INVASION POTENTIAL AND COLONIZATION DYNAMICS OF *FUSARIUM PROLIFERATUM*

by

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B.S., Wichita State University, 2007
M.S., Wichita State University, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

The trade of food, plant, and animal products has increased the worldwide movement and establishment of exotic pathogens with dramatic negative impacts on plant systems. *Fusarium proliferatum* is a broad host-range pathogen and among the most common maize pathogens globally. It is often seed-borne and symptomless in maize, making it a high risk for introduction in maize and other grains. Considering the global distribution of maize and the wide host range and production of mycotoxins by *F. proliferatum*, a better understanding of its life history is needed. To provide markers for tracking *F. proliferatum* in laboratory experiments, strains of *F. proliferatum* were transformed to express a green fluorescent protein (GFP). Active dispersal (at least 1.5cm at 25°C and -50mb soil matric potential) and colonization of organic matter in nonsterile field soil was demonstrated in soil microcosms. *Fusarium verticillioides* is commonly isolated from maize seed also colonized by *F. proliferatum*. A red fluorescent (mRFP) *F. verticillioides* transformant was developed to study competition with *F. proliferatum*. For quantification in host tissues, a TaqMan multiplex qPCR protocol was developed using primer and probe sets targeting fragments of the green and red fluorescence genes to detect *F. proliferatum* and *F. verticillioides*, respectively. Prior colonization of maize tissues by *F. verticillioides* ($p=0.6749$) and other seed-borne microorganisms ($p=0.1910$) did not affect subsequent colonization by *F. proliferatum*. Genotyping-by-sequencing (GBS) was used to identify genetic markers in *F. proliferatum*. Primer sets based GBS markers were designed to allow detection of specific isolates in field experiments. *F. proliferatum* populations were characterized from maize seed prior to planting and again after harvest. End-point PCR identified *F. proliferatum* isolates containing the GBS marker. AFLP-fingerprinting indicated that 23 of the 817 *F. proliferatum* isolates contained the molecular marker and were genetically related to the original isolate. Based on the subclade and percentage similarity in UPGMA phylogenetic trees, and the population grouping observed in STRUCTURE and Principal Coordinate Analysis, these isolates could have a single origin and be clonal. Understanding the life cycle of *F. proliferatum* is critical for learning more about the risk of introducing seed-borne exotic isolates into new environments.

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Dedication

To my wife and son for always showing me unconditional love.

Chapter 1 - **Literature Review**

Biosecurity in Agriculture

Agriculture in the 21st century is under pressure to provide sufficient food for billions of people worldwide and farmers are highly dependent on crop protection to sustain or increase such production (Mujerki and Chincholkar, 2007). The challenge to feed 9.6 billion people by 2050 (2.6 billion more than now or 37% increase in population) involves increasing the yield and quality of staple crops, increasing resistance to biotic and abiotic stresses, and protecting crops in the field and after harvest. Crop yields have increased significantly due to improved pest control management as well as plant breeding and genomic approaches seeking for resistance and higher yields. However, at present, the rate of increase in yield potential is much less than the expected increase in demand for maize, wheat, and rice, which provide two-thirds of energy in human diets (Cassman, 1999). Global rates of yield increases have declined for most major cereal crop species since the start of the green revolution in the 1960s (Grassini *et al*., 2013, Cassman, 1999; Fischer and Edmeades, 2010; Hafner, 2003).

Chemical pesticides have improved the quality and quantity of food production worldwide (Tilman *et al.*, 2002). Nevertheless, we have also seen an increase in concern for the environment and non-target organisms with the increase in pesticide use (Flexner *et al.*, 1986; Pimentel, 1995). The increase of long distance commodity trade worldwide is increasing the introduction of exotic pathogens and pests into new environments with known and potentially unknown consequences for which there may not be pesticides for management (Elmer *et al.*, 1999; Elmer, 2001). The misuse and over use of chemical pesticides can result in the evolution of new pathogen and pest genotypes with acquired resistance to the chemicals; resistant pathogen and pest genotypes become hard to manage (Koenraadt *et al.*, 1992; Jones and Walker, 1976; Stanis and Jones, 1985; Avenot and Michailides, 2010).

Invasive species are often introduced in imported goods, or as stowaways on products, ships or shipping containers (Elmer *et al.*, 1999; Elmer, 2001, Anagnostakis, 1987). For example, it is suspected that the bacterium *Candidatus Liberibacter asiaticus*, which causes the disease huanglongbing in citrus, was introduced in Florida in 2005 by the illegal importation of plant

material (Michaud, 2004). Moreover, there is a positive correlation between the magnitude of imports and the number of invasive species and their rate of introduction (Dalmazzone, 2000). For this reason, the interest in agricultural biosecurity has risen considerably over the last years in parallel with the increased trade in plant products (Stack and Fletcher, 2007; Stack *et al.*, 2006).

The introduction of pathogens can also result from legal trade of asymptomatic but contaminated plant material (Fletcher *et al.*, 2006; Kim *et al.*, 2003; Williamson *et al.*, 2002). This can occur when a pathogen is seed-borne and/or symptomless so inspection at ports of entry does not detect it. For example, it is suspected that the oomycete *Phytophthora ramorum*, which causes sudden oak death, was accidentally introduced into the United States by the importation of plants from Europe (Rizzo *et al.*, 2002). Furthermore, the introduction of a pathogen can also happen inadvertently on shoes or clothing, trade commodities, migrating wildlife, and other moving entities (Stack *et al.*, 2006).

Early detection of exotic pathogens introduced in seed or symptomless plant material is often complicated by the large geographical areas that are devoted to agriculture. For example, every year the US plants on average 90 million acres (364,217 km^2) of maize (United States Department of Agriculture - USDA, 2015) (Figure 1.1); approximately the size of Japan or Norway, and bigger than Germany and Malaysia. In addition, the 2012 U.S. census of agriculture revealed that there were 2.1 million farms covering an area of 3,698,827 km^2 (USDA Census, 2012).

As a consequence, new diseases caused by introduced pathogens may not be detected until after several generations of the pathogen are produced in the field (Madden and Wheelis, 2003). For example, *Candidatus Liberibacter asiaticus* was first detected in Brazil in 2004, but researchers believe that it was introduced 10 years prior to 2004 (Gottwald et al., 2007).

The impacts of exotic pathogens introduced into the U.S. are disastrous, as it could result in a ban placed upon imports of plant materials from the U.S. by members of the World Trade Organization (Nutter and Madden, 2005), which could result in huge economic losses. In

addition, introduced pathogens have had significant social impacts; e.g., the introduction of *Phytophthora infestans* into Ireland caused the devastating potato famine in the 1840s.

Biosecurity in plant pathosystems

Humans have been moving plants and plant products for thousands of years, perhaps from 8000 BC (Huxley, 1978), to the first nomadic cultures (Zhang *et al.,* 2007), to explorers and colonists moving exotic crops between continents (Wyman, 1968), to the current globalized trade practices (Anderson *et al.,* 2004). These human activities have affected the global distribution and diversity of plant pathogens (Goss, 2015), to such large extent that some crops and countries have been exposed to most of the known plant pathogens (Bebber *et al.,* 2014). Although many plant pathogens are cosmopolitan in distribution, populations often differ in virulence, resistance to chemical controls, and genetic diversity (Goss, 2015).

The use of new genotyping techniques and whole-genome sequencing has facilitated the study of the movement of these pathogens (Goss, 2015). Genome sequencing allows complete genotyping of plant pathogens, allowing the generation of high-quality molecular markers that can be used for analysis of migration of pathogens or study emerging and clonal pathogens with little genetic variation (Goss, 2015).

Baysal *et al.,* (2010) used inter-simple sequence repeats (ISSR) markers to study the dissemination of bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in southern Turkey. ISSR primers that showed high polymorphism ratios were used to characterize Cmm strains and study their dissemination by seeds and seedlings. Another investigation used ISSR and sequence-related amplified polymorphism (SRAP) markers to study the genetic diversity of races of *Fusarium oxysporum* f. sp. l*ycopersici* and *F. oxysporum* f. sp. *radicis*, the causal agents of root rot and crown rot diseases (Baysal *et al.,* 2009).

The development of these advanced genotyping methods and whole-genome sequencing allow the study of the introduction, establishment, and dispersal of plant pathogens. These tools are important considering the global movement and trade of plants and plant products. The information provided by these tools is valuable in identifying sources of plant pathogens, and

pathways of dispersal that can be used to reduce pathogen movement, or monitor their movement (Goss, 2015).

The host: *Zea mays*

Maize is an annual row crop believed to have originated from a Mexican grass called teosinte in prehistoric times (Iltis, 1983). Its specialized physiology, C4 photosynthesis, makes it well suited to hot, dry climates, and breeding has made it possible to grow it in colder climates as well. Maize is one of the most important crops worldwide with an annual cultivation area of more than 1.50 million km^2 and an annual harvest of more than 800 million metric tons of grain (Food and Agriculture Organization Statistics - FAO STAT, 2009).

According to the International Grains Council (IGC, 2013), maize production has increased dramatically over the last 40 years worldwide and has become the number one grain cereal crop over wheat and rice with respect to gross production (Table 1.1). Around 200 million people in developing countries consume maize directly as their staple food.

Maize is grown in most U.S. states, but production is concentrated in the Midwest region including Illinois, Iowa, Indiana, Nebraska, eastern portions of South Dakota, western Kentucky and Ohio, and the northern part of Missouri. Iowa and Illinois are the top maize-producing states and their production is about one-third of the total U.S. maize production (Graham *et al.,* 2007). The production of maize has risen over time as a result of improvements in technology and in production practices. In addition, its production has expanded to non-traditional growing areas as adapted hybrids have been developed.

Maize is the primary U.S. feed grain, accounting for 95 percent of total feed grain production and use. Other uses of maize include the production of ethanol, food, seed, and industrial uses (USDA, 2015) (Figure 1.2). Maize is the most produced agricultural commodity in the U.S. and fourth in revenue behind cattle and chicken meat, and cow milk (FAO, 2012) (Figure 1.3).

Breeding of maize hybrids in the U.S. has allowed a steady increase in yield at a rate of 100.88 to 134.5 kilograms per hectare per year since the 1950s (Figure 1.1) (Edgerton *et al.,* 2009). Maize

improvement is based on several traits including yield, quality, time to maturity and resistance or tolerance to biotic and abiotic stresses. For example, DuPont Pioneer has 120 research locations in 30 countries devoted to maize breeding and testing of hybrids. By the time a DuPont Pioneer hybrid is offered for sale, it has been tested at more than 1,500 locations in more than 200 customer fields (DuPont Pioneer website - https://www.pioneer.com/CMRoot/Pioneer/About_Global/news_media/media_library/articles/maize_hybrid.pdf), which highlights the importance given to maize breeding programs. In addition, traditional breeding programs for different crops, including maize, are starting to use molecular tools (e.g. genetic modification to introduce R genes) to accelerate the development of crops resistant to different virus, bacteria, oomycetes, nematodes, and fungi (Hammond-Kosack and Parker, 2003; Fitch *et al.*, 1992; Scofield *et al.*, 2005; Martin *et al.*, 1993; Steeves *et al.*, 2006; Aarts *et al.*, 1998).

Associations between fungi and plants are both ancient and ubiquitous (Alexopoulos et al., 1996; Berbee 2001; Heckman et al., 2001). These interactions can have many different outcomes that have different effects on the fungus and the plant (Faust and Raes, 2012). Certain species of *Fusarium* are known pathogens of maize; some are seed-borne and some produce mycotoxins that can affect human and animal health. Because *Fusarium* spp. can be seed-borne and symptomless in maize seed, they are hard to detect at ports of entry which facilitates the distribution of these fungi worldwide (Elmer, 2001).

Fusarium proliferatum is often found in maize seeds, a potentially important source of inoculum in the field (Cotten and Munkvold, 1998; Postic *et al.*, 2012). This species has been associated with symptomatic and asymptomatic maize plants and is considered at times to be a primary causal agent of disease, a secondary invader, or a seed-borne organism. As a seed-borne organism, this fungus is suspected to colonize the emerging seedlings, the maturing plant, and the new ear and kernels (Logrieco *et al.*, 1995). Multiple strains are often isolated from seed; however, some strains may be dominant (Jurado *et al.*, 2010; Chulze *et al.*, 2000). Research suggests that some of the mating populations of *F. moniliforme* may have co-evolved with the host with which they are most commonly associated, in this case, *G. fujikuroi* mating population D (i.e., *F. proliferatum*) and maize in America (Leslie, 1996).

Fusarium proliferatum is considered an endophyte organism that systemically colonizes maize plants, including the kernels (Olah *et al.*, 2006). Endophytes are organisms that live within their host plants without causing any noticeable symptoms of disease (Carroll, 1988), except when the host is under stress conditions (Stone et al., 2000). Moreover, endophytes have the potential to confer protection to host plants against pests and to provide greater resistance to stress (e.g. drought), but that relationship has not been demonstrated in the maize – *F. proliferatum* association (Saunders and Kohn, 2009). Moreover, there is evidence that correlates the appearance of disease symptoms in maize plants with the presence of *F. proliferatum* under optimal conditions for the host (Saunders and Kohn, 2009).

Fusarium proliferatum is thought to commonly exist systemically and asymptomatically in most field maize (in roots, stalk, tissues, and kernels) and to be passed from parent to progeny by seed-borne infection (Wilke et al., 2007). In addition, kernel rot caused by *F. proliferatum* is often associated with feeding of insects (Munkvold and Hellmich, 2000) or mechanical harvesting (Munkvold, 2003). The mechanisms by which otherwise undamaged and uninfected plants are invaded remain unclear but may be significant (Maiorano *et al.*, 2009).

The fungus: *Fusarium proliferatum*

The genus *Fusarium*, described by Link in 1809, contains a large number of plant pathogenic fungi. The members of the genus can directly cause disease in plants, humans, and animals (Leslie and Summerell, 2006). The genus *Fusarium* belongs to the Ascomycota phylum, Ascomycetes class, and Hypocreales order, and its teleomorphs are classified in 3 genera including *Gibberella*, *Hemanectria*, and *Albonectria*. The genus *Fusarium* consists of species that attack many economically important crops, including wheat and maize, which are of high importance in the Midwest of the U.S.

Fusarium proliferatum (Matsushima) Nirenberg ex Gerlach & Nirenberg is a fungal plant pathogen. Prior to the Nirenberg description, most isolates of *F. proliferatum* were identified as *F. moniliforme*. The sexual stage of this fungal species, known as *Gibberella intermedia (fujikuroi)* (Kuhlman), belongs to the section Liseola of *Fusarium* species (Nelson *et al.*, 1983).

Fusarium proliferatum has worldwide distribution and has been recovered from numerous environments; it is considered to be a moderately aggressive pathogen (Stępień *et al.,* 2011). It has an extraordinarily broad host range, causing disease in economically important plants as diverse as maize, asparagus, banana (Jimenez *et al.,* 1993), citrus fruits (Hyun *et al.,* 2000), orchids (Ichikawa *et al.,* 2000; Benyon *et al.,* 1996), rice (Desjardins *et al.,* 2000) and sorghum (Leslie *et al.,* 1990), to name a few. It is a cause of root rot of pine seedlings (Ocamb *et al.,* 2002), Fusarium crown and root rot of asparagus (Elmer *et al.,* 1995) and date palm decline (Abdalla *et al.,* 2000). It also causes stalk and cob rot of maize (Logrieco *et al.,* 1995). There are two maize ear rots that are most commonly described: pink ear rot, which is caused by *F. verticillioides* in association with *F. proliferatum* and *F. subglutinans*, and red fusariosis, which is caused by *F. graminearum.*

The ideal environmental conditions for *F. proliferatum* growth are water activity (aw) of 0.994-0.98, temperature range of 20°C to 35°C and a pH of 5.5 (Marin *et al.,* 1995). Maximum linear growth for *F. proliferatum* is reported to occur at 25°C and an osmotic potential of -1.0 MPa and conidia germinate optimally at 30°C (Marin *et al.,* 1996).

This fungus produces macroconidia and microconidia but not chlamydospores. The macroconidia are slender, thin-walled and relatively straight, usually with 3-to-5 septa. The microconidia are club shaped (clavate) with a flattened base and 0-septate in false heads and in chains that often form in pairs from polyphialides. Pyriform microconidia also may occur but are generally rare (Leslie and Summerell, 2006).

Fusarium proliferatum has been identified as a colonizer of maize plants worldwide and is considered to be an increasingly important component of maize ear rot in Europe (Logrieco *et al.,* 1995). Among *Fusarium* strains from 42 maize ear rot samples collected in Italy in 1992 and 1993, for example, *F. proliferatum* infected a mean of 78% of the kernels in northern areas, 42% in central areas, 47% in southern areas, and 30% in Sardinia (Logrieco *et al.,* 1995). Consistent isolation of *F. proliferatum* from symptomatic and asymptomatic plant tissues suggests that the

fungus can systematically colonize maize plants. *F. proliferatum* can persist in maize stalk debris either on the surface of soil or buried in a field for at least 21 months (Cotten *et al.,* 1998).

Along with *F. verticillioides, F. proliferatum* is considered to be the most common maize pathogen (Stepien *et al.,* 2011) and the most effective producer of the polyketide-derived fumonisin mycotoxin: fumonisin B_1 (FB_1) being the most prevalent (Rheeder *et al.,* 2002). Fumonisins were first identified and described by Gelderblom *et al.,* (1988), who reported that they had cancer-promoting activity. Fumonisin B_1 is toxic to both humans and animals due to inhibition of sphingolipid metabolism and cell cycle regulation (Riley *et al.,* 1996). It has been associated with esophageal cancer, liver cancer, and neural tube defects (Desjardins, 2006). Some strains of *F. proliferatum* are reported to produce FB_1 in culture at more than 6,000 µg/g (for each gram of fungal culture) (Leslie *et al.,* 2004). Fumonisin contamination has been associated with *F. proliferatum* infection of maize, animal feeds, and other agricultural commodities (Leslie *et al.,* 2004; Logrieco *et al.,* 1998; Thiel *et al.,* 1991; Pascale *et al.,* 2002). In horses, it causes leukoencephalomalacia, and in swine it causes pulmonary edema (Ross *et al.* 1990). Divergence among strains from the same host plant has been demonstrated through variation in mycotoxin profiles. Among *F. proliferatum* from Nepalese rice, for example, some strains produced both fumonisins and moniliformin, whereas other strains produced only fumonisins or only moniliformin on laboratory culture media (Desjardins *et al.,* 2000). Antioxidants can be used to treat grain and retard growth by *F. proliferatum* and reduce the amount of fumonisins produced *in situ* (Etcheverry *et al.,* 2002; Reynoso *et al.,* 2002). *Fusarium proliferatum* also produces a wide range of other mycotoxins and biologically actives metabolites, including beauvericin, enniatin, fusaric acid, fusarin, fusaproliferin, and moniliformin (Leslie and Summerell, 2006).

The role fumonisins play in endophytic or pathogenic growth of *Fusarium* is poorly understood (Munkvold, 2003; Marin *et al.,* 1999; Munkvold and Desjardins, 1997). Early work found that fumonisins likely play a role in seedling blight, but that they are not necessary to cause disease (Desjardins *et al.,* 1995; Jardine and Leslie, 1999). Other research found that fumonisin is a phytotoxin responsible for disease symptoms on seedlings and other plant tissue (Nelson *et al.,* 1993; Glenn et al. 2004; Williams et al. 2007).

It has also been reported that the production of fumonisins (FB_1, FB_2, and/or FB_3) is not required by *F. verticillioides* to cause maize ear infection and ear rot (Desjardins *et al.,* 2002, Desjardins, and Plattner 2000). Recent work has shown that the roots of maize seedlings exposed to fumonisins have elevated levels of free sphingoid bases (Williams *et al.,* 2006). In both plant and animal model systems, intermediates in the ceramide biosynthetic pathway (e.g. sphingoid bases) are important secondary messengers in stress responses (Ng *et al.,* 2001; Hannun and Obeid 2002; Chalfant and Spiegel 2005). The role, if any, of the increase in sphingoid bases in *Fusarium*–maize interaction remains to be determined.

The life cycle of seed-borne *F. proliferatum* has not been fully studied, but it is suspected to share some similarities to that of *F. verticillioides* (Munkvold and Desjardins, 1997; Battilani and Rossi, 2003, Figure 1.4). The *F. verticillioides*-maize pathosystem is complex (Munkvold and Desjardins, 1997), consisting of three main infection pathways for kernel infection: the air- or splash-borne infection by conidia through silks or wounds; the systemic growth of the pathogen via the stalk, and the infection carried by spore-carrying insects (Munkvold *et al*., 1997; Battilani and Rossi, 2003).

Macroconidia and microconidia produced on crop residues and on tassels (Logrieco and Bottalico, 1987) are air and splash-dispersed and infect ears through silks or wounds (Ooka and Kommendal, 1977). Systemic development of the pathogen within the plant can result from root, stalk or leaf sheath penetration, or from seed transmission (Marin *et al*., 1996; Munkvold and Carlton, 1997). Spore-carrying insects, like European corn borer larvae, *Ostrinia nubilalis* (Le Bars *et al*., 1994), can transmit *F. proliferatum* providing wound sites for penetration and facilitating dissemination within systemically infected stalks (Munkvold *et al.,* 1999).

Phylogenetically, *F. proliferatum* is very closely related to *Fusarium fujikuroi* and *Fusarium globosum* in clade 3 of the *Gibberella fujikuroi* species complex (Geiser *et al.,* 2003). The sexual stage of this heretothallic *Fusarium* species was first established in 1982 by crossing strains of *F. proliferatum* and was designated *Gibberella fujikuroi* variety *intermedia* (Kuhlman, 1982). The sexual stage was then identified as *G. fujikuroi* mating population D (Kerenyi *et al.,* 1999;

Leslie, 1991). Phylogenetic analysis of DNA sequences of different and diverse strains of *F. proliferatum* has demonstrated that they are highly diverse but monophyletic and show mating compatibility despite their high levels of divergence (Geiser *et al.,* 2003).

Fusarium fujikuroi is also a species of the *Gibberella fujikuroi* complex and the causal agent of bakanae disease on rice (Carter *et al.,* 2008). *F. fujikuroi* is seed-borne and produces fumonisin B_1, moniliformin, beauvericin, fusaric acid, and fusarin (Carter *et al.,* 2008, Leslie and Summerell 2006). *F. proliferatum* has been isolated from rice, but it does not cause bakanae disease (Amatulli *et al.,* 2010 and 2012, Desjardins *et al.,* 1997). Despite its close phylogenetic relationship with *F. fujikuroi*, *F. proliferatum* does not produce gibberellins. The potential for crossing and the formation of interspecific hybrids has been reported between *F. proliferatum* and *F. fujikuroi* (Leslie *et al.,* 2004; Nor, 2014), increasing the risk of the unregulated introduction of *F. proliferatum* isolates into new environments.

Development of diagnostic and detection methods in *Fusarium*

Developments in molecular biology and genetics, along with the development of more advanced technologies, have allowed for more detailed studies into the diversity and classification of fungi. The identification of *Fusarium* species is one of the most critical issues in fungal taxonomy given that the number of species recognized in the genus constantly changes due to different taxonomic systems (Chandra *et al.,* 2011).

Detection and diagnosis of *Fusarium* species was traditionally based on the combination of diagnostic symptoms on the host with the presence of the fungus in the affected tissues (Baayen, 2000). This approach can be problematic when relying only on morphological traits for pathogen identification (Balajee *et al.,* 2006; Cai *et al.,* 2011; Chandra *et al.,* 2011). For that reason, the use of molecular tools for the detection and diagnosis of pathogens has become widely used (Cai *et al.,* 2011; Chandra *et al.,* 2011).

Conventional PCR has emerged as a major tool for the identification and study of fungi and has helped with detection and diagnosis at family, genus, and species level by targeting specific genome regions (Ristaino *et al.,* 1998; Cullen *et al.,* 2002; Glass *et al.,* 1995). The use of nucleic

acid sequencing and online databases (e.g. NCBI BLAST) can further help in this endeavor. PCR-based protocols that can detect specific *forma specialis* (Moricca *et al.,* 1998) and specific races within *forma specialis* (Jimenez-Gasco *et al.,* 2003) also have been developed. However, the detection of specific and unique strains within a species requires more specific primers that target unique loci in specific strains.

Discriminating among isolates of the same *Fusarium* species isolated from the same host genotype can be very difficult. However, it may be the only way to understand the life cycle of species such as *F. proliferatum*. Since *F. proliferatum* is seed-borne in maize and is introduced to new environments often, it is crucial to learn more about the fate of introduced *F. proliferatum* strains. The amount of genetic variation that occurs in a plant pathogen may have a direct impact on its biological activity and its role in the environment (Zabalgogeazcoa, 2008). It is important to monitor populations for shifts in virulence with changes in environmental factors and host cultivars. The variability within and between populations from different geographical locations is important to understand how different environments, host genotypes, and other factors impact virulence. Continuous evolutionary pressure for better adaptation to different ecosystems and hosts further increases the importance of having a better understanding of *F. proliferatum* populations for variation in biological activities.

Various molecular markers are used to identify *Fusarium* species via PCR. These markers target different genomic regions such as the β-tubulin gene, the internal transcribed spacers (ITS 1/2) region of ribosomal genes, and the translation elongation factor 1-α (TEF-1α) gene; TEF-1α is the most useful in taxonomic studies of the *Gibberella fujikuroi* species complex, as well as in other members of the *Fusarium* genus (Geiser *et al.,* 2004; Kristensen *et al.,* 2005). However, this approach is not sensitive enough when trying to detect specific strains of *F. proliferatum* from other isolates of the same species. It has been reported that amplified fragment length polymorphism (AFLP) analysis might provide the best option for strain discrimination (Baayen *et al.,* 2000; Zeller *et al.,* 2003; Belabid *et al.,* 2004).

To discriminate among isolates of the same species it is necessary to identify unique markers or loci that are neutral (i.e. mutation on these markers do not have an impact on organism fitness)

and mitotically conserved in specific isolates (Leiononen *et al.*, 2008; Ballard and Kreitman, 1995). Different techniques are available to identify such loci, like amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), multi locus sequence typing (MLST), and other techniques including restriction fragment length polymorphism (RFLP) and rapid amplified polymorphic DNA (RAPD). These different methods differ in discriminatory power, reproducibility (within and between labs and users), interpretation, and cost. Accurate classification of fungal species can be obtained by comparing DNA sequences (Mule *et al.*, 2005). In the same way, discrimination, identification, and characterization of isolates from the same species can be obtained by comparing DNA sequences found throughout the genome; genotyping-by-sequencing (GBS) is one such approach to identify strain-specific DNA sequences (Elshire *et al.*, 2011).

GBS libraries are constructed using restriction enzyme digests of whole genomic DNA. GBS is simple, quick, specific and highly reproducible (within and between laboratories). Restriction enzymes are methylation-sensitive and thus avoid repetitive regions of genomes. In addition, the whole genome is subjected to sequencing, which increases the discrimination power of GBS.

To better understand *F. proliferatum* epidemiology in maize production systems, knowledge of the population genetic structure is required. Strain-level molecular characterization is necessary to analyze the potential for new isolates to establish in new environments. This can be accomplished by using strain-specific DNA markers that target unique polymorphic genome regions.

Objectives

- Objective 1: To determine the potential for *F. proliferatum* to actively disperse from colonized maize seeds and establish in soil environments into which the colonized seed is planted (Chapter 3).
- Objective 2: To determine the relative competitive ability of *F. proliferatum* and *F. verticillioides* with respect to colonization of maize tissues (Chapter 4).
- Objective 3: To determine if specific strains of *F. proliferatum* introduced via colonized maize seed into a field environment are present in the seeds of the plants

grown from that seed and thus positioned for dispersal from that environmental site (Chapter 5).

Figures and Tables

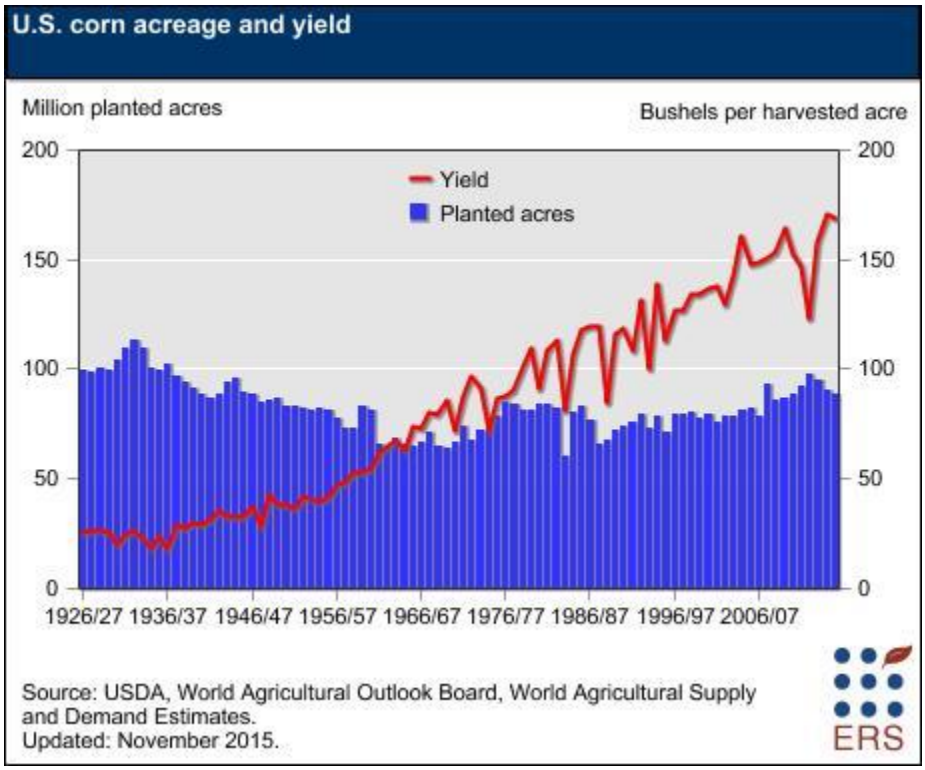

Figure 1.1 United States maize acreage and yield production (in bushels per harvested acre). Figure taken from the Economic Research Service (ERS) component of the United States Department of Agriculture (USDA).

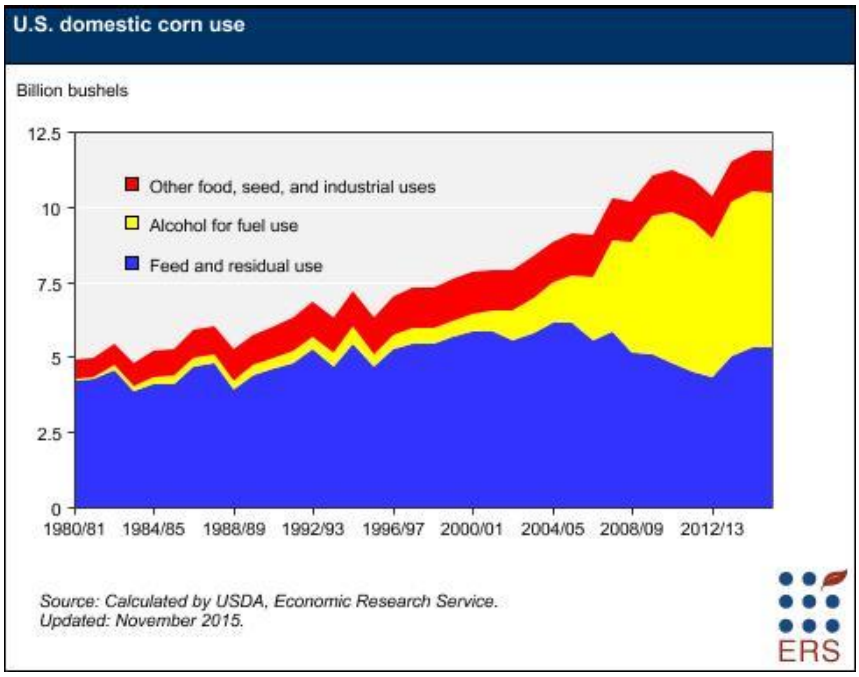

Figure 1.2 United States domestic use of maize. Figure taken from ERS-USDA.

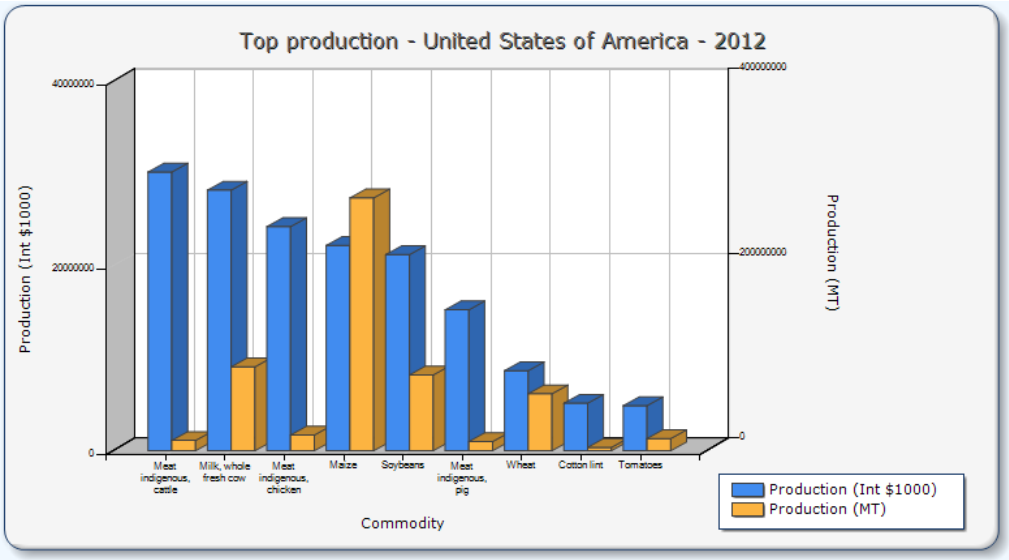

Figure 1.3 Production and revenue produced by top commodities in the United States in 2012. Figure taken from the Food and Agriculture Organization (FAO) of the United Nations.

