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MYCOTOXICOLOGY TO ACCOMPANY PHYLOGENETIC REVISIONS TO THE GENUS *FUSARIUM*

A Thesis in

Plant Pathology

by

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ABSTRACT

Fusarium species traditionally have been and still are problematic to identify using morphology. This is an issue of concern since many fusaria are toxigenic, producing such toxins as trichothecenes, fumonisins and zearalenone. Fumonisins are sphingolipid analogues associated primarily with the *Gibberella fujikuroi* species complex (GFC). *Fusarium* trichothecenes are sesquiterpenoid mycotoxins, and are generally divided into two categories: type A, which lack oxygen at the C-8 position and include T-2 toxin and diacetoxyscirpenol, and type B, which include nivalenol and deoxynivalenol. Zearalenone is an estrogenic mycotoxin. Phylogenetically characterized isolates were subjected to mycotoxin analysis via high performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS), HPLC and electrospray ionization mass spectrometry (HPLC/ESI-MS), or HPLC using fluorescence detection. Isolates with previous reports of toxin production outside of the groups analyzed were also included in the mycotoxin analyses. Our results reveal scattered toxin production within the GFC (fumonisin) and the trichothecene-producing fusaria (trichothecenes and zearalenone), as well as by a few isolates outside of these groups. Phylogenetic placement in either group is predictive of toxin production potential, but not toxin production, level or toxin class (type A or type B trichothecenes).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1: Introduction and Literature Review

I. Literature Review

A. History of *Fusarium* Taxonomy

The members of the fungal genus *Fusarium* have been a chronic threat to the consumers of agriculturally important crops, such as wheat, barley, and corn, throughout history. In addition to being pervasive plant pathogens, fusaria produce numerous toxic metabolites termed mycotoxins. Due to the threat mycotoxins pose to both agriculture and human and animal health, *Fusarium* toxicology has been of distinct interest to plant pathologists. Traditionally, it has been a challenging subject for many reasons, the principal problem being that species of *Fusarium* are extremely difficult to identify morphologically.

This project, however, presents a novel approach to *Fusarium* toxicology using newly developed phylogenetic species concepts to systematically determine mycotoxin production within two groups of *Fusarium:* (1) the organisms that make up the *Gibberella fujikuroi* species complex and other closely related species and (2) the trichothecene-producing fusaria. Since the phylogenetic framework used in this project differs from more traditional systems based upon morphology, a summary of previous reports is necessary.

Wollenweber and Reinking (1935) presented a taxonomic system to deal with the fusaria. This system addressed what was then approximately 1000 described species and organized them into 65 species, 55 varieties, and 22 forms, mostly through the recognition of synonyms. Characters used to differentiate fusaria were the presence or absence of macroconidia and the presence, absence, and/or position of chlamydospores.

Macroconidium morphology, sclerotium presence or absence, and stroma color, where available, were also used to clarify taxonomic position.

Snyder and Hansen (1940, 1941, 1945) presented an alternate look at the genus *Fusarium*. They took Wollenweber and Reinking's 65 species and reduced them down to nine species. Their determinations were made primarily by using morphology of macroconidia. One of the main arguments against Wollenweber and Reinking's system was that their concept allowed for little variation within a species, and that some recognized species were really cultural variants. Snyder and Hansen addressed this by performing a detailed study of variation within the fusaria to aid their taxonomic system.

Booth (1971) took a less extreme approach when he proposed a revision to *Fusarium* taxonomy. His system had fewer species than Wollenweber and Reinking, but still significantly more than Snyder and Hansen's approach. Booth proposed 44 species, using differences in conidiogenous cells as important characteristics, reflecting the contemporary trend in using developmental characters in the systematics of fungal anamorphs(Kendrick, 1971).

Nelson, Toussoun, and Marasas (1983) presented yet another system to deal with fusaria, performing what was essentially a conservative expansion of Snyder and Hansen's system. They ended up cataloguing 30 species, plus an additional 16 species viewed as having insufficient documentation to warrant firm support.

Since most previous research has been performed using morphological identification, as per the systems mentioned above, reports of toxin production in the primary literature cannot be relied upon. Many toxicology researchers have not had adequate training in *Fusarium* species identification, and thus report toxin production

from species that may or may not produce those toxins. One further confounding issue is that recent molecular data have shown that morphological species concepts within *Fusarium* vastly underestimate the true diversity present in this genus.

Our current understanding of *Fusarium* biology represents a sum of many decades of knowledge including the sum of all of the changes that have occurred over that time. Isolates with particular toxicological characteristics were identified to species, often incorrectly, using one of these previous morphological taxonomic systems, any of which may have been considered state-of-the-art at the time, but are now known to be outmoded.

B. New Phylogenetic Concepts in *Fusarium*

The identification of *Fusarium* species associated with particular mycotoxins has been extremely problematic due to the general instability in *Fusarium* taxonomy, coupled with inconsistent reports of toxin production associated with particular isolates. Marasas *et al*. (1984) dealt with this problem broadly by confirming and debunking previous reports of mycotoxin production in a wide spectrum of *Fusarium* species, adopting specific morphological criteria for species identification (Nelson *et al*., 1983). However, since the publication of that important volume, *Fusarium* taxonomy has undergone a revolution due to the influence of molecular phylogenetics. This has led to a major expansion in the number of recognized species and reorganization of groups of species around the concept of clades. These advances necessitate a re-evaluation of associations

between the production of specific mycotoxins and taxa newly recognized within the genus *Fusarium* based on phylogenetic criteria.

Examples of fusaria that have had varying taxonomic histories and reports of toxin production include *Fusarium nivale* and *F. graminearum*. In some cases, isolates and their associated toxins have a checkered taxonomic history because of changing and incorrect taxonomic recognition. For example, the toxin nivalenol was initially reported as being produced by *F. nivale* (Tatsuno *et al.,* 1968). *Fusarium nivale* has since been moved to the genus *Microdochium* (Samuels and Hallett, 1983). To further complicate matters, the initial toxin report was based on a misidentification, and the fungus actually producing the toxin was then identified as *F. tricinctum* and *F. sporotrichioides* by two independent researchers. This also was incorrect, as molecular phylogenetics has now placed this organism as the new species *F. kyushuense* (Aoki and O'Donnell, 1998). In other cases, what appears to be a single species morphologically can often represent a complex of species. The causal agent of head scab of wheat, *F. graminearum* (teleomorph *Gibberella zeae*), has recently been shown to be, in fact, a clade of many species, nine of which have been described (O'Donnell *et al.,* 2004). The toxin-production profiles of these species are still being determined. These two cases clearly illustrate the need to combine molecular phylogenetic data with toxicological data to obtain a realistic profile of a species and its toxin-producing capabilities.

Fusarium moniliforme is an example of a problematic species with varying toxin production reported. The name fumonisin is derived from the species name *F. moniliforme*, yet the species itself is no longer recognized. It has been determined that

this name represents a complicated and diverse species complex. Within this complex, some species are fumonisin producers, and others are not.

In an effort to standardize and make publicly available resources to aid in correct identification of *Fusarium* species, Geiser *et al.* (2004) have developed a public database called Fusarium-ID. v.1.0. This database consists of partial DNA sequences for the translation elongation factor 1-α gene of various and diverse *Fusarium* species. This allows researchers to more correctly identify isolates of *Fusarium* with minimal work (generating PCR products and sequencing) (O'Donnell *et al.,* 1998a).

One goal of this project is to provide a similar publicly available database of toxin production and strains and species associated with them. These data will be added to the information held in the Fusarium-ID database.

C. *Fusarium* Mycotoxins

i. Fumonisins

Fumonisins are aminopolyalcohols that are produced by some species of *Fusarium.* Chemically, the fumonisins consist of a 20-carbon backbone, with methyl groups at carbons 12 and 16, an amino group at carbon 2, and two tricarballylic esters at carbons 14 and 15 (see Fig. 1.1). There are three series of fumonisins, A, B and C, with multiple forms in each class (e.g., B_1, B_2).

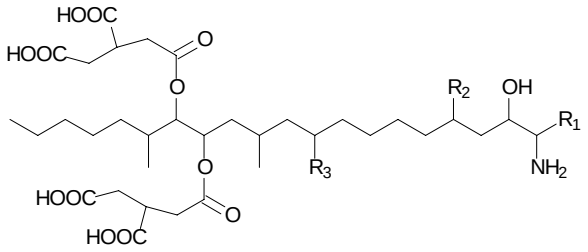
Fumonisins were first discovered in South Africa in 1988, after an investigation of the disease equine leukoencephalomalacia (ELEM) associated with *F. verticillioides,* then recognized as *F. moniliforme* (Marasas, 2001). There was also observed during this time an association with increased risk of esophageal cancer in humans and consumption

of *F. verticillioides*-infected corn. The discovery of the toxins involved an 18-year search for the compound(s) associated with the disease, and also implicated in cancer in rats. After the discovery of the fumonisins, it was quickly determined that they were the cause of ELEM, as well as porcine pulmonary edema. When it was also found that fumonisins cause cancers in rats, the discovery was justification for a more complete analysis of the cancer-causing potential of this toxin. This analysis was performed (IARC, 1993), and the study resulted in the classification of the fumonisins as Group 2B carcinogens (i.e., *possibly carcinogenic to humans*).

Fumonisins also have been implicated in neural tube defects (NTD) in mammals. They have been shown to induce NTD and craniofacial defects in cultured mouse embryos (Marasas *et al*., 2004). In addition, a correlation between incidence of NTD and consumption of fumonisin-contaminated foodstuffs has been shown. In areas of high fumonisin consumption, there is higher than normal incidence of NTD (Marasas *et al*., 2004).

Fumonisins exist naturally in numerous chemical forms. The most common of these are the B-series fumonisin, and, of those, the toxins fumonisin B_1 and B_2 are the most often encountered in foods and feeds.

Fig. 1.1: The Chemical Structure of Select Fumonisins



Fumonisin Analog	R_1	R_2	R_3	Molecular Weight
FB_1	CH_3	OH	OH	721
FB_2	CH_3	OH	H	705
FB_3	CH_3	H	OH	705
FC_1	H	OH	OH	707
FC_2	H	OH	H	691
FC_3	H	H	OH	691
FC_4	H	H	H	675

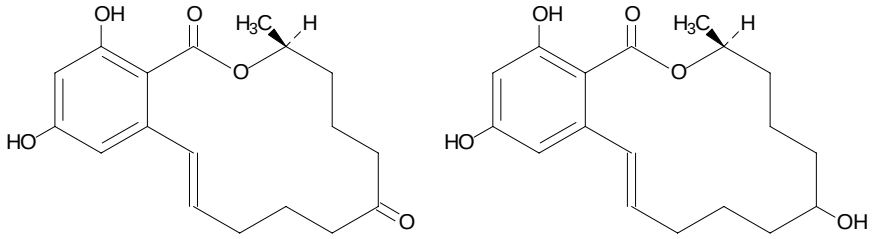
Based upon Sewram *et al.* 2005.

ii. Zearalenone

Zearalenone (chemical name: 6-[(10S)-10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcyclic acid lactone; see Fig. 1.2) is an estrogenic mycotoxin, meaning that it elicits effects similar to those of mammalian estrogen. A zearalenone analog, zearalenol, exhibits similar effects, though the compound is active at lower levels (Mirocha *et al*., 1978). In 1927 and 1928, there were the first indications that uterotrophic responses could be induced in pigs when they were fed moldy corn (Hagler, 2001). The causative agent of this syndrome was later identified as zearalenone (Urry *et al.,* 1966). This toxin

is extremely heat-stable, and survives most cooking and processing methods intact and unchanged.

Fig. 1.2: The Chemical Structures of Zearalenone (left) and Zearalenol (right)



iii. Trichothecenes

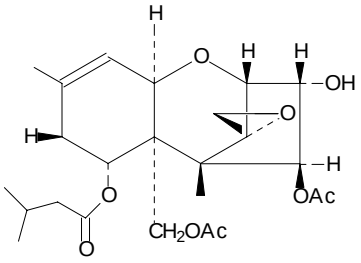
Trichothecenes are a large group of sesquiterpenes produced by hypocrealean (Phylum: Ascomycota) fungi, including certain species of *Fusarium*. These fungi naturally occur on living cereal crops and act as decomposers in agricultural debris. Grain crops such as wheat and barley are often infected with these fusaria and are contaminated by trichothecenes and other toxins. This occurs as the fungi colonize and degrade the plants. Fusaria are the causal agents of many plant diseases, including head scab of wheat and *Gibberella* ear rot of corn. There are several studies indicating that trichothecenes are involved in the process of plant pathogenesis or influence disease severity (Proctor *et al.,* 1995; Proctor *et al.,* 2002).

Trichothecenes inhibit protein translation by direct binding to the ribosome (Cannon *et al.,* 1976). Mammalian exposure to trichothecenes results in immunosupression and disturbances of the gastrointestinal system such as diarrhea, vomiting, and ulceration, among other effects. Members of the genus *Fusarium* produce

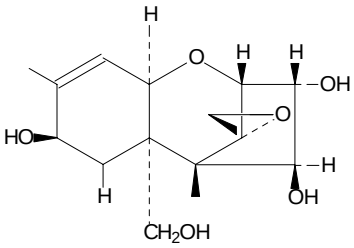
two classes of trichothecenes, referred to as type A (e.g., diacetoxyscirpenol, T-2 toxin, T-2 tetraol, HT-2 toxin, and neosolaniol) and type B (e.g., deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenon-X). These types differ at the C-8 position, where a keto group is found in the type B trichothecenes (see Figs. 1.3 and 1.4). Type A trichothecenes are generally considered more toxic than type B and can cause blistering upon contact with skin or mucous membranes.

The first report of isolation of a trichothecene was in 1948 (Freeman and Morrison, 1948) during a survey for antifungal compounds produced by molds. It is likely, however, based upon descriptions of the physical ailments associated with contact of skin to culture filtrates, that Brian and McGowan (1946) had actually discovered trichothecenes in a similar search. The next flurry of activity on trichothecenes appears in the 1960s, when researchers discovered and characterized diacetoxyscirpenol (Brian *et al.,* 1960), fusarenon-X (Ueno *et al.,* 1962), T-2 toxin (Bamburg *et al.,* 1968), and HT-2 toxin (Bamburg and Strong, 1969).

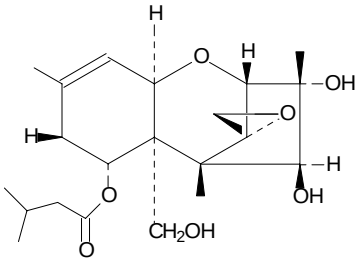
Fig. 1.3: The Chemical Structures of Five Type A Trichothecenes



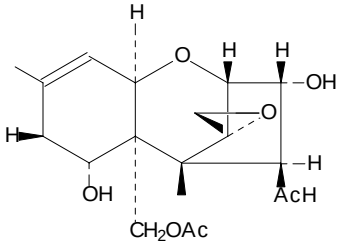
T-2 Toxin



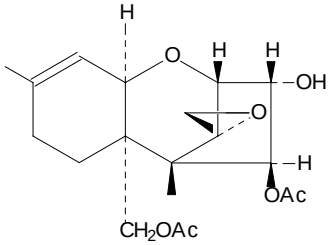
T-2 Tetraol



HT-2 Toxin

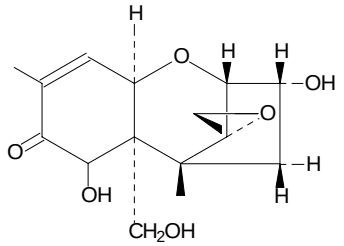


Neosolaniol

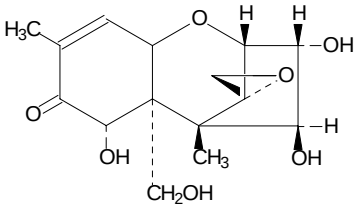


Diacetoxyscirpenol

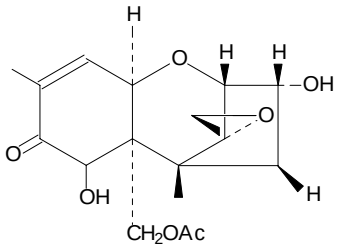
Fig. 1.4: The Chemical Structures of Five Type B Trichothecenes



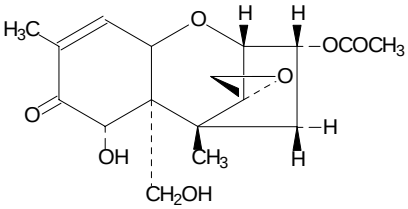
Deoxynivalenol



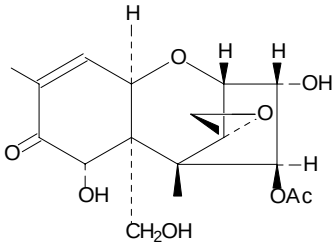
Nivalenol



15-acetyl-Deoxynivalenol



3-acetyl-Deoxynivalenol



Fusarenon-X

D. Mycotoxicology of *Fusarium*

Fumonisin production has been reported from numerous species within the genus *Fusarium*. Most of these fall into the group known as the *Gibberella fujikuroi* species complex, though there are a few reports from species outside of this group. The reports of fumonisin production from fusaria are summarized in Table 1.1.

Table 1.1: *Fusarium* Species with Reported Fumonisin Production

Fungal species	Reported toxins	References
F. acutatum	FB_1, FB_2, FB_3[1]	Norred *et al.* unpublished, Fotso *et al.*, 2002
F. andiyazi	FB_1	Rheeder *et al.*, 2002
F. annulatum	FB_1, FB_2, FB_3	Norred *et al.* unpublished
F. anthophilum	FB_1	Nelson *et al.*, 1992
F. begoniae	FB_1	Fotso *et al.*, 2002
F. brevicatenulatum	FB_1	Fotso *et al.*, 2002
F. dlaminii	FB_1	Nelson *et al.*, 1992
F. fujikuroi	FB_1	Desjardins *et al.*, 2000a
F. globosum	FB_1, FB_2, FB_3	Syndenham *et al.*, 1997
F. napiforme	FB_1	Nelson *et al.*, 1992
F. nygamai	FB_1, FB_2	Thiel *et al.*, 1991
F. oxysporum	C-series fumonisins	Seo *et al.*, 1996
F. oxysporum var. redolens	FB_1, FB_2, FB_3	Abbas, 1995
F. polyphialidicum	FB_1	Abbas, 1995
F. proliferatum	FB_1, FB_2, FB_3	Nelson *et al.*, 1992
F. phyllophilum	FB_1	Fotso *et al.*, 2002
F. pseudocircinatum	FB_1, FB_2	Fotso *et al.*, 2002
F. pseudonygamai	FB_1, FB_2	Leslie *et al.*, 2005
F. ramigenum	FB_1, FB_2, FB_3	Norred *et al.* unpublished
F. subglutinans	FB_1	Desjardins *et al.*, 2000b
F. sacchari	FB_1	Leslie *et al.*, 1992
F. thapsinum	FB_1	Klittich *et al.*, 1997
F. verticillioides	FB_1, FB_2, FB_3, FB_4	Gelderblom *et al.*, 1988

FB_1= Fumonisin B_1, FB_2= Fumonisin B_2, FB_3= Fumonisin B_3, FB_4= Fumonisin B_4

The reports of trichothecene production within the fusaria are numerous and convoluted. The commonplace misidentification of species has resulted in numerous reports that cannot be relied upon. Table 1.2 represents a summary of the reports of trichothecene production within the genus.

Table 1.2: *Fusarium* Species Within the Trichothecene-Producing Clade and Their Reported Trichothecene and Zearalenone Production

Fungal species	Reported toxins	References
F. acaciae-mearnsii	DON, 15-ADON, NIV[1]	O'Donnell *et al.* 2000
F. acuminatum	T2-4ol	Wing *et al.* 1993b
F. armeniacum	T2-4ol, T2, HT2	Wing *et al.* 1993b Moss *et al.* 2004
F. asiaticum	DON, 3-ADON, NIV, ZEA	O'Donnell *et al.* 2000
F. austroamericanum	DON, 3-ADON, NIV, ZEA	O'Donnell *et al.* 2000
F. avenaceum	ZEA	Langseth *et al.* 1999
F. boothii	DON, ZEA	O'Donnell *et al.* 2000
F. brachygibbosum	no reports	
F. brasilicum	no reports	
F. cerealis	NIV, ZEA	O'Donnell *et al.* 2000
F. crookwellense	NIV	Moss *et al.* 2004
F. culmorum	DON, 15-ADON, NIV, FUS-X, ZEA	Mirocha *et al.* 1994 Moss *et al.* 2004
F. equiseti	NIV, DAS, NEO, ZEA	Morrison *et al.* 2001 Moss *et al.* 2004
F. graminearum	DON, 3-ADON, 15-ADON, NIV, FUS-X, ZEA	Mirocha *et al.* 1994 O'Donnell *et al.* 2000
F. kyushuense	NIV	Moss *et al.* 2004
F. langsethiae	DAS, T2, HT2, NEO, T2-4ol	Thrane *et al.* 2004
F. longipes	no reports	
F. lunulosporum	ZEA	O'Donnell *et al.* 2000
F. meridionale	NIV, ZEA	O'Donnell *et al.* 2000
F. mesoamericanum	DON, ZEA	O'Donnell *et al.* 2000
F. musarum	T2, HT2, DAS	Moss *et al.* 2004
F. poae	NIV, FUS-X, DAS, T2, HT2, NEO, T2-4ol	Thrane *et al.* 2004 Moss *et al.* 2004

F. pseudograminearum	DON, 3-ADON, ZEA	Moss *et al.* 2004 O'Donnell *et al.* 2000
F. sambucinum	DAS, NEO, T2, HT2, ZEA	Marasas *et al.* 1984 Moss *et al.* 2004
F. sporotrichioides	T2, T2-4ol, DON (1 report), DAS, Nivalenol diacetate, FUS-X, HT2, NEO, NIV, ZEA	Marasas *et al.* 1984 Moss *et al.* 2004
F. tumidum	NEO, T2, HT2, DAS	Altomare *et al.* 1995 Moss *et al.* 2004
F. venenatum	DAS, NIV	Moss *et al.* 2004 O'Donnell *et al.* 1998a

[1]Type-B trichothecenes: DON=deoxynivalenol, NIV=nivalenol, 3-ADON=3-acetyldeoxynivalenol, 15-ADON=15-acetyldeoxynivalenol, FUS-X=fusarenon-X
Type-A trichothecenes: T2=T-2 toxin, T2-4ol=T-2 tetraol, HT2=HT-2 toxin, DAS=diacetoxyscirpenol, NEO=neosolaniol

i. Regulation of Toxin Production

The regulation of mycotoxin production has been of considerable interest recently. There are two main foci to the investigation of mycotoxin regulation: environmental conditions eliciting toxin production and the molecular genetic basis of toxin biosynthesis. While these two factors are inexorably linked, the approach used to examine each is decidedly different. The study of environmental conditions is more important to this project, as the conditions under which the isolates are grown certainly influences toxin production.

