheeler (1990), a species is differentiated from other species by being smallest phylogenetic subgrouping (clade) of individuals or populations that share a diagnostic character. The important change proposed by Harrington and Rizzo 1999 was that other phenotypic character such as morphological and biological characteristic also play a rule to indicate the relevance of the observed phylogenetic clustering.

Starting 20th century, phylogenetic tree analyses was broadly applied in identification in genus *Fusarium*, especially very effective for those species that not produce a sexual stage and homothallic fungi, for example *Aspergillus nidulans*, *Neurospora tetrasperma*, *Podospora anserina* and *F. graminearum* (Gale *et al.*, 2002; Zeller *et al.*, 2003). Development of phylogenetic species concepts vary in terms of whether a single isolate is used to characterize the species or to distinguish populations and the variation that they may harbour are to be considered. Generally, combination of phylogenetic and biological species concept has greatest potential for classifying species-level grouping in fungi. Within genus *Fusarium*, this species concept has been used to assist in resolving several species that have unclear, high similarities and interfertile of morphological and biological character, for example in

differentiate species between *Fusarium fujikuroi* and *Fusarium proliferatum* which known that can be crossly fertile to each other and also have similar morphological characters.

2.3 *Fusarium fujikuroi*

Fusarium fujikuroi is an anamorph of *Gibberella fujikuroi* (Sawada) Ito in Ito and K. Kimura and is a member of *Fusarium fujikuroi* species complex (formerly known as *G. fujikuroi* species complex (GFSC)) (O'Donnell *et al.*, 1998). FFSC is a polyphyletic group include of species from sections *Liseola* and *F. fujikuroi* under mating population C (MP-C) (Leslie and Summerell, 2006). Refer to The *Fusarium* manual laboratory by Leslie and Summerell (2006), *F. fujikuroi* can be identified through observing the presence and shapes of conidia and chlamydo-spores. Macroconidia have uniform shape and size. It has slender, medium length with no significant curvature, contained 3 to 5 septate and tapered apical structure and poorly developed at basal. Microconidia have oval or club shape with flattened base and 0 to 1 septa. False heads and short to medium length of microconidia will appear via observing under compound microscope on CLA agar. Usually, it also have polyphialides and monophialides. Chlamydo-spore is absent. This fungus have been well known as causal agent of Bakanae disease in rice and have widely spread at Asia, Africa, North America and Italy (Ou, 1985; Prà *et al.*, 2010).

2.4 *Fusarium proliferatum*

Fusarium proliferatum belongs to *Liseola* section of the *Fusarium* genus (Nelson *et al.*, 1983) and its teleomorph, *Gibberella intermedia*, belongs to the *G. fujikuroi* species complex composed of several reproductively isolated biological species designated by separate *Gibberella* species names (syn. mating population (MPs)). Previously, *F. proliferatum* was mentioned as a *Cephalosporium* species by Matsushima (Matsushima, 1971) and mentioned as *Fusarium* species by Nirenberg (1976). Then, it was known as a species by Gerlach and Nirenberg (1987) and Nelson *et al.* (1983). Refer to The *Fusarium* manual laboratory by Leslie and Summerell (2006), *F. proliferatum* can be identified via observe the conidia and chlamyospore. General morphology macroconidia, it has slender, thin wall, relatively straight, curved apical cell and basal cell is poor develop with 3 to 5 septate. Microconidia are club shaped with a flattened base with 0 septa. False heads and chains of moderate length are produced from monophialides and polyphialides, whereas, chlamyospore are absent. *F. proliferatum* is a well know causal agent for wide host plant such as maize (Logrieco *et al.*, 1995), rice (Desjardins *et al.*, 1997), asparagus (Elmer, 1990), date palm (Abdalla *et al.*, 2000) and ornamental palms (Armengol *et al.*, 2005).

2.5 Interspecific hybrids between *F. fujikuroi* and *F. proliferatum*

The history of *F. fujikuroi* and *F. proliferatum* is not easy as these two species are difficult and hard to determine based on morphology. These two species are closely related to one another (Leslie and Summerell, 2006). A decade past, there are suspected that hybrid between *F. fujikuroi* and *F. proliferatum* have being found from native tall grass prairie which a large area of grassland in the United States (Leslie *et al.*, 2004) and dragon fruit (Masratul Hawa, 2014). The presence of these hybrids could tell us that evolution is occurring or another possibility the speciation process are incomplete. As for laboratory condition, hybrids can be produced by crossing strains between opposite mating type on the carrot agar (Leslie and Summerell, 2006). The viable progeny from this cross could have unique phenotypic combination in term of pathogenicity and produce uncommon secondary metabolites (Studt *et al.*, 2012).