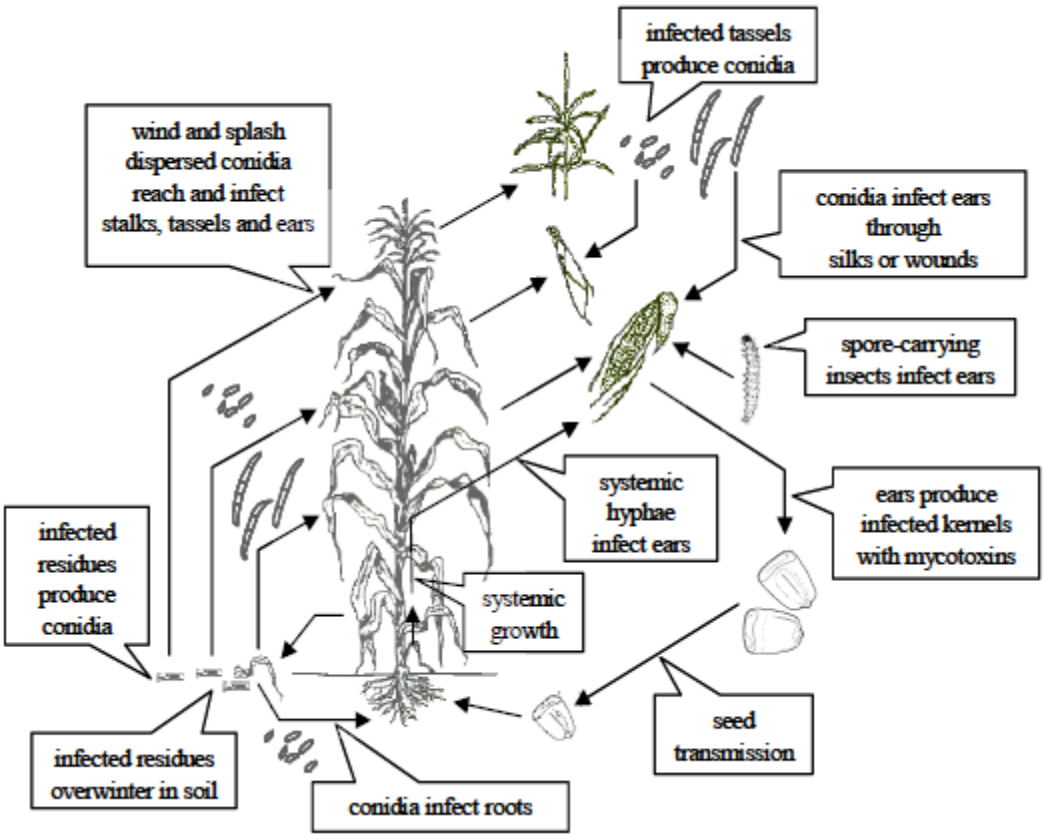

Figure 1.4 Three pathways of maize infection by *Fusarium verticillioides*. Figure taken from Battilani and Rossi, 2003.

Table 1.1 World production (tons) estimates for maize, wheat and rice production. Table generated using FAO data.

Crop	Estimated production of maize, wheat and rice in million tons					
	2007/08	2008/09	2009/10	2010/11	2011/12	2012/13
Maize	795	798	820	830	877	850
Wheat	609	686	679	653	696	656
Rice	433	448	441	448	465	466

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Chapter 2 - **Significance and Description of Methodology**

The fungus *Fusarium proliferatum* is seed-borne in maize seeds. Until 20 years ago, it was placed within a species complex known as *Fusarium moniliforme*, therefore not much is known about it. *F. proliferatum* has a broad host range with worldwide distribution. Moreover, this fungus is a prolific producer of mycotoxins (i.e. fumonisins) that are toxic to humans and animals.

The introduction of this cryptic seed-borne organism into new environments can have devastating consequences, which include disruption of trade if the pathogen is detected post-entry, and result in economic problems to the country producing the commodity (temporary or permanent trade bans), and expansion of the geographical range of undesirable populations/isolates (e.g., high mycotoxin producing isolates).

In this research project, seven different maize hybrids were used to isolate seed-borne *Fusarium* species including *F. proliferatum*, *F. verticillioides*, *F. andiyazi*, *F. fujikuroi*, and *F. thapsinum*. The identities of the *Fusarium* species were confirmed morphologically and by polymerase chain reaction (PCR) amplification, followed by sequencing of TEF-1α and β-tubulin genes.

After characterization and identification of the *Fusarium* species, *Agrobacterium tumefaciens* mediated transformation of a *F. proliferatum* strain was conducted to introduce hygromycin resistance and expression of green fluorescence (GFP). The transformants were used in active dispersal experiments from a source of inoculum colonized with GFP-*F. proliferatum* to a bait organic matter, under different temperature (10°C, 25°C and 35°C), soil matric potential (-50mb, -150mb, -330mb, and -1000mb) and distance (0cm, 0.5cm, 1cm, and 1.5cm) combinations. The presence/absence of the transformed *F. proliferatum* in the baits was determined by plating them in Nash-Snyder medium amended with hygromycin.

Agrobacterium tumefaciens mediated transformation of a *Fusarium verticillioides* strain was conducted to introduce hygromycin resistance and expression of red fluorescence (mRFP). This transformant was used to study its competition with GFP-*F. proliferatum* for maize plants. A

TaqMan multiplex real-time qPCR protocol was developed by designing sensitive and specific primer and probe sets targeting a fragment of the respective fluorescence gene, GFP for *F. proliferatum* and mRFP for *F. verticillioides.* This protocol was used to identify and quantify these two *Fusarium* species in roots and stem segments of maize plants.

Genotyping-by-sequencing (GBS) was used to identify unique polymorphisms in maize seed-borne *F. proliferatum* isolates from specific maize hybrids. The polymorphisms were used to design specific primer sets to accurately detect specific isolates of *F. proliferatum* by end-point PCR. In field experiments, a molecularly characterized seed-borne isolate of *F. proliferatum* was studied. Ears of maize were collected from the field and kernels plated on Nash-Snyder medium. DNA of the growing *Fusarium* was extracted and the specific primer set was used in end-point PCR to detect the GBS-characterized *F. proliferatum* isolates. The isolates that tested positive for the presence of the molecular marker were subjected to further fingerprinting using amplified fragment length polymorphism (AFLP) to assess their genetic relatedness to the original isolates that were characterized with GBS. The AFLP binary data was analyzed in NTSYSpc (to generate a UPGMA tree), STRUCTURE (to determine population structure), and GenAlEx (to do Principal Coordinate Analysis, and AMOVA).

Chapter 3 - **Active dispersal through soil and colonization of organic matter by *Fusarium proliferatum***

Abstract

Fusarium proliferatum is a broad host-range, mycotoxin-producing fungus and among the most common maize colonizers globally. It is seed-borne in maize, providing an efficient vehicle for introduction into new soil environments. The ability of *F. proliferatum* to grow from colonized maize seed through nonsterile soil and colonize non-viable maize seeds was investigated. To provide markers to track the fungus in soil microcosms, *F. proliferatum* was transformed to express a green fluorescent protein and hygromycin-resistance (Fp-GH). Maize seeds were heat-killed (75°C water bath for 20 minutes) and re-colonized with Fp-GH ($1*10^6$ spores/ml suspension overnight). Re-colonized seeds served as sources of inoculum and were added to non-sterile soil with heat-killed, non-colonized maize seeds (baits) at several soil temperature, soil matric potential, and source-to-bait distance combinations. Controls included sources of inoculum consisting of non-viable maize seeds that were not re-colonized (negative control) and non-viable maize seeds that were re-colonized with non-transformed parental type Fp (positive control). Baits were retrieved over time from soil and plated on Nash-Snyder medium amended with hygromycin (1µl/ml). Fp-GH grew at least 1.5cm through non-sterile sieved soil and colonized the bait seeds. Growth through soil and colonization of baits was less in soil with intact field structure; Fp-GH grew at least 0.5cm after 7 days. In addition to plant and seed colonization, *F. proliferatum* may have an active soil resident phase in its life history.

Introduction

Over the past few decades the trade of food, plant, and animal products has resulted in the worldwide movement, introduction and establishment of exotic pests and pathogens that in some cases had dramatic negative impacts on native and cultivated plant systems (Jeger et al. 2011). Because *F. proliferatum* can be symptomless and seed-borne in maize, it has a high risk of being introduced into new environments. Introduction of seed-borne organisms into new environments

can disrupt trade if the pathogen is detected post-entry, and it can extend the geographical range of undesirable populations and/or strains (e.g., high toxin producing strains).

Fusarium proliferatum (Matsushima) Nirenberg ex Gerlach & Nirenberg, also known as *Gibberella fujikuroi* mating population D, is a fungal plant pathogen with worldwide distribution. It has an extraordinarily broad host range, colonizing and causing disease in economically important plants as diverse as asparagus (Elmer, 1990), banana (Jimenez *et al*., 1993), citrus fruits (Hyun *et al*., 2000), onion (Stankovic et al. 2007), orchids (Benyon *et al*., 1996), maize (Munkvold 2003), rice (Desjardins *et al*., 2000) and sorghum (Leslie, 2008).

Fusarium proliferatum is seed-borne in maize and colonizes maize plants worldwide (Logrieco *et al*., 2002). This fungus is considered to be the most effective producer of polyketide-derived fumonisin mycotoxins, fumonisin B_1 (FB_1) being the most prevalent (Rheeder *et al*., 2002). Fumonisin B_1 is toxic to animals, including humans, due to inhibition of sphingolipid metabolism and cell cycle regulation. It has been associated with esophageal cancer, liver cancer, and neural tube defects in humans (Desjardins, 2006). Strains of *F. proliferatum* are reported to produce FB_1 at more than 6000 µg/g (ppm) in culture (Leslie *et al*., 2004). In humans, there is little information on the acute toxicity of FB_1 and the LD_{50} of FB_1 is unknown. No information is available on the toxicological effects of single dose exposure to FB_1 by inhalation or dermal routes (EHC, 2000). However, based on the potential adverse effects caused by fumonisins to humans and animals, the U.S. Food and Drug Administration (FDA) issued a recommendation for maximum levels of fumonisins in human food (4 ppm) and in animal feed (100 ppm) (FDA, 2001).

Fusarium proliferatum also produces a wide range of other mycotoxins and biologically active metabolites, including beauvericin, enniatin, fusaric acid, fusarin, fusaproliferin, and moniliformin (Desjardins *et al*., 2000; Leslie *et al*., 2004; Bacon *et al*., 1996; Herrmann *et al*., 1996; Marasas *et al*., 1986; Moretti *et al*., 1996; Ritieni *et al*., 1995).

The life cycle of seed-borne *F. proliferatum* has not been fully studied, but it is suspected to share some similarities to that of *Fusarium verticillioides* (Battilani et al. 2003). For this reason it

is assumed that dispersal of seed-borne *F. proliferatum* in the field is passive by conidia movement in water, air, or by insect-vector. This research presents evidence that active hyphal growth through soil is another means of dispersal for *F. proliferatum*.

Materials and Methods

Fusarium proliferatum isolates

Fusarium proliferatum was isolated from maize seed (DuPont Pioneer® hybrid 32N70). Seed were surface sterilized with 10% sodium hypochlorite solution for 1 minute, rinsed in distilled water for 30 seconds, and plated onto isolation medium. Isolates were single-spored and grown on Nash-Snyder (NS) medium at 27°C for 7 days. Isolates were identified as *F. proliferatum* by morphological and molecular characteristics, including the amplification and sequencing of the *TEF-1α* and *FUM* genes. One isolate (Fp-70-2-5) confirmed as *F. proliferatum* was used in this study.

Agrobacterium tumefaciens-mediated transformation of isolate Fp-70-2-5

A plasmid (pBV126, provided by Dr. Barbara Valent) carrying the green fluorescent protein (GFP) and antibiotic-resistance genes was used for the transformation of *F. proliferatum* following published protocols (Mullins *et al*., 2001; Rho *et al*., 2001). Transformations were considered successful when individual isolates expressed the GFP and hygromycin-resistance. Nineteen hygromycin resistant-colonies were confirmed as green transformants and stored in 15% glycerol in cryovial tubes at -80°C.

Transformant characterization

The nineteen transformed strains were tested to confirm that the insertion was mitotically stable by comparing them to the parent isolate, Fp-70-2-5. Characterization included morphology, hyphal growth rate, pathogenicity in apples, and colonization of maize seeds. The stability of the insertion was tested by sub-culturing five generations on Spezieller Nährstoffarmer Agar (SNA) medium (Kunitake *et al*., 2011); the fifth generation was characterized for morphology, hyphal growth rate, hygromycin resistance, and fluorescence.

Hyphal growth rate was determined by placing a colonized agar plug (~ 8mm diameter) in the center of NS and SNA agar medium plates and the radial hyphal growth measured for 7 consecutive days.

Pathogenicity was tested in organic Granny Smith apples; 5 puncture wounds (5 mm in diameter and 5 mm in depth) were made in each apple and 50µL of a spore suspension ($1*10^6$ spores/ml) was applied to the wounds. Two of the wounds were used for the negative control (sterile-distilled water) and the positive control (the apple pathogen *Penicillium expansum*). The three other wounds were inoculated with *F. proliferatum*; one wound with the wild type (Fp-70-2-5) and two wounds with the transformed strain being tested. The inoculated apples were placed in a chamber with wet paper towels to maintain high humidity and the container placed in an incubator at 25°C.

Colonization ability was tested using Dupont Pioneer hybrid 32N70 seeds. Seeds were hydrated for 5 hours in distilled water and then placed in a hot water-bath (65°C) for 3 minutes. Heat-treated seeds were subjected to one of 3 treatments: 1) distilled water (negative control), 2) Fp-70-2-5 (positive control), and 3) a transformed strain (spore suspension of $1*10^6$ spores/ml) for 20 hours. The seeds were then surface sterilized (10% sodium hypochlorite for one minute) and rinsed in distilled water for 30 seconds. The seeds were plated onto NS medium, incubated at 25°C for 5 days and the percentage seed colonization determined.

Soil collection, characterization, and determination of soil water retention curve

Soil was collected from a Kansas State University experimental field in Hutchinson, KS; maize had been grown the previous season. Soil was collected from the surface horizon (approximately the top 20cm) and sieved (1.5cm) to remove large debris. The following soil characterization was performed by the Kansas State University Soil Testing Lab: pH = 7.1; texture = 72% sand, 16% silt and 12% clay; total nitrogen content = 0.0901% (917ppm); total carbon content = 1.268%; total phosphorous content = 210ppm; and organic matter content = 2.3%. The soil characteristics were consistent with a sandy loam Shellabarger soil series (35% - 80% sand content, 2% - 19% clay content, sandy loam texture, acidic to neutral pH).

Water retention characteristics were determined experimentally at -15,000mb, -10,000mb, -5,000mb, -1,000mb, -330mb, -100mb, and 0mb by the Kansas State University Soil Testing Lab and the Soil, Water and Plant Testing Lab at Colorado State University by using a published protocol (Klute, 1986). Similar results were obtained for measurements at both labs.

The soil water retention curve was determined using the van Genuchten model in the RETC (version 1.0) software. The van Genuchten model parameters are ideal to precisely describe a curve for a broad range of soils including disturbed and undisturbed soils (van Genuchten *et al*., 1991). Finally, an equation was developed to calculate the amount of water needed to add to the soil to reach the target soil matric potentials.

Source of inoculum and bait generation

Maize seeds were heat-killed in water at 75°C for 20 minutes. Nonviable *Fusarium*-free seeds were placed in the following spore suspensions ($1*10^6$ spores/ml) and placed on the rotary shaker (80 RPM) at 25°C for 16 hours to allow colonization: (i) wild-type *F. proliferatum* Fp-70-2-5 (hygromycin-sensitive, non-fluorescent) or (ii) *F. proliferatum* Fp-GH (hygromycin-resistant, green fluorescent). Nonviable Fusarium-free seeds placed in sterile water served as the negative control. *Fusarium proliferatum*-colonized seeds were surface sterilized (10% sodium hypochlorite for 1 minute) and rinsed in distilled water (30 seconds). Seeds that served as sources of inoculum were tested for the presence of *F. proliferatum*, and it was found all of them were colonized with this fungus. These *F. proliferatum*-colonized seeds served as the sources of inoculum. Non-colonized, non-viable seeds were surface sterilized (10% sodium hypochlorite) and rinsed with distilled water (30 seconds). These non-colonized, non-viable seeds were used as bait organic matter.

Colonization of baits and statistical analyses

Percentage of baits colonized by *F. proliferatum* Fp-GH was determined as a function of temperature (10°C, 25°C, and 35°C), soil matric potential (-50mb, -150mb, -330mb, and -1000mb), and source-to-bait distance (0cm, 0.5cm, 1cm, and 1.5cm). Five replicates were used

for each temperature-soil matric potential-distance combination and the experiment was conducted twice. Preliminary data demonstrated that *F. proliferatum* did not actively disperse more than 2cm from the source of inoculum and that the optimum sampling times were 5 days for 0 cm, 7 days for 0.5 cm, 10 days for 1.0 cm and 14 days for 1.5 cm. At the appropriate sampling times, the baits were retrieved from the soil, surface sterilized (10% sodium hypochlorite for one minute), rinsed in distilled water for 30 seconds, plated onto NS agar amended with hygromycin (1µl/ml), and incubated at 27°C. Presence or absence of *F. proliferatum* Fp-GH was used to determine the percentage colonization of baits.

A split-plot experimental design was used: each whole-plot was temperature (10°C, 25°C, or 35°C) and each split-plot was a specific distance - soil-matric potential combination. A total of 48 combinations (3 temperatures * 4 distances * 4 soil matric potentials = 48) were tested. Soil matric potential values were linearized by logarithm transformations. The data were analyzed using the covariance model of SAS® mixed procedure (Version 9.3; SAS Institute Inc., Cary, NC).

To better mimic field conditions, soil columns with an intact soil structure were obtained using a golf cup cutter from the same field in Hutchinson, Kansas at the same sampling time. Small wells were sunk into the soil column to a depth of 2cm; baits were placed into wells at 0cm, 0.5cm, 1cm, or 1.5cm from the source well. Constant temperature (25°C) and approximate soil moisture conditions (-50mb) were used. Five replicates were used for each distance and the experiment was repeated twice.

Results

Characterization of the transformed strain

One transformed strain (Fp-GH) expressed strong fluorescence (GFP) and hygromycin resistance; it was selected for further characterization. With respect to morphology and pathogenicity, the transformed strain and the wild-type strain were indistinguishable. Morphologically, the transformed strain, Fp-GH, and the wild type, Fp-70-2-5, were identical: macroconidia were slender and 3- to 5- septate and microconidia were club shaped and formed in

chains from mono- and poly-phialides (Leslie and Summerell, 2006). Radial hyphal growth rate for the transformed strain and the wild type were indistinguishable on the two media tested, SNA (R^2 = 0.99880) and NS (R^2 = 0.99661).

After 5 successive generations, the morphology of the transformed strain Fp-GH and the radial hyphal growth rate on the two media, NS (R^2 = 0.99606) and SNA (R^2 = 0.99830) remained identical to the wild type (Fp-70-2-5) with no change from the 1st generation. After 5 successive generations, there were no changes in fluorescence or hygromycin-resistance: the transformation was stable.

Pathogenicity to apples and colonization of maize seeds by the transformed strain was identical to the wild type. After 5 successive generations, there were no changes in pathogenicity to apples or colonization of maize seeds. Both the transformant Fp-GH and wild type developed lesions in apples which had 3.6cm diameter on average twelve days after inoculation. In addition, maize seeds showed 100% colonization by both the transformant Fp-GH and the wild type.

Colonization of baits at different temperatures, distances, and soil matric potentials

Growth through soil and colonization of baits was significantly affected by temperature ($p=0.0365$) and was linearly related to both distance ($p<0.0001$) and soil matric potential ($p<0.0001$). The optimum conditions for growth and colonization was 25^{o} C and -50mb matric potential. There was a linear decrease in colonization of baits with increasing distances between source of inoculum and baits and a similar trend occurred with decreasing soil matric potentials. The interaction of soil matric potential and temperature ($p=0.0015$) and the interaction of distance and soil matric potential ($p<0.0001$) were significant, indicating that the slopes associated with soil matric potential varied with both temperature and distance (Table 3.1). To illustrate this significant interaction, statistical models were developed to demonstrate the change in colonization of baits in the interaction of soil matric potential and temperature, and soil matric potential and distance (Figure 3.1). These models were built using the colonization estimates of the least square means table of the SAS mixed output. The slopes for soil matric potential had the greatest spread at smaller distances for the 3 temperatures. In addition, the slopes converged at greater distances due to the interaction of soil matric potential and distance. The average decline

in colonization with increasing distance was similar for all temperatures. Finally, the soil matric potential slopes are shifted upward when temperature is 25°C, which reflects the significant effect of temperature on hyphal growth through soil. This is evident because under same conditions (same soil matric potential, and distance between source of inoculum and bait) more baits are colonized at 25°C than at 10°C or 35°C. Furthermore, the models validate that the colonization of the baits decrease as the soil matric potential decreases for all temperatures, as demonstrated for the slopes. In addition, as the distance between the source of inoculum and the bait increases, the colonization decreases as demonstrated in the models (Figure 3.1).

The models used to analyze the colonization of baits represented 78.8% of the variation in the data set (Figure 3.2) which demonstrates the fitness of the models to explain the differences in colonization for the different temperatures, distances and soil matric potentials.

Sieving soil alters its natural structure, which could influence the hyphal growth and colonization of baits. Therefore, to determine if colonization could occur in soil with field structure, soil columns were obtained using a golf cup cutter. The experiments were conducted twice at -50mb, and 25°C. In the first experiment, *F. proliferatum* colonized 80% of the baits at 0 cm and 20% of the baits at 0.5 cm. In the second experiment *F. proliferatum* colonized 60% of the baits at 0 cm and 40% of the baits at 0.5 cm.

Discussion

Fusarium proliferatum grew from colonized maize seed through nonsterile soil (sieved reconstituted soil and intact soil columns) and colonized organic matter. This demonstrates the capability of *F. proliferatum* for post-entry establishment in new locations subsequent to planting. Optimal soil conditions for hyphal growth and colonization of organic matter were 25°C and -50mb. The colonization of baits decreased as the distance between the source of inoculum and the bait increased. The baits used in this experiment were non-viable heat-treated maize seeds free of seed-borne organisms. In natural field soils, organic matter is more diverse with respect to chemical composition and microbial communities which may influence the colonization potential of *F. proliferatum*. However, *F. proliferatum* has a wide host range that

includes monocots and dicots and is a successful saprophyte (Cotten and Munkvold, 1998), which makes it fit to compete for available organic matter with other microorganisms.

A recent study (Wu *et al*., 2016) reported that GFP and DsRed transformants of *F. verticillioides* had decreased growth at different pH compared to the parental type; as well as fewer colony forming units (CFUs) in root samples. They found that in two cases, the integration of GFP had occurred in non-coding regions but the transformant still had an impact on its fitness. A different study reported that the insertion of GFP in the *F. verticillioides* genome did not have an impact in the ability of the fungus to colonize maize stalk (Wilke *et al*., 2007). Another investigation reported that the insertion of GFP and DsRed did not have an impact in the fitness of transformants in *Fusarium oxysporum* f. sp. *lycopersici* (Nahalkova and Fatehi, 2003). Based on the characterization of the transformant we performed that tested morphological characteristics, growth in different media and at different temperatures, stability of the insertion, and pathogenicity, we concluded that there were no observable differences between the transformed strain and the parental type. For this reason, we do not think the insertion had a large fitness impact in the behavior or ecology of the transformed strain in our investigation.

Many organisms associated with the soil microbial community, including fungi, bacteria, and nematodes (Fierer *et al*., 2005; Neher and Campbell, 1994), may compete with *F. proliferatum* for available organic matter. A previous study performed *in-vitro* found that different fungi, including *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, and *Penicillium implicatum*, have a mutual inhibitory effect on the colonization of maize grain by *F. proliferatum* (Marin *et al*., 1998). However, additional research demonstrated that *F. proliferatum* is very competitive and dominant against *Penicillium* spp. and *A. flavus* when competing for a maize seed (Marin *et al*., 1998). In our experiments, to simulate field conditions of competition, these experiments were performed in non-sterile soil microcosms. The ability of *F. proliferatum* to grow through soil and colonize organic matter under different temperatures and soil matric potentials may provide an advantage in the competition for available resources in soil with organisms more sensitive to different environmental conditions.

There is evidence that *F. proliferatum* can survive in soil for extended periods of time (Leslie *et al*., 1990; Logrieco and Bottalico, 1988; Logrieco *et al*., 1995), at least 630 days in surface or buried maize residue (Cotten and Munkvold, 1998). Colonized residue has been shown to serve as a source of inoculum (Cotten and Munkvold, 1998). For this reason, ears of maize with kernels colonized by *F. proliferatum* could also serve as residue within which it can survive and from which it can disperse. The colonization of organic matter in soil from introduced sources of inoculum can enhance the survival capacity of *F. proliferatum* in the field, resulting in establishment in new environments. Furthermore, during different stages of field work, including tillage, sowing, harvest, and rotation, a vast amount of organic matter is incorporated into the soil, likely increasing the available substrates for utilization by *F. proliferatum*.

These characteristics increase the risk that *F. proliferatum* can be introduced into new environments by contaminated maize seed. As a result, high-consequence strains of this fungus (i.e. high toxin producers, or highly aggressive) may become invasive in these new environments, where they could affect native and cultivated plant species as well as the native fauna. Since maize is the most produced agricultural commodity in the United States (FAO, 2011) and is a major export and import commodity, there is a high chance that new strains are being introduced into new environments where they can establish (Elmer 1995).

In previous study, which investigated the effects of water activity (a_w), pH, and temperature on growth of *F. proliferatum* isolates from maize, growth was optimum at 0.994-0.90 a_w in the temperature range of 20-35°C (optimum of 25°C) and pH of 5.5. Growth was reported also to occur at 4°C and 0.994-0.96 a_w, but no growth was recorded at 40 and 45°C even under ideal a_w (Marin *et al*., 1995). Germination of microconidia of *F. proliferatum* was optimal at 30°C (Marin *et al*., 1996). These studies were conducted *in-vitro* in artificial systems. This research demonstrated that hyphal growth of *F. proliferatum* occurs in non-sterile soil under different temperature and soil matric potential combinations. *Fusarium proliferatum* was capable of growing through both sieved and intact soil. The proportion of baits colonized was much lower in the latter possibly due to more restrictive pore size distribution and to natural physical barriers present in soil (Christensen, 2001).

Despite its global distribution and multiple hosts in diverse environments, the life cycle of *F. proliferatum* is still not well understood. Dispersal of seed-borne *F. proliferatum* in the field can be passive, by conidia movement in water, air, or by insect-vectors (Munkvold, 2003). This research demonstrated that hyphal growth through soil can be a means of short-range active dispersal for *F. proliferatum* and might suggest that it has the capacity for an active soil resident phase in its life history.

Figures and Tables

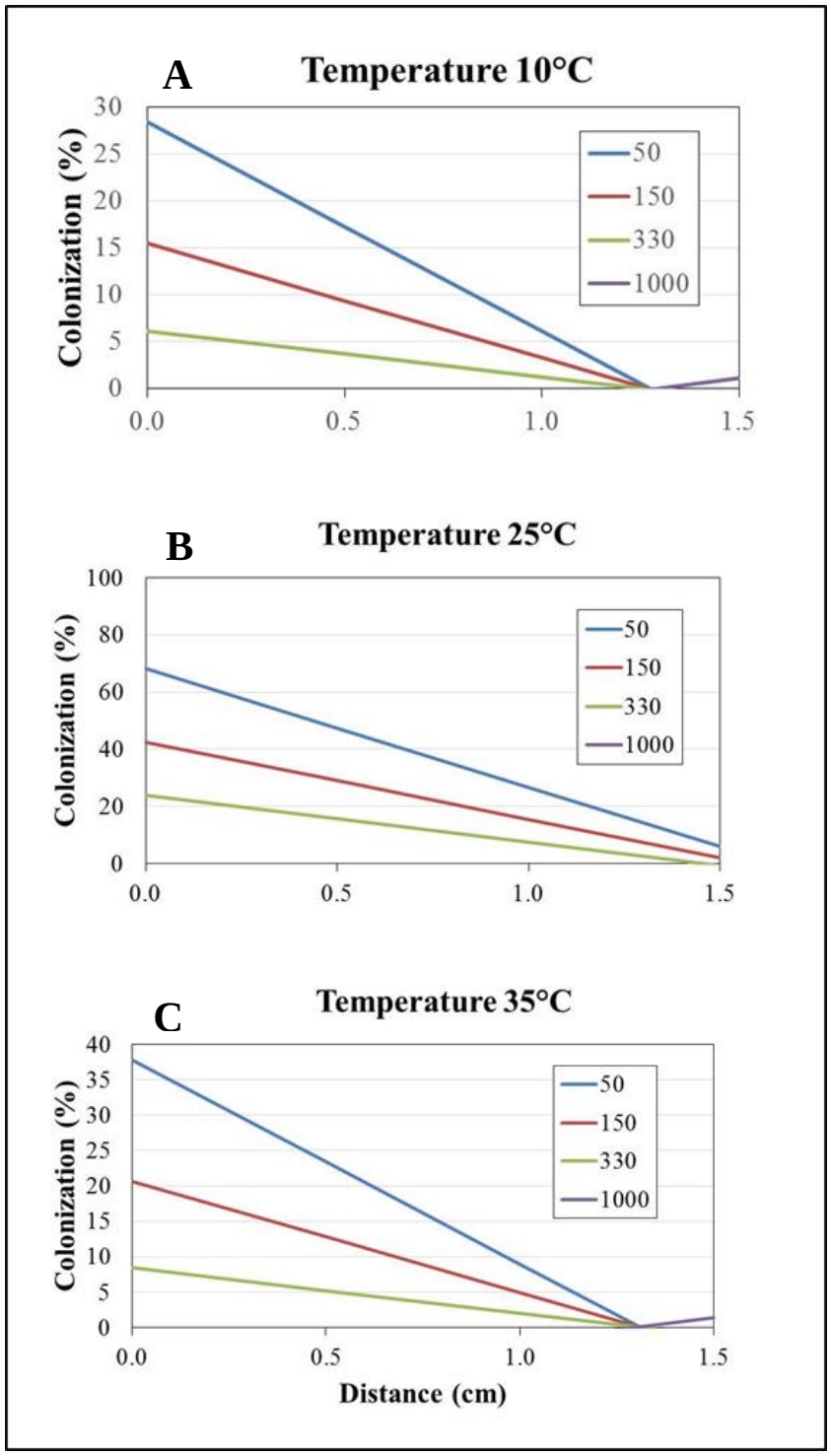

Figure 3.1 The relationships among soil temperature, source-to-bait distance, soil matric potential on hyphal growth through non-sterile soil and colonization of organic matter by *Fusarium proliferatum* strain Fp-GH. The main effects (soil temperature, source-to-bait distance, soil matric potential) and their interactions were statistically significant.

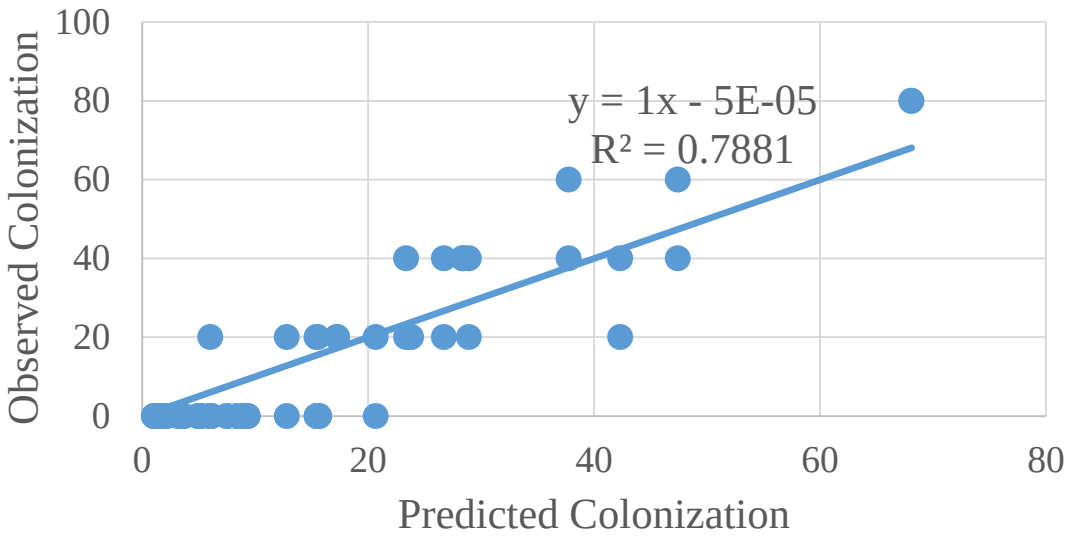

Figure 3.2 Covariance model fit for bait colonization data explains 78.8% of the variance (R^2=0.7881). Predicted colonization was based on linear models (Figure 3.1) of bait colonization by distance, log-transformed soil matric potential (SMP), and the distance by SMP interaction for each of three temperatures.