Numerous researchers have studied the environmental factors influencing fumonisin production. Shim and Woloshuk (1999) demonstrated that the presence of high concentrations of nitrogen in the media used to grow fumonisin-producing strains resulted in a repression of production. Keller *et al.* (1997) demonstrated that fumonisin

production by *F. proliferatum* was highest at pH between 5.0 and 2.5 under well-aerated conditions. Alberts *et al.* (1990) showed that at 25°C, fumonisin production by '*F. moniliforme'* was at its highest.

Multiple genes that appear to play a regulatory function in the production of fumonisins have been identified. The gene *PAC1* has an apparent role in repressing fumonisin production under alkaline conditions (Flaherty *et al.,* 2003). *FCC1* is a cyclin-like gene that seems to be involved in translational activation/repression. Mutants of *F. verticillioides* with disrupted *FCC1* do not produce fumonisins on maize kernels, but will produce fumonisin on acidified media (Shim and Woloshuk, 2001). Flaherty and Woloshuk (2004) have identified a third gene, *ZFR1*, which appears to act as a positive regulator of fumonisin biosynthesis. This gene has sequence similarity to the family of genes of the zinc binuclear cluster type.

Shim, Flaherty, and Woloshuk (2003) have shown that more fumonisins are produced in the degermed portion of a kernel of corn. After growth on the degermed material, the pH was lowered, and fumonisin production was high. In the germ portion, the pH increased, and there was little fumonisin production. This observation was consistent with the genetics work described above.

Research on the influence of cultural conditions on zearalenone production is confusing at best. Differences in production are commonly noted for isolates grown under different temperature regimes (Eugenio *et al.,* 1970; Di Menna *et al.,* 1991). There are no obvious trends evident concerning temperature and zearalenone production, but there does seem to be some correlation with cold temperature stress and an increase in zearalenone production, though this varies by species and isolate (Naik *et al.,* 1978). In

1970, an isolate then called *Fusarium roseum* (actual species unknown) grown on rice at 60-65 percent moisture at 15°C produced the highest levels of zearalenone (Eugenio *et al.*). *Fusarium cerealis* (herein called *F. crookwellense*) grown on rice at 16-29°C in daylight produced higher levels than in the dark at 18-23°C or at constant temps of 11°C, 20°C, or 25°C in the dark (Menna *et al.,* 1991).

The regulation of trichothecene production is extremely complicated, and the mechanisms and conditions supporting production are in most cases unclear. An overview of the regulation of trichothecenes is presented by Desjardins *et al.* (1993). Trends are difficult to discern in the published research. The most useful trend may be that there are no set conditions that favor trichothecene production in all species or strains. Variation in production, both in amounts and diversity of toxins produced, vary greatly by species, strain, and sometimes even replicate.

ii. Biosynthesis

The media upon which the strains are grown (rice cultures) has a direct influence on the nutrients available to the fungus for biosynthetic pathways, including mycotoxin production. The physical characteristics of the media (water content, pH, etc.) may also play a role in toxin biosynthesis.

The biosynthesis of fumonisins has not been completely elucidated to date. Feeding studies have shed light on the precursor molecules of fumonisins, including methionine, alanine, acetate, and molecular oxygen (Blackwell *et al.,* 1994; Branham and Plattner, 1993; Plattner and Shackleford, 1992; Caldas *et al.,* 1992). The fumonisins are

polyketide mycotoxins, and the polyketide synthetase gene required for fumonisin production has been identified (Proctor *et al.,* 1999). Many of the components of fumonisin biosynthesis, as is the case in numerous other fungal secondary metabolites, are encoded in a gene cluster. This cluster contains at least 15 genes, including the polyketide synthetase (Proctor *et al.,* 2003).

Zearalenone is also a polyketide and its biosynthesis involves the incorporation of nine acetate molecules. The acetate is incorporated in an every-other-carbon fashion, such that nine of the carbons originate from acetate, and nine carbons are from another source (Blackwell *et al.,* 1985).

Trichothecenes are sesquiterpene mycotoxins. The first precursor for the biosynthesis of trichothecenes is farnesyl pyrophosphate. This molecule is modified by the gene product of TRI-5 into trichodiene in the first committed step of trichothecene biosynthesis (Hohn and Middlesworth, 1986). Trichodiene is then modified in a series of steps (at least nine) into the compound calonectrin. Calonectrin can then be modified in two ways, one of which leads to the type A trichothecenes, and the other to type B trichothecenes. If calonectrin is modified by the gene product of TRI-13, it is converted into 3,15-DAS, a type A trichothecene (Lee *et al.,* 2002). Calonectrin can also be modified by another enzyme, TRI-1, to be converted into 8-hydroxycalonectrin, a type B trichothecene (McCormick *et al.,* 2004). A review of trichothecene biosynthesis has been performed by Desjardins *et al.* (1993).

E. Mycotoxin Detection and Quantification

i. Trichothecenes

The analysis of trichothecene toxins has been performed in most cases using gas chromatography (GC) followed by electron capture or mass spectrometry (MS) as a detection method (Koch, 2004). Less frequently used in trichothecene analysis is high-pressure liquid chromatography (HPLC) followed by MS or fluorescence detection. Thin layer chromatography (TLC) has been employed in the past, but seems to have been utilized less as GC and HPLC methods have been developed (Koch, 2004).

GC/MS has been employed in the analysis of trichothecenes. The method often used is electron impact mass spectrometry (GC/EI-MS) (Rodrigues-Fo *et al.,* 2002; Tanaka *et al.,* 2000). Gas chromatography of trichothecenes is a somewhat tedious process as compared to LC/MS, involving derivatization of samples with N-trimethylsilyl-imidazole and trimethylchlorosilane (Rodrigues-Fo *et al.,* 2002).

Liquid chromatography (LC) has also been employed using various methods to analyze these toxins. Atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) and electrospray ionization mass spectrometry (LC/ESI-MS) were compared under various conditions by Lagana *et al.* (2003). This study determined LC/ESI-MS to be a slightly better method than LC/APCI-MS, having lower detection limits.

A more complete analysis of eight trichothecenes plus zearalenone has been performed using HPLC-tandem MS by Berthiller *et al.* (2005). The method employed in this study was HPLC/APCI-MS/MS. While the study by Lagana *et al.* (2003) showed

HPLC/ESI-MS to be better suited to trichothecene analysis, it should be noted that this study only analyzed four type B trichothecenes. Berthiller *et al.* (2005) suggests that for mixtures of type A and type B trichothecenes LC/APCI-MS is preferable. A thorough review of methods used historically and their utility has been compiled by Koch (2004).

ii. Fumonisins

High-pressure liquid chromatography (HPLC) with fluorescence detection of OPA-derivatized samples has been used for detection and quantification of fumonisins (Sydenham *et al.,* 1996). Other methods also have been used with some success for the detection and quantification of fumonisin. Akiyama *et al.* (1998) described a variation of Syndenham's method involving LC/MS with a post-HPLC column derivatization. Preis and Vargas (2000) describe a method utilizing an immunoaffinity cleanup followed by TLC. Abouzied and Pestka (1994) have utilized a line immunoblot assay to detect and quantify fumonisins, aflatoxins, and zearalenone.

iii. Zearalenone

Zearalenone is often detected using methods that can also detect other *Fusarium* mycotoxins. The most common of these techniques (based upon available literature) is HPLC, which is sometimes coupled with mass spectrometry. Berthiller *et al.* (2005) described a method that can detect zearalenone, type A, and type B trichothecenes simultaneously using HPLC-MS. Another common method of detecting zearalenone is

through the use of TLC. Numerous researchers have employed this method, as it is relatively fast and inexpensive when compared to HPLC or HPLC-MS (Quiroga *et al.*, 1994; Jimenez *et al.*, 1996; Blaney *et al.*, 2002). However, there are potential problems with using TLC to detect zearalenone, as other compounds may sometimes interfere with its detection. Smith *et al.* (2004) described the interference of bostrycoidin, an analog of zearalenone, in TLC analyses. Abouzied and Pestka (1994) have utilized a line immunoblot assay to detect and quantify fumonisins, aflatoxins, and zearalenone.

II. Research Objectives

A. Project Rationale

The state of knowledge with regard to mycotoxin production by members of the genus *Fusarium* is tenuous at best. Misidentifications based on morphology have led to a plethora of incorrect reports of toxin production. Confounding this issue, many new lineages recently have been identified within groups of organisms previously thought of as one species. This leads to a situation where many toxin reports have been generated on fungi whose identification is suspect, thus making the association of toxin production with that species suspect as well.

The aim of this project was to clarify the toxin-production potential of many species within the genus *Fusarium*. Specifically, the *Gibberella fujikuroi* species complex (GFC) was re-evaluated phylogenetically and toxin production potential (fumonisins) determined based upon the new phylogenetic relationships observed. The trichothecene-producing clade of *Fusarium* was analyzed to determine type A and type B trichothecene

production within this group. Also within the trichothecene-producing clade, zearalenone production was analyzed.

B. Research Objectives

i. Survey of Fumonisin Production in the *Gibberella fujikuroi* Species Complex

The objective of surveying the GFC and some strains of *F. oxysporum* for fumonisin production was to determine which species in this group had the ability to produce fumonisins under our test conditions. One hypothesis of this work was that a phylogenetic pattern with regard to toxigenicity would be able to be discerned. The *F. oxysporum* isolates were included, as previously they had been reported to be fumonisin producers (Seo *et al.,* 1996). A phylogenetic tree of isolates within the GFC was developed using the translation elongation factor 1-α gene sequences and neighbor joining analysis (Fig. 4.2). By selecting representative strains from across the phylogeny of the GFC, we hoped to associate fumonisin production with phylogenetically defined isolates. In the process of performing this survey, we generated fumonisin production information on each strain used. See Appendix 1 for a list of strains used in this survey.

ii. Evaluation of Trichothecene Production Using New Phylogenetic Data

As with the fumonisin study, isolates were chosen for analysis based upon phylogenetics, host, and geographic diversity. A phylogeny was developed by Ward and O'Donnell that incorporated sequence information from seven genetic regions:

translation elongation factor 1- α (tef); phosphate permease; the 28s rDNA; β-tubulin; and three genes involved in trichothecene production: tri4, tri5, and tri101. The dataset (6,968 nucleotides) was analyzed by maximum parsimony using PAUP to generate a phylogenetic tree (Fig. 2.4) (Swofford, 2003). This phylogeny was then used to select isolates for toxin analysis of 10 trichothecene mycotoxins. Other isolates not in this phylogenetic study that are interesting for historical or toxigenicity reasons were also included. These isolates have had relevant gene sequences generated in order to include them in the Ward/O'Donnell phylogeny. The goal of this was to determine any possible patterns of toxin association with phylogenetic placement, as well as to re-evaluate toxin-production potential.

iii. Evaluation of Zearalenone Production Using New Phylogenetic Data

The production of zearalenone was surveyed for in all isolates tested in the trichothecene analysis. Zearalenone production mainly has been reported from species in the *F. graminearum* group. This survey included those species, as well as other species used in the trichothecene survey. This allowed for a test of the many other confusing reports of fusaria outside of the *F. graminearum* group producing zearalenone. This includes a few reports of isolates completely outside of the trichothecene-producing fusaria. The objective was to determine the phylogenetic species and species groups with potential to produce zearalenone.

CHAPTER 2: The Phylogenetics of Trichothecene Production in *Fusarium*

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II. Abstract

Trichothecenes are sesquiterpene mycotoxins produced by a number of *Fusarium* species as well as several other fungal genera, including *Trichothecium*, *Stachybotrys*, and others. This work is a reassesment of the relative trichothecene production potential of *Fusarium* species using a foundation of recently developed phylogenetic concepts and robust strain identification technologies. Phylogenetic relationships among trichothecene-producing fusaria were inferred based on DNA sequences from seven gene regions: translation elongation factor EF1-α, beta-tubulin, phosphate permease, nuclear large ribosomal subunit, and three genes associated with trichothecene biosynthesis, Tri101, Tri4, and Tri5. Sixty-four isolates were examined spanning the known species diversity of *Fusarium* trichothecene producers. A monophyletic group was identified that contains all trichothecene-producing fusaria analyzed. Gene genealogies independently supported

the existence of four major clades within the trichothecene-producing clade, one of which represented a species complex that includes the major type B trichothecene producers *Fusarium graminearum* and its relatives. Across all four clades, a minimum of 30 species lineages were identified based on genealogical concordance. The production of four type A and five type B trichothecenes was analyzed using high performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS) in rice cultures of 78 isolates representing the phylogenetic breadth of trichothecene-producing fusaria. In contrast to current understanding of Fusarium toxin prevalence, the production of type B trichothecenes was found to be widespread, occurring in species across the entire trichothecene-producing clade, including species known only to be type A producers.

III. Introduction

Trichothecenes are a large group of sesquiterpenes produced by hypocrealean (Phylum: Ascomycota, Class: Sordariomycetes) fungi, including certain species of *Fusarium*. Many of these fungi naturally occur on living cereal crops as endophytes and parasites and act as decomposers in dead plant debris. Fusaria are ubiquitous plant pathogens on a wide range of host plants, with diseases such as head scab of wheat and barley, crown rot of wheat, and stalk rot of corn among the most significant. In these cereal diseases, damage to the plant and its seed is accompanied by the production of trichothecenes and other toxins, which can reduce the value of their yield.

Exposure to trichothecene mycotoxins through the diet of both humans and animals is known to occur. The U.S. Food and Drug Administration (FDA) has set contamination limits of deoxynivalenol in foods at 1.0 μg/g for those commodities destined for human consumption, and between 5.0 and 10.0 μg/g for food products intended for animal consumption (depending on the animal species the food is meant for) (CAST 2003). A variety of symptoms may occur upon ingestion or exposure to trichothecenes, including vomiting, diarrhea, blistering, and ulceration of mucus membranes, among others. This work aims to identify which organisms represent significant threats to crops via contamination with trichothecene mycotoxins.

Trichothecenes inhibit protein translation by direct binding to the ribosome (Cannon *et al.,* 1976). Mammalian exposure to trichothecenes results in immunosupression and disturbances of the gastrointestinal system, among other effects (Joffe, 1986). Members of the genus *Fusarium* produce two classes of trichothecenes, referred to as type A (e.g., diacetoxyscirpenol, T-2 toxin, HT-2 toxin, and neosolaniol) and type B (e.g., deoxynivalenol and its 3-acetylated and 15-acetylated forms, nivalenol, and fusarenon-X) (Figs. 2.1 and 2.2). These types differ at the C-8 position, where a keto group is found in the type B trichothecenes. Type A trichothecenes generally are considered more toxic than type B and can cause blistering upon contact with skin or mucous membranes (Joffe, 1986). As with many secondary metabolite biosynthetic pathways, most of the genes involved in trichothecene production are clustered (Brown *et al.,* 2004; Hohn *et al.*, 1993).

The biosynthesis of trichothecenes has not been completely elucidated. A key branch point in the conversion of the intermediate calonectrin into either a type A or type

B trichothecene is still being characterized. It appears that the two key genes involved in this branch point are *LH-1*, which appears to add the C-8 keto group to calonectrin in *F. graminearum* (McCormick *et al.*, 2004), and *TRI-13*, which hydroxylates C-4 of the backbone, leading to further type A trichothecene transformations (Lee *et al.*, 2002). Modifications to these two initial products downstream lead to the observed diversity of trichothecenes in both classes.

For the most part, type A and B trichothecenes are known to be associated with separate groups of *Fusarium* species. However, there are a few taxa that have been reported to produce both type A and type B trichothecenes. These include *F. kyushuense*, which produces nivalenol (Thrane *et al.,* 2004; Tatsuno *et al.,* 1968 as *F. nivale*), fusarenon-X (Ueno *et al.,* 1962 as *F. nivale*), and T-2 toxin (Joffe and Yagen, 1977). *Fusarium poae* also has been known to produce both type A and B trichothecenes, including nivalenol, fusarenon-X, 15-monoacetoxyscirpenol, diacetoxyscirpenol, T-2 toxin, HT-2 toxin, neosolaniol, and culmorin (Thrane *et al.,* 2004).

The identity of *Fusarium* species associated with particular mycotoxins has been extremely problematic due to the general instability in *Fusarium* taxonomy, coupled with inconsistent reports of toxin production associated with particular isolates. Marasas *et al*. (1984) dealt with this problem broadly by confirming and debunking previous reports of mycotoxin production in a wide spectrum of *Fusarium* species, adopting specific morphological criteria for species identification (Nelson *et al*., 1983). However, since the publication of that important volume, *Fusarium* taxonomy has undergone a revolution due to the influence of molecular phylogenetics, which has led to a major expansion in the number of recognized species and reorganization of groups of species around clades.

These advances necessitate a reevaluation of associations between specific mycotoxins and phylogenetically defined taxa within the genus *Fusarium*.

Poor morphological species concepts, as well as misidentification of toxigenic isolates, have been a major source of confusion. For example, the toxin nivalenol was initially reported as being produced by *F. nivale* (Tatsuno *et al.,* 1968). *F. nivale* has since been moved to the genus *Microdochium* (Samuels and Hallett, 1983; Gams, 1989). A further complication is that the initial toxin report was based on a misidentification, and the fungus actually producing the toxin was then identified as *F. tricinctum* and *F. sporotrichioides* by two independent researchers. This was again incorrect, as molecular phylogenetics has now placed this organism as the new species *F. kyushuense* (Aoki and O'Donnell, 1998). In other cases, morphological species that seem well defined are, in fact, complexes of phylogenetic species within which toxin production may have a complicated evolutionary history. For example, the causal agent of head scab of wheat, *F. graminearum* (teleomorph *Gibberella zeae*), recently has been shown to be a monophyletic group comprising multiple species, nine of which have been formally described (O'Donnell *et al.,* 2004). Both cases clearly illustrate the need to combine molecular phylogenetic data with toxicological data to form more concrete links.

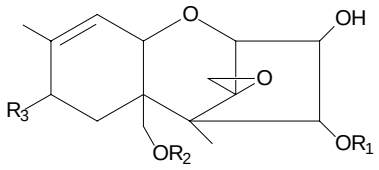
The detection and quantification of trichothecene toxins has been performed previously by liquid chromatography (LC) or gas chromatography (GC) followed by electron impact mass spectrometry (GC/EI-MS) (Rodrigues-Fo *et. al.,* 2002), electrospray ionization mass spectrometry (LC/ESI-MS) (Plattner *et al.*, 1999), and by atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) (Lagana *et al.,* 2003). Lagana *et al.* analyzed four toxins: nivalenol, deoxynivalenol, fusarenon-X,

and 3-acetyldeoxynivalenol. A more complete analysis of eight trichothecenes plus zearalenone has been performed by Berthiller *et al.* (2004). Gas chromatography coupled with mass spectrometry also has been employed in the analysis of trichothecenes. These methods include electron impact mass spectrometry (GC/EI-MS) (Rodrigues-Fo *et al.,* 2002). Liquid chromatography is a more straightforward approach to analysis of trichothecenes, as GC/MS often involves derivatization of samples with reagents such as N-trimethylsilane and trimethylchlorosilane (Rodrigues-Fo *et al.,* 2002).

We sought to place the production of nine different trichothecenes in a robust phylogenetic context, though only eight trichothecenes were separated, and 3- and 15-acetyldeoxynivalenol were quantified as a sum. A phylogenetic analysis based on seven different gene regions was performed on 66 isolates representing the known spectrum of trichothecene producers, plus two outgroups. The isolates used in this study were obtained from the *Fusarium* Research Collection (The Pennsylvania State University, University Park, PA) and the USDA Northern Regional Research Laboratory (NRRL, Peoria, IL). For each isolate of the trichothecene- producing group included in the phylogenetic analyses, cultures were grown on rice, and extracts were subjected to HPLC/APCI-MS to determine presence and quantity of nine different type A and B trichothecenes (3- and 15-acetyldeoxynivalenol determined as a sum).

