GENETICS OF SOUTHEAST ASIAN POPULATIONS AND INTERSPECIFIC HYBRIDS OF *FUSARIUM* SPP.

by

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B.S., Universiti Sains Malaysia, Penang, Malaysia, 2005
M.S., Universiti Sains Malaysia, Penang, Malaysia, 2008

AN ABSTRACT OF A DISSERTATION

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College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Members of the genus *Fusarium* are widely distributed in many geographic regions of the world. This genus includes plant pathogens of many important cereal crops, *e.g.*, wheat, maize, rice and sorghum, and of other native and economically important plants. From culture collections at Kansas State University and Universiti Sains Malaysia, strains from Southeast Asia, primarily from Malaysia and Thailand, associated with mango malformation disease, bakanae disease of rice, and stalk rot of sorghum were analyzed in sexual crosses and molecular diagnostics, *e.g.*, Amplified Fragment Length Polymorphisms (AFLPs). *Fusarium proliferatum* was recovered from all three crops, with each crop also yielding some species unique to the crop, *e.g. F. fujikuroi* from rice, *F. thapsinum* from sorghum, and *F. mangiferae* from mango. These results are consistent with hypotheses that *F. proliferatum* has a wide host range while other species have much more limited host preferences. The absence from our samples of species associated with these diseases in other parts of the world suggests policies should be developed to reduce the chances of introduction of novel pathogens into Southeast Asia. *Fusarium fujikuroi* and *F. proliferatum* are closely related. They usually can be separated by sexual cross-fertility and DNA sequence analysis. However, some strains can cross irregularly and with poor fertility to produce viable interspecific hybrids. From a laboratory cross between *F. fujikuroi* FGSC8932 and *F. proliferatum* FGSC7615, 533 progeny were collected. These progeny were characterized for their AFLP genotype, mating type, gibberellic acid production, and pathogenicity on rice, onions, and apples. A recombination-based map from this interspecific cross was constructed. QTLs associated with gibberellic acid production, rice pathogenicity, and onion pathogenicity were identified. Gene segregation amongst the progeny of the *F. fujikuroi* × *F. proliferatum* cross was distorted towards *F. proliferatum*. Both novel and transgressive pathogenicity

phenotypes were detected. Overall, this research demonstrates the potential threats that can result from an interspecific cross. These threats include pathogens with novel toxin profiles, new pathogenicity phenotypes, and more virulent strains. The variation observed among the progeny may enable isolation and characterization of genetic factors that have a role in pathogenicity, toxin production, and host specificity.

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Dedication

This dissertation is dedicated to my beloved parents Mohamed Nor Abdul Samat and Tengku Hamimah Tengku Abdul Majid, my loving wife Nurulsyaida Mat Saman, and my precious children Nik Izz Ramadhan and Nik Jannah Az-zahra.

Chapter 1 - Literature Review

Fusarium in Southeast Asia

Countries in Southeast Asia (SEA) lie near the equator and have predominantly tropical climates. These climates provide the region with hot, humid weather year round. The predominant food crop in this region is paddy rice. Other food crops grown in SEA are maize, sugarcane, cacao, coffee, coconuts, sorghum, spices, and numerous tropical fruits (www.fao.org). Oil palm and rubber also are important introduced agricultural commodities that are economically important in this region. Both of these crops are cash crops, widely grown in Malaysia, Indonesia, and Thailand. Given the diverse agriculturally important crops and the conducive climate, the study of plant diseases and pathogens in this region is very important to protect the growers and their plant products.

Fusarium spp. are ubiquitous and cosmopolitan across different crops in SEA (Leslie and Summerell 2006; Summerell et al. 1993, 2003). Some of the *Fusarium* species in this region are not found in temperate and sub-temperate regions (Table 1.2) (Burgess and Summerell 1992; Gordon 1960; Summerell et al. 2003). Multiple *Fusarium* species can cause the same disease, e.g. mango malformation, and stalk rot of maize and sorghum (Leslie 2002; Marasas et al. 2006; Otero-Colina et al. 2010). Intensive research on *Fusarium* spp. in SEA has been in progress since the 1970s. Major changes in nomenclature and identification of new *Fusarium* spp. over the past 20 years resulted in misidentification in culture collections, governmental regulations, and academic literature (Table 1.1) (Leslie and Summerell 2006; Salleh 1994; Summerell et al. 2003). Thus, correct identification using modern diagnostic tools and current nomenclature is very important for research on animal and plant diseases, and making risk estimates of potential secondary metabolite contamination.

Fusarium spp. associated with mango

Mango (*Mangifera indica*) is an important commercial fruit that originated in Southeast Asia (Srivastava 1998). It has been cultivated for more than 4000 years in India. The distribution of mango to other parts of the world began in the 1650s by the Portuguese, who spread it to Mexico, Hawaii, South and Central America, the Middle East and Africa (Srivastava 1998). Mango malformation is an important disease of mango caused by *Fusarium* spp. This disease has been known for more than one hundred years and is still a significant economic problem in mango orchards. The disease was first reported in India in 1891 (Kumar et al. 1993), and in the ensuing 120 years has been reported from almost all mango growing countries: Australia (Summerell et al. 2011), Brazil (Lima et al. 2009a, 2012), China (Zhan et al. 2010), Egypt (Youssef et al. 2007), Israel (Britz et al. 2002), Mexico (Otero-Colina et al. 2010), Oman (Kvas et al. 2008), Pakistan (Iqbal et al. 2011), and South Africa (Britz et al. 2002). Several *Fusarium* spp. have been suggested as the causal agent of this disease. *F. mangiferae* is the species most commonly associated with this disease, but other *Fusarium* spp., e.g. *F. sterilihyphosum*, *F. mexicanum*, *F. proliferatum* and *F. tupiense*, are associated with geographically limited outbreaks (Lima et al. 2012; Marasas et al. 2006; Otero-Colina et al. 2010).

Mango malformation disease can cause losses of up to 86% in individual groves (Kumar et al. 1993). The disease may result in vegetative or floral malformation. Vegetative malformation usually occurs on seedlings. Infected seedlings exhibit symptoms such as swollen buds in the leaf axil, scaly leaves with a bunch-like appearance, or branch dieback to give a witches' broom-like appearance. Eventually, severely infected seedlings become stunted and die. Floral malformation occurs during the inflorescence stage. Infected flowers are enlarged, panicle growth increases, and no fruit is produced (Kumar et al. 1993; Marasas et al. 2006).

Fusarium spp. associated with rice

Fusarium fujikuroi is commonly known as the causal agent of Bakanae disease of rice. Bakanae disease was first recognized in Japan in 1828. "Bakanae" is a Japanese word that means "foolish seedling", which describes the elongated seedlings that are a typical symptom of this disease. This disease is widely distributed in Asia, but was first observed in California in 1999 (Carter et al. 2008).

Bakanae-infected rice has multiple symptoms. The most common symptoms are chlorotic, elongated, thin seedlings (Hwang et al. 2013; Wulff et al. 2010; Zainuddin et al. 2008a). Infected rice seedlings are several inches taller than healthy seedlings and are easily observed in the field. Other symptoms of bakanae include stunted, chlorotic seedlings and crown rot in older plants. Usually, the infected seedlings die. However, if older plants are infected, then abnormal elongation of the plants, or normal growth with an empty or no panicle may occur (Ou 1985; Wulff et al. 2010), with yield losses of up to 40% (Desjardins et al. 2000a). Bakanae maybe seedborne, if the ovules are infected during flowering, or soilborne, if the fungus penetrates the germinating seed (Ou 1985).

Other *Fusarium* spp. reported from rice include *F. andiyazi*, *F. verticilliodes*, *F. proliferatum*, *F. sacchari*, *F. subglutinans*, and *F. graminearum* (Amoah et al. 1995; Desjardins et al. 2000a; Hsuan et al. 2011; Kim et al. 2012; Wulff et al. 2010; Zainuddin et al. 2008a). All of these species, except *F. graminearum*, are members of the *Fusarium fujikuroi* species complex. Although *F. fujikuroi* is the primary causal agent for bakanae disease, the presence of other *Fusarium* spp. on bakanae-infected rice raises concerns regarding secondary metabolite production. Excess gibberellic acid (GA_3), a known plant growth hormone, is responsible for elongated seedling growth in rice plants with bakanae disease (Desjardins 2006). Rice also may

be contaminated with mycotoxins such as fumonisins, moniliformin, fusaric acid, and beauvericin produced by *Fusarium* spp. that occur incidentally on rice (Cruz et al. 2013; Wulff et al. 2010).

Fusarium spp. associated with sorghum

Numerous *Fusarium* spp. have been associated with sorghum including: *F. andiyazi*, *F. thapsinum*, *F. proliferatum*, *F. napiforme*, *F. nygamai*, *F. pseudonygamai*, *F. sacchari*, and *F. verticillioides* (Klittich et al. 1997; Leslie et al. 2005a; Marasas et al. 1987, 2001; Palmero et al. 2012). The species most commonly associated with sorghum diseases is *F. thapsinum* (Jardine and Leslie 1992; Leslie et al. 2005a). *F. thapsinum* is the most important causal agent for stalk rot which is one of the most important diseases of sorghum. High incidence of stalk rot in sorghum can result in yield losses of up to 90% (Leslie 2002). Typical symptoms of stalk rot caused by *Fusarium* include shredding and discoloration of the internal stem tissue. The shredded tissue may be red or salmon (Jardine 2006). The diseased stalk usually dies prematurely and often lodges in the field.

Fusarium fujikuroi and *Fusarium proliferatum*

Fusarium fujikuroi and *Fusarium proliferatum* are both members of the *Fusarium fujikuroi* species complex. Based on morphological characteristics, these species are very closely related and often cannot be distinguished from one another (Leslie and Summerell 2006). Phylogenetically, *F. fujikuroi* and *F. proliferatum* are closely related sister taxa (Kvas et al. 2009; O'Donnell et al. 1998b). Differences between these species occur in three categories – sexual cross-fertility, host range, and secondary metabolite production (Table 1.3). *F.*

proliferatum can synthesize numerous secondary metabolites, of which the best known are the fumonisins.

Fumonisins are mycotoxins that can inhibit sphingolipid biosynthesis, induce hepatotoxicity, and elevate serum cholesterol concentrations in animals (Desjardins 2006; Haschek et al. 2001). These mycotoxins can cause pulmonary edema in pigs, and leukoencephalomalacia in horses fed contaminated feed (Desjardins 2006).

Gibberellic acid (GA) is the best known secondary metabolite produced by *F. fujikuroi.* GAs are plant growth hormones, with 136 different GAs described (GA_1 – GA_{136}) (MacMillan 2001). Only a few of these compounds, however, have significant biological activity, including GA_1, GA_3, GA_4, and GA_7, (Tudzynski 1999). GA_3 is the form of gibberellic acid produced by *F. fujikuroi,* and is responsible for the elongated seedlings observed in bakanae rice plants (Aytoun et al. 1959; Sunder and Satyavir 1998). GA_3 also helps *F. fujikuroi* invade the symplasts of parenchyma cells of rice epidermis and cortex (Wiemann et al. 2013). Thus, GA_3 has an important role in the pathogenicity phenotype that results from fungal infection.

Relative to *F. fujikuroi*, *F. proliferatum* has a broad host range and has been recovered from many different crops grown in different environments worldwide. Plants that can be successfully attacked by *F. proliferatum* include important crops such as: apples, asparagus, date palm, mango, onions, orchids, rice, and maize (Abdalla et al. 2000; Hsuan et al. 2011; Konstantinou et al. 2011; Logrieco et al. 1995; Martinez et al. 2002; Stankovic et al. 2007; Tsavkelova et al. 2008; Zhan et al. 2010).

Interspecific hybrids in *Fusarium*

In general, crosses between strains of *F. fujikuroi* and *F. proliferatum* are not cross-fertile, thus, they are grouped in different biological species or mating populations (MP) (Table

1.3). Members of the same biological species are populations that are actually or potentially interbreeding (Mayr 1940). Although a few strains of *F. fujikuroi* and *F. proliferatum* are poorly cross-fertile and can produce viable sexual progeny, their fertility is low enough (Perkins 1994) that they are still considered separate species. Leslie et al. (2004a) recovered 47 viable progeny from a *F. fujikuroi* × *F. proliferatum* cross in which the *F. proliferatum* strain served as the female parent, and found that the segregation ratios of many markers were not the expected 1:1 amongst the progeny. This cross was repeated in 2012 and an additional 486 viable progeny were collected.

The viable progeny from these crosses may have unique phenotypic combinations that can be transgressive in terms of pathogenicity and produce unusual profiles of secondary metabolites (Studt et al. 2012). Evaluation of these progeny enables analysis of genes for pathogenicity on various hosts by generating unusual genotypes that would be rare under field conditions, and are not normally associated with either parental species. Microscopic observations of perithecial contents suggest that the fertility of the *F. fujikuroi* × *F. propliferatum* crosses is approximately 0.01% that of a "normal" *F. fujikuroi* × *F. proliferatum* cross. .

Research Objectives

The objectives of this study were: (i) to determine the species composition and population genetic characters of *Fusarium* from mango, sorghum, and rice in Malaysia and Thailand; (ii) to characterize the genotypes and phenotypes of the progeny from an interspecific cross between *F. proliferatum* and *F. fujikuroi*; (iii) to analyze the segregation of pathogenicity towards apples, onions, and rice amongst the progeny of the interspecific cross; and (iv) to identify regions of the fungal genomes that could be critical for pathogenicity or host specificity.

Table 1.1 *Fusarium* species available in the *Fusarium* Collection, School of Biological Sciences, Universiti Sains Malaysia in December 1994 (Salleh 1994)

Fusarium species	Source
beomiforme	Soil
bugnicourtii	Mangrove, tobacco, watermelon
camptoceras	Asparagus, mango, mullberry, rice, soil
chlamydosporum	Asparagus, banana, broad bean, mango, rice, soil, sorghum, and tobacco
concolor	Mangrove, rice, tobacco
decemcellulare	Durian, eggplant, mango, soil, vanilla
dimerum	Soil
equiseti	Asparagus, banana, broad bean, broccoli, chili, Chinese cabbage, coconut, long bean, mulberry, paprika, peanut, rice, soil, sorghum, soybean, sugarcane, tobacco, tomato, vanilla, watermelon
graminum	Soil, vanilla
lateritium	Eggplant, long bean, corn, mangrove, rice, tobacco
longipes	Asparagus, banana, bitter gourd, cassava, cucumber, long bean, melon, oil palm, rice, soil, soybean, tobacco, watermelon
*moniliforme**	Asparagus, banana, corn, mango, pineapple, rice, soil
nygamai	Asparagus, banana, chili, fire worm, grapes, mango, orchid, rattan, rice, soil, sorghum, sugarcane, watermelon, wheat
oxysporum	Asparagus, banana, cassava, coconut, coffee, cotton, durian, garlic, long bean, oil palm, orchid, passion fruit, potato, pumpkin, red bean, radish, rice, star fruit, sugarcane, tobacco, vanilla, tomato, watermelon, weeds, yam
semitectum	Asparagus, banana, cassava, cempaka, chili, Chinese cabbage, coconut, coffee, eggplant, long bean, corn, mango, mangrove, melon, mulberry, nutmeg, oil palm, onion, papaya, paprika, passion fruit, peanut, purut lime, radish, rice, soil, sorghum, soybean, spinach, star fruit, sugarcane, tea, tomato, watermelon
polyphialidicum	Soil
proliferatum	Asparagus, banana, chili, coconut, corn, mango, orchid, rice, soil,

	sorghum, sugarcane, tobacco, vanilla, watermelon, wheat
scirpi	Asparagus, mango, rice, soil, tobacco
tumidum	Cocoa, rubber, soil

*Name no longer in current use (Seifert et al. 2003)

Table 1.2 Distribution of *Fusarium* species in relation to climate after Burgess et al. (1994)

Species which occur in most climatic regions	Species which occur mainly in temperate regions	Species which occur mainly in subtropical and tropical regions
F. chlamydosporum	*F. acuminatum*	*F. beomiforme*
F. equiseti	*F. avenaceum*	*F. compactum*
F. proliferatum	*F. crookwellense*	*F. decemcellulare*
F. oxysporum	*F. culmorum*	*F. longipes*
F. poae	*F. graminearum*	
F. semitectum	*F. sambucinum*	
F. solani	*F. sporotrichioides*	
F. tricinctum	*F. subglutinans*	

Table 1.3 Major differences between *F. fujikuroi* and *F. proliferatum*

	F. fujikuroi	*F. proliferatum*
Host range	Narrow, specific to rice	Wide host range
Secondary metabolites[a]	Beauvericin, fusaric acid, **gibberellic acid**, moniliformin	Beauvericin, eniatin, **fumonisin**, fusaproliferin, fusaric acid, fusarins, moniliformin

[a]Bold indicates the most important secondary metabolite produced by the species (Desjardins 2006).

Chapter 2 - *Fusarium* Species Associated with Mango Malformation in Peninsular Malaysia

Abstract

Mango malformation has become the most important global disease on mango. *Fusarium* species previously associated with this disease include *F. mangiferae*, *F. mexicanum*, *F. sterilihyphosum*, *F. proliferatum*, *F. subglutinans*, and *F. tupiense*. The strains recovered from Malaysian mango populations were evaluated with morphology, mating tester strain cross-fertility, amplified fragment length polymorphisms (AFLPs), and partial DNA sequences of the genes encoding translation elongation factor 1-α (*tef-1α*) and β-tubulin (*tub-2*). Amongst the 43 strains evaluated three species were identified – *F. proliferatum*, *F. mangiferae*, and *F. subglutinans* – with *F. proliferatum* being the most frequent (29). None of the *Fusarium* species that appear to originate in the Americas were recovered in Malaysia, which suggests special measures may be warranted to keep these species from entering the country.

Introduction

Mango malformation (MM) is the most important disease affecting mango trees (*Mangifera indica*) and was first described in India in 1891 (Kumar et al. 1993; Marasas et al. 2006). The disease has been reported in all areas where mango is cultivated (Kumar et al. 2011; Kvas et al. 2008; Marasas et al. 2006; Youssef et al. 2007; Zhan et al. 2010). There are two stages of MM – vegetative malformation, and inflorescence malformation. Vegetative malformation usually occurs in young seedlings particularly in nurseries. The symptoms in the seedlings include loss of apical dominance, swollen axillary buds, and vegetative buds that

sprout at the internodes. Inflorescence malformation occurs on mature trees at flowering. This form of malformation results in enlargement of the inflorescence, increased panicle growth, and the abortion of fruit production (Kumar et al. 1993; Marasas et al. 2006). Yield loss from this form of the disease can reach 83% (Kumar et al. 1993). This disease is distinct from the galls formed on trunks and branches by *Fusarium decemcellulare* (Ploetz et al. 1996), which is not known to occur in Malaysia.

Several *Fusarium* species have been suggested as the causal agent of mango malformation. Most of the earlier literature identifies *Fusarium subglutinans* or *Fusarium moniliforme* as the causal agent (Kumar et al. 1993; Marasas et al. 2006; Ploetz 1994). These identifications, however, were based solely on morphological characters, which are insufficient to resolve the currently known species. In the Americas, particularly in Brazil and Mexico, *F. mangiferae*, *F. mexicanum*, *F. sterilihyphosum*, *F. subglutinans*, and *Fusarium tupiense* are all associated with this disease (Lima et al. 2009a, 2012; Steenkamp et al. 2000b, Otero-Colina et al. 2010). *F. mangiferae* has been reported to be a causal agent of mango malformation in at least Egypt, Israel, Oman, Spain and the United States (Britz et al. 2002; Crespo et al. 2012; Kvas et al. 2008; Youssef et al. 2007) and *F. proliferatum* as a causal agent of mango malformation in South China (Zhan et al. 2010). In Malaysia, a few strains associated with mango malformation were identified as *F. proliferatum* and *Fusarium* sp. although Koch's postulates were not completed with any of these strains (Britz et al. 2002; Zheng and Ploetz 2002). All of the species associated with mango malformation belong to the *Gibberella fujikuroi* species complex and have similar morphological characters (Kvas et al. 2009; Leslie and Summerell 2006; Lima et al. 2012). These species can be differentiated by using Amplified Fragment Length Polymorphisms (AFLPs), sexual cross-fertility, and DNA sequences of diagnostic genes (Geiser et al. 2004;

Leslie and Summerell 2006; Lima et al. 2012). The correct identification of species associated with mango malformation in Malaysia is very important because the presence and distribution of the various species within the country will affect the country's quarantine regulations.

In this study, we evaluate a broader sample of mango strains collected from Peninsular Malaysia. Thus, the objectives of the study were: (i) to identify the *Fusarium* species associated with mango malformation in Peninsular Malaysia; (ii) to determine the genetic relationship amongst the strains; and (iii) to analyze the genetic diversity within the identified species. We hypothesized that several *Fusarium* species within the *G. fujikuroi* species complex would be present and that *F. mangiferae* would be the dominant species. Our results will guide local horticulturists in their evaluation of mango varieties and the development of regulatory policies for mangoes and related tropical fruits.

Materials and Methods

Strains and morphological studies

Fusarium spp. were isolated from mango inflorescence tissue collected across Peninsular Malaysia. Forty-three strains were recovered and purified by subculturing microconidia separated by micromanipulation. The resulting cultures were preserved as spore suspensions in 15% glycerol at -70ºC.

Morphological characteristics were observed on cultures grown on carnation leaf agar (CLA) (Fisher et al. 1982) for 7 to 10 days at 25ºC with a 12-hour photoperiod under a combination of fluorescent white light and black light. Pigmentation was observed in cultures grown on potato dextrose agar (PDA) (Leslie and Summerell 2006).

DNA isolation

Strains were cultured on complete medium (Correll et al. 1987) slants for seven days. One ml of a spore suspension in a 0.25% Tween 60 solution (~10^6 spores/ml) was used to inoculate 30 ml of complete medium broth in a 125-ml Erlenmeyer flask that was incubated for two days at room temperature (22–26°C) on an orbital shaker (150 rpm). Mycelia were harvested by filtration through a Milk Filter disk (KenAG, Ashland, Ohio), dried by blotting with paper towels, and ground to a powder in a mortar with a pestle under liquid nitrogen. DNA was extracted by using a CTAB procedure (Leslie and Summerell 2006). DNA extracts were stored in a 1.5-ml microcentrifuge tubes at 4ºC until used. The quality of the DNA was evaluated following resolution in a 1% agarose gel, while the DNA concentration was measured with a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware).

Mating type PCR

DNA solutions were diluted to ~20 ng/µl with sterile double-distilled water. The procedure used to identify the mating type alleles was that of Leslie and Summerell (2006), which follows that of Steenkamp et al. (2000a). The products of the PCR amplification were separated on a 1% agarose gel. The size of the amplified DNA products were used to identify the mating type of the strain.

Biological species and female fertility

Strains of *F. proliferatum* from the samples were crossed with standard tester strains FGSC 7614 and FGSC 7615, and strains of *F. subglutinans* were crossed with standard tester strains FGSC 7616 and FGSC 7617. The sexual stage of *F. mangiferae* is not known and no crosses were made with the strains assigned to this species. Standard tester strains are available

from the Fungal Genetics Stock Center (Dept. of Plant Pathology, Kansas State University). Field strains were tested as both male and female parents in crosses with the standard testers after the mating-type alleles were determined through PCR analyses. Sexual crosses were made on carrot agar as described by Klittich and Leslie (1988). Fertility was determined by the presence of numerous perithecia with a cirrus of ascospores oozing from the perithecia 2 – 4 weeks after fertilization.

AFLP reaction and analysis

DNA fingerprinting was conducted by using AFLPs (Vos et al. 1995) following the protocol described in Leslie and Summerell (2006). Three primer pairs were used in the selective amplification: *Eco*RI + GG/*Mse*I + CT, *Eco*RI + AA/*Mse*I + TT, and *Eco*RI + TT/*Mse*I + AC. Bands 200–500 bp in size were scored manually based on the presence or absence of a band. Fragments of the same size were assumed to be homologous. Similarities between all strains were analyzed by the neighbor joining (NJ) clustering option of PAUP (version 4.10b; D. L. Swofford, Sinauer Associates, Sunderland, Mass.) with 1000 bootstrapping replications. The genetic distance and similarities between the species were calculated with the Dice coefficient by using SAS (SAS Institute, Cary, N.C.). Reference strains in the AFLP analysis included: *F. proliferatum* (FGSC 7614 and FGSC 7615), *F. fujikuroi* (KSU 1993 and KSU 1994), *F. mangiferae* (KSU 11781), *F. pseudocircinatum* (KSU 10761), *F. sterilihyphosum* (KSU 16215), *F. subglutinans* (FGSC 7616 and FGSC 7617), and *F. tupiense* (KSU 16197 and KSU 16231).

DNA sequencing

Partial gene sequences of translation elongation factor 1α (*tef-1α*) and β-tubulin (*tub-2*) were analyzed for strains other than *F. proliferatum*. DNA samples were diluted to ~20 ng/µl.

The primer sequences used for *tef-1α* were EF-1 (forward: 5′-ATGGGTAAGGAGGACAAGAC-3′) and EF-2 (reverse: 5′-GGAAGTACCAGTGATCATGTT-3′) (O'Donnell et al. 1998a). The conditions for the *tef-1* amplification were: 94ºC for 1 min., followed by 34 cycles of 94ºC for 30 sec., 62ºC for 45 sec., and 72ºC for 1 min., and then 4ºC until analyzed. The primer sequences for *tub-2* were T1 (forward: 5′-AACATGCGTGAGATTGTAAGT-3′) and T2 (reverse: 5′-TAGTGACCCTTGGCCCAGTTG-3′) (O'Donnell and Cigelnik 1997). The amplification program for *tub-2* was: 94ºC for 1 min., followed by 34 cycles of 94ºC for 30 sec., 61ºC for 45 sec., and 72ºC for 1 min., and then 4ºC until analyzed. The amplification products for *tef-1* and *tub-2* were cleaned with ExoSAP-IT (Affymetrix, Cleveland, OH). We used an Applied Biosystems 3730 DNA Analyzer for sequencing at the Kansas State University sequencing facility. These sequences were analyzed by using BioEdit version 7.0.5.3 (Hall 1999) and BLASTed against GenBank (http://blast.ncbi.nlm.nih.gov/) and *Fusarium* Database (http://isolate.fusariumdb.org/).

Results

Species Identification

Three species were identified based on morphology, molecular characters, and mating population. *F. proliferatum* was the most common species found from the mango population in peninsular Malaysia composing 69% of the population followed by *F. mangiferae* (26%) and *F. subglutinans* (5%). Morphologically, *F. proliferatum* produces chains of microconidia while *F. mangiferae* and *F. subglutinans* produce microconidia only in false heads (Leslie and Summerell 2006). Neither *F. mangiferae* nor *F. subglutinans* produce coiled hyphae. *F. mangiferae* and *F.*

subglutinans were distinguished based on AFLP fingerprints (Figure 2.1), and the differences confirmed by sequencing diagnostic genes. One hundred and twenty-four AFLP bands were evaluated by scoring all of the fragments between 200 and 500 bp in length produced following amplification with any of the three primer pairs. Strains in the same species shared ≥ 70% of the AFLP bands. Sexual crosses with *F. proliferatum* and *F. subglutinans* testers also were used to confirm species identifications.

Based on AFLP patterns, there were two clonal pairs of *F. proliferatum* strains – 21170 & 21181, and 21171 & 21175 (Figure 2.1). Strains 21170 and 21181 are both *MAT-1* but are from different locations, Penang and Johor, respectively, and differ in their fertility, as 21170 is female-fertile and 21181 is not (Table 2.1). Strains 21171 and 21175 are both *MAT-2* and are from the same location in Penang. These strains differ in fertility as 21171 is fertile as neither a male nor a female parent while 21175 is fertile as a male parent (Table 2.1). The apparent clonal identity of 21171 and 21175 is another reason to classify 21171 as *F. proliferatum*, even though it did not cross with the tester strains.

Mating type and cross fertility

Both mating types were detected amongst the strains of *F. proliferatum*, of which 17 were *MAT-1* and 12 were *MAT-2* (Table 2.1). Twenty of the *F. proliferatum* strains were male-fertile and female-sterile, six were fertile as both the male and the female parent in a cross, and three were not fertile as either the male or the female parent and produced barren perithecia without ascospores. Two of the non-fertile strains (21143 and 21145) are both *MAT-1* and from Johor, while the third non-fertile strain (21171) was *MAT-2* and from Penang. These strains are not closely related to one another based on their AFLP profiles, which are all consistent with them being members of *F. proliferatum* and not members of another species (Figure 2.1).

Both mating types also were present amongst the *F. mangiferae* strains, of which two were *MAT-1* and nine were *MAT-2*. No fertility tests were conducted with the *F. mangiferae* strains as no sexual stage is known for this fungus and no mating type tester strains are available. Both of the strains of *F. subglutinans* were *MAT-2* and fertile as the male parent, but infertile as the female parent, in crosses with FGSC 7616 (Table 2.1).