Table 3.1 The effects of soil temperature, source-to-bait distance, and soil matric potential and their interactions on hyphal growth through non-sterile soil and colonization of organic matter by *Fusarium proliferatum* strain Fp-GH.

Individual Effects and Interactions of Variables	p-value	Distance (cm)	Soil Matric Potential (mb)	Colonization (%)		
				10°C	25°C	35°C
Temperature	0.0365	0	50	40	80	50
Distance	<0.0001	0	150	10	30	10
Distance*Temperature	0.1802	0	330	0	20	0
Log(Soil Matric Potential)	<0.0001	0	1000	0	10	0
Log(Soil Matric Potential)*Temperature	0.0015	0.5	50	20	50	30
Distance*Log(Soil Matric Potential)	<0.0001	0.5	150	0	30	10
Distance*Log(Soil Matric Potential)*Temperature	0.4789	0.5	330	0	0	0
		0.5	1000	0	0	0
		1	50	0	30	0
		1	150	0	10	0
		1	330	0	0	0
		1	1000	0	0	0
		1.5	50	0	10	0
		1.5	150	0	0	0
		1.5	330	0	0	0
		1.5	1000	0	0	0

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Chapter 4 - **Interspecific competition for colonization of maize between *Fusarium proliferatum* and *Fusarium verticillioides***

Abstract

Fusarium proliferatum and *Fusarium verticillioides* are seed-borne pathogens of maize. They are often asymptomatic in seed, eluding symptom-based detection. Although co-colonization of individual seed occurs, some seed lots are colonized predominantly by a single species. Experiments were conducted in non-sterile soil to determine if interspecific competition influenced establishment in maize plants of an introduced isolate of *F. proliferatum* or *F. verticillioides*. Green fluorescent protein (GFP-tagged), hygromycin resistant *F. proliferatum* (Fp-G) and monomeric red fluorescent protein (mRFP-tagged) hygromycin resistant *F. verticillioides* (Fv-R) strains were developed to provide molecular markers to track fungal establishment. Heat-killed (75°C water bath for 20 minutes) *Fusarium*-free maize seeds, colonized with Fp-G or Fv-R by immersion in a spore suspension for 16 hours, served as source of inoculum. The ability of Fp-G and Fv-R to colonize viable seed already colonized by the other species was determined. Controls included non-colonized cured *Fusarium*-free seeds and naturally colonized viable seeds. Maize plants were retrieved from soil after 14 days and DNA extracted from three consecutive root segments (3cm each) and three consecutive stem segments (3cm each). A TaqMan multiplex real-time qPCR protocol was developed to identify and quantify Fp-G and Fv-R for each plant segment from each treatment; the experiment was repeated three times. This experiment confirmed that Fp-G and Fv-R effectively colonize roots and stems of the maize plant already colonized with the other species. Prior colonization did not preclude subsequent colonization by the challenger species. This investigation demonstrated that *F. proliferatum* can grow from seed in soil and effectively compete with a species (*F. verticillioides*) having a similar life cycle and occupying a common niche, as well as with other naturally occurring seed microorganisms to colonize a maize plant.

Introduction

Fusarium proliferatum and *Fusarium verticillioides* belong to the Liseola section of *Fusarium* (Leslie and Summerell, 2006). These two fungal species share many morphological characteristics and misidentification can occur (Leslie and Summerell, 2006). In addition, they are both seed-borne in maize and important colonizers of maize plants (Logrieco *et al*., 2002; Munkvold 2003). They have the ability to produce fumonisins and other mycotoxins in contaminated grain.

To colonize maize plants, these *Fusarium* species compete against each other, as well as with other microorganisms, including other fungi and bacteria (Marin *et al*., 1998a; Marin *et al*., 1998b; Marin *et al*., 1998c). The competition for a niche in the maize plant comes from other seed-borne microorganisms, as well as from microorganisms outside the seed. These studies were all done *in vitro* on culture media or on seed in chambers. Extrapolation of activity and competitive ability to natural environments (e.g., soils in agroecosystems) is not possible, though essential to estimating the significance to invasion potential.

In addition, abiotic conditions can enhance or diminish the colonization ability of these organisms. Previous studies in synthetic media demonstrated the importance of water activity, pH, and temperature for the growth of *F. proliferatum* and other *Fusarium* species and fungi (Marin *et al*., 1995). The influence of abiotic factors on fungal interactions has been extensively demonstrated in a range of ecosystems (Magan and Lacey, 1984; Magan and Lacey, 1985; Ramakrishna *et al*., 1993). *Fusarium* species from the section Liseola (*F. proliferatum* and *F. verticillioides*) have similar abiotic requirements (Leslie and Summerell, 2006), which makes them ideal for interspecific competition experiments.

Moreover, past investigations done *in-vitro* (Marin *et al*., 1998a) suggested that strains of *F. proliferatum* and *F. moniliforme* (now called *F. verticillioides*) were very competitive against a range of other maize colonizers. Niche overlap indices, based on patterns of carbon/nitrogen sources found that *F. proliferatum* was more competitive than *F. moniliforme*, and that, depending on abiotic factors (water potential and temperature), different niches were occupied by other species (Marin *et al*., 1998a).

Considering the ecological similarities of *F. proliferatum* and *F. verticillioides*, it is likely that they also have similarities in their life cycles. In the colonization of maize plants, it is likely that they compete against each other for the same niche. In Chapter 3, it was demonstrated that *F. proliferatum* hyphae can grow up to 1.5cm from a source of inoculum and colonize available organic matter under different abiotic conditions. In this study, we investigated how well *F. proliferatum* competes with *F. verticillioides* and other naturally occurring seed-borne microorganisms for a niche in maize plants. To facilitate strain discrimination of the two *Fusarium* species, they were transformed using *Agrobacterium tumefaciens*-mediated transformation; the *F. proliferatum* strain was transformed to express a green fluorescence protein (GFP-tagged) and hygromycin resistance, and the *F. verticillioides* strain was transformed to express a monomeric red fluorescence protein (mRFP-tagged) and hygromycin resistance.

Detection of fungi in plant tissues using nucleic acid-based techniques can be hampered by the presence of plant DNA and DNA from other microorganisms (Arif *et al.,* 2013). For this reason, we developed a multiplex TaqMan real-time qPCR assay with high specificity and sensitivity for the detection and quantification of Fp-G and Fv-R using their respective fluorescence genes as targets.

Materials and Methods

Fusarium isolates

F. proliferatum and *F. verticillioides* were isolated from maize seed (DuPont Pioneer® hybrids 32N70 and 33B54). Seeds were surface sterilized with a 10% sodium hypochlorite solution for 1 minute, rinsed in distilled water for 30 seconds, and plated onto Nash-Snyder (NS) medium. Isolates were single-spored and grown on NS medium at 27°C for 7 days, and identified as *F. proliferatum* or *F. verticillioides* by morphological and molecular characteristics, including the amplification and sequencing of the *TEF-1α* and *FUM* genes. One isolate (Fp-70-2-5) confirmed

as *F. proliferatum* and another isolate (Fv-54-3-5) confirmed as *F. verticillioides* were used in this study.

Agrobacterium tumefaciens-mediated transformation of isolates and characterization of transformants

A plasmid (pBV126, provided by Dr. Barbara Valent) carrying the green fluorescent protein (GFP) and hygromycin resistance was used for the transformation of *F. proliferatum* and a plasmid (pBV216, provided by Dr. Barbara Valent) carrying the monomeric red fluorescent protein (mRFP), and hygromycin resistance was used for the transformation *F. verticillioides*. Transformations were done following published protocols (Mullins *et al*., 2001; Rho *et al*., 2001). Transformations were considered successful when *F. proliferatum* expressed the GFP and hygromycin-resistance and *F. verticillioides* expressed the mRFP and hygromycin-resistance. Nineteen hygromycin-resistant colonies were confirmed as green *F. proliferatum* transformants, and four hygromycin resistant-colonies were confirmed as red *F. verticillioides* transformants.

The transformed strains were tested to confirm that the insertion was mitotically stable by comparing them to the parent isolates, Fp-70-2-5 and Fv-54-3-5. Characterization included morphology, hyphal growth rate, pathogenicity in apples, and colonization of maize seeds as described in Chapter 3.

Southern blot analysis

The number of insertions of the fluorescent genes in the transformants was determined by Southern blot analysis of DNA of the transformed strains. For each reaction, 5µg of genomic DNA was digested with EcoRI-HF (New England Biolabs) overnight, separated in a 0.7% agarose gel, and blotted to a positively charged nylon membrane using an upward alkaline capillary transfer. Probe preparation and labeling, hybridization of the probe, and washes of the nylon membrane were done using the AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare Life Sciences). A 557 base-pair long gene fragment was selected as the probe for the green fluorescence gene and a 647 base-pair long gene fragment was selected as the probe for the red fluorescence gene, to detect the gene copy number. The two probes were

hybridized onto the membrane at the same time. Probes were synthesized as gBlocks® gene fragments by IDT (Table 4.1) (Integrated DNA Technologies, Inc., Corelville, IA).

Source of inoculum and bait plants generation

Maize seeds were heat-killed and disinfected in water at 75°C for 20 minutes. These nonviable, *Fusarium*-free seeds were placed in the following spore suspensions ($1*10^6$ spores/ml) and placed on the rotary shaker (80 RPM) at 25°C for 16 hours to allow colonization: (i) *F. proliferatum* Fp-70-2-5-G2 (hygromycin-resistant, green fluorescent), or (ii) *F. verticillioides* Fv-54-3-5-R1 (hygromycin-resistant, red fluorescent); and (iii) nonviable, *Fusarium*-free seeds placed in sterile water which served as the negative control. These colonized seeds were surface sterilized (10% sodium hypochlorite for 1 minute) and rinsed in distilled water (30 seconds), and they served as the sources of inoculum.

Non-colonized, and naturally or artificially colonized, viable seeds were surface sterilized (10% sodium hypochlorite) and rinsed with distilled water (30 seconds), and used as bait plants. To artificially colonize viable maize seeds, we modified a published protocol to eradicate and cure maize seeds (Daniels, 1983) of naturally occurring *Fusarium* by exposing the seeds to 65°C water-bath for 4 minutes, and then re-colonized the seeds by placing them in spore suspensions as described above. These viable seeds were surface sterilized with 10% sodium hypochlorite for 1 minute and rinsed in distilled water for 30 seconds.

Colonization of plants and analyses from RT-PCR data

Colonization of plants was investigated in 9 different treatment groups (Table 4.2) to study Fp-G and Fv-R performance within different competition events. The experiment used a split-plot design in which each experiment was treated as the whole plot, and each individual treatment group was treated as a subplot. The experiment was repeated three times in the same growth chamber, and the environmental conditions were kept at 25°C day and 21°C night temperature, and 16 hours of light and 8 hours of dark, to reduce the variability among experiments.

Treatment 7, the negative control for each experiment, was included to ensure that no cross-contamination occurred among treatment groups. Treatment groups 1, 3 and 5 were used to compare the ability of Fp-G to grow from a source of inoculum and colonize a maize plant under different competition conditions, including maize plants colonized with Fv-R, colonized with naturally occurring microorganisms, or cured. Treatments 2, 4 and 6 were used to compare the ability of Fv-R to grow from a source of inoculum and colonize a maize plant under different competition conditions, including maize plants colonized with Fp-G, colonized with naturally occurring microorganisms, or cured. Treatment 8 was compared to treatment 1 to assess whether the presence of Fp-G had an effect on Fv-R. Treatment 9 was compared to treatment 2 to assess whether Fv-R had an effect on Fp-G.

Two weeks after planting, plants were retrieved from the soil and rinsed with water to remove soil and other particles which had adhered to the plant. Then, for each plant, the root and the stem were divided in three equal segments of 3cm each. The segments were labeled R1, R2, R3, for segments collected from the root, R1 being closest to the seed and R3 furthest away, and S1, S2 and S3, for segments collected from the stem, S1 being closest to the seed and S3 furthest away (Figure 4.1).

These segments were sterilized with 10% sodium hypochlorite for 1 minute and rinsed with water for 1 minute. Genomic DNA was extracted from the root and stem segments using the GeneJet Plant Genomic DNA Purification kit (ThermoFisher Scientific) according to the manufacturer's instructions. Detection and quantification of Fp-G and Fv-R in the plant segments was done using real-time qPCR.

The data were logarithmically transformed, and analysis was done using GLIMMIX procedure from SAS® (Version 9.3; SAS Institute Inc., Cary, NC).

Primer and probe design

Sequences of the green fluorescent protein (GFP) and red fluorescent protein (mRFP) genes were used to design two primer and probe sets specific to each gene (Table 4.1). The two fluorescence genes were aligned using Geneious to find polymorphic regions to ensure specificity in the

design of the primer and probe sets, and they were designed using Primer3 (Rozen and Skaletsky, 1999). The specificity was also confirmed *in-silico* by using BLASTn (Altschul *et al*., 1990) to screen the primer and probe sequences (Arif *et al*., 2013). Primer thermodynamics, internal structures and self-dimer formation were examined *in-silico* with mFold (Zuker, 2003). Primers and double-quencher probes sets were synthesized by IDT. The probe for the detection of GFP in the transformed *F. proliferatum* was 5'-/6-carboxyfluorescein (6-FAM)/ZEN/3' Iowa Black FQ, and the probe for the detection of mRFP in the transformed *F. verticillioides* was 5'-/cyanine5 (Cy5)/TAO/3' Iowa Black RQ.

Real-time qPCR amplification of treatments

The amplification reactions were carried out in 20µl mixtures containing 10µl Sso Advanced Universal Probes Supermix (Bio-Rad), 1µl (5µM) of mixture of forward and reverse primer for GFP, 1µl (5µM) of mixture of forward and reverse primer for mRFP, 1µl (5µM) of probe for GFP, 1µl (5µM) of probe for mRFP, 5µl of nuclease-free water, and 1µl of genomic DNA. Negative control (nuclease-free water) was used in each run to control for cross-contamination. In addition, for each run, one of the genomic DNA samples was run in three replicates. Cycling parameters included an initial hold for 3 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 58°C for 30 seconds. The assays were performed in a Bio-Rad CFX96 Real-Time System thermocycler, and data analysis was done using Bio-Rad CFX Manager software 2.1.

Real-time qPCR sensitivity and spiked assays

The detection limits and accuracy of the detection were tested for the two primer and probe sets. Genomic DNA from Fp-G or Fv-R was diluted 10-fold and tested from 1ng to 10fg per mixture. Initial DNA concentrations were determined using Qubit, and the quality was determined using a NanoDrop v.2000 spectrophotometer. Each reaction was performed in five replicates and the average data were used to build a standard curve with Ct in the y-axis and amount of DNA (in ng) in the x-axis, using a linear fit. In addition, spiked assays including known quantities of Fp-G and Fv-R genomic DNA were performed in triplicate in reactions mixed with root or stem extracts from maize as described in Arif *et al.*, 2013, and without root or stem extracts from maize to assess reactions efficiency.

Results

Transformants characterization

One transformed strain of *F. proliferatum* (Fp-G) and one transformed strain of *F. verticillioides* (Fv-R) having strong fluorescence and stable hygromycin-resistance were selected for further characterization. The analyses were performed as previously described (Chapter 3) using morphological and pathogenicity traits to compare the transformed strains to the parental types. In all characteristics measured, the transformed strains of both species were stable and indistinguishable from their respective parental types.

Southern blot analysis

End-point PCR using the primers developed for the real-time qPCR reactions confirmed the presence of the respective fluorescence genes, GFP in the *F. proliferatum* strain and mRFP in the *F. verticillioides* strain. For the Southern blot analysis, each transformed strain, Fp-G and Fv-R, was replicated three times to ensure accuracy of the results. The results showed that they each contained one copy of the respective fluorescence gene; the parental types (negative control) of both transformed strains did not have the fluorescence gene (Figure 4.2). This indicated that the Ct values obtained in the real-time qPCR assays could be directly compared and were not confounded by different copy numbers of the fluorescence genes.

Sensitivity and specificity assay of primers and probes and spiked assays

Primer and probe sets designed for Fp-G and Fv-R showed high specificity as the real-time qPCR successfully detected Fp-G or Fv-R without cross-reactions. For the specificity assays, both primer and probe sets detected as little as 10 fg of genomic DNA. Spiked assays which included known amounts of genomic DNA from Fp-G and Fv-R in single reactions also showed high sensitivity (10 fg) and high specificity (no cross-reaction). In addition, the spiked assays, that included known amounts of genomic DNA from Fp-G and Fv-R and extracts either from maize roots or maize stems, also showed high specificity and no inhibition or reduction in sensitivity caused by inhibitors in the plant (Figure 4.3). The presence of root or stem extracts

had no detectable effect on the real-time qPCR reactions for the detection of Fp-G and/or Fv-R. The double-quenched probes increased signal detection (assay sensitivity) and decreased background fluorescence.

The real-time qPCR data-generated standard curves for Fp-G and Fv-R were similar ($R^2 \geq 0.98$) for the non-spiked and spiked assays, which indicated that the reactions were accurate, specific and sensitive (PCR reaction efficiencies between 90-105%) (Figure 4.4).

Colonization of plants and analyses from RT-qPCR data

The data from the three experiments were analyzed together as the results indicated that there was no statistically significant interaction between experiment and treatments. To analyze the significance of the interactions among variables a Bonferroni correction of p-values was calculated; the critical p-value (α) was divided by the number of comparisons. This step was necessary to account for the multiple comparisons (statistical tests) of data that increased the probability of obtaining a significant result due to chance. The Bonferroni adjustments reduced the chance of obtaining false-positive results (type I errors) (Verhoeven *et al.,* 2005).

Real-time qPCR data for Fp-G treatments 1, 2, 3, 5 and 9 were analyzed together, and real-time qPCR data for Fv-R treatments 1, 2, 4, 6 and 8 were analyzed together.

The real-time qPCR data for *F. proliferatum* indicated that the colonization of maize plants from sources of inoculum showed significant p-value differences from the colonization of maize plants from the inoculated seeds (Table 4.3). This was evidenced by the amount of Fp-G biomass calculated in the plant tissues in the different treatments (values shown in parenthesis in the comparisons). There were no significant differences among treatments 1 (1.28 picograms), 3 (2.22 picograms) and 5 (0.94 picograms), however they were significantly different from treatments 2 (5.39 picograms) and 9 (5.45 picograms); there was no significant difference between treatments 2 and 9. These results (comparison G) indicate that subsequent colonization by *F. verticillioides* (treatment 2) did not significantly impact *F. proliferatum* colonization of that plant (treatment 9). Also, interestingly, colonization by Fp-G in treatment 3 (cured plants) was not significantly different from that in treatments 1 (Fv-R-colonized plants; comparison B)

and 5 (natural microflora; comparison H). The latter data suggest that the presence of *F. verticillioides* (treatment 1), or of naturally occurring seed-borne microorganisms (treatment 5), in the plant does not inhibit *F. proliferatum* from colonizing the maize plants from sources of inoculum in soil.

Colonization of roots by Fp-G (4.43 picograms) was greater than colonization of stems by Fp-G (1.68 picograms) by a 2.64-fold difference ($p<0.0001$) (Table 4.4). There was a significant inverse gradient in the degree of colonization (fungal biomass) of the plant tissues as a function of the distance from the seed; the most fungal biomass was found in the root and stem segments closest to the seed (Table 4.4). The amount of Fp-G biomass in the segment closest to the seed (segment 1) was 5.24 picograms, the amount of Fp-G biomass in the second segment closest to the seed (segment 2) was 3.21 picograms, and the amount of Fp-G biomass in the segment farthest away from the seed (segment 3) was 0.72 picograms; a 7.3-fold difference in Fp-G biomass across that gradient.

Analysis of the combination of plant parts (root or stem) and plant segments (1, 2 or 3) was done to study the colonization of maize plants by Fp-G. There were significant differences, supporting the conclusion that roots are more colonized than stems, and that plant segments closer to the seed are more colonized than those far away (Table 4.5).

The real-time qPCR data for *F. verticillioides* indicated that the colonization of maize plants from sources of inoculum showed significant p-value differences from the colonization of maize plants from the inoculated seeds (Table 4.6). This was evidenced by the amount of Fv-R biomass calculated in the plant tissues in the different treatments (values shown in parenthesis in the comparisons). There were no significant differences among treatments 2 (0.92 picograms), 4 (1.80 picograms) and 6 (0.39 picograms), however they were significantly different from treatments 1 (3.50 picograms) and 8 (3.39 picograms); there was no significant difference between treatments 1 and 8. These results (comparison N) indicate that subsequent colonization by *F. proliferatum* (treatment 1) did not significantly impact *F. verticillioides* colonization of that plant (treatment 8). Also, interestingly, colonization by Fv-R in treatment 4 (cured plants) was not significantly different from that in treatments 2 (Fp-G-colonized; comparison O) and 6

(natural microflora; comparison R). The latter data suggest that the presence of *F. proliferatum* (treatment 2), or of naturally occurring seed-borne microorganisms (treatment 6), in the plant do not inhibit *F. verticillioides* from colonizing the maize plants from sources of inoculum in soil.

Colonization of roots by Fv-R (2.14 picograms) was greater than colonization of stems by Fv-R (1.86 picograms) by a 1.15-fold difference ($p<0.0001$) (Table 4.7). There was a significant inverse gradient in the degree of colonization (fungal biomass) of the plant tissues as a function of the distance from the seed; the most fungal biomass was found in the root and stem segments closest to the seed (Table 4.4). The amount of Fv-R biomass in the segment closest to the seed (segment 1) was 3.61 picograms, the amount of Fv-R biomass in the second segment closest to the seed (segment 2) was 1.53 picograms, and the amount of Fp-G biomass in the segment farthest away from the seed (segment 3) was 0.86 picograms; a 4.2-fold difference in Fv-R biomass across that gradient.

Analysis of the combination of plant parts (root or stem) and plant segments (1, 2 or 3) was done to study the colonization of maize plants by Fv-R. There were significant differences, supporting the conclusion that roots are more colonized than stems, and that plant segments closer to the seed are more colonized than those far away (Table 4.8).

Discussion

This investigation demonstrated that *F. proliferatum* can grow from a source of inoculum in soil, infect a maize plant, and effectively compete against *F. verticillioides* and the natural maize microflora for a niche in the roots and stems of developing maize plants. We used a TaqMan multiplex real-time qPCR approach with two primer and probe sets which were highly sensitive and specific to detect the fluorescence genes that were targeted, GFP for *F. proliferatum* and mRFP for *F. verticillioides*. Southern blot analysis indicated a single insertion of GFP in the Fp-G genome and a single insertion of mRFP in the Fv-R genome; hence, the real-time qPCR data did not require transformation for analysis. Sensitivity and specificity of the primer and probes were important to obtain accurate and reliable results of the colonization of maize plants by Fp-G and Fv-R. No non-specific or cross-reactions were observed for the two primer and probe sets. In

addition, we found that the presence of maize tissue suspension did not inhibit or decreased the detection of Fp-G or Fv-R.

In Chapter 3 it was found that *F. proliferatum* can actively grow from a source of inoculum and colonize available non-colonized organic matter in a non-sterile soil under different temperature and soil matric potential combinations; this was true in a sieved, reconstituted field soil as well as in non-sterile soil with an intact field structure. This research complements those findings by demonstrating that *F. proliferatum* can also colonize living plants already colonized with other microorganisms, even those likely to compete for the same substrate/niche. For this project, *F. verticillioides* was selected to compete against *F. proliferatum* because of their ecological and life cycle similarity and common co-existence in maize plants (Chulze *et al*., 2000; Leslie and Summerell, 2006). These two fungi are known for systemically colonizing maize plants, causing disease in different plant tissues, and producing mycotoxins in kernels.

F. proliferatum can survive in soil for extended periods of time (Leslie *et al*., 1990; Logrieco and Bottalico, 1988; Logrieco *et al*., 1995) and at least 630 days in surface or buried maize residue (Cotten and Munkvold, 1998). Therefore, *F. proliferatum* can potentially act as source of inoculum for at least 2 growing seasons. In addition, this fungus has worldwide distribution and a wide host range that includes both monocots and dicots. These factors may increase the risk of infection for plants in a field with multiple sources of inoculum.

Population genetics research on *F. proliferatum* indicated that strains vary in aggressiveness in certain crops (Elmer, 1991; Iglesias *et al.,* 2010) and that some strains can be predominant in some fields (Elmer, 1991; Elmer *et al.,* 1999). Moreover, there is genetic and phenotypic variation among strains from different plant hosts (Stepien *et al*., 2011), but host specialization has not been reported. Therefore, the introduction of *F. proliferatum* on one plant species may be an important source for infection/colonization of other plant species. This can occur where crop rotations of different plant species, such as maize with wheat, soybean, or alfalfa are practiced. In addition, because the presence of *F. proliferatum* in plant and seed can be asymptomatic, this fungus can be inadvertently introduced into new environments, thereby extending the geographical range of these undesirable strains.

In interspecific competition between fungi, environmental factors play an important role and influence the dominance of species (Magan and Lacey, 1985). *F. proliferatum* and *F. verticillioides* have similar abiotic requirements for optimal growth (Nelson *et al.*, 1990; Marin *et al.*, 1996) and commonly occur together in plants, which makes them ideal to study competition. These previous studies were conducted in vitro. Understanding the competitive ability of *F. proliferatum* under more natural conditions is essential to assessing the risk of entry and establishment of new strains into new environments via colonized seed.

Figures and Tables

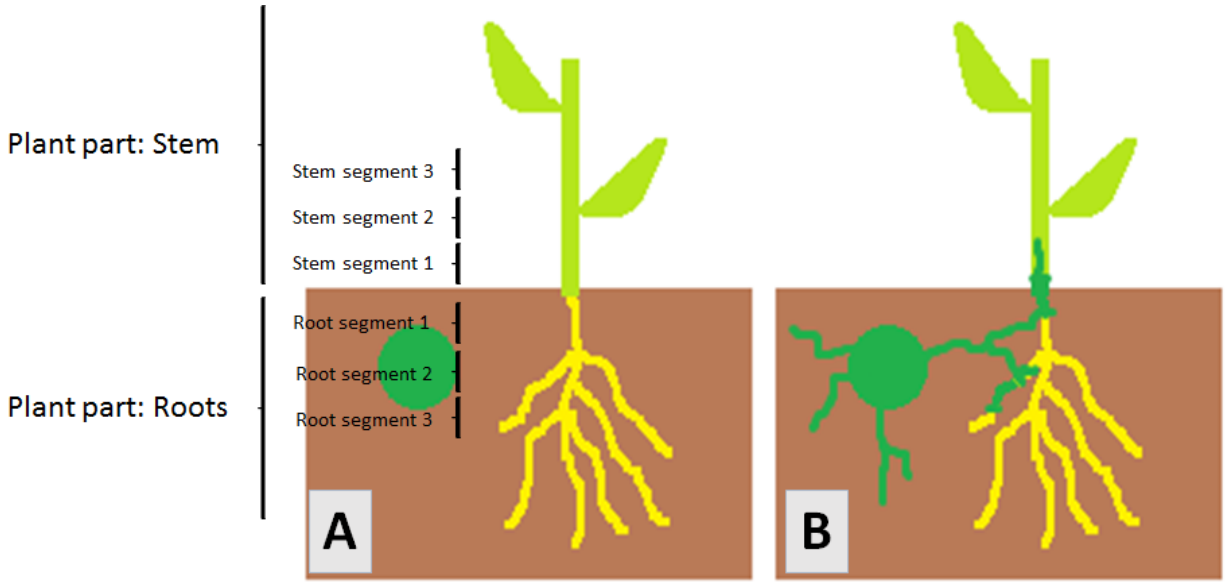


Figure 4.1 Graphical representation of Treatment 3 showing the plant parts (stem and roots) and the 3 plant segments for roots and stem. (A) Heat-treated cured seeds are planted with non-viable heat-killed seed re-colonized with Fp-G. (B) The heat-killed re-colonized seed (sources of inoculum) introduces Fp-G effectively into the soil and colonizes tissue of the bait plant.

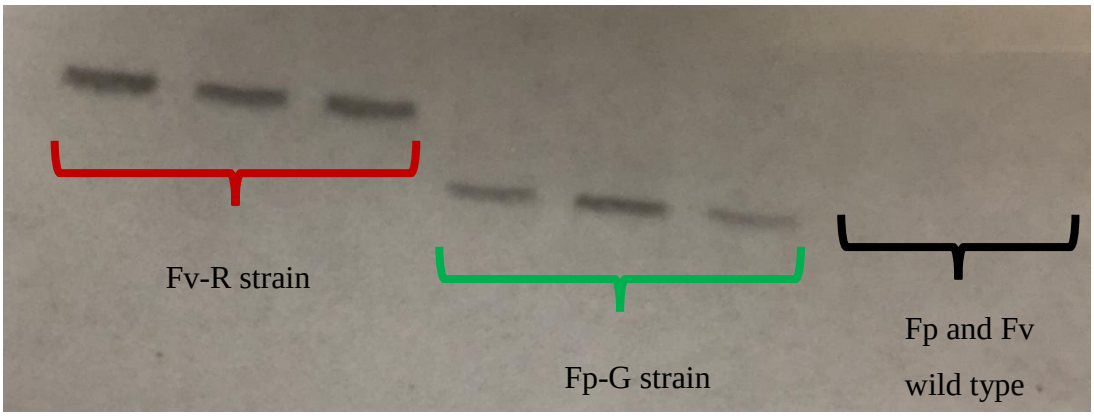
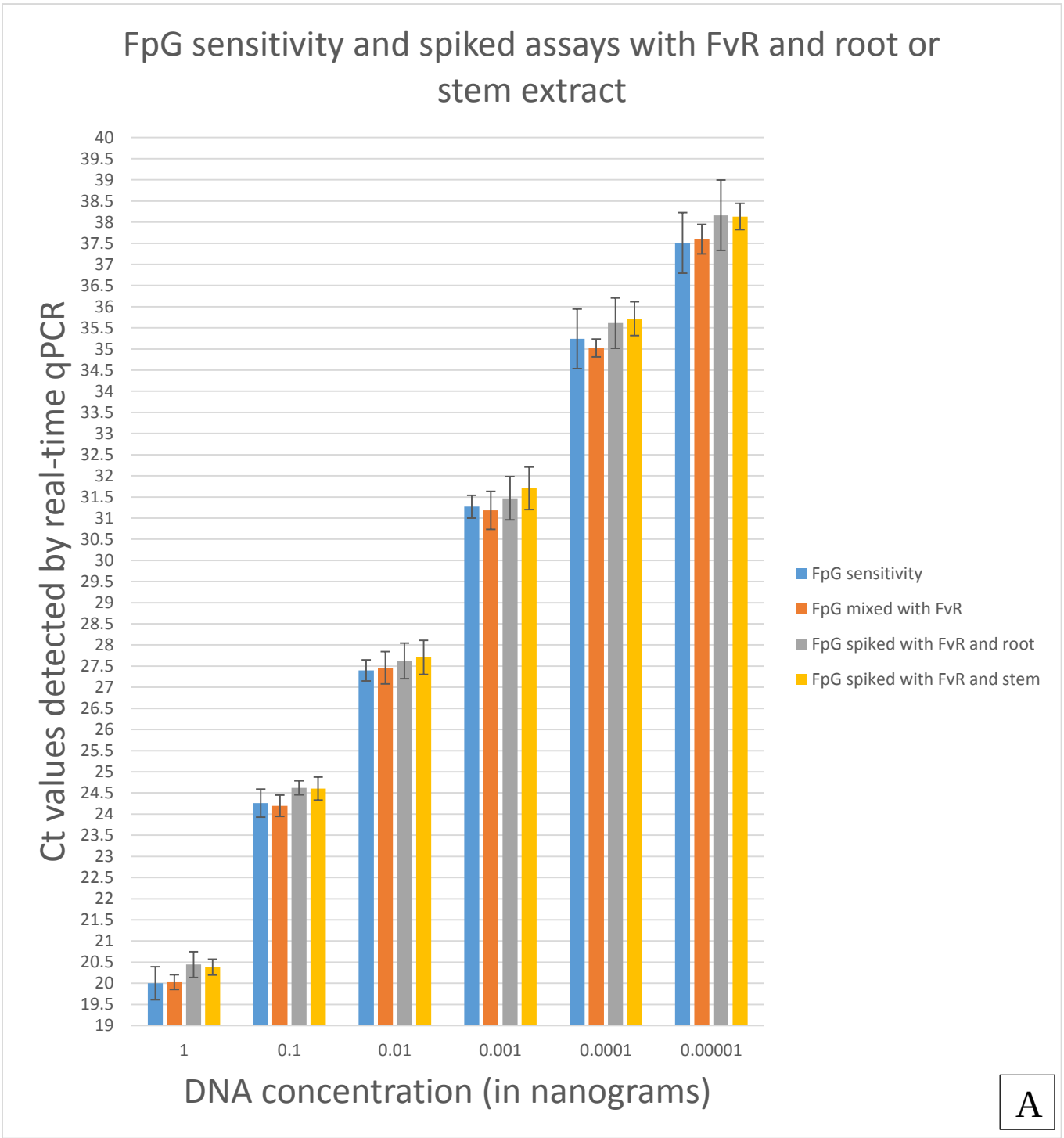


Figure 4.2 Southern blot results for Fp-G and Fv-R suggest one insertion of the respective fluorescence gene in each genome. This suggests that GFP is found one time in the *F. proliferatum* genome and mRFP is found one time in the *F. verticillioides* genome. The respective parental types of Fp-G and Fv-R did not have the respective fluorescence gene.