Fig. 2.1: Structure of Type A Trichothecenes with R-Groups for Each Toxin

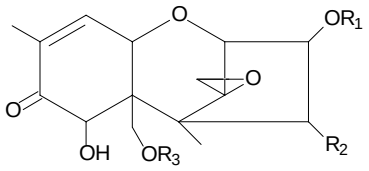
Type A Trichothecenes



T2: R_1= CH_3CO, R_2= CH_3CO, R_3= $OCOCH_2CH(CH_3)_2$
HT2: R_1= H, R_2= CH_3CO, R_3= $OCOCH_2CH(CH_3)_2$
Neosolaniol: R_1= CH_3CO, R_2= CH_3CO, R_3= H
Diacetoxyscirpenol: R_1= CH_3CO, R_2= CH_3CO, R_3= H

Fig. 2.2: Structure of Type B Trichothecenes with R-Groups for Each Toxin

Type B Trichtothecenes



Deoxynivalenol: R_1= H, R_2= H, R_3= H
Nivalenol: R_1= H, R_2= OH, R_3= H
3-Acetyl-deoxynivalenol: R_1= CH_3CO, R_2= H, R_3= H
15-Acetyl-deoxynivalenol: R_1= H, R_2= H, R_3= CH_3CO
Fusarenon-X: R_1= H, R_2= OCH_3CO, R_3= H

IV. Methods

A. Chemicals and Reagents

Standards of deoxynivalenol (DON [CAS# 51481-10-8]), 15-O-acetyl-4-deoxynivalenol (15ADON [CAS# 88337-96-6]), 3-acetyldeoxynivalenol (3ADON [CAS# 50722-38-8]), fusarenon-X (FUS-X [CAS# 23255-69-8]), neosolaniol (NEO [CAS# 36519-25-2]), T-2 toxin (T2 [CAS# 120467-83-6]), nivalenol (NIV [CAS# 23282-20-4]), T-2 tetraol (T2ol [CAS# 34114-99-3]), and hydrocortisone (HYC [CAS# 50-23-7]) were purchased from Sigma (St. Louis, MO). Standards of HT-2 toxin (HT2 [CAS# 26934-86-2]) and diacetoxyscirpenol (DAS [CAS# 2270-40-8]) were purchased from Romer Labs (Union, MO). Standard mixtures for use during HPLC-MS were prepared in 90:10 water (W [Milli-Q UV-Plus]):acetonitrile (ACN [HPLC-grade, CAS# 75-05-8]) such that each toxin had a concentration of 20.0 μg/ml, and the internal standard hydrocortisone had a concentration of 140 μg/ml.

B. Culturing

Fusarium isolates representing the known diversity of trichothecene production were obtained from The Pennsylvania State University Fusarium Research Center (FRC) or the USDA National Regional Research Laboratory (NRRL) (see Appendix B). To prepare inoculum for trichothecene production, these isolates were grown on Carnation Leaf Agar (CLA) (Fisher *et al.*, 1982) for 7-12 days at 25ºC with a 12 h alternating light and dark cycle. Spore suspensions were made by adding 2 ml of sterile water to the CLA plate surface and scraping with a plate spreader. Spore suspensions (100 μl) were used to

inoculate each of at least two tubes of rice media. Rice media was prepared by adding 2 ml dH_2O to 1 g converted long grain rice (Uncle Ben's [TM] Food Company, Vernon, CA) in 14 ml polypropylene round-bottom tubes (VWR, West Chester, PA) with polystyrene foam plugs and aluminum foil caps. The rice was allowed to imbibe the water for approximately 4 h and then was autoclaved at 121ºC for 15 min at 20 lbs. pressure. Following inoculation, rice cultures were incubated in the dark for 3 weeks at 25ºC. Prior to extraction and analysis, the rice cultures were stored at –20ºC.

C. DNA Analyses

DNA extracts from *Fusarium* isolates were prepared as described previously (O'Donnell *et al.,* 1998b). PCR was performed to amplify seven different gene regions: translation elongation factor 1-alpha (EF1); phosphate permease (PHO); nuclear ribosomal RNA (rDNA); TRI101; TRI4 and TRI5 genes encoding portions of the trichothecene biosynthetic pathway; and beta-tubulin (TUB). PCR products were cleaned using Millipore Montage PCR 96-well kits (Bedford, MA) and sequenced using ABI BigDye Terminator sequencing kits on an ABI 3730 automated DNA sequencer (Foster City, CA). Sequences were edited and aligned using Sequencher v.4.1.2 (GeneCodes Corporation, Ann Arbor, MI) and imported into PAUP* v.4.0.b10 (Swofford, 2003) for phylogenetic analyses.

A parsimony analysis of the combined data from seven genes was performed using TBR branch-swapping under a heuristic search with random sequence addition. Boostrap analyses were performed with 1000 pseudoreplicates of the dataset.

D. Extraction and Sample Preparation

Toxins were extracted by adding 7.00 ml of 84:16 acetonitrile:water to the growth tubes followed by maceration with a metal spatula to enhance extraction. The macerates were then placed on an orbital shaker for 2 h at 350 rpm, shaken vigorously and vortexed for several seconds, and then returned to the orbital shaker for an additional 2 h at 350 rpm. Macerates were then centrifuged at 3500 rpm (2537 G) for 5 min and the supernatant separated from the solid portion as the crude extract. Four (4.00) ml of each crude extract were cleaned by passing through an adsorption column containing 0.20g of a mixture of 46.7% w/w Darco G-60 activated carbon, 33.3% neutral alumina, and 20% Celite 545 (VWR, West Chester, PA). Twenty (20) μl of a 0.175% w/v hydrocortisone solution in acetonitrile were then added as an internal standard to 1.78 ml aliquots of cleaned extract. These aliquots were concentrated for HPLC-MS by desolvating under heated nitrogen gas purge to a final volume of 0.2-0.1 ml.

E. HPLC-MS

Liquid chromatography followed by positive ion atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) of the fungal culture extracts or standards was used for semi-quantitative analysis of nine trichothecenes (though 3- and 15-acetydeoxynivalenol were quantified as a sum of both forms). Separations were accomplished with a water-acetonitrile gradient for 24 min: 100% H_2O → 65% H_2O;

flow gradient 0.35 ml/min → 0.70 ml/min. This was followed by a 4 min methanol wash at 0.70 ml/min and a 2 min water equilibration. Each chromatogram required 31 minutes of instrument time. Mass data (*m*/*z* 150 to 800) were recorded for the first 28 minutes after injection. The column used was a Thermo Betasil C18 150 X 2.1 mm with 5µm particle size with an Agilent Eclipse XDB-C8 12.5 X 2.1 mm with 5µm particle size guard column. Instrumentation was Micromass MS (Quattro II with MassLynx software) with APCI Interface (Milford, MA), PAL CTC autosampler and Shimadzu LC-10ADVP liquid chromatographic instrument (Kyoto, Japan). Some samples were run on a Waters 2695 HPLC coupled to a Waters/Micromass LCT Premier electrospray ionization time-of-flight mass spectrometer (Milford, MA). The two systems showed similar response to samples introduced to both. This method successfully separated and detected eight trichothecenes: NEO, DAS, HT-2, T-2, NIV, FUS-X, DON, and 3ADON/15ADON (see Fig. 2.3). Typically 50-100 specimens were run as a batch. The standard solution was run at least once every 10 samples. Blanks of extracts from rice cultures inoculated with sterile water were also run, as were extracts from known toxin producers.

Fig. 2.3: An Example LC/MS Chromatogram Showing Separation of Trichothecene Mycotoxins in Run of Standards

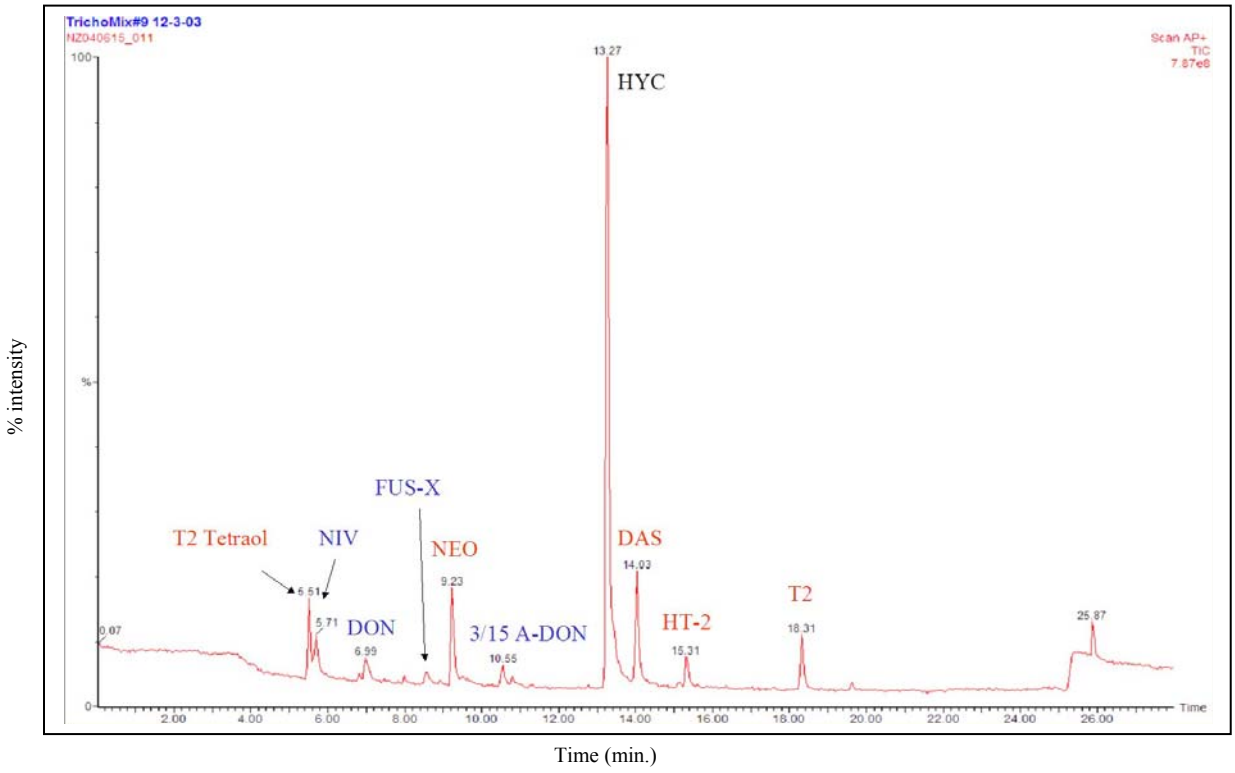


Figure shows relative total ion current on the y-axis and time on the x-axis. NIV=nivalenol, DON=deoxynivalenol, FUS-X=fusarenon X, NEO=neosolaniol, 3/15 A-DON=3/15 acetyldeoxynivalenol, HYC=hydrocortisone (the internal standard), DAS=diacetoxyscirpenol, HT-2=HT-2 toxin, T2=T-2 toxin.

F. Data Analysis

MassLynx v3.5 was used to search various retention time windows for mass signals corresponding to known compounds and fragments observed in the spectra of standards for the nine mycotoxins investigated (Table 2.1). For each mycotoxin, four to

six masses were chosen as indicators for the presence or absence of a particular toxin in a given chromatogram. The area of the chromatogram peak for each quantification mass was compared to that of the internal standard hydrocortisone to determine the concentration of the toxin. Limits of detection were determined by dividing the area of an average noise peak (selected by visual inspection of the baseline from blanks) by the area of a standard, then multiplying by the concentration of that standard. As there were multiple runs of standard solution in every batch, the average limit of detection was used. Since the samples were introduced to the instrument in multiple batches, there was a range of detection limits determined for each toxin analyzed.

Table 2.1: Masses of Trichothecene Ions and Fragment Ions Used for Mass Spectrometry Quantification and Verification

Compound	Quantification Mass	Verification Masses and Proposed Fragment
NIV	295.12 $[M - OH^-]^+$	247.10 $[M - COH_2 - 2H_2O + H^+]^+$ 277.00 $[M - OH^- - H_2O]^+$ 336.14 $[M + H^+ + CH_3CN - H_2O]^+$ 313.13 $[M + H^+]^+$ 265.11 $[M - COH_2 - H_2O + H^+]^+$
DON	249.11 [M - COH2 - H2O + H+]+	231.10 $[M - COH_2 - 2H_2O + H^+]^+$ 267.12 $[M - COH_2 + H^+]^+$ 279.12 $[M - OH^-]^+$
FUS-X	355.14 $[M + H^+]^+$	265.11 $[M - CH_3COO^- - COH_2]^+$ 247.10 $[M - CH_3COO^- - H_2O - COH_2]^+$ 229.09 $[M - CH_3COO^- - 2H_2O - COH_2]^+$ 211.08 $[M - CH_3COO^- - 3H_2O - COH_2]^+$
NEO	305.14 $[M - CH_3COO^- - H_2O]^+$	185.10 $[M - 2CH_3COOH - 2COH_2 - H_2O + H^+]^+$ 197.10 $[M - 2CH_3COOH - 2H_2O - COH_2 + H^+]^+$ 245.12 $[M - CH_3COO^- - CH_3COOH - H_2O]^+$ 257.12 $[M - CH_3COO^- - 2H_2O - COH_2]^+$ 323.15 $[M - CH_3COO^-]^+$
3 and 15 ADON	339.14 $[M + H^+]^+$	231.10 $[M - CH_3COO^- - H_2O - COH_2]^+$ 279.12 $[M - CH_3COO^-]^+$ 291.14 $[M - 2CH_3COO^- + OH^- + 3H_2O]^+$ 380.17 $[M + H^+ + CH_3CN]^+$ 321.13 $[M - OH^-]^+$
DAS	307.15 $[M - CH_3COO^-]^+$	247.13 $[M - CH_3COO^- - CH_3COOH]^+$ 229.12 $[M - CH_3COO^- - CH_3COOH - H_2O]^+$ 349.17 $[M - OH^-]^+$ 384.2 $[M + NH_4^+]^+$ 289.14 $[M - CH_3COO^- - H_2O]^+$
HT2	425.22 $[M + H^+]^+$	215.11 $[M - C_5H_9O_2^- - CH_3COOH - COH_2 - H_2O]^+$ 263.13 $[M - C_5H_9O_2^- - CH_3COOH]^+$ 245.12 $[M - C_5H_9O_2^- - CH_3COOH]^+$ 323.15 $[M - C_5H_9O_2^-]^+$ 407.21 $[M - OH^-]^+$
T2	305.14 $[M - C_5H_9O_2^- - CH_3COOH]^+$	215.11 $[M - C_5H_{10}O_2 - 2CH_3COOH - COH_2 + H^+]^+$ 245.12 $[M - C_5H_{10}O_2 - 2CH_3COOH + H^+]^+$ 197.10 $[M - C_5H_{10}O_2 - 2CH_3COOH - COH_2 - H_2O + H^+]^+$ 185.10 $[M - C_5H_{10}O_2 - 2CH_3COOH - 2COH_2 + H^+]^+$ 257.12 $[M - C_5H_{10}O_2 - - CH_3COOH - COH_2 - H2O]^+$

V. Results and Discussion

A. Phylogenetics of the Trichothecene-Producing Fusaria

The combined seven-gene DNA sequence alignment was composed of 6968 sites for the 66 isolates, 511 of which were deleted from the analysis due to ambiguous alignment. Of the remaining 6457 characters, 2202 were variable, and 1811 were parsimony-informative. Two most-parsimonious trees of 5800 steps were generated, with Consistency Index of 0.53 and Homoplasy Index of 0.47. One of the two most parsimonious trees is shown in Fig. 2.4. Four major clades with strong bootstrap support were evident.

The trichothecene-producing fusaria were shown to be a monophyletic group comprising four individual clades (Fig. 2.4). Trichothecene production was evident in at least some members of each of the four clades, though toxin class and amount of production varied. The phylogenetic placement of *Fusarium* species in one of these four clades is indicative of toxin production potential. The placement of species in these clades alone is not enough evidence to state the organism is a toxin producer, but rather that investigation with regard to its toxin profile should be performed. This work has provided a framework within which *Fusarium* species may be placed as a means to guide toxin production investigations and thus evaluate the threats via toxin contamination that these species and isolates may pose to agriculture.

B. Detection Levels of Trichothecenes and Toxin Responses

The trichothecene detection limits varied per batch and thus are reported as a range. They are as follows: 0.16 – 2.3 μg/ml nivalenol, 0.35 – 1.6 μg/ml deoxynivalenol, 0.34 – 12 μg/ml fusarenon-X, 0.39 – 11 μg/ml 3/15-acetyl-deoxynivalenol, 0.11 – 5.4 μg/ml neosolaniol, 0.05 – 1.3 μg/ml diacetoxyscirpenol, 0.20 – 12 μg/ml HT2 Toxin, 0.12 – 2.7 μg/ml T2 Toxin. In order to determine presence/absence of a toxin in a given sample, the concentration determined by the software was compared to the individual limit of detection for the batch that included that sample. If the concentration was determined to be below the limit of detection, it was reported as a non-producer of that toxin. These detection limits are given as per our extract, and so do not represent detection limits per gram of rice.

The separation of nine trichothecenes occurred in the order shown in Fig. 2.3. The separation of 3- and 15-acetyl-deoxynivalenol was not affected using our chromatographic system, and, as such, the sum of the two forms is reported. The separation occurs in such a way that smaller, more polar molecules, such as NIV, elute from the column in advance of larger, less polar compounds, such as T-2.

Initially, in our analysis for trichothecene production, the toxin T2-tetraol was included. After completing analysis of all isolates in our study, this toxin was detected in very low levels in almost every run. The most likely reason for this observation is that this toxin is a small, highly polar molecule, and in our system, such molecules are persistent in the LC despite washes (likely binding to worn areas in seals or perhaps lingering on the column itself) and are slowly released with subsequent runs. As such, we

have elected to discount our data regarding this toxin. The toxin NIV is also very often present at low levels. Given that NIV is also a smaller, more polar molecule, it is possible that this toxin is overrepresented in our data set. Caution shoud be used when analyzing the significance of NIV production, as some of these lower-level producers may be false positives. This may have resulted from a chemical degeneration of other trichothecenes present in a given sample, or perhaps a persistence of low-level amounts of NIV in the LC-MS system itself. These data may be more accurately interpreted by including a higher limit of detection to filter out false positives, but determining this value would be mostly arbitrary, and as such has not been performed. With the exception of clade 2, it was often the case that where NIV was found, other type B trichothecenes were found as well. This would imply that the NIV observed is likely real, since the pathway resulting in type B production is apparently active in these organisms.

Figure 2.5 shows that only a minor fraction of replicates show high levels of production of any given toxin. The individual distributions of production shown in Fig. 2.5 indicate that the normal level of production for any toxin in any clade is at or below the limit of detection, meaning that zero-production tends to be the normal state for most isolates. For this reason, the interpretation of data such as shown in Fig. 2.6 needs to be explained. For instance, it would seem logical to describe toxin production using the mean values for each toxin (in each clade). These data are represented by the graph of the 50% production in Fig. 2.6. This graph shows that the mean level of production is often zero, and this is not likely an accurate representation of the threat these organisms may pose due to toxin production. For this reason, the 75% graph and the 100% graph (that showing the highest levels observed) are more useful in determining a “spectra” of toxin

production to be used as a possible profile of toxin production for each of the clades. The concept that the typical level of production for many of these isolates is zero indicates that extreme caution needs to be used when performing studies of the sort presented here. If only a few replicates are performed, the observed levels may indicate that an organism is not a toxin producer. This may be untrue as infrequent producers are evident in our data, but the levels observed when these isolates do produce may be significantly high to warrant concern due to crop contamination. This type of situation may be observed in the graph for DON in clade 2 from Fig. 2.5. The vast majority of isolates show zero-level production of DON. There are a few isolates, however, that show at or around the 100ppm level, which may represent significant threats to agriculture. The profile of this production is evident as a narrow, sharp peak with an otherwise flat baseline in Fig. 2.5. Other examples as seen in the figure include FUS-X and ADON from clade 2 and HT-2 from clade 1. The more bell-shaped these graphs are, the more evidence there is for frequent overall production of a given toxin, but care should be taken to consider the potentially high detection limits for some of these toxins. As the limit of detection is not displayed on Fig. 2.5 (for ease of displaying the data), caution is advised in interpreting overall levels, as the baseline values (those below the detection limit) are displayed on these graphs along with those values that represent levels above the detection limit.

It is also important to note that all of the data shown here are the result of one growth condition. There is no doubt that different culturing regimes will result in different toxin production profiles. This needs to be considered when comparing these data to other data sets that potentially used different culturing methods.

The trends displayed in Figs. 2.5, 2.6, and 2.7 illustrate a need for future work to investigate the correspondence between production of multiple toxins. It would be possible to analyze all of the data presented here to determine how toxins group with one another, both at the isolate and clade levels. This information may be potentially useful in elaborating metabolic pathways and control points. Furthermore, the mass data collected potentially include information regarding production of many other metabolites, such as other trichothecenes like scirpene and scirpene-triol. These data essentially represent a collection that could be used as a starting point for a metabolomics study that may shed light on subtleties of metabolic control of trichothecene production. This, for the sake of maintaining an economically and temporally feasible work, must be relegated to future studies.