Discussion

This report extends the evaluation of *Fusarium* spp. isolated from mangoes in Malaysia that was begun by Britz *et al*. (2002). These authors also identified two *Fusarium* strains isolated from malformed mango inflorescences in Malaysia as *F. mangiferae*. In the current study, *F. proliferatum* was the dominant species followed by *F. mangiferae* and then *F. subglutinans*. Morphologically, it is difficult to distinguish *F. mangiferae* and *F. subglutinans*; however, these species were clearly distinguished with AFLP fingerprints by their clustering patterns on a tree (Figure 2.1). We included other *Fusarium* spp. associated with mango malformation, *e.g.*, *F. tupiense* and *F. sterilihyphosum*, and other closely related species, *e.g.*, *F. fujikuroi* and *F. pseudocircinatum*, as controls in this tree. We also confirmed the identification of *F. subglutinans* and *F. mangiferae* strains with partial sequences of the *tef-1α* gene. *F. sterilihyphosum* is known so far only from Brazil and South Africa (Britz et al. 2002; Lima et al. 2009a, 2012; Zheng and Ploetz 2002), while *F. mangiferae* has been isolated from mango malformation in many locations worldwide. Koch's postulates confirming pathogenicity have been completed with both *F. mangiferae* and *F. sterilihyphosum* (Lima et al. 2009a). A new species, *F. mexicanum*, has been described and shown to cause vegetative malformation on mango in Mexico (Otero-Colina et al. 2010). *F. proliferatum* has been reported to cause mango malformation in South China (Zhan et al. 2010); however, these authors used only morphology

and rDNA-ITS sequences to make their identifications. Some species in the *Fusarium fujikuroi* species complex have non-orthologous copies of the rDNA-ITS region that can lead to identification errors if this region is used for species identification purposes (O'Donnell and Cigelnik 1997). Based on our results, *F. proliferatum* may be the most important causal agent for mango malformation in Malaysia and in neighboring countries.

The role of *F. subglutinans* in mango malformation remains unsettled. With the description of four *Fusarium* species that are morphologically similar to *F. subglutinans* – *F. mangiferae*, *F. mexicanum*, *F. sterilihyphosum* and *F. tupiense* – most researchers decided that the *F. subglutinans* name had been misapplied to strains from one or more of these species. At present, the name *F. subglutinans* is used for members of mating population E of the *G. fujikuroi* species complex, also known as *Gibberella subglutinans* (Leslie 1991; Samuels et al. 2001). The current usage of the name *F. moniliforme* var. s*ubglutinans* for the strains that cause mango malformation, *e.g.*, Kumar et al. (2011) should be discontinued, as recommended for the name *F. moniliforme* (Seifert et al. 2003), and the strains so named identified as one of the four described sibling species known from mango or as a new species. We identified two strains of *F. subglutinans* in this study, which again raises the question of the ability of members of this species to induce or contribute to mango malformation. We confirmed the identity of these strains with both sexual crosses and partial sequences of the *tef-1α* gene. As these strains were collected from different locations, the association between *F. subglutinans* and mango malformation may be biologically significant. Koch's postulates now need to be completed for these strains to determine if strains that have been accurately described as *F. subglutinans* can cause mango malformation.

Genetic variation has not been well-studied in populations of *Fusarium* species that cause mango malformation. Lima et al. (2009b) and Zheng & Ploetz (2002) both evaluated VCG diversity in populations of *F. mangiferae*, *F. sterilihyphosum*, and *F. tupiense*. Zheng & Ploetz (2002) identified six VCGs within *F. mangiferae* and one in *F. sterilihyphosum*. Members of a single VCG of *F. mangiferae* were found in as many as four countries, and there was heterogeneity for RAPD bands within some of the *F. mangiferae* VCGs. Lima et al. (2009b) identified a second VCG in *F. sterilihyphosum* and six VCGs in *F. tupiense*, all from Brazil. Strains in the same VCG with the same AFLP banding pattern could be recovered from geographically distant locations. We did not use VCGs as a measure of diversity in the present study. Our AFLP analyses identified considerably more genotypic variation than did the earlier studies, particularly within *F. mangiferae*, for which we identified no two strains that were clones based on AFLPs. This result is quite different from the previously reported results where putatively identical strains were recovered from different continents. In general, our results are consistent with the hypothesis that the clones that do exist are unlikely to dominate a population and that most strains will differ genetically from one another. The recovery of putative clones of *F. proliferatum* from sites in Penang and Johor separated by 800 km is consistent with the hypothesis that human movement of either infected plants or contaminated fruit play an important role in the dispersal of the pathogens associated with mango malformation.

Most of the strains of *F. proliferatum* that we recovered were not hermaphrodites (79%), but were fertile only as males, which is a somewhat higher percentage than that observed for a larger, more cosmopolitan sample of strains of *F. proliferatum* (70%; Leslie 1995). The lower number of hermaphrodites (21%) in the present study results in a N_e (effective population number), as calculated with the equations of Leslie and Klein (1996), of 57% of the count, and

suggests ~1%, or less, of the population is participating in sexual reproduction at any one time. Thus, sexual reproduction by *F. proliferatum* strains on mango probably is not a significant part of the life cycle on this host. The genotypic variation we observed, which normally would occur as a result of sexual reproduction, could easily arise if strains of *F. proliferatum* from other native or cultivated hosts are moving to mango and can cause mango malformation. The relative lack of sexual reproduction by *F. proliferatum* from mango is consistent with the life cycles of several other *Fusarium* species that are pathogenic to mango. None of *F. mangiferae*, *F. mexicanum* or *F. sterilihyphosum* has a reported sexual stage, and female-fertile strains of *F. tupiense* are rare.

F. mangiferae is firmly established as a cause of mango malformation through the completion of Koch's postulates and through detailed cytological examinations of infected tissues (Freeman et al. 1999; Iqbal et al. 2010). Spores are dispersed by wind and travel up to 35 m, with the number of spores released sensitive to the time of year and relative humidity, but not to the time of day (Gamliel-Atinsky et al. 2009). Spores can survive in soil for six months and on the soil surface for shorter periods of time (Youssef et al. 2007), especially under hot, dry conditions. Mango fruits from a tree with mango malformation usually are contaminated on the surface with *Fusarium* conidia, and mango seedlings growing under trees with mango malformation usually also are infected by the fungus. Vegetative contamination is most common at the apical growing point and lessens as the distance to the apical bud increases. Root infection has no clear role in mango malformation as the fungus apparently does not systemically colonize the plant and is not known to be seedborne. Thus, human movement of contaminated fruit and infected seedlings is likely a very important mechanism for the long distance dispersal of these pathogens.

Malaysia produced ~23,000 metric tonnes of mangoes in 2010, ranking 45th in world mango production, and imported a similar amount (25,700 tonnes in 2007) from Thailand and India (http://agriexchange.apeda.gov.in/Market). There are no estimates available for the losses due to mango malformation in Malaysia, or in other countries in Southeast Asia. Losses of 50-80% due to mango malformation, such as those reported by Kumar et al. (2011), however, are very rarely reported. Free cross border agricultural trade between countries in South and Southeast Asia could help disperse the pathogens already in the region and those that might enter from elsewhere. With Malaysia's neighbors, Thailand and Indonesia, ranked 3rd and 4th, respectively in world production (www.faostat.fao.org/) the opportunity of movement between countries in the Southeast Asian region is good. *F. mexicanum*, *F. sterilihyphosum*, and *F. tupiense* are not currently known in Southeast Asia, and their introduction from the Americas, mostly likely through asymptomatic plant cuttings or seedlings, should be avoided through strict quarantine and enforcement.

Australia also is increasing its share of the mango export market. Mango malformation has been reported sporadically in Queensland and the Northern Territories (http://www.dpi.qld.gov.au/4790_15965.htm). The causal agent was identified as *F. mangiferae* and the affected trees were destroyed, presumably solving the problem and removing the pathogen from the country. Australia faces the same concerns as Malaysia with respect to risks posed by imports from the Americas for the three *Fusarium* species reported only from there. *F. proliferatum* is endemic to Australia (Summerell et al. 2011) and could cause mango malformation there, which would not present any new or unusual risk for Malaysia. *Fusarium* sp. (NRRL 25807) is closely related phylogenetically to *F. sterilihyphosum* and originates from Australian forest soil (Marasas *et al.*, 2006). Thus, there is a risk that this closely related species

could move from its native host to mangoes in a manner paralleling that which occurred when strains in unique VCGs of *F. oxysporum* f. sp. *vasinfectum* moved from native Australian *Gossypium* spp. to Australian cultivated cotton in the early 2000s (Wang et al. 2004, 2010). Consequently, Australian outbreaks of mango malformation are of particular significance for Malaysia as they could result from a *Fusarium* species not already known in the country.

In conclusion, mango malformation in Malaysia can occur as a result of at least three different *Fusarium* species. Transport within the country is probably through the movement of infected plantings or contaminated fruit. The lack of recovery of any of the three species so far reported from only the Americas, suggests that particular care should be exercised in the movement of plant materials from the Americas to Malaysia (and neighboring countries) to reduce the opportunity for the introduction of novel plant pathogens into the region. Movement of plant materials and fruits from Australia also should be monitored to determine if the cause of an outbreak of mango malformation there is the result of an already described species. Finally, the ability of *F. proliferatum* to cause disease on plants as diverse as maize, onions and mangoes is worthy of further study to discern how this fungus causes so much damage to such a broad array of plants.

Table 2.1 Characters of *Fusarium* strains collected from malformed mango inflorescences in peninsular Malaysia.

KSU Number	*MAT* Allele	Fertility	Mango Variety	Geographic Origin
F. proliferatum				
21143	1	Barren[a]	Chokkanan	Kg. Gambut, Penawar, Johor
21145	1	Barren	Chokkanan	Kg. Gambut, Penawar, Johor
21146	1	♂, ♀	Chokkanan	Kg. Gambut, Penawar, Johor
21147	1	♂, ♀	Chokkanan	Kg. Gambut, Penawar, Johor
21149	2	♂	Epal	Relau, Penang
21150	2	♂	Epal	Relau, Penang
21151	2	♂	Chokkanan	Kg. Gambut, Penawar, Johor
21153	1	♂, ♀	Epal	Sg. Nibong, Penang
21156	1	♂	Magolba	Air Hitam, Penang
21157	1	♂, ♀	Chokkanan	Kg. Gambut, Penawar, Johor
21159	1	♂	Epal	Relau, Penang
21160	2	♂	Epal	Air Itam, Penang
21161	2	♂	Epal	Century Garden, Penang
21164	1	♂, ♀	Epal	Century Garden, Penang
21165	1	♂	Epal	Century Garden, Penang
21166	1	♂	Epal	Relau, Penang
21170	1	♂, ♀	Telur	Brown Garden, Penang
21171	2	Barren	Telur	Brown Garden, Penang
21172	2	♂	Telur	Brown Garden, Penang

KSU Number	*MAT* Allele	Fertility	Mango Variety	Geographic Origin
21173	1	♂	Telur	Brown Garden, Penang
21174	1	♂	Telur	Brown Garden, Penang
21175	2	♂	Chokkanan	Kg. Gambut, Penawar, Johor
21177	2	♂	Siam	Century Garden, Penang
21178	1	♂	Siam	Century Garden, Penang
21179	2	♂	Epal	Paya Terubung, Penang
21180	1	♂	Epal	Paya Terubung, Penang
21181	1	♂	Chokkanan	Kg. Gambut, Penawar, Johor
21183	2	♂	Chokkanan	Kg. Gambut, Penawar, Johor
21185	2	♂	Chokkanan	Kg. Gambut, Penawar, Johor
F. mangiferae				
21144	2	-[b]	Chokkanan	Kg. Gambut, Penawar, Johor
21148	2	-	Chokkanan	Kg. Gambut, Penawar, Johor
21154	1	-	Epal	Sg. Nibong, Penang
21155	2	-	Chokkanan	Kg. Gambut, Penawar, Johor
21158	2	-	Siam	Century Garden, Penang
21162	2	-	Epal	Century Garden, Penang
21163	2	-	Epal	Century Garden, Penang
21168	1	-	Telur	Permatang Tinggi, Penang
21169	2	-	Telur	Tanjung Bunga, Penang
21176	1	-	Chokkanan	Kg. Gambut, Penawar, Johor
21182	2	-	Chokkanan	Kg. Gambut, Penawar, Johor

KSU Number	*MAT* Allele	Fertility	Mango Variety	Geographic Origin
21184	2	-	Chokkanan	Kg. Gambut, Penawar, Johor
F. subglutinans				
21142	2	♂	Chokkanan	Kg. Gambut, Penawar, Johor
21167	2	♂	Epal	Sg. Ara, Penang

[a]Barren – numerous perithecia, but no ascospore cirrhi. [b]No data.

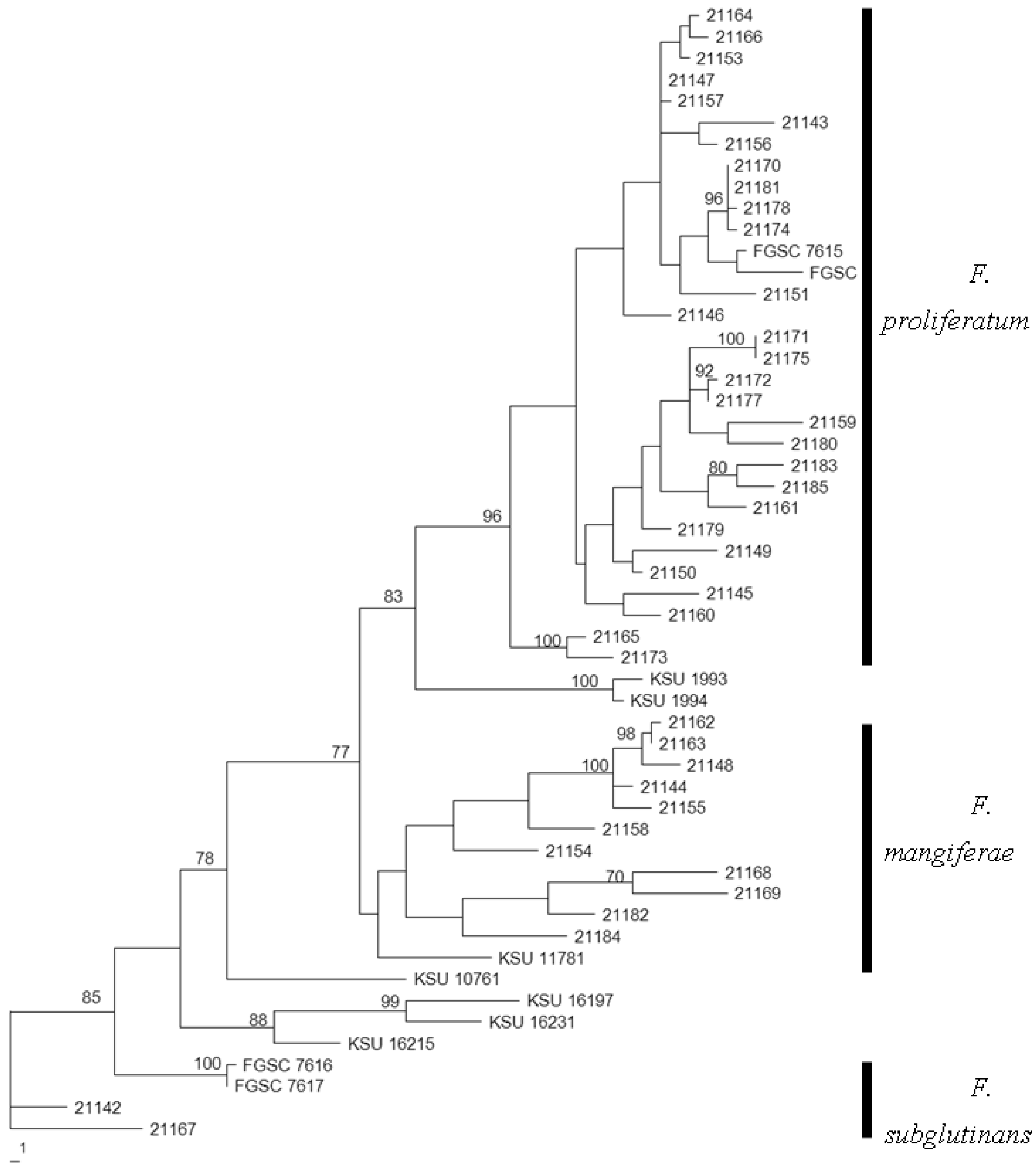


Figure 2.1 Tree generated by the neighbor-joining distance method based on similarities of AFLP marker profiles of strains collected from malformed mango inflorescences.

Bootstrap values ≥ 70% based on 1000 replications are noted. In addition to the species labeled in the diagram the following additional species are represented: *F. fujikuroi* (KSU 1993 and KSU 1994), *F. pseudocircinatum* (KSU 10761), *F. sterilihyphosum* (KSU 16215) and *F. tupiense* (KSU 16197 and KSU 16231).

Figure 2.2 Relationship of the strains collected from malformed mango inflorescences to reference strains.

Reference strains: *F. fujikuroi* (KSU 1993 and KSU 1994), *F. mangiferae* (KSU 11781), *F. proliferatum* (FGSC 7614 and FGSC 7615), *F. pseudocircinatum* (KSU 10761), *F. sterilihyphosum* (KSU 16215), *F subglutinans* (FGSC 7616 and FGSC 7617), and *F. tupiense* (KSU 16197 and KSU 16231). This unrooted tree was generated by the neighbor-joining distance method based on the AFLP markers.

Chapter 3 - *Fusarium* species from Sorghum in Thailand

Abstract

Some of the most important diseases of sorghum are stalk rot and grain mold, both of which have a *Fusarium* causal agent. Numerous *Fusarium* species also have been isolated from sorghum, including *F. andiyazi*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, *F. pseudonygamai*, *F. thapsinum*, and *F. verticillioides*, and several as yet undescribed species. The general lack of information on sorghum pathogens in Southeast Asia and the revisions to the nomenclature of the relevant *Fusarium* species over the past 20 years are both strong reasons to re-evaluate *Fusarium* isolates from Southeast Asia and to determine if the species distribution in Southeast Asia is comparable to that observed in other better-studied locations where sorghum is grown. The objective of this study was to identify the *Fusarium* species present on sorghum growing in Thailand. Sixty-eight isolates were identified and assigned to one of five species: *F. proliferatum*, *F. verticillioides*, *F. thapsinum*, *F. beomiforme* and *F. sacchari*. This report is the first of *F. thapsinum* from Southeast Asia. Three species were common – *F. proliferatum* (35%), *F. verticillioides* (31%), and *F. thapsinum* (29%), all of which are known on sorghum elsewhere. These species are known as toxin producers, thus, increasing the risk of toxin-contaminated seeds. The absence of some species commonly associated with sorghum e.g. *F. andiyazi*, suggests that the movement of sorghum into Thailand and other neighboring countries should be monitored to avoid the introduction of new pathogens into the region.

Introduction

Sorghum (*Sorghum bicolor*) is an important crop for human consumption, animal feed, and bioenergy. It is the fifth most important cereal in the world after wheat, rice, maize, and barley. In Thailand, sorghum is the 3rd most important cereal crop and is grown in all regions of the country, but most commonly in the central and northeastern regions. Much of the sorghum grown in Thailand is exported, the country ranks 18th in the world for sorghum exports (http://www.indexmundi.com/agriculture/). Some also is used as animal feed and for ethanol production (Ariyajaroenwong et al. 2012; Boon Long 1992; Nuanpeng et al. 2011). The average production of sorghum from 2006 to 2012 was 54,500 tons per year, a decrease of more than 50% since 2000 (http://faostat.fao.org). *Fusarium* spp. from sorghum have been poorly differentiated and described at the field level, although there has been great progress in identifying and differentiating species on this host over the last 20-30 years. In many parts of the world, the only *Fusarium* species reported to occur on sorghum is *Fusarium moniliforme*. This name was retired in 2003 (Seifert et al. 2003), as is now known to refer to some 15-50 different species. From Thailand, only *F. moniliforme* has been reported (Boon Long 1992; Salleh et al. 1995).

Some of the most important diseases of sorghum are stalk rot and grain mold, both of which have a *Fusarium* causal agent (Bandyopadhyay et al. 2000; Little et al. 2012; Marasas et al. 2001). Stalk rot can cause yield losses up to 90%, although year-to-year losses are usually much less (Leslie 2002). Numerous *Fusarium* species also have been isolated from sorghum, including *F. andiyazi*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, *F. pseudonygamai*, *F. thapsinum*, and *F. verticillioides* (Leslie et al. 1990; Leslie et al. 2005a; Marasas et al. 1987, 2001; Nelson et al. 1987) and several other species that have not yet been described. Some of

these species produce fumonisins and other secondary metabolites, which are toxic to humans and domesticated animals (Desjardins 2006). Sorghum growing in Southeast Asia is grown under very different climatic conditions than found in Africa, the Americas or Australia, where most work on sorghum pathology has occurred. The indigenous fungal populations in Southeast Asia also differ from those recovered elsewhere, as do the rice-focused cropping systems. The general lack of information on sorghum pathogens in this region and the revisions to the nomenclature of the relevant *Fusarium* species over the past 20 years are both strong reasons to re-evaluate *Fusarium* isolates from Southeast Asia and to determine if the species distribution in Southeast Asia is comparable to that observed in other better-studied geographic locations where sorghum is grown.

The objective of this study was to identify the *Fusarium* species present on sorghum growing in farmers' fields in Thailand. We hypothesize that *Fusarium* species within the *Fusarium fujikuroi* species complex associated with sorghum in other parts of the world will be present, although many of these species and their relative frequencies have not previously been reported from Southeast Asia. Our data can be used to develop or adapt disease management strategies and to estimate risks of mycotoxin contamination to sorghum grain produced in this region.

Materials and Methods

Isolates and culture condition

Fusarium spp. were isolated from heads and stalks of healthy sorghum plants at five locations in Thailand (Table 3.1). Sixty-eight isolates were recovered and purified by

subculturing microconidia separated by micromanipulation. The resulting cultures were preserved as spore suspensions in 15% glycerol at -70ºC.

Reference strains used in this study were *F. verticillioides* (FGSC7415 and FGSC7416), *F. sacchari* (FGSC7419 and FGSC7420), *F. fujikuroi* (KSU1993 and KSU1994), *F. proliferatum* (FGSC7422 and FGSC7421), *F. subglutinans* (FGSC7616 and FGSC7617), *F. thapsinum* (FGSC7056 and FGSC7057) and *F. nygamai* (KSU5112), and *F. circinatum* (FGSC9022 and FGSC9023) [FGSC – Fungal Genetics Stock Center, Kansas State University; KSU – Department of Plant Pathology, Kansas State University].

DNA isolation

Isolates were cultured on complete medium (Leslie and Summerell 2006) slants for seven days. One ml of a spore suspension in 0.25% Tween® 60 solution (~10^6 spores/ml) was used to inoculate 30 ml of complete medium broth in a 125 ml flask, and then cultured for two days at room temperature (24-26°C) on an orbital shaker (150 rpm). Mycelia were harvested by filtration through a Milk Filter disk (KenAG, Ashland, Ohio), dried by blotting with paper towels and ground to a powder in a mortar with a pestle under liquid nitrogen. DNA was extracted by using a CTAB protocol (Leslie and Summerell 2006). DNA extracts were stored in 1.5-ml microcentrifuge tubes at 4ºC until used. The quality of DNA was evaluated following separation on a 1% agarose gel. DNA concentrations were measured with a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Delaware).

Mating type PCR

DNA solutions were diluted to ~20 ng/µl with sterile double-distilled water. The MAT alleles were amplified by PCR as previously described (Leslie and Summerell 2006), with the

primers developed by Steenkamp et al. (2000a) and Kerényi et al. (2004). PCR amplification products were resolved on a 1% agarose gel, and the bands present were used to identify the mating type.

Biological species and female fertility

Isolates of *F. proliferatum, F. sacchari, F. thapsinum*, and *F. verticillioides* were crossed with standard, female-fertile, tester isolates. Sexual crosses were made on carrot agar as previously described (Leslie and Summerell 2006). Fertility was determined by the presence of perithecia exuding a cirrhus of ascospores 2-4 weeks after fertilization. Positive crosses were repeated twice and negative crosses were repeated three times.

Field isolates were tested as both male and female parents in crosses with the standard testers after the *MAT* allele in the field isolate was determined. Male fertility was scored in crosses in which the field isolate was the male and the standard tester strain was the female parent. Female fertility was tested in crosses in which the field isolate was the female parent and the standard tester strain was the male.

AFLP reactions and analysis

DNA fingerprints were generated by using AFLPs (Vos et al. 1995) following the protocol of Leslie and Summerell (2006). Three primer pairs were used in the selective amplification: *Eco*RI + GG/*Mse*I + CT, *Eco*RI + AA/*Mse*I + TT, and *Eco*RI + TT/*Mse*I + AC. Bands 200-500 bp in size were scored manually based on the presence or absence of the band. Individual bands were presumed to represent alleles at single loci. Similarities between all strains were analyzed by the neighbor joining clustering option of PAUP (version 4.10b; D. L. Swofford, Sinauer Associates, Sunderland, Massachusetts) with 1000 bootstrapping replications.

The estimates of variance for the AFLP genotypes within and among populations were calculated by using analysis of molecular variance (AMOVA) as installed in GenAlEx 6.5 (Peakall and Smouse 2012).

DNA sequencing

Partial gene sequences of translation elongation factor 1α (*tef-1*α) were analyzed for several selected isolates in each major cluster identified following AFLP analysis. DNA samples for sequencing were diluted to ~20 ng/µl. The primer sequence used for *tef-1*α was EF-1 (forward: 5′-ATGGGTAAGGAGGACAAGAC-3′) and EF-2 (reverse: 5′-GGAAGTACCAGTGATCATGTT-3′) (O'Donnell et al. 1998a). The conditions for the *tef-1*α fragment amplification were: 94ºC for 1 min., followed by 34 cycles of 94ºC for 30 sec., 62ºC for 45 sec., and 72ºC for 1 min., and then 4ºC indefinitely. The amplification products obtained were cleaned with the ExoSAP-IT (Affymetrix, Cleveland, Ohio) following the manufacturer's protocol. DNA sequences were obtained by using an Applied Biosystems 3730 DNA Analyzer at the K-State Sequencing facility. The DNA sequences were analyzed with BioEdit and BLASTed against NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Fusarium Database (http://isolate.fusariumdb.org/index.php).

Results

Identification and species distribution

All 68 isolates could be assigned to one of five species: *F. proliferatum*, *F. verticillioides*, *F. thapsinum*, *F. beomiforme* and *F. sacchari*. Species identity was based on at least two of: AFLP analysis, partial gene sequence of *tef-1,* and cross fertility with standard tester strains. Three species were common – *F. proliferatum* (35%)*, F. verticillioides* (31%), and *F. thapsinum*

(29%) (Table 3.4). *Fusarium proliferatum* and *F. thapsinum* were the only species recovered from sorghum heads (seeds) (Table 3.4), although all five species were recovered from stalks. *Fusarium proliferatum* dominated in samples from sorghum heads, but was found at only three of the five locations. *Fusarium thapsinum* and *F. verticillioides* were more widely distributed, being recovered from four locations (Table 3.4), but only *F. thapsinum* was recovered from the head. *Fusarium beomiforme* and *F. subglutinans* were both recovered from only a single site – Phai Sali (Table 3.4). Four species were recovered at this site, while only two or three species were recovered from each of the other sites.

AFLP analyses

Seventy-eight polymorphic bands were scored manually as the presence or absence of a band. All of the isolates from the same species clustered in unique clades in an unrooted tree (Figure 3.1). Reference strains were included in the analysis. Identify of isolates that did not cluster with the reference strains, e.g. strains of *F. beomiforme*, were confirmed by using the partial sequence of the *tef-1* genes.

Analysis of molecular variance (AMOVA) based on the AFLP bands was calculated for *F. verticillioides*, *F. proliferatum*, and *F. thapsinum* (Table 3.3). The genetic variation for *F. proliferatum* and *F. thapsinum* was higher within locations than it was among them at 68% and 83%, respectively. In contrast, the variation observed for *F. verticillioides* was almost equally distributed with 53% within and 47% among locations.

Mating type and female fertility

Mating type and female fertility was scored for all species except *F. beomiforme*, which lacks a known sexual stage (Table 3.2). Both isolates of *F. beomiforme* carry the *MAT-2* allele.