FpG sensitivity and spiked assays with FvR and root or stem extract
Ct values detected by real-time qPCR
40
39.5
39
38.5
38
37.5
37
36.5
36
35.5
35
34.5
34
33.5
33
32.5
32
31.5
31
30.5
30
29.5
29
28.5
28
27.5
27
26.5
26
25.5
25
24.5
24
23.5
23
22.5
22
21.5
21
20.5
20
19.5
19
1
0.1
0.01
0.001
0.0001
0.00001
DNA concentration (in nanograms)
FpG sensitivity
FpG mixed with FvR
FpG spiked with FvR and root
FpG spiked with FvR and stem
A

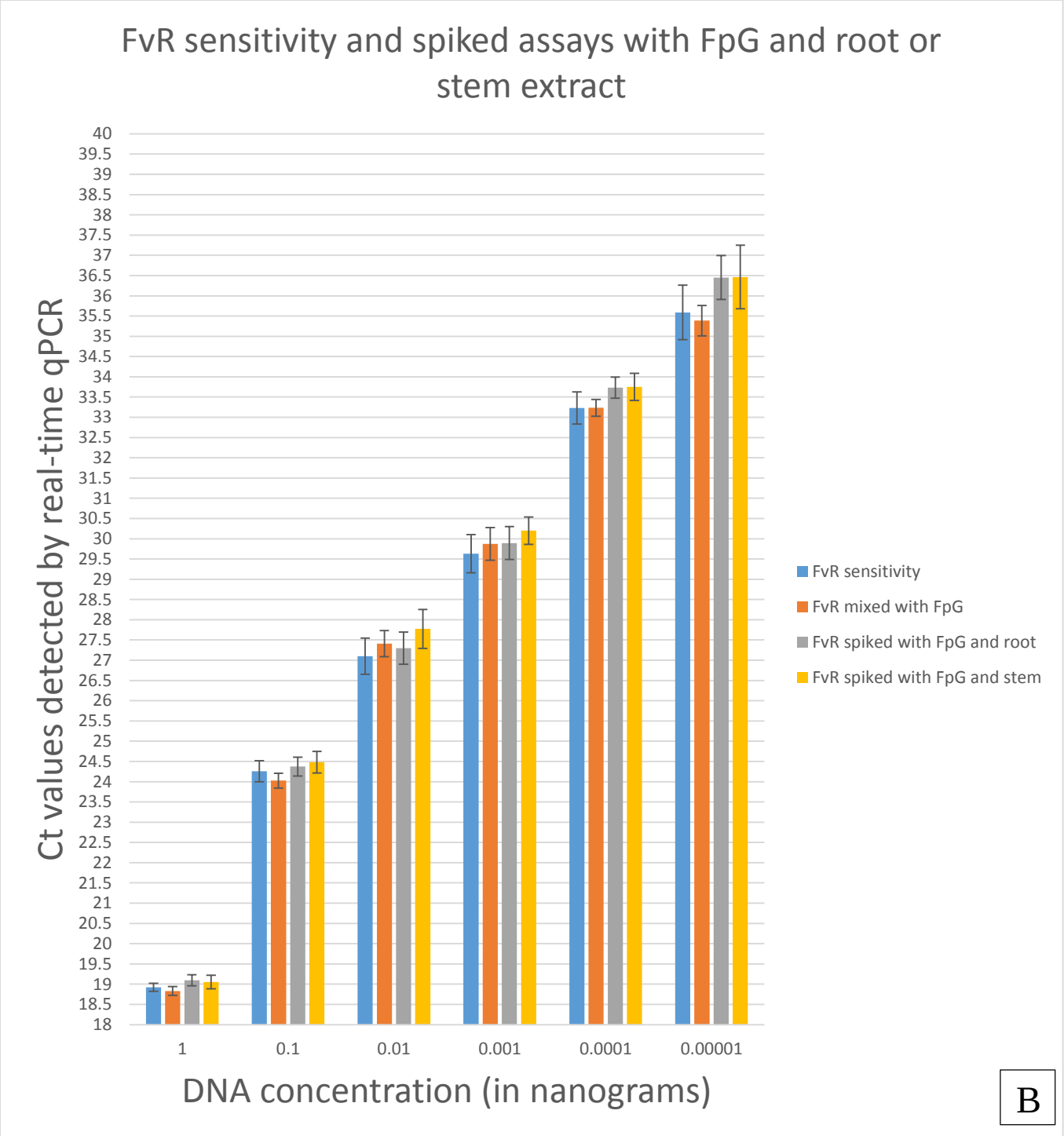
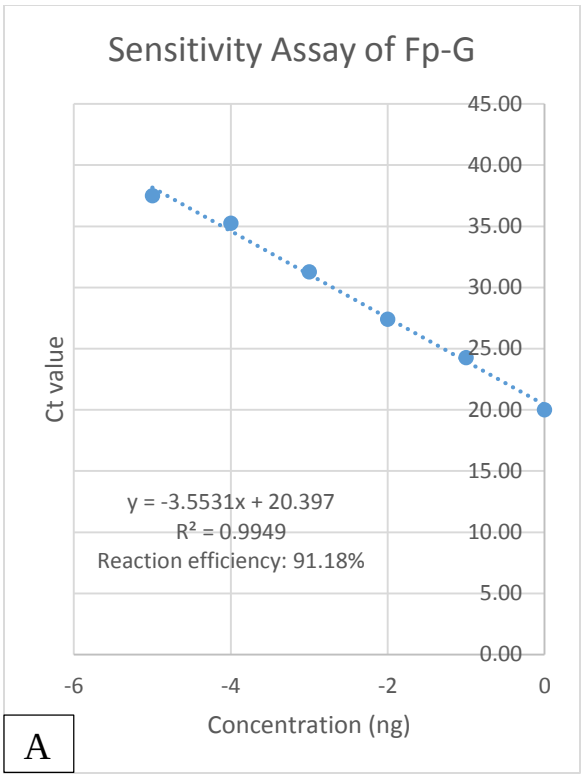
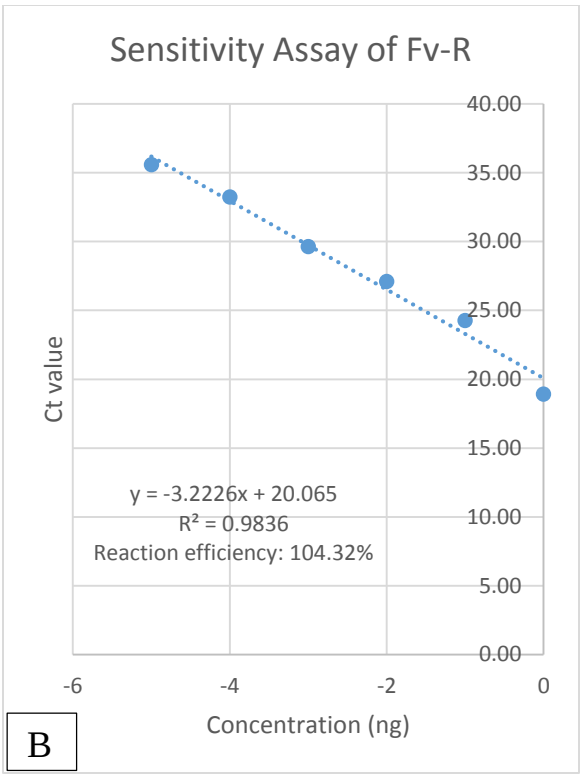


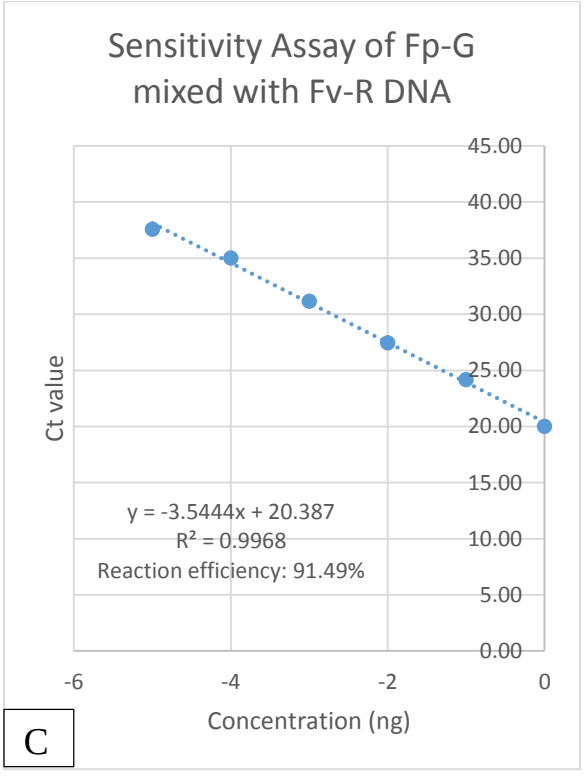
Figure 4.3 Ct values and standard deviation detected for the assays for (A) Fp-G and (B) Fv-R. Fp-G assays show high sensitivity and specificity (no cross-reaction with Fv-R) on its detection in spiked and non-spiked assays. Fv-R assays show high sensitivity and specificity (no cross-reaction with Fp-G) on its detection in spiked and non-spiked assays.


Sensitivity Assay of Fp-G
Ct value
Concentration (ng)
y = -3.5531x + 20.397
R² = 0.9949
Reaction efficiency: 91.18%
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-6
-4
-2
0

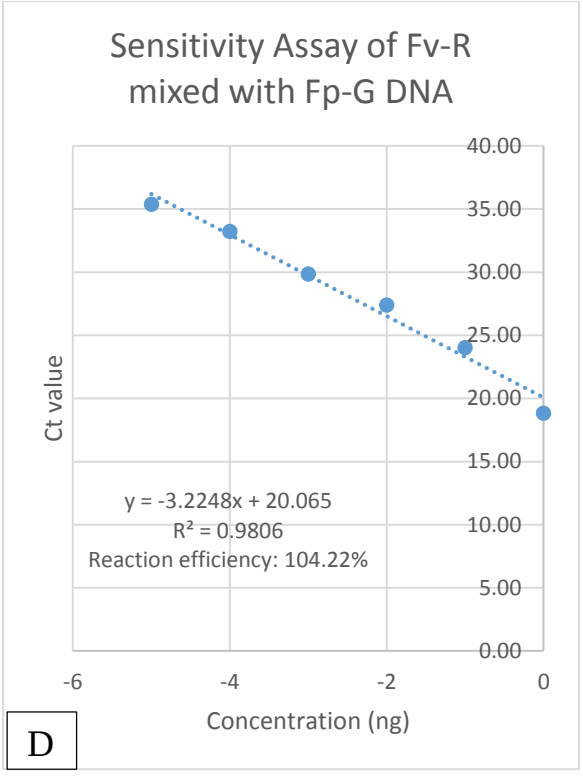
A


Sensitivity Assay of Fv-R
Ct value
Concentration (ng)
y = -3.2226x + 20.065
R² = 0.9836
Reaction efficiency: 104.32%
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-6
-4
-2
0

B


Sensitivity Assay of Fp-G mixed with Fv-R DNA
Ct value
Concentration (ng)
y = -3.5444x + 20.387
R² = 0.9968
Reaction efficiency: 91.49%
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-6
-4
-2
0

C


Sensitivity Assay of Fv-R mixed with Fp-G DNA
Ct value
Concentration (ng)
y = -3.2248x + 20.065
R² = 0.9806
Reaction efficiency: 104.22%
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-6
-4
-2
0

D

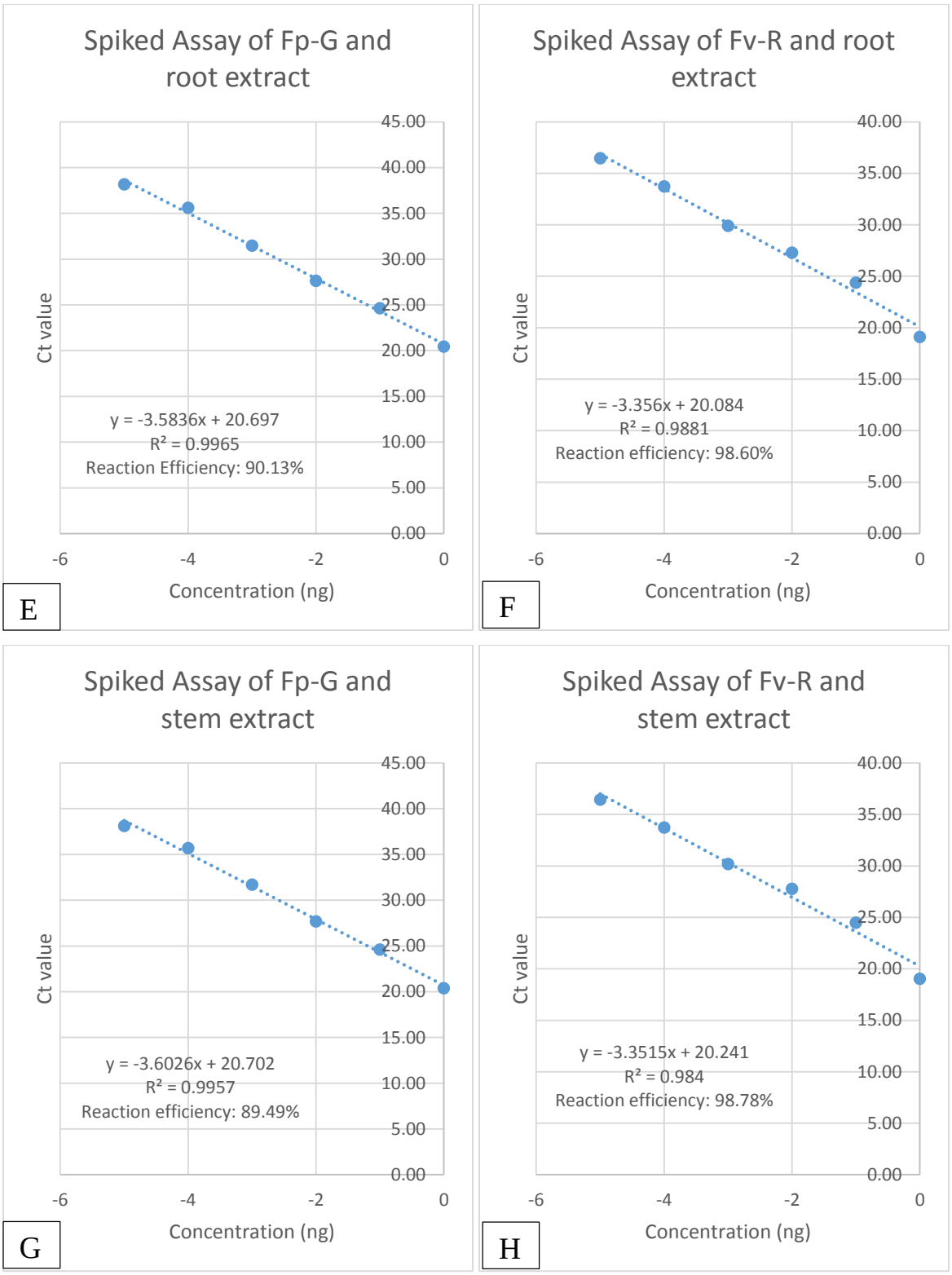


Figure 4.4 Standard curves were obtained for all real-time qPCR reactions. On the x-axis DNA concentrations were logarithmically transformed to obtain a linear graph. Reaction efficiency was calculated as described in the Bio-rad real-time PCR applications guide. (A) Fp-G and (B) Fv-R sensitivity assays, (C) Fp-G and (D) Fv-R sensitivity and specificity

assays, (E) Fp-G and (F) Fv-R sensitivity and specificity inhibition-assays spiked with maize root extract, and (G) Fp-G and (H) Fv-R sensitivity and specificity inhibition-assays spiked with maize stem extract.

Table 4.1 Real-time qPCR primer and probe sequences for Fp-G and Fv-R, and Southern blot probe sequences for Fp-G and Fv-R. The Fp-G primers produced a 134bp amplicon, and the Fv-R primers produced a 132bp amplicon.

Real-time qPCR primers and probes and Southern blot probes	Sequences
Fp-G real-time qPCR forward primer	5'-GAACGGCATCAAGGTGAACT-3'
Fp-G real-time qPCR reverse primer	5'-AGCTCAGGTAGTGGTTGTCG-3'
Fv-R real-time qPCR forward primer	5'-ATGAGGCTGAAGCTGAAGGA-3'
Fv-R real-time qPCR reverse primer	5'-CTCGTTGTGGGAGGTGATG-3'
Fp-G real-time qPCR probe	5'-/6-carboxyfluorescein (6-FAM)-AACATCGAG/ZEN/GACGGCAGCGTG-3'-Iowa Black FQ
Fv-R real-time qPCR probe	5'-/cyanine5 (Cy5)-ACGACGCCG/TAO/AGGTCAAGACCAC-3'-Iowa Black RQ
Fp-G Southern blot probe (double stranded)	5'-AAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCAT-3'
Fv-R Southern blot probe (double stranded)	5'-TCCTCCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCCAGTACGGCTCCAAGGCCTACGTGAAGCACCCCG

	CCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACC GTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAG GTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATG CAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGGATGTA CCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGATGAGGCTGA AGCTGAAGGACGGCGGCCACTACGACGCCGAGGTCAAGACCACC TACATGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAAGAC CGACATCAAGCTGGACATCACCTCCCACAACGAGGACTACACCA TCGTGGAACAGTACGAGCGCGCCGA-3'

Table 4.2 Competition between *Fusarium proliferatum* (Fp-G) and *F. verticillioides* (Fv-R) for colonization of maize seedlings. Maize seed were heat-cured in treatments 1, 2, 3, 4, 8, and 9.

Treatments	Source of inocula*	Target maize seedlings (colonized with)**
1	Fp-G	Fv-R
2	Fv-R	Fp-G
3	Fp-G	Non-colonized
4	Fv-R	Non-colonized
5	Fp-G	Natural seed microflora
6	Fv-R	Natural seed microflora
7	Non-colonized	Non-colonized
8	Non-colonized	Fv-R
9	Non-colonized	Fp-G

*For source of inoculum, maize seeds were heat-killed and then subsequently colonized by Fp-G or Fv-R; non-colonized heat-killed maize seeds were used in some control treatments (7, 8, 9).

**For target seedlings, maize seeds were heat-cured and colonized by Fp-G or Fv-R (treatments 1, 2, 8, 9), heat-cured and not colonized by Fp-G or Fv-R (treatments 3, 4, 7), or not heat-cured (treatments 5, 6).

Table 4.3 Competitive colonization of maize plants by *Fusarium proliferatum* strain Fp-G in treatments 1, 2, 3, 5 and 9. Colonization of maize plants from sources of inoculum (treatments 1, 3 and 5) showed significant differences from the colonization of maize plants from the inoculated seeds (treatments 2 and 9). Colonization in treatment 3 (cured plants) was not significantly different from that in treatments 1 (comparison B) and 5 (comparison H). Comparisons were done using amount of Fp-G biomass which was calculated from the Ct values detected by real-time qPCR.

Comparison	TRT	Source	Bait	TRT	Source	Bait	Adj p-value*
A	1	Fp-G	Fv-R	2	Fv-R	Fp-G	0.0009
B	1	Fp-G	Fv-R	3	Fp-G	Non-colonized	0.6749
C	1	Fp-G	Fv-R	5	Fp-G	Natural seed microflora	1.0000
D	1	Fp-G	Fv-R	9	Non-colonized	Fp-G	0.0013
E	2	Fv-R	Fp-G	3	Fp-G	Non-colonized	0.0089
F	2	Fv-R	Fp-G	5	Fp-G	Natural seed microflora	0.0004
G	2	Fv-R	Fp-G	9	Non-colonized	Fp-G	1.0000
H	3	Fp-G	Non-colonized	5	Fp-G	Natural seed microflora	0.1910
I	3	Fp-G	Non-colonized	9	Non-colonized	Fp-G	0.0148
J	5	Fp-G	Natural seed microflora	9	Non-colonized	Fp-G	0.0006

*p-values were adjusted with a Bonferroni correction

Table 4.4 Colonization of roots by Fp-G was higher than that of stems. Colonization of plant segments by Fp-G decreased as the distance from the seed increased.

Effect	PART		PART		Adj p-value*
PART	Root		Stem		<.0001
Effect		**SEGMENT**		**SEGMENT**	**Adj p-value***
SEGMENT		1		2	<.0001
SEGMENT		1		3	<.0001
SEGMENT		2		3	<.0001

*p-values were adjusted with a Bonferroni correction

Table 4.5 Colonization of plant parts (roots and stems) and segments (1, 2 and 3) by Fp-G. Roots were more colonized than stems, and segments closer to the seed were more colonized than those farther away.

PART	SEGMENT	PART	SEGMENT	Adj p-value*
Root	1	Root	2	0.0339
Root	1	Root	3	<.0001
Root	1	Stem	1	0.0011
Root	1	Stem	2	<.0001
Root	1	Stem	3	<.0001
Root	2	Root	3	<.0001
Root	2	Stem	1	0.8744
Root	2	Stem	2	<.0001
Root	2	Stem	3	<.0001
Root	3	Stem	1	0.0111
Root	3	Stem	2	0.6293
Root	3	Stem	3	<.0001
Stem	1	Stem	2	<.0001
Stem	1	Stem	3	<.0001
Stem	2	Stem	3	0.0004

*p-values were adjusted with a Bonferroni correction

Table 4.6 Competitive colonization of maize plants by *Fusarium verticillioides* strain Fv-R in treatments 1, 2, 4, 6 and 8. Colonization of maize plants from sources of inoculum (treatments 2, 4 and 6) showed significant differences from the colonization of maize plants from the inoculated seeds (treatments 1 and 8). Colonization in treatment 4 (cured plants) was not significantly different from that in treatments 2 (comparison O) and 6 (comparison R). Comparisons were done using amount of Fv-R biomass which was calculated from the Ct values detected by real-time qPCR.

Comparison	TRT	Source	Bait	TRT	Source	Bait	Adj p-value*
K	1	Fp-G	Fv-R	2	Fv-R	Fp-G	0.0009
L	1	Fp-G	Fv-R	4	Fv-R	Non-colonized	0.0092
M	1	Fp-G	Fv-R	6	Fv-R	Natural seed microflora	0.0004
N	1	Fp-G	Fv-R	8	Non-colonized	Fv-R	1.0000
O	2	Fv-R	Fp-G	4	Fv-R	Non-colonized	0.7032
P	2	Fv-R	Fp-G	6	Fv-R	Natural seed microflora	1.0000
Q	2	Fv-R	Fp-G	8	Non-colonized	Fv-R	0.0013
R	4	Fv-R	Non-colonized	6	Fv-R	Natural seed microflora	0.1447
S	4	Fv-R	Non-colonized	8	Non-colonized	Fv-R	0.0142
T	6	Fv-R	Natural seed microflora	8	Non-colonized	Fv-R	0.0005

*p-values were adjusted with a Bonferroni correction

Table 4.7 Colonization of roots by Fv-R was higher than that of stems. Colonization of plant segments by Fv-R decreased as the distance from the seed increased.

Effect	PART		PART		Adj p-value*
PART	Root		Stem		<.0001
Effect		**SEGMENT**		**SEGMENT**	**Adj p-value***
SEGMENT		1		2	<.0001
SEGMENT		1		3	<.0001
SEGMENT		2		3	<.0001

*p-values were adjusted with a Bonferroni correction

Table 4.8 Colonization of plant parts (roots and stems) and segments (1, 2 and 3) by Fv-R. Roots were more colonized than stems, and segments closer to the seed were more colonized than those farther away.

PART	SEGMENT	PART	SEGMENT	Adj p-value*
Root	1	Root	2	0.3514
Root	1	Root	3	<.0001
Root	1	Stem	1	0.1143
Root	1	Stem	2	<.0001
Root	1	Stem	3	<.0001
Root	2	Root	3	<.0001
Root	2	Stem	1	1.0000
Root	2	Stem	2	<.0001
Root	2	Stem	3	<.0001
Root	3	Stem	1	0.0014
Root	3	Stem	2	0.1332
Root	3	Stem	3	<.0001
Stem	1	Stem	2	<.0001
Stem	1	Stem	3	<.0001
Stem	2	Stem	3	<.0001

*p-values were adjusted with a Bonferroni correction

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Chapter 5 - **Introduction and dissemination of *Fusarium proliferatum* in maize seed**

Abstract

Fusarium proliferatum has a broad host range with worldwide distribution and is a prolific producer of mycotoxins that are toxic to humans and animals. Strains of *F. proliferatum* vary substantially in toxigenicity. The global movement of maize seed harboring *F. proliferatum* may promote geographic range expansion of undesirable populations/strains of these pathogens (e.g., high mycotoxin producers).Tests for introduction and seed dissemination of maize seed-borne *F. proliferatum* were assessed in field experiments. Genotyping-by-sequencing (GBS) was used to identify unique strain-specific genetic markers in isolates of seedborne *F. proliferatum* collected from two Dupont Pioneer maize hybrids, 33D49 and P1395R. The strain-specific markers allowed accurate detection of specific isolates of *F. proliferatum* by end-point PCR. Extensive exclusivity panels were performed to validate the accuracy of the strain-specific primer sets; two *F. proliferatum* isolates from the two maize hybrids were uniquely identified. Using a randomized complete block design, two plots each containing six subplots (three of each hybrid) were planted. At maturity, six ears were collected arbitrarily from each subplot and isolations made from 50 kernels from each ear. A total of 1855 isolates from 3600 kernels from 72 ears were collected (914 from hybrid 33D49, and 941 from hybrid P1395R); 817 isolates were identified as *F. proliferatum*, 751 isolates as *F. verticillioides*, and 23 isolates were idenfitied as having the unique genetic marker. Isolates that tested positive for the unique genetic marker were subjected to DNA fingerprinting using amplified fragment length polymorphism to determine genetic relatedness to the original *F. proliferatum* isolates that were characterized with GBS. Phylogentic analyses indicated that 19 of the field isolates with the unique genetic marker were genetically indistinguishable from the originally GBS characterized isolates, suggesting a single origin. Strains of *F. proliferatum* introduced via seed into a new environment may be disseminated within that environment to the next generation of seed produced thus facilitating dissemination from that environment.

Introduction

Fusarium proliferatum (Matsushima) Nirenberg ex Gerlach & Nirenberg is a fungal plant pathogen with worldwide distribution. It has been recovered from numerous environments (Leslie and Summerell, 2006) and has an extraordinarily broad host range, causing disease in economically important plants (Stępień *et al*., 2011).

Fusarium proliferatum is often found in maize seeds, constituting an important source of inoculum in the field (Cotten and Munkvold, 1998). Associated with symptomatic and asymptomatic plants, it is considered to be a primary causal agent of disease (Munkvold, 2003) and a seed-borne organism in maize (Christensen and Kaufmann, 1965; McGee, 1988). As a seed-borne organism, this fungus can colonize emerging seedlings, the maturing plant, and the newly developed ear (Al-Juboory and Juber, 2013). Although seed can contain a diversity of isolates; a few isolates are usually dominant (Elmer, 1995).

Fusarium proliferatum has been identified as a colonizer of maize plants worldwide and is considered to be an increasingly important component of maize ear rot in Europe (Logrieco *et al*., 2002). Consistent isolation of *F. proliferatum* from symptomatic and asymptomatic plant tissues suggests that the fungus can systemically colonize maize plants. *F. proliferatum* can persist in maize stalk debris either on the surface of soil or buried in soil for at least 21 months (Cotten and Munkvold, 1998).

In addition, *F. proliferatum* is considered to be the most effective producer of the polyketide-derived fumonisin mycotoxins: fumonisin B_1 (FB_1) is the most prevalent (Rheeder et al., 2002). Fumonisin B_1 is toxic to both humans and animals due to inhibition of sphingolipid metabolism and cell cycle regulation. It has been associated with esophageal cancer, liver cancer, and neural tube defects (Desjardins, 2006).

Considering the global distribution and trade of maize seed, the wide host range of *F. proliferatum,* and the potential production of mycotoxins by *F. proliferatum,* there is risk for the introduction, dispersal, and seed dissemination of exotic isolates of this fungus into new environments. For this reason, it is important to have a better understanding of the life cycle of

seed-borne *F. proliferatum*. In this investigation, we used genotyping-by-sequencing (GBS) (Elshire *et al.*, 2011) and molecular detection tools to study the introduction and dissemination of *F. proliferatum* in field experiments.

Materials and Methods

Fusarium isolates collected from maize seeds

Fusarium species were isolated from two different maize hybrids (33D49 and P1395R) provided by Dupont Pioneer Hybrid Seeds®. Seed were surface sterilized with 10% sodium hypochlorite solution for 1 minute, rinsed in distilled water for 30 seconds, and plated onto Nash-Snyder (NS) medium. Isolates were single-spored and grown on NS medium at 27°C for 7 days. Isolates were identified as *F. proliferatum*, *F. verticillioides*, *F. andayazi*, *F. fujikuroi* or *F. thapsinum* using morphological and molecular characteristics, including the amplification and sequencing of the translation elongation factor 1-*α* (*TEF-1α)* and β-tubulin genes. Ninety-six isolates of *F. proliferatum* and *F. verticillioides* were arbitrarily selected and characterized using GBS.

Genotyping-by-sequencing

Restriction-Digestion and Ligation

Genomic DNA (100ng) was restriction-digested in 20µl of 10X CutSmart Buffer with 8U of PstI-HF and 8U of MspI (New England BioLabs). The digestion was conducted at 37°C for 2 hours and then the samples were incubated at 65°C for 20 minutes to inactivate the enzymes.

Ligation was completed in the same tube as the digestion in a 36µl reaction containing T4 DNA ligase (200U) in 10X NEB Buffer 4 with additional ATP (final concentration of 1mM). For each reaction, 2µl of a mix containing 0.1pmol of Adapter 1 and 15pmol of Adapter 2 (common Y adapter) was added as described in Poland *et al* (2012). The ligation was completed at 22°C for 2 hours and then the samples were incubated at 65°C for 20 minutes to inactivate the enzyme.

Multiplexing and Amplification

The 96-samples were pooled together (5µl per sample) and cleaned using the QIAquick PCR Purification Kit (Qiagen). The amplification was completed in a 25µl reaction containing 10µl of the cleaned pooled sample, 5X Taq NEB Master Mix (New England Biolabs), and 1µl of 10µM of Illumina primers (forward and reverse) as described by Poland *et al* (2012). The library was amplified for 24 cycles: 95°C for 30 seconds, 62°C for 20 seconds and 68°C for 90 seconds. The sample was cleaned using the QIAquick PCR Purification Kit (Qiagen). The library was checked using BioRad Experion automated electrophoresis system, and quantified using Qubit 3.0 (Invitrogen). The library was sequenced on a single lane of Illumina MiSeq using 300 cycles, single-end reads. The unfiltered and raw data had ".fastq" format.

Filtering of MiSeq (GBS) data

Geneious® v. 7.1.7 was used to sort the raw reads (sequences) using the barcode adapters to group the sequence reads by isolate.

Galaxy (Goecks *et al.*, 2010) was used to design a pipeline to analyze the raw sequence data. Quality trimmer was used to trim 3' ends of reads based on a PHRED quality score of at least 20 (99% base call accuracy). Reads with unknown bases (N) and reads shorter than 15 nucleotides were discarded. The reads were then filtered by quality with a minimum PHRED score of 25 in at least 50% of the bases of all reads. Finally, Bowtie 2 (Langmead and Salzberg, 2012) was used to align the reads with a draft genome of *F. proliferatum* (Yue and Toomajian, unpublished).

Design of specific primers using Stacks

Stacks (Catchen *et al.*, 2013) was used to analyze all reads from all samples and to identify samples with unique polymorphic loci and single nucleotide polymorphisms (SNPs) (Figure 5.1). Stacks grouped and aligned similar DNA sequence reads from all isolates and identified polymorphisms that were used as molecular markers for these isolates. It also provided information about the coverage depth (14x in Figure 5.2) and the number of isolates represented in the comparison. These polymorphic reads were aligned to a draft complete genome sequence of *F. proliferatum* (Yue and Toomajian, unpublished) and used to design strain-specific primers. The specificity of the primers was confirmed by obtaining unique amplicons in end-point PCR

for those isolates. Extensive exclusivity panels including isolates of *F. proliferatum*, *F. verticillioides* and *F. fujikuroi* were tested to increase confidence on the specificity of the primers.

Additional *Fusarium* isolates were obtained from the same seed lots (33D49 and P1395R) used to collect isolates for GBS and used to test the specificity of the primers. Amplification of the partial TEF-1α gene was used to confirm that the isolates belonged to the genus *Fusarium* (O'Donnell *et al.,* 1998) (Table 5.1).

Field experiment and data collection

Seeds from two maize hybrids (33D49, P1395R) were planted (May 1st & June 9th) in a randomized complete block design (2 plots, 6 subplots, 8 rows per subplot) at the K-State research farm in Manhattan, KS. Each row was 15 feet long, seeds were planted 6 inches apart, and rows spacing was 30 inches. At maturity (October 6th), 20 ears were harvested per subplot; 15 ears from the center 4 rows in the middle of each subplot and 5 ears from the rows adjacent to subplots with the other hybrid.