Fig. 2.4: Phylogenetic Tree Resulting from Maximum Parsimony Analysis
(Bootstrap values from 863 pseudoreplicates)

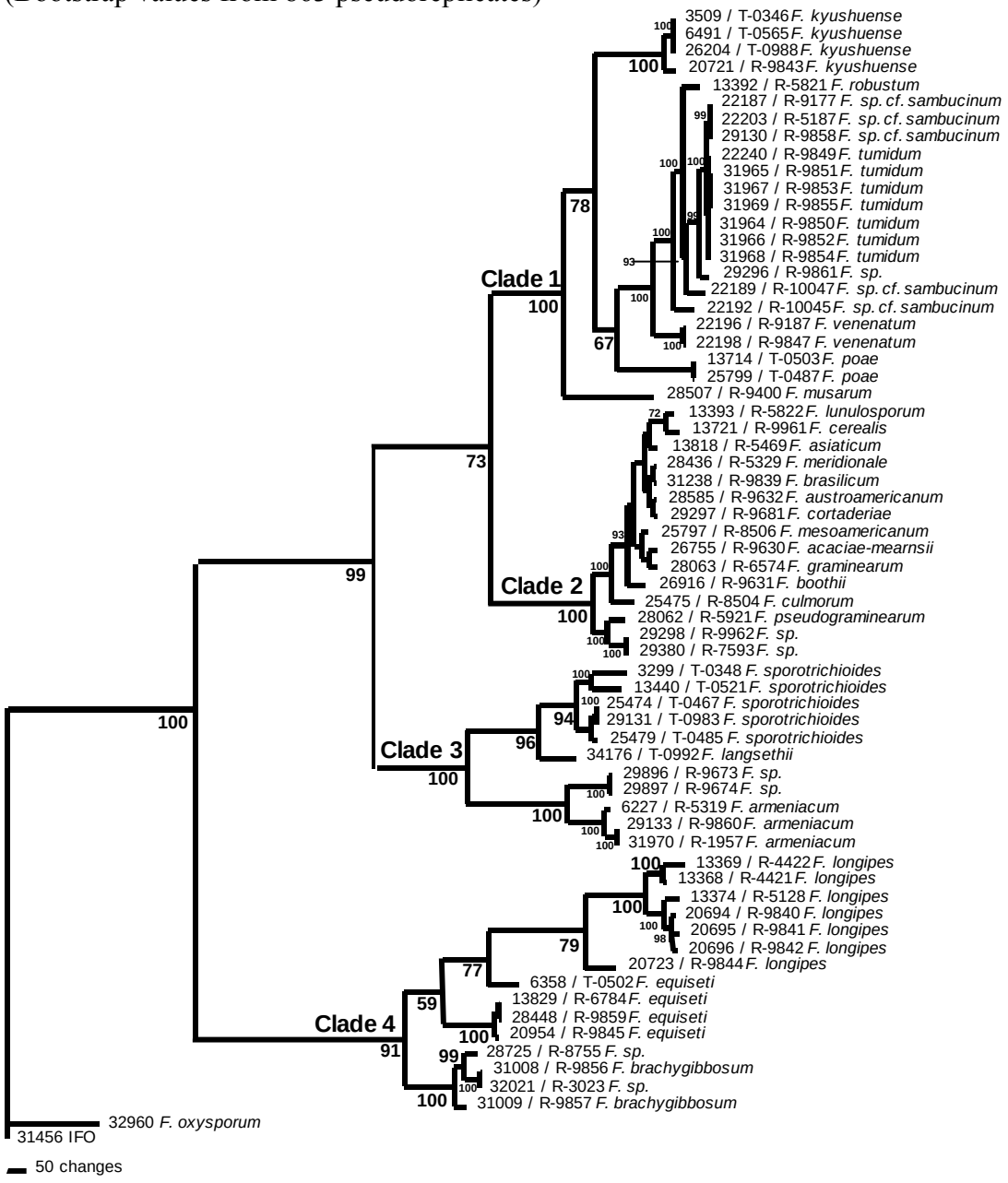


Fig. 2.5: Profiles of Individual Toxin Production Within Each Clade of the Trichothecene-Producing Fusaria

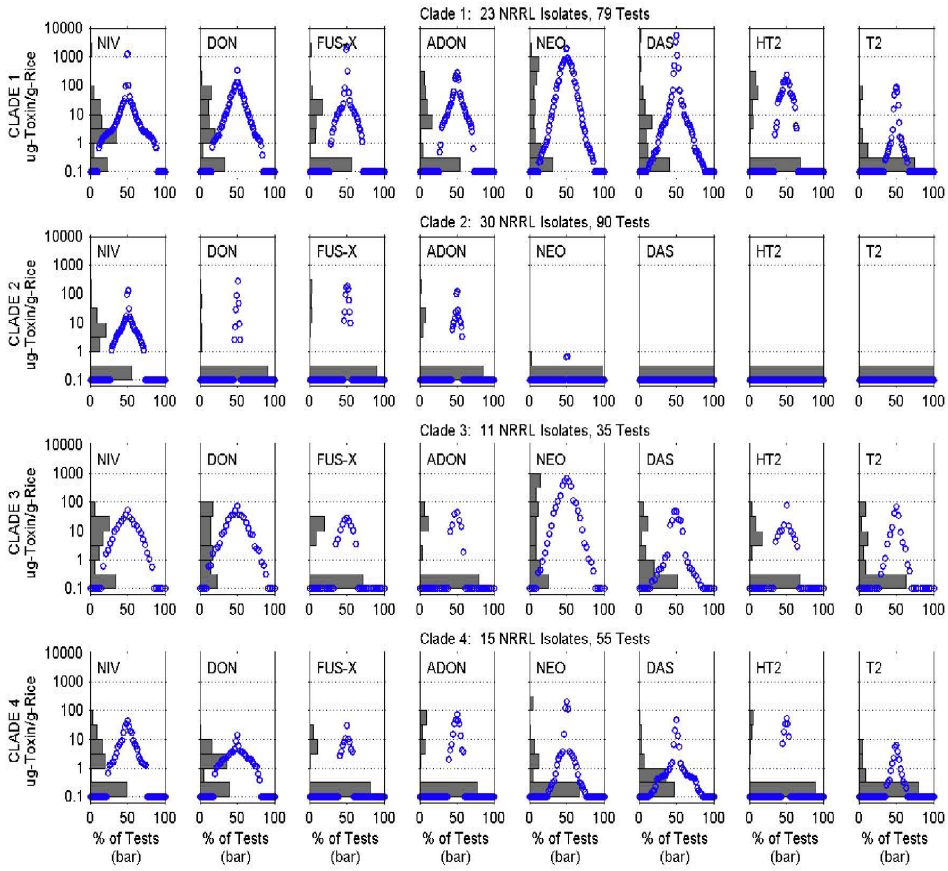


Fig 2.6: The Observed Toxin Response of all Isolates Within Each Clade Displayed by Percent Total of Toxin Amounts Produced

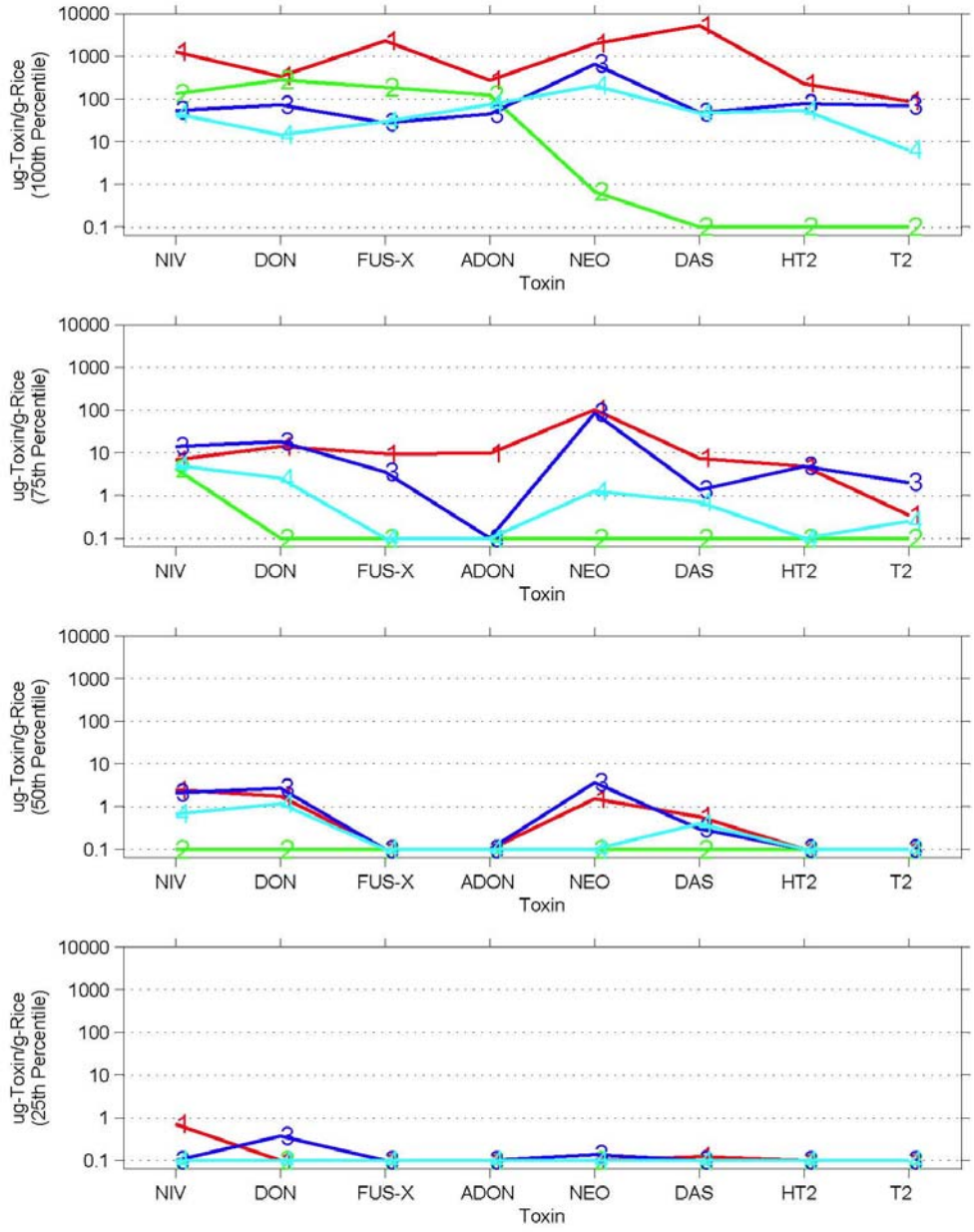
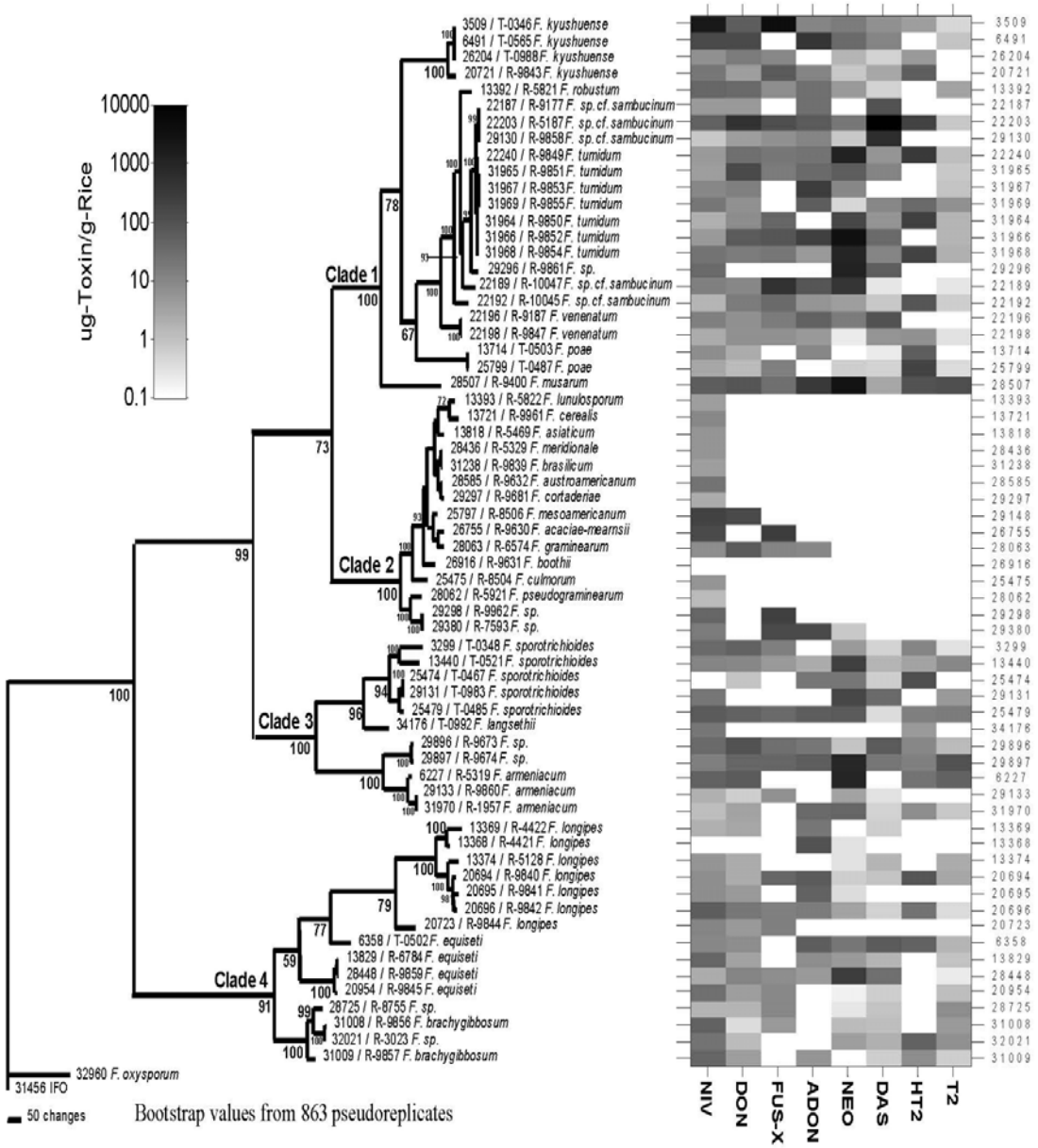


Fig. 2.7: Phylogenetic Tree Resulting from Maximum Parsimony Analysis Showing Trichothecene Concentrations
(Bootstrap values from 863 pseudoreplicates)



*Shading represents maximum determined concentration of each toxin for all reps of each isolate.

C. Trichothecene Production in Clade 1

Clade 1 (Fig. 2.4) contained isolates that previously have been identified as *F. kyushuense*, *F. robustum*, *F. sambucinum*, *F. tumidum*, *F. venenatum*, *F. poae*, and *F. musarum*, as well as one unnamed lineage. *Fusarium venenatum* and *F. sambucinum* are both known to be type A trichothecene producers, and type B production has been seen only in from *F. kyushuense* and *F. poae* within this clade. This work demonstrates that species in Clade 1 often produce both type A and type B toxins, suggesting that exposure to a wider range of toxins is possible when food crops become contaminated by these fungi than would have previously been expected.

Clade 1 showed the highest overall ability to produce many different trichothecenes at high levels. Figure 2.7 shows that this clade, compared to the other three, has a high propensity for producing type B trichothecenes, and these are often in the same isolates producing large amounts of type A trichothecenes. Figure 2.5, in separating out the overall production of individual toxins within each clade, clearly demonstrates that clade 1 has fewer non-producing isolates for any given toxin, and higher potential production of those toxins in most cases (i.e., FUS-X, NEO, DAS, HT-2, and T-2). The typical toxin profile for organisms in clade 1 shows broad toxin diversity and higher production levels than the other three clades. Figure 2.6 illustrates clearly that clade 1 is capable of the highest overall production (100% graph), but also illustrates that even at lower percentage level productions (75% and 50% graphs) this clade often is still producing more than the other three clades.

Fusarium kyushuense has been shown to produce nivalenol (Thrane *et al.*, 2004; Tatsuno *et al.,* 1968 as *F. nivale*), fusarenon-X (Ueno *et al.,* 1962 as *F. nivale*), as well as T-2 toxin (Joffe and Yagen, 1977, NRRL 3509 in that paper). Our results agree with these reports that *F. kyushuense* is capable of production of both A and B trichothecenes, though our data indicate that a much broader range of toxins are produced by this species, encompassing all toxins analyzed for here (Appendix B).

Fusarium robustum has not been investigated previously for trichothecene production. Our analysis shows NRRL 13392 to produce NIV, DON, FUS-X, ADON, NEO, DAS, and T2. This isolate showed consistent production of both DON and DAS in all replicate rice cultures.

Isolates previously identified as *Fusarium sambucinum* were paraphyletic and resolved into three separate lineages. The first lineage comprised isolates NRRL 22187, 22203, and 29130. This lineage was strongly supported with 99% bootstrap support. Two other divergent isolates, 22189 and 22192, grouped with the other three isolates with 99 and 100% bootstrap support, respectively. *Fusarium sambucinum* is known to produce type A trichothecenes such as DAS, T-2, and NEO (Altomare *et al*., 1995a; Marasas *et al.,* 1984). The lineage comprising isolates NRRL 22187, 22203, and 29130 showed the ability to produce all trichothecenes here investigated. The lineage defined by NRRL 22189 produced NIV, DON, FUS-X, ADON, NEO, and DAS. The lineage defined by NRRL 22192 produced NIV, DON, FUS-X, ADON, NEO, DAS, HT-2 toxin, and T-2 toxin. The first lineage described (that of isolates NRRL 22187, 22203, and 29130) appears to tend more towards type A trichothecene production, as all three isolates produced relatively large amounts of DAS. NRRL 22189 produced more total type B

trichothecenes, mainly FUS-X, though that replicate did also show copious production of NEO. NRRL 22192 showed less overall toxin production than the other two lineages. *Fusarium sambucinum* warrants additional phylogenetic and toxigenic study to accurately address which lineages previously identified as *F. sambucinum* are valid and what the actual toxin production potentials of those strains are.

Isolates previously identified as *Fusarium tumidum* compose a distinct phylogenetic lineage. This species has been shown to produce neosolaniol and T-2 tetraol in previous studies (Altomare *et al.,* 1995b; Morin *et al.,* 2000). This is consistent with our observations of type A production, though our data also show evidence of type B production including all type B toxins analyzed for here, which has not previously been described. In this species, type A trichothecene production was observed at higher levels than type B trichothecenes in four of seven isolates. A phylogenetically distinct isolate of *Fusarium* (NRRL 29296) was evident in clade 1. This isolate only produced NIV, DAS , and NEO of the toxins analyzed for here.

Fusarium venenatum represented a distinct lineage with 100% bootstrap support. Isolate 22198 has been shown to produce 911 μg/g diacetoxyscirpenol when grown in rice culture (O'Donnell *et al*., 1998). Our analyses have confirmed this strain as a diacetoxyscirpenol producer in three of four replicates. We also witnessed production of NIV, DON, FUS-X, ADON, NEO, HT-2 toxin, and T-2 toxin. The other isolate of *F. venenatum* analyzed here (NRRL 22196) showed a similar toxin profile, though it never produced HT-2 or T-2 toxin in any replicate tested.

Isolates previously identified as *Fusarium poae* formed a distinct phylogenetic group with 67% bootstrap support. This species has been shown to produce both type A

and type B trichothecenes, including nivalenol, fusarenon-X, neosolaniol, diacetoxyscirpenol, T-2 toxin, HT-2 toxin, T-2 tetraol, T-2 triol, among others (Thrane *et al.,* 2004). Our results are consistent with the findings of Thrane *et al.* (2004) in that all isolates of *F. poae* analyzed produced a range of both type A and B trichothecenes, including NIV, DON, FUS-X, ADON, NEO, DAS, HT-2, and T-2. The majority of type A production for both of these isolates was from HT-2 toxin, though lesser amounts of NEO, DAS, and T-2 were also observed.

The one isolate included in our study previously identified as *Fusarium musarum* was the basal-most lineage in clade 1. This species has been shown to produce both T-2 and HT-2 toxins (Mule *et al.,* 1997, there referred to as *F. camptoceras* R). The one isolate analyzed in our study showed consistent (six of six replicates) production of NIV, NEO, T-2 and HT-2 toxins, but also showed production of other DON, FUS-X, ADON, and DAS. This isolate produced more type A toxins than type B, specifically producing large amounts of NEO in every replicate.

Clade 1 contained isolates capable of producing all of the toxins analyzed. For instance, NRRL 31964, *F. tumindum,* and NRRL 28507, *F. musarum*, both produced some of each toxin analyzed. Both classes of trichothecenes (As and Bs) were found to be produced by each isolate of this clade. The highest type B trichothecene production from clade 1 was NRRL 3509, *F. kyushuense*, which mainly produced nivalenol and fusarenon-X. The highest type A trichothecene production from clade 1 was NRRL 22203, *F. sp. cf. sambucinum*, which mainly produced diacetoxyscirpenol. This clade evidenced more widespread type B trichothecene production than would have been expected based upon previous toxin reports for species in the clade. Overall, our data

support clade 1 as representing a group of species capable of making both type A and type B trichothecenes.

Figures 2.5 and 2.6 display profiles of toxin production for the entire clade. Clade 1, moreso than any of the other three clades, shows a high frequency of production of all of the toxins analyzed for, and at higher levels than the other three clades. This can be seen clearly in Fig. 2.7, in that the shading next to clade 1 is more frequent and darker here than in the other clades. It should be noted that Fig. 2.7 represents only the highest level production seen for each toxin, and so this may be somewhat misleading, but the overall picture of toxin production in clade 1, as seen in all of the figures presented, is that of frequent and high level production of multiple toxins.

D. Trichothecene Production in Clade 2

This clade contains the notorious DON and NIV producers *F. graminearum* and *F. culmorum*, both of which are frequently found causing disease on grain crops. These species have long been associated with type B trichothecene production, and, as such, have been thoroughly examined for their ability to produce trichothecenes many times in the past. The unexpected widespread production of type B trichothecenes by species previously not known to produce these toxins (in the other three clades) led us to question whether species in clade 2 were, in fact, limited to type B trichothecene production. As such, we chose to include these species in our toxin analysis to determine extent of toxin

diversity by these species, and the potential toxin exposure likely to result when these species are found contaminating food crops.

Figure 2.7 very clearly illustrates the fact that isolates from clade 2 are predominantly type B trichothecene producers. Looking at the toxin production in this clade, it is clear that NIV is the most frequently produced toxin in this clade. However, as noted previously, the high prevalence of NIV in this data set should be carefully considered, as there is a possibility of false positives for the reasons mentioned above. The profiles shown in Figs. 2.5 and 2.6 also clearly illustrate the lack of type A production, with one exception, explained below. The overall levels of production in clade 2 were low compared to the other three clades. Looking at Fig. 2.6, it is clear that even at the 75th percentile, the amount of toxin is still zero, with the exception of NIV, discussed above. Only a very few replicates showed toxin production in this clade, and these figures illustrate this trend. Clade 2 is a good example of why care needs to be taken in the interpretation of data generated from a low number of replication. Had only two or three replicates been performed for some of these isolates, toxin production easily could have been missed. This group, in particular, should likely be highly replicated if toxin profiles are to be determined, as replicates frequently show no production.