The *MAT* alleles in *F. proliferatum* and *F. verticillioides* were both present in roughly equal frequencies. In *F. thapsinum*, there were significantly ($\chi^2 = 5.0$; $p \leq 0.05$) more strains with the *MAT-1* genotype than with the *MAT-2* genotype. The only isolate of *F. subglutinans* was *MAT-1*. Female fertility in *F. verticillioides* was relatively high, with 62% of the strains being female fertile, while 38% were female sterile. Amongst strains of *F. proliferatum* and *F. thapsinum*, female fertility was low, at 17% and 15%, respectively.

Discussion

All five species recovered in this study are known to be associated with sorghum, but not necessarily pathogens. The three dominant species, *F. proliferatum*, *F. verticillioides*, and *F. thapsinum,* recovered from this research are commonly associated with sorghum (Leslie et al. 1990; Leslie 2002; Lincy et al. 2011; Sharma et al. 2011). *Fusarium thapsinum* is a major pathogen that can cause stalk rot and grain mold of sorghum (Jardine and Leslie 1992; Klittich et al. 1997; Leslie et al. 2005a; Onyike and Nelson 1992). Sorghum also is a preferred host for *F. thapsinum*.

The higher frequencies of *F. proliferatum* and *F. verticillioides* observed in this study differ from previous studies of sorghum grown in the United States and Tanzania (Leslie 2002), where these frequencies were much lower. In Thailand, sorghum usually is planted as a second crop following maize (Salleh et al. 1995). Thus, the increased frequency of *F. proliferatum* and *F. verticilloides* could result from inoculum build up in the previous crop. In maize, both *F. proliferatum* and *F. verticillioides* can cause stalk and cob rots (Logrieco et al. 1995, 2002). In sorghum, *F. verticillioides* can cause stalk and root rot in the greenhouse (Jardine and Leslie 1992; Palmero et al. 2012), and *F. proliferatum* can cause grain mold (Martinez et al. 2002). *F. proliferatum* has a wide host range and could adapt and survive on many hosts commonly

planted in rotation. The sorghum/maize crop rotation practiced in Thailand could increase the incidence of stalk and cob rot in maize, and a systematic evaluation of diseases in this system is needed to define disease frequency and risks. The frequent recovery of these two species may indicate that these species are of pathogenic importance to sorghum grown in Southeast Asia.

Small numbers of *F. sacchari* and *F. beomiforme* were isolated in this study. *F. sacchari* is not well-known for its pathogenicity to sorghum, however, the species has commonly been isolated from diseased sorghum (Leslie et al. 2005b; Sharma et al. 2011), and could be a potential pathogen (Petrovic et al. 2013). Thus, the *F. sacchari* strains recovered in this study also could potentially be sorghum pathogens. *F. beomiforme* is the only species recovered in this study not belonging to the *Fusarium fujikuroi* species complex. This species is excluded from the species complex based on the phylogenetic analysis. This species is common in soil and soil debris in Australia, South Africa, and Papua New Guinea (Nelson et al. 1987; O'Donnell et al. 1998b). In Papua New Guinea, *F. beomiforme* was found in soil in which sorghum had been cultivated (Nelson et al. 1987). To date, this species has not been reported to be pathogenic to sorghum. This report is the first of the recovery of *F. beomiforme* from sorghum tissue.

Isolates from the same species cluster together in the phylogenetic tree and share more than 70% of the AFLP bands. Based on the AFLPs, two strains of *F. verticillioides* and *F. thapsinum,* and 4 strains of *F. proliferatum*, all appear to be clonal. Members of clonal groups are all from the same location which is consistent with a hypothesis that there is little or no movement of strains amongst these locations. Based on population analysis, sexual reproduction is important for *F. verticillioides*, as the N_e for female fertility is 94% of the count. Frequent sexual reproduction could increase the genotypic diversity of the population and could lead to more virulent strains towards maize, sorghum or other hosts. In comparison to the composite

global population of Leslie and Klein (1996), the effective population number based on female-fertility for *F. thapsinum* is relatively higher in this study ($N_{e(f)}$ = 45%), but lower for *F. proliferatum* at 49%. Based on the AMOVA analysis, most of the genetic variation in the AFLP-based loci is found within populations for *F. proliferatum* and *F. thapsinum* (Table 3.3). This suggests that gene flow occurs through sexual recombination or frequent movement of strains of these species between populations. The variation observed within and among populations of *F. verticillioides* suggests that these strains are more isolated and that there is less migration between the populations

All three of the dominant species, i.e. *F. proliferatum*, *F. verticillioides*, and *F. thapsinum*, can produce fumonisins, although the amounts produced by *F. thapsinum* usually are very limited (Desjardins 2006; Leslie et al. 2005a). Sorghum contaminated with fumonisins could be hazardous to humans and the domesticated animals that consume it. *F. proliferatum* and *F. thapsinum* also produce a second mycotoxin, moniliformin, and *F. thapsinum* is a prolific moniliformin producer (Leslie et al. 2005a). Moniliformin is toxic to chickens, and has been associated with Keshan disease in China (Desjardins 2006). Thus, sorghum produced in Thailand has the potential to be contaminated with either the fumonisin or the moniliformin mycotoxins.

An important sorghum pathogen, *F. andiyazi*, was not recovered in this study (Leslie et al. 2005a). This species has been isolated from South Africa, Ethiopia, Nigeria, and the United States (Marasas et al. 2001). The absence of this species from Thailand implies that sorghum imported from these countries should be subject to stricter screening and quarantine measures to prevent the introduction of this pathogen into Southeast Asia.

In conclusion, we recovered five *Fusarium* spp. from sorghum fields in Thailand: *F. beomiforme*, *F. thapsinum*, *F. proliferatum*, *F. sacchari*, and *F. verticillioides*. Three of these

species (*F. thapsinum*, *F. proliferatum*, and *F. verticilliodes*) contain potentially toxigenic strains, and should be managed to reduce the risk of toxin contamination in the field. An assessment of the disease frequency and causal agent of cob and stalk rot in maize is needed to determine *F. proliferatum* is associated with this disease in Thailand and whether it benefits from a sorghum/maize rotation. Finally, stricter quarantine procedures for sorghum material coming from the Americas and the Africa should be implemented to prevent the introduction of *F. andiyazi* into Thailand and neighboring countries.

Table 3.1 Source and species of *Fusarium* isolates collected from sorghum fields in Thailand.

Location	Plant Part	Total isolates	Fp	Ft	Fv	Fb	Fs
Hat Yai	Seed	21	20	1	0	0	0
Phai Sali	Stalk	7	0	2	2	2	1
Ban Chai Badan	Stalk	7	0	2	5	0	0
Ban Wang Phong	Stalk	23	3	15	5	0	0
Tak Fa	Stalk	10	1	0	9	0	0
Total	-	68	24	20	21	2	1

Fp: *F. proliferatum*; Ft: *F. thapsinum*; Fv: *F. verticillioides*; Fb: *F. beomiforme*; Fs: *F. sacchari*

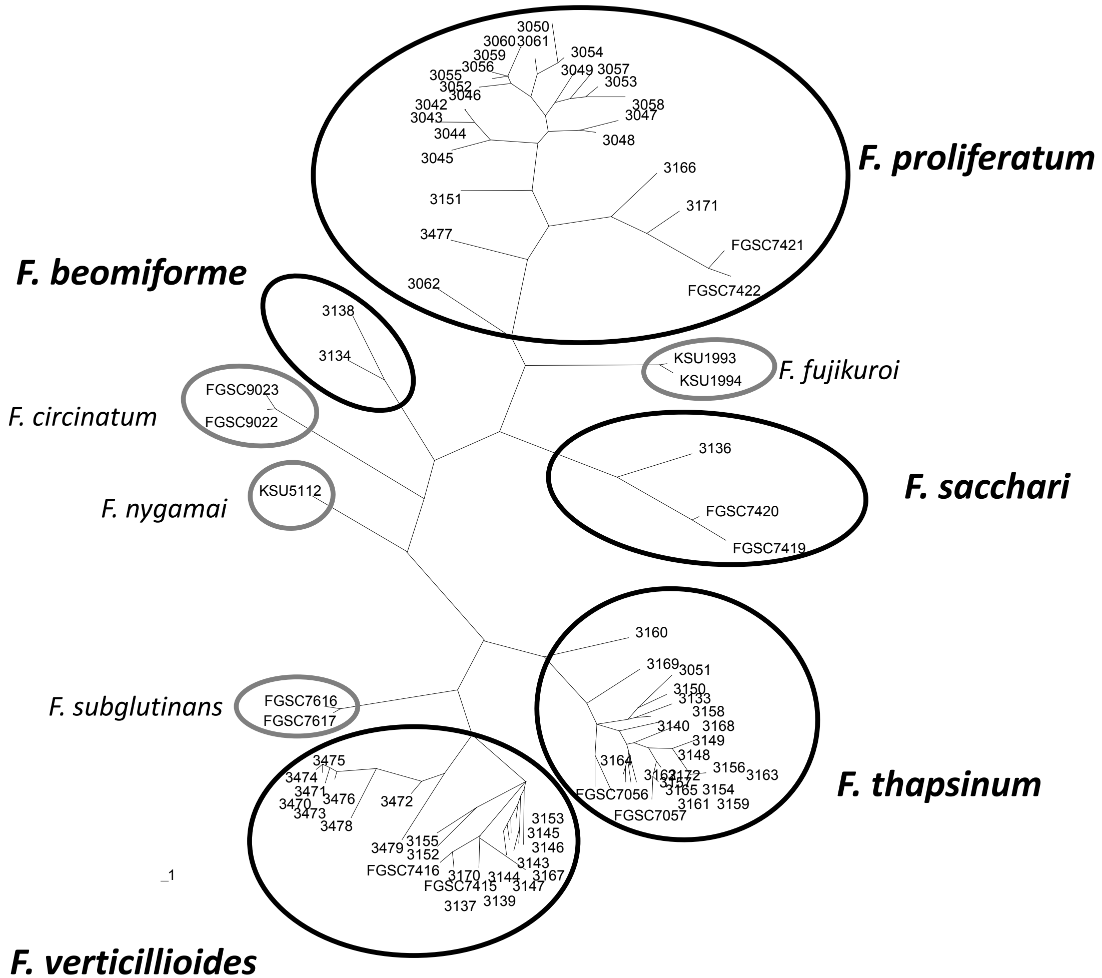


Figure 3.1 Unrooted phylogenetic tree generated with UPGMA based on AFLP markers.
Black circles encompass strains from species identified in the field populations. Gray circles encompass reference strains for related species.

Table 3.2 Mating type and fertility of isolates of *Fusarium* collected from sorghum grown in Thailand.

Fusarium species	Mating type		[a]$N_{fs} : N_h$
	MAT-1	*MAT-2*	
F. verticillioides	9	12	8:13
F. proliferatum	10	14	20:4
F. thapsinum	15	5	17:3

[a]N_{fs} is the number of female sterile strains; N_h is the number of hermaphrodites

Table 3.3 Analysis of molecular variance (AMOVA) of AFLPs for *F. verticillioides*, *F. proliferatum*, and *F. thapsinum* from sorghum.

Source of variation	Degrees of freedom	Variance	% of total variance
(a) *F. verticillioides*			
Among populations	3	4.138	47
Within populations	17	4.718	53
Total	20	8.857	100
(b) *F. proliferatum*[a]			
Among populations	1	2.816	32
Within populations	21	6.060	68
Total	22	8.875	100
(c) *F. thapsinum*[a]			
Among populations	2	0.932	17
Within populations	16	4.488	83
Total	18	5.419	100

[a]One location is not included in the calculation since only one isolate was present at that location.

Table 3.4 *Fusarium* isolates from sorghum in Thailand.

KSU Strain number	*MAT* allele	Fertility	Sorghum tissue	Geographic origin (Thailand)
F. proliferatum				
3042, 3048, 3049, 3053, 3058, 3059	1	♂	Head (seed)	Experimental Farm, Prince Songkhla University, Hat Yai
3046, 3047	1	♂/♀	Head (seed)	
3061	1	♂	Head (seed)	
3043, 3060	2	♂/♀	Head (seed)	
3044, 3045, 3054, 3055, 3056, 3057	2	♂	Head (seed)	
3050, 3052, 3062	2	♂	Head (seed)	
3477	1	♂	Stalk	Highway 11, Takfa
3151, 3166, 3171	2	♂	Stalk	Highway 205, Ban Wang Phong
F. verticillioides				
3143, 3144, 3146	2	♂/♀	Stalk	Highway 205, Ban Chai Badan
3152	2	♂	Stalk	Highway 205, Ban Wang Phong
3137, 3139	1	♂/♀	Stalk	Highway II, Phai Sali

KSU Strain number	*MAT* allele	Fertility	Sorghum tissue	Geographic origin (Thailand)
3145	1	♂	Stalk	Highway 205, Ban Chai Badan
3153	1	♂/♀	Stalk	Highway 205, 206, Ban Wang Phong
3155	1	♂	Stalk	
3473	1	♂	Stalk	Highway 11, Takfa
3476, 3478, 3479	1	♂/♀	Stalk	
3470	2	♂/♀	Stalk	
3471, 3472, 3474, 3475	2	♂/♀	Stalk	
3147	2	♂	Stalk	Highway 205, Ban Chai Badan
3167, 3170	2	♂	Stalk	Highway 205, Ban Wang Phong
F. thapsinum				
3051	2	♂	Head (seed)	Experimental Farm, Prince Songkhla University, Hat Yai
3133, 3140	1	♂	Stalk	Highway II, Phai Sali
3148, 3149	1	♂	Stalk	Highway 205, Ban Chai Badan
3154, 3158, 3161, 3162, 3169	1	♂	Stalk	Highway 205, Ban Wang Phong
3159, 3160, 3164	1	♂	Stalk	

KSU Strain number	*MAT* allele	Fertility	Sorghum tissue	Geographic origin (Thailand)
3163, 3165, 3172	1	♂/♀	Stalk	
3150, 3156, 3157, 3168	2	♂	Stalk	
F. sacchari				
3136	1	♀	Stalk	Highway II, Phai Sali
F. beomiforme				
3134, 3138	2	- [a]	Stalk	Highway II, Phai Sali

[a]-, No data due to lack of mating testers for this species.

Chapter 4 - Population Structure of *Fusarium fujikuroi* Species Complex Associated with Bakanae Disease of Rice in Malaysia and Thailand

Abstract

Several species in the *Fusarium fujikuroi* species complex are commonly associated with bakanae disease of rice. Rice is an important agricultural export for both Malaysia and Thailand. The movement of rice may be accompanied by the movement of fungal strains in the *F. fujikuroi* species complex. We analyzed 182 *Fusarium* isolates from bakanae-infected rice in Malaysia and Thailand – 76 from Malaysia and 106 from Thailand. Among the isolates, *F. fujikuroi* (87%) was the most common followed by *F. proliferatum* (9%), *F. sacchari* (3%), *F. verticillioides* (1%) and *F. concentricum* (<1%). An unrooted tree was constructed based on amplified fragment length polymorphisms (AFLPs). There were two subclusters within *F. fujikuroi*, one containing strains from Malaysia and another containing strains from Thailand, indicating that there is some genetic structure to populations of *F. fujikuroi*. There is considerable genotype variation within and between these populations, but none of the strains were female fertile under laboratory conditions. We conclude that the populations of *Fusarium fujikuroi* in Malaysia and Thailand are genetically isolated. Additional samples from other populations in the region are critical to determine the level of genetic isolation within this species.

Introduction

The *Fusarium fujikuroi* species complex has a widespread host and geographic distribution (Kim et al. 2012; Leslie et al. 1990; Lima et al. 2009a; Mansuetus et al. 1997). Morphologically, most of these species are very similar, making identification of the species

based on morphological characters alone difficult (Leslie and Summerell 2006). Most of these species can be separated based on sexual fertility by using the biological species concept. To date, there are 12 biological species (mating populations A – L) within this species complex (Kvas et al. 2009; Leslie and Summerell 2006). These biological species also can be distinguished based on amplified fragment length polymorphisms (AFLPs) and partial DNA sequences of genes such as β-tubulin, histone *H3*, and translation elongation factor 1α (Leslie and Summerell 2006; O'Donnell and Cigelnik 1997; Steenkamp et al. 1999; Wulff et al. 2010).

Rice is a very important crop in Thailand and Malaysia, as it is the staple food for most of the people in this region. Although Malaysia produces 1.75 million metric tons of rice, it also imports 1.2 million tons of rice from neighboring countries, with 33% of these imports coming from Thailand (http://www.indexmundi.com; http://www.riceimportexport.com). The movement of rice also could move and mix the *Fusarium* spp. associated with rice. A well-known disease caused by *Fusarium* spp. is bakanae, or "foolish seedling" disease. Bakanae disease can cause yield losses of up to 40% (Desjardins et al. 2000a). This disease has been known since 1828 in Japan. This disease was very serious in Malaysia and Thailand from 1960 to the 1980s (Kanjanasoon 1965; Saad 1986). In Malaysia, disease incidence up to 12.5% was reported during the main growing season in 2005 (Zainuddin et al. 2008b). Typical symptoms of bakanae include elongated seedlings, chlorosis, and visible fungal mycelium on dried-up seedlings above the water level (Amoah et al. 1995; Zainuddin et al. 2008b). Elongated seedlings result from the excess gibberellic acid produced by the pathogen (Desjardins et al. 2000a).

Two *Fusarium* species are associated with bakanae disease on rice: *F. fujikuroi* and *F. proliferatum* (Amoah et al. 1995; Desjardins et al. 2000a; Zainuddin et al. 2008b). *F. andiyazi*, *F.*

sacchari, *F. subglutinans* and *F. verticillioides* also have been isolated from bakanae-infected rice (Wulff et al. 2010; Zainuddin et al. 2008b).

In this study, we evaluated fungal isolates from bakanae-infected rice growing in Thailand and Malaysia. Our objectives were: (i) to identify *Fusarium* species associated with bakanae disease of rice; (ii) to determine the genetic relationship amongst the strains; and (iii) to evaluate the population structure of the collected strains. We hypothesize that *F. fujikuroi* is the dominant species in these populations. Due to the long history of bakanae disease in this region, we further hypothesize that the genetic variation of *F. fujikuroi* in this region is higher than in California. Furthermore, the movement of rice between Malaysia and Thailand may lead to homogenization of the genetic composition of the strains in both countries. This study will guide plant pathologists and local breeders to improve their current strategy to manage bakanae.

Materials and Methods

Isolates and culture

One-hundred-and-eighty-two isolates were obtained from bakanae-infected rice in Thailand and Malaysia – 106 from northern Thailand and 76 from peninsular Malaysia. All isolates were single-spored by using a micromanipulator and cultured in complete medium at 25°C (Correll et al. 1987). The isolates were preserved as aqueous spore suspensions made with 15% glycerol and kept at -70°C (Leslie and Summerell 2006). Reference strains were obtained from the Kansas State University culture collection. Reference strains used in this research were *F. circinatum* (FGSC9022 and FGSC9023), *F. fujikuroi* (FGSC8931 and FGSC8932), *F. nygamai* (KSU5112), *F. proliferatum* (FGSC7615 and FGSC7614), *F. sacchari* (FGSC7610 and

FGSC7611), *F. subglutinans* (FGSC7616 and FGSC7617), and *F. verticillioides* (FGSC7600 and FGSC7603) (FGSC – Fungal Genetics Stock Center, Kansas State University).

Morphological characters were observed on cultures grown on carnation leaf agar (CLA) (Fisher et al. 1982) for 7 to 10 days at 25ºC with a 12-hour photoperiod under a combination of cool white fluorescent light and black light. Pigmentation was observed in cultures grown on potato dextrose agar (PDA) (Leslie and Summerell 2006).

Extraction of DNA

Strains were cultured on complete medium (Correll et al. 1987) slants for seven days. One ml of spore suspension in a 0.25% Tween 60 solution (~10^6 spores/ml) was used to inoculate 30 ml of complete medium broth in a 125-ml Erlenmeyer flask that was incubated for two days at room temperature (22 – 26°C) on an orbital shaker (150 rpm). Mycelia were harvested by filtration through a Milk Filter disk (KenAG, Ashland, Ohio), dried by blotting with paper towels, and ground to a powder in a mortar with a pestle under liquid nitrogen. DNA was extracted by using a CTAB procedure (Leslie and Summerell 2006). DNA extracts were stored in 1.5-ml microcentrifuge tubes at 4ºC until used. The quality of the DNA was evaluated following separation in a 1% agarose gel. The DNA concentration was measured with a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware).

Nucleic Acid Analyses – Mating-Type, AFLPs, and sequenced loci

DNA obtained from the extractions was diluted, to approximately 20 ng/µl, with sterile double-distilled water. The procedures used to identify the mating type idiomorphs were described by Leslie and Summerell (2006) and used the reactions and primers described by Steenkamp et al. (2000a). The amplification program for the *MAT* alleles was 94°C for 60 sec.,

followed by 29 cycles of 94°C for 30 sec., 65°C foor 45 sec., and 72°C for 30 sec., followed by 72°C for 5 min, and then 4°C indefinitely. The PCR amplification products were resolved on 1% agarose gels, with the presence of bands indicating the mating type.

DNA fingerprinting was conducted by using AFLPs (Vos et al. 1995) with the reaction conditions described by Leslie and Summerell (2006). Three primer pairs were used: *Eco*RI + GG/*Mse*I + CT, *Eco*RI + AA/*Mse*I + TT, and *Eco*RI + TT/*Mse*I + AC. Bands between 200 bp and 500 bp in size were scored manually. A tree based on AFLP bands was generated by using the neighbor-joining clustering option of PAUP (version 4.10b; D. L. Swofford, Sinauer Associates, Sunderland, Mass.) with 1000 bootstrap replications. The Dice coefficient with CLUSTER procedure of SAS (v 6.12, SAS Institure, Cary, North Carolina) was used to estimate genetic similarity between strains.

Partial DNA sequences of the translation elongation factor 1α (*tef-1*) gene for a few strains were used for confirmation of species identity. The DNA solutions were diluted to ~20 ng/μl. The primers used for *tef-1* amplification were EF-1 (forward: 5′-ATGGGTAAGGAGGACAAGAC-3′) and EF-2 (reverse: 5′-GGAAGTACCAGTGATCATGTT-3′) (O'Donnell et al. 1998a). The conditions for the *tef-1* sequence amplification were: 94ºC for 1 min., followed by 34 cycles of 94ºC for 30 sec., 62ºC for 45 sec., and 72ºC for 1 min., and then 4ºC until analyzed. The amplification products for *tef-1* were cleaned with ExoSAP-IT (Affymetrix, Cleveland, Ohio). We used an Applied Biosystems 3730 DNA Analyzer to produce DNA sequences. These sequences were analyzed by using BioEdit and BLASTed against NCBI GenBank (http://blast.ncbi.nlm.nih.gov/) and Fusarium-ID (http://isolate.fusariumdb.org/).

Sexual crosses and female fertility

Isolates of *F. fujikuroi*, *F. proliferatum*, *F. sacchari*, and *F. verticillioides* were crossed with tester isolates (*F. fujikuroi*: FGSC8931 & FGSC8932; *F. proliferatum*: FGSC7422 & FGSC7421; *F. sacchari*: FGSC7419 & FGSC7420; *F. verticillioides*: FGSC7415 & FGSC7416) to test for sexual fertility. Crosses were made on carrot agar as previously described in Klittich and Leslie (1988). Field isolates were tested as both male and the female parents in crosses with standard tester strains of the opposite mating type. Fertility was determined by the presence of numerous perithecia with a cirrhus of ascospores oozing from the perithecia 2 – 4 weeks after fertilization.

Population structure analysis

The analysis of molecular variance (AMOVA) was calculated by using GenAlEx 6.5 (Peakall and Smouse 2012) based on the frequency of the AFLP alleles. The STRUCTURE program (version 2.3.4; available online), a Bayesian model-based clustering method, was used to infer population structure (Pritchard et al. 2000). Individuals were assigned to K populations that are characterized by specific allele frequencies at examined loci. Exploratory runs for K-values were allowed from 1 to 4 with a 10,000 iteration burn-in period and 100,000 iterations of Markov Chain Monte Carlo (MCMC). The number of clusters (K) was determined by using the modal value of ΔK based on the rate of change in the log probability of data between successive K-values (Evanno et al. 2005).

Results

Species identification and distribution

Isolates of five *Fusarium* species were identified in this study: *F. fujikuroi* (87%), followed by *F. proliferatum* (9%), *F. sacchari* (3%) and *F. verticillioides* (1%), *F. concentricum* (< 1%) (Table 4.1). Both *F. fujikuroi* and *F. proliferatum* were found in Thailand and Malaysia, while *F. sacchari* and *F. concentricum* were isolated only from Malaysian samples and *F. vertillioides* was isolated only from Thai isolates. Sixty-three polymorphic AFLP bands were identified based on PCR amplifications with the three primer pairs. The resulting unrooted tree (Figure 4.1) contains four major clusters, each representing a different species and containing known reference strains. Within the *F. fujikuroi* cluster, there were two sub-clusters, one containing strains isolated from Thailand and a second containing strains isolated from Malaysia (Figure 4.1). Species identifications were confirmed for some strains based on partial *tef-1* gene sequences and/or sexual crosses.

Based on AFLP patterns, there were 48 *F. fujikuroi* haplotypes in the Thai population and 40 haplotypes in the Malaysian population. The number of clonal groups in the Thai population (20) was higher than that in the Malaysian population (5). None of the clonal groups contained strains from both Malaysia and Thailand. The average similarity of the *F. fujikuroi* strains was 80%.

Mating types and fertility

Strains of both mating types were found in the *F. fujikuroi*, *F. proliferatum*, and *F. sacchari* populations. Only *MAT-2* strains were recovered for *F. verticillioides* and *F. concentricum*. The distribution of *MAT* idiomorphs within the *F. fujikuroi* population was 32

MAT-1:117 *MAT-2*. Within country populations, the MAT idiomorph ratio was 9 *MAT-1*:92 *MAT-2* in Thailand, and 23 *MAT-1*:24 *MAT-2* in Malaysia. None of the *F. fujikuroi* strains were female fertile. Only one strain of *F. proliferatum* was female fertile, and the remaining were female sterile. As for *F. sacchari*, three strains were male fertile, but not female fertile. Both strains of *F. verticillioides* were both male and female fertile. No fertility tests were conducted with the *F. concentricum* strain as no sexual stage is known.

Molecular variance and STRUCTURE analysis of F. fujikuroi

The AMOVA indicates that variation within *F. fujikuroi* was almost equally distributed between the within population (49%) and the among population (51%) components (Table 4.3). In STRUCTURE analysis, initial simulations were performed assuming cluster numbers from $K = 1$ to 4. The maximum value of ΔK occurs at $K = 2$, and divides the sample into Thai and Malaysian population (Figure 4.2). Seven of the strains from Thailand (3350, 3356, 3388, 3427, 3438, 3451, and 3454) contained some allels characteristic of the Malaysian population (Figure 4.2).

Discussion

All of the *Fusarium* species recovered were members of the *F. fujikuroi* species complex. Although *F. verticillioides* and *F. sacchari* are not common in rice fields, both have previously been recovered from rice (Amatulli et al. 2010; Hsuan et al. 2011; Kim et al. 2012; Zainuddin et al. 2008a). The pathogenicity of these two species towards rice is not thought to be significant. *F. verticillioides* and *F. proliferatum* can both synthesize relatively high levels of fumonisins, which are harmful to humans and domesticated animals (Desjardins 2006). *F. sacchari* is not known to make high levels of mycotoxins and the few strains of *F. sacchari* isolated from rice in

Malaysia that have been tested for secondary metabolite production do not produce fumonisins, moniliformin, or fusaric acid (Zainuddin et al. 2008b). One strain of *F. concentricum* was recovered in this study. This species also has been isolated from rice in South Korea (Kim et al. 2012). The South Korean strain produces symptoms in rice that are similar to bakanae disease (Jeon et al. 2013), but its ability to produce gibberellic acid is not known. This report of *F. concentricum* from rice is a first report for Malaysia.

As hypothesized, *F. fujikuroi* is the most frequent *Fusarium* species recovered from rice. This finding is consistent with numerous previous reports (Amatulli et al. 2010; Carter et al. 2008; Cruz et al. 2013; Kim et al. 2012). Based on AFLP banding patterns, isolates are considered to be members of the same species if they share > 70% of the bands (Leslie and Summerell 2006). The average genetic similarity of *F. fujikuroi* strains in this population was 80%, which is lower than the 94% similarity found in California populations (Carter et al. 2008). Although both mating types are found in this population, none of the *F. fujikuroi* strains were female fertile under laboratory conditions. The effective population number based on mating type was 68% of the count which is the lowest $N_{e(mt)}$ reported for any *Fusarium* species (Leslie and Klein 1996). The lack of strains with observable fertility and the skewing of the *MAT* allele frequencies are both consistent with the hypothesis that the populations of *F. fujikuroi* primarily reproduce asexually.