Four weeks prior to harvest, a pre-sampling was done to collect *Fusarium* isolates from the two hybrids (33D49 and P1395R) to further test the specificity of the primers. Amplification of the partial TEF-1α gene was used to confirm that the isolates belonged to the genus *Fusarium* (O'Donnell *et al.,* 1998) (Table 5.1).

From the harvested ears, 6 were arbitrarily selected per subplot and 50 kernels were arbitrarily collected per ear for isolation of *Fusarium*. Kernels were surface sterilized (10% sodium hypochlorite solution for 1 minute), rinsed in distilled water (30 seconds) and plated onto NS medium (27°C). After 7 days, the following data were recorded: the percentage germination of seeds, the percentage *Fusarium* infection of seeds, the number of *F. proliferatum* isolates and the number of *F. verticillioides* isolates. The identity of *F. proliferatum* and *F. verticillioides* isolates was confirmed by endpoint PCR using specific primers published in the literature (Jurado *et al*., 2006; Patino *et al*., 2004) (Table 5.2). Mycelia samples of all isolates were stored in 30%

glycerol at -80°C. Genomic DNA was collected for all isolates using the GeneJet Genomic DNA Purification kit (ThermoFischer) and stored at -20°C.

Amplified Fragment Length Polymorphism Analysis

Amplified Fragment Length Polymorphism (AFLP) fingerprinting was used to determine the genetic relatedness of the field-collected isolates that tested positive for the presence of the strain-specific molecular marker (identified with GBS). Two replicates (Fp1-49-16-4 and Fp2-49-16-4) of the GBS-characterized *F. proliferatum* isolate Fp-49-16-4 were included as positive controls. Additional *F. proliferatum* and *F. verticillioides* isolates lacking the strain-specific molecular marker were used for comparison to the isolates having the strain-specific molecular marker.

Restriction-Digestion and Ligation

The digestion and ligation reactions were done in a single step. Genomic DNA (120ng) was digested and ligated in 20µl reactions of 10X OPA restriction digest buffer (50mM K acetate, 10mM Mg acetate and 10mM Tris-acetate with a final pH of 7.4), 4U of EcoRI-HF, 2U of MseI, 80U of T4 DNA ligase (New England Biolabs), T4 DNA ligase buffer, 0.4µl of 5µM EcoRI adaptor mix, 0.4µl of 50µM MseI adaptor mix and water. The EcoRI and MseI adaptor mix was designed and prepared as in Leslie and Summerell (2006). The digestion-ligation mixture was incubated at room temperature overnight to ensure complete digestion and ligation and then diluted 10X in water.

Pre-selective Amplification

Pre-amplification reactions were done using primers that were complementary to the DNA restriction sites and adaptor pair (EcoRI=5'-CTCGTAGACTGCGTACCAATTC-3'and MseI=5'-GACGATGAGTCCTGAGTAA-5'). Individual pre-amplification PCRs were prepared in a final volume of 25µL per reaction and included: 5µl of diluted digestion-ligation template, 0.205µl dNTPs (25mM each nucleotide), 0.65µl of EcoRI core primer (50ng/µl), 0.65µl of MseI core primer (50ng/µl), 5.1µl 5X GoTaq Buffer, 0.2µl (1U) GoTaq polymerase (Promega) and water. The pre-amplification PCR steps were 94°C for 5 minutes, 25 cycles of 94°C for 30

seconds, 56°C for 1 minute, and 72°C for 2 minutes, a final extension step at 72°C for 5 minutes, and a hold at 4°C. The pre-amplified templates were diluted 20X with water.

Selective Amplification

For the selective amplification, 8 primer combinations were tested that included two labeled 5'-HEX EcoRI (E) primers, E-AA and E-TG, and four non-labeled MseI (M) primers, M-TT, M-CA, M-CC, and M-CT. Primer combinations were tested with five *F. proliferatum* isolates and one *F. verticillioides* isolate to identify the primer pair with the best discriminatory power. Primers 5'-HEX-E-TG and M-CT were chosen for further fingerprinting analysis of all the isolates because it gave a better discriminatory resolution of the isolate of interest (isolates with strain-specific marker). Individual selective amplification PCRs in a final volume of 20µL per reaction included 3µL diluted pre-selection amplification DNA template, 1.6µl 25mM $MgCl_2$, 0.4µL 10mM dNTPs, 5µM EcoRI selective primer, 5µM MseI selective primer, 2µL 10X PCR Buffer (Takara Clontech), 0.3µL GoTaq polymerase (5U/µL) (Promega) and water. The touchdown PCR steps were 94°C for 2 minutes, 10 cycles of 94°C for 20 seconds, 66°C for 30 seconds (-1°C per cycle) and 72°C for 2 minutes, followed by 24 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 3 minutes, a final extension at 60°C for 30 minutes, and a hold at 4°C. The selective amplification template was diluted 10X in water.

Preparation of DNA samples for analysis

A well-mixed solution of 9.5µl formamide and 0.5µl GeneScan-500 ROX internal size standard (Applied Biosystems) was equally distributed throughout the PCR plate and 2µl of the diluted selective amplification template added to each well. Samples were then centrifuged at 2100 RPM for 1 minute, denatured at 95°C for 5 minutes, cooled in ice for 5 minutes, and centrifuged at 2100 RPM for 1 minute. Analysis of the samples was performed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

AFLP Marker Scoring and Error Rate Estimation

AFLP profiles were scored using GeneMarker software v. 1.97 (SoftGenetics LLC, State College, PA). Size Standard (size calling) peaks were manually calibrated for every sample to

obtain consistent results. A 500 relative fluorescent unit (RFU) minimum peak height was used for peak scoring as this was reliably above the noise of negative controls (water) and 20,000 RFU was used as the maximum peak height. In addition, only bands between 60bp and 600bp were scored.

To verify the consistency of the AFLP technique, DNA from the same isolate was included in every AFLP reaction and genotyping run. As reported by Bonin *et al* (2004), the AFLP technical error rate estimation was calculated by dividing the total number of mismatched bands by the total number of AFLP bands produced overall in the fingerprint.

AFLP Data Analysis

Analyses of the AFLP binary data were done using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.2 to build an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree to cluster the isolates (1000 bootstrap runs, data not shown on NTSYS). In addition, the full AFLP marker data set was analyzed using STRUCTURE version 2.3.4 with 10,000 burn-in and 50,000 Markov chain Monte Carlo (MCMC) steps (Pritchard *et al.*, 2000; Falush *et al.*, 2007) and 20 iterations per k to analyze the structure of the population. Admixture was included in the model and correlated allele frequencies assumed. STRUCTURE HARVESTER (Earl, 2012) was used for the calculation of delta K (Evanno *et al*., 2005) to determine the number of clusters/structures (k). GenAlEx was used to perform Analysis of Molecular Variance (AMOVA) and Principal Coordinates Analysis (PCoA) of the populations (Peakall and Smouse, 2006); the calculations and algorithms were performed using previously published methods (Peakall and Smouse, 2007).

Results

Isolates collected from maize seeds

Fusarium species were isolated and single-spored from two Pioneer® maize hybrids. The hybrids were from the same geographical location and showed different percentages of seed-borne *Fusarium* species, 30% in hybrid 33D49 and 63% in hybrid P1395R, but similar percentages of seed-borne *F. proliferatum* isolates, 15% (Stack, personal communication).

Use of Stacks to analyze GBS data and preliminary data

The GBS run had a raw output of 21,792,630 reads. After quality control and filtering, approximately 50% of the reads were discarded. Stacks identified seven *F. proliferatum* isolates having unique molecular markers (Table 5.3). Stacks also provided information about the coverage depth and the number of samples represented (sequenced at that locus). Primers were designed for these unique molecular markers for use in end-point PCR amplification. Extensive exclusivity panels were tested using the primers in end-point PCR; two of the isolates (Fp-49-16-4 and Fp-95-8-4) had unique molecular markers (Figure 5.3). The specificity of the primers was confirmed by end point PCR (Table 5.4) and was also confirmed *in-silico* by screening the primer sequences with BLASTn against the NCBI GenBank database (Altschul *et al.*, 1990). The other molecular markers failed the exclusivity panels; and hence, they were not further used in this investigation. The exclusivity panels included isolates of *F. proliferatum*, *F. verticillioides* and *F. fujikuroi*.

The unique amplicons for the two *F. proliferatum* strains were screened with BLASTn against the NCBI GenBank database; both (for isolates Fp-49-16-4 and Fp-95-8-4) had similarity to sequences of a draft genome of *Fusarium fujikuroi* (Table 5.5). This can be explained by the high similarity of *F. proliferatum* and *F. fujikuroi* which are often hard to distinguish morphologically and molecularly (Leslie and Summerell, 2006); hence this could be a genomic region shared by both species. However, the polymorphisms in that region are still useful to identify specific strains of *F. proliferatum*.

Based on the results obtained in Stacks and the exclusivity panels, isolate Fp-49-16-4 was only found in maize hybrid 33D49, and isolate Fp-95-8-4 was only found in maize hybrid P1395R. These two maize hybrids were used in field experiments.

Additional *Fusarium* isolates were obtained, 63 from hybrid 33D49 and 61 from hybrid P1395R, and tested using the strain-specific primers to amplify the unique locus (Figure 5.4). This increased the power of the exclusivity panels and the confidence in the strain-specific primers. Using the primers designed for the Fp-49-16-4 isolate, 3 out of the 63 isolates from hybrid

33D49 (4.8%) and 0 out of the 61 isolates from hybrid P1395R (0%) had the Fp-49-16-4-unique locus. The primers designed for isolate Fp-95-8-4 amplified sequence from isolates collected from hybrid 33D49 (Figure 5.5). Therefore, these primers were not used in the field experiments.

Field Experiments

For the collection of *F. prolfieratum* isolates, 20 ears were harvested from each subplot in the field (240 ears total). Six ears were arbitrarily selected from each subplot and the kernels collected for further studies. From each ear, 50 arbitrary kernels were plated on NS medium (3,600 seeds) and infection and germination rates were scored (Table 5.6). Similar infection and germination rates were found for both hybrids in every plot and subplot (Table 5.7). A total of 1855 isolates of seed-borne *Fusarium* were cultured; 817 isolates were identified as *F. proliferatum*, 751 were identified as *F. verticillioides*, and 287 were not determined. Of the 817 *F. proliferatum* isolates, 23 had the unique strain-specific molecular marker (Table 5.8). The number of *F. proliferatum* and *F. verticillioides* isolates collected from both hybrids in every plot and subplot were recorded (Table 5.9).

AFLP fingerprinting of isolates

Amplified fragment length polymorphism (AFLP) analysis was used to determine the genetic relatedness among isolates collected from the field with the strain-specific molecular marker (Fp-49-16-4).

For selective amplification, 8 primer combinations were tested (Table 5.10) on 6 isolates to optimize discrimination of the strain (Fp-49-16-4) of interest (Table 5.11). Primers 5'-HEX-EcoRI-TG-3' and 5'-MseI-CT-3' were chosen for the selective amplification because they generated a consistent and high number of alleles and provided good discrimination of isolate Fp-49-16-4.

Isolate Fp-49-16-4 was used in three runs to estimate the technical error rate as described by Bonin *et al.*, (2004). The AFLP error rate estimation was done using binary data generated with primers 5'-HEX-EcoRI-TG-3' and 5'-MseI-CT-3' and counting 241 loci as the total number of

loci produced (Table 5.12).The error rate for the three AFLP runs were 4.15%, 10.37%, and 4.56%.

Analyses of AFLP data

All analyses were performed twice; 1) all *F. proliferatum* and *F. verticillioides* isolates included and 2) only *F. proliferatum* isolates included. NTSYSpc v. 2.2 was used to cluster isolates and generate a UPGMA-derived phylogenetic tree. The isolates were bootstrapped 1,000 times using the "re-sampling" option in NTSYS (the bootstrap data is not shown on NTSYS trees). Variation was assessed by calculating the simple matching (SM) similarity coefficient, which is an informative measure of similarity when working with closely related taxa (Kosman and Leonard, 2005; Dalirsefat *et al.,* 2009; Sesli and Yegenoglu, 2010; Balestre *et al.,* 2008). The SM matrix was used to cluster the isolates using the Sequential, Agglomerative, Hierarchical, and Nested (SAHN) clustering methods (Sneath and Sokal, 1973) by UPGMA. The UPGMA analysis which included all *F. proliferatum* and *F. verticillioides* isolates grouped the isolates into two major clades; the *F. proliferatum* clade was divided into one outlier (Fp-49-2-2-666) and a subclade containing all the other isolates (Figure 5.6). In addition, all the isolates that contained the unique molecular marker grouped into the same subclade with the two positive control replicates (Fp-49-16-4).

The UPGMA analysis that included only the *F. proliferatum* isolates showed results similar to the one that included all isolates; it was divided into one outlier (Fp-49-2-2-666) and a clade containing all the other isolates. Similarly, isolates with the unique molecular marker grouped into a subclade separated from the other isolates (Figure 5.7).

STRUCTURE and STRUCTURE HARVESTER analyses that included all isolates revealed the presence of two populations (Figure 5.8) based on delta K calculations (Figure 5.9). These populations divided *F. proliferatum* and *F. verticillioides* isolates. Isolate Fp-49-2-2-666 showed the greatest variation within the *F. proliferatum* isolates. STRUCTURE and STRUCTURE HARVESTER analyses with only *F. proliferatum* isolates identified two populations (Figure 5.10) based on delta K calculations (Figure 5.11), 1) *F. proliferatum* isolates with the unique

strain-specific molecular marker and 2) all other *F. proliferatum* isolates that lacked the strain-specific marker.

Principal Coordinates Analysis (PCoA) of all isolates further showed how *F. proliferatum* and *F. verticillioides* isolates clustered separately (Figure 5.12). In addition, *F. proliferatum* isolates related to Fp-49-16-4 with the unique molecular marker grouped closer to each other than with the other *F. proliferatum* isolates within the cluster (Figure 5.13).

PCoA analysis of just *F. proliferatum* isolates also clustered the isolates in two groups, 1) *F. proliferatum* isolates related to Fp-49-16-4 with the unique strain-specific molecular marker, and 2) all the other *F. proliferatum* isolates (Figure 5.14).

The analysis of molecular variance (AMOVA) for all *F. proliferatum* and *F. verticillioides* isolates revealed that there was slightly greater molecular variation among populations (52%) than within populations (48%) (Figure 5.15). The PhiPT (ΦPT) obtained was 0.524 and highly significant (p=0.001), suggesting there were two populations (Table 5.13). AMOVA for the *F. proliferatum* isolates revealed greater molecular variation within populations (64%) than between populations (36%) (Figure 5.16). The PhiPT (ΦPT) obtained was 0.363 and highly significant (p=0.001), suggesting there were two populations (Table 5.14). To understand the high molecular variation (AMOVA) within and among populations and get a graphic representation of the molecular differences, STRUCTURE analysis was done using 8 populations (k=8) for all isolates (Figure 5.17) and for only *F. proliferatum* isolates (Figure 5.18). This analysis showed the high variation present in the *F. proliferatum* isolates lacking the unique strain-specific molecular marker compared to the *F. verticillioides* isolates, and to the *F. proliferatum* isolates having the unique strain-specific molecular marker.

Maize hybrid 33D49 yielded a total of 914 *Fusarium* isolates, of which 405 (44.3%) were identified as *F. proliferatum* and 380 (41.6%) were identified as *F. verticillioides*. Twenty-three of the 914 (2.52%) *Fusarium* isolates were identified as having the unique strain-specific molecular marker and likely had a single origin and were clonal.

Discussion

The results of this investigation demonstrate that specific *F. proliferatum* strains introduced into a new environment by planting maize seeds were detected in the newly formed kernels on the ear of the maize plant and hence are likely to be disseminated from that environment in the harvested seed. The unregulated movement of this cryptic fungus in maize seed may extend the geographical range of undesirable exotic populations or strains (e.g., high mycotoxin producers).

Long-distance movement and establishment of microorganisms in new environments are favored by asymptomatic colonization of the seed, which allows repeated entry into new sites that may go undetected for years (Elmer, 2001). Considering the global distribution and trade of maize seed and the seed-borne nature of *F. proliferatum*, the introduction of maize seed-borne isolates into new locations is likely occurring. Because of the wide host range of *F. proliferatum*, crops other than maize can be affected as well. For example, *F. oxysporum* f.sp. *apii*, which causes Fusarium yellows of celery, was detected 3 years after its first introduction into Michigan. By the time it was detected, it comprised over 20% of all *F. oxysporum* isolates in that state (Elmer and Lacy, 1987) and was isolated from other plant species (Elmer and Lacy, 1987). During the time a microorganism goes undetected, it can build up inoculum, become established as the dominant pathogen in a field, and start colonizing other plant species that provide adequate niches.

Our results demonstrate that the seed of different maize hybrids were colonized by different *F. proliferatum* strains. The association between a seed-borne microorganism and a plant host can be influenced by the host genotype (Ahlholm *et al.*, 2002) and environmental factors (Ahlholm *et al.*, 2002; Pamphile *et al*., 2002), which suggests the potential for genetic variability among seed-borne microorganisms including maize seed-borne *F. proliferatum*.

High genetic variability among seed-associated *F. proliferatum* isolates used in this investigation was demonstrated (STRUCTURE analysis with k=8, Figure 5.17). Higher genetic variability was detected in the *F. proliferatum* population than in the *F. verticillioides* population. High divergence among *F. proliferatum* isolates from the same host plant has been demonstrated through analyzing variation in mycotoxin profiles (Desjardins et al., 2000), suggesting that the introduction of a single maize seed or plant can greatly increase the diversity and genetic pool of

F. proliferatum. AMOVA analysis found 48% variation within species and 52% variation between species (Figure 5.15).

Moreover, STRUCTURE HARVESTER identified 2 major populations when using all isolates (k=2 based on Delta K graph, Figure 5.9) and STRUCTURE divided those 2 populations into *F. proliferatum* isolates and *F. verticillioides* isolates (Figure 5.8). The *F. proliferatum* isolates having the unique molecular marker grouped with the other *F. proliferatum* isolates. Further analysis was done with only the *F. proliferatum* isolates; STRUCTURE HARVESTER identified 2 major populations (k=2 based on Delta K graph, Figure 5.11). The *F. proliferatum* isolates having the genetic marker related to Fp-49-16-4 formed one cluster while the other *F. proliferatum* isolates formed the second cluster. This analysis supports the hypothesis that isolates introduced into new environments in seed can then be disseminated from that environment in the seed that is subsequently harvested. The PCoA analyses also demonstrated that the *F. proliferatum* isolates having the molecular marker grouped separately from the other *F. proliferatum* isolates, thus supporting the same conclusion.

Phylogenetic analyses grouped all 23 isolates having the molecular marker (related to Fp-49-16-4) in the same subclade as the two replicate positive-controls (Fp-49-16-4 and Fp2-49-16-4). A previous study that grouped *F. proliferatum* isolates by VCGs (Elmer *et al.,* 1999) found that the different VCG groups had different virulence in asparagus, and that the isolates from group VCG US5, which could colonize asparagus residue faster (Elmer *et al*., 1991), were found in higher proportion in the United States and in Australia than isolates from other VCG groups. Elmer *et al.,* (1999) hypothesized that group VCG US5 was likely introduced to Australia from the U.S. by importation of asparagus seeds or crowns, supporting the conclusion that seed transmission of *F. proliferatum* in asparagus is an important pathway of dispersal. The introduction of maize seed colonized by *F. proliferatum* isolates having high genetic variability may result in the introduction and establishment of undesirable isolates into new areas.

Different technologies were used in this investigation including basic plant pathology techniques, basic and advanced molecular tools (AFLP), and next-generation sequencing (GBS) technology. The results obtained in GBS and AFLP were verified by estimation of their error rates. Both

techniques depend on uniform and precise restriction-digestion, ligation, amplification of fragments, and sequencing (GBS) or display (AFLP) of the fragments.

GBS analysis of the *F. proliferatum* isolates allowed the generation of a uniform and reduced representation of their genomes (Elshire *et al.*, 2011, Poland *et al.*, 2012). Stacks analysis detected seven unique *F. proliferatum* isolates (Table 5.3) by finding isolate-specific polymorphic genomic regions (i.e. SNPs). However, the shallow depth of coverage in GBS (Table 5.3) resulted in SNP variation among identical samples that were used as controls; it has been reported that variant calling error rates are higher in GBS than in standard sequencing (Cooke *et al.*, 2015). These errors in sequencing (SNP calls) were corroborated when primers were designed to detect the unique isolates in end-point PCR. In some cases, there were no amplifications, while in others the polymorphisms were not unique, as the primers failed the exclusivity panels. More quality control and filters of the raw data (GBS reads) can be used to increase the accuracy of the final reads. Furthermore, using a more powerful sequencer (i.e. Illumina HiSeq 2000/2500) can increase the representation of the genome, the final number of reads obtained and the coverage depth.

AFLP fingerprinting of the isolates gave an electropherogram which displayed peaks representing loci of different size. The presence of the locus of a particular size was counted as "1", and its absence counted as "0". Therefore, the quality control of the peaks that represented loci was extensive to avoid the false inclusion or exclusion of loci. DNA fingerprinting technology is a powerful tool to compare isolates and has previously been used with a high success rate (Chiocchetti *et al*., 1999). AFLP has certain error rates with respect to reproducibility (Jones *et al.*, 1997, Pei *et al.*, 2007, Bonin *et al.*, 2004) that can complicate the interpretation of results. The use of AFLP to study the population genetics of different *Fusarium* species, *forma speciales*, strains, and isolates is common (Baayen *et al.*, 2000; Abdel-Satar *et al.*, 2003; Groenewald *et al.*, 2006; Belabid *et al.*, 2004; Moretti *et al.*, 2004; Qu *et al.*, 2008; Perchepied *et al.*, 2005; Lee *et al.*, 2009); however, the error rate of the AFLP results is not commonly reported, which complicates the interpretation of results and their reproducibility (Crawford *et al.*, 2012). Other AFLP population studies have reported error rates of 2-10% and have an average of 5-6% for fungal species (Voyron *et al.*, 2009; Avolio *et al.*, 2011; Rouse *et*

al., 2011; Gray *et al.*, 2014). This is consistent with the 6.36% error estimation in this study. Therefore, all isolates with the unique molecular marker that have a similarity of 94% or greater are likely to be clonal.

It is important to recognize the role of seeds as vectors in spreading microorganisms to derive a more complete understanding of the epidemiology of some diseases (Elmer, 2001). Furthermore, many of the plant disease epidemics that occurred during the 1900s are correlated to the creation of seed industries and the movement of seed worldwide (Elmer, 2001). The dispersal of *F. proliferatum* in maize seed may be the result of the demand for seed in different markets. Moreover, *F. proliferatum* often goes undetected because it does not cause any visual disease symptoms in the seed nor does it hinder germination. This dispersal greatly increases the genetic variability of populations in new areas and can result in an aggressive, high toxin producing isolate becoming dominant. Therefore, early detection and identification of infested seed lots could slow dispersal (Elmer, 2001) and the movement of undesirable isolates.

Figures and Tables

Stacks version 1.04

RAD-Tag Samples for batch #907 [2015-02-12;]

Id	Type	Unique Stacks	Polymorphic Loci	SNPs Found	Source
702	Sample	12336	3	3	Bowtie2_on_data_25_and_data_1001__aligned_reads
703	Sample	11659	2	2	Bowtie2_on_data_25_and_data_1009__aligned_reads
704	Sample	12018	4	6	Bowtie2_on_data_25_and_data_1017__aligned_reads
705	Sample	11969	3	3	Bowtie2_on_data_25_and_data_1025__aligned_reads
706	Sample	10351	2	14	Bowtie2_on_data_25_and_data_1033__aligned_reads
707	Sample	9826	2	17	Bowtie2_on_data_25_and_data_1041__aligned_reads
708	Sample	12640	7	25	Bowtie2_on_data_25_and_data_1049__aligned_reads
709	Sample	9649	3	18	Bowtie2_on_data_25_and_data_1072__aligned_reads
710	Sample	10291	3	13	Bowtie2_on_data_25_and_data_1080__aligned_reads
711	Sample	10978	2	2	Bowtie2_on_data_25_and_data_1088__aligned_reads
712	Sample	10268	2	2	Bowtie2_on_data_25_and_data_1096__aligned_reads
713	Sample	10607	3	24	Bowtie2_on_data_25_and_data_1112__aligned_reads
714	Sample	12420	2	25	Bowtie2_on_data_25_and_data_1120__aligned_reads
715	Sample	12018	2	24	Bowtie2_on_data_25_and_data_1128__aligned_reads
716	Sample	9213	0	0	Bowtie2_on_data_25_and_data_1136__aligned_reads
717	Sample	10628	4	25	Bowtie2_on_data_25_and_data_1144__aligned_reads
718	Sample	13271	7	28	Bowtie2_on_data_25_and_data_1152__aligned_reads
719	Sample	10000	2	12	Bowtie2_on_data_25_and_data_115__aligned_reads
720	Sample	14284	13	51	Bowtie2_on_data_25_and_data_1160__aligned_reads
721	Sample	11722	4	24	Bowtie2_on_data_25_and_data_1168__aligned_reads
722	Sample	9250	0	0	Bowtie2_on_data_25_and_data_139__aligned_reads
723	Sample	8220	1	1	Bowtie2_on_data_25_and_data_147__aligned_reads
724	Sample	8863	0	0	Bowtie2_on_data_25_and_data_155__aligned_reads
725	Sample	3103	0	0	Bowtie2_on_data_25_and_data_177__aligned_reads
726	Sample	8128	0	0	Bowtie2_on_data_25_and_data_185__aligned_reads
727	Sample	10940	2	12	Bowtie2_on_data_25_and_data_209__aligned_reads
728	Sample	10341	4	16	Bowtie2_on_data_25_and_data_233__aligned_reads

Figure 5.1 Identification of polymorphic loci and single nucleotide polymorphisms (SNPs) of GBS samples in Stacks.

Figure 5.2 Stacks aligns a consensus sequence to the complete genome of *F. proliferatum* to identify polymorphisms (not shown). The identified polymorphisms are highlighted in light blue for easy interpretation of results.

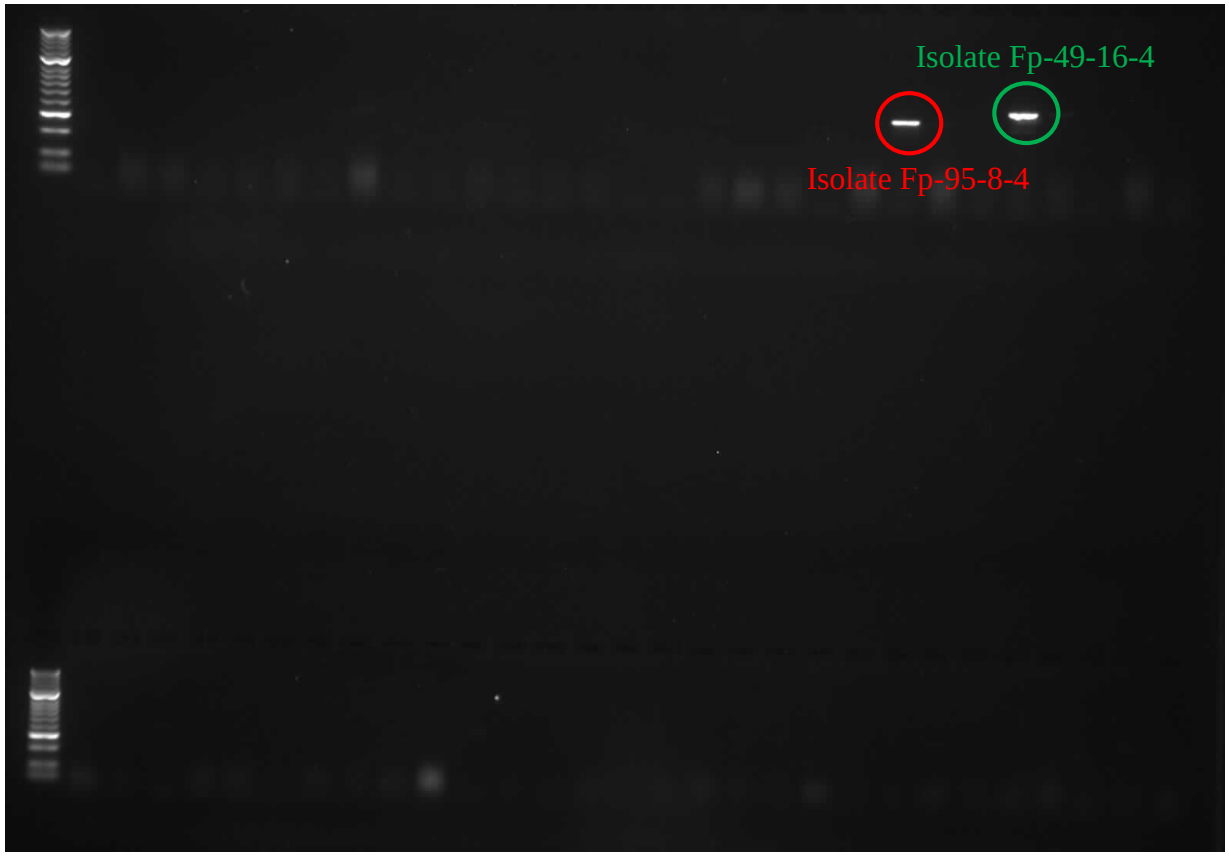
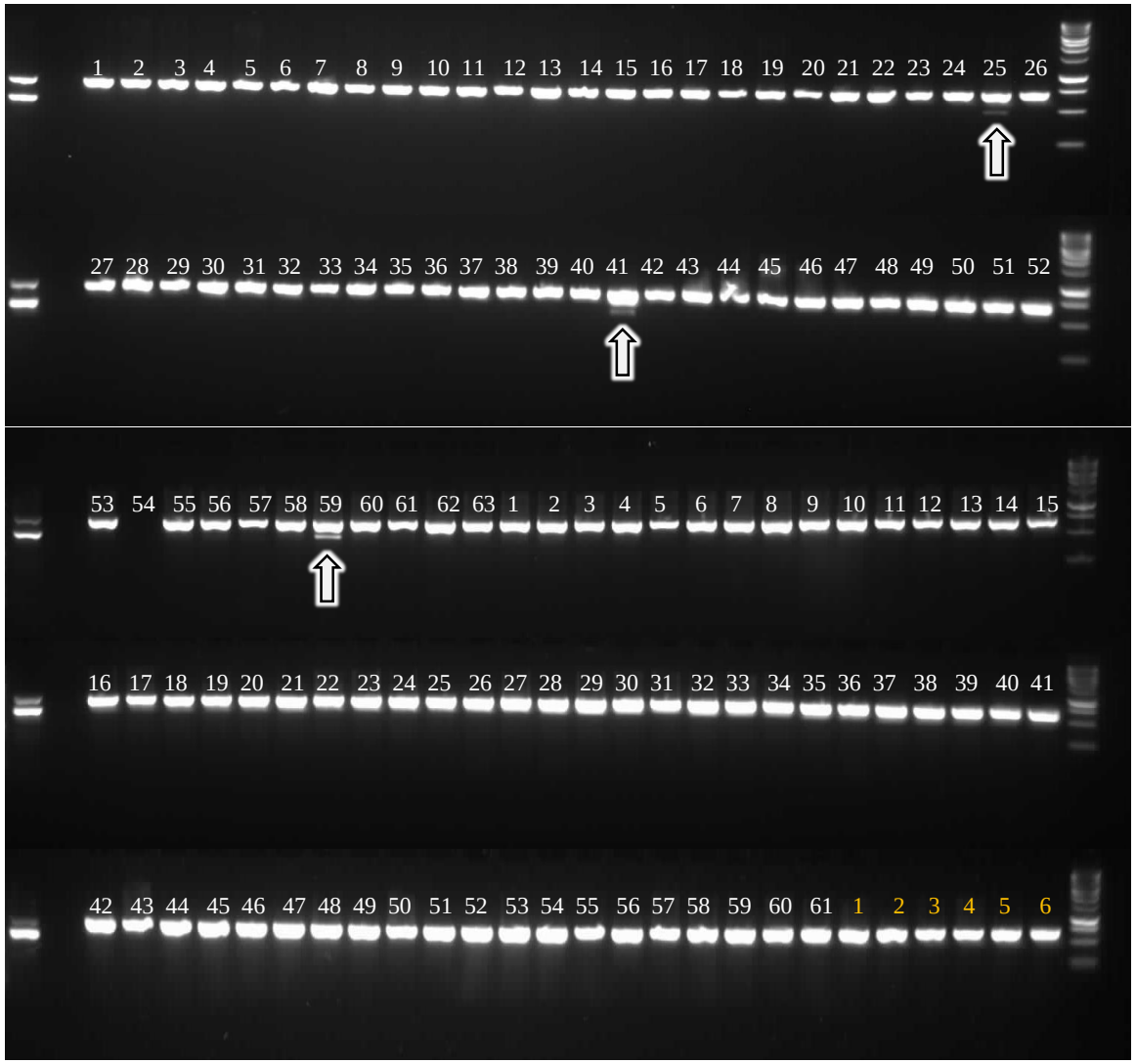