Clade 2 (Fig. 2.4, Fig. 2.5) was composed of isolates previously identified as the following species: *F. lunulosporum*, *F. cerealis*, *F. asiaticum*, *F. meridionale*, *F. brasilicum*, *F. austroamericanum*, *F. cortadieriae*, *F. mesoamericum*, *F. acaciae-mearnsii*, *F. graminearum*, *F. boothii*, *F. culmorum*, *F. pseudograminearum,* as well as three unnamed lineages (NRRL 29298 and 29380 represent one of these lineages; the other two lineages are not included in the phylogeny shown, but are composed of NRRL

34498, 34197, 34461, and 34502). Most of the isolates investigated here have been the subject of detailed phylogenetic analyses previously (O'Donnell *et al.,* 2004).

Fusarium lunulosporum (NRRL 13393) evidenced no trichothecene production in O'Donnell *et al.* (2000), but showed nivalenol production in our work. No other trichothecenes were detected from this isolate. This may be due to a false positive in our system, or it may be the result of different culturing methods. O'Donnell *et al*. used a method involving inoculating 50g rice media and incubating for 30 days. Our smaller-scale method may have induced toxin production differently than the larger-scale culturing.

Fusarium cerealis (syn. *F. crookwellense*) is known to produce NIV and acetyl-NIV (De Nijs *et al.,* 1996). This species also has been known to produce DON and DAS (Abramson *et al.,* 1993; Vesonder *et al.,* 1991). The observation of DAS production by this species may be due to misidentifications, though, as Thrane *et al.* (2004) has found some isolates previously identified as *F. cerealis* to be *F. venenatum*. *Fusarium cerealis* was shown to produce only NIV by O'Donnell *et al.* (2000). Strain NRRL 13721 displayed nivalenol production in the work by O'Donnell *et al.* (2000), as well as in our current study. Also in agreement with the O'Donnell *et al.* (2000) work, we found strain NRRL 25805 to be a non-producer of trichothecenes.

Fusarium asiaticum previously was shown to have potential to make DON, 3ADON, and NIV (O'Donnell *et al*., 2000). Isolate NRRL 13818 was shown to be a trichothecene non-producer in O'Donnell *et al*. (2000). In our study, one of four replicates showed low-level production of NIV. This discrepancy may again be due to a potential false positive for NIV in our work or different toxin induction caused by

different culturing regimes. Isolate NRRL 28720 was shown to be a producer of DON in the O'Donnell study. Our data are in agreement with this observation, with one of two replicates producing DON.

Fusarium meridionale was shown previously to be able to produce NIV and acetyl-NIV (O'Donnell *et al.,* 2000). Specifically, isolate NRRL 29010 produced NIV and acetyl-NIV, while NRRL 28436 produced only acetyl-NIV of the trichothecenes investigated. Our study also shows isolate NRRL 28436 can produce NIV.

Fusarium brasilicum has not been analyzed previously for production of trichothecenes. In our study, isolates NRRL 31238 produced NIV, and NRRL 31281 produced NIV, DON, and ADON, respectively. The production of type B trichothecenes is in agreement with the overall trend observed in clade 2.

Fusarium austroamericanum was shown to produce DON, NIV, and 3ADON *in planta* by Goswami and Kistler (2005). O'Donnell *et al.* (2000) showed the same toxin production potential when this species was grown in rice culture. Isolate NRRL 28718 was shown in both previously mentioned studies to produce DON and 3ADON. In the current work, this isolate was shown to produce DON and ADON (we could not reliably distinguish 3 and 15 ADON), an observation in agreement with both prior analyses. The absence of NIV production by this isolate in our work may be due to differences in culture conditions (again, 50g rice cultures with a 30 day incubation in the O'Donnell *et al.* work, as opposed to 1g rice culture with 21 day incubations here). Isolate NRRL 28585 produced only NIV in our study.

Fusarium cortaderiae has been shown to produce both NIV and DON *in planta* (Goswami and Kistler, 2005). Monds *et al.* (2005) have shown production of NIV and

15ADON from this species in New Zealand. Isolate NRRL 29297 was analyzed for toxin production in Goswami and Kistler (2005) and was shown to produce DON. In the current analysis, this isolate was found to produce NIV but not DON. In the Goswami and Kistler study (2005), the isolates were examined *in planta*, and this very likely influenced toxin production in different ways than growing them on rice, as in our work. Another isolate (NRRL 31185) was here found to produce NIV and not DON.

Fusarium mesoamericanum (isolate NRRL 29148) was shown to produce both DON and NIV *in planta* (Goswami and Kistler, 2005). This same isolate evidenced no trichothecene production when cultured on rice (O'Donnell *et al.,* 2000), though another isolate (NRRL 25797) did show DON production. Our results are in agreement with Goswami and Kistler (2005) with regard to isolate NRRL 29148, as we show both NIV and DON production, and no other trichothecenes.

Fusarium acaciae-mearnsii was shown to be a producer of DON, 15ADON, and NIV in O'Donnell *et al.* (2000), whereas we witnessed NIV and FUS-X production only. Fusarenon-X was not included in the analysis of O'Donnell *et al.* (2000). Again, this difference may be due to the different culturing methods mentioned previously.

Fusarium graminearum has been reported to produce type B trichothecenes numerous times (Marasas *et al*., 1984). Specifically, DON, 3ADON, 15ADON, NIV, and acetylated forms of NIV often have been reported from strains of *F. graminearum*. *Fusarium graminearum* (NRRL 28063) was shown previously to be a producer of DON, but a non-producer of NIV and acetyl-DON (O'Donnell *et al.,* 2000). Our study confirms the production DON, but also shows production of NIV, acetyl-DON, and FUS-X. Isolate

NRRL 28063 produced NIV, DON, FUS-X, and ADON. Isolate NRRL 29169 produced NIV, DON, and ADON.

Fusarium boothii has been shown to produce DON in previous work by O'Donnell *et al.* (2000). One strain of this species (NRRL 26916) was shown to be a trichothecene non-producer in this previous analysis. Our results for this isolate are in agreement with this observation. Other *F. boothii* isolates, such as NRRL 29020, showed production of DON previously (O'Donnell *et al.,* 2000), but evidenced no trichothecene production in this study. As other work in our laboratory has shown DON production by isolates of *F. boothii* (Eunice Mutitu, personal communication, unpublished results), it is likely that the isolate examined in our work is simply a non-producer under our test conditions.

Fusarium culmorum has been reported to produce NIV, DON, and ADON (Langseth *et al.,* 2001; Marasas *et al.,* 1984). *Fusarium culmorum* strains NRRL 25475 and 3288 showed no trichothecene production in a study by O'Donnell *et al.* (2000), but in our work, both showed production of NIV. Again, this may be the result of different culturing conditions, as mentioned previously.

Fusarium pseudograminearum has been shown to produce DON and 3ADON in a study by Blaney and Dodman (2002). O'Donnell *et al.* (2000) showed the same toxins produced by one (NRRL 28338) of four isolates investigated. Monds *et al.* (2005) showed production of NIV and both 3 and 15 ADON from this species in New Zealand. The current investigation shows NRRL 28062 as a producer of NIV and ADON. The other isolate of *F. pseudograminearum* (NRRL 28065) investigated here was not a trichothecene producer under our conditions.

One new lineage is evident as a close relative of *F. pseudograminearum* (composed of isolates NRRL 29298, isolated from *Dactylis glomerata* in New Zealand, and 29380, isolated from orchard grass in Corvalis, OR). These two isolates both produced NIV and FUS-X, and NRRL 29380 also produced ADON and a small amount of NEO. The production of NEO in clade 2 is unique to this isolate, and as such, warrants more detailed attention. This isolate should be investigated further to determine if this result can be replicated. It is interesting to note that this lineage is one of the more ancestral lineages within clade 2, and that it is from this lineage only that type A trichothecene production was seen. This may suggest that the loss of type A production did not occur prior to clade 2 but perhaps within it.

Four other isolates from clade 2 (NRRL 34498, 34197, 34461, and 34502) were analyzed for toxin production potential, though they were not included in the phylogenetic analyses. NRRL 34498 and 34502 were isolated from wheat in Rock Springs, PA; NRRL 34197 was isolated from soil in Australia; and NRRL 34461 was isolated from a soil dilution in South Africa. These isolates show very little overall trichothecene production, with NRRL 34498, 34461, and 34502 each making only a relatively small quantity of NIV in one of two replicates each. Isolate NRRL 34197 showed no production of any trichothecene analyzed.

Almost no type A trichothecene production was identified in Clade 2. Highest production of type B trichothecenes was evidenced by NRRL 26755, *F. acacieae-mearnsii*, mainly producing fusarenon-X. The only amount of type A trichothecenes was a small quantity of neosolaniol produced by NRRL 29380, an unnamed lineage. As this observation is not in accordance with most previous toxin association with regard to

species within clade 2, further research should address the potential for type A trichothecene production by this isolate. Recently DAS production by 17 of 127 isolates of *F. pseudograminearum* has been reported (Clear *et al.*, 2006). Our data represent the second report of type A trichothecenes being produced by organisms within clade 2.

The organisms that are included in clade 2 have been well characterized both for mycotoxin productin and for various molecular characteristics. One key work has shown that the organisms in this group are likely under the influence of balancing selection for the type of type B trichothecenes they produce (Ward *et al.*, 2002). More specifically, Ward *et al.* have shown that there exist three distinct chemotypes for type B trichothecene production within the organisms of clade 2: a NIV chemotype, a 3ADON chemotype, and a 15ADON chemotype. It is these three chemotypes that have been hypothesized to be under the influence of balancing selection throughout the evolution of the organisms of clade 2.

Our data include toxin profiles for many of the isolates that were investigated for chemotype information by Ward *et al.* (2002). For the most part, our data are consistent with the Ward *et al.* study in that the determined chemotype matches the toxins found to be produced here. The following isolates were found to be in agreement between these two data sets in showing NIV production by NIV chemotype isolates: NRRL 13393, *F. lunulosporum*; NRRL 13721, *F. cerealis*; NRRL 13818, *F. asiaticum*; NRRL 28723, *F. meridionale*; NRRL 28436, *F. meridionale*; NRRL 28585, *F. austroamericanum*; and NRRL 29297, *F. cortaderiae*.

Determining correspondence between the 3ADON and 15ADON chemotype isolates with our data is more difficult, as we did not distinguish these two forms, and

rather report the sum of both. However, the following isolates were at least in agreement between our data and that of Ward *et al.* (2002) with regard to production of acetylated DON forms overall: NRRL 28718, *F. austroamericanum* (3ADON chemotype); NRRL 29169, *F. graminearum* (15ADON chemotype); and NRRL 28062, *F. pseudograminearum* (3ADON chemotype).

Correspondence between chemotype of an isolate and toxin production in our work was not possible for the following isolates, as we saw no toxin production at all: NRRL 25805, *F. cerealis* (NIV chemotype); NRRL 29169, *F. boothii* (15ADON chemotype); NRRL 29020, *F. boothii* (15ADON chemotype); and NRRL 28065, *F. pseudograminearum* (3ADON chemotype). These isolates may very well fit with the chemotype concept as set forth in Ward *et al.* (2002), but our data do not provide the means to draw conclusions to that end.

Only three of the 18 isolates that overlap from these two datasets were in disagreement. Our cultures of NRRL 29148, *F. mesoamericanum,* evidenced no production of acetylated DON forms but did produce both NIV and DON. This isolate was placed as a 3ADON chemotype by the work of Ward *et al.* (2002). The one replicate of isolate NRRL 25475, *F. culmorum*, included in our study produced NIV but no other toxins. In the Ward *et al.* (2002) work, this isolate was shown to produce 3ADON and no NIV. This same discrepancy—the production of NIV and no other toxins in our work, as opposed to production of 3ADON and no NIV in the Ward *et al.* (2002) study— is true for isolate NRRL 3288, *F. culmorum.* These discrepancies may be due to cultural condition differences inducing different toxin biosynthetic pathways. Another possible explanation is that there is a redundant copy of this gene present in the genome of some

of the members of clade 2, though we would expect the chemotype concepts to be less well supported by available evidence were this the case.

E. Trichothecene Production in Clade 3

This clade contains isolates that previously have been identified as numerous species, including *F. sporotrichioides*. This species often has been reported to be a type A producer, though misidentifications have long plagued the reports of toxin production by this species (Marasas *et al.* 1984). Our data suggest that misidentifications of species in clade 3 may have been complicated further by the fact that *F. sporotrichioides* represents two or more cryptic species.

Clade 3 (Fig. 2.4) is composed of isolates previously identified as the following species: *F. sporotrichioides*, *F. langsethii*, and *F. armeniacum*, as well as one unnamed lineage composed of two isolates, NRRL 29896 and 29897. The phylogenetic data show three separate lineages of isolates that have been identified previously as *F. sporotrichioides*. There does not appear to be a clear separation of these lineages with regard to toxin production, though two isolates (NRRL 3299 and 13440) did show consistent DON production, while the other lineages were not as consistent in production of this toxin. *Fusarium sporotrichioides* has been known to produce T-2 toxin, neosolaniol, and diacetoxyscirpenol, among other type A trichothecenes (Thrane *et al.,* 2004; Marasas *et al.,* 1984). Our data show a variety of type A trichothecenes being produced by the isolates identified as *F. sporotrichioides,* including NEO, DAS, HT-2

and T-2, but a range of type B trichothecenes as well, including NIV, DON, FUS-X, and ADON.

Fusarium langsethii was shown in a comprehensive survey of 23 isolates to produce DAS, T-2, HT-2, NEO, and T2ol (Thrane *et al.,* 2004). The one isolate of *F. langsethii* included in our study produced NIV and HT-2. The production of NIV is inconsistent with the study by Thrane *et al.* (2004), though the production of HT-2 by this species is in agreement with that work. This may be due, again, to a false positive as described previously, or it may be due to culturing differences.

Fusarium armeniacum has been noted to produce T-2 and HT-2 toxins by Moss and Thrane (2004). Our data show that the isolates analyzed that have been previously called *F. armeniacum* have the ability to produce the entire range of trichothecenes tested. Isolate NRRL 6227 showed production of NIV, DON, NEO, HT-2, and T-2. Isolate 29133 showed production of NIV, DON, FUS-X, NEO, and DAS. Isolate NRRL 31970 showed production of NIV, DON, ADON, NEO, DAS), and T-2.

The two isolates that make up the new lineage evident in clade 3 were NRRL 29896 and 29897. Isolate NRRL 29896 showed production of NIV, DON, FUS-X, ADON, NEO, DAS, HT-2, and T-2. Isolate NRRL 29897 showed production of NIV, DON, FUS-X, ADON, NEO, DAS, and T-2.

Clade 3 contained isolates capable of production of both type A and B trichothecenes as well. Highest production of total type B trichothecenes was found in NRRL 25479 (identified as *F. sporotrichioides*), and highest production of type A trichothecenes in clade 3 was by NRRL 6227 (*F. armeniacum*), which produced mainly neosolaniol.

Clade 3 showed fairly consistent but low production of NIV, DON, NEO, and DAS. This can be seen in the 50th percentile graph of Fig. 2.6. Figure 2.5 also shows that the profiles for most toxins in this clade show a wide bell shape, as opposed to the sharp peak with flat baselines one would see if a toxin was only produced infrequently. For instance, NEO seems to be widespread in its production within clade 3, whereas in clade 4, by comparison, there are a larger number of replicates producing zero NEO. Comparitively, clade 3 had capacity for higher toxin production than either clades 2 or 4, but less than clade 1. This is visible in Fig. 2.7, in that the general shading for clade 3 is darker than both clades 2 and 4, but not as dark as clade 1 (looking at the clade as a whole).

The isolates composing clade 3 show high phylogenetic and toxin-production diversity. As such, contamination by fungi belonging to this clade could significantly increase the threat of exposure to trichothecenes. This is specifically true with regard to the type B trichothecenes, which would typically not be expected or even screened for when these fungi were found contaminating foodstuffs.

The fusaria of clade 3 have been isolated from many different substrates, including barley, pampas grass, fescue hay, and soil. Many of the isolates included in our work did not have any substrate information associated with them in the collections from which they were received. Given the available information and the nature of the species within clade 3 to be commonly associated with grain crops, the threat to human and animal health posed by these fungi should not be underestimanted. Future studies should strive to more completely characterize exposures due to contamination by these organisms.

F. Trichothecene Production in Clade 4

Clade 4 (Fig. 2.4) was the most basal lineage and comprised isolates identified previously as the following species: *F. longipes*, *F. equiseti*, and *F. brachygibbosum*, as well as one unnamed lineage. Isolates previously identified as *Fusarium longipes* represent three phylogenetically distinct lineages, each with 100% bootstrap support. The first of these three lineages comprised NRRL 13369 and 13368. Isolate NRRL 13369 produced NIV, DON, ADON (110 μg/g in one of six replicates) and DAS. Isolate NRRL 13368 showed production of ADON and NEO. Another distinct lineage was composed of isolates NRRL 13374, 20694, 20695, and 20696. These isolates varied in toxin profile from isolate NRRL 13374, which produced small amounts of NIV, DON, NEO, DAS, and T-2, to isolate NRRL 20694, which produced NIV, DON, FUS-X, ADON, NEO, DAS, HT-2, and T-2. The remaining lineage was represented by isolate NRRL 20723. This isolate produced only NIV, DON and DAS.

Fusarium longipes was shown to be generally non-toxic in a chick bioassay (Wing *et al.,* 1993a). Only one of five isolates demonstrated toxicity in the assay. Unfortunately, no further chemical identification of toxins present was performed for this study. Isolates of *F. longipes* included here show the ability to produce all toxins analyzed.

Isolates previously identified as *F. equiseti* include two distinct phylogenetic groups, one of which produces trichothecenes, and one which is unrelated to the trichothecene-producing clade (Michele Mansfield, personal communication). The true

F.equiseti is likely that lineage unrelated to the trichothecene-producing fusaria, and thus reports of trichothecene production from this species are probably due to production witnessed from misidentified isolates belonging to the trichothecene-producing clade. The group of isolates previously identified as *F. equiseti* included in the trichothecene-producing clade composed two lineages. The first lineage is represented by isolate NRRL 6358, which produced NIV, DON, ADON, NEO, DAS, HT-2, and T-2. The second lineage of isolates previously identified as *F. equiseti* was composed of NRRL 13829, 28448, 20954. These isolates together showed the ability to produce all toxins analyzed for with the exception of HT-2. Isolate NRRL 28448 produced the most total trichothecenes of these three, producing large amounts of NEO, as well as lesser amounts of NIV, DON, FUS-X, ADON, and DAS. Previous reports of trichothecenes from *F. equiseti* include DAS (Brian *et al.* 1961), NIV, as well as other precursors and derivatives of type A trichothecenes (Marasas *et al.* 1984). Together, the four isolates of *F. equiseti* included in our study demonstrated the ability to produce all of the toxins analyzed, and thus are not likely the "true" *F. equiseti*.

The phylogenetic analysis revealed that isolates previously identified as *Fusarium brachygibbosum* represent a paraphyletic group. This species has not been studied previously for trichothecene production potential. Our data show production of NIV, DON, FUS-X, NEO, DAS, T-2, and HT-2 by isolates identified as this species, even though they are phylogenetically distinct. For instance, isolate NRRL 31008 produced NIV, DON, FUS-X, NEO, DAS, and T-2. NRRL 31009, identified previously as *F. brachygibbosum* but from a different lineage, produced NIV, DON, DAS, HT-2, and T-2.

Two unidentified isolates, NRRL 28725 and 32021, are closely related to those isolates previously identified as *F. brachygibbosum*. Isolate NRRL 28725 produced NIV, DON, NEO, DAS, and T-2. Isolate NRRL 32021 produced NIV, NEO, DAS, HT-2, and T-2.

Clade 4 also contained isolates capable of producing both classes of trichothecenes. Highest total type B production was by NRRL 13368 (previously identified as *F. longipes*), which produced high levels of 3/15-acetyl-deoxynivalenol. Highest total type A production was by NRRL 28448 (identified as *F. equiseti*), which produced mainly neosolaniol.

While the toxin diversity and amounts produced in clade four were occasionally high, it should be noted that in this clade, as opposed to the preceding three, the toxin amounts were generally much lower. This, combined with the fact that most of the isolates in this group were isolated originally from soil or had no substrate information associated with them in their respective databases, would indicate that clade 4 is of lesser threat to human health through toxin exposure than the other three clades.

Figure 2.7 shows that even the highest level production witnessed in this clade is relatively low compared to the amounts produced in the other three clades. The overall total toxin amounts from this clade tended to be the lowest of the four clades analyzed. This is also clear when looking at Fig. 2.6's 100^{th} percentile graph. The line for clade 4 is below all of the other clades for all toxins except ADON (being just higher than clade 3) and the type A trichothecene being present whereas they are mainly absent in clade 2 (as expected). Figure 2.5 shows that most of the replicates for clade 4 were non-producers of

any given toxin, and, again, the levels of production were low compared to the other clades.

G. Conclusions

One of the major advances that our data provide is that of showing that the trichothecene-producing fusaria represent a monophyletic group made up of four distinct monophyletic clades. This phylogeney has shown that multiple new, undescribed lineages are present within this group of organisms (i.e., NRRL 29296, 29298, 29380, 29896, 29897, 28725, and 32021). It has also shown that many previously described morphological species are polyphyletic and are likely multiple distinct species (i.e., *F. sporotrichioides*, *F. longipe*, *F. sp. cf. sambucinum*, and *F. brachygibbosum*).