The Thai and Malaysian populations of *F. fujikuroi* are clearly distinct from each other (Figures 4.1, 4.2). This conclusion is supported by the relatively low proportion of variation within the populations (Table 4.3) and the relatively high levels of between population variations. In Thailand, the *MAT-2* allele dominates, while in Malaysia, the *MAT* alleles are approximately equally frequent. The effective population number based on mating type was

32.5% in the Thai subpopulation and effectively 100% in the Malaysian population. The higher effective population number in Malaysia suggests that sexual recombination could be occurring in the field populations. This conclusion also is supported by the lower number of clonal strains in the Malaysian population. To date, there has been no report of perithecia occurringunder field conditions in either Malaysia or Thailand. Field occurrence of perithecia has been reported in Taiwan and Japan (Sung and Snyder 1977; Watanabe and Umehara 1977).

Bakanae disease has a much longer history in Southeast Asia than in California. Thus, finding more haplotypes in the Malaysian and Thai populations than in the California population (Carter et al. 2008) supports the hypothesis that *F. fujikuroi* is a relatively recent clonal introduction to California. Clonal strains can be very informative when analyzing the movement of pathogen strains within a population. In our study, none of the clonal groups contained strains from both Malaysia and Thailand, which suggests little migration of *F. fujikuroi* between these two populations. Geographic barriers could play a role in the isolation of these subpopulations as more than 1000 km separate the collection sites in peninsular Malaysia and northern Thailand. The Thai and Malaysian populations also appear to have accumulated quite a few mutations as there are clear lineages within each population. Loci that are polymorphic in both locations probably were polymorphic in the population from which both the Thai and Malaysian populations were derived. Samples from additional populations are needed to determine whether geographic distance is related to genetic distance and whether local populations of *F. fujikuroi* primarily reproduce asexually.

F. proliferatum is the second most commonly isolated species from rice with Bakanae. *F. proliferatum* can produce mycotoxins such as fumonisins, beauvericin, and moniliformin, and also can stunt the growth of rice seedlings (Desjardins 2006; Jeon et al. 2013). *F. proliferatum* is

very closely related to *F. fujikuroi*. Some strains of *F. proliferatum* and are cross-fertile with *F. fujikuroi*. Interspecific hybrids between *F. proliferatum* and *F. fujikuroi* can be generated in the laboratory (Chapter 5), but have been reported only once from field conditions (Leslie et al. 2004b). Such interspecific hybrids can produce sets of secondary metabolites not observed in either of the parental species (Studt et al. 2012). A naturally occurring hybrid between these two species that was viable could pose a threat to rice and other plant hosts in terms of pathogenicity and secondary metabolite production.

In conclusion, we identified five *Fusarium* species from rice in Malaysia and Thailand. The genetically diverse *F. fujikuroi* strains evaluated in this study should be tested for fungicide resistance to determine the effectiveness of current bakanae disease management program. We observed two distinct populations in *F. fujikuroi*, i.e. Malaysian and Thail, that warrant further study in comparing their pathogenicity on rice and the amount of GA_3 produced. An understanding of pathogen population structure will help determine the deployment of resistant varieties in the field. Finally, additional samples from other countries in Southeast Asia will be crucial to determine if the isolation of *F. fujikuroi* is due to geographic distance.

Table 4.1 Location and species of *Fusarium* isolates from rice.

Location	*F. fujikuroi*	*F. proliferatum*	*F. sacchari*	*F. verticillioides*	*F. concentricum*
Thailand	102	2	0	2	0
Malaysia	57	14	4	0	1
Total	149	16	4	2	1

Table 4.2 *Fusarium* isolates from rice in Malaysia and Thailand

Strains	*MAT*	Fertility	Geographic origin
F. fujikuroi			
3356, 3361, 3362, 3364	1	NF[a]	Chiang Mai, Thailand
3345-3350, 3352-3355, 3357-3360, 3363, 3365-3367	2	NF	Chiang Mai, Thailand
3368	1	NF	Sri Lanna National Park, Thailand
3369, 3371-3385, 3387-3391	2	NF	Sri Lanna National Park, Thailand
3454, 3456, 3458, 3460	1	NF	Highway 106, KM Post 46, Li, Thailand
3410-3440, 3442-3452, 3455, 3457, 3459, 3461-3468	2	NF	Highway 106, Li, Thailand
21067	2	NF	Seberang Perak (FELCRA), Perak, Malaysia
21069	2	NF	Kg. Apal, Jabi, Terengganu, Malaysia
21093, 21102-21106, 21108-21112	1	NF	Sekinchan, Selangor
21082, 21084, 21087, 21088, 21091, 21094, 21099-21101, 21107	2	NF	Sekinchan, Selangor
21115, 21116	1	NF	Sungai Leman, Selangor
21117-21122, 21125-	2	NF	Sungai Leman, Selangor

21128			
21129	2	NF	Cuping, Perlis
21130	2	NF	Kg. Paya, Kedah
21131	1	NF	Jitra, Kedah
21132	1	NF	Bagan Serai, Perak
21134	1	NF	Sungai Baru 3, Perak
21135	1	NF	Kg. Banggol, Petaling, Kelantan
21136	1	NF	Kg. Lapa, Peringat, Kelantan
21137	1	NF	Kemubu 1, Kelantan
21138-21141	1	NF	Kg. Paya, Kedah
F. proliferatum			
3351	2	♂	Chiang Mai, Thailand
3386	2	♀, ♂	Sri Lanna National Park, Thailand
21064	1	♂	Seberang Perak (FELCRA), Perak
21065, 21066	2	♂	Seberang Perak (FELCRA), Perak
21070	2	♂	Padang Pak Amat, Pasir Puteh, Kelantan
21071, 21072	2	♂	Palekbang, Tumpat, Kelantan
21073	2	♂	Ladang Ana Fasa 2, Tumpat, Kelantan
21075, 21080	2	♂	Padang Sungai Laka, LKPP, Rompin, Pahang
21081	1	♂	Padang Sungai Laka, LKPP, Rompin, Pahang
21085	2	♂	Sekinchan, Selangor
21113, 21114	2	♂	Sungai Nibong, Selangor
F. sacchari			

21076-21078	2	♂	Padang Sungai Laka, LKPP, Rompin, Pahang
21079	1	♂	Padang Sungai Laka, LKPP, Rompin, Pahang
F. verticillioides			
3441, 3453	2	♀, ♂	Highway 106, Li, Thailand
F. concentricum			
21074	2	NA[b]	Padang Sungai Laka, LKPP, Rompin, Pahang

[a]NF – Non-fertile; [b]NA – Fertility data is not available due to lack of tester strain.

Table 4.3 Analysis of molecular variance (AMOVA) of AFLPs for *F. fujikuroi* from rice.

Source of variation	Degrees of freedom	Variance	% of total variance
Among populations	1	4.012	51
Within populations	147	3.903	49
Total	148	7.916	100

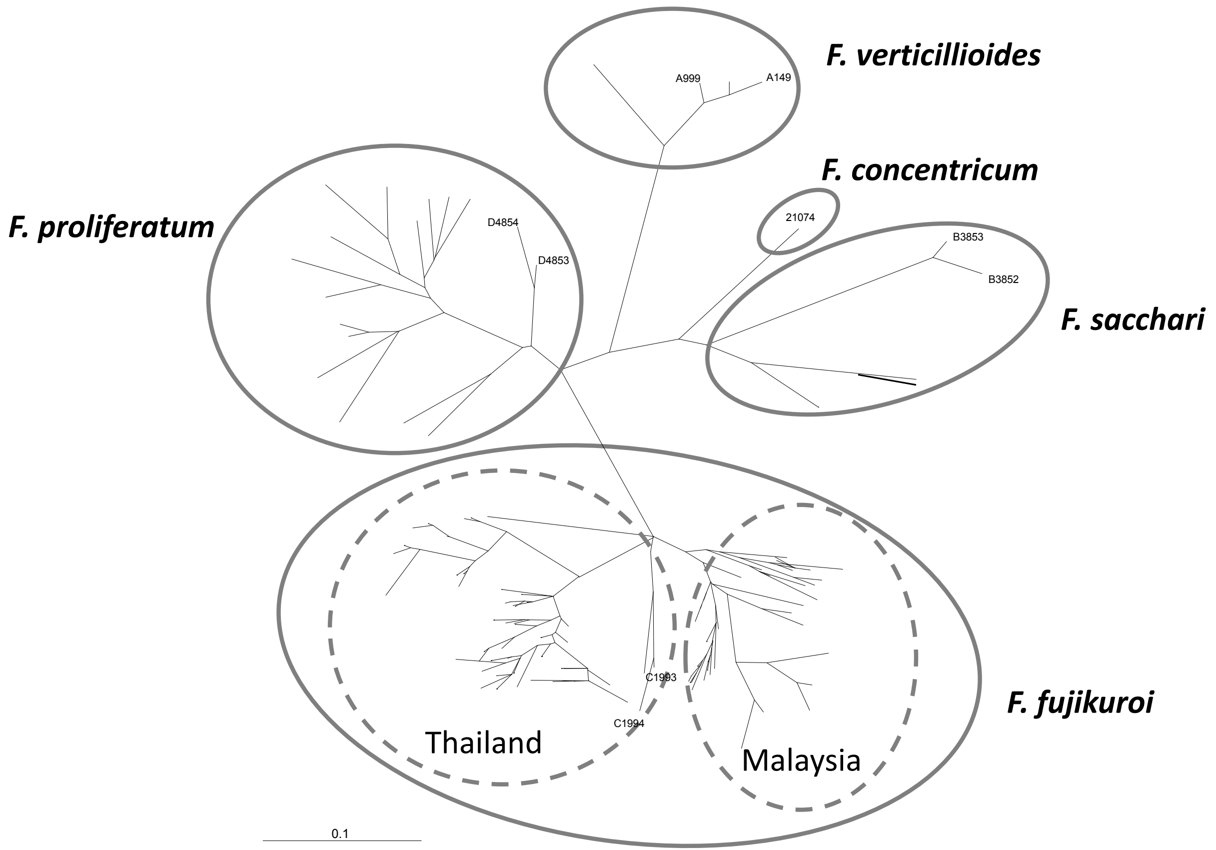


Figure 4.1 Unrooted phylogenetic tree generated based on AFLP markers.

Circles with solid lines delineate species clusters. Circles with dotted lines delineate clusters based on countries, i.e. Malaysia and Thailand. The strain numbers on the branches of *F. fujikuroi*, *F. proliferatum*, *F. sacchari*, and *F. verticillioides* are the reference strains.

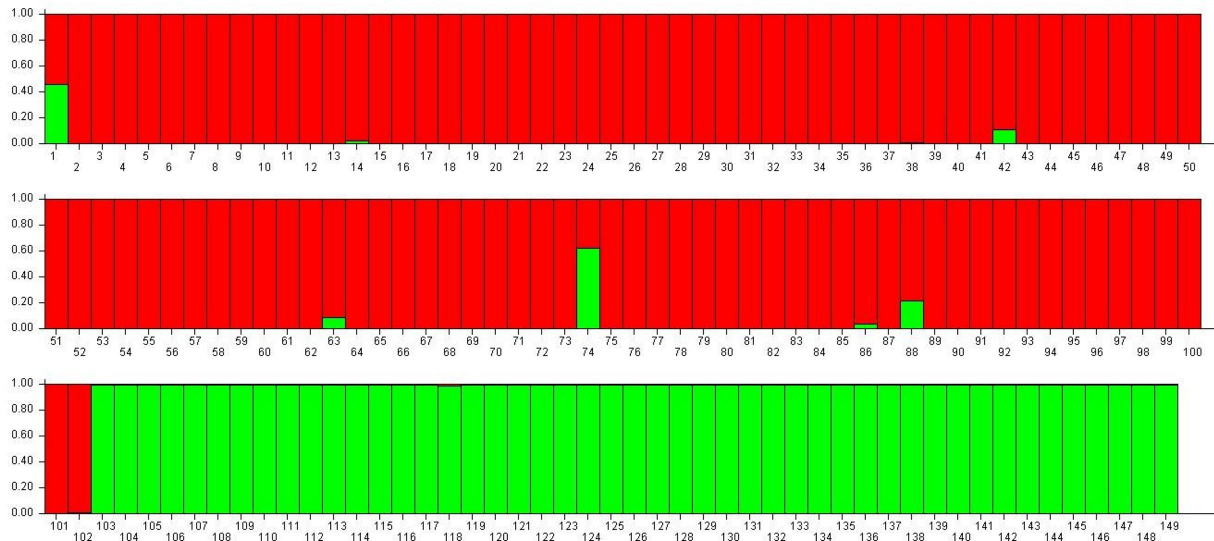


Figure 4.2 Genetic structure of individual strains of *F. fujikuroi* in both populations. Strains 1 – 102 are from Thailand. Strains 103 – 149 are from Malaysia.

Chapter 5 - Genetic map of the interspecific hybrids between *F. proliferatum* and *F. fujikuroi,* segregation of Gibberellic acid production and its pathogenicity towards rice

Abstract

F. fujikuroi and *F. proliferatum* are closely related species. Some strains are cross-fertile with members of the other species and those crosses produce viable progeny in limited numbers. As a result of interspecific crosses, novel pathogenicity and secondary metabolite combinations may occur. An interspecific cross between *F. fujikuroi* and *F. proliferatum* was made and 533 ascospore progeny collected. Amplified Fragments Length Polymorphisms (AFLPs) were used to genotype the progeny and the parental strains. Eighty-six AFLPs markers were scored and used to construct a recombination-based map. Seventy-three of the markers were distorted towards the *F. proliferatum* parent. The recombination-based map was aligned with the physical map. The genomic location of GA_3 production was on chromosome 5, which is consistent with earlier reports. QTL analysis of GA_3 production with a nonparametric method identified several genomic regions associated with GA_3 production. Pathogenicity tests of the progeny on rice seeds resulted in two phenotypes: seedling elongation, and seed germination. Both pathogenicity phenotypes varied continuously amongst the progeny. Several of the clonal progeny resulting from this cross represent potentially novel biological events. The novel combinations of secondary metabolite production phenotypes and pathogenicity profiles evident in some of the progeny could threaten rice varieties and other crops if such progeny occur under field conditions.

Introduction

The genus *Fusarium* is one of the most important fungal genera containing plant pathogens due to its cosmopolitan distribution and the wide array of hosts attacked. One of the best studied species complexes within the genus is the *Fusarium fujikuroi* species complex (Geiser et al. 2013). This species complex has been subdivided into three clades with multiple biological and phylogenetic species. *F. fujikuroi*, and *F. proliferatum* are sister species in this species complex. Morphologically, these two species are almost indistinguishable, but a polyphasic approach that uses both biological and phylogenetic species concepts can resolve them clearly (Kvas et al. 2009; Leslie and Summerell 2006).

Biological species concepts have been used in *Fusarium* to identify sibling species (Leslie and Summerell 2006). Under this concept, strains in the same species are sexually cross-fertile and produce viable, fertile progeny at a "normal" rate. In some cases, strains in different species may be poorly cross-fertile with one another (Desjardins et al. 2000b; De Vos et al. 2013, Leslie et al. 2004a). This cross-fertility includes some strains of *F. fujikuroi* and *F. proliferatum*. Although *F. fujikuroi* and *F. proliferatum* are very closely related, the two species differ in many characters. Strains of *F. fujikuroi* are most commonly recovered from rice, while *F. proliferatum* strains can be recovered from many quite different plant hosts (Kvas et al. 2009; Leslie and Summerell 2006). The two species also differ in their secondary metabolite production profiles. *F. fujikuroi* strains commonly produce gibberellic acid, while *F. proliferatum* strains can produce fumonisins, fusaproliferin and moniliformin (Desjardins 2006; Kvas et al. 2009).

Sexual crosses between *F. fujikuroi* and *F. proliferatum* were first reported in 1997 (Desjardins et al. 1997), and progeny were collected from several crosses in 2004 (Leslie et al. 2004a). These interspecific crosses are consistent with the conclusions that the genomes of *F.*

fujikuroi and *F. proliferatum* are closely related (Ellis 1988; O'Donnell et al. 1998b). The interspecific laboratory cross produced progeny with novel secondary metabolite profiles (Studt et al. 2012). In 2012, we repeated one cross between the two species and increased the number of progeny to enable the construction of a robust genetic linkage map and to study the segregation of traits such as pathogenicity and secondary metabolite productions.

The objectives of this study are: (i) to evaluate segregation of AFLP loci in an interspecific cross between *F. fujikuroi* and *F. proliferatum*; (ii) to generate a genetic linkage map from the interspecific cross; (iii) to study the segregation of gibberellin production and pathogenicity towards rice; and (iv) to locate a region(s) within the genome involved in gibberellin production and pathogenicity towards rice. The recombination-based map from this study provides a basis for further assembly of the genome of both parental strains. This study will facilitate other genetic research on these interspecific hybrids, such as identification of genetic factors involved in pathogenicity, host specificity, and speciation.

Materials and Methods

Interspecific cross and collection of progeny

A cross between FGSC8932 (*F. fujikuroi*, *MAT-2*) and FGSC7615 (*F. proliferatum*, *MAT-1*) was made on multiple occasions. The parental strains are available from the Fungal Genetics Stock Center (Department of Plant Pathology, Kansas State University). Crosses were made on carrot agar as described by Klittich and Leslie (1988). FGSC7615 was used as the female parent and FGSC8932 was the male parent. Ascospores were recovered from perithecia 4-6 weeks after fertilization. Ascospores were separated by micromanipulation on 3% water agar slides and incubated right side up at 25˚C overnight. Germinated spores were examined,

identified with a dissecting microscope. Individual germinated spores were cut from the agar slab and transferred to a complete medium (Correll et al. 1987) slant. All progeny were preserved by freezing spore suspensions in 15% glycerol at -70˚C. An example of the numbering code for the progeny used for these progeny isCD010203, which indicates – CD: progeny from cross between *F. fujikuroi* (MP-C) and *F. proliferatum* (MP-D); 01: ascospore isolated from petridish no. 1; 02: ascospore isolated from perithecia no. 2; 03: number of the individual ascospore. Progeny with code numbers beginning with the letter "Z" were obtained from crosses of the same strains made in 2004.

DNA isolation

Strains were cultured on complete medium (Correll et al. 1987) slants for seven days. One ml of a spore suspension in a 0.25% Tween 60 solution (~10^6 spores/ml) was used to inoculate 30 ml of complete medium broth in a 125-ml Erlenmeyer flask that was incubated for two days at room temperature (22–26°C) on an orbital shaker (150 rpm). Mycelia were harvested by filtration through a Milk Filter disk (KenAG, Ashland, Ohio), dried by blotting with paper towels, and ground to a powder in a mortar with a pestle under liquid nitrogen. DNA was extracted by using a CTAB procedure (Leslie & Summerell, 2006). DNA extracts were stored in 1.5-ml microcentrifuge tubes at 4ºC until used. The quality of the DNA was evaluated following resolution of a sample in a 1% agarose gel, while the DNA concentration was measured with a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware).

Mating type PCR

DNA solutions were diluted to ~20 ng/µl with sterile double-distilled water. The procedure used to identify the mating type alleles was that of Leslie & Summerell (2006), which

follows that of Steenkamp et al. (2000). The products of the PCR amplification were separated on a 1% agarose gel. The size of the amplified DNA products were used to identify the mating type of the strain.

AFLP reactions

DNA fingerprinting was conducted by using AFLPs (Vos et al. 1995) following the protocol described in Leslie & Summerell (2006). Four primer pairs were used in the selective amplification: *Eco*RI + CC/*Mse*I + CG, *Eco*RI + AA/*Mse*I + AA, and *Eco*RI + GG/*Mse*I + TG, *Eco*RI + TT/*Mse*I + AC. Bands 200 – 500 bp in size were scored manually based on the presence or absence of a band. Only polymorphic bands based on the parental strains were scored. Fragments of the same size were assumed to be homologous. Both parental strains were included in each AFLP gel: *F. proliferatum* (FGSC 7615), *F. fujikuroi* (FGSC8932) for reference on band scoring. The size of the AFLP fragments was estimated by comparisons with the low mass ladder 200 to 500 bp.

Gibberellic acid extraction

Strains were cultured in optimized production media (OPM) (Tsavkelova et al. 2008) on a rotary shaker (130 rpm) for 7 days. The culture was filtered through filter paper (Whatman #1) and the filtrate collected in a flask. The pH of the filtrate was adjusted to pH 2.8 with 1N HCl. Eight hundred microliters of the filtrate was extracted with ethyl acetate at a 1:2 (filtrate:ethyl acetate) ratio. The mixture was vortexed for 2 minutes. Two layers formed after vortexing. The ethyl acetate layer (top layer) was transferred to a 1.5 ml microcentrifuge tube and evaporated by using a speed vacuum (DyNA-Vap, Labnet International Co., Woodbridge, New Jersey). The

residue was dissolved in 30 µl of absolute ethanol. The extractions were stored at 4˚C until analyzed. Each sample was filtered through a PVDF 0.45 µm filter prior to analysis.

Gibberellic acid analysis

Gibberellic acid production was analyzed with an incomplete block design. Each set of runs was treated as a block. Within each run, both parental strains and blanks were included. Spiked samples were used to assess the recovery rate. Extracts from the parental strains were mixed with 100 µl/µg GA_3 as spiked samples. Gibberellic acid extracts were analyzed by using an HPLC (Shimadzu HPLC system consisting of a CBM-20A controller, SIL-20A HT auto-sampler, LC-20AT pump, SPD-20AV UV/VIS detector and CTO-20AC column ovens, Canby, Oregon). The HPLC column was protected with a universal guard column (SecurityGuard Guard Cartridge System Column Protection with C18 guard cartridge, 4 × 2.0 mm ID, Phenomenex Inc., Torrance, California). The column used was a Supelco Discovery C18, 150 × 4.6 mm, particle size = 5 µm, at a temperature of 25˚C. The mobile phase was 20% methanol containing 10 mM H_3PO_4 adjusted with KOH to pH = 2.3 (Barendse et al. 1980). The injection volume was 10 µl. The flow rate was set at 1.0 ml/min, with column oven temperature at 25˚C. Each sample was run for 50 minutes with the UV detector at 203 nm. All of the solutions used in the experiment were HPLC grade. For each set of runs, a range (100, 250, 500, 1000, 5000, 10000) of standard GA_3 solutions was included to develop a standard curve. All samples were diluted 1:100 with methanol.

Rice seedling pathogenicity assay

The rice seedling germination procedure was based on that of Ellis et al. (2011), which uses a rolled towel assay. Rice seed (*Oryza sativa* L. ssp. *japonica*) was obtained from Kitazawa

Seed Company (Oakland, California). Seeds were surface-sterilized with 10% bleach and rinsed three times with tap water. Seeds were heat treated in a water bath at 55˚C for 15 minutes and then dried overnight. Prior to inoculation, strains were cultured in 5 ml of liquid complete medium in glass tubes (Pyrex, No. 9820) on a rotary shaker (120 rpm) for 48 hrs. The spore concentration was adjusted to $1x10^5$ spores/ml and kept at 4˚C overnight. Twenty-five seeds per strain were soaked in one ml of the spore suspension for 4 hours at room temperature. Seeds were placed on a moistened towel and the towels were rolled. Rolled towels were placed in a closed plastic container containing 100 ml of sterile double-distilled water and incubated at 25˚C for 7 days.

The seed germination rate and the length of the germinated shoots were measured. The experiment was designed as a randomized complete block with replications treated as blocks. There were four experimental replications conducted at different times. The blocks were treated as a random effect while strains were treated as fixed effects. Statistical analyses were performed with SAS software, version 9.3 (SAS Institute Inc., Cary, North Carolina). The analysis used the "Proc mixed" procedure. Ranking of the strains based on seedling shoot length was used to compare the treatments.

Genetic linkage map construction and alignment to the physical map

The physical map and the genetic map were constructed concurrently. Based on the estimated size of the AFLP fragments, the sequence of the fragment was predicted with AFLPinSilico (Rombauts et al. 2003) based on partially assembled genome sequences of the two parental genomes (Toomajian, unpublished). The location of the predicted fragments in the genome was obtained by BLASTing the sequences against available *F. fujikuroi* genomic sequences (Wiemann et al. 2013; Toomajian, unpublished).

The genetic linkage map was constructed and QTL analysis was performed by using the QTL package in R, version 3.1 (www.rqtl.org) (Broman et al. 2003; Broman and Sen 2009). The AFLP and phenotypic data were imported as a backcross from Microsoft Excel. All of the AFLP markers and progeny were included in the analysis to form linkage groups. Using R/qtl, we checked for missing data, duplications, and distortions. Fifty-one strains with > 95% genotype similarity to other strains were removed from further analysis. Segregation of most markers was distorted, so the function "markerlrt( )" was used to form the linkage groups. This function uses the LOD score of a general likelihood ratio test of each pair of markers to assess their association. The functions "orderMarkers( )" and "ripple( )" were used to find the best marker order for each linkage group based on the number of crossovers and the likelihoods of the given order. The initial linkage groups were compared and aligned to the *in silico* physical map. The linkage groups and unlinked markers could be consolidated to 12 chromosomes based on known physical sequences. The procedure to establish marker order was then repeated. The marker order resulting from the function "orderMarker( )" also was compared to the marker order based on the physical map, when they differed, by using the function "compareorder( )". Three-point test crosses were analyzed manually for all markers that were not well-aligned with the physical map. The genetic length of the map was estimated by using the Haldane mapping function, which is the default for this program and assumes that there is no crossover interference.

Analysis of QTLs

QTLs for gibberellic acid production and rice pathogenicity were detected by using the R/qtl program (Broman and Sen 2009). With the single-QTL model, all of the markers were subjected to an interval mapping analysis. Initially, the function "calc.genoprob( )" was used to calculate conditional genotype probabilities. Three methods were used to search for QTLs:

parametric, nonparametric, and binary. The phenotype data were log-transformed when using the parametric method to generate a normal distribution. A genome scan for QTLs based on single marker analysis was performed by using the Haley-Knott regression (Haley and Knott 1992). For nonparametric interval mapping, no data transformation was needed. The nonparametric protocol uses rank-based methods in an extension of the Kruskal-Wallis test for interval mapping to search for QTLs. In the binary method, the phenotype data were transformed into binary data, e.g. band or no band, pathogenic or nonpathogenic. Binary methods used maximum likelihood estimates (MLEs) similar to standard interval mapping to produce a LOD curve on a grid covering the genome.

A permutation test with 1000 replicates was performed to obtain a genome-wide significance threshold. In the permutation test, phenotypes were randomized relative to genotypes and the test repeated 1000 times. Based on the permutation test, the significance threshold level for logarithm of odds (LOD) was set at $\alpha = 0.05$ for the detection of QTLs.

Results

AFLP and Linkage map analysis

Eighty-six polymorphic AFLP bands were scored manually from the four AFLP primer pairs. The segregation of most markers was distorted toward one of the parental types (Table 5.1). Seventy-three markers were distorted towards *F. proliferatum*, and eight markers towards *F. fujikuroi*, and five segregated at the Mendelian ratio of 1:1. Among the progeny, 355 progeny had more than 50% of their markers from the *F. proliferatum* parent, 31 progeny carried more than 50% *F. fujikuroi* alleles, and 147 progeny had an equal number of alleles from both parents in their genome.

Ten clonal groups containing 29 progeny also were observed (Table 5.5). Seven perithecia produced clonal progeny in one of these clonal haplotypes. Six of the 10 haplotypes were recovered from multiple perithecia and must have originated independently during meiosis. All of these clonal groups were highly distorted towards the *F. proliferatum* alleles in their genomic composition.

Eighty-three AFLP markers and the mating type locus were used to establish a genetic linkage map. Three problematic AFLP markers were dropped from the linkage analysis due to the ambiguity of their location in the physical genome. The in silico analysis indicated that these bands could each represent 2 segregating loci. After the initial analysis, there were 14 linkage groups and 15 unlinked markers at minimum LOD = 13. This recombination-based map was aligned to the physical map to identify the chromosomes associated with each linkage group and to confirm marker order. Linkage groups were re-constituted by combining two or more linkage groups and the unlinked markers associated with the same chromosome. Five AFLP markers were unlinked to any other marker and were not identifiable in the *F. fujikuroi* reference genome. Twelve linkage groups corresponding to the twelve chromosomes present in the reference genome of *F. fujikuroi* were used for further analysis (Figure 5.5). In the linkage groups, five AFLP loci segregated in a Mendelian manner, and four loci were significantly distorted towards *F. fujikuroi* (Figure 5.5). The location of markers on the linkage map was confirmed by making three-point cross calculations.

Gibberellic acid production

An HPLC was used to detect gibberellic acid (GA_3) at 33 minutes after injection (Figure 5.4). As expected, the *F. fujikuroi* parental strain produced GA_3 and the *F. proliferatum* parent did not. Two-hundred-fifty-one progeny (58%) produced no GA_3 and the remaining progeny

produced GA_3 at various levels (Figure 5.3). The segregation of the GA_3 production was not Mendelian amongst the progeny ($\chi^2 = 11.34$, $P < 0.01$). Only 4/13 strains that cause severe stunting in rice seedlings produced GA_3 (Table 5.2). The progeny (CD220111) that caused the most severe stunting did not produce GA_3 (Table 5.2). The concentration of GA_3 produced by each strain was determined based on a calibration curve developed for each run.