Figure 5.3 Multiplex end-point PCR assay using strain-specific primers for isolates Fp-49-16-4 and Fp-95-8-4 in 58 different *F. proliferatum* isolates showed specificity of the primers. Fp-49-16-4 had a 498bp amplicon and Fp-95-8-4 had a 454bp amplicon. 60 additional *F. proliferatum* isolates were tested in the exclusivity panels.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52
53 54 55 56 57 58 59 60 61 62 63 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 1 2 3 4 5 6

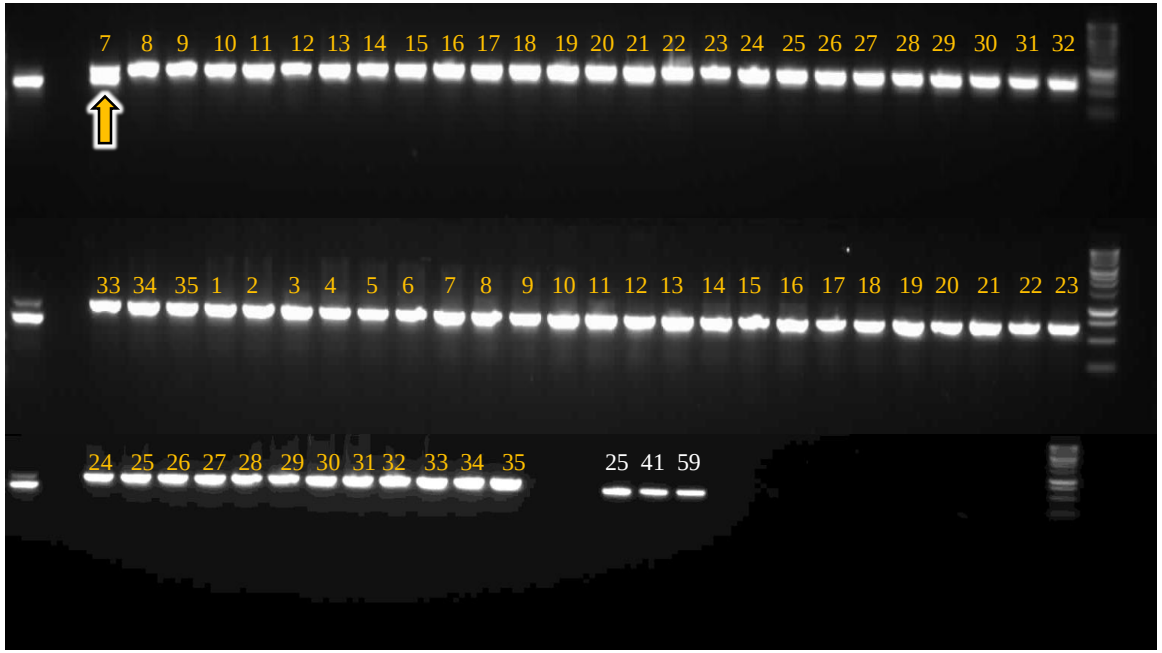
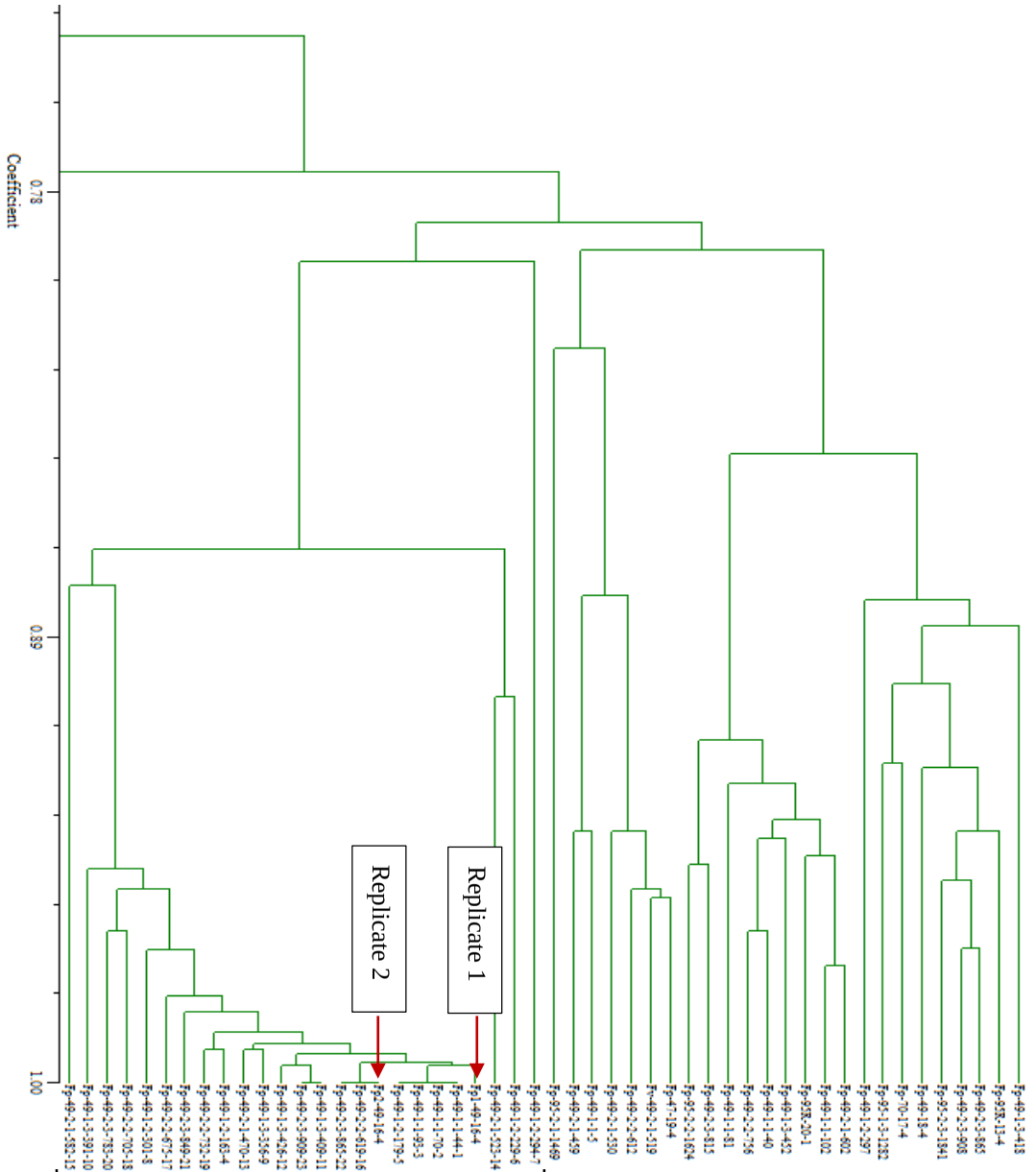


Figure 5.4 Additional *Fusarium* isolates were collected from DuPont Pioneer maize hybrid 33D49 and hybrid P1395R; the same seed lots that were used for the original GBS studies (white numbers). Of the 63 isolates collected from hybrid 33D49, three (numbers 25, 41 and 59) contained the strain-specific molecular marker. Of the 61 isolates collected from hybrid P1395R, none had the molecular marker unique to isolate Fp-49-16-4. Additional *Fusarium* isolates were collected from the ears of the maize plants from the field experiment (yellow color) to test for the presence of the strain-specific molecular markers identified with GBS. Of the 35 isolates collected from hybrid 33D49, one of them (number 7) had the strain-specific molecular marker. Of the 35 isolates collected from hybrid P1395R, none had the molecular marker unique to isolate Fp-49-16-4. The molecular marker for isolate Fp-49-16-4 was 498 base pairs. Every gel had a positive control in the first lane (isolate Fp-49-16-4) and a negative control in the second lane (water) to detect potential cross-contamination between samples. Isolates were confirmed to be *Fusarium* by partial amplification of the TEF-1α gene (750 base pairs).

Figure 5.5 Primers designed for isolate Fp-95-8-4 amplified a 454bp fragment in all the isolates collected from maize hybrid 33D49. This set of primers was not used in the field experiments as it was not specific to isolate Fp-95-8-4.



23 *F. proliferatum* isolates collected from the field grouped with the 2 replicates

Fusarium proliferatum isolates

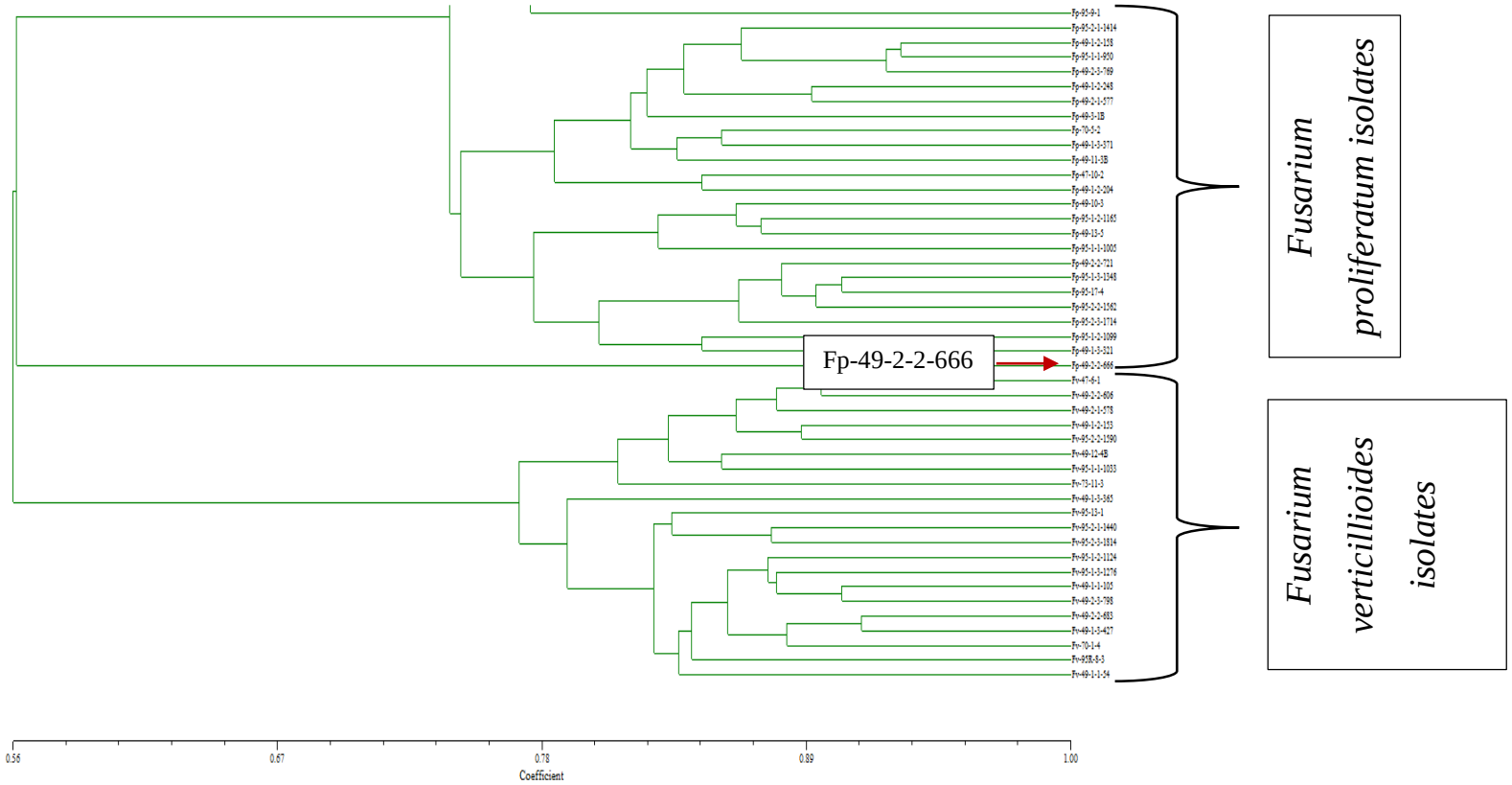
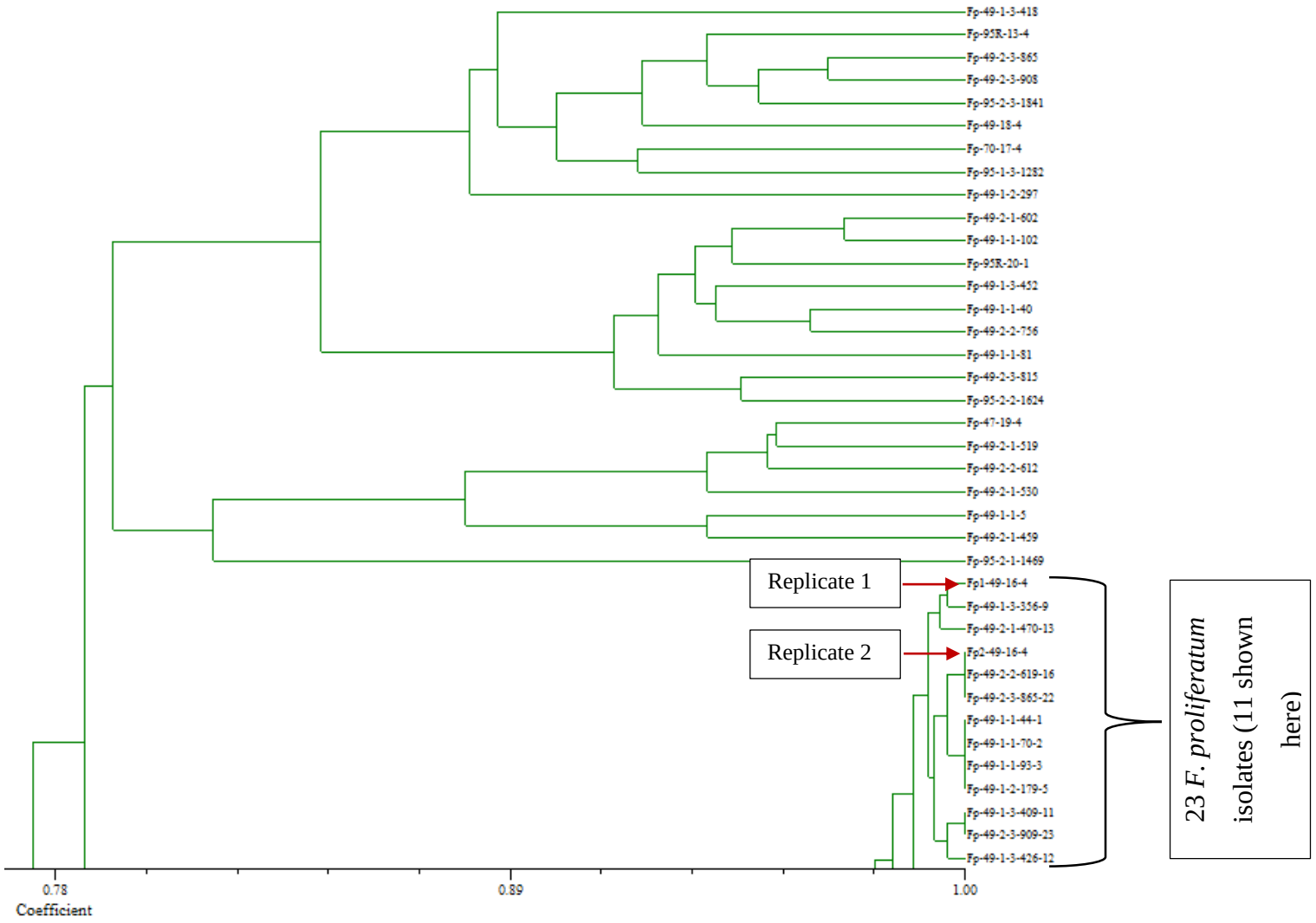


Figure 5.6 UPGMA clustering tree identified two major clades: A) all *F. proliferatum* isolates and B) all *F. verticillioides* isolates. The clade with *F. proliferatum* isolates was divided into one outlier (Fp-49-2-2-666), and a subclade with all the other isolates. All isolates with the strain-specific molecular marker grouped in the same subclade with the two positive control replicates (Fp1-49-16-4 and Fp2-49-16-4) and separate from the other *F. proliferatum* isolates. 19 of those 23 isolates had at least 95% similarity with the two replicates. AFLP error rate was calculated as 6.36%; therefore, all isolates having the strain-specific molecular marker that have a similarity of 94% or greater are likely to be clonal.


Fp-49-1-3-418
Fp-95R-13-4
Fp-49-2-3-865
Fp-49-2-3-908
Fp-95-2-3-1841
Fp-49-18-4
Fp-70-17-4
Fp-95-1-3-1282
Fp-49-1-2-297
Fp-49-2-1-602
Fp-49-1-1-102
Fp-95R-20-1
Fp-49-1-3-452
Fp-49-1-1-40
Fp-49-2-2-756
Fp-49-1-1-81
Fp-49-2-3-815
Fp-95-2-2-1624
Fp-47-19-4
Fp-49-2-1-519
Fp-49-2-2-612
Fp-49-2-1-530
Fp-49-1-1-5
Fp-49-2-1-459
Fp-95-2-1-1469
Fp1-49-16-4
Fp-49-1-3-356-9
Fp-49-2-1-470-13
Fp2-49-16-4
Fp-49-2-2-619-16
Fp-49-2-3-865-22
Fp-49-1-1-44-1
Fp-49-1-1-70-2
Fp-49-1-1-93-3
Fp-49-1-2-179-5
Fp-49-1-3-409-11
Fp-49-2-3-909-23
Fp-49-1-3-426-12
Replicate 1
Replicate 2
23 F. proliferatum isolates (11 shown here)
0.78
0.89
1.00
Coefficient

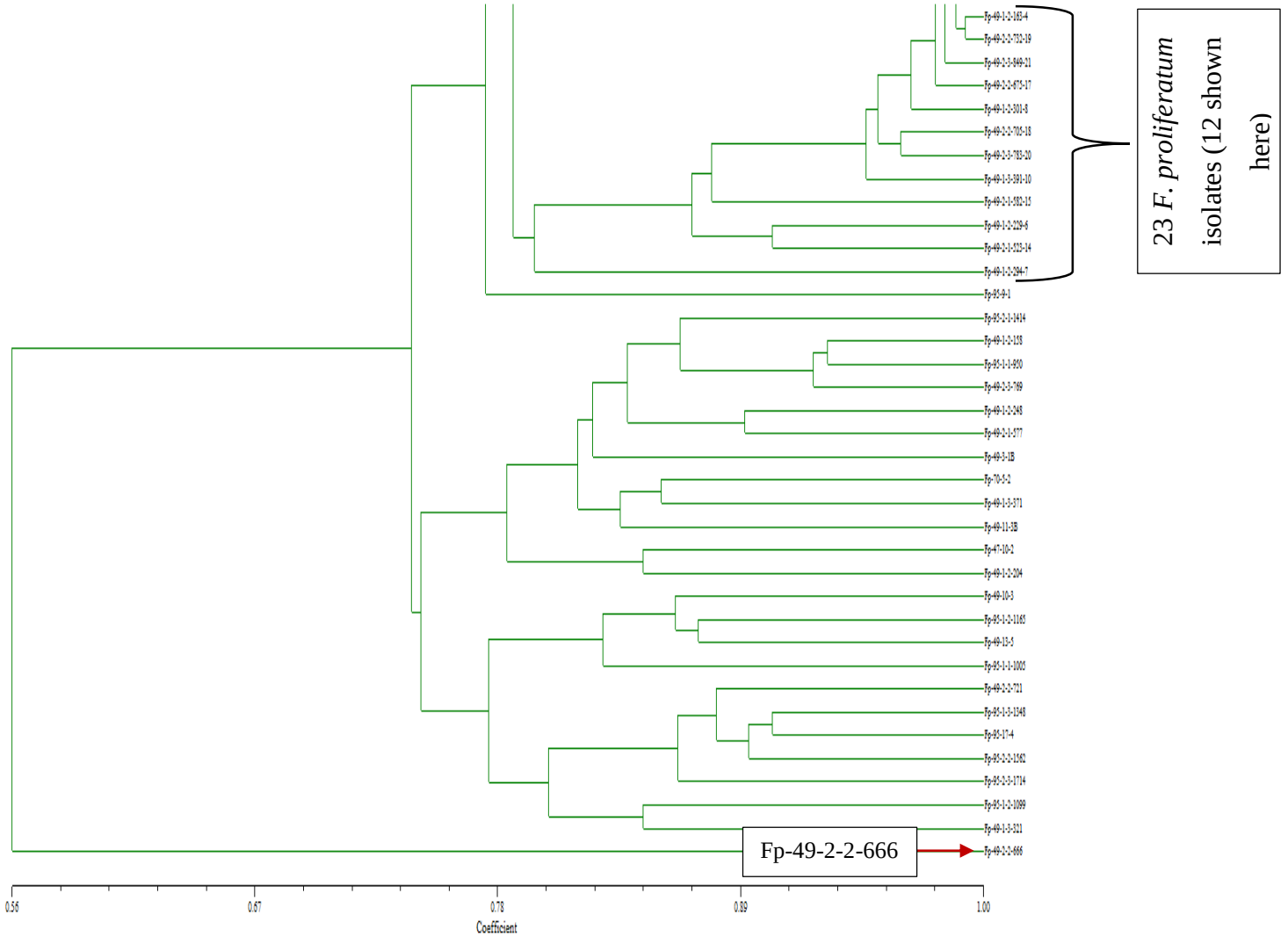


Figure 5.7 UPGMA clustering only including *F. proliferatum* isolates showed the same results as the UPGMA analysis including all isolates. The isolates were divided into one outlier (Fp-49-2-2-666), and a clade with all the other isolates. The 23 isolates with the unique molecular marker grouped in the same subclade with the two positive control replicates (Fp1-49-16-4

and Fp2-49-16-4). 19 of those 23 isolates had at least 95% similarity with the two replicates and they likely have a single origin and are clonal. This is explained by the AFLP error rate which was calculated to be 6.36%.

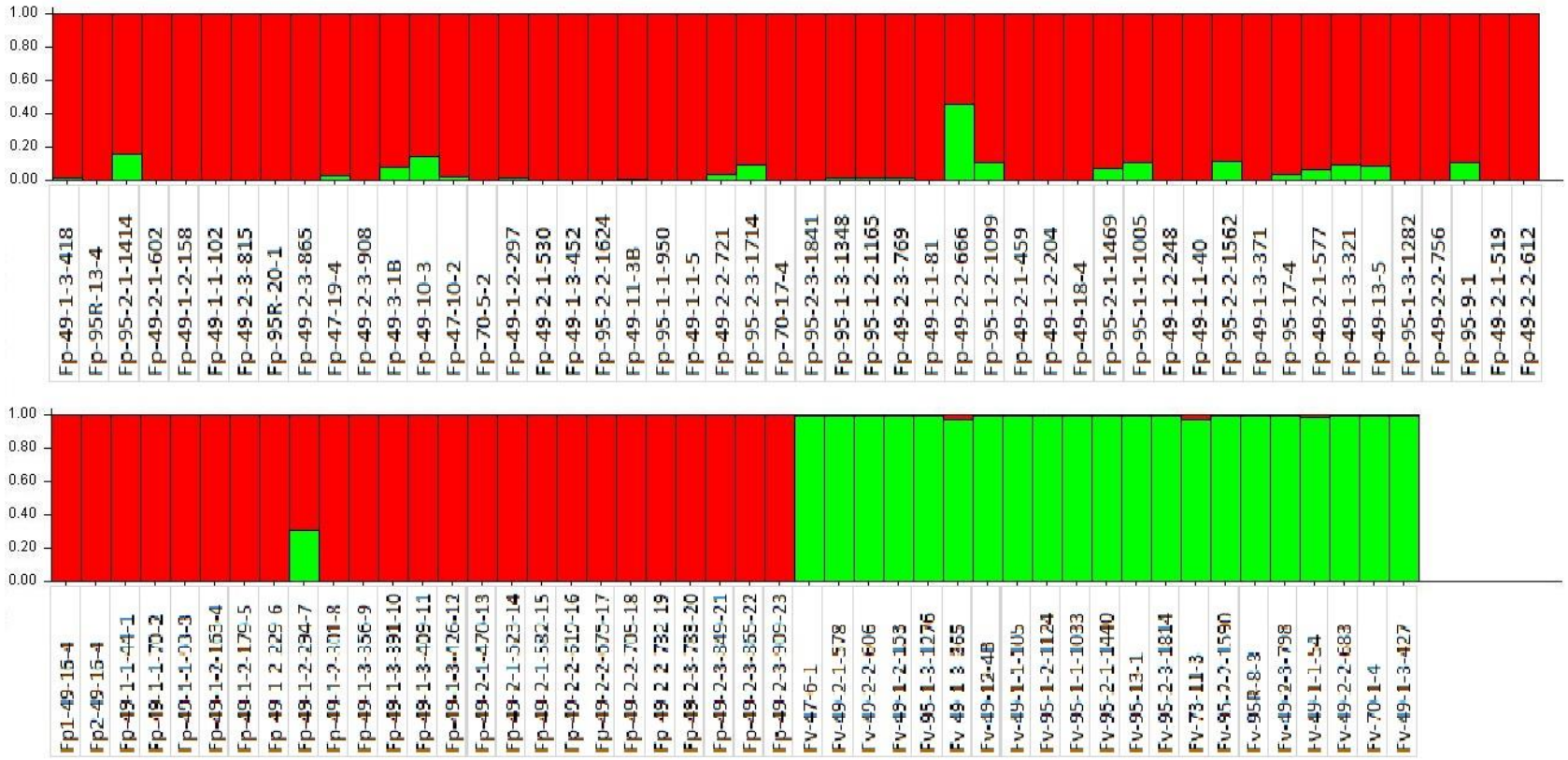


Figure 5.8 STRUCTURE analysis determined the presence of two populations; *F. proliferatum* population is predominantly red and *F. verticillioides* population is predominantly green. Bars with both green and red indicate similarity of some loci.

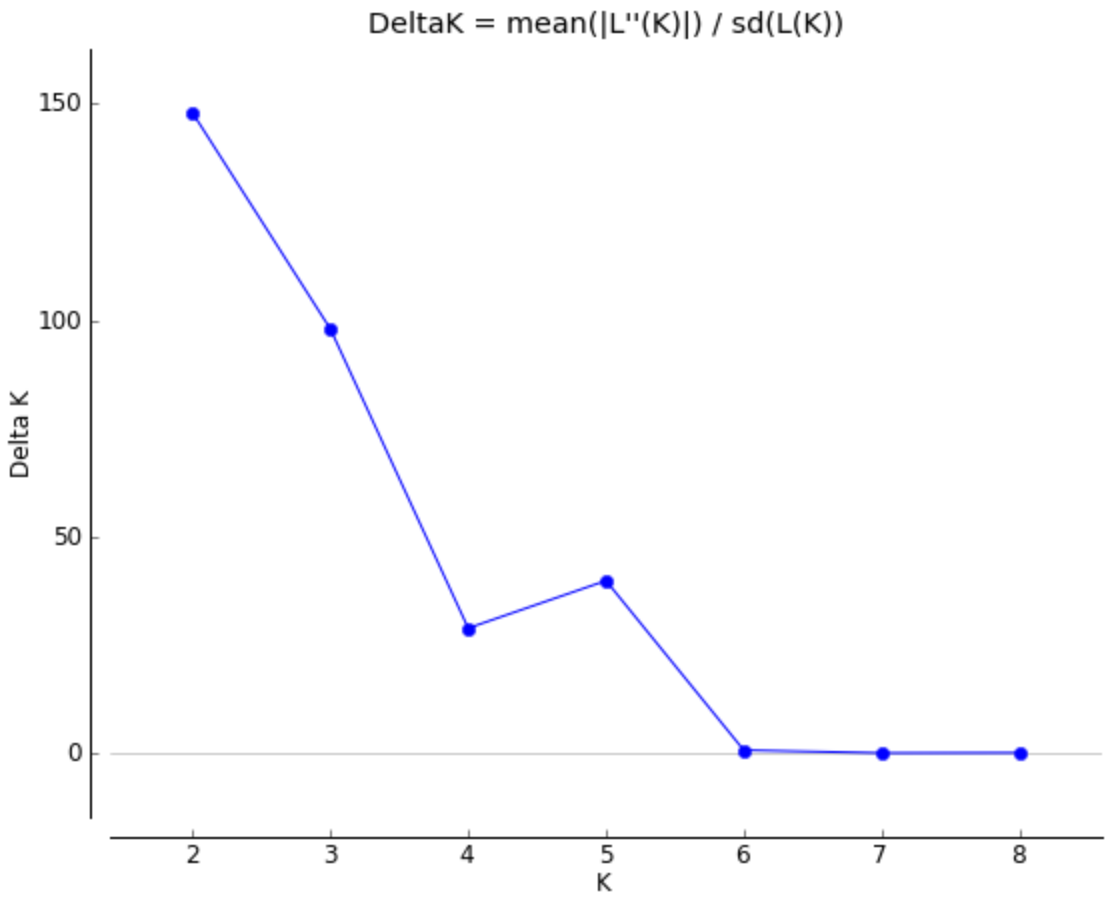


Figure 5.9 Evanno's delta K plot indicates two populations; STRUCTURE analyses of all isolates were done assuming two population.

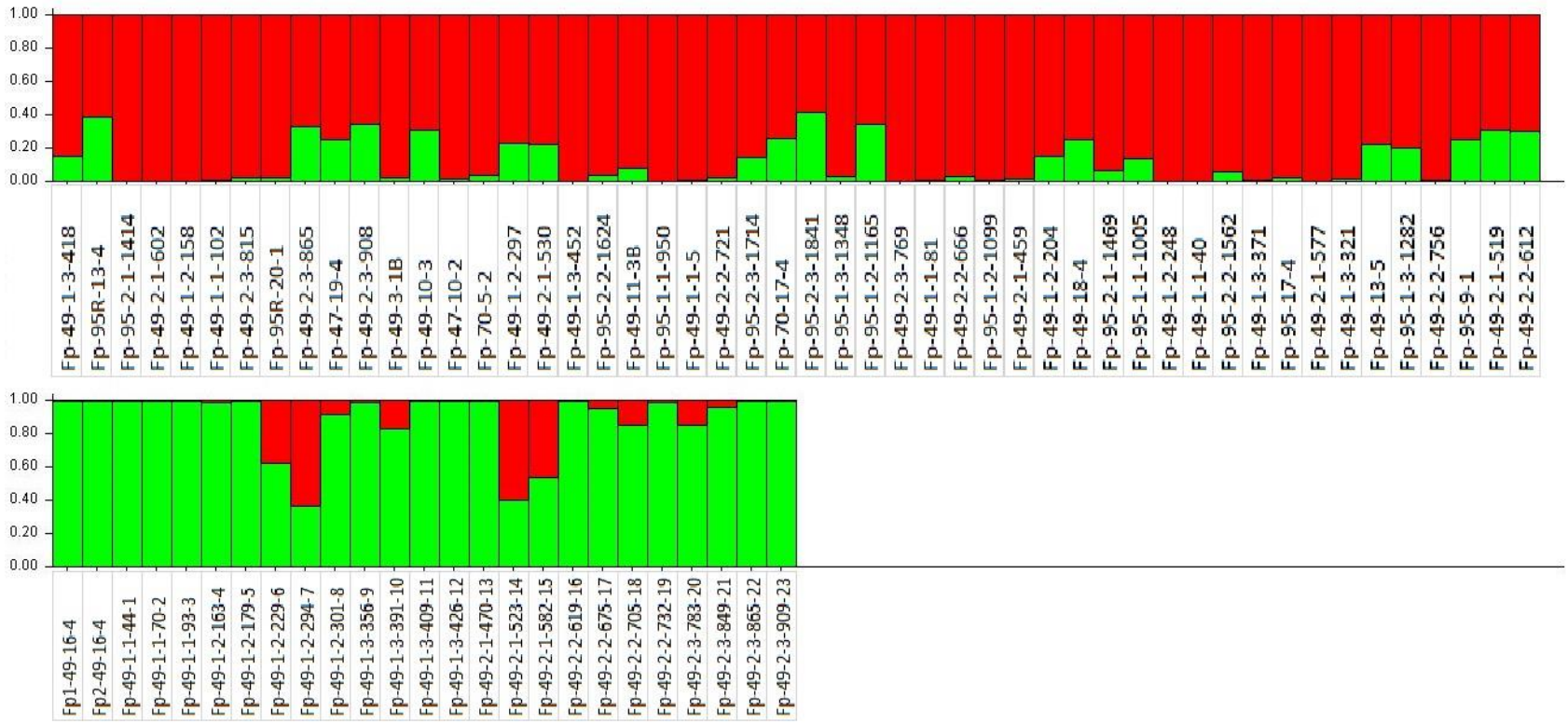


Figure 5.10 STRUCTURE analysis of just *F. proliferatum* isolates determined the presence of two populations, one that includes the isolates with the strain-specific molecular marker (green), and the other one that includes all the other *F. proliferatum* isolates (red). Bars with both green and red indicate similarity of some loci.