Another significant finding of our study was the evidence showing widespread type B trichothecene production. When beginning this work, we suspected the type B trichothecenes would be produced only in clade 2, with the few previously known exceptions from clade 1 (*F. poae* and *F. kyushensis*). Our data support a broader concept of type B trichothecene production, as there is evidence of these toxins in all four of the clades revealed upon phylogenic analysis. Whereas previously only *F. kyushuense* and *F. poae* have been known to produce both type A and type B trichthecenes, we have provided evidence of dual production by isolates of species identified previously as *F. tumidum*, *F. sambucinum*, *F. venenatum*, *F. robustum*, *F. musarum*, *F. sp.* (NRRL 29380, clade 2), *F. sporotrichioides*, *F. langsethi*, *F. armeniacum*, *F.sp.* (NRRL 29896 and 29897, sister taxa to *F. armeniacum*), *F. longipes*, *F. equiseti*, and *F. brachygibbosum*.

With a now broader concept of what the source species of trichothecne contamination are, the threat these fungi pose to agriculture may be more thoroughly investigated. Crops found to be compromised by these fungi should likely be analyzed more frequently for contamintation with type B trichothecenes. Further investigation into the prevalence of toxin production in field and stored crops by these fungi previously not known as type B producers may expose routes of toxin exposure not previously addressed.

The biosynthetic genes involved in trichothecene biosynthesis have been the subject of much recent research (McCormick *et al.*, 2004; Lee *et al*., 2002; Kimura *et al*., 2003; Ward *et al*., 2002). The full story of the evolution of the genes of this cluster across the many organisms in the trichothecene-producing fusaria is still being elucidated, however. This work sheds some light on what genes are likely present in certain organisms that would not have been expected previously. The prevalence of type B trichothecenes in this phylogeny indicate that the gene responsible for the conversion of calonectrin into 8-hydroxycalonectrin, *Fg TRI-1* (or *LH-1*, as per McCormick *et al.*, 2004), is more widespread than previously known. Future studies may be able to use the results presented here to direct the search for relevant biosynthetic genes in previously uninvestigated lineages and individuals, potentially shedding light on some of the unclear details of the production of these diverse toxins.

CHAPTER 3: The Phylogenetics of Zearalenone Production in *Fusarium*

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II. Abstract

Zearalenone is an estrogenic mycotoxin produced by a variety of *Fusarium* species, specifically those known also to produce trichothecene mycotoxins, particularly type B trichothecenes such as nivalenol and deoxynivalenol. Phylogenetic studies have shown that trichothecene-producing fusaria are a monophyletic group comprising four clades, in one of which reside the major species traditionally associated with type B trichothecene and zearalenone production, including *F. graminearum*. As is the case for most *Fusarium* mycotoxins, zearalenone production has been reported in a variety of species outside of its core group of established producers. We surveyed the extent of zearalenone production in all four known trichothecene-producing clades using high performance liquid chromatograph analysis of rice cultures of 62 isolates. These isolates represented the phylogenetic breadth of trichothecene-producing fusaria and other previously reported producers. The analysis of fusaria for the ability to produce zearalenone was here performed in a more rigorous phylogenetic framework than has

been reported on previously. The production of zearalenone was determined to be limited mainly to those isolates in the clade encompassing *F. graminearum* and its relatives, though other producers were also identified. The effects of incubation temperatures on the production of zearalenone were also studied, and this revealed varied responses to temperature treatment by different species.

III. Introduction

Zearalenone (6-[(10S)-10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcyclic acid lactone, Fig. 3.1) is an estrogenic mycotoxin, meaning that its effects are similar to those of mammalian estrogen. A zearalenone analog, zearalenol, exhibits similar effects, though the compound has higher activity (Mirocha *et al*., 1978).

Zearalenone is one of a large group of chemicals in the environment termed endocrine disruptors (ED). Endocrine disruptors are steadily gaining recognition as hazardous compounds in the environment, as they are often found in wastewater and in industrial pollutants (Falconer *et al.*, 2006). These chemicals have often been overlooked in the environment, as other more prominent pollutants have been the focus of monitoring and clean-up efforts (Falconer *et al.*, 2006). Zearalenone and zearalenol are compounds that humans are exposed to through diet (Eriksen *et al.*, 2000), but that have not been traditionally of major concern due to their low toxicity as compared to other mycotoxins. The exact effects of zearalenone and zearalenol exposure to humans are not clearly known, though there are data from numerous animal systems. Both of these toxins bind competitively to mammalian estrogen receptors, and can result in such effects as

decreased fertility, adrenal gland weight changes, and decreased reproductive ability, among others (Creppy, 2002).

Given the fact that zearalenone is an endocrine disruptor that many humans will be challenged with in their diet, it is of great importance to understand what species and groups of species within *Fusarium* have the ability to produce it. It was our goal to survey the known diversity of trichothecene-producing fusaria in order to develop a concept of zearalenone production potential within the framework of well-defined phylogenetic species. The trichothecene-producing fusaria were the focus of this survey, as there is known to be overlap of toxin production potential with regards to the trichothecenes and zearalenone (Table 3.1).

There are many varied and conflicting reports of the effect of incubation temperature, water activity, and duration on the accumulation of zearalenone in both culture and grain storage conditions. For instance, there are reports of incubation at 25-30ºC followed by a lower temperature incubation inducing high zearalenone production in *F. graminearum* and *F. oxysporum* (Jimenez *et al.*, 1996). The identification of these strains was performed based on morphology, and as such, may not be reliable for use in associating toxin-production potential. In this same study (Jimenez *et al.*, 1996), *F. culmorum* showed decreased zearalenone production when incubated with the "cold-snap" treatment. Ryu and Bullerman (1999) showed higher zearalenone production when incubating with cold treatment for *F. graminearum* strain NRRL 5883. On the other hand, Llorens *et al.* (2004) report that this "cold-snap" incubation inhibits zearalenone production in *F. graminearum* and *F. culmorum.* The association of a "cold snap" with a decrease in zearalenone production was observed by Milano and Lopez (1991) when

investigating isolates identified as *F. graminearum* and F. *oxysporum*. This decrease in zearalenone production was also witnessed by Di Menna *et al.* (1991) when investigating toxin production by strains of *F. crookwellense* (syn. *F. cerealis*). This variation in experimental results is likely due to inherent differences in the propensities of individual species and strains to produce zearalenone under variable conditions. The wide differences in response to temperature treatment at the species level is likely influenfed by the adaptation of individual strains to specific environments. The discovery that the morphospecies *F. graminearum* actually comprises nine phylogenetically diagnosable species (O'Donnell *et al.*, 2004) raises further questions about variation in this character partitioned among species.

These issues arising from variation of zearalenone production with regard to culturing conditions, as well as the difficulties presented by cryptic species, illustrate the need to establish more concrete concepts of toxin production linked to well-defined species of fusaria. Without phylogenetic confirmation of species and subsequent association of toxin production to these defined species concepts, toxin reporting becomes suspect, as the pitfalls of morphologically identifying species within fusaria often result in misidentifications. Consequently, these misidentifications may then lead to incorrect association with toxin production. Ultimately, these problems may lead to unforeseen exposure to higher levels of estrogenic mycotoxins than would be anticipated based upon previous species-toxin associations. Table 3.1 (below) indicates previously reported zearalenone producers by species, along with reported trichothecene production by those species.

Fig. 3.1: The Chemical Structures of Zearalenone (left) and Zearalenol (right)

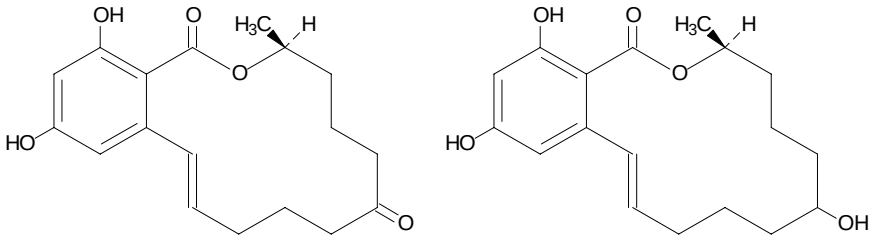


Table 3.1: Reported Zearalenone Producers

Fungal species	Reported toxins[1]	References
*F. asiaticum**	DON, 3-ADON, NIV, ZEA	O'Donnell *et al.* 2000
*F. austroamericanum**	DON, 3-ADON, NIV, ZEA	O'Donnell *et al.* 2000
F. avenaceum	ZEA	Langseth *et al.* 1999
*F. boothii**	DON, ZEA	O'Donnell *et al.* 2000
*F. cerealis***	NIV, ZEA	O'Donnell *et al.* 2000
F. culmorum	DON, 15-ADON, NIV, FUS-X, ZEA	Mirocha *et al.* 1994 Moss and Thrane 2004
*F. equiseti****	NIV, DAS, NEO, ZEA	Morrison *et al.* 2001 Moss and Thrane 2004
*F. graminearum**	DON, 3-ADON, 15-ADON, NIV, FUS-X, ZEA	Mirocha *et al.* 1994 O'Donnell *et al.* 2000
F. lunulosporum	ZEA	O'Donnell *et al.* 2000
*F. meridionale**	NIV, ZEA	O'Donnell *et al.* 2000
*F. mesoamericanum**	DON, ZEA	O'Donnell *et al.* 2000
*F. pseudograminearum*****	DON, 3-ADON, ZEA	Moss and Thrane 2004 O'Donnell *et al.* 2000
*F. sambucinum****	DAS, NEO, T2, HT2, ZEA	Marasas *et al.* 1984 Moss and Thrane 2004
F. semitectum	ZEA	Marasas *et al*. 1984
*F. sporotrichioides******	T2, T2-4ol, DON (1 report), DAS, Nivalenol diacetate, FUS-X, HT2, NEO, NIV, ZEA	Marasas *et al.* 1984 Moss and Thrane 2004

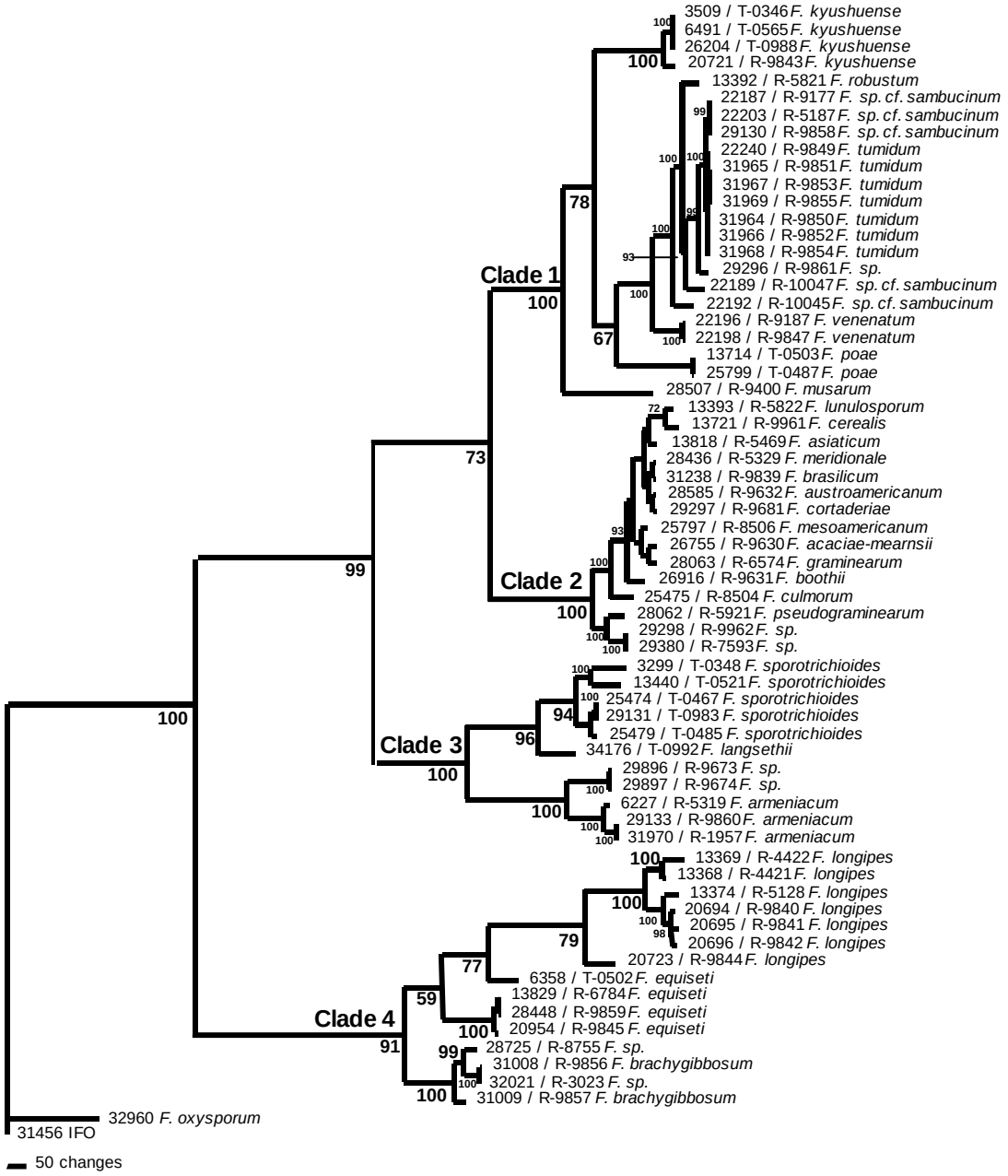
[1]Type-B trichothecenes: DON=deoxynivalenol, NIV=nivalenol, 3-ADON=3-acetyldeoxynivalenol, 15-ADON=15-acetyldeoxynivalenol, FUS-X=fusarenon-X
Type-A trichothecenes: T2=T-2 toxin, T2-4ol=T-2 tetraol, HT2=HT-2 toxin, DAS=diacetoxyscirpenol, NEO=neosolaniol

* Species previously considered to be *F. graminearum*, now separated based on multilocus phylogenetics
** Same as *F. crookwellense*, proposed to be a synonym of *F. cerealis* (Nirenberg 1990)
*** Species poorly defined phylogenetically
**** Previously recognized as *F. graminearum* Group 1
***** Comprises at least three related phylogenetically distinct groups in the trichothecene clade

A multigene phylogeny of the trichothecene-producing fusaria (Fig. 3.2) showed that trichothecene-producing species resided in a monophyletic group comprising four major clades, one of which (clade 2) contained the known major zearalenone producers (Zitomer *et al.,* 2006). The discovery that type B trichothecenes are produced more widely than previously known in the entire group of trichothecene producing fusaria, and the fact that the most notable zearalenone producing species are those also known to specifically produce the type B trichothecenes warrants a thorough investigation of the entire group of trichothecene-producing fusaria for their ability to produce zearalenone. The study reported here was designed to investigate the range of zearalenone production within the trichothecene-producing fusaria and thus provide insight into the potential for human exposure to this toxin when commodities become contaminated by these fungi. Isolates representing the diversity within this clade were grown in small scale rice cultures to determine zearalenone production. Zearalenone was found to be mostly, but not entirely, limited to the clade encompassing *Fusarium graminearum* and its close relatives.

Fig. 3.2: Phylogenetic Tree Resulting from Maximum Parsimony Analysis of Multiple Gene Regions from Species Representing the Diversity of the Trichothecene-Producing Fusaria (from Zitomer *et al.* 2006)

(Bootstrap values from 863 pseudoreplicates)



IV. Methods

A. Chemicals and Reagents

One-hundred µg/ml solutions of zearalenone (ZEA [CAS# 17924-92-4]) in 70:30 methanol:water and zearalenol (ZOL [CAS# 26538-44-3]) in methanol (ZEA purchased in solution, ZOL prepared from solid) were purchased from Romer Labs (Union, MO) to be used as standards in HPLC. Culture extracts were cleaned and concentrated using C18 3ml 15mg extraction disks (Varian, Lake Forest, CA). Methanol used for extraction, cleanup, and LC was HPLC grade. Water used was purified using a Milli-Q system (Milli-Q UV-Plus).

B. Culturing

Fusarium isolates were obtained from The Pennsylvania State University Fusarium Research Center (FRC) and/or the USDA National Regional Research Laboratory (NRRL) located in Peoria, IL (See Table 3.2). Rice cultures were prepared by adding 2 ml dH_2O to 1 g converted long grain rice (Uncle Ben's [TM] Food Company, Vernon, CA) in 14 ml polypropylene round-bottom tubes (VWR, West Chester, PA) with polystyrene foam plugs and aluminum foil caps. The rice was allowed to imbibe the water for approximately 4 h then was autoclaved at 121 ºC for 15 min. at 20 lbs. pressure. To generate inoculum for zearalenone production, isolates were grown on Carnation Leaf Agar (CLA) (Fisher, 1982) for 7-12 days at 25 ºC with a 12 h alternating light (including both white and black lights) and dark cycle. Spore suspensions for inoculation were made

by adding 2 ml of sterile water to the CLA plate surface and scraping with a plate spreader. 100 µl of spore suspension was added to each of six rice cultures per isolate. Following inoculation, the six replicate rice cultures were separated into three different time and temperature treatment groups (two cultures each), all incubated in darkness: 1) 3 weeks at 25 ºC; 2) 3 weeks at 25 ºC, followed by 3 weeks at 15ºC; 3) 2 weeks at 25 ºC, followed by 2 weeks at 15 ºC, followed by 2 weeks at 25 ºC. Prior to extraction and analysis, the rice cultures were frozen at –20 ºC.

C. Extraction and Sample Preparation

Compounds were extracted by adding 10.00 ml of a 75:25 methanol:water mixture to the culture tubes followed by maceration with a metal spatula to enhance extraction. The crude macerate was placed on an orbital shaker overnight (12h) at 200 rpm. Crude macerates were then centrifuged at 2537 X G for 5 min and the supernatant separated from the solid portion. The supernatant was saved for analyis and the solid macrate was disposed of.

Two separate analyses were then performed. First, each isolate had 2 ml from each of the three temperature treatments pooled. This resulted in two (duplicate) pooled extracts from each isolate. Two ml of the pooled extract was then mixed with 5.50 ml of 2.0 mM acetic acid, which was then passed through a C18 cleanup cartridge. The cartridge was washed with 0.5 ml of 30:70 methanol:water (v/v) and then dried by pulling vacuum across it for 3 min. Following drying, zearalenone and zearalenol were eluted from the cartridge by passing 0.50 ml 70:30 methanol:water through it. For those

pooled extracts found to be positive for zearalenone, the unpooled extracts were then analyzed separately in the same manner.

D. HPLC

The HPLC method was based partly after methods developed by George Rottinghaus (personal communication). An Agilent 1100 Series HPLC system with a quaternary pump and fluorescence detector (Agilent, Palo Alto, CA) was used for detection of the compounds studied. The column was a Zorbax SB-C18 2.1 mm X 15 cm with a 5 micron particle size and 80 angstrom pore size (Agilent, Palo Alto, CA) with an Agilent Zorbax Reliance guard column of the same particle and pore size. Fluorescence detection (excitation λ = 274nm, emission λ = 465nm) was used to quantify zearalenone and zearalenol levels in the fungal extracts. HPLC was run isocratically at 0.60 ml/min with a mobile phase of 50:50 methanol:water for 24 min, followed by a 95:5 methanol:water wash for 4.5 min, then 4.5 min re-equilibration to the isocratic initial column condtions, for a total of 31 min per sample. Zearalenone and zearalenol were were quantified in culture extracts by comparison to chromatograms of standards which were run approximately once every ten sample runs. The retention times of ZOL and ZEA were approximately 14.8min and 16.5min, respectively.

V. Results

Zearalenone and zearalenol production was found to be associated mainly with the species residing in Clade 2 of the previously identified trichothecene-producing clade

of *Fusarium*, the major group previously associated with zearalenone production (Fig. 3.3). All members tested within this clade were positive for zearalenone, with the exception of the two related species *F. graminearum* (R-6574) and *F. asiaticum* (R-5469). Outside of this clade, three isolates identified as *F. sporotrichioides* (T-521, T-336, T-341) were also positive for both zearalenone and zearalenol production. One of these (T-521) was included in the phylogenetic analyses, while the other two are isolates previously reported to be producers (Marasas, 1984). One isolate (T-988) of three analyzed of *F. kyushuense* was also positive for both zearalenone and zearalenol. Table 3.2 below shows positive and negative producers of zearalenone. Of all isolates positive for zearalenone production, only *F. culmorum* (R-8504) was negative for zearalenol production at all three temperature regimes tested. Species found to be negative for zearalenone production under our culture conditions were as follows: *F. tumidum*, *F. robustum*, *F. venenatum*, *F. poae*, *F. musarum*, *F. graminearum*, *F. asiaticum*, *F. armeniacum*, *F. longipes*, *F. brachygibbosum*, and *F. oxysporum*.

Two isolates analyzed seemed to consistently produce zearalenone regardless of temperature incubation. These were *F. pseudograminearum* (R-5291) and *F. graminearum* (R-4485) (Table 3.3). Three isolates showed highest production of zearalenone under the conditions of temperature regime one (3 weeks at 25 ºC). These were *F. meridionale* (R-5329), *F. lunulosporum* (R-5822), and *F. culmorum* (R-7593). Three isolates showed highest production of zearalenone under the conditions of temperature regime two (3 weeks at 25 ºC, followed by 3 weeks at 15ºC). These were *F. culmorum* (R-8504), *F. cerealis* (R-9961), and *F. sporotrichioides* (T-336). Only one isolate in this study showed highest zearalenone production when cultured under

temperature regime three (2 weeks at 25 ºC, followed by 2 weeks at 15 ºC, followed by 2 weeks at 25 ºC). This was *F. kyushuense* (R-5470). The results of the temperature treatments are shown in Table 3.3.