2.6 Host of research (Dragon fruit)

Dragon fruit is relationship with Cactaceae family and come with several common names such as pitaya or pitahaya (Latin America) (Le Bellec *et at.*, 2006), pānīnikapūnahou or pāpīpi pua (Hawaii) (Zee *et al.*, 2004) kaeo mangkon and luk mangkon (Thailand) (Clark *et al.*, 2005), paw wong fa kor (China) (Feng-Ru and Chung-Ruey, 1997), night-blooming cereus (South America) (Mizrahi *et al.*, 1997), nanettikafruit or thanhlong (Vietnam) (N' Guyen, 1996) and mata naga (Malaysia) (Cheah and Zulkarnain 2008). Today, it is one of the important exotic fruit market worldwide due to the promising condition at certain country, the qualities of the fruit, characteristic, good nutritional values and health interest.

2.6.1 Origin, distribution and ecology

Mata naga or pitaya was originated from South, Central and Northern America, nowadays this crop is already well known all over the world, including the subtropical and tropical regions. This crop have been tremendously cultivated in Australia (Jacobs, 1999), Taiwan (Wu et al., 2005), Argentina (Wright et al., 2007), Brazil (de Andrade et al., 2007), China (Feng-Ru and Chung-Ruey, 1997), Columbia (Le Bellec *et al.*, 2006), Costa Rica (Esquivel, 2004), Egypt (Mohammed-Yasseen, 2002), Germany (MoBhammer et al., 2005), Hawaii (Zee *et al.*, 2004), Mauritius (Govinden, 2007), Mexico (Valiente-Banuet et al., 2006), Nicaragua (Barbeau, 1990), Vietnam, Thailand and Malaysia (Zainuudin, 2011). It has been introduced to Malaysia since 1990's and was cultivated in Perak, Pahang, Pulau Pinang, Johor, Negeri Sembilan and Sabah (Nurul *et al.*, 2014).

Dragon fruit crop can survive at tropical and subtropical climate with approximately temperature between 21-29 °C, but can resist at 38-40 °C temperature, and at cold temperature which 0 °C for a short period. At Neveg Desert, Israel, the most preferable conditions for growth and fruit production were found to be 30 % shade for *H. polyrhizus* (Raveh et al., 1998), at French West Indies, cultivation of *H. trigonus* have optimum production about 50 % shade. In Vietnam, *H. undatus* has undergone big development with nearly 2000 ha under plantation. Dragon fruit need a less water treatment, because too much water will lead to abscission of flower and young fruit. This crop also can fit with different kind of well-drained soil (Perween *et al.*, 2018).

2.6.2 Botanical classification and *Hylocereus undatus*

Dragon fruit is a under family of Cactaceae with several genera which this study focusses on genus *hylocereus* and *undatus* species. This crop is primarily characterized by climbing plants with aerial roots that bear a glabrous berry with large scales (Fournet, 2002). *Hylocereus* species are diploid ($2n = 22$) (De Dios, 2004). Member of Cactaceae family primarily important because of their ornamental qualities, but also include about 250 cultivated species of fruit-bearing and industrial crops. However, only a certain species have an economic value (Perween *et al.*, 2018).

In classification of *Hylocereus* species, many disagreements that probably explained by the similarities of the morphological characteristic and / or environmental conditions (Mizrahi *et al.*, 1997). Refer to Britton and rose (1918) determination of *Hylocereus* genus, particularly *undatus* species has very long (25-30 cm) flowers with white in colour and bell shaped, stamens and lobed stigmas are cream coloured. Fruit is a fleshy berry, oblong to ovoid, up to 6-12 cm long, 4-9 cm thick, red with large bracteoles, pulp white and embedded with many small black seeds. Average fruit weight is 350-400 g (Merten, 2003).