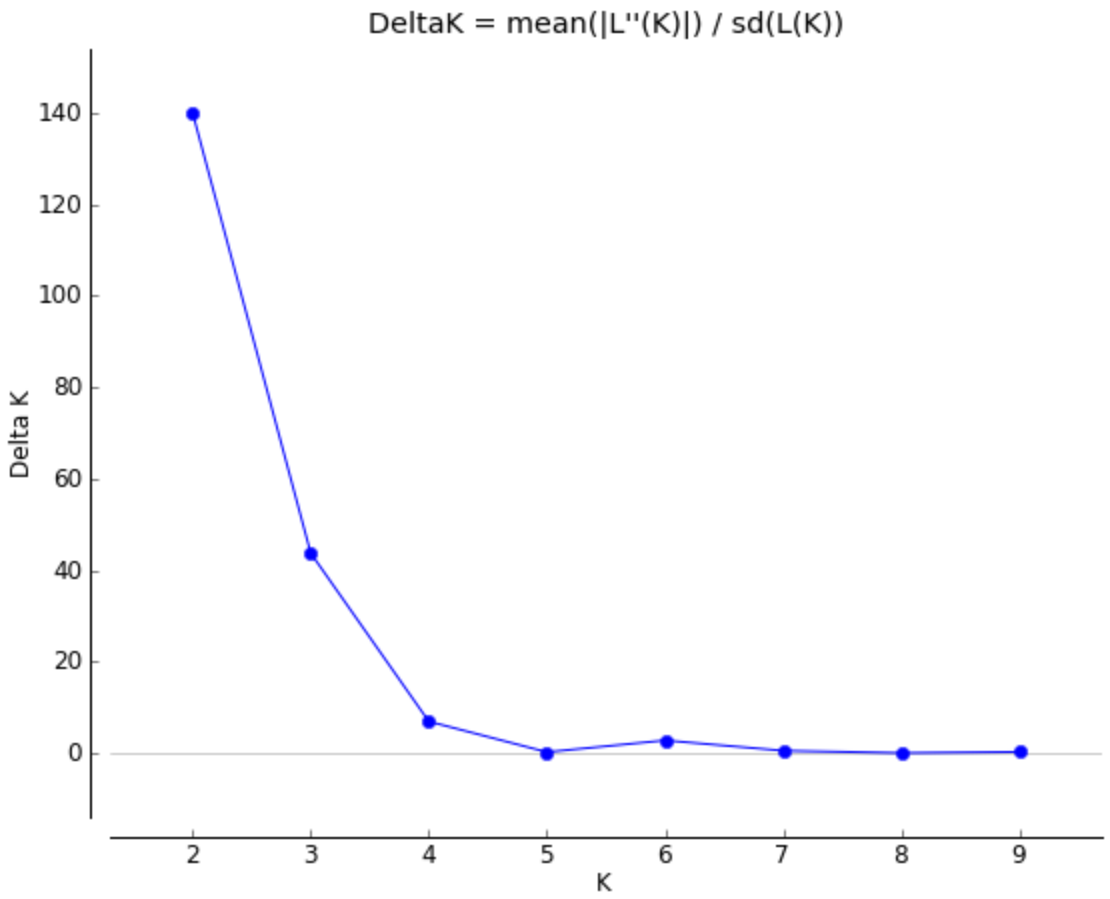


Figure 5.11 Evanno's delta K plot indicates two populations; STRUCTURE analyses of *F. proliferatum* isolates were done assuming two population.

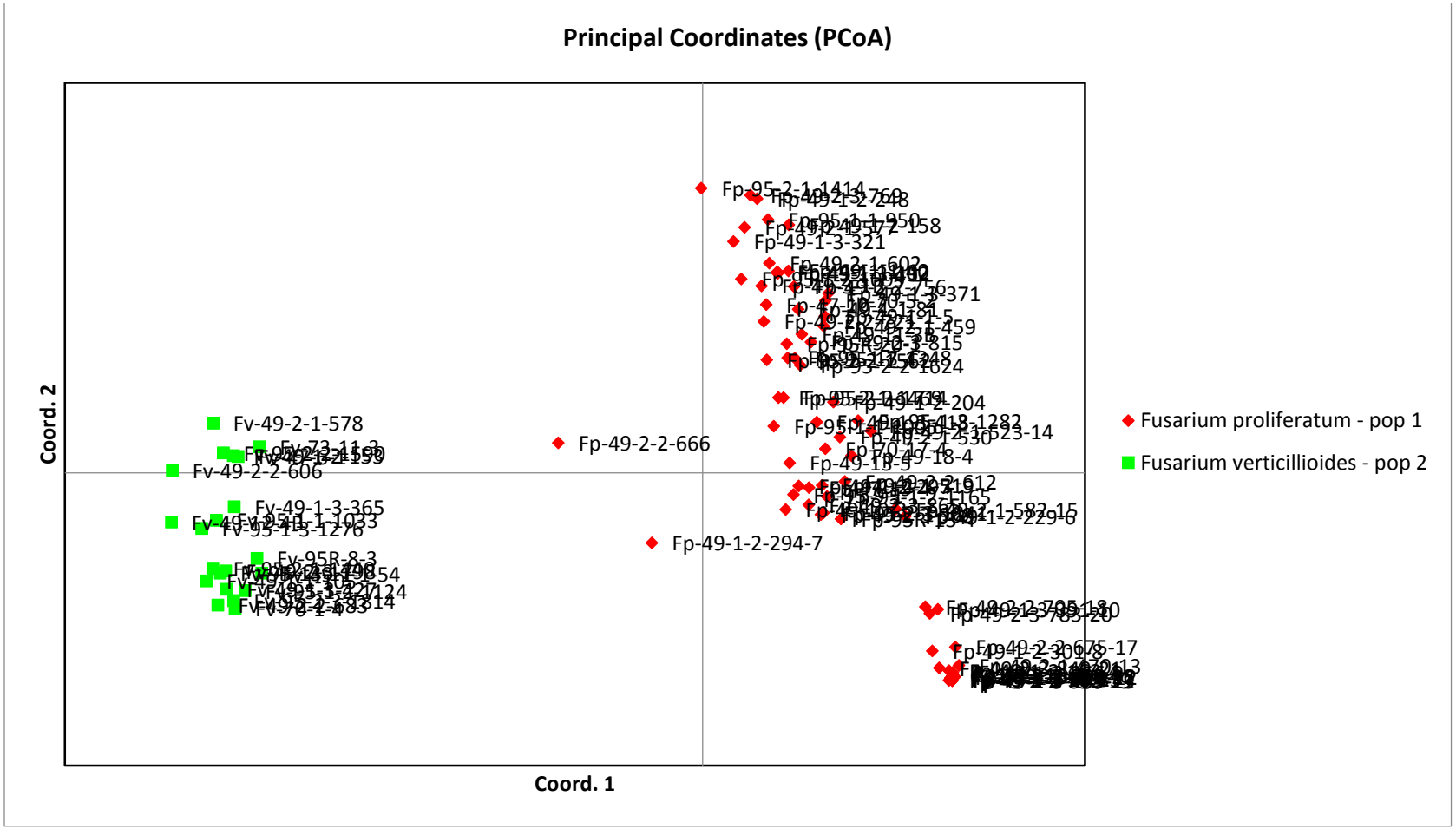


Figure 5.12 Principal coordinates analysis divided all the isolates into two clusters, *F. proliferatum* isolates (red) and *F. verticillioides* isolates (green). Coordinate 1 accounts for 29.9% of the variation and coordinate 2 accounts for 15.36 % of the variation.

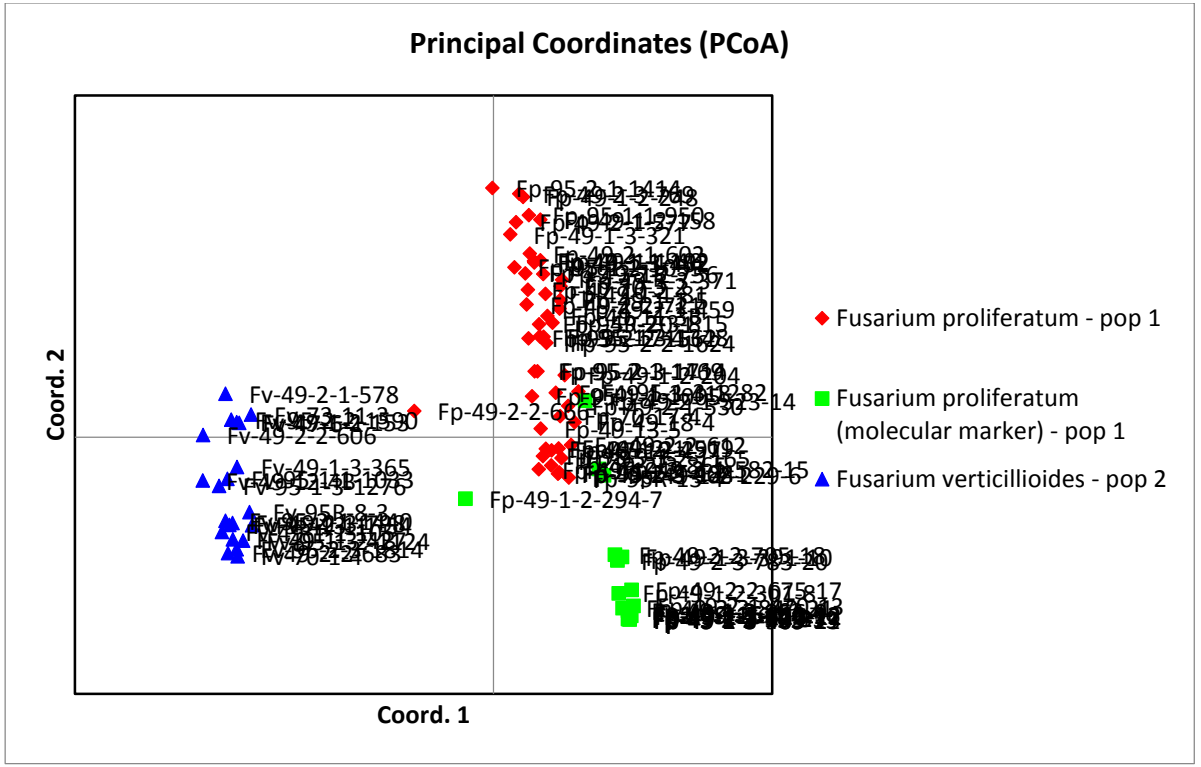


Figure 5.13 Principal coordinates analysis divided the *F. proliferatum* isolates in two clusters. Isolates with the strain-specific molecular marker (related to Fp-49-16-4; green) grouped close to each other in the same cluster with the other *F. proliferatum* isolates (red). Coordinate 1 accounts for 29.9% of the variation and coordinate 2 accounts for 15.36 % of the variation.

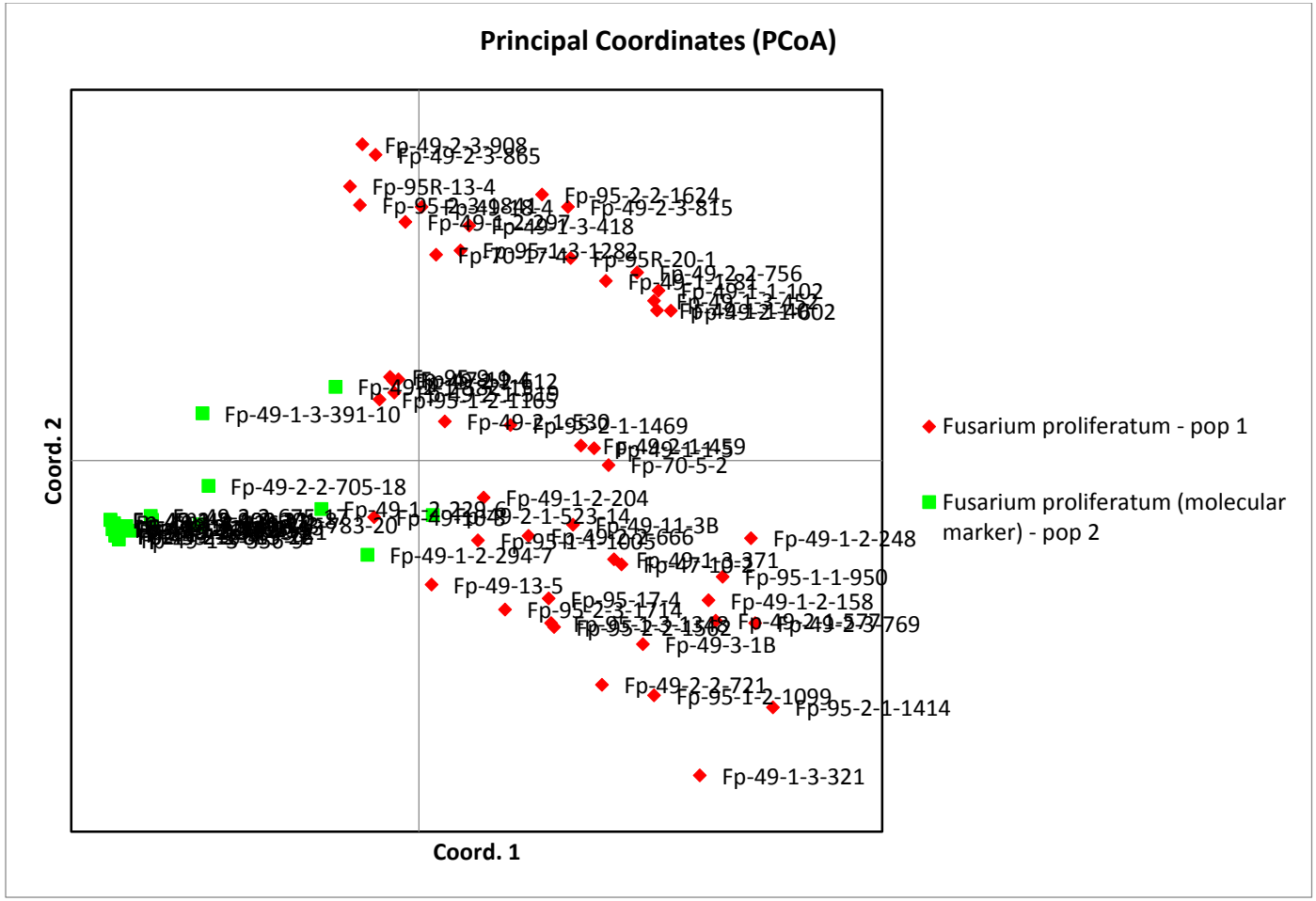


Figure 5.14 Principal coordinates analysis divided the *F. proliferatum* isolates in two clusters, *F. proliferatum* isolates related to Fp-49-16-4 (green), and the other *F. proliferatum* isolates (red). Coordinate 1 accounts for 27.26% of the variation and coordinate 2 accounts for 11.84% of the variation.

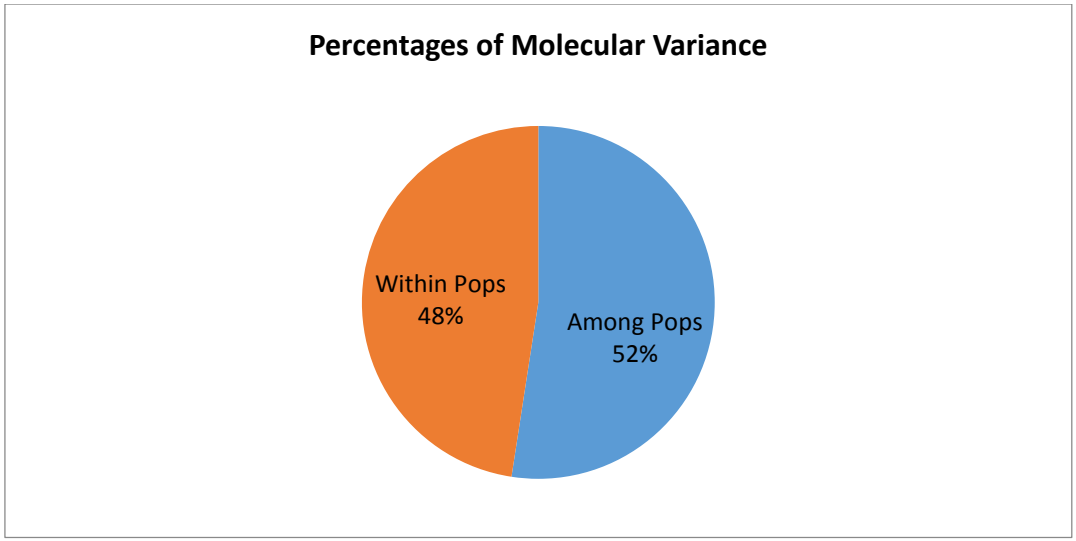


Figure 5.15 AMOVA analysis revealed that there was slightly more molecular variation among isolates between populations (species) (52%) than among isolates within population (species) (48%) for all isolates.

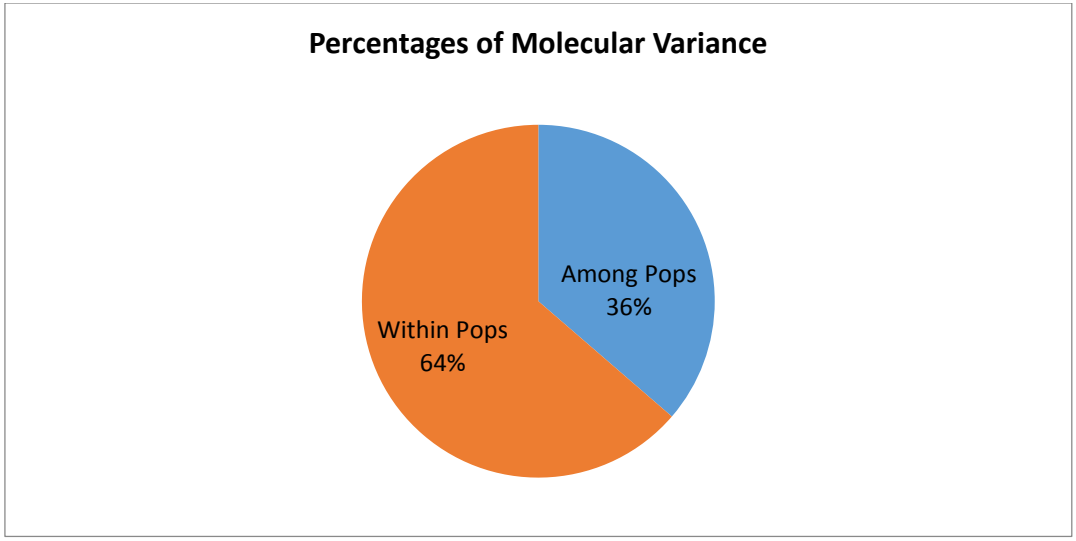


Figure 5.16 AMOVA analysis for the *F. proliferatum* isolates revealed that there was more molecular variation within *F. proliferatum* populations (64%) than among *F. proliferatum* populations (36%). The two populations are *F. proliferatum* isolates related to Fp-49-16-4 (unique molecular marker) and all the other *F. proliferatum* isolates..

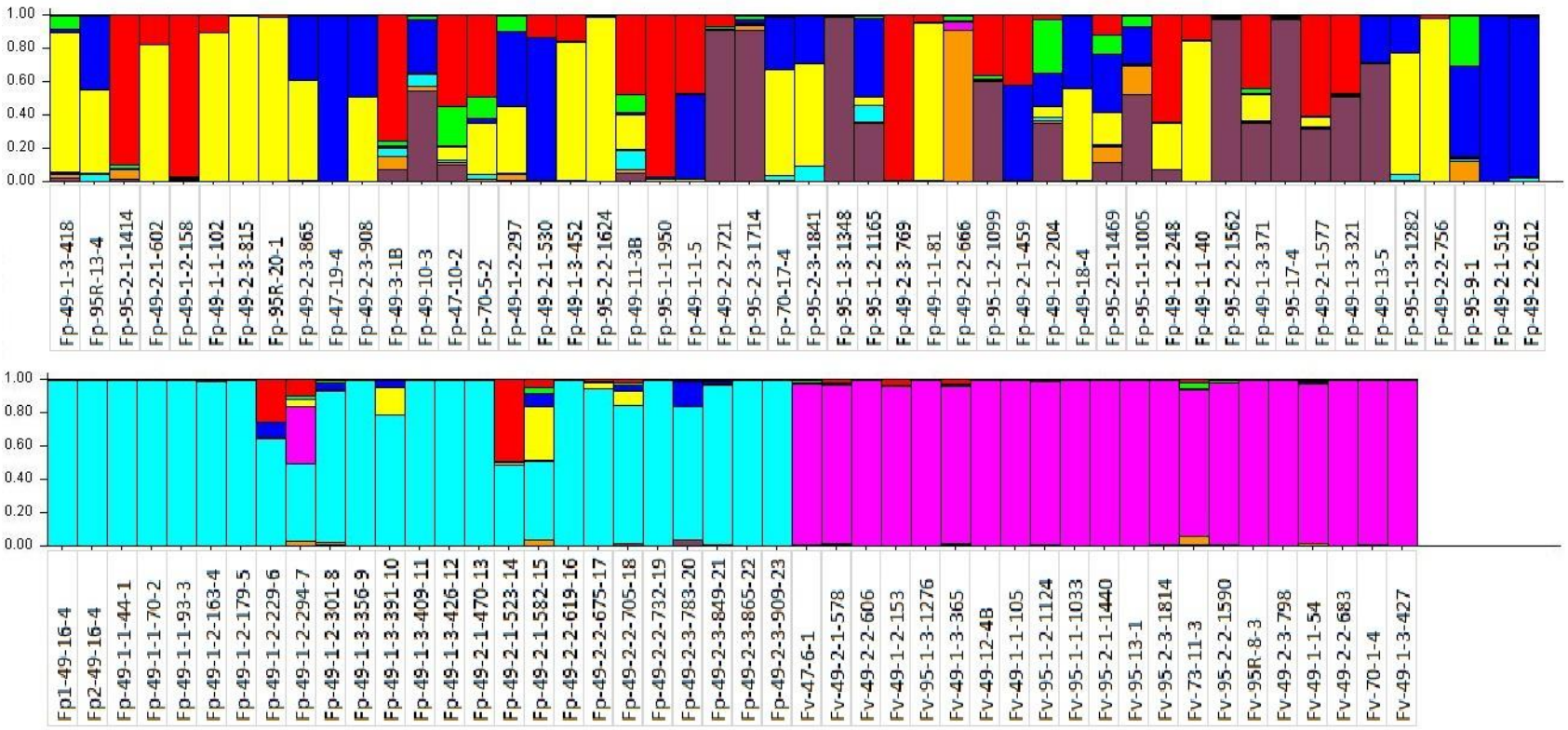


Figure 5.17 STRUCTURE analysis of all isolates using k=8 (8 populations) gives a graphic representation of the diversity and molecular differences within and among populations. The variation among *F. proliferatum* isolates is higher when compared to the variation found among the *F. verticillioides* isolates.

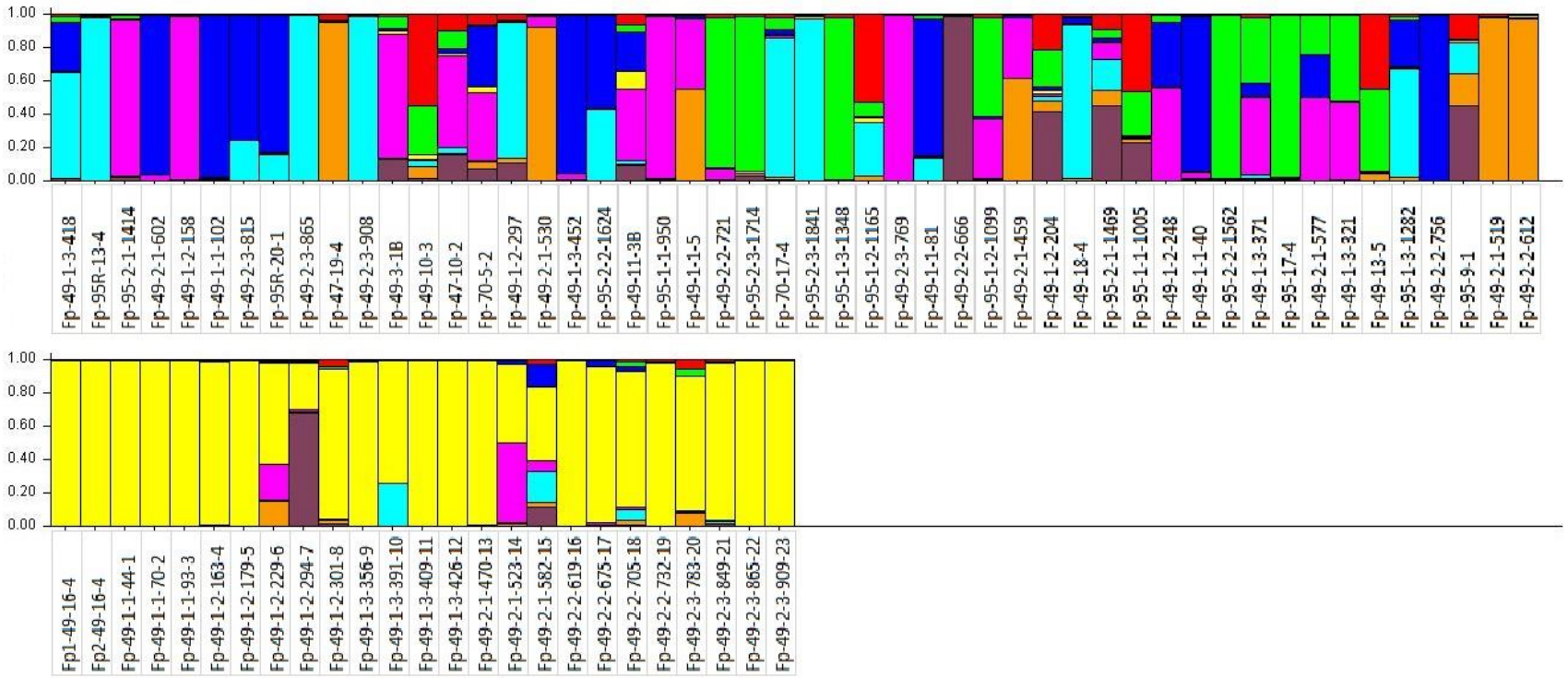


Figure 5.18 STRUCTURE analysis of only *F. proliferatum* isolates using k=8 (8 population structures) gives graphic representation of molecular differences within and among populations. The variation among the *F. proliferatum* isolates is higher when compared to the variation found among the *F. proliferatum* isolates with the unique molecular marker.

Table 5.1 Primers for partial amplification of the TEF-1α gene were used in end-point PCR to confirm that the isolates belong to the genus *Fusarium*.

Primer/Genus	*Fusarium*
Forward Primer	5'-ATGGGTAAGGAGGACAAGAC-3'
Reverse Primer	5'-GGAAGTACCAGTGATCATGTT-3'
Amplicon size (bp)	750

Table 5.2 Primers for partial amplification of the intergenic spacer (IGS) of rDNA were used in end-point PCR to identify *F. proliferatum* and *F. verticillioides* isolates.

Primer/Species	*Fusarium proliferatum*	*Fusarium verticillioides*
Forward Primer	5'-CGGCCACCAGAGGATGTG-3'	5'-CGCACGTATAGATGGACAAG-3'
Reverse Primer	5'-CAACACGAATCGCTTCCTGAC-3'	5'-CACCCGCAGCAATCCATCAG-3'
Amplicon size (bp)	230	700

Table 5.3 Stacks identified seven *F. proliferatum* isolates (Fp) having unique molecular markers. Note that Fp-49-16-4 had the strain-specific locus with 6 SNPs not found in other isolates.

Isolates	Depth	Number of samples represented	Number of samples with unique locus	# SNPs	Position of SNPs with respect to each other
Fp-47-4-2	6	53	1	1	12
Fp-95-14-5	6	70	1	1	50
Fp-49-11-1	6	96	2	5	101,102,110,114,119
Fp-95-8-4	2	64	1	1	68
Fp-47-6-1	7	95	3	3	5,15,17
Fp-49-11-3B	2	96	1	1	11
Fp-49-16-4	5	96	1	6	8,10,13,18,22,24

Table 5.4 GBS was used to identify loci unique to *F. proliferatum* strains. These strain-specific loci were used to design specific primers for use in end-point PCR for their identification (Fp-49-16-4 and Fp-95-8-4). Exclusivity panels revealed specificity of the primers.

Strains	Fp-49-16-4	Fp-95-8-4
Forward Primer	5'-TTCTCTCAGAGCCGCGAGT-3'	5'-CGCCCGCTACTGGAAAAA-3'
Reverse Primer	5'-GACAGCAGAGGACCTTGGAG-3'	5'-CTCGGTCATGATTTGGTTGG-3'
Amplicon Size (in bp)	498	454

Table 5.5 The amplicons generated by the strain-specific primers for Fp-49-16-4 and Fp-95-8-4- revealed similarity to *F. fujikuroi* draft genome. This can be explained by the high similarity between *F. proliferatum* and *F. fujikuroi*; however, the polymorphisms in the strain-specific molecular marker can still be used to identify the *F. proliferatum* isolates.

Isolate	Fp-49-16-4	Fp-95-8-4
Amplicon Sequence	5'-TTCTCTCAGA GCCGCGAGTCCATCTGCTGCAGAT GCTATGGGTCTGTAAATGTTTCCTT AGGTTGTTTCGGTAGTTTGAGGAG AGTTTGGTGTTTTCTCGTGGCCTCA TGGAAGGCGCCAGACACTTTCC ACTCGGGAGTCTGTAGTTTATTGA AATCTAGTTTAATTAAAAGGTAAG TACATCAACCCA ATTTTCGAATCGTACCACTGTGAG GTAAATAAGATAGTGACCGAGATA CTAACGTTGCAA TAACCAGGATGTGAACCCCGCACG ACAACCGCCGATCCCACTCAAATT CGGGGAACCTTG ATCATATCCTTCCTGATTGAGTATA CGGAACCGACGCTTATCGGGCGGT CATGTTTCTCC ATGATTGTTGAGCGAATCTCGGTG CGGCTCTACAATTGAAGCCCGGAT CCTTCTCGTCGCGCTGACTCGCTAT AAAAGATCGACAGCGAGGCTTCCT CGCTTTTCCTCCTCCAAGGTCCTCT GCTGTC-3'	5'-CGCCCGCTACTGG AAAAACAACAACGTCCAGTA CAACGAACTTCGCATCTCTC AATCAATCATTGTTGGCCGG GCAGTGATTCTCGCTCAACC TATGATGGCCCTTCCCTCACC GCAAACTTTTAACCCCGAG GCTGAAGCAGGCAGGTTCAG CTGGCTGCTCTATGGAACAG AGAAAGATATGTTTGAAATC CATGGAGGTTGTGGCTTTTC GAAGAAATTGTTGCATCTTA TGAGCCAGGTAACGTATTGT GCAGGTCGATTGCAGCAAGA GCCTGAATCCACCATCGTTC CAATCACGGCAAAATTCCTC TTGCGCGAACTATCAGAAAT GCGACAATGGAGCCGTGAGG GCAAAGACTGGGAGCTGGCT CGAAAGTACCCACCAACGAT AGACTGGGTGCGCGACAAAG CAGACGAAGTGATAATCGAT TCCAACCAAATCATGACCGA G-3'
Amplicon Size	498 base pairs	454 base pairs
% Similarity to *F. fujikuroi*	91% Identity to *Fusarium fujikuroi* IMI 58289 draft genome, chromosome FFUJ_chr11	99% Identity to *Fusarium fujikuroi* IMI 58289 draft genome, chromosome FFUJ_chr06

Table 5.6 Germination and infection rates of seeds collected from the field. Infection and germination rates were determined by plating seed on Nash-Snyder medium.

#	Hybrid	Plot #	Subplot #	# Infected seeds	% Infection	# Germinated Seeds	% Germination
1	33D49	1	1	23	46	49	98
2	33D49	1	1	25	50	44	88
3	33D49	1	1	28	56	47	94
4	33D49	1	1	27	54	43	86
5	33D49	1	1	21	42	48	96
6	33D49	1	1	23	46	47	94
7	33D49	1	2	26	52	50	100
8	33D49	1	2	27	54	50	100
9	33D49	1	2	31	62	42	84
10	33D49	1	2	29	58	46	92
11	33D49	1	2	21	42	45	90
12	33D49	1	2	20	40	47	94
13	33D49	1	3	32	64	49	98
14	33D49	1	3	20	40	43	86
15	33D49	1	3	32	64	46	92
16	33D49	1	3	20	40	46	92
17	33D49	1	3	23	46	43	86
18	33D49	1	3	26	52	46	92
19	33D49	2	1	30	60	48	96
20	33D49	2	1	21	42	41	82
21	33D49	2	1	25	50	47	94
22	33D49	2	1	21	42	46	92
23	33D49	2	1	30	60	46	92
24	33D49	2	1	24	48	45	90
25	33D49	2	2	25	50	41	82
26	33D49	2	2	30	60	44	88
27	33D49	2	2	27	54	50	100
28	33D49	2	2	27	54	50	100
29	33D49	2	2	29	58	48	96
30	33D49	2	2	23	46	49	98
31	33D49	2	3	23	46	45	90
32	33D49	2	3	30	60	41	82
33	33D49	2	3	25	50	45	90
34	33D49	2	3	26	52	42	84
35	33D49	2	3	23	46	50	100
36	33D49	2	3	21	42	48	96

37	P1395R	1	1	32	64	43	86
38	P1395R	1	1	27	54	50	100
39	P1395R	1	1	23	46	41	82
40	P1395R	1	1	24	48	50	100
41	P1395R	1	1	22	44	44	88
42	P1395R	1	1	25	50	47	94
43	P1395R	1	2	20	40	42	84
44	P1395R	1	2	25	50	49	98
45	P1395R	1	2	20	40	40	80
46	P1395R	1	2	24	48	49	98
47	P1395R	1	2	30	60	45	90
48	P1395R	1	2	32	64	50	100
49	P1395R	1	3	32	64	50	100
50	P1395R	1	3	32	64	45	90
51	P1395R	1	3	20	40	44	88
52	P1395R	1	3	22	44	44	88
53	P1395R	1	3	29	58	43	86
54	P1395R	1	3	31	62	44	88
55	P1395R	2	1	30	60	46	92
56	P1395R	2	1	20	40	48	96
57	P1395R	2	1	27	54	43	86
58	P1395R	2	1	21	42	48	96
59	P1395R	2	1	30	60	44	88
60	P1395R	2	1	20	40	46	92
61	P1395R	2	2	26	52	45	90
62	P1395R	2	2	27	54	50	100
63	P1395R	2	2	27	54	42	84
64	P1395R	2	2	29	58	43	86
65	P1395R	2	2	22	44	50	100
66	P1395R	2	2	30	60	49	98
67	P1395R	2	3	28	56	42	84
68	P1395R	2	3	26	52	47	94
69	P1395R	2	3	22	44	42	84
70	P1395R	2	3	27	54	48	96
71	P1395R	2	3	27	54	50	100
72	P1395R	2	3	32	64	41	82

Table 5.7 Percentage infection and percentage germination of kernels collected from hybrids 33D49 (49) and P1395R (95) in each plot and subplot.