Table 3.2: Summary of Pooled Zearalenone Analysis by HPLC
+ indicates zearalenone was detected in the pooled extracts; - indicates none detected

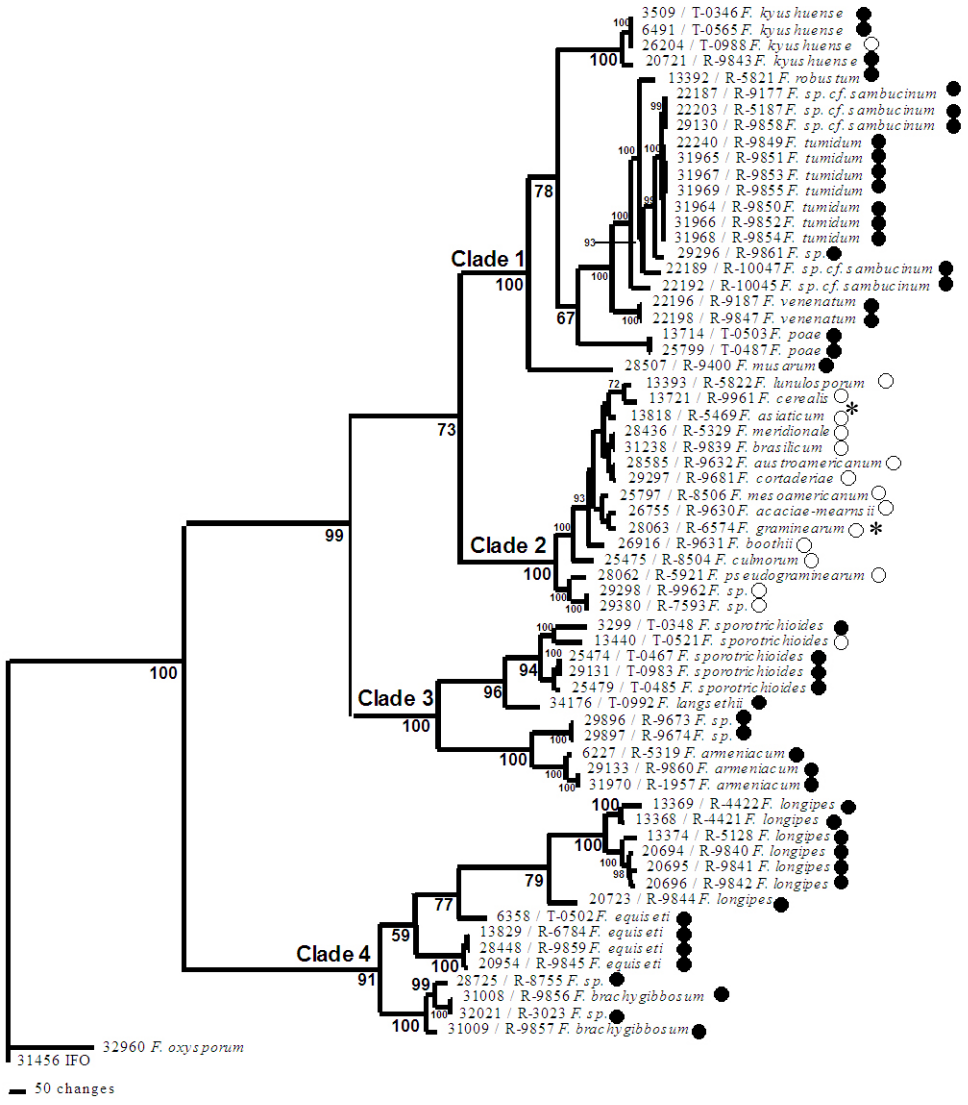
NRRL #*	FRC #**	Identified as***	Zearalenone
31965	R-9851	*F. tumidum*	-
31967	R-9853	*F. tumidum*	-
31969	R-9855	*F. tumidum*	-
22240	R-9849	*F. tumidum*	-
31964	R-9850	*F. tumidum*	-
31966	R-9852	*F. tumidum*	-
31968	R-9854	*F. tumidum*	-
22203	R-5187	*F. tumidum*	-
29130	R-9858	*F. tumidum*	-
22187	R-9177	*F. tumidum*	-
13392	R-5821	*F. robustum*	-
22198	R-9847	*F. venenatum*	-
13714	T-0503	*F. poae*	-
25799	T-0487	*F. poae*	-
3509	T-0346	*F. kyushuense*	-
26204	T-0988	*F. kyushuense*	+
20721	R-9843	*F. kyushuense*	-
28507	R-9400	*F. musarum*	-
26755	R-9630	*F. acaciae-mearnsii*	+
28063	R-6574	*F. graminearum*	-
26916	R-9631	*F. boothii*	+
28436	R-5329	*F. meridionale*	+
31238	R-9839	*F. brasilicum*	+
28585	R-9632	*F. austroamericanum*	+
29297	R-9681	*F. cortaderiae*	+
13818	R-5469	*F. asiaticum*	-
13393	R-5822	*F. lunulosporum*	+
13721	R-9961	*F. cerealis*	+
25475	R-8504	*F. culmorum*	+
29298	R-9962	*F. pseudograminearum*	+
29296	R-9861	*F. culmorum*	+
29380	R-7593	*F. pseudograminearum*	+
28062	R-5291	*F. pseudograminearum*	+
25474	T-0467	*F. sporotrichioides*	-
3299	T-0348	*F. sporotrichioides*	-
13440	T-0521	*F. sporotrichioides*	+
29133	R-9860	*F. armeniacum*	-
31970	R-1957	*F. armeniacum*	-
6227	R-5319	*F. acuminatum*	-
29896	R-9673	*F. sp.*	-
29897	R-9674	*F. sp.*	-

20695	R-9841	*F. longipes*	-
20696	R-9842	*F. longipes*	-
20694	R-9840	*F. longipes*	-
13374	R-5128	*F. longipes*	-
13369	R-4422	*F. longipes*	-
13368	R-4421	*F. longipes*	-
20723	R-9844	*F. longipes*	-
6358	T-0502	*F. equiseti*	-
20954	R-9845	*F. equiseti*	-
31008	R-9856	*F. brachygibbosum*	-
32021	R-3023	*F. culmorum*	-
28725	R-8755	*F. brachygibbosum*	-
31009	R-9857	*F. brachygibbosum*	-
	O-1174	*F. oxysporum*	-
	O-1166	*F. oxysporum*	-
	R-6784	*F. compactum*	+
	R-6354	*F. cerealis*	+
	R-4485	*F. equiseti*	+
	T-249	*F. sporotrichioides*	-
	T-625	*F. sporotrichioides*	-
	T-336	*F. sporotrichioides*	+
	T-341	*F. sporotrichioides*	+

*NRRL indicates strain number from the USDA National Regional Research Laboratory.
**FRC indicates strain number from the Fusarium Research Center.
***Isolates were not identified in this work; these designations are as the isolates have been identified previously.

Fig. 3.3: Phylogenetic Tree Representing the Diversity of the Trichothecene-Producing Fusaria with Zearalenone-Producing Species Identified Upon It (tree from Zitomer *et al.* 2006)

(Bootstrap values from 863 pseudoreplicates)



Isolates with a closed circle were here found to be ZEA non-producers.
Isolates with an open circle were here found to be ZEA producers.
*the isolates of *F. graminearum* and *F. asiaticum* used in our study were found to be ZEA non-producers, but these species are known producers. The isolates used here have been previously shown to be non-producers.

Table 3.3: Zearalenone and Zearalenol Amounts (in µg/g rice) Produced Under Three Temperature Regimes by *Fusarium* Species

			Zearalenone			Alpha-Zearalenol		
FRC #	Identified As	ZEA TOTALS*	Temperature Treatment**					
			1	2	3	1	2	3
R-5291***	*F. pseudograminearum*	139.38	47.03	43.47	48.88	0.00	30.21	0.00
R-5291		188.68	44.75	81.94	61.99	0.00	55.76	0.00
R-5329	*F. meridionale*	41.30	27.89	4.12	9.29	0.00	0.00	6.32
R-5329		7.33	2.93	1.99	2.41	0.00	0.00	4.32
R-5470	*F. kyushuense*	24.16	0.11	0.76	23.29	0.00	0.00	13.50
R-5470		67.20	0.51	0.00	66.69	0.00	0.00	1.32
R-5822	*F. lunulosporum*	10.92	6.90	2.17	1.85	0.00	0.00	0.00
R-5822		14.15	12.69	1.38	0.08	2.45	0.00	0.00
R-6784	*F. compactum*	0.01	0.01	0.00	0.00	0.00	0.00	0.00
R-6784		0.27	0.13	0.14	0.00	11.56	0.00	0.00
R-7593	*F. culmorum*	362.37	307.18	35.80	19.39	14.36	12.96	2.65
R-7593		159.02	111.87	18.19	28.96	11.65	14.96	1.49
R-8504	*F. culmorum*	11.21	2.91	5.62	2.68	0.00	0.00	0.00
R-8504		9.91	1.90	6.10	1.91	0.00	0.00	0.00
R-9630	*F. acaciae mearnsii*	11.04	4.56	4.46	2.02	0.00	0.00	4.24
R-9630		17.31	2.21	1.52	13.58	0.00	0.00	6.05
R-9631	*F. boothii*	17.46	4.48	6.05	6.93	0.00	0.00	1.88
R-9631		17.11	10.03	5.94	1.14	0.00	0.00	2.22
R-9632	*F. austroamericanum*	5.55	2.95	1.56	1.04	0.00	0.00	2.38
R-9632		3.77	2.53	0.70	0.54	0.00	0.00	0.00
R-9681	*F. cortaderiae*	5.05	0.00	1.55	3.50	0.00	0.00	2.25
R-9681		26.83	0.00	23.23	3.60	0.00	0.00	3.39
R-9839	*F. brasilicum*	409.94	122.77	285.76	1.41	20.68	0.00	5.96
R-9839		53.85	47.80	5.00	1.05	7.23	0.00	1.76
R-9861	*F. culmorum*	0.00	0.00	0.00	0.00	0.00	0.00	0.00
R-9861		0.03	0.03	0.00	0.00	6.17	0.00	0.00
R-9961	*F. cerealis*	22.75	2.03	19.73	0.99	0.00	0.00	2.85

R-9961		48.13	15.54	31.87	0.72	23.70	0.00	0.00
R-9962	*F. pseudograminearum*	3815.87	1412.40	1170.42	1233.05	1.31	112.92	0.00
R-9962		2274.21	1568.15	543.16	162.90	0.00	19.39	4.61
T-0521	*F. sporotrichioides*	5.20	0.49	4.07	0.64	0.00	0.00	5.34
T-0521		2.51	0.05	1.82	0.64	0.00	0.00	1.38
R-6354	*F. cerealis*	8.05	0.38	1.42	6.25	0.00	0.00	0.00
R-6354		18.50	10.00	0.61	7.89	95.00	0.00	69.75
R-4485	*F. equiseti*	22108.56	7065.79	7006.12	8036.65	2050.64	1508.43	167.88
R-4485		20604.93	4969.92	7930.03	7704.98	1371.68	1039.38	34.22
T-336	*F. sporotrichioides*	3.08	0.36	1.94	0.78	0.00	0.00	4.30
T-336		2.12	0.00	1.70	0.42	0.00	0.00	2.09
T-341	*F. sporotrichioides*	0.41	0.00	0.41	0.00	0.00	0.00	0.00
T-341		3.04	0.00	2.39	0.65	0.00	0.00	1.13

*the sum of the individual temperature treatment values

**Temperature Treatment 1: 3 weeks at 25 ºC

Temperature Treatment 2: 3 weeks at 25 ºC, followed by 3 weeks at 15ºC

Temperature Treatment 3: 2 weeks at 25 ºC, followed by 2 weeks at 15 ºC, followed by 2 weeks at 25 ºC

***The two lines per isolate represent within batch replicates

VI. Discussion

Based upon the total accumulated knowledge with regards to zearalenone production by isolates within the trichothecene producing fusaria, it may seem logical to suggest that clade 2 represents the only group capable of producing this toxin. Certainly it is the organisms belonging to clade 2 that have the most impact on human and animal food crops via contamination with both trichothecene mycotoxins as well as zearalenone, as this group includes *F. graminearum* and *F. culmorum*, the two most notorious zearalenone producing fusaria. The miscellaneous reports of zearalenone production outside of this clade can then be viewed as very likely the results of misidentifications of the species producing zearalenone. This view of ZEA production within this group of organisms is incorrect, however.

This survey has provided a detailed view of zearalenone and zearalenol production within a robust phylogenetic framework. The production of these toxins is clearly most strongly correlated with clade 2 of the trichothecene-producing fusaria. Our data show that production of these toxins is not limited to this clade as occasional high-producing strains were evident outside of it. Many previous reports of the production of zearalenone by species traditionally not associated with trichothecene production, and thus perhaps residing outside of the trichothecene-producing clade, are likely the result of mis-identifications. However, as we show occasional production outside of the *F. graminearum* clade, these results should be investigated further (Table 3.2, Fig. 3.3). Specifically, we saw the following species outside of the clade 2 producing zearalenone

and zearalenol: *F. kyushuense* (T-0988) and *F. sporotrichioides* (T-0521, T-336, and T341). We also saw zearalenone production by R-4485, previously identified as *F. equiseti*. This isolate was further examined, as it showed very high levels of both zearalenone and zearalenol under our test conditions. The identity of this isolate was determined by sequence analysis of partial sequence of the elongation factor gene to represent a new species outside of the trichothecene-producing fusaria. The capability to produce zearalenone may be ancestral within the three core clades of the trichothecene producers, but largely has been lost in Clades 1 and 3 while being maintained in Clade 2, and lost completely from Clade 4. The ability to produce these toxins may, in fact, be ancestral outside of the trichothecene-producing fusaria, as we have shown one isolate with very high production to be separate from this group of species. This observation indicates that a more widespread search for zearalenone-producing species may be warranted, as the number of species able to contaminate food commodities is unknown and potentially large.

It should be noted that there exists even in this small data set a temperature preference discrepancy within the species *F. culmorum*. Three isolates of this species were analyzed, and each responded uniquely to the temperature treatments. For this reason, and for the generally artificial nature of laboratory culture experiments, prudence should be used in interpreting these types of data for inference into ecological/field significance.

The following species of *Fusarium* within clade 2 were shown here to be ZEA producers: *F. acaciae-mearnsii*, *F. boothii*, *F. meridionale*, *F. brasilicum*, *F. austroamericanum*, *F. cortaderiae*, *F. lunulosporum*, *F. cerealis*, *F. culmorum*, *F.*

pseudograminearum. Outside of clade 2, isolates previously identified as the following species were also shown to be ZEA producers: *F. kyushuense*, *F. sporotrichioides*, *F. equiseti*, and *F. compactum. Fusarium kyushuense* has not been investigated for zearalenone production to date. This report is thus the first evidence of zearalenone production by this species. This work also represents the first report of zearalenone and zearalenol production by both *F. brasilicum* and *F. cortaderiae*, both members of Clade 2.

O' Donnell *et al.* (2000) surveyed much of the clade within the trichothecene-producing fusaria encompassing *F. graminearum* and its relatives (Clade 2). Both this study and our data are in agreement with regards to showing the following species as zearalenone producers: *F. boothii*, *F. meridionale*, *F. austroamericanum*, *F. lunulosporum*, *F. cerealis*, *F. culmorum*, and *F. pseudograminearum*. Isolate R-6354, *F. cerealis*, was investigated previously as well (Scott *et al.*, 1980) and our work and this study are also in agreement showing this isolate as a producer.

O' Donnell *et al.* (2000) found *Fusarium acaciae-mearnsii* to be a ZEA non-producer (3/3 negative for zearalenone and zearalenol by O' Donnell *et al.* in 2000). Our data are not in agreement with this observation. In this study *F. acaciae-mearnsii* produced both zearalenone and zearalenol. This represents the first report *F. acaciae-mearnsii* producing zearalenone and zearalenol.

There was a lack of zearalenone production by *F. graminearum* and *F. asiaticum*, two species from which zearalenone production would have been highly expected. O'Donnell *et al*. (2000) showed the same isolate of *F. graminearum* (NRRL 28063) to be a zearalenone non-producer, though other isolates of this species were producers in that

work. *Fusarium asiaticum* was also included in the O' Donnell *et al.* (2000) study, and the isolate NRRL 13818 was shown to be a low producer (5-50 ppm). In the current work, this same isolate did not produce zearalenone.

Overall, our work supports the previously held observation that the majority of ZEA producers are found within clade 2 of the trichothecene producing fusaria. All isolates from within clade 2 included in our study were shown to be ZEA producers, with the exception of *F. graminearum* and *F. asiaticum*, and these results have been addressed above. The organisms within clade 2 are also closely associated with type B trichothecene production (those trichothecenes lacking a keto group at the C-8 position, notably dexynivalenol, nivalenol, and related metabolites). The evolutionary pressure that resulted in these organisms showing such a predilection towards type B trichothecenes over type A trichothecenes may also have had a role in selecting for ZEA production. The exact nature of the selective forces involved in shaping these organisms and populations towards the metabolic diversity now observed is unknown, and the correlation of ZEA production with type B trichothecene production may be no more than evolutionary coincidence.

Two isolates shown to be ZEA producers outside of clade 2 were also included in the previous phylogenetic analysis and trichothecene production survey by Zitomer *et al*. (2006) (*F. kyushuense* T-0988 and *F. sporotrichioides* T-0521). These two isolates had very different trichothecene profiles: T-0988, *F. kyushuense*, showed higher overall production of type B trichothecenes than type A, though both types were produced; T-0521, *F. sporotrichioides*, showed much higher type A production (particularly large amounts of neosolaniol, from 57 to 1200μg/g rice) than type B production. While

relatively a small amount of data in comparison to the larger number of isolates in clade 2, this comparison does at least illustrate that ZEA production is not always correlated with higher type B trichothecene production than type A production. Again, the ecological and evolutionary pressures that have resulted in the observed metabolic profiles for these organisms may never be fully elucidated, but the data presented here may be useful towards these ends.

Fusarium equiseti has been reported to be a zearalenone producer in previous studies (Hestbjerg *et al.*, 2002). The isolates used in that study were identified using morphology and physiological tests, as well as comparisons of metabolite profiles with previous work. While this identification system is robust, there is confusion as to the exact identities of isolates named *F. equiseti*. Those isolates that have been identified as *F. equiseti* compose two distinct phylogenetic groups. One of these groups produces trichothecenes, and the other is unrelated to the trichothecene-producing clade (Michele Mansfield, personal communication). The isolates of *F. equiseti* included in the current study are those belonging to the clade within the trichothecene-producing fusaria, and are zearalenone non-producers. The one exception to this is R-4485, an isolate previously identified as *F. equiseti*, but here determined to represent a new species outside of the trichothecene-producing clade. This isolate produced very high levels of both zearalenone and zearalenol.

Fusarium semitectum has been described previously as a zearalenone producer (Marasas *et al*., 1984). No isolates named *F. semitectum* were included in our study, and so no comparison may be made. The same situation is true of *F. avenaceum*. Langseth *et al.* (1999) reported zearalenone production from this species, but no isolates were

included in the current study so no comparison may be made. Further work is needed to confirm or deny zearalenone production by isolates of these species.

Fusarium sporotrichioides has been demonstrated to produce zearalenone previously (Cullen, 1981). Our data confirms the observation that this species is a zearlaneone producer. This is one of the two lineages identified in this work to be a Zea producer that is outside of clade 2.

Fusarium compactum has not been reported previously to be a zealenone producer. It was included in our work as it is known to be an occasional producer of type A trichothecenes. We found very low levels of zearalenone produced by the one isolate included here.

Fusarium oxysporum has been reported to be a zearalenone producer multiple times (Milano and Lopez, 1991; Ueno *et al.*, 1977). The two strains identified by Ueno *et al.* (1977) as zearalenone producers (O-1174 and O-1166, described then as *F. lateritium* and later reidentified by Marasas *et al*. [1984] as *F. oxysporum* based upon morphology) were shown in the current study to be zearalenone non-producers.

This work represents a significant advance in the understanding of zearalenone and zearalenol production by fusaria within a robust phylogenetic framework. It also addresses some of the complexity seen regarding variation of zearalenone production under diverse temperature conditions. The data shown indicate that more research is needed on a strain-by-strain basis to tease out the idiosyncrasities of temperature effects on zearalenone production. We have not addressed every species previously reported to produce zearalenone (for instance, *F. semitectum*). Such reports also warrant further investigation in order to determine potential foodstuff contamination. The complete

picture of the evolution of ZEA production within the members of the trichhothecene producing fusaria is still unclear, but these observations shed light on some previously dark areas.

CHAPTER 4: Fumonisin Production Investigated Within a Phylogenetic Analysis of the *Gibberella fujikuroi* Species Complex

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II. Abstract

Fumonisins are sphingolipid analogues associated with the *Gibberella fujikuroi* species complex (GFC) that cause fatal diseases in horses and swine and are associated with cancers and neural tube defects. The goal in this study was to make precise connections between phylogenetically well-defined *Fusarium* species and the production of fumonisins, with the aim of determining threats to exposure via food crop contamination. We first generated a database of translation elongation factor 1-alpha sequences from putative isolates in the GFC to accurately identify isolates to known and potentially new phylogenetic species. Members of new and previously uncharacterized species were then analyzed for fumonisin production by high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI MS). Fumonisin producers were scattered throughout the phylogenetic diversity of the GFC, and new groups were identified with the potential for fumonisin production. Many of

these groups showed association with food crops including, but not limited to corn, sorghum, and mango.

III. Introduction

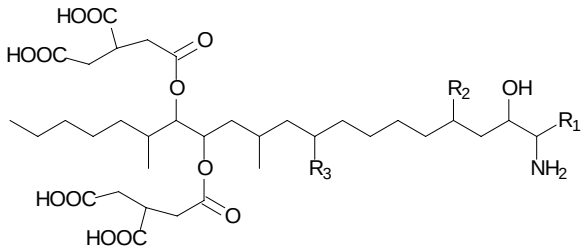
Fumonisins are aminopolyalcohols that are produced by some species of *Fusarium.* Chemically, the fumonisins consist of a 20-carbon backbone, with methyl groups at carbons 12 and 16, an amino group at carbon 2, and two tricarballylic esters at carbons 14 and 15 (see Fig. 4.1). There are many series of fumonisins, including the A, B and C series, with multiple forms in each class (e.g., B_1, B_2) (Rheeder *et al*., 2005).