2.6.3 Nutrition and important of dragon fruit

Fruit and vegetables contain numerous natural antioxidant that offer protection against chronic disease and nutritional values of dragon fruit have been well researched and being promoted abroad. Recently study by Cheah *et al.*, (2016) nutritional composition in *H. undatus* was 87 ± 0.11 g/100 g of moisture, 0.5 ± 0.01 g/100 ash, 11.0 ± 0.11 g/100 g of carbohydrate, 1.1 ± 0.01 g/100 g of protein and 3.0 ± 0.03 g/100 g total dietary fibre. Next, it also contains a lot mineral contained which important for maintaining health body, for instead it may assist in reducing risk for hypertension and decrease blood pressure. Macroelement in *H. undatus* such as magnesium have 26.40 mg, sodium have 35.63 mg, calcium have 8.5 mg while trace element like iron have 1.9 mg and zinc have 0.40 mg (Nurul and Asmah, 2014)

The red pitaya also has a good source of vitamin C (ascorbic acid) as reported by Ruzainah *et al.*, (2009) who found 20.5 mg of vitamin C in raw fruit. In addition, there are also found vitamin A in red pitaya juice with 85.22 μ g/100 g. The red pigment of *H. undatus* have been reported contain betanin, betacyanin and lycopene (Wu *et al.*, 2006), which are antioxidants and good for metabolism. Betanin is a red glycosidic food dye that contains nitrogen and degrades when there are present of heat, light and oxygen (Strack *et al.*, 1993). Betacyanin is the phytochemical that gives moderate purple colour that help reduce homocysteine levels in the body (Wybraniec *et al.*, 2001) while lycopene is a red fat-soluble pigmentation and under family carotenoids. Important of lycopene is help in preservation of macular degenerative which causes of blindness. Other important of an antioxidant is enhance the immune system, helps in digestion and blood circulation systems, controlling emotional pressure and neutralized toxins as well as reduces cholesterol

level in the blood (Sonawane, 2017).

The *H. undatus* not only rich in term of nutritional composition to the fruit, it also have a lot importance at the whole part. For the young stems of *H. undatus* are edible as well as fresh flower buds that are eaten as vegetables, while dried ones are used for traditional medicine. In Taiwan, dry flowers are consumed as vegetables besides it also taken in the form of juice, jam, or preserves according to the taste needed. It is widely used as juice and in fruit salads at restaurants. The edible black seed contained oil of linoleic and linolenic acid which this acid is essential fatty acids but cannot produced by the body (Ariffin *et al.*, 2009). The pulp and the skin of dragon fruit can act as natural food colouring and dye because the present of betacyanins (Harivaindaran *et al.*, 2008).

2.6.4 Diseases of dragon fruit

Everything that live must be die with their own way, good management is needed so that the life span will be longer and healthy. Dragon fruit one of the organism that infected with diseases caused by fungi, bacteria and pest invasion. The most common disease that found on dragon is anthracnose which the causal agent were *C. gloeosporioides* and *C. truncatum* (Vijaya *et al.*, 2015). Both species have similar symptom which reddish-brown lesions with chlorotic diameter. Masyahit *et al.*, (2009) reported that the inoculated fruit became completely decaying on seventh day after inoculation, while the stem severely rotting on tenth day after inoculation. Next, stem necrosis reported have infected most pitaya plantations in Malaysia which Kelantan, Melaka, Negeri Sembilan, Pulau Pinang and Perak with 41% disease incidence and 25% disease severity. Causal agent that responsible this disease is *Culvaria lunata* with a spots or small, circular, faint pink to beige

necrotic lesions symptom. Stem canker was acknowledged as the most destructive disease on pitaya in Malaysia. Causal agent for this disease was *Neoscytalidium dimidiatum* with the symptom initially were brown sunken lesion and the lesion became dark brown with age and as the disease continue, the stem rotted. There were also orange spot and black pycnidia on the surface of the canker (Masratul Hawa *et al.*, 2013).