# Infected - 49	914	# Germinated – 49	1657
% Infected - 49	50.8	% Germinated – 49	92.1
# Infected - 95	941	# Germinated – 95	1644
% Infected - 95	52.3	% Germinated – 95	91.3

# Infected - 49 (plot 1)	454	# Germinated - 49 (plot 1)	831
% Infected - 49 (plot 1)	50.4	% Germinated - 49 (plot 1)	92.3
# Infected - 49 (plot 2)	460	# Germinated - 49 (plot 2)	826
% Infected - 49 (plot 2)	51.1	% Germinated - 49 (plot 2)	91.8
# Infected - 95 (plot 1)	470	# Germinated - 95 (plot 1)	820
% Infected - 95 (plot 1)	52.2	% Germinated - 95 (plot 1)	91.1
# Infected - 95 (plot 2)	471	# Germinated - 95 (plot 2)	824
% Infected - 95 (plot 2)	52.3	% Germinated - 95 (plot 2)	91.6

# Infected - 49 (plot 1 - subplot 1)	147	# Germinated - 49 (plot 1 - subplot 1)	278
% Infected - 49 (plot 1 - subplot 1)	49.0	% Germinated - 49 (plot 1 - subplot 1)	92.7
# Infected - 49 (plot 1 - subplot 2)	154	# Germinated - 49 (plot 1 - subplot 2)	280
% Infected - 49 (plot 1 - subplot 2)	51.3	% Germinated - 49 (plot 1 - subplot 2)	93.3
# Infected - 49 (plot 1 - subplot 3)	153	# Germinated - 49 (plot 1 - subplot 3)	273
% Infected - 49 (plot 1 - subplot 3)	51.0	% Germinated - 49 (plot 1 - subplot 3)	91.0
# Infected - 49 (plot 2 - subplot 1)	151	# Germinated - 49 (plot 2 - subplot 1)	273
% Infected - 49 (plot 2 - subplot 1)	50.3	% Germinated - 49 (plot 2 - subplot 1)	91.0
# Infected - 49 (plot 2 - subplot 2)	161	# Germinated - 49 (plot 2 - subplot 2)	282
% Infected - 49 (plot 2 - subplot 2)	53.7	% Germinated - 49 (plot 2 - subplot 2)	94.0
# Infected - 49 (plot 2 - subplot 3)	148	# Germinated - 49 (plot 2 - subplot 3)	271
% Infected - 49 (plot 2 - subplot 3)	49.3	% Germinated - 49 (plot 2 - subplot 3)	90.3
# Infected - 95 (plot 1 - subplot 1)	153	# Germinated - 95 (plot 1 - subplot 1)	275
% Infected - 95 (plot 1 - subplot 1)	51.0	% Germinated - 95 (plot 1 - subplot 1)	91.7
# Infected - 95 (plot 1 - subplot 2)	151	# Germinated - 95 (plot 1 - subplot 2)	275
% Infected - 95 (plot 1 - subplot 2)	50.3	% Germinated - 95 (plot 1 - subplot 2)	91.7
# Infected - 95 (plot 1 - subplot 3)	166	# Germinated - 95 (plot 1 - subplot 3)	270
% Infected - 95 (plot 1 - subplot 3)	55.3	% Germinated - 95 (plot 1 - subplot 3)	90.0
# Infected - 95 (plot 2 - subplot 1)	148	# Germinated - 95 (plot 2 - subplot 1)	275
% Infected - 95 (plot 2 - subplot 1)	49.3	% Germinated - 95 (plot 2 - subplot 1)	91.7
# Infected - 95 (plot 2 - subplot 2)	161	# Germinated - 95 (plot 2 - subplot 2)	279
% Infected - 95 (plot 2 - subplot 2)	53.7	% Germinated - 95 (plot 2 - subplot 2)	93.0
# Infected - 95 (plot 2 - subplot 3)	162	# Germinated - 95 (plot 2 - subplot 3)	270
% Infected - 95 (plot 2 - subplot 3)	54.0	% Germinated - 95 (plot 2 - subplot 3)	90.0

Table 5.8 A total of 1,855 isolates were collected from the field. 817 were found to be *F. proliferatum* (Fp) and 751 were found to be *F. verticillioides* (Fv). 287 isolates could not be identified as *F. proliferatum* or *F. verticillioides*. 23 of the *F. proliferatum* isolates coming from hybrid 33D49 collected in the field were found to have the strain-specific molecular marker.

#	Maize Hybrid	Plot	Subplot	Primer 49	*Fusarium proliferatum*	*Fusarium verticillioides*	Unknown	Result
1	33D49	1	1		0	1	0	Fv
2	33D49	1	1		0	1	0	Fv
3	33D49	1	1		0	0	1	unknown
4	33D49	1	1		0	1	0	Fv
5	33D49	1	1		1	0	0	Fp
6	33D49	1	1		0	1	0	Fv
7	33D49	1	1		0	1	0	Fv
8	33D49	1	1		0	1	0	Fv
9	33D49	1	1		0	1	0	Fv
10	33D49	1	1		1	0	0	Fp
11	33D49	1	1		0	1	0	Fv
12	33D49	1	1		0	1	0	Fv
13	33D49	1	1		0	0	1	unknown
14	33D49	1	1		1	0	0	Fp
15	33D49	1	1		1	0	0	Fp
16	33D49	1	1		0	1	0	Fv
17	33D49	1	1		0	0	1	unknown
18	33D49	1	1		1	0	0	Fp
19	33D49	1	1		1	0	0	Fp
20	33D49	1	1		0	0	1	unknown
21	33D49	1	1		0	0	1	unknown
22	33D49	1	1		1	0	0	Fp
23	33D49	1	1		1	0	0	Fp
24	33D49	1	1		1	0	0	Fp
25	33D49	1	1		1	0	0	Fp
26	33D49	1	1		1	0	0	Fp
27	33D49	1	1		1	0	0	Fp
28	33D49	1	1		1	0	0	Fp
29	33D49	1	1		1	0	0	Fp
30	33D49	1	1		1	0	0	Fp
31	33D49	1	1		1	0	0	Fp
32	33D49	1	1		1	0	0	Fp
33	33D49	1	1		1	0	0	Fp

34	33D49	1	1		1	0	0	Fp
35	33D49	1	1		1	0	0	Fp
36	33D49	1	1		1	0	0	Fp
37	33D49	1	1		1	0	0	Fp
38	33D49	1	1		1	0	0	Fp
39	33D49	1	1		1	0	0	Fp
40	33D49	1	1		1	0	0	Fp
41	33D49	1	1		1	0	0	Fp
42	33D49	1	1		1	0	0	Fp
43	33D49	1	1		1	0	0	Fp
44	33D49	1	1	1	1	0	0	Fp
45	33D49	1	1		1	0	0	Fp
46	33D49	1	1		1	0	0	Fp
47	33D49	1	1		1	0	0	Fp
48	33D49	1	1		1	0	0	Fp
49	33D49	1	1		1	0	0	Fp
50	33D49	1	1		1	0	0	Fp
51	33D49	1	1		1	0	0	Fp
52	33D49	1	1		1	0	0	Fp
53	33D49	1	1		0	1	0	Fv
54	33D49	1	1		0	1	0	Fv
55	33D49	1	1		0	1	0	Fv
56	33D49	1	1		0	1	0	Fv
57	33D49	1	1		0	1	0	Fv
58	33D49	1	1		0	1	0	Fv
59	33D49	1	1		0	1	0	Fv
60	33D49	1	1		1	0	0	Fp
61	33D49	1	1		0	1	0	Fv
62	33D49	1	1		0	1	0	Fv
63	33D49	1	1		0	1	0	Fv
64	33D49	1	1		0	1	0	Fv
65	33D49	1	1		0	1	0	Fv
66	33D49	1	1		1	0	0	Fp
67	33D49	1	1		0	1	0	Fv
68	33D49	1	1		0	1	0	Fv
69	33D49	1	1		0	1	0	Fv
70	33D49	1	1	1	1	0	0	Fp
71	33D49	1	1		0	1	0	Fv
72	33D49	1	1		0	1	0	Fv
73	33D49	1	1		1	0	0	Fp
74	33D49	1	1		0	0	1	unknown

75	33D49	1	1		0	0	1	unknown
76	33D49	1	1		0	0	1	unknown
77	33D49	1	1		1	0	0	Fp
78	33D49	1	1		1	0	0	Fp
79	33D49	1	1		0	0	1	unknown
80	33D49	1	1		1	0	0	Fp
81	33D49	1	1		1	0	0	Fp
82	33D49	1	1		0	0	1	unknown
83	33D49	1	1		0	0	1	unknown
84	33D49	1	1		1	0	0	Fp
85	33D49	1	1		1	0	0	Fp
86	33D49	1	1		1	0	0	Fp
87	33D49	1	1		0	1	0	Fv
88	33D49	1	1		1	0	0	Fp
89	33D49	1	1		1	0	0	Fp
90	33D49	1	1		1	0	0	Fp
91	33D49	1	1		1	0	0	Fp
92	33D49	1	1		0	1	0	Fv
93	33D49	1	1	1	1	0	0	Fp
94	33D49	1	1		0	0	1	unknown
95	33D49	1	1		0	0	1	unknown
96	33D49	1	1		1	0	0	Fp
97	33D49	1	1		1	0	0	Fp
98	33D49	1	1		1	0	0	Fp
99	33D49	1	1		1	0	0	Fp
100	33D49	1	1		0	1	0	Fv
101	33D49	1	1		1	0	0	Fp
102	33D49	1	1		1	0	0	Fp
103	33D49	1	1		1	0	0	Fp
104	33D49	1	1		1	0	0	Fp
105	33D49	1	1		0	1	0	Fv
106	33D49	1	1		0	1	0	Fv
107	33D49	1	1		0	1	0	Fv
108	33D49	1	1		0	0	1	unknown
109	33D49	1	1		0	1	0	Fv
110	33D49	1	1		0	1	0	Fv
111	33D49	1	1		0	1	0	Fv
112	33D49	1	1		0	0	1	unknown
113	33D49	1	1		0	0	1	unknown
114	33D49	1	1		0	1	0	Fv
115	33D49	1	1		0	0	1	unknown

116	33D49	1	1		0	1	0	Fv
117	33D49	1	1		0	1	0	Fv
118	33D49	1	1		0	1	0	Fv
119	33D49	1	1		0	1	0	Fv
120	33D49	1	1		0	1	0	Fv
121	33D49	1	1		0	1	0	Fv
122	33D49	1	1		0	0	1	unknown
123	33D49	1	1		0	1	0	Fv
124	33D49	1	1		0	1	0	Fv
125	33D49	1	1		0	1	0	Fv
126	33D49	1	1		0	1	0	Fv
127	33D49	1	1		0	1	0	Fv
128	33D49	1	1		0	1	0	Fv
129	33D49	1	1		0	0	1	unknown
130	33D49	1	1		0	1	0	Fv
131	33D49	1	1		0	1	0	Fv
132	33D49	1	1		0	1	0	Fv
133	33D49	1	1		0	1	0	Fv
134	33D49	1	1		0	1	0	Fv
135	33D49	1	1		0	1	0	Fv
136	33D49	1	1		0	1	0	Fv
137	33D49	1	1		0	1	0	Fv
138	33D49	1	1		0	1	0	Fv
139	33D49	1	1		0	1	0	Fv
140	33D49	1	1		0	1	0	Fv
141	33D49	1	1		0	1	0	Fv
142	33D49	1	1		0	1	0	Fv
143	33D49	1	1		0	1	0	Fv
144	33D49	1	1		0	1	0	Fv
145	33D49	1	1		0	1	0	Fv
146	33D49	1	1		0	1	0	Fv
147	33D49	1	1		0	1	0	Fv
148	33D49	1	2		0	1	0	Fv
149	33D49	1	2		0	1	0	Fv
150	33D49	1	2		0	1	0	Fv
151	33D49	1	2		0	1	0	Fv
152	33D49	1	2		0	1	0	Fv
153	33D49	1	2		0	1	0	Fv
154	33D49	1	2		0	1	0	Fv
155	33D49	1	2		0	1	0	Fv
156	33D49	1	2		0	1	0	Fv

157	33D49	1	2		0	1	0	Fv
158	33D49	1	2		1	0	0	Fp
159	33D49	1	2		1	0	0	Fp
160	33D49	1	2		1	0	0	Fp
161	33D49	1	2		1	0	0	Fp
162	33D49	1	2		1	0	0	Fp
163	33D49	1	2	1	1	0	0	Fp
164	33D49	1	2		1	0	0	Fp
165	33D49	1	2		1	0	0	Fp
166	33D49	1	2		1	0	0	Fp
167	33D49	1	2		1	0	0	Fp
168	33D49	1	2		1	0	0	Fp
169	33D49	1	2		1	0	0	Fp
170	33D49	1	2		1	0	0	Fp
171	33D49	1	2		1	0	0	Fp
172	33D49	1	2		1	0	0	Fp
173	33D49	1	2		1	0	0	Fp
174	33D49	1	2		1	0	0	Fp
175	33D49	1	2		1	0	0	Fp
176	33D49	1	2		1	0	0	Fp
177	33D49	1	2		1	0	0	Fp
178	33D49	1	2		1	0	0	Fp
179	33D49	1	2	1	1	0	0	Fp
180	33D49	1	2		0	1	0	Fv
181	33D49	1	2		0	1	0	Fv
182	33D49	1	2		0	1	0	Fv
183	33D49	1	2		0	1	0	Fv
184	33D49	1	2		0	1	0	Fv
185	33D49	1	2		0	1	0	Fv
186	33D49	1	2		0	1	0	Fv
187	33D49	1	2		0	1	0	Fv
188	33D49	1	2		0	1	0	Fv
189	33D49	1	2		0	1	0	Fv
190	33D49	1	2		0	1	0	Fv
191	33D49	1	2		0	1	0	Fv
192	33D49	1	2		0	1	0	Fv
193	33D49	1	2		0	1	0	Fv
194	33D49	1	2		0	1	0	Fv
195	33D49	1	2		0	1	0	Fv
196	33D49	1	2		0	1	0	Fv
197	33D49	1	2		0	1	0	Fv

198	33D49	1	2		0	1	0	Fv
199	33D49	1	2		0	1	0	Fv
200	33D49	1	2		0	1	0	Fv
201	33D49	1	2		0	1	0	Fv
202	33D49	1	2		0	1	0	Fv
203	33D49	1	2		0	1	0	Fv
204	33D49	1	2		1	0	0	Fp
205	33D49	1	2		1	0	0	Fp
206	33D49	1	2		1	0	0	Fp
207	33D49	1	2		0	1	0	Fv
208	33D49	1	2		1	0	0	Fp
209	33D49	1	2		1	0	0	Fp
210	33D49	1	2		1	0	0	Fp
211	33D49	1	2		0	1	0	Fv
212	33D49	1	2		1	0	0	Fp
213	33D49	1	2		1	0	0	Fp
214	33D49	1	2		1	0	0	Fp
215	33D49	1	2		1	0	0	Fp
216	33D49	1	2		0	1	0	Fv
217	33D49	1	2		1	0	0	Fp
218	33D49	1	2		0	1	0	Fv
219	33D49	1	2		1	0	0	Fp
220	33D49	1	2		0	1	0	Fv
221	33D49	1	2		1	0	0	Fp
222	33D49	1	2		0	0	1	unknown
223	33D49	1	2		1	0	0	Fp
224	33D49	1	2		1	0	0	Fp
225	33D49	1	2		1	0	0	Fp
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605	33D49	2	1		0	1	0	Fv
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612	33D49	2	2		1	0	0	Fp
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653	33D49	2	2		0	1	0	Fv
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969	P1395R	1	1		0	1	0	Fv
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975	P1395R	1	1		1	0	0	Fp
976	P1395R	1	1		0	1	0	Fv

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981	P1395R	1	1		0	1	0	Fv
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983	P1395R	1	1		1	0	0	Fp
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991	P1395R	1	1		0	1	0	Fv
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1707	P1395R	2	3		1	0	0	Fp
1708	P1395R	2	3		1	0	0	Fp
1709	P1395R	2	3		1	0	0	Fp
1710	P1395R	2	3		1	0	0	Fp
1711	P1395R	2	3		0	1	0	Fv
1712	P1395R	2	3		1	0	0	Fp
1713	P1395R	2	3		1	0	0	Fp
1714	P1395R	2	3		1	0	0	Fp

1715	P1395R	2	3		1	0	0	Fp
1716	P1395R	2	3		1	0	0	Fp
1717	P1395R	2	3		1	0	0	Fp
1718	P1395R	2	3		0	1	0	Fv
1719	P1395R	2	3		0	1	0	Fv
1720	P1395R	2	3		0	1	0	Fv
1721	P1395R	2	3		0	1	0	Fv
1722	P1395R	2	3		0	1	0	Fv
1723	P1395R	2	3		0	1	0	Fv
1724	P1395R	2	3		0	1	0	Fv
1725	P1395R	2	3		1	0	0	Fp
1726	P1395R	2	3		0	0	1	unknown
1727	P1395R	2	3		1	0	0	Fp
1728	P1395R	2	3		1	0	0	Fp
1729	P1395R	2	3		1	0	0	Fp
1730	P1395R	2	3		0	1	0	Fv
1731	P1395R	2	3		1	0	0	Fp
1732	P1395R	2	3		1	0	0	Fp
1733	P1395R	2	3		0	1	0	Fv
1734	P1395R	2	3		0	1	0	Fv
1735	P1395R	2	3		1	0	0	Fp
1736	P1395R	2	3		0	1	0	Fv
1737	P1395R	2	3		1	0	0	Fp
1738	P1395R	2	3		1	0	0	Fp
1739	P1395R	2	3		0	0	1	unknown
1740	P1395R	2	3		1	0	0	Fp
1741	P1395R	2	3		1	0	0	Fp
1742	P1395R	2	3		1	0	0	Fp
1743	P1395R	2	3		1	0	0	Fp
1744	P1395R	2	3		1	0	0	Fp
1745	P1395R	2	3		0	1	0	Fv
1746	P1395R	2	3		1	0	0	Fp
1747	P1395R	2	3		1	0	0	Fp
1748	P1395R	2	3		1	0	0	Fp
1749	P1395R	2	3		1	0	0	Fp
1750	P1395R	2	3		1	0	0	Fp
1751	P1395R	2	3		0	0	1	unknown
1752	P1395R	2	3		1	0	0	Fp
1753	P1395R	2	3		1	0	0	Fp
1754	P1395R	2	3		1	0	0	Fp
1755	P1395R	2	3		1	0	0	Fp

1756	P1395R	2	3		1	0	0	Fp
1757	P1395R	2	3		1	0	0	Fp
1758	P1395R	2	3		0	0	1	unknown
1759	P1395R	2	3		1	0	0	Fp
1760	P1395R	2	3		1	0	0	Fp
1761	P1395R	2	3		1	0	0	Fp
1762	P1395R	2	3		1	0	0	Fp
1763	P1395R	2	3		1	0	0	Fp
1764	P1395R	2	3		1	0	0	Fp
1765	P1395R	2	3		0	1	0	Fv
1766	P1395R	2	3		0	1	0	Fv
1767	P1395R	2	3		0	1	0	Fv
1768	P1395R	2	3		0	1	0	Fv
1769	P1395R	2	3		0	1	0	Fv
1770	P1395R	2	3		0	1	0	Fv
1771	P1395R	2	3		0	1	0	Fv
1772	P1395R	2	3		0	1	0	Fv
1773	P1395R	2	3		0	1	0	Fv
1774	P1395R	2	3		0	1	0	Fv
1775	P1395R	2	3		0	1	0	Fv
1776	P1395R	2	3		1	0	0	Fp
1777	P1395R	2	3		1	0	0	Fp
1778	P1395R	2	3		1	0	0	Fp
1779	P1395R	2	3		1	0	0	Fp
1780	P1395R	2	3		0	1	0	Fv
1781	P1395R	2	3		1	0	0	Fp
1782	P1395R	2	3		1	0	0	Fp
1783	P1395R	2	3		1	0	0	Fp
1784	P1395R	2	3		1	0	0	Fp
1785	P1395R	2	3		1	0	0	Fp
1786	P1395R	2	3		0	0	1	unknown
1787	P1395R	2	3		0	1	0	Fv
1788	P1395R	2	3		0	0	1	unknown
1789	P1395R	2	3		0	0	1	unknown
1790	P1395R	2	3		0	0	1	unknown
1791	P1395R	2	3		0	0	1	unknown
1792	P1395R	2	3		0	0	1	unknown
1793	P1395R	2	3		0	0	1	unknown
1794	P1395R	2	3		0	0	1	unknown
1795	P1395R	2	3		0	0	1	unknown
1796	P1395R	2	3		0	1	0	Fv

1797	P1395R	2	3		0	0	1	unknown
1798	P1395R	2	3		0	1	0	Fv
1799	P1395R	2	3		0	0	1	unknown
1800	P1395R	2	3		0	0	1	unknown
1801	P1395R	2	3		0	0	1	unknown
1802	P1395R	2	3		0	0	1	unknown
1803	P1395R	2	3		0	0	1	unknown
1804	P1395R	2	3		0	0	1	unknown
1805	P1395R	2	3		1	0	0	Fp
1806	P1395R	2	3		1	0	0	Fp
1807	P1395R	2	3		0	1	0	Fv
1808	P1395R	2	3		0	0	1	unknown
1809	P1395R	2	3		0	0	1	unknown
1810	P1395R	2	3		0	0	1	unknown
1811	P1395R	2	3		0	0	1	unknown
1812	P1395R	2	3		0	0	1	unknown
1813	P1395R	2	3		1	0	0	Fp
1814	P1395R	2	3		0	1	0	Fv
1815	P1395R	2	3		0	0	1	unknown
1816	P1395R	2	3		0	0	1	unknown
1817	P1395R	2	3		0	0	1	unknown
1818	P1395R	2	3		0	0	1	unknown
1819	P1395R	2	3		0	0	1	unknown
1820	P1395R	2	3		0	1	0	Fv
1821	P1395R	2	3		0	1	0	Fv
1822	P1395R	2	3		0	0	1	unknown
1823	P1395R	2	3		0	0	1	unknown
1824	P1395R	2	3		1	0	0	Fp
1825	P1395R	2	3		1	0	0	Fp
1826	P1395R	2	3		1	0	0	Fp
1827	P1395R	2	3		1	0	0	Fp
1828	P1395R	2	3		1	0	0	Fp
1829	P1395R	2	3		0	1	0	Fv
1830	P1395R	2	3		1	0	0	Fp
1831	P1395R	2	3		1	0	0	Fp
1832	P1395R	2	3		0	1	0	Fv
1833	P1395R	2	3		0	1	0	Fv
1834	P1395R	2	3		0	1	0	Fv
1835	P1395R	2	3		0	1	0	Fv
1836	P1395R	2	3		0	1	0	Fv
1837	P1395R	2	3		0	1	0	Fv

1838	P1395R	2	3		0	1	0	Fv
1839	P1395R	2	3		0	1	0	Fv
1840	P1395R	2	3		1	0	0	Fp
1841	P1395R	2	3		1	0	0	Fp
1842	P1395R	2	3		0	1	0	Fv
1843	P1395R	2	3		0	1	0	Fv
1844	P1395R	2	3		0	1	0	Fv
1845	P1395R	2	3		0	1	0	Fv
1846	P1395R	2	3		0	1	0	Fv
1847	P1395R	2	3		1	0	0	Fp
1848	P1395R	2	3		0	1	0	Fv
1849	P1395R	2	3		0	1	0	Fv
1850	P1395R	2	3		0	1	0	Fv
1851	P1395R	2	3		0	0	1	unknown
1852	P1395R	2	3		0	0	1	unknown
1853	P1395R	2	3		0	0	1	unknown
1854	P1395R	2	3		0	0	1	unknown
1855	P1395R	2	3		1	0	0	Fp

Table 5.9 Number of *F. proliferatum* and *F. verticillioides* isolates cultured from kernels of hybrids 33D49 and P1395R from each plot and subplot.

Both Hybrids	**Plot 1**		**Plot 2**		**Total per hybrid**		**Total**
	33D49	P1395R	33D49	P1395R	33D49	P1395R	
Total *F. proliferatum*	206	210	199	202	405	412	817
Total *F. verticillioides*	181	190	199	181	380	371	751
Total Unknown	67	70	62	88	129	158	287
Total	454	470	460	471	914	941	1855

Maize Hybrid 33D49	**Plot 1**			**Plot 2**			**Total**
	Subplot 1	Subplot 2	Subplot 3	Subplot 1	Subplot 2	Subplot 3	
Total *F. proliferatum*	61	85	60	63	67	69	405
Total *F. verticillioides*	67	64	50	79	55	65	380
Total Unknown	19	5	43	9	39	14	129
Total	147	154	153	151	161	148	914

Maize Hybrid P1395R	**Plot 1**			**Plot 2**			**Total**
	Subplot 1	Subplot 2	Subplot 3	Subplot 1	Subplot 2	Subplot 3	
Total *F. proliferatum*	72	73	65	63	67	72	412
Total *F. verticillioides*	58	51	81	51	77	53	371
Total Unknown	23	27	20	34	17	37	158
Total	153	151	166	148	161	162	941

Table 5.10 Eight primer combinations were tested with 6 isolates for use in selective amplification in the AFLP assay.

Primer Combinations	**5'-HEX-EcoRI**	
5'-MseI	AA-3'	TG-3'
TT-3'	*1*	*5*
CA-3'	*2*	*6*
CC-3'	*3*	*7*
CT-3'	*4*	*8*

Table 5.11 Number of alleles found in each of the 6 different isolates (5 *F. proliferatum* (Fp) and 1 *F. verticillioides* (Fv)) tested.

	Primer Combination							
Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Fp-70-15-3	178	154	146	158	134	162	157	156
Fp-49-16-4	152	174	205	174	119	159	147	165
Fp-95-8-4	197	147	197	183	176	172	155	152
Fp-47-15-5	201	198	178	197	179	156	165	161
Fp-49-19-5B	205	175	161	173	156	194	161	160
Fv-73-26-4B	147	168	177	163	160	155	146	169
Total Alleles	480	385	415	410	388	391	363	385

Table 5.12 Error rate estimation in 3 AFLP runs was found to average 6.36%.

Isolate Fp-49-16-4	Same bands	Mismatched bands	% Error rate
Run 1	231	10	4.15
Run 2	216	25	10.37
Run 3	230	11	4.56

Table 5.13 Summary of analysis of molecular variance (AMOVA) of *F. proliferatum* and *F. verticillioides* isolates. Probability for ΦPT is based on standard permutation across the full data set. ΦPT = AP / (WP + AP) = AP / TOT (where AP, Est. Var among populations; WP, Est. Var. within populations). Levels of significance are based on 999 iterations. ΦPT is significant (p-value 0.001) which suggests the two populations (*F. proliferatum* and *F. verticillioides*) are distinct.

Source	df	SS	MS	Est. Var.	%	Stat	Value	p-value
Among Pops	1	948.979	948.979	28.143	52%			
Within Pops	94	2400.084	25.533	25.533	48%			
Total	95	3349.063		53.676	100%	PhiPT	0.524	0.001

Table 5.14 Summary of analysis of molecular variance (AMOVA) of *F. proliferatum* isolates. Levels of significance are based on 999 iterations. ΦPT is significant (p-value 0.001) which suggests the two populations are distinct.

Source	df	SS	MS	Est. Var.	%	Stat	Value	p-value
Among Pops	1	415.140	415.140	11.832	36%			
Within Pops	73	1513.420	20.732	20.732	64%			
Total	74	1928.560		32.564	100%	PhiPT	0.363	0.001

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Chapter 6 - **Conclusions and Future Directions**

The objective of this investigation was to improve our understanding of the life cycle of *Fusarium proliferatum*, specifically, the fate of seed-borne strains subsequent to introduction into new environments, to learn about the invasion process and the potential for establishment and dissemination. Knowledge gained from this research should allow the estimation of risk associated with the introduction of exotic strains in seed into new environments. It is important to acknowledge the function of seeds as vectors of pests and pathogens.

In Chapter 3, the results demonstrated that seed-borne *F. proliferatum* can actively grow from a source of inoculum through non-sterile soil to colonize available organic matter within a 1.5 cm sphere of the source. Growth through soil and colonization of organic matter by *F. proliferatum* occurred over a temperature range of 10°C to 35°C with an optimum of 25°C. Colonization of organic matter was linearly related to both the source-to-bait distance and the soil water matric potential; the percentage colonization decreased as the distance increased and as the soil matric potential decreased. Soil structure was also important; the percentage colonization was less in soils with an intact structure compared to sieved reconstituted soils. Future experiments to increase the knowledge of the impact of environmental conditions in the active dispersal and colonization of organic matter by *F. proliferatum* could include the control of other abiotic conditions such as soil pH and soil composition (sand, silt and clay percentages). Moreover, different sources of organic matter (e.g. other plant species) could be used as baits to learn about the colonization capabilities and limitations of *F. proliferatum*. Also, transformation of additional *F. proliferatum* strains with different traits (e.g. aggressiveness to different hosts, mycotoxin production, mycotoxin profile) could help us understand the colonization as a function of those traits and weigh their importance. This research found that *F. proliferatum* actively dispersed at least 1.5 cm from a source of inoculum to a bait, and it would be important to learn if this fungus could disperse longer distances if baits are introduced at spatial and temporal intervals. This experiment would lead to the investigation of "chain-colonization", in which the colonization from a source of inoculum to bait A, and then from bait A (new source of inoculum) to bait B, and then from bait B (new source of inoculum) to bait C, etc. The active

dispersal research will help generate more complete models about the risk of *F. proliferatum* active dispersal in field conditions.

The research in Chapter 4 demonstrated that *F. proliferatum* can effectively compete with the microflora naturally found in the same niche, including *F. verticillioides* and microorganisms naturally occurring in maize seed. Roots were more colonized than stem (qPCR quantification) and tissue segments of the maize plant that were closer to the seed were more colonized than segments that were more distant. In all competition events, the species present in the plant first had a colonization advantage over the challenging species. Future experiments should include intraspecific competition between different strains of *F. proliferatum* for a niche in the maize plant using similar methods. This research would determine the significance of competition among strains with different aggressiveness and mycotoxin production and profile to identify traits that impact colonization of the plant. Similar interspecific and intraspecific competition experiments could be performed using other hosts including wheat and sorghum which are hosts of these two fungal species and also important commodities. Furthermore, an additional experiment could include the control of environmental variables such as temperature and soil matric potential to analyze how they impact the ability of the isolates to colonize the host plant and if they provide advantages or disadvantages to the resident or to the challenging species.

Maize seed-borne *F. proliferatum* was transmitted to the kernels in the newly formed ear under field conditions (Chapter 5). This suggests that *F. proliferatum* strains, which can include high mycotoxin producers or highly aggressive strains, can disseminate from the planted seed into the new seed to be used in following planting seasons at distant locations. Additionally, considering global trade of commodities, these seeds can be introduced into new environments without consideration of its potential to be a vector of *F. proliferatum* strains. Moreover, once the strains are disseminated into the newly formed ear, they can be maintained as debris within the field during the harvest process. They can be further spread by other means such as insect-vectors. Future experiments should include the use of Illumina HiSeq sequencing of *F. proliferatum* isolates for genotyping-by-sequencing (GBS) to get more coverage depth, better representation of the genome and a higher number of reads. This would identify a higher number of polymorphisms that would allow tracking of multiple isolates simultaneously in field

experiments. A similar approach could be used with *F. verticillioides*. Moreover, establishment of the GBS-characterized *F. proliferatum* isolates could be monitored in the field in subsequent seasons. This could be done not only with maize but also with other hosts of *F. proliferatum* that are commonly used in crop rotation such as soybeans.

The trade of food, plant, and animal products has increased the worldwide movement and establishment of exotic pathogens with dramatic negative impacts on plant systems as it was documented with examples in Chapter 1. *Fusarium proliferatum* is a broad host-range pathogen and among the most common maize pathogens globally. Therefore, it is critical to understand the life cycle of *F. proliferatum* to evaluate the risk of strains of *F. proliferatum* becoming invasive in new environments. Most invasion research is done at the species level; however, because *F. proliferatum* has worldwide distribution the greater risk is the introduction of exotic strains.