Rice pathogenicity assessed as seed germination and seedling elongation

Based on the rolled towel assay, both the *F. fujikuroi* and *F. proliferatum* parents stunted shoot elongation of rice seedlings. In this experiment, only 79 progeny were tested. Experimental replications of this study were consistent with acceptable correlation coefficients (Table 5.3). The *F. fujikuroi* parent was in the severe stunting phenotypic range, while the *F. proliferatum* parent was in the intermediate stunting phenotypic range (Figure 5.1). Seedling growth, as assessed by the mean length of shoots, was a continuous phenotype (Figure 5.1). Transgressive phenotypes, relative to parental level of inhibition, were observed among the progeny. Fungal progeny that permitted more growth than either parent and progeny that limited growth more severely than either parent were both observed. The reduction of rice seedling growth was positively correlated ($R = 0.3445$, $P = 0.0019$) with GA_3 production (Table 5.4).

Decrease in seed germination rates also were evaluated as a pathogenic effect of the fungus on rice seeds. Germination rates for seeds treated with fungal progeny ranged from 54 to 96% (Figure 5.2). Germination of seeds treated with the parental strains was 78% (*F. fujikuroi*) for one parent and 89% (*F. proliferatum*) for the other parent. Transgressive phenotypes relative to parental level of seed germination also were observed. Seedling elongation and seed germination were positively correlated ($R = 0.7478$, $P < 0.0001$). However, seed germination rates were not significantly correlated ($R = 0.1687$, $P = 0.1374$) with GA_3 production (Table 5.4).

Detection of QTLs for GA_3 and rice pathogenicity

Single marker analysis was performed by using parametric, non-parametric, and binary methods for GA_3 production, and by using parametric and nonparametric methods for germination and seedling elongation. QTLs were identified for GA_3 production and rice seedling elongations (Figure 5.6; 5.7, 5.8). QTLs for these characters occurred on Chromosome 5 near one end of the chromosome. These QTLs could explain some of the variation observed for GA_3 production and seedling growth. Three AFLP markers were associated with these traits - ETTMAC-4, EAAMAA-13, and EAAMAA-10 (Figure 5.6). Additional QTLs associated with GA_3 production were detected by using the nonparametric and binary methods (Figure 5.7). With the nonparametric analysis, significant ($\alpha = 0.05$) QTLs were found on chromosomes 2, 3, 4, 5, 8, 11, and 12. Similarly using binary analysis, QTLs were detected on chromosomes 2, 3, 4, 5, 8 and 12. There were no differences between the parametric and nonparametric methods in the QTLs detection for rice seedling elongation. No significant QTLs were detected for seed germinations (Figure 5.8).

Discussion

We constructed a recombination-based map for this interspecific cross with 83 AFLP markers and the mating type locus. Based on available genomic sequences for the parental strains and the published *F. fujikuroi* genome, we correlated physical and recombination-based maps (Figure 5.5). The mating type locus is in the same relative position in most *Fusarium* genomic sequences and maps. Adding this marker to the map assists us in validating the linkage group/physical chromosome correlation. Segregation for most of markers was distorted towards *F. proliferatum*. Through sexual recombination, the genomes of the two species were mixed, but

the number of spores surviving meiosis was between 0.1 and 0.01% of the normal number. Thus, the viable progeny could be selected for multi-locus genotypes required for viability and not necessarily be completely random in nature. The distortion towards *F. proliferatum* that we observed could mean that there are portions of the *F. proliferatum* genome that need to be more or less intact or to co-segregate with one another for the progeny to survive. Three AFLP loci that were distorted towards *F. fujikuroi* suggest that this genomic region may be important for their viability. Segregation distortion also was observed in the interspecific hybrids resulting from a cross between *F. circinatum* and *F. subglutinans* (De Vos et al. 2013). We think that progeny fitness was improved by the presence of the alleles from *F. proliferatum* that caused this distortion.

Currently, two research groups have published whole genome sequences for different strains of *F. fujikuroi* (Jeong et al. 2013; Wiemann et al. 2013). Both strains can cause bakanae disease of rice and produce gibberellic acid. The gibberellic acid gene cluster is located on one end of chromosome 5 in both sequences. Our results are consistent with their findings, as there is a QTL for GA_3 production on the end of chromosome 5. We expected the segregation of this gene cluster amongst the progeny to be 1:1 (GA_3 producer:GA_3 non-producer), but GA_3 non-producers were more common. Important regulators of GA_3 located elsewhere in the genome could be polymorphic and segregating in a manner that reduces GA_3 biosynthesis. The QTL analysis based on nonparametric and binary analyses identifies genetic factors associated with GA_3 production on several other chromosomes. These QTLs could be regulators involved in GA_3 production. Thus, the non-Mendelian segregation of GA_3 production amongst the progeny is due to the skewed segregation of the GA_3 gene cluster and the segregation of other genes unlinked to the cluster that are associated with GA_3 production.

F. fujikuroi is a well-known cause of the bakanae disease of rice. Infected seeds also can exhibit two other disease symptoms, i.e. reduced germination and stunting of seedling growth (Amoah et al. 1995; Wulff et al. 2010). Shoot elongation symptoms expressed as bakanae disease are due to excessive production of gibberellic acid by *F. fujikuroi*. Stunted seedlings also may result from the production of fusaric acid (Desjardins 2006). *F. fujikuroi* stunted the growth of rice seedlings in the rolled towel assay. *F. proliferatum* also stunted seedling growth, but not as severely as did *F. fujikuroi*. Seed germination rates were lower when treated with the *F. fujikuroi* parent than with the *F. proliferartum* parent. The results from the rolled towel assay suggest that the pathogenicity of *F. fujikuroi* on rice seedlings results from growth inhibition. This character is not a result of GA_3 being present. Most of the progeny that cause severe stunting of rice seedlings do not produce GA_3 (Table 5.2). Thus, excessive GA_3 is not the cause of this phenotype and other factors, e.g. fusaric acid, could be responsible for the growth stunting and reduced germination that we observed in this study. Both *F. fujikuroi* and *F. proliferatum* can produce fusaric acid, which is a known phytotoxin (Desjardins 2006).

A QTL for stunted rice seedling growth was detected in the same region as the GA_3 gene cluster on chromosome 5, so there is a pathogenicity factor for stunting located near the GA_3 gene cluster. The rolled towel assay may not be the best method to observe the seedling elongation associated with bakanae, but it did show a range of effects on rice seedling germination and growth. Reduced seedling germination and seedling stunting were highly correlated, suggesting that the same genes could be involved in both traits. No significant QTL for seed germination was detected. However, there is a relatively strong although not statistically significant, QTL on chromosome 5 associated with marker ECCMCG-18, which is further away from the GA_3 cluster than marker EAAMAA-10 (Figure 5.8). This region could potentially be

one containing pathogenicity determinants towards rice in this fungus. The ability to detect QTLs would be greater if more samples had been evaluated in the rolled towel assay, if the genetic map were denser, and if the segregation of the markers was less skewed towards the *F. proliferatum* parent.

The presence of the clonal progeny was unexpected because the probability of getting clones when assessed with large a number (80+) of markers on 12 independent chromosomes following meiosis is very small. We observed 10 clonal groups where some of the groups consist of more than two clonal strains (Table 5.5). During the formation of ascospores, the spores go through mitosis after the second division of meiosis resulting in two clones. Based on the biology of ascospore formation, it should be possible to obtain two clones from the same ascus, especially since the number of surviving ascospores/perithecium often was small. However, we also found clonal strains that originated from different perithecia. Many of the clones were isolated from different perithecia on the same plate. The perithecia on this plate also yielded more progeny than did those on other plates. We do not understand the reason for the discrepancies on this plate, but think that the number of clones observed here is unusually high. Members of some clonal groups also were recovered from perithecia on other Petri dishes.

In conclusion, we identified multiple genomic regions associated with GA_3 production that warrant further analysis to determine their function(s). Potential pathogenicity factors responsible for stunting rice seedling growth were located on chromosome 5. Comparing this sequence with other species that are not rice pathogens, e.g. *F. verticillioides*, could lead to the identification of unique genes on this chromosome that have a role in pathogenicity towards rice. The interspecific cross, and the unusual characters segregating amongst the progeny, provide a novel approach to understanding the regulation of GA_3 biosynthesis and the genes required for

rice pathogenicity. Alleles from the *F. proliferatum* parent could be of interest in showing how pathogenicity loci could be rendered new and functionals.

Table 5.1 AFLP markers used for map construction.

Marker	Segregation (F.f. : F.p.)[a]	Band size (bp)[d]
ECCMCG-1	206:327[b]	514*
ECCMCG-2	114:419[b]	505
ECCMCG-3	170:363[b]	495
ECCMCG-4	205:328[b]	490
ECCMCG-5	237:296[b]	431
ECCMCG-6	267:266	414
ECCMCG-7	157:376[b]	395
ECCMCG-8	157:376[b]	393*
ECCMCG-9	83:450[b]	386*
ECCMCG-10	195:338[b]	352*
ECCMCG-11	82:451[b]	349*
ECCMCG-12	105:428[b]	343
ECCMCG-13	147:386[b]	341*
ECCMCG-14	129:404[b]	300*
ECCMCG-15	142:391[b]	292
ECCMCG-16	139:394[b]	264*
ECCMCG-17	527:6[c]	249*
ECCMCG-18	97:436[b]	247
EAAMAA-1	160:373[b]	536
EAAMAA-2	169:364[b]	496*
EAAMAA-3	206:327[b]	477
EAAMAA-4	317:216[c]	445*
EAAMAA-5	146:387[b]	435
EAAMAA-6	148:385[b]	403
EAAMAA-7	349:184[c]	396
EAAMAA-8	307:226[c]	375*
EAAMAA-9	250:283	370
EAAMAA-10	151:382[b]	363*
EAAMAA-11	84:449[b]	348*
EAAMAA-12	233:300[b]	346
EAAMAA-13	136:397[b]	332
EAAMAA-14	138:395[b]	316*
EAAMAA-15	521:12[c]	309*
EAAMAA-16	319:214[c]	287
EAAMAA-17	181:352[b]	273
EAAMAA-18	162:371[b]	271
EAAMAA-19	152:381[b]	261*
EAAMAA-20	162:371[b]	255
EAAMAA-21	278:255	222
EAAMAA-22	157:376[b]	210*

EAAMAA-23	140:393[b]	206
EGGMTG-1	201:332[b]	536
EGGMTG-2	110:423[b]	518
EGGMTG-3	189:344[b]	488
EGGMTG-4	150:383[b]	477
EGGMTG-5	172:361[b]	474*
EGGMTG-6	47:486[b]	463
EGGMTG-7	217:316[b]	442
EGGMTG-8	170:363[b]	435*
EGGMTG-9	336:197[c]	400
EGGMTG-10	154:379[b]	397
EGGMTG-11	241:292[b]	390*
EGGMTG-12	209:324[b]	373*
EGGMTG-13	208:325[b]	368*
EGGMTG-14	147:386[b]	362
EGGMTG-15	251:282	357*
EGGMTG-16	246:287	354
EGGMTG-17	111:422[b]	348
EGGMTG-18	124:409[b]	324
EGGMTG-19	157:376[b]	315
EGGMTG-20	154:379[b]	309*
EGGMTG-21	120:413[b]	307
EGGMTG-22	122:411[b]	297
EGGMTG-23	172:361[b]	246*
EGGMTG-24	140:393[b]	207*
ETTMAC-1	270:263	518*
ETTMAC-2	205:328[b]	500
ETTMAC-3	137:396[b]	468
ETTMAC-4	138:395[b]	443
ETTMAC-5	180:353[b]	428*
ETTMAC-6	139:394[b]	422
ETTMAC-7	181:352[b]	394*
ETTMAC-8	291:242[c]	384
ETTMAC-9	163:370[b]	358*
ETTMAC-10	151:382[b]	355*
ETTMAC-11	114:419[b]	342
ETTMAC-12	145:388[b]	336
ETTMAC-13	148:385[b]	331*
ETTMAC-14	163:370[b]	316
ETTMAC-15	154:379[b]	298*
ETTMAC-16	212:321[b]	287
ETTMAC-17	183:350[b]	268
ETTMAC-18	150:383[b]	262

ETTMAC-19	121:412[b]	255*
ETTMAC-20	70:463[b]	226*
ETTMAC-21	206:327[b]	210*

[a]Segregation ratio alleles of *F. fujikuroi* : *F. proliferatum*

[b]Segregation of the marker is significantly different from 1:1 and distorted towards *F. proliferatum*.

[c]Segregation of the marker is significantly different from 1:1 and distorted towards *F. fujikuroi*.

[d]Band size with "*" indicates source of the band is from *F. proliferatum*.

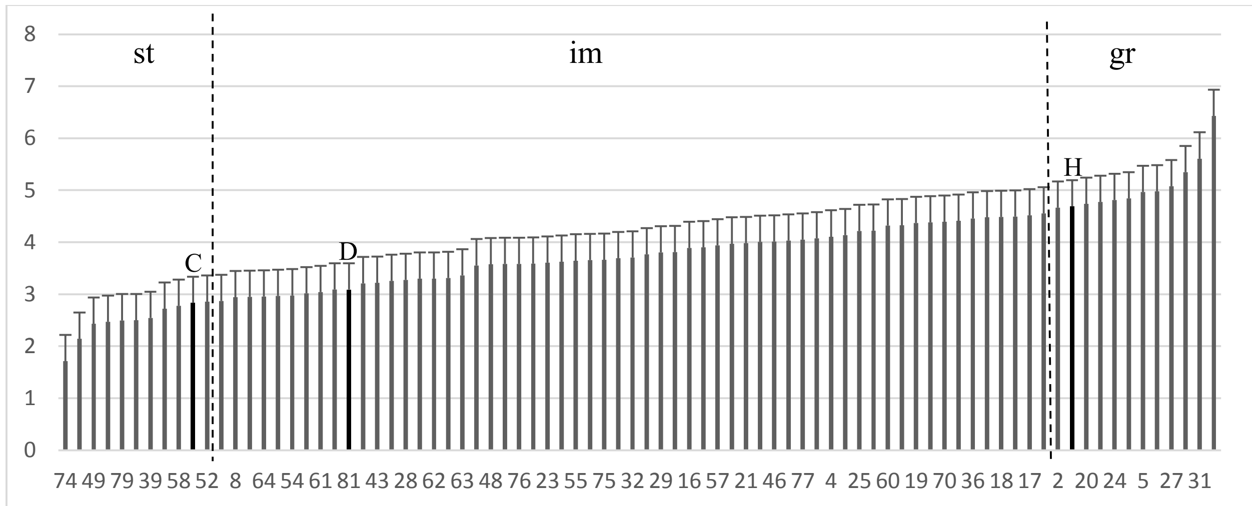


Figure 5.1 Rice seedling growth as altered by fungal strains.

Vertical dashed line divides strains into three categories; st = stunting, im = intermediate, gr = growth enhancing. Letters above bars identify controls used in the experiment: C = *F. fujikuroi*, D = *F. proliferatum*, H = ddH_2O. Bars in group st are significantly different than group gr ($P < 0.05$). The y-axis represents the shoot length (cm), and the x-axis represents the individual strains.

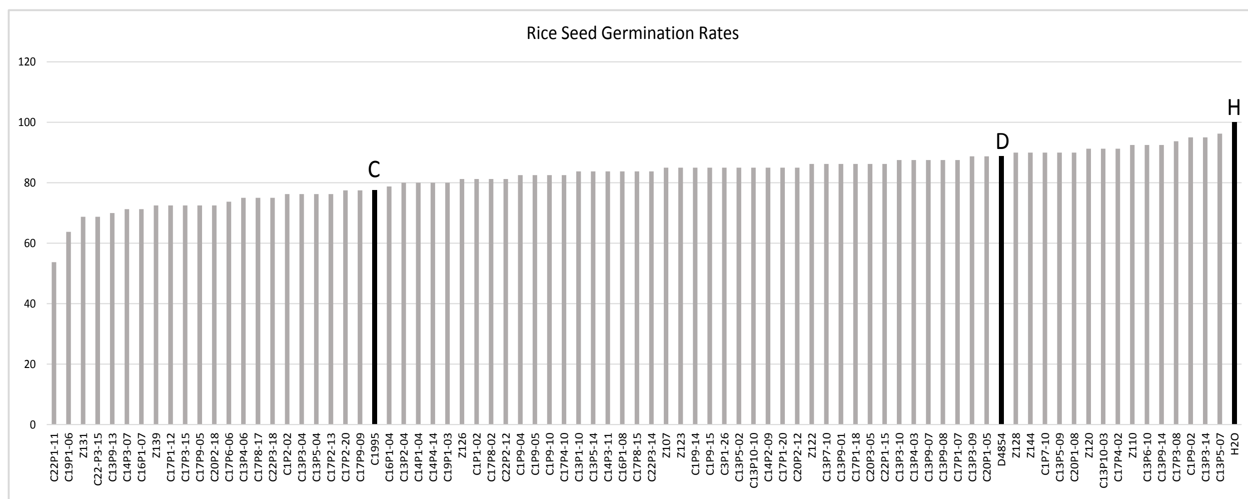


Figure 5.2 Germination rate of rice seeds as altered by fungal strains.

Dark bars with a letter above them indicate controls: C = *F. fujikuroi*, D = *F. proliferatum*, H = ddH_2O. The y-axis represents percent seed germination (%), whereas the x-axis represents the individual strains.

Table 5.2 Strains that cause stunting of rice seedlings.

The length of rice seedlings are compared to negative control (H_20) at P=0.05.

Strain	% F.f.	% F.p.	GA_3
[a]CD170909	14	86	-
[a]CD170315	15	85	+
[a]CD220111*	17	83	-
[a]CD170817	22	78	-
[a]CD190106	26	74	-
[a]CD220318	26	74	-
[a]CD130913	28	72	-
[a]Z131	28	72	+
[a]CD170112	29	71	-
[a]CD220315	30	70	-
[b]CD010202	47	53	-
[b]CD160107	57	43	+
[b]CD130504	60	40	+
C1995	100	0	+
D4854	0	100	-

[a]Segregation ratio of AFLP alleles is distort towards *F. proliferatum*

[b]Proportion of alleles from each parent is not significantly different from 1:1 based on a Chi-square test at P=0.05

*Strains that cause the most stunted growth on rice seedlings

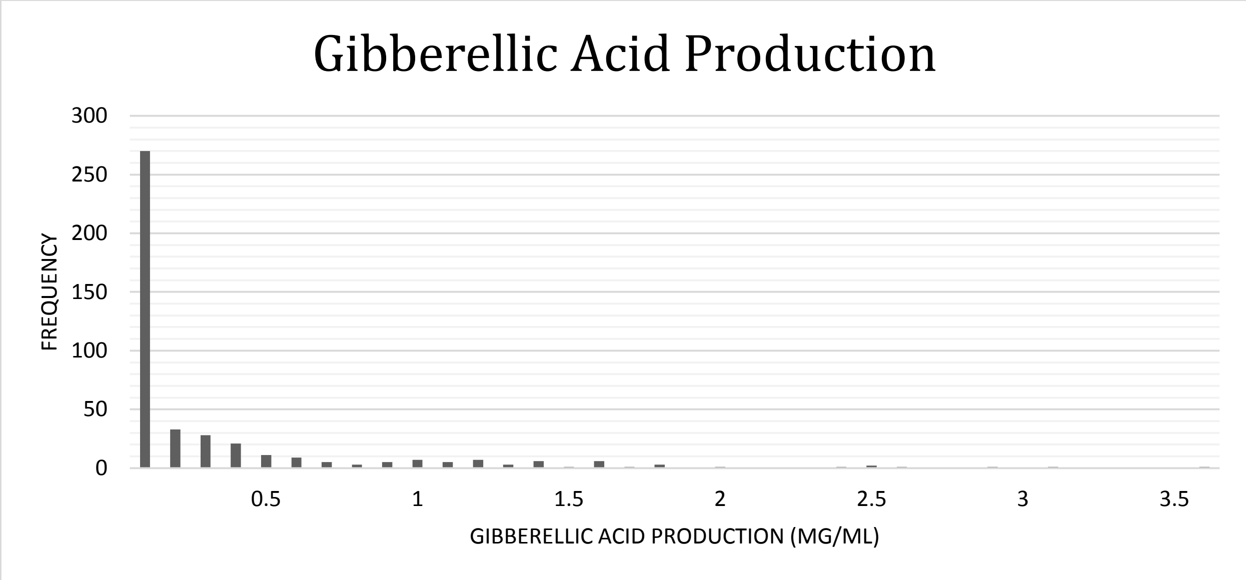


Figure 5.3 Production of gibberellic acid by progeny.

Table 5.3 Correlation (R) between the experimental replications of the rice pathogenicity assay

	Rep. 1	Rep. 2	Rep. 3	Rep. 4
Rep. 1	1	0.45574 (P < 0.0001)	0.40073 (P = 0.0002)	0.49931 (P < 0.0001)
Rep. 2			0.33366 (P = 0.0022)	0.48653 (P < 0.0001)
Rep. 3				0.38525 (P = 0.0004)

Table 5.4 Correlation (R) between GA_3 production, rice seedling growth, and seed germination rates.

	GA_3 production	Rice seedling growth	Seed germination rate
GA_3 production	1	0.34452 ($P = 0.0019$)	0.16865 ($P = 0.1374$)
Rice seedling growth		1	0.74783 ($P < 0.0001$)
Rice germination rates			1

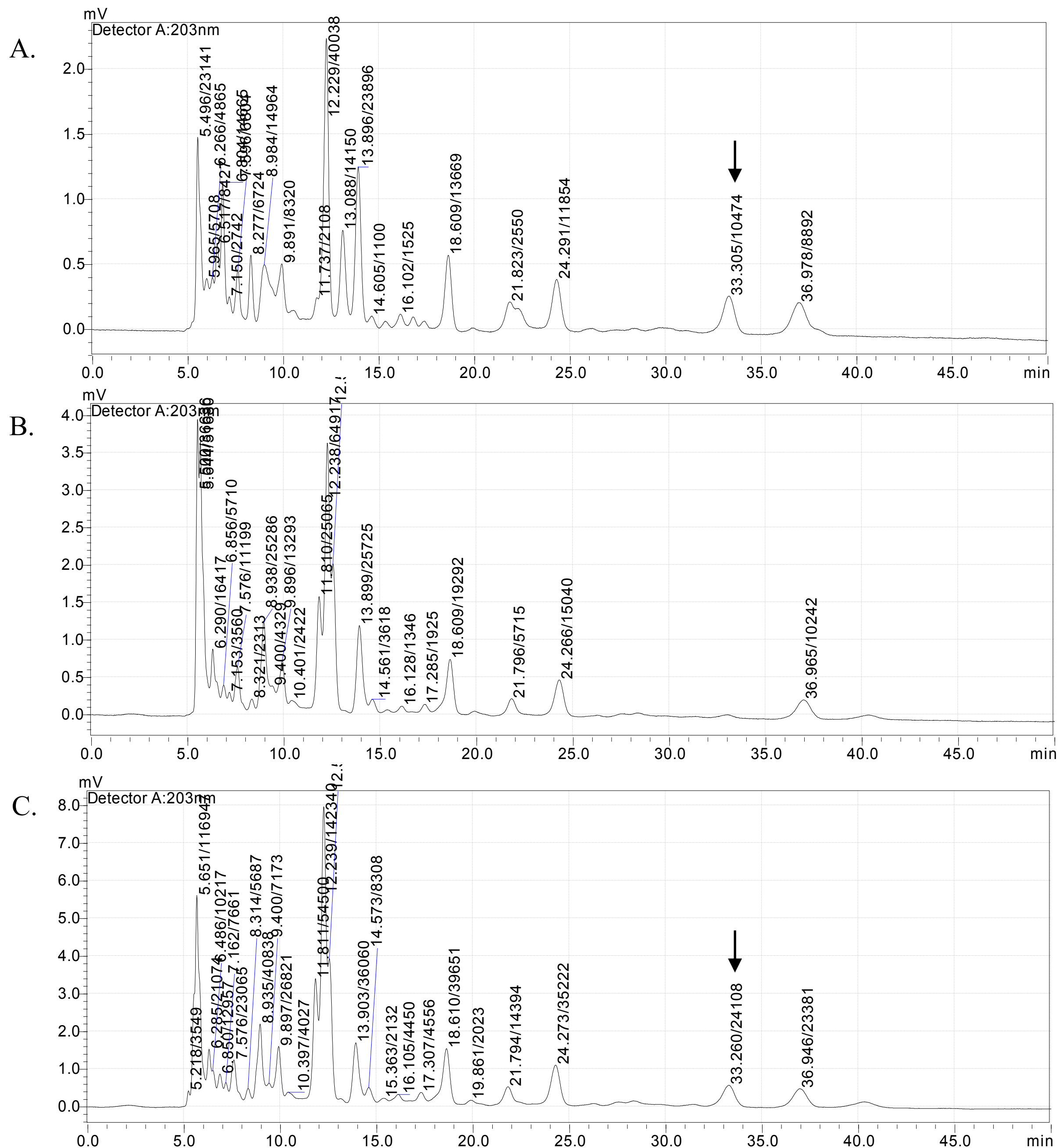


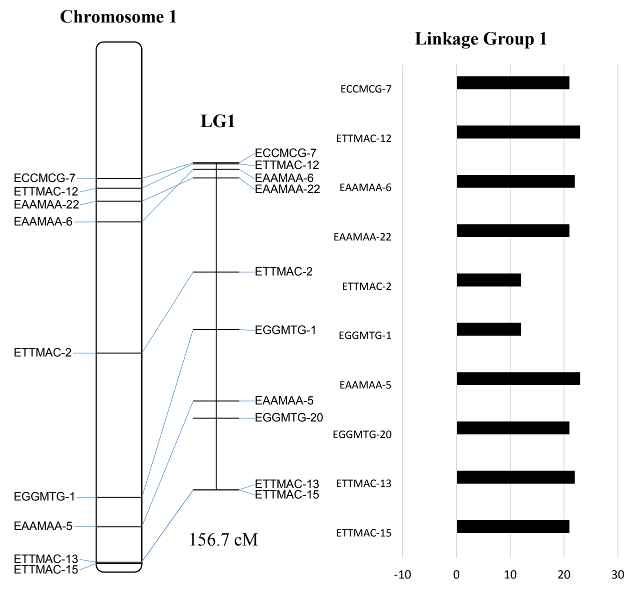
Figure 5.4 Chromatogram showing separation of GA_3 from other compounds in the gibberellin extraction.

GA_3 molecule is detected at 33 minutes. (A) GA_3 analysis of the extract of the *F. fujikuroi* parental strain. (B) GA_3 analysis of the extract of the *F. proliferatum* parental strain. (C) GA_3 analysis of the spiked *F. proliferatum* sample. Arrows indicate GA_3 peak.

Table 5.5 Clonal groups of progeny from the interspecific cross.
Haplotype based on AFLP markers.