In 2012, He *et al.*, (2012) reported that *Bipolaris cactivora* was the causal agent to fruit rot disease as the disease progressed, it will continue rotted the stem. The symptom was water-soaked, olive to black powdery spots to coalescing soft as the disease progressed. In addition, *Dothiorella* has been reported can infected dragon fruit that caused brown spots on stem and the fruits plant in Malaysia, Hawaii and Florida (Zee *et al.*, 2004). The symptom appearance were small, circular with yellow to brown spots. However, group *Gibberella fujikuroi* species complex (GFSC) also play the important role in dragon fruit invasion which this group are among the most common disease reported in agricultural and horticultural worldwide. *Fusarium oxysporum* was reported that can caused stem blight on dragon fruit and the first report invasion in Malaysia was by Hafifi *et al.*, (2019). The infected stem showed symptom as brown lesions surrounded by yellow halos, as the infection continue the brown lesion become darker at the centre with formation white mycelia on the lesion surface and the end the stem turned soft and watery. Stem rot disease with symptom appeared as circular, brown sunken lesion with orange sporodochia and present of white mycelium. The causal agent that identified was *Fusarium proliferatum* (Hawa *et al.*, 2013) and *Fusarium fujikuroi* (Hawa *et al.*, 2017) in Malaysia and *Fusarium solani* (Rita *et al.*, 2013) in Bali, Indonesia, respectively.

Besides fungal diseases, dragon fruit also infected with bacterial disease such as *Xanthomonas compestris*, *Enterobacter cloacae* and *Erwinia* species. Several studies have recognised, *Xanthomonas campestris* was the causal agent of stem rot on dragon fruit in Malaysia, Central America, Vietnam and Australia (Zee *et al.*, 2004). The infection initially from an injured stem and afterwards the injured area became yellow soften and rot. Masyahit *et al.*, (2009), reported that *Enterobacter cloacae* caused soft rot disease on dragon fruit at Peninsular Malaysia. The rot symptom appeared as yellowish to brownish soft and watery on infected stem and fruit. On the other hand, *Erwinia carotovora* also reported can lead to soft rot on dragon fruit with water soaked lesion and later becoming a soft rot (Cheah and Zulkaarnain, 2008).

Dragon fruit also can be infected by viral disease. Several studies have shown that *Cactus Virus X (CVX)* caused mottled disease to dragon fruit stems (Masanto *et al.*, 2018). Symptom that will appeared was at early stage is appearance of necrotic small spot on young shoot and unripe fruit which later turned to orange. The disease can be found in Malaysia, Florida (Gazis *et al.*, 2018), Taiwan (Liou *et al.*, 2001) and Korea (Kim *et al.*, 2016).

2.6.5 Disease management

There are several strategies that can be apply to control plant disease at the field and in plant house. The incidence and severity of dragon fruit disease have been found to be reduced by temperature control, hot water treatment, and gamma irradiation. Hot water treatment for 2 minutes at 50 °C before storage in 12 °C for 21 days can reduced lesion diameter by 63.1 % without significantly changing the fruit's sensory qualities (Vilaplana *et al.*, 2017). Fruit that was exposed to radiation at 800 Gy or less was also beneficial in preventing or slowing down fruit degradation, however only slight changes in fruit quality were noticed. (Wall and Khan, 2008). Next, the effectiveness of sodium carbonate and potassium sorbate have been investigated against several fruit rot causing by *C. gloesporioides*, *C. capsici* and *Fusarium* species (Jitareerat *et al.*, 2018). Potassium sorbate totally inhibited the spore germination of all fungal pathogens in culture media. Sodium carbonate completely prevented the germination of *C. gloessporiodes*, *C. capsici* and *Fusarium* species spores in media with the chemical at 2 % and 3 %, respectively.

Furthermore, it has been discovered that employing rain tree (*Samanea saman*) leaf extract as a biopesticide can prevent *F. solani* from growing in culture media and at a concentration of 2.5% can prevent *F. solani* from developing stem rot in dragon fruit stems. (Rita *et al.*, 2016). There have been reports of the usage of silicon to reduce the incidence and severity of several fungal diseases in dragon fruit plants. Plant that treated with silicon (5.0 mL/L) had lower disease incidence compared to the control treatment and lower silicon treatment (1.5 and 2.5 mL/L) (Nurul Faziha *et al.*, 2019). Biological control such as spent mushroom sawdust can reduced the incidence of basal stem rot to 3-12 % compared to the control which 44-59 % (Choi *et al.*, 2007). The sawdust's bacterial and fungal population was the key

to the disease-suppressing properties. In addition, It has been suggested that a combination of azoxystrobin (200 g/L) and difenoconazole (124 g/L) be used to treat dragon fruit diseases, particularly anthracnose and stem canker (Noegrohati *et al.*, 2019). Another biocontrolling agent that can reduce severity on dragon fruit were *Bacillus methylotrophicus*, *Bacillus subtilis* and *Penicillium oxalicum* T3.3 (Meetum *et al.*, 2017; Mamat *et al.*, 2018).

2.7 Pathogenicity test

Pathogenicity is the measurable capacity that can cause disease which the overall disease will inducing capacity of biotic, abiotic factor and determined simultaneously by the aggressiveness and virulence of the pathogen (Bos & Parlevliet, 1995). Pathogenicity also defined as the outcome of a complex interaction in time between a host and pathogen, every possible variable in a changing environment to differentiate between host specificity and the severity of disease. The pathogen can have wide range or narrow range virulence is refer to the degree of pathology caused by the microbes.

Pathogenicity test also can be conducted to recognize the host range of plant pathogen. The pathogen can have the capability to infect widely range or narrow range. Several studies of host range have been conducted for *Gibberella Fujikuroi species complex* (GFSC) such as *F. solani* from avocado, cow bean (Silbernagel *et al.*, 1990), cocoyam (Ofoegbu, 1993), pepper (Fletcher, 1994) and potato (Secor & Gudmestad, 1999), while *F. proliferatum* can be found on asparagus (Liu *et al.*, 2007), banana (Li *et al.*, 2017), tomato (Gao *et al.*, 2016), orchids (Wang *et al.*, 2018) and rice (Quazi *et al.*, 2013). Next, *F. circinatum* from pine plant family, that cause pitch canker disease in USA, Japan, Mexico, South Africa, Australia and New

Zealand (Leslie and Summerell, 2006). Knowledge and data regarding the host range is important in developing control strategies especially for farmer and researcher.

Pathogenicity test also functioned to measure or to determine virulence of different fungal isolates (*Zeller et al.*, 2003). Degree of virulence can be influenced by the pathogen, environment and host. For instead, the pathogen may be more or less virulent as it may be present in small or extremely large number or it may be in dormant state, or it may need water or specific host. Abiotic factor also affect the degree of virulence of the pathogen. Information on degree of virulence will be useful in toxicology study of fungal pathogens and also useful in determination how severe of particular pathogen on the field.

Those fungi isolate in the field or from the plants could be the main causal agent or pathogen that cause the disease or it could be a saprophyte that grow in the dead tissues of infected plant and not harmful to healthy plants. There are also certain pathogen can be more virulent toward their host if there are harsh condition such as insufficient soil nutrient, extreme temperature or excess usage of herbicides (*Burgess et al.*, 1994).

2.8 Molecular characterization of *Fusarium* species

A correct and precise for pathogen identification and time consumable when detection could provide a very beneficial tool for understanding the disease epidemiology and developing strategies to control the occurrence disease. Recently, molecular approaches based on DNA analysis have been widely used in an identification of *Fusarium* species (Amatulli *et al.*, 2012). PCR-based technique and PCR species-specific primers method was demonstrated as good for recognizing *Fusarium* species. Species-specific primer was designed from IGS region, ITS region, calmodulin gene, β -tubulin gene and translation elongation factor gene (*TEF1-a*) have been broadly used by a lot researchers due to the primers specificity to detect and differentiate *Fusarium* species from difference hosts (Mulè *et al.*, 2004; Sampietro *et al.*, 2010).

DNA sequence gene of fungal one of the practicable molecular techniques as well as to determine phylogenetic relationship. In phylogenetic species concept, a species can be differentiated via the smallest phylogenetic clade of individuals that share similar characteristics (Nixon and Wheeler, 1990). This method required the concordance of more than one gene for the success analysis. Phylogenetic species concept also used in taxonomic studies, by comparing DNA sequences of protein sequence and connected among the isolates also can be recognised and estimated from phylogenetic tree (Leslie and Summerell, 2006)

Based on phylogenetic study, about 40 different phylogenetic species of GFSC were recognized by using 28S rDNA, ITS region, mtSSU and β -tubulin gene (O'Donnell *et al.*, (1998). In other study, total nice of phylogenetically distinct species of *F. graminearum* were clearly discriminated using of 11 nuclear genes