Strains	Number of strains	Perithecia	Genome ratio *(*F. fujikuroi*:*F. proliferatum*)
CD160210, CD160608	2	C16P2, C16P6	12:74
CD160616, CD160620	2	C16P6	7:79
CD160601, CD160604, CD160607, CD160613	4	C16P6	9:77
CD160610, CD160611	2	C16P6	10:76
CD171110, CD171112	2	C17P11	3:83
CD160310, CD160602	2	C16P3, C16P6	10:76
CD160301, CD160302, CD160615	3	C16P3, C16P6	10:76
CD160306, CD160311, CD160502, CD160605	4	C16P3, C16P5, C16P6	11:75
CD160401, CD160606	2	C16P4, C16P6	11:75
CD160209, CD160305, CD160307, CD160308, CD160309, CD160501	6	C16P2, C16P3, C16P5	12:74
Unique individuals	504	-	-

*F.f = Alleles from *F. fujikuroi* parent; F.p. = Alleles from *F. proliferatum* parent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LG1
Linkage Group 1
ECCMCG-7
ETTMAC-12
EAAMAA-22
EAAMAA-6
ETTMAC-2
EGGMTG-1
EAAMAA-5
ETTMAC-13
ETTMAC-15
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EAAMAA-6
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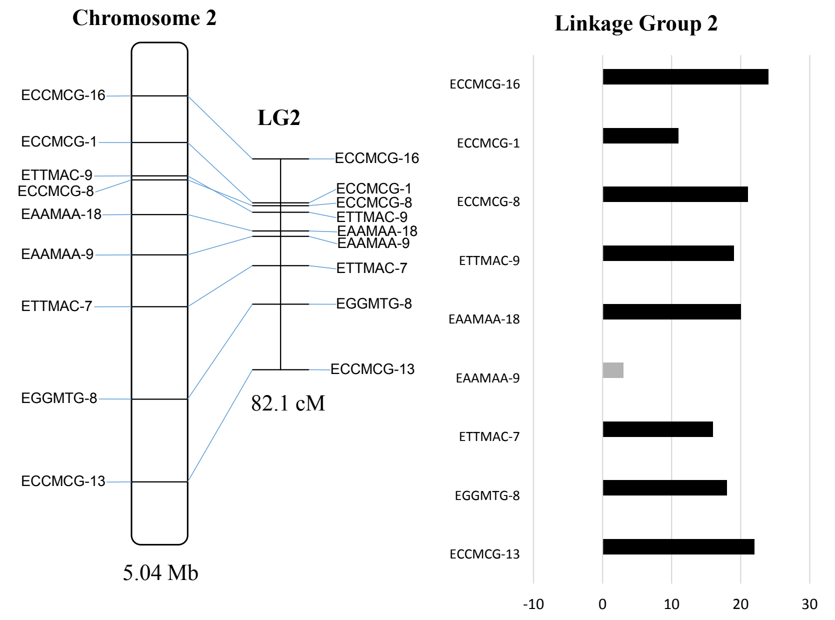
0
10
20
30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Linkage Group 2
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82.1 cM
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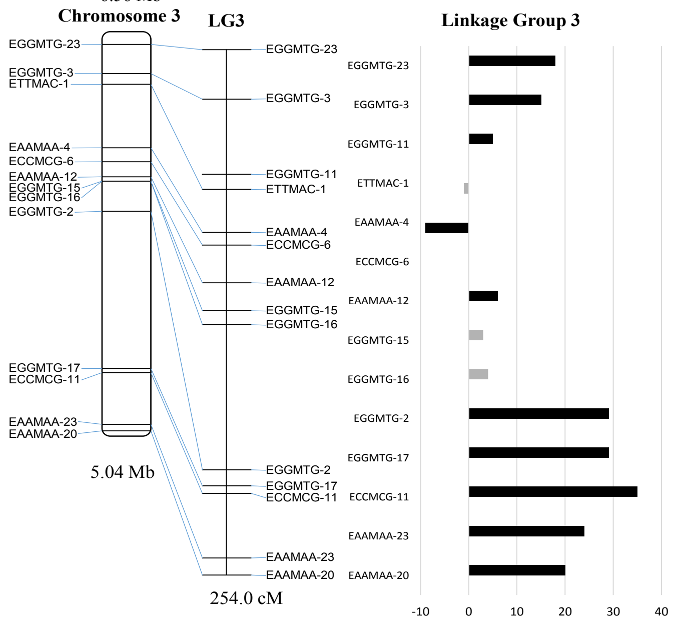
0
10
20
30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Linkage Group 3
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EGGMTG-2
EGGMTG-17
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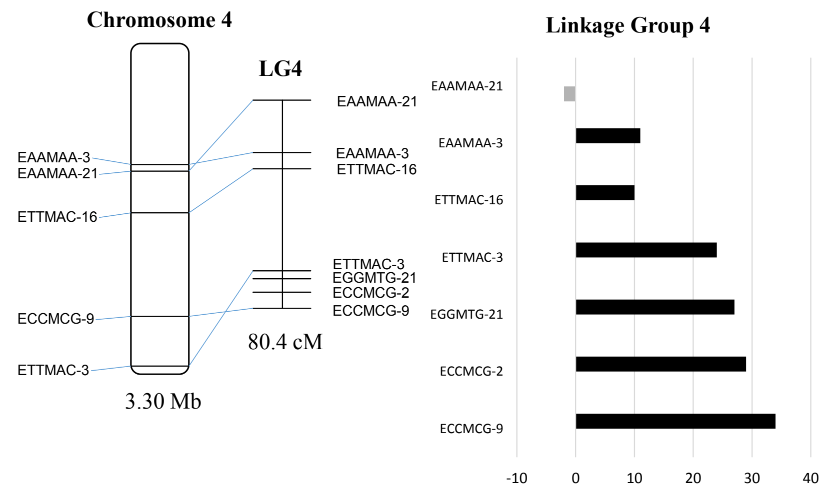
0
10
20
30
40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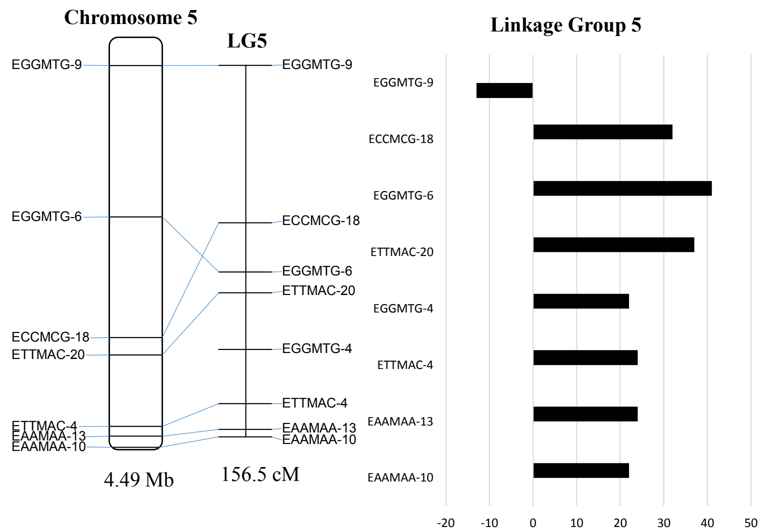
0
10
20
30
40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Linkage Group 5
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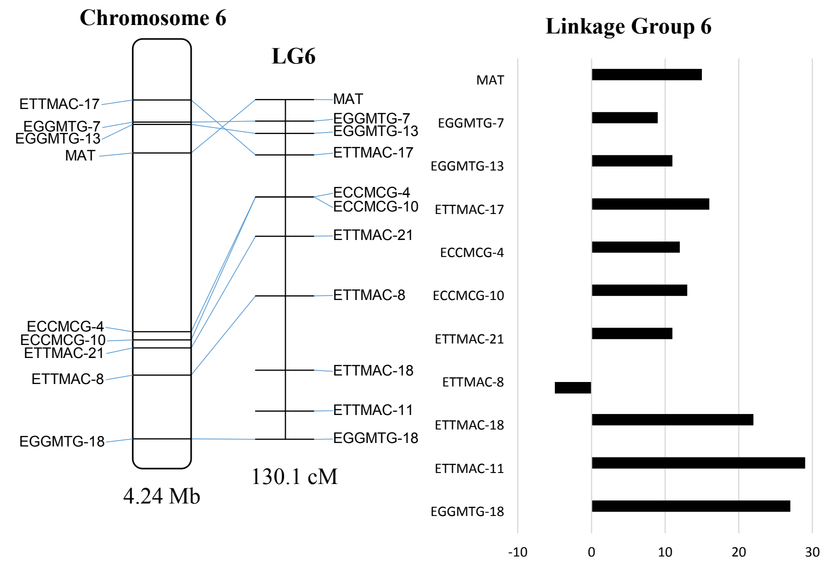
EAAMAA-10
EGGMTG-9
ECCMCG-18
EGGMTG-6
ETTMAC-20
EGGMTG-4
ETTMAC-4
EAAMAA-13
EAAMAA-10
4.49 Mb
156.5 cM
EGGMTG-9
ECCMCG-18
EGGMTG-6
ETTMAC-20
EGGMTG-4
ETTMAC-4
EAAMAA-13
EAAMAA-10
-20
-10
0
10
20
30
40
50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LG6
Linkage Group 6
ETTMAC-17
EGGMTG-7
EGGMTG-13
MAT
ECCMCG-4
ECCMCG-10
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ETTMAC-8
EGGMTG-18
MAT
EGGMTG-7
EGGMTG-13
ETTMAC-17
ECCMCG-4
ECCMCG-10
ETTMAC-21
ETTMAC-8
ETTMAC-18
ETTMAC-11
EGGMTG-18
4.24 Mb
130.1 cM
MAT
EGGMTG-7
EGGMTG-13
ETTMAC-17
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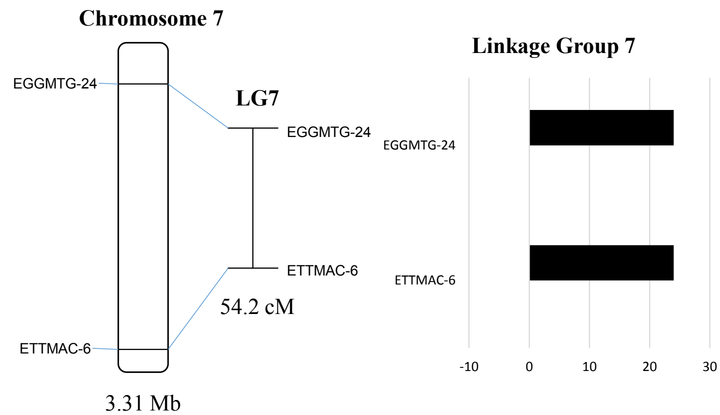
-10
0
10
20
30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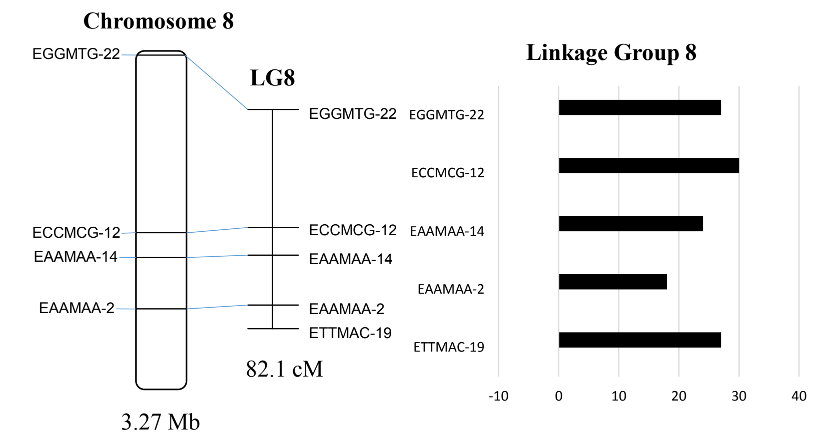
Linkage Group 7
EGGMTG-24
ETTMAC-6
EGGMTG-24
ETTMAC-6
3.31 Mb
54.2 cM
EGGMTG-24
ETTMAC-6
-10
0
10
20
30


Chromosome 8
LG8
Linkage Group 8
EGGMTG-22
ECCMCG-12
EAAMAA-14
EAAMAA-2
EGGMTG-22
ECCMCG-12
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EAAMAA-2
ETTMAC-19
3.27 Mb
82.1 cM
EGGMTG-22
ECCMCG-12
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EAAMAA-2
ETTMAC-19
-10
0
10
20
30
40

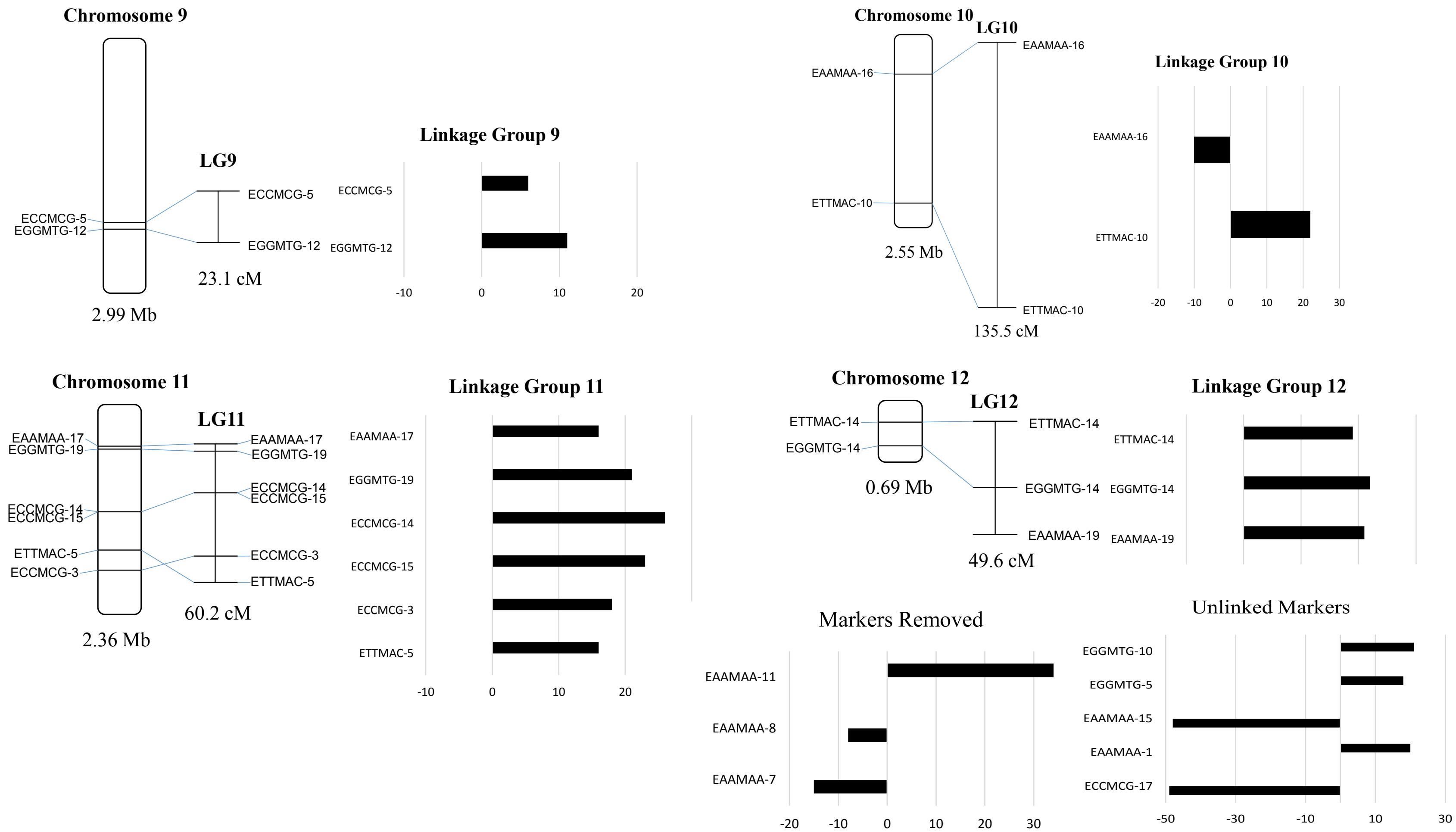
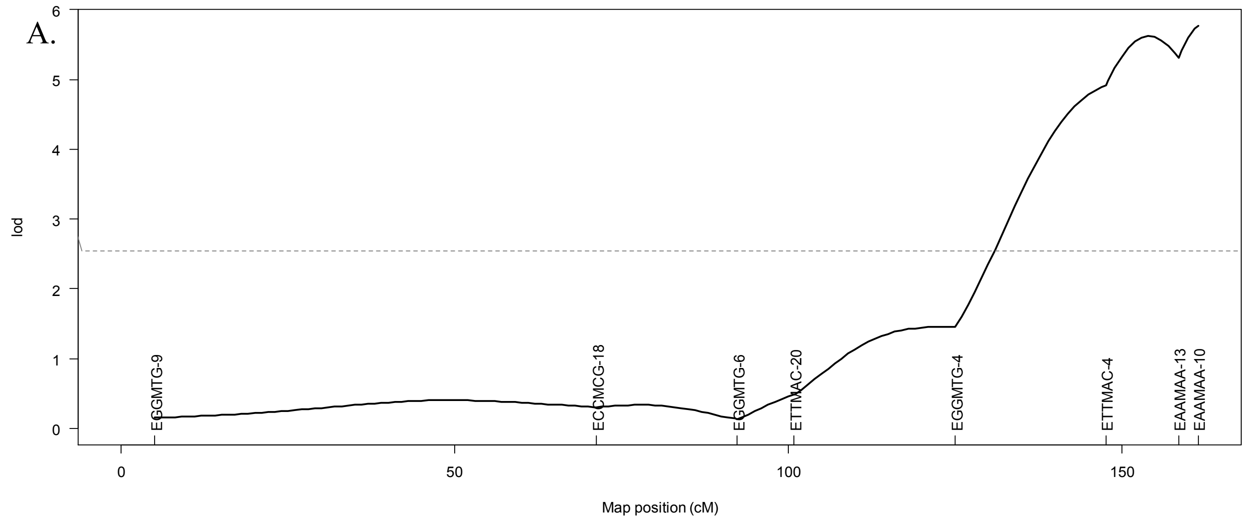


Figure 5.5 Alignment between the physical map of *F. fujikuroi* and the genetic map from the interspecific cross.

LG indicates linkage group. The horizontal bar represent the percentage and the direction of marker distortion. Positive direction represent distortion towards *F. proliferatum*. A gray bar indicates that the distortion does not deviate from a 1:1 segregations based on χ^2-test(P=0.05).



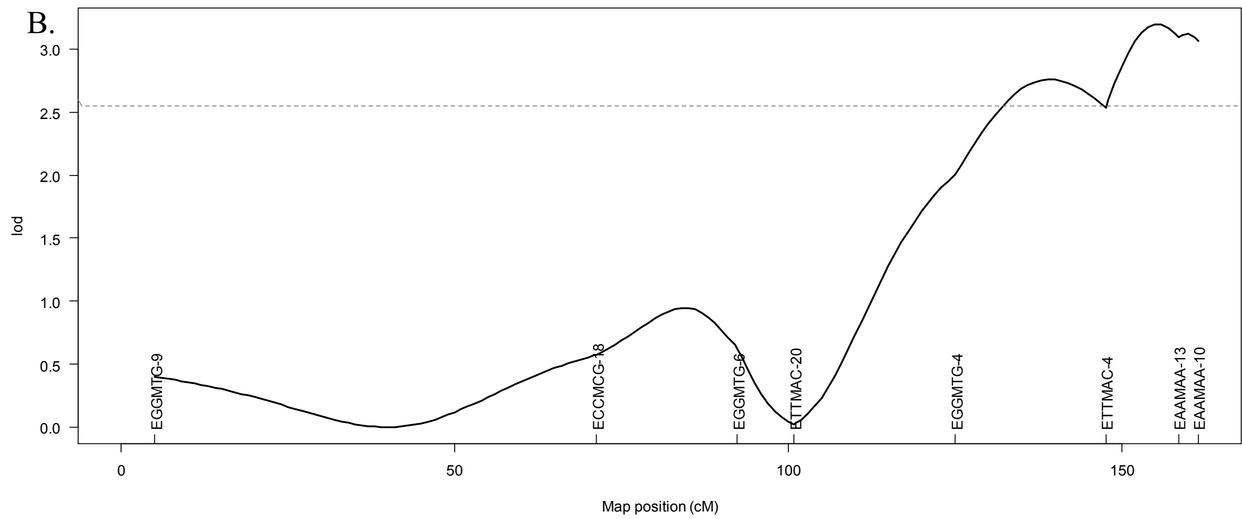


Figure 5.6 QTLs on chromosome 5.

The dotted line indicates the LOD significance level at α=0.05. (A) Location of QTL for GA_3 production on chromosome 5. (B) Location of QTL for rice pathogenicity on shoot elongation on chromosome 5.

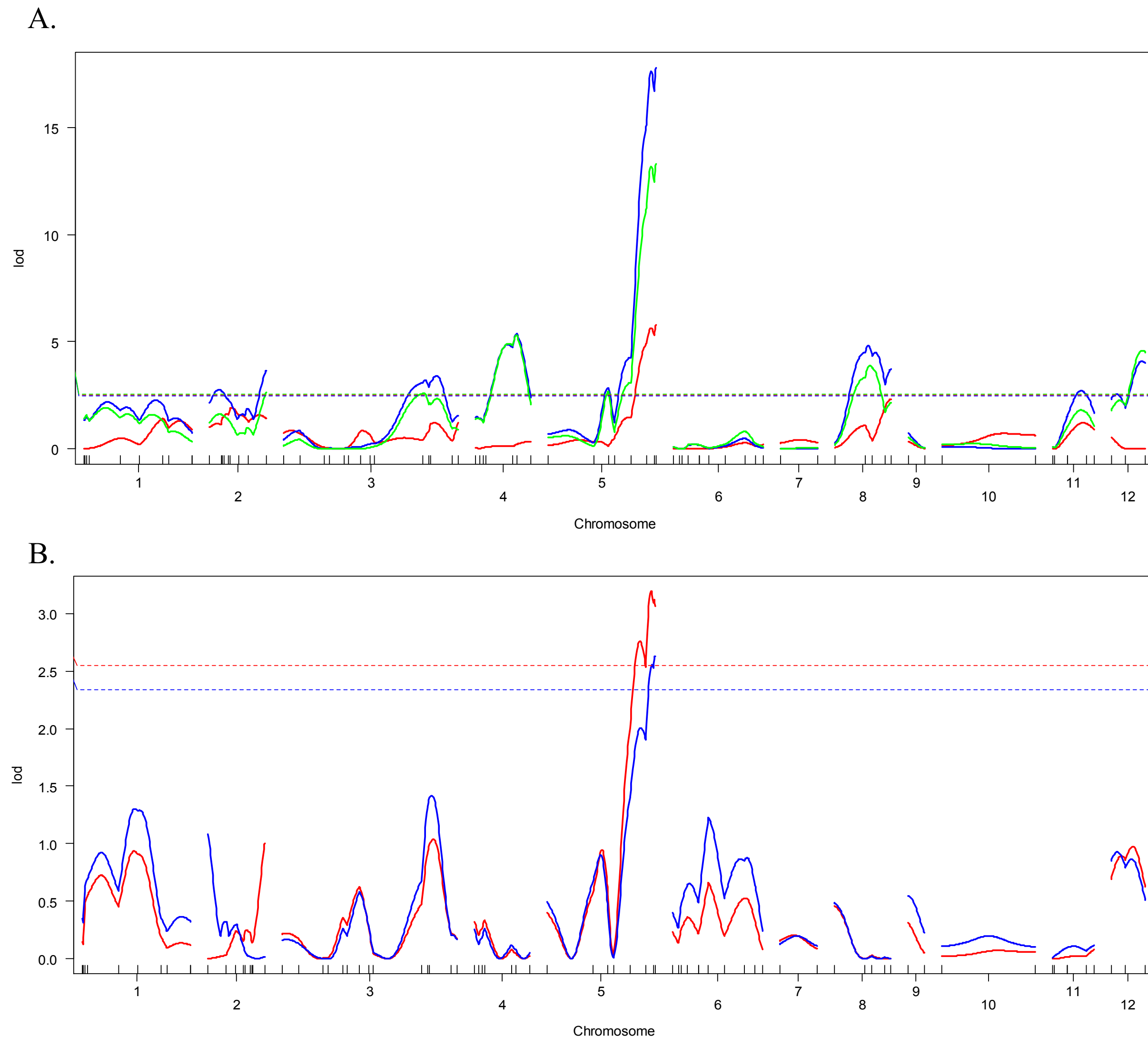


Figure 5.7 Comparison of QTL detection methods.

(A.) QTLs for Gibberellic acid production across the genetic map. The solid red line represents the parametric method, the solid blue line represents the nonparametric method, and the solid green line represents the binary method. The dotted line indicates the significance threshold level at α=0.05. (B) QTLs for rice seedling elongation pathogenicity across the genetic map. The solid blue line represents the parametric method, and the solid red line represents the nonparametric method.

A.

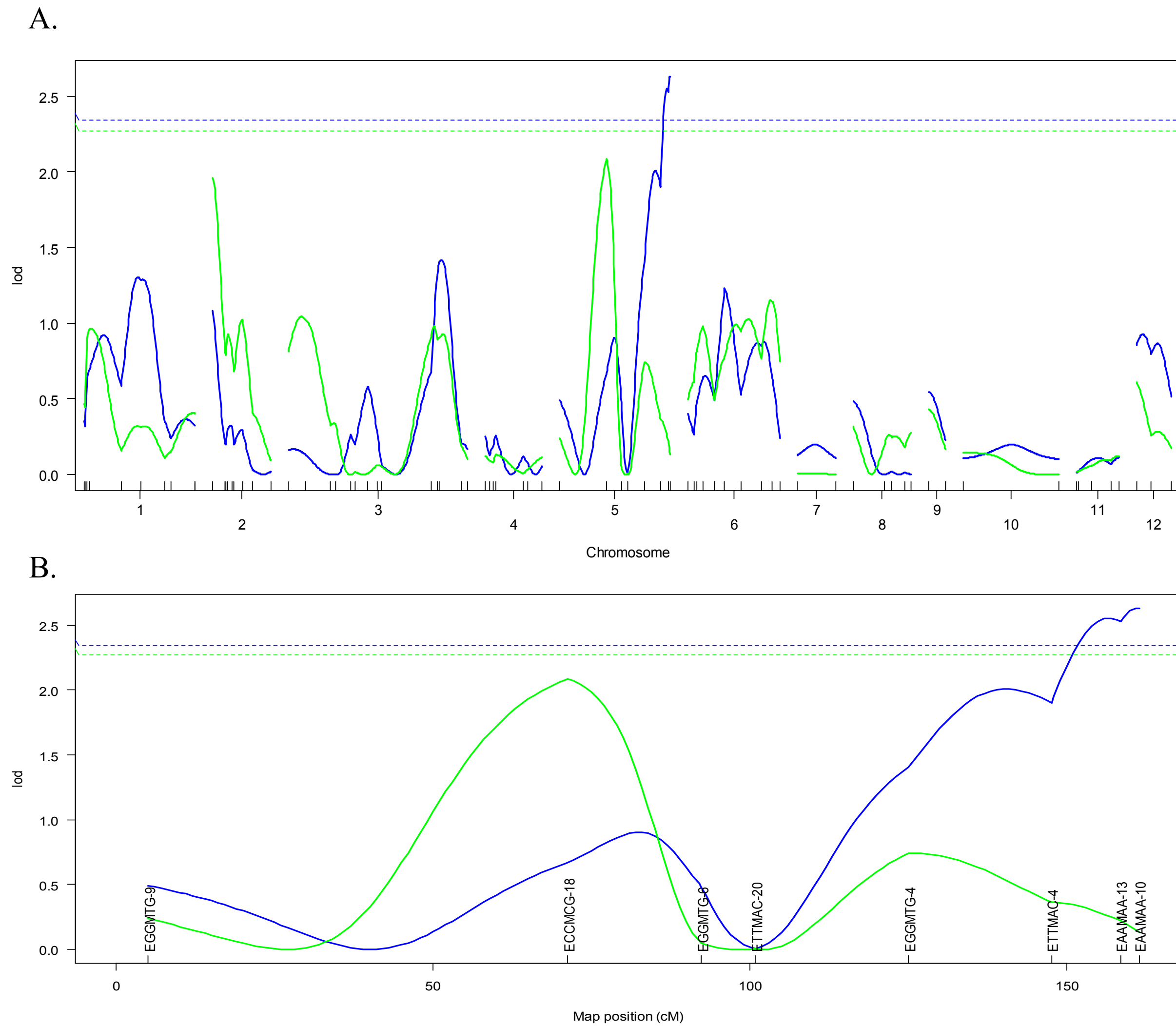


Figure 5.8 Detection of QTLs for rice seedling elongation and seed germination.

Blue - Rice elongation, Green - Seed germination. (A.) QTL detection for both traits across the genetic map; (B.) QTL detection on Chromosome 5. The solid blue line represents the QTL for rice seedling growth, and the solid green line represents QTLs for rice seed germination.. The dotted line represents the LOD significance level at α=0.05.

Chapter 6 - Pathogenicity of the interspecific hybrids between *F. proliferatum* and *F. fujikuroi* towards onions

Abstract

Fusarium prolifeatum can cause disease on a wide range of economically important plants. Onion rot has been reported to be caused by this species. The disease symptoms include pink discoloration, necrosis on roots and leaves, reduced bulb size, water-soaking, and tan lesions on the bulbs. A successful interspecific cross between *F. fujikuroi* and *F. proliferatum* yielded 533 viable progeny for a study of the segregation of pathogenicity traits. A genetic linkage map with 86 AFLP markers based on 432 progeny was used to detect Quantitative Trait Loci (QTLs) associated with pathogenicity on white pearl onions. The onion bulbs were wounded, inoculated with a fungal strain and then incubated in a humid chamber at 25°C for 14 days. Three pathogenic phenotypes were observed - external, internal, and blister. The external and internal phenotypes varied in lesion size and detectable pathogen growth. Both external and internal phenotypes were associated with the same QTLs located on chromosomes 6 and 7 ($P < 0.05$). Blister is a novel phenotype expressed by neither parental strain and segregated 3:1 (non-blister:blister) amongst the progeny. There were no QTLs associated with the blister phenotype and blister was negatively correlated with the external and internal pathogenicity phenotypes. These results enable the identification of loci associated with onion pathogenicity in *F. proliferatum* and alleles associated with lack of pathogenicity in *F. fujikuroi*. The occurrence of the blister phenotype demonstrates that fungal strains with novel pathogenicity characters can arise from interpecific crosses under field conditions and that such crosses could be important in the development and evolution new pathogen traits and capabilities.

Introduction

Fusarium proliferatum rots onions and garlic bulbs in Argentina, Israel, Japan, Spain, Serbia, Turkey, and the United States (Bayraktar and Dolar 2011; Dissanayake et al. 2009; du Toit et al. 2003; Lebiush-Mordechai et al. 2014; Palmero et al. 2010; Salvalaggio and Ridao 2013; Stankovic et al. 2007) This pink rot of onion (Carrieri et al. 2013) results in pink discoloration of the outer scale of the bulbs, necrosis on roots and leaves, and reduced bulb size. In storage, the infected bulbs have water-soaked, tan lesions, and white or light pink fungal mycelium covering the cloves (du Toit et al. 2003; Jepson 2008; Palmero et al. 2010). Reports of this disease have been increasing and *F. proliferatum* has emerged as an important pathogen of onions due to its wide distribution and the resulting economic losses.

F. fujikuroi, a species closely related to *F. proliferatum*, is a well-known rice pathogen that causes bakanae disease (Amatulli et al. 2010; Cruz et al. 2013; Kim et al. 2012; O'Donnell et al. 1998b). Plants with bakanae disease have abnormal growth elongation, dry, yellowing leaves, and partially filled or empty grains (Cruz et al. 2013; Ou 1985; Zainuddin et al. 2008a). *F. fujikuroi* also can stunt the growth of rice seedlings and reduce seed germination rates (Chapter 5). Morphologically, *F. fujikuroi* and *F. proliferatum* are effectively indistinguishable (Leslie and Summerell 2006), and phylogenetically they are very closely related (Ellis 1988; O'Donnell et al. 1998b). Sexual crosses usually clearly distinguish these species, but some strains from these species may occasionally cross with one another to produce a few perithecia containing 0.1 – 0.01% of the normal number of ascospores (Leslie and Summerell 2006). The two species also differ in their host range and in the profile of secondary metabolites produced (Desjardins 2006; Kvas et al. 2009).

The first interspecific crosses between *F. proliferatum* and *F. fujikuroi* to be analyzed were made in 2004 (Leslie et al. 2004a) and analyzed for traits such as GA production (Studt et al. 2012). Additional progeny from one cross were collected (533 total), characterized with AFLPs (Chapter 5), used to construct a genetic map, and align the genetic map with the physical map. Chromosome 5 carries a QTL for rice pathogenicity (Chapter 5).

The availability of the progeny from this interspecific cross enabled us to study the segregation of the genes associated with onion pathogenicity. The objectives of this study were: (i) to determine the segregation of pathogenicity factors within the interspecific progeny; and (ii) to identify QTLs for onion pathogenicity. We hypothesize that multiple onion pathogenicity factors will segregate independently to yield progeny with a range of aggressiveness in pathogenicity that may include transgressive progeny. This study will enable molecular identification of genes responsible for onion pathogenicity.

Materials and Methods

Sexual cross and collection of interspecific progeny

Two mating type tester strains, *F. fujikuroi* (FGSC8932, *MAT-2*) and *F. proliferatum* (FGSC7615, *MAT-1*), were crossed, and progeny collected as described Chapter 5.

Onion pathogenicity assay and analysis

White pearl onions were obtained from the local grocery store (Dillons). The dried skin and the outer layer of the onions were removed. The surface was sterilized by dipping the onion in 70% ethanol, then 10% bleach, and finally washed twice with sterile water for one minute each. The onions were dried overnight in a laminar flow hood. Onions were wounded at the

equator with a hole punch (one wound 5 mm in diameter and 5 mm in depth per onion). Strains used for the pathogenicity assay were cultured on a potato dextrose agar plug for 3-days prior to inoculation. Each strain plug was inserted into the wound of an onion. All of the inoculated onions were placed on wet filter paper in small Petri dishes. The inoculated onions were kept in a humid chamber and incubated at 25˚C for 14 days.

Pathogenicity was assessed based on the lesion produced and the aggressiveness of the fungal strain (Table 6.1; Table 6.2; Figure 6.1; Figure 6.2). The average of the three replicates was used for the QTL analysis in both parametric and non-parametric analyses. Binary scores of nonpathogenic (progeny with grades of 0 and 1) or pathogenic (progeny with grades of 2 and 3) were used for QTL analyses that employed a binary method.

The experiment was conducted in an incomplete block design with three replicates. Each replicate was treated as a block. Within each replicate, samples were divided into two runs (216 strains) due to the large number of progeny to assay. Each run was conducted at a different time under the same conditions. In the analysis, the replications and runs were treated as random effects. Strains were treated as a fixed effect. Statistical analyses were performed with the "Proc mixed" procedure implemented in SAS, version 9.3 (SAS Institute Inc., Cary, North Carolina). Strains were ranked based on pathogenicity scores, and the ranking used to compare the treatments.

QTL Analysis for Onion Pathogenicity

Detection of QTLs for onion pathogenicity was performed by using R/qtl version 3.1 (Broman and Sen 2009). With the single-QTL model, all markers were subjected to an interval mapping analysis. Initially, the function "calc.genoprob( )" were used to calculate conditional genotype probabilities. Then, a genome scan for QTLs based on single marker analysis was

performed with different calculation methods, i.e. parametric, non-parametric, and binary. The parametric and non-parametric analyses were made with quantitative data, i.e. the average scores from the three replications. For the binary method, the data were converted to a pathogenic or non-pathogenic form. Permutation tests were performed to establish a genome-wide significance threshold based on one thousand replicates. The significance threshold level for logarithm of odds (LOD) was set at $\alpha = 0.05$ for the detection of QTLs.

Results

Onion pathogenic assay

Three phenotypes were observed on onions 14 days after inoculation: external, internal, and blister. The external phenotype was based on the fungal growth on the outer tissue of the onion bulbs. The pathogenic symptoms included water-soaking, soft tissue, yellowing and brown tissue in the outer layer of the bulb. Based on the grading scale, there were 150 progeny with grade 0, 88 grade 1, 183 grade 2, and 11 grade 3. For the internal phenotype, the onion bulb was cut in half and the symptoms were observed in the internal layers of the onion. The distribution of the progeny for the internal phenotype was 167 grade 0, 68 grade 1, 189 grade 2, and 8 grade 3. A third onion pathogenicity phenotype was blister, which is seen as swollen onion tissue produced on the outer scale around the inoculation site. 101/432 progeny produced the blister phenotype on the onion bulb.

Based on chi square analysis, these three phenotypes are not independent ($P < 0.01$). If grades 0 and 1 are combined (non-pathogen) and grades 2 and 3 are combined (pathogen), then segregation for the external phenotype departs slightly from a 1:1 segregation ratio (marginally significant, $0.05 > P > 0.01$), while the internal phenotype did not segregate significantly

different from 1:1 (0.1 > P > 0.05). The segregation of the blister phenotype was significantly different from 1:1 (P < 0.05) but not significantly different from a 3:1 ratio (non-blister:blister) (0.2 > P > 0.05). Some of the inoculated onion bulbs had a pink discoloration that resembles the pink rot disease, but the phenotype was not consistent across replications and was not analyzed further.

Of the 32 possible phenotypes, only 14 were observed (Table 6.4). Forty percent of the progeny had the *F. proliferatum*, parental phenotype, 19% had the *F. fujikuroi* parental type, and 41% were recombinant (Table 6.4). When the scoring grading system was used, 7/8 of the possible phenotypes were observed. If the binary scores were used, then42% of the progeny had the *F. proliferatum* parental type, 33% had the *F. fujikuroi* parental type and 23% were recombinant (Table 6.5).

The external and internal phenotypes were strongly correlated (R = 0.9726, P < 0.0001). These two phenotypes both were negatively correlated with the blister phenotype (Table 6.3).

AFLP and QTL analysis

The genetic map with 12 chromosomes developed in chapter 5 was used for the QTL analysis. The associations between the markers and the phenotypic characters were analyzed for the identification of QTLs. The same QTLs were identified for both the external and the internal pathogenicity traits. When a non-parametric method was used for the analysis, two QTLs were detected, one each on chromosomes 6 and 7. If the binary scoring system is used, then there is one significant QTL, located on chromosome 6. The markers associated with the external and internal pathogenicity trait were ETTMAC-18 on chromosome 6 and ETTMAC-6 on chromosome 7 (Figure 6.3; Figure 6.4). No significant QTLs were associated with the blister phenotype (Figure 6.5).

Discussion

Our results confirm that *F. proliferatum* is pathogenic to onions, and that *F. fujikuroi* is not. This result is consistent with other reports that *F. proliferatum* can cause onion rot in the field (Carrieri et al. 2013; Lebiush-Mordechai et al. 2014). A common symptom associated with onion infection by *F. proliferatum* is pink discoloration of the outer scale (du Toit et al. 2003). We observed this discoloration in our study but did not analyze it further due to its inconsistency during replication.

F. proliferatum can produce many secondary metabolites, particularly mycotoxins (Desjardins 2006). Infected onion bulbs that are kept in storage may be contaminated with one, or more, mycotoxins that threaten consumer health. The results from this study suggest that mycotoxin levels on onion bulbs infected by *F. proliferatum* should be evaluated. Both transgressive pathogenicity traits and novel secondary metabolite profiles were observed amongst the progeny of the interspecific cross. It is possible that *F. fujikuroi* would become an onion pathogen through vertical gene transferred from this type of cross.

With respect to onion pathogenicity traits, there were three pathogenic phenotypes and a non-pathogenic phenotype. The strong correlation between the internal and external onion pathogenicity phenotypes suggests that similar sets of genes underlie these traits. The number of genes segregating for these pathogenicity traits in this cross probably is small, probably only one or two. The chromosomal regions identified, however, are relatively large since the number of markers on the map is relatively small. More detailed maps are needed to localize and identify the loci responsible for these traits. The difference between the binary phenotypes and the more continuous phenotypes suggests that there may be genes that enhance, but are not essential for

pathogenicity. Using chi square analysis, the segregation of the internal ($0.05<P<0.1$) and external ($0.01<P<0.05$) pathogenicity traits are weakly, but significantly different from 1:1 (non-pathogenic:pathogenic). In a haploid fungus, 1:1 segregation means that only a single gene is segregating for the trait. Apparent Mendelian segregation also could occur if the genes segregating for the traits are tightly linked to one another. The chi-square analysis suggests that multiple genes underlie the internal and the external pathogenicity traits. The interspecific nature of this cross and the limited number of viable progeny both suggest caution against over-interpretation of the segregation data, especially since only a single QTL is associated with this trait when the binary data are analyzed.

The QTL analysis identifies a region in the genome associated with the onion pathogenicity traits. As expected from the high correlation that we observed between external and internal phenotype, similar QTLs are detected in the analysis of both the internal and external pathogenicity traits. Two significant QTLs associated with external and internal pathogenicity were detected when using the non-parametric method. The detection of two QTLs means that multiple unlinked loci control these phenotypes.

Most of the AFLP marker segregation ratios were distorted towards the *F. proliferatum* alleles. The segregation pattern for pathogenicity may have been affected by these distortions, and more of the progeny have the *F. proliferatum* parental phenotype than *F. fujikuroi* type. This pattern is consistent with the distortion that we observed in the AFLP marker segregation. The distortion suggests that there are genomic regions within each species that must remain intact to prevent incompatible (lethal) genetic combinations within the progeny.

Blister is a novel onion pathogenicity phenotype that we observed in about a quarter of the progeny from this cross. Infection of onions by the smut fungi in the genus *Urocystis* can

result in black blisters on the infected leaves or the outer scale of the onion bulbs (Horst 2013). These black blisters appear as an elongated, swollen, raised portion of the outer scale of the onion bulb. The segregation ratio for the blister phenotype in this study was about 3 non-blister-producing strains to 1 blister-producing strain. This ratio is consistent with two genesinteracting to produce the blister phenotype. This phenotype might result from a defense reaction by the onions to inhibit fungal invasion and perhaps limit the spread of (toxic) secondary metabolites produced by the fungus. The negative correlation between blister and the external and internal pathogenicity phenotypes is consistent with the hypothesis that blister is the expression of a successful defense response by the onions. Neither parent of the cross can cause blister formation. Recombination that occurs during meiosis may result in novel gene combinations in the progeny that produce the blister phenotype.

In conclusion, our study illustrates risks that could occur from the interspecific cross between *F. proliferatum* (pathogenic) and *F. fujikuroi* (nonpathogenic) in terms of producing pathogens that are more aggressive or that have pathogenicity characters that differ from both parental strains. Thus, progeny from this interspecific cross could result in higher economic losses by onion growers. The transgressive progeny observed in this study suggests that *F. fujikuroi* may contain genes that can enhance pathogenic aggressiveness towards onions. The QTLs identified in this study are an important first step towards identifying the fungal genes involve in this disease.

Table 6.1 Grading scale for pathogenicity of *Fusarium* strains toward onions.

Grade	External behavior	Internal behavior	Classification of strains
0	Absence of lesion; No mycelial growth on tissue.	Absence of lesion; Tissue discoloration only at the edge of the wound	Non-pathogenic
1	Brown/yellow lesion spread up to 3 mm from point of inoculation; Mycelial growth on the edge of the wounded tissue	Yellowing tissue spread up to 3 mm from point of inoculation; Only the first internal layer is affected	Less aggressive
2	Mycelial growth on the outer tissue 4 – 10 mm from the point of inoculation; Yellowing, browning, and soft tissue where the mycelia grow	Yellowing, browning, and water-soaking tissue spread 4 – 10 mm from the point of inoculation; 1 – 3 layers of onion infected	Aggressive
3	Thick mycelial growth on outer tissue over 10 mm from the point of inoculation; Outer tissue is soft and at least half of the bulb is water-soaked	Soft and water-soaked inner tissue over 10 mm from the point of incoculation that spreads to at least half of the bulb; 1 – 3 layers affected	Very aggressive

Table 6.2 Grading scale for blister phenotype on onions.

Grade	Blister behavior	Classification
0	No swollen tissue around the inoculation point; brown, dry edge of wounded tissue	No blister
1	Swollen tissue present around point of inoculation; yellowish- brown color to the swollen tissue	Blister

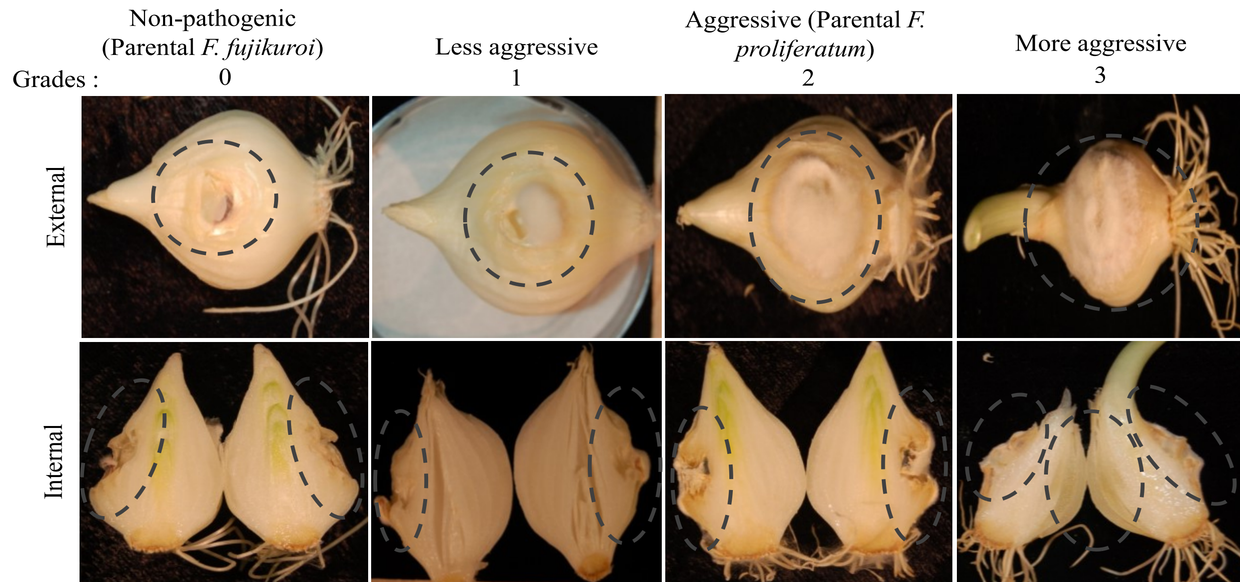


Figure 6.1 Onion pathogenicity phenotypes.

Relative aggressiveness of the progeny of the interspecific cross between *F. fujikuroi* and *F. proliferatum* on onion.

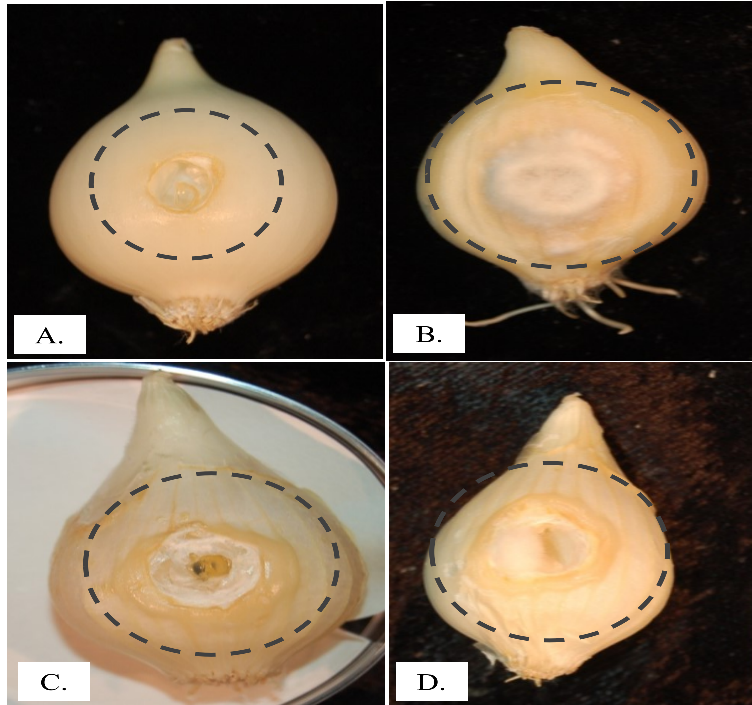


Figure 6.2 Blister phenotype of white pearl onions.

(A) No blisters on onions inculated with non-pathogenic strains. (B) No blister on onions inculated with pathogenic strains; (C) Blisters on onions inoculated with non-pathogenic strains non-pathogenic strain; (D) Blister on onions inoculated with a less aggressive strain.

Table 6.3 Correlation (R) among the external, internal and blister onion phenotypes.

	External	Internal	Blister
External	1	0.97262	- 0.46367
		(P<0.0001)	(P<0.0001)
Internal		1	- 0.46145
			(P<0.0001)
Blister			1

Table 6.4 Onion phenotype combinations based on the full grading scale and their frequency in progeny.

Type[a]	External	Internal	Blister	Frequency
Parental 1	2	2	0	174
Parental 2	0	0	0	82
Rec 1	0	0	2	66
Rec 2	1	1	0	50
Rec 3	1	1	2	17
Rec 4	1	0	0	12
Rec 5	2	2	2	8
Rec 6	3	3	0	8
Rec 7	1	0	2	7
Rec 8	3	2	0	3
Rec 9	0	2	2	2
Rec 10	2	1	0	1
Rec 11	1	2	2	1
Rec 12	1	2	0	1

[a]Parental 1 = *F. proliferatum* parent phenotype; Parental 2 = *F. fujikuroi* parent phenotype; Rec = Recombinant

Table 6.5 Phenotype combinations based on the binary grading scale and their frequency.

Type[a]	External[b]	Internal[b]	Blister[b]	Frequency
Parental 1	P	P	NP	185
Parental 2	NP	NP	NP	144
Rec 1	P	P	P	8
Rec 2	P	NP	NP	1
Rec 3	NP	P	P	3
Rec 4	NP	NP	P	90
Rec 5	NP	P	NP	1
Rec 6	P	NP	P	0

[a]Parental 1 = *F. proliferatum* parent phenotype; Parental 2 = *F. fujikuroi* parent phenotype; Rec = Recombinant.

[b]P = Pathogenic; NP = Non Pathogenic

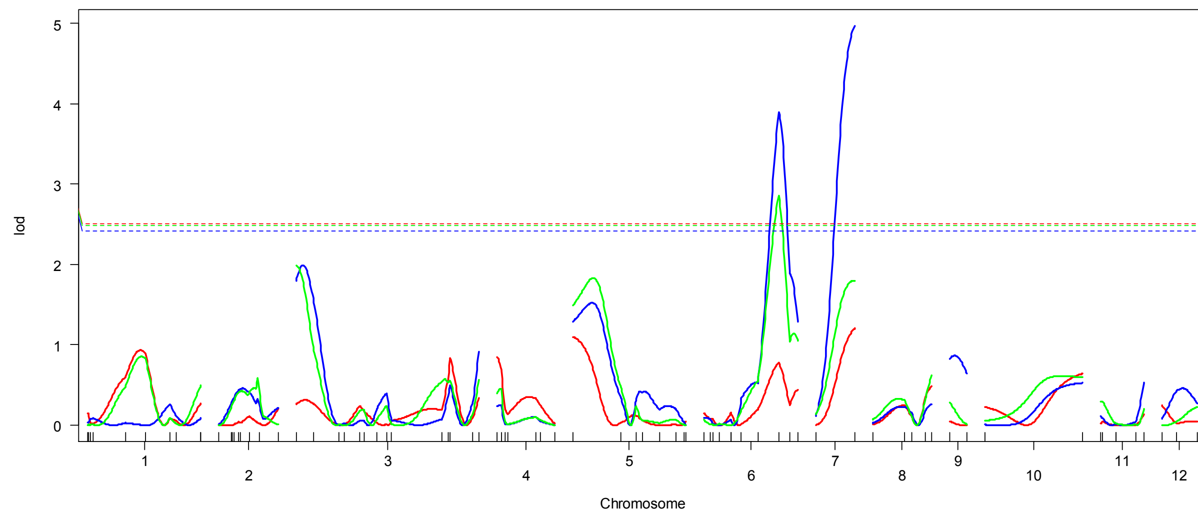


Figure 6.3 QTLs for the external phenotype of onion pathogenicity.

QTLs detected significantly over the threshold level at α = 0.05 located on chromosomes 6 and 7; Red = Parametric method, Blue = Non-parametric method, Green = Binary method.

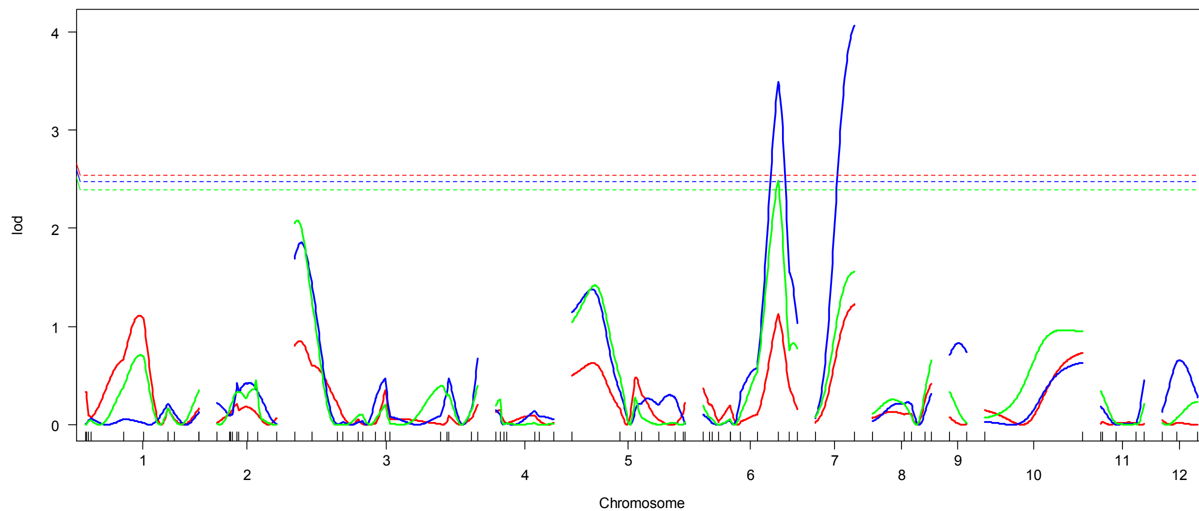


Figure 6.4 QTL for the internal onion pathogenicity phenotype.

QTLs detected significantly over the threshold level at α = 0.05 located at chromosome 6 and 7; Red = Parametric method, Blue = Non-parametric method, Green = Binary method. The significance threshold for each phenotype is indicated by its color.

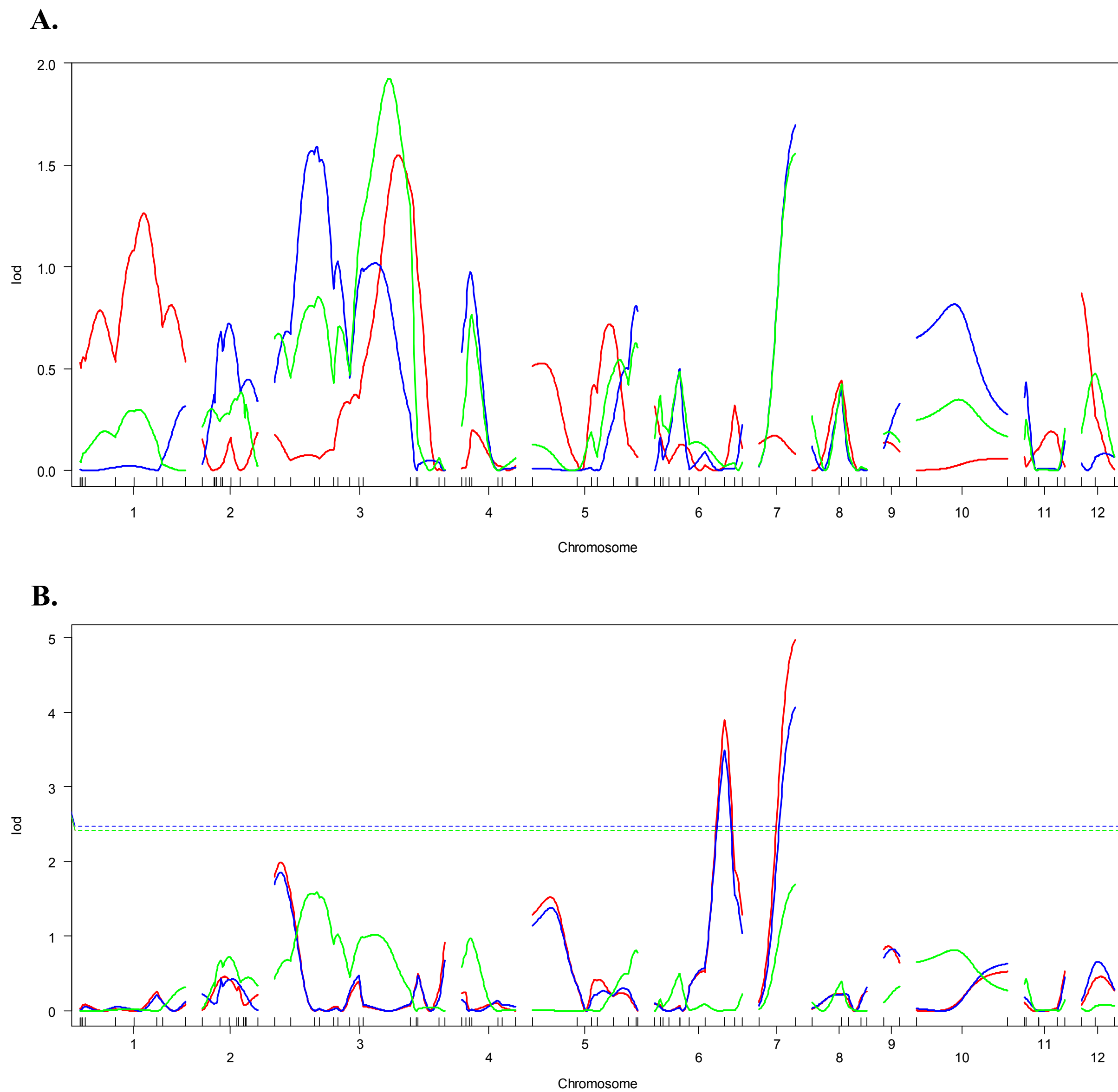


Figure 6.5 QTLs of all phenotypes on onions.

(A) QTL for blister scanned on all markers in the genome. None of the peaks are significant. (B) Comparison of QTL detection among three phenotypes. Red = external phenotype; Blue = internal phenotype; Green = blister phenotype. The significant threshold line for each phenotype matches its color.

Chapter 7 - Pathogenicity of interspecific hybrids towards Apples

Abstract

432 progeny of a cross between *F. fujikuroi* and *F. proliferatum* were tested for pathogenicity on Golden Delicious apples. The experiment was designed as an incomplete block with replication treated as a block. However, the experiment stopped after two replications due to inconsistency between replications (R^2 = 0.05). The apples were the largest source of variation. Although the experiment failed in its goal to identify pathogenicity genes in the fungus, information gained from this experiment is valuable as a reference for similar types of experiments with apples and other fruits.

Introduction

Apples (*Malus domestica*) are prone to post-harvest diseases and particularly to fungal infections. One of the most important post-harvest diseases of apples is wet core rot. This disease can be caused by several fungal pathogens, including *Trichothecium* spp., *Fusarium* spp. and *Penicillium* spp. (Gao et al. 2013; van der Walt et al. 2010). The symptoms associated with this disease are necrotic flesh and white mycelia or pink conidia in the core region of the apple. Fungal infection associated with post-harvest disease on apple may be latent or through wounds. Although *Fusarium* spp. are not the most important pathogens causing wet apple core rot, they have been isolated from infected tissue either alone or in combination with other pathogens (Gao et al. 2013). *F. avenaceum* is the most common *Fusarium* species associated with this disease, especially in Europe (Sanzani et al. 2013; Schroers et al. 2008). Other *Fusarium* spp. recovered

from wet core rot disease include: *F. proliferatum*, *F. equiseti*, and *F. solani* (Gao et al. 2013; Konstantinou et al. 2011).

Reports of the isolation of *F. proliferatum* from diseased apples lead to this study of the pathogenicity of interspecific hybrids between *F. proliferatum* and *F. fujikuroi* towards apple. The objectives of our study were (i) to analyze the segregation pattern of pathogenicity towards apples amongst the interspecific hybrids; and (ii) to identify QTLs for apple pathogenicity on the existing genetic map of an interspecific cross between *F. fujikuroi* and *F. proliferatum*. We hypothesize that multiple genes are involved in apple pathogenicity and segregate independently resulting in a continuous pathogenicity phenotype that include, the possibility of transgressive phenotypes. From this study, we could identify and locate QTLs involved in pathogenicity towards apple. This study also provides us the pathogenicity data on a third host, in addition to rice and onion, for this set of progeny. Thus, we may observe progeny with novel combinations of pathogenicity traits that could concern researchers and growers if this interspecific cross occured in the field.

Materials and Methods

Sexual cross and interspecific progeny collection

Refer to Chapter 5 for the procedure and collection of the interspecific hybrid progeny used in this study.

Apple Pathogenicity Assay

Initial tests were conducted with only 28 fungal progeny. The number of progeny tested was later expanded to include 432 of the progeny. Golden Delicious apples were obtained from a local grocery store (Dillon's). The apples were wounded by creating a hole with a blunt-end nail

at the equator (five wounds 5 mm in diameter and 5 mm deep per apple). Each wound was inoculated with 20 µl of 1 x 10^5 spores/ µl spore suspension. Both parental strains (FGSC8932 and FGSC7615) were inoculated on every apple. Water was used as a negative control on every apple. Two progeny were inoculated on each apple. Inoculated apples were placed in moist chambers (35.6 × 20.3 × 11.7 cm plastic shoe box) and incubated at 25˚C for 8 days. The moisture level was maintained by placing a wet towel on the bottom of the chamber and covering the box with plastic wrap. Water was added every three days to keep the humidity up. The diameter and depth of the resulting lesions were measured. The diameter of the lesion was used to evaluate pathogenicity, the depth was used to measure the volume of the lesion using the formula for a right circular cone ($V = \pi r^2(h/3)$; h = depth, r = radius). The correlation between diameter and volume was determined.

Initially, the parental strains were tested on different varieties of commercially available apples. Nine apple varieties including: Sage Rome, Jazz, Fuji, Red delicious, Braeburn, American Cameo, Gala, Golden Delicious, and Granny Smith, were inoculated with the parental strains. The experiment was repeated three times. The apple variety that displayed the clearest differences and the greatest consistency across the replicates was selected for pathogenicity tests with the rest of the progeny.

Analysis of pathogenicity

The experiment followed an incomplete block design with two replications. The experimental design is block by replications. Within each replicate, three sets of runs consisted of 144 progeny per run tested at different times. Four apples were placed in a humid chamber for every run. Replications, runs, boxes, and individual apples were treated as random effects. Strains were treated as fixed effects. Statistical analyses were performed with SAS software,

version 9.3, (SAS Institute Inc., Cary, North Carolina). The analyses were made with the "Proc mixed" procedure. The ranking of the strains based on lesion diameter was used to compare the treatments.

Results

Apple pathogenicity

Twenty-eight progeny were selected for an initial test of pathogenicity towards apple. After three replications, the results had an acceptable correlation between the replicates (Table 7.2). The diameter, depth, and volume of the lesions were measured 8 days after inoculation. The lesions appeared brown and necrotic on the flesh of the apple. Lesion diameter, depth, and volume measurements were highly correlated (Table 7.1). So, the lesion diameter was used to assess pathogenicity. Based on the initial test, the mean of the lesion diameter ranged from 0.6 cm for the least pathogenic to 1.8 cm for the most pathogenic (Figure 7.1). Transgressive progeny that were more pathogenic than the parents and less pathogenic than the parents were observed. The parental strains had lesions of consistent size on different apple varieties, with the *F. proliferatum* parent consistently more aggressive than *F. fujikuroi* in terms of pathogenicity (Figure 7.2). Pathogenicity was easiest to measure on Golden Delicious apples. Pathogenicity tests were then expanded to 432 progeny. After two replications, the results for most (95%) of the progeny were not consistent (Figure 7.3). Individual apples were the highest source of variation for the pathogenicity measurements (Table 7.3).

Discussion

We obtained promising results in our initial experiments with 28 progeny. Both the parental strains and the progeny performed consistently across replications. Furthermore, there

was a continuous range in the degree of pathogenicity amongst the progeny and clear differences between the parental strains. The initial results showed that the experimental design used was appropriate for this type of study. However, we could not duplicate this consistency when working with a larger number of strains.

High variation was observed between replicates with R^2=0.05. The sources of the variation were the block (replication), the runs (subset of replication), the boxes (4 apples per box), and the apples, with the apples contributing the most to the variation. Although we purchased all of the apples from the same grocery store, the source of their apples could vary. Washington State is the largest producer of apples in the United States. However, Golden Delicious apples are grown in many locations in the US from coast to coast. We ordered three cases of apples for each run, and each run took 5 weeks to complete. The supplier of the apples could vary from run to run. One way to possibly reduce the variation would be to use apples sourced from the same location and preferably from the same orchard.

The symptoms that we observed are consistent with the wet apple core rot disease. Brown, necrotic lesions of the apple flesh were observed from the point of inoculation (Konstantinou et al. 2011; Sorensen et al. 2009). The lesion expands in diameter as well as in depth, moving towards the core of the apple. All of the apple varieties that we tested could be used for this study. We chose Golden Delicious apples because the lesions were easy to observe and measure. Golden Delicious apples also are reported to be one of the most susceptible varieties to fungal infections (Konstantinou et al. 2011)

This research could potentially be used to identify fungal loci involved in pathogenicity towards apple, but testing variation and consistency problems must be resolved first. Smaller

samples would reduce the margin of error and variations, but might lack the power provided by a larger sample to identify critical genetic properties.

Table 7.1 Correlation between diameter, depth, and volume of apple lesions.

	Diameter	Depth	Volume
Diameter	1.0000		
Depth	0.8560 (P<0.0001)	1.0000	
Volume	0.9209 (P<0.0001)	0.8509 (P<0.0001)	1.0000

Table 7.2 Correlation between replications 1, 2, and 3 of the apple pathogenicity tests.

	Rep. 1	Rep. 2	Rep. 3
Rep. 1	1.0000		
Rep. 2	0.6567	1.0000	
	(P<0.0001)		
Rep. 3	0.7909	0.6362	1.0000
	(P<0.0001)	(P<0.0001)	

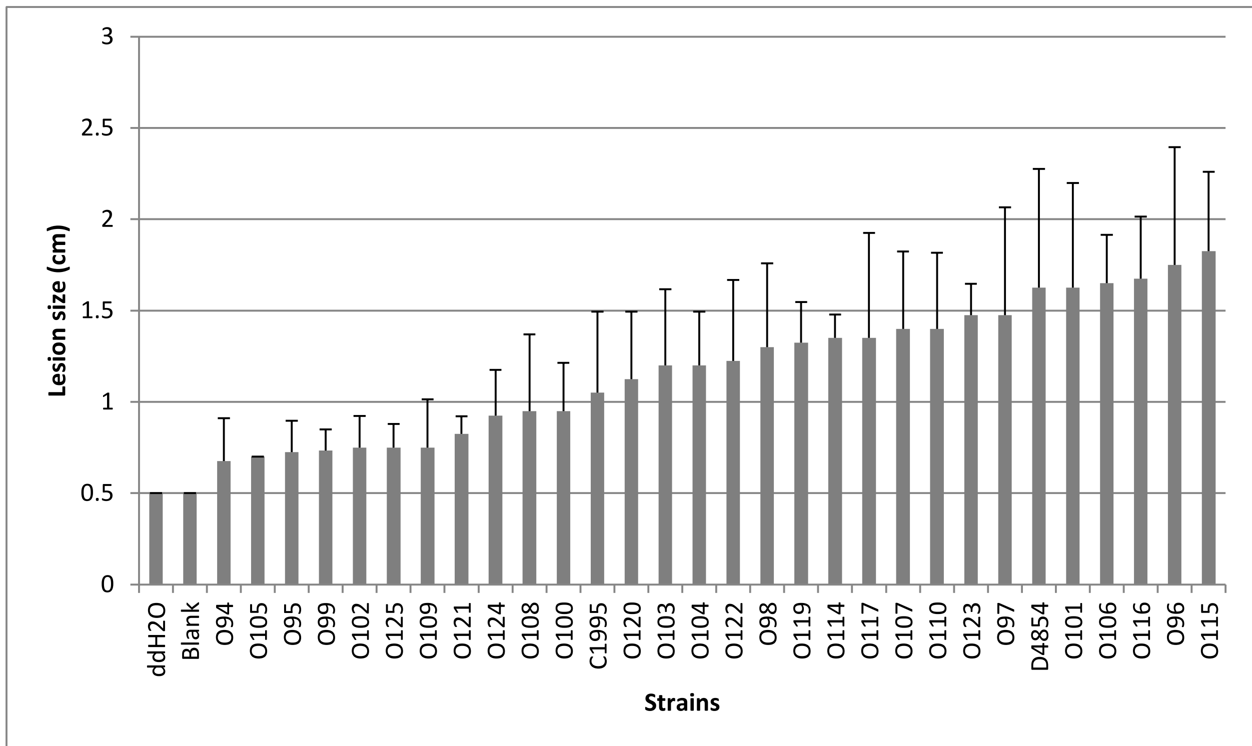


Figure 7.1 Means of lesion diameter (three replicates) on Golden Delicious apples.

Apples were inoculated with hybrid progeny of *F. fujikuroi* × *F. proliferatum*, both parental strains, and double distilled water (ddH_2O).

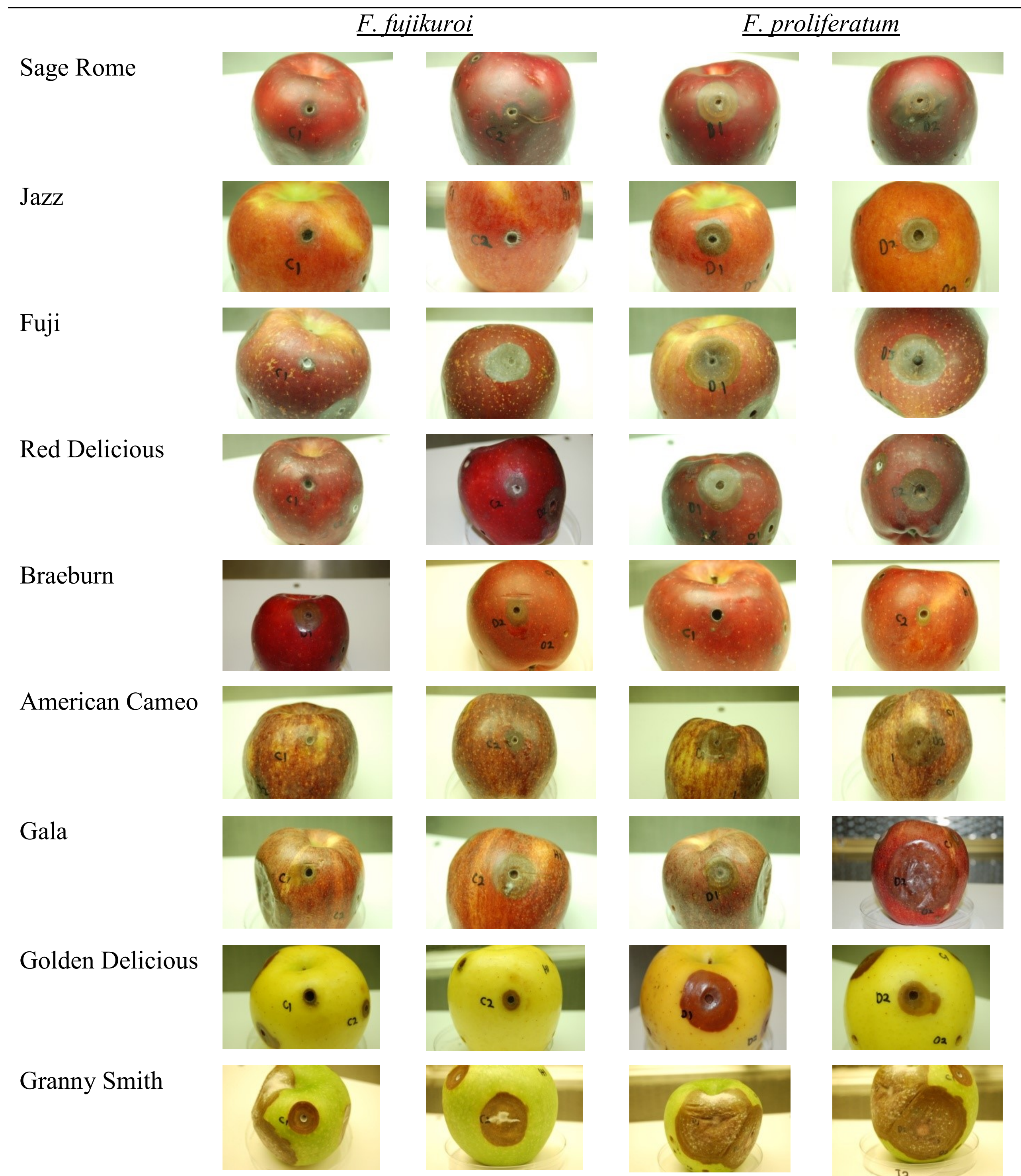


Figure 7.2 Apple pathogenicity phenotypes.

Each parental strain had two replicates. The photo was taken 20 days after inoculation. F.f = *F. fujikuroi* parent, F.p. = *F. proliferatum* parent.

Table 7.3 Estimated sources of variation in the apple pathogenicity assays.

Covariance parameter	Estimate	Standard Error	Z Value	Pr > Z
REP[a]	0	-	-	-
RUN(REP)[b]	4045.91	2835.03	1.43	0.0768
BOX(REP*RUN)[c]	6282.75	1153.04	5.45	<0.0001
Residual[d]	31032	1094.17	28.36	<0.0001

[a]Replication of experiments treated as a block
[b]Runs of experiment within each block treated as incomplete block
[c]Boxes of apples within each Runs treated as random effects
[d]Individual apples with inoculation treated as random effects

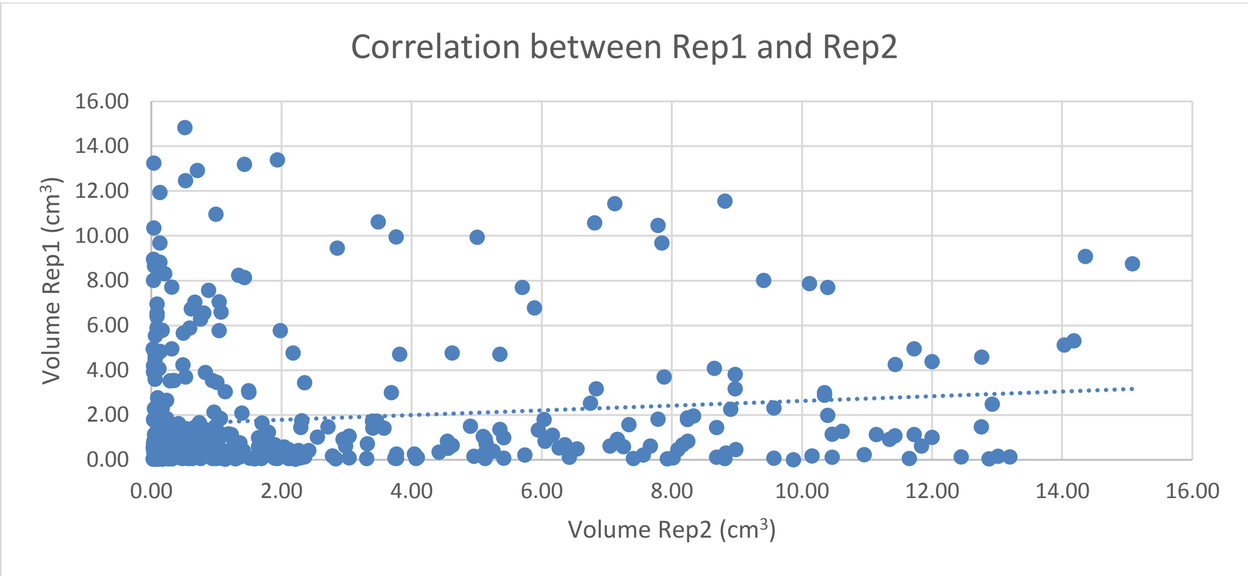


Figure 7.3 Correlation between Rep1 and Rep2 based on the volumes of the lesion in apple pathogenicity.

$R^2 = 0.05$

Chapter 8 - Conclusions

Summary of results and general discussion

The *Fusarium fujikuroi* species complex remains an important and interesting group within the genus *Fusarium* that contains many important plant pathogens. All three of the population studies focused on species within this complex. The possibility of successful sexual crosses between closely related species within the *F. fujikuroi* species complex makes this group of fungi even more interesting.

Populations of *Fusarium* spp. from mangoes, sorghum and rice

In population studies, identification of *Fusarium* spp. is a key step in correctly associating species with the diseases that they cause. In past research in Southeast Asia, *Fusarium* spp. usually were identified based on morphology. Some species within the *F. fujikuroi* species complex are difficult to distinguish based solely on morphology. We used multiple characters – cross fertility, AFLP phylogeny, and diagnostic gene sequences (tef-1α and β-tubulin) to ensure correct identification of the isolates.

Amplified Fragment Length Polymorphisms (AFLPs) are useful tools for species identification, phylogenetic analysis, and population genetic study. AFLPs clearly group strains into clusters corresponding to species. We successfully grouped the strains from mango, sorghum, and rice into different species following phylogenetic analysis based on AFLPs. Within a species, the strains can be grouped into different populations. In the rice population, there are distinct subpopulations of *F. fujikuroi* in Malaysia and Thailand. AFLPs also can be used to evaluate variation within and among populations and to infer the degree of migration or isolation of subpopulations within the species. In the sorghum population, *F. thapsinum* which is a known

sorghum pathogen, appears to be panmictic in Thailand as 83% of variation within the population is found within the subpopulation. Thus, there appears to be extension migration by members of this species within the region.

Mating type and fertility also are important characters in studies of population genetics of *Fusarium*. Most *Fusarium* species are heterothallic. For the sexual cycle to occur, two strains of the same species that carry opposite mating type alleles cross to produce viable meiotic progeny. The *Fusarium* life cycle allows researchers to use the relative frequency of female-sterile and female-fertile strains to calculate the effective population number. The effective population number can be used to compare populations and to determine the similarity of the population to a randomly mating population. Amongst the species evaluated, *F. verticillioides* has the highest effective population number suggesting that this species is the most active participant in sexual recombination evaluated in this study.

The studies of *Fusarium* populations in mango, sorghum, and rice from Thailand and Malaysia reinforce the importance of members of the *Fusarium fujikuroi* species complex as plant pathogens. Species from this species complex are found in all of the populations surveyed, and *F. proliferatum* was recovered from all of the hosts sampled. Other species were limited to just a single host, e.g. *F. fujikuroi* on rice, *F. mangiferae* on mango, and *F. thapsinum* on sorghum. Malaysia and Thailand were taken as representatives of Southeast Asia due to their location. In both the mango and sorghum populations, there were species associated with these hosts that had not previously been reported from this region. The number of new reports suggests that more careful inspections or quarantine procedures related to the movement of plant materials from other continents such as Africa and the Americas to Malaysia and its neighboring countries may be needed to reduce the opportunity for the introduction of novel pathogens into the region.

All of the species in the *F. fujikuroi* species complex recovered in this study are potential mycotoxin producers. The presence of *F. proliferatum* in mangoes, sorghum and rice could lead to contamination of these products with fumonisins. In the rice population, *F. fujikuroi* was the most common species. Interestingly, the *F. fujikuroi* strains separate clearly into Malaysian and Thai populations. Significant levels of variation between these populations from rice suggests that these populations between species are genetically relatively isolated from one another in genetic terms.

An Interspecific cross between *F. fujikuroi* and *F. proliferatum*

Progeny from an interspecific cross between *F. fujikuroi* and *F. proliferatum* can offer insights into speciation, pathogenicity, and mycotoxin biosynthesis. These hybrids also may have unique phenotypes not present in either parent strain or parental species. We analyzed a relatively large number of progeny to help ensure that significant rare events could be clearly identified. We constructed a genetic map based on 83 AFLP markers and aligned this map with the physical sequence. Regions containing genes involved in secondary metabolite biosynthesis and plant pathogenicity segregate and can be mapped to chromosomes.

Segregation of the AFLP markers usually was not 1:1, with alleles from *F. proliferatum* usually more common than alleles from *F. fujikuroi*. There were 29 putatively clonal progeny, some of which came from different perithecia and thus arose independently following meiosis. These clonal lines do not suffice to explain the observed segregation bias and were removed prior to construction of the genetic map. The segregation distortion was not sufficient to prevent assembly of the genetic map. For 32/83 fragments there were more double recombinants than expected, gene conversion probably explains some of this excess, but microinversions and fragments with poorly paired homologs or that contain genes that must remain together also

could explain some of this excess. The relatively poor fertility of the cross (0.1 – 0.01%) of a normal cross also is consistent with this conclusion.

QTLs for rice and gibberellic acid production occur on the same chromosome, although there is no correlation between rice pathogenicity and GA_3 production. The results suggest that the pathogenicity genes causing the symptoms that we observed are not involved in GA biosynthesis, but are still located on chromosome 5. A denser genetic map of chromosome 5 and testing a larger number of progeny for rice pathogenicity should decrease the size of the genomic region to which the QTLs map.

Segregation distortion also occurred for the onion pathogenicity traits. There were more progeny with the *F. proliferatum* parental phenotype than with the *F. fujikuroi* parental phenotype, with about a third of the progeny having a recombinant phenotype. The segregation of pathogenicity phenotypes within the progeny suggests that the genes segregating for pathogenicity are independent, including QTLs for the pathogenicity traits that result in external and internal lesions of onions. We also observed a novel, i.e. not seen in either parent, onion pathogenicity phenotype termed blister in some of the progeny. Thus, the interspecific hybrids may have pathogenicity profiles unlike either of the parental species.

The apple pathogenicity assay is potentially useful as a fast, easy, and reliable pathogenicity test when used with a relatively small number of fungal isolates. The apple pathogenicity assay requires only 7 days for results and yields a quantitative measure that can be used to detect QTLs. The disadvantage to this method is the variation amongst the apples used as substrates for the test. To reduce this variation all of the apples should be obtained from the same orchard. Due to the excessive variation, our pathogenicity assay results were not reliable and could not be interpreted in a meaningful manner.

In summary, my work on population genetics of *Fusarium* and the interspecific cross between *F. fujikuroi* and *F. proliferatum* broadened the boundaries of knowledge of the genus *Fusarium*. This research has opened new research venues for understanding the threat of *Fusarium* spp. to several Southeast Asian crops, and the pathogenic threat of interspecific hybrids between *F. fujikuroi* and *F. proliferatum* wherever they might occur.

Future research and perspectives

The results obtained from my research could serve as the basis of future work by others. Some of these topic, include:

1. Mango malformation: The frequent recovery of *F. proliferatum* from mangoes and its association with diseased mango inflorescences warrant further study. Pathogenicity assays for *F. proliferatum* and *F. mangiferae* on mango seedlings are needed to determine if the association observed in Southeast Asia is pathogenic or opportunistic.
2. *Fusarium* spp. on sorghum: The strains isolated from this population not normally associated with sorghum should be tested for the completion of Koch's postulates on sorghum. The mycotoxins produced by the strains should be determined as should the extent of mycotoxin production occurring on sorghum under field conditions. The number of isolates and the number of sites at which they are recovered should both be expanded within Thailand and in neighboring countries. Such expansion will not be easy as sorghum is not widely grown in Southeast Asia.
3. *Fusarium* spp. on rice research: All of the strains isolated from rice should be tested for their ability to cause bakanae. The common symptoms, e.g. yellowing and shoot elongation, should be observed for this disease. These strains also should be tested for pathogenicity in rolled towel assays to measure shoot growth and germination rates.

Analysis of these pathogenicity traits in our *F. fujikuroi* field strains will extend our understanding of the impact of *Fusarium* spp. on rice from seedling to maturity.

4. Segregation of pathogenicity factors on different hosts: Following the pathogenicity studies on onions and rice, increasing the number of hosts evaluated could answer questions regarding pathogenicity factors, e.g. how general or specific are the pathogenicity factors? For example, *F. proliferatum* is a confirmed pathogen of dragonfruit (*Hylocereus* sp.) in Malaysia. A pathogenicity assay that can be used for such tests is available and can be used to test this hypothesis. Rice pathogenicity testing also should be expanded to include a method other than seedling germination, and to test explicitly for bakanae.
5. Segregation of fertility within the progeny of interspecific hybrids between *F. fujikuroi* and *F. proliferatum*: The genetic compatibility of two strains is an important in fertility parameter. The progeny obtained from this cross should be backcrossed to the parents, and intercrossed to some extent with one another, to identify fertility factors segregating in the cross. The progeny should be used as both female and male parents in crosses with the parents. The relative number of perithecia produced and the relative numbers of ascospores produced are both important fertility indicators, as is the ability to act as both a male and a female parent in the crosses. It should be possible to identify QTLs involved in fertility from such studies.
6. Increase genetic map resolution: The genetic map constructed in this study is based on only 83 AFLP markers. Adding more markers would increase the utility of the map. Genotyping-by-sequencing (GBS) provides a large number of SNPs that will increase the

saturation of the map. A more detailed map would facilitate identification of genes (or regions) associated with pathogenicity, speciation, and secondary metabolite production.

7. Localize pathogenicity genes: Following an increase in map saturation, the distance between neighboring markers on the chromosomes should decrease. Thus, QTLs for pathogenicity identified in this study can be localized with higher confidence. The sequence of the region associated with pathogenicity phenotype could be compared with other known genome sequences, e.g. *F. verticillioides* and *F. oxysporum* to identify genes with potential roles in the pathogenicity processes.
8. Segregation of other secondary metabolites amongst progeny: Besides GA_3 production, studies on the segregation of other important secondary metabolites specific to one parent, e.g. fusarin C and bikaverin for *F. fujikuroi*, fumonisin and fusarubins for *F. proliferatum*, are needed. Novel metabolite profiles among the progeny will result from this segregation. QTLs associated with these metabolites would be detected. This study would build on the earlier report of Studt et al. (2012).
9. Double crossover analysis: Based on existing markers and the existing genetic map, the number of expected and observed crossovers can be calculated. This analysis could identify potential recombination hot spots in the genome which result in negative interference. To the extent these regions are specific to the interspecific cross and not found in crosses within either *F. fujikuroi* or *F. proliferatum,* they could provide insights into genome organization as well.
10. Analysis of the clonal strains: Further analysis of the characterization and genetic make-up of the clonal strains identified in this study may facilitate an understanding of the

method that leads to the independent production of these apparently clonal progeny at levels for more frequent than would be expected by chance alone.

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