Fumonisins were first discovered in South Africa in 1988, after an investigation of the disease equine leukoencephalomalacia (ELEM) associated with *F. verticillioides,* then recognized as *F. moniliforme* (Marasas, 2001). There was also observed during this time an association with increased risk of esophageal cancer in humans and consumption of *F. verticillioides*-infected corn. The discovery of the toxins involved an 18-year search for the compound/s associated with the disease, and also associated with cancer in rats. After the discovery of the fumonisins, it was quickly determined that they were the cause of ELEM, as well as porcine pulmonary edema (Marasas *et al.*, 1988; Ross *et al.*, 1990). The fact that fumonisins cause cancers in rats was justification for a more complete analysis of the cancer-causing potential of this toxin. This analysis was performed (IARC, 1993), and the study resulted in the classification of the fumonisins as Group 2B carcinogens (i.e., *possibly carcinogenic to humans*).

Fumonisins have also been implicated in neural tube defects (NTD) in mammals. They have been shown to induce NTD and craniofacial defects in cultured mouse embryos (Marasas *et al*., 2004). There has also been shown a correlation of incidence of NTD and consumption of fumonisin contaminated foodstuffs. In areas of high fumonisin consumption there is higher than normal incidence of NTD (Marasas *et al*., 2004; Mismer *et al.*, 2006).

Fumonisins exist naturally in numerous chemical forms. The most common of these are the B-series fumonisin, and, of those, the toxins fumonisin B_1 and B_2 are the most often encountered in foods and feeds.

Fig. 4.1: The Chemical Structure of Select Fumonisins



Fumonisin Analog	R_1	R_2	R_3	Molecular Weight
FB_1	**CH_3**	**OH**	**OH**	**721**
FB_2	**CH_3**	**OH**	**H**	**705**
FB_3	**CH_3**	**H**	**OH**	**705**
FC_1	**H**	**OH**	**OH**	**707**
FC_2	**H**	**OH**	**H**	**691**
FC_3	**H**	**H**	**OH**	**691**
FC_4	**H**	**H**	**H**	**675**

Based upon Sewram *et al.* 2005.

The production of fumonisins has been associated mainly with species contained within the *Gibberella fujikuroi* species complex (GFC) (Desjardins, 2006). The reports of fumonisin production from fusaria are summarized in Table 4.1. Molecular systematics and phylogenetics revealed a biogeographic pattern within the phylogenetic structure of the GFC (O'Donnell *et al.,* 1998b). This work shows three clades within the GFC with some biogeographic associations to Asian, African and American origins. However this observation is only apparent under specific phylogenetic parameters, i.e. gene regions used. As such this hypothesis needs further inquiry to determine its validity.

This work aimed to use a combination of phylogenetic analysis of gene sequences and HPLC-MS analysis of culture extracts to develop a comprehensive picture of fumonisin production in the GFC.

Table 4.1: *Fusarium* Species with Reported Fumonisin Production

Fungal species	Reported toxins[1]	References
F. acutatum	FB_1, FB_2, FB_3	Norred *et al.* unpublished Fotso *et al.* 2002
F. andiyazi	FB_1	Rheeder *et al.* 2002
F. annulatum	FB_1, FB_2, FB_3	Norred *et al.* unpublished
F. anthophilum	FB_1	Nelson *et al.* 1992
F. begoniae	FB_1	Fotso, 2002
F. brevicatenulatum	FB_1	Fotso *et al.* 2002
F. dlaminii	FB_1	Nelson *et al.* 1992
F. fujikuroi	FB_1	Desjardins *et al.* 2000a
F. globosum	FB_1, FB_2, FB_3	Syndenham *et al.*1997
F. napiforme	FB_1	Nelson *et al.* 1992
F. nygamai	FB_1, FB_2	Thiel *et al.* 1991

F. oxysporum	C-series fumonisins	Seo *et al.* 1996
F. oxysporum var. redolens	FB_1, FB_2, FB_3	Abbas 1995b
F. polyphialidicum	FB_1	Abbas 1995a
F. proliferatum	FB_1, FB_2, FB_3	Nelson *et al.* 1992
F. phyllophilum	FB_1	Fotso *et al.* 2002
F. pseudocircinatum	FB_1, FB_2	Fotso *et al.* 2002
F. pseudonygamai	FB_1, FB_2	Leslie *et al.* 2005
F. ramigenum	FB_1, FB_2, FB_3	Norred *et al.* unpublished
F. subglutinans	FB_1	Desjardins *et al.* 2000b
F. sacchari	FB_1	Leslie *et al.* 1992
F. thapsinum	FB_1	Klittich *et al.* 1997
F. verticillioides	FB_1, FB_2, FB_3 FB_4	Gelderblom *et al.* 1988

[1] FB_1 = Fumonisin B_1, FB_2 = Fumonisin B_2, FB_3 = Fumonisin B_3, FB_4 = Fumonisin B_4

The objective of surveying the GFC and some strains of *F. oxysporum* for fumonisin production was to determine which species in this group had the ability to produce fumonisins under our test conditions. This will result in a more comprehensive concept of the threat that certain species of fusaria pose to food crops with regard to fumonisin contamination. This may facilitate a greater understanding of how to reduce fumonisin exposure through diet. As these toxins undoubtedly enter the foodstream and result in human exposure and the associated health risks therein, work such as this offers a potential way to bottleneck fumonisin exposure. The source of greatest human exposure to fumonisin toxins is through contaminated corn products (CAST 2003). The FDA has set guidance levels for fumonisin B_1 in human foodstuffs at 2 to 4μg/g depending on food type, and from 5 to 100μg/g for animal foodstuffs, again based on food type and target

animal species (CAST 2003). One hypothesis of this work was that a phylogenetic pattern with regard to toxigenicity would be discerned. The *F. oxysporum* isolates were included as they had been previously reported to be fumonisin producers (Seo *et al.,* 1996). Another goal of this work was to determine the extent of agricultural commodities at risk for fumonisin contamination. Currently, the main food of concern is corn and corn products, but as many of the species noted to produce fumonisins occur on other crops, this work aimed to determine the extent of possible risk of exposure.

IV. Materials and Methods

A. Fungal Strains

Isolates were chosen based upon representative diversity in both phylogenetic (as determined by preliminary phylogenetic analysis), as well as geographic, origin (See Appendix B for strain list). In total, 164 isolates were examined for fumonisin production. The African clade was represented by 89 isolates, the American clade by 16 isolates, and the Asian clade by 31 isolates. Twenty-eight other isolates were also included from outside of the GFC.

B. Culture Preparation

Fusarium species isolates, representing diversity across the phylogeny, were obtained from The Pennsylvania State University Fusarium Research Center (FRC) located in University Park, PA or the USDA National Regional Research Laboratory

(NRRL) located in Peoria, IL. These isolates were grown on Carnation Leaf Agar (CLA) (Fisher, 1982) for 7-12 days at 25 ºC with a 12 h alternating light (both white and UV lights) and dark cycle. Spore suspensions were made by adding 2 ml of sterile water to the CLA plate surface and scraping with a plate spreader. Spore suspensions (100 µl) were used to inoculate each of two rice cultures. Rice cultures were prepared by adding 2 ml dH_2O to 1 g converted long grain rice (Uncle Ben's [TM] Food Company) in 14 ml polypropylene round-bottom tubes (VWR, West Chester, PA) with polystyrene foam plugs and aluminum foil caps. The rice was allowed to imbibe the water for approximately 4 h then was autoclaved at 121 ºC for 15 min. at 20 lbs. pressure. Following inoculation, rice cultures were incubated in the dark for three weeks at 25 ºC. Prior to extraction and analysis, the rice cultures were frozen at –20 ºC.

C. Extraction and Cleanup

Cultures were extracted in the polypropylene tubes they were grown in with 8.00 ml of 3:1 methanol:water and were broken up with a metal spatula to enhance extraction. The cultures were then placed on an orbital shaker for 30 min at 310 rpm, shaken vigorously and vortexed for several seconds before being shaken for an additional 30 min. Cultures were then centrifuged at 1294 X G for 10 min at 20 °C and the supernatant separated from the solid portion. Extracts were adjusted to pH 5.8-6.5 using NaOH and/or HCl. The entire volume of extract (~8ml) was then passed through a cleanup column containing 500 mg of SAX resin (Bondesil – SAX, Varian, Walnut Creek, CA) between two polypropylene frits with a 20µm pore size. The column was washed with 5 ml of 3:1

methanol:water, then 3 ml of methanol. The fumonisins were eluted by washing the cartridge with 10 ml 1:99 acetic acid:methanol. The elutant was desolvated under nitrogen gas purge to dryness, then resolvated in 200 µl methanol.

D. LC-MS

The cleaned concentrates in methanol were introduced to a Waters 2695 HPLC coupled to a Waters/Micromass LCT Premier electrospray ionization time-of-flight mass spectrometer (Milford, MA) in positive ion mode. In some cases, positive samples were diluted 1/300 (v/v) in methanol and then run a second time. The method used was a variation on Mansfield *et al.* in process. Separation of fumonisins was affected using the following solvent gradient: 0 – 10 min, the gradient was changed from 70% H_2O:acetic acid (99:1 v/v):30% acetonitrile:acetic acid (99:1 v/v) to 100% acetonitrile:acetic acid (99:1 v/v). The solvent conditions were then held at 100% acetonitrile:acetic acid (99:1 v/v) for three min, followed by a methanol wash for five min. The system was then reequilibrated at 70% H_2O:acetic acid (99:1 v/v):30% acetonitrile:acetic acid (99:1 v/v) for three min. Twenty minutes of instrumentation time was required for each sample. The column used was a Thermo Betasil C18 150 X 2.1 mm with 5µm particle size with an Agilent Eclipse XDB-C8 12.5 X 2.1 mm with 5µm particle size guard column, both with pore sizes of 80 angstroms. This method successfully separated FB_1, FB_2, and compounds with masses corresponding to FB_3, FC_1, FC_2, FC_3, and FC_4.

V. Results and Discussion

A. Impetus and Methodological Results

The GFC contains a wide diversity of species that are found on food products. For instance, the isolates used in this study were originally isolated from substrates as diverse as corn, sugar cane, coffee, tree nuts, mangoes, millet, sorghum, rice, sweet potato, asparagus, barley, and bananas. Identifying which species and lineages within the GFC are toxin producers is of great importance in the effort to control fumonisin contamination of these food crops. The FDA has set guidance levels for human food consumption at from 2 to 4μg/g depending on the food source. Studies such as the one presented here allow for a greater understanding of which species and on what crops fumonsins might be expected. This will allow the overall exposure of both humans and animals to fumonisins to be better evaluated, and potentially controlled.

The detection limit of fumonisins in this study was determined to be 5μg/g rice for both FB_1 and FB_2. This value is slightly higher than theoretically possible on the instrumentation used, as carryover of fumonisins was observed in blanks run after samples containing fumonisins (either standards or positive samples). We determined an average level of carryover to normally be in the range of 200 ppb as per the samples introduced to the instrument (themselves five times more concentrated than as per one gram of rice). This value therefore corresponds to a carryover level of around 1μg/g rice. Therefore, the detection limit was set at five times this observed carryover (5μg/g rice) to account for potential carryover. Some samples contained a large amount of fumonisins, and this caused column overloading in the LC-MS system. These samples were diluted

1/300 (v/v) and run again. The system used was very effective at determining presence/absence of fumonisin toxins, but was sensitive to amount of toxin present, often showing effects such as column overloading and detector saturation. These effects reduced the accuracy of quantification, particularly at higher levels, but was useful in determining ranges of toxin production, which were placed into four categories: Category 1 produced no detectable fumonisins up to 5 µg/g rice; Category 2 produced 5-50 µg/g rice, Category 3 produced 50-1500µg/g rice, Category 4 produced over 1500µg/g rice. Quantification in this manner was only possible for $FB_1$and FB_2, as these were the only two toxins for which standards were available. For all other fumonisin analogues reported here, it was assumed that ionization efficiency was similar to that of $FB_1$and FB_2, though this may not be the case. To avoid false positives as often as possible, an isolate was deemed a fumonisin producer if, and only if, both replicate cultures showed production of the same toxin.

The retention time for FB_1 was 5.1 min, and for FB_2 was 6.9 min. The retention times for the C-series fumonisins were determined by searching the chromatogram of a known producer for corresponding masses. The isolate investigated in this manner was O-2137, which has been demonstrated previously to produce the C-series analogues (Seo *et al.,* 1996, there referred to as isolate CAR). The retention times of the peaks corresponding to C-series analogues were then used in the analysis of all other fumonisin assays on the LC-MS. The mass corresponding to FC_1 (m/z 708 $[M+H]^+$) showed two peaks, one at 4.7 min, the other at 5.17 min. Since no standard was available for this toxin, data for both peaks were collected. The determination of which peak is correctly representing FC_1 could not be made without a standard for comparison. The mass

corresponding to FC_2 (m/z 692 $[M+H]^+$) is also the mass corresponding to FC_3, as they are isomeric. The two retention times found for these two compounds were 6.03 min and 6.97 min. The determination of which peak represents which form could not be performed without a standard, so the presence or absence of peaks at these retention times is reported. The mass corresponding to FC_4 (m/z 676 $[M+H]^+$) showed a peak at 7.7 min. The same method was used to determine the retention time of FB_3. The chromatogram resulting from analysis of M-8335 was investigated for presence of a peak at mass 706 (m/z $[M+H]^+$). This isolate has been known previously as a producer of FB_3. The retention time for this compound was determined to be 6.3 min. Again, exact identity of the peak at this retention time can not be explicitly stated as FB_3, but the fact that the expected mass is appearing in the chromatogram of a known producer at this retention time is strong evidence that this is the correct assignment of this peak.

An isolate was determined to be a producer of any of these other analogues if there was an instrument response at the expected mass of a quantifiable area similar to that corresponding to 5μg/g rice FB_1 and FB_2 (approximately 2000 area units). If this same threshold was exhibited in the diluted samples, the isolate was categorized as a high producer of the given analogue (as this corresponds approximately to a concentration of 1500μg/g rice and higher).

A number of species have been analyzed for fumonisin production previously, and were not included in our analysis because we were mainly interested in identifying production characteristics in new or less well-defined lineages. Other species have not been investigated previously, but were also not included. That being said, many species with well-defined fumonisin production characteristics were included in order to ensure

our data were in agreement with previous observations. Some species which were not included were *F. sacchari* (indicated as a non-producer by Leslie *et al.,* 1992), *F. bactridioides* (no available data on fumonisin production), *F. succisae* (no available data on fumonisin production), *F. bulbicola* (no available data on fumonisin production), *F. circinatum* (indicated as a non-producer by Desjardins *et al*., 2000b), *F. begoniae* (indicated as a low producer by Fotso *et al*., 2002), *F. guttiforme* (indicated as a non-producer by Fotso *et al*., 2002), *F. xylarioides* (no available data on fumonisin production), *F. acutatum* (production of fumonisins reported by Fotso *et al.*, 2002), *F. napiforme* (A low percentage of isolates of this species have been shown to be fumonisin producers previously by Nelson *et al.,* 1992 and Thiel *et al.,* 1991), *F. ramigenum* (shown to produce B-series fumonisins by Norred, personal communication), *F. pseudoanthophilum* (no available data on fumonisin production), *F. brevicatenulatum* (no available data on fumonisin production), *F. lactis* (a known non-producer of fumonisins ccording to Fotso *et al*., 2002), *F. pseudocircinatum* (a known producer of FB_1 and FB_2 according to Fotso *et al*., 2002), and *F. denticulatum* (known to be a non-producer of fumonisins according to Fotso *et al*., 2002).

B. Fumonisin Production in the African Clade of the GFC

The species included as members of the African clade of the GFC are among the most significant fumonsisn producers with regard to the contamination of food crops. *Fusarium verticillioides* is known to occupy an ecological niche as either an endophyte or pathogen of corn, depending on conditions, but it is known to occur on many other hosts,

particularly grains and cereals. This species, as well as others in the African clade of the GFC are known producers of fumonisins. Isolates examined from this group were originally isolated from a variety of food crop sources, including but not limited to sorghum, millet, corn, banana, sugar cane, and barley. The wide range of food crops from which these species are known to occur, in combination with their high propensity to produce fumonisins, makes this clade a significant threat to food safety via exposure to mycotoxins.

The African clade, which is supported as monophyletic in multilocus analyses, is paraphyletic with regard to the TEF gene region. The species included as belonging to the African clade include: *F. andiyazi, F. pseudonygamai*, *F. pseudoantholphilum*, *F. brevicatenulatum*, *F. verticillioides, F. napiforme*, *F. ramigenum, F. lactis, F. thapsinum*, *F. nygamai*, *F. pseudocircinatum*, *F. udum*, *F. phyllophilum*, *F. acutatum*, *F. xylarioides*, *F. dlaminii*, *F. denticulatum*, and 10 undescribed lineages.

Our results suggest that the following species from this clade are non-producers or category 1 fumonisin producers: *F. andiyazi*, *F. pseudonygamai*, *F. udum*, *F. dlaminii*, four lineages closely related to *F. andiyazi*, and three unnamed lineages. *Fusarium andiyazi* has been known to be a low level fumonsin producer from one study previously (Rheeder *et al.*, 2002). Eighteen isolates of this species were analyzed for toxin production, and only isolate M-3162 was found to be category 3 producer of FB_1 and isolate M-5820 a category 2 producer of FB_1 and FB_2. All other isolates of this species analyzed were fumonisin non-producers. This observation suggest *F. andiyazi* is not a significant threat to agriculture via fumonisin contamination.

Fusarium pseudonygamai has been reported to produce FB_1 and FB_2 in one previous investigation (Leslie *et al*., 2005). We have included six isolates from this species in the fumonisin analysis, and all isolates were negative for all analogues investigated. Our data suggest *F. pseudonygamai* is not a significant threat to agriculture with regards to fumonisin contamination. The discrepancy between our observations and that of previous work may be due to different culturing conditions.

Fusarium udum is not known to be a fumonisin producer. The only isolate of *F. udum* analyzed for fumonisin production in our work was found to be a non-producer. This isolate was originally from sorghum. This observation supports the concept that this species is not a threat due to fumonisin production.

Fusarium dlaminii has been shown to produce FB_1 previously (Nelson *et al*., 1992). Seven isolates of this species have been included in the fumonisin production survey here. Only B-series fumonisins were detected from one isolate of this species, and this was at category 2 levels. All other isolates of this species were found to be negative for fumonisin production. The only isolate producing fumonisins here was originally from a soil isolation. This species is not likely a threat to agricultural commodities based upon our data.

Four undescribed lineages showed a close relationship to *F. andiyazi*. These four lineages included a total of 14 isolates, and only one of these was a fumonisin producer (category 1). These lineages do not appear to pose a threat to agricultural due to fumonisin contamination. The one isolate found to produce fumonisin, however, was originally from sorghum, and so the threat of contamination may exist. Overall, however, that threat appears to be minimal.

One undescribed lineage evident in the African clade was represented by isolate M-3210 (previously identified as *F. anthophilum*), originally recovered from soil. This lineage was closely related to *F. napiforme* and *F. ramigneum*. Isolate M-3210 was surveyed for production of fumonisins, and was determined to be a non-producer of fumonisins. This lineage does not appear to be a threat to agriculture.

Another unnamed lineage was evident near *F. lactis.* This lineage was represented by isolates M-3072 (from soil near wheat), M-3074 (from soil near wheat), M-3073 (from soil near wheat), and NRRL 25615 (=M-8660, from rice seed). All of these isolates were included the fumonisin survey, and determined to be fumonsins non-producers. These isolates do appear to be associated with food crops, but do not appear to represent a threat, as they all were negative for fumonisin production.

Two new lineages were evident closely related to *Fusarium lactis*. Each lineage was represented by only one isolate in our phylogenetic analysis, and these were M-7533 and NRRL 26793 (=M-8664). Isolate M-7533 (originally isolated from corn kernels) was not investigated here. Isolate NRRL 26793 (=M-8664) was found to be a category 4 producer of FB_2, and a producer of FB_3 and one of the compounds indicated at the mass corresponding to FC_1. This isolate was originally isolated from the parasitic plant *Striga*. The fact that this lineage was recovered from *Striga*, and that high producers of fumonisin exist, suggest that this species is not likely a threat. Future studies should address the host range of this lineage, howver, to determine if potential for food crop contamination does exist.

Our data suggest the following species are potentially threats to food crops via contamination with fumonisins: *F. verticillioides*, *F. thapsinum*, *F. nygamai*, *F.*

phyllophillum, and one unnamed lineage. *Fusarium verticillioides* is a well-characterized fumonisin producing species. It was from this species that fumonisins were first isolated and described (Marasas, 2001). Many studies have been done showing this species is often a high producer of B-series fumonisins, and that many isolates are also capable of producing other analogues as well (Sewram *et al.,* 2005; Desjardins *et al*., 2000b; Thiel *et al.,* 1991; Leslie *et al*., 1992). This species was found to be a monophyletic group with high diversity in our phylogenetic analysis, consisting of 21 isolates. Many of the isolates of this species were found to be category 3 or 4 producers of B-series fumonisins, in agreement with previous studies. Three isolates (M-1552, M-5500 and M-6715) were found to be negative for B-series production, though isolate M-1552 was positive for production of either FC_2 or FC_3. Isolates M-6715 and M-5500 were found not to produce any of the analogues investigated here. Isolate M-5500 has been previously shown to be a fumonisin non-producer (Desjardins, 2000a). The lack of production evidenced by the other two species may be due to culture degeneration from repeated subculturing, or simply a lack of induction in our culturing conditions. This species is a well-known threat to agricultural crops, specifically corn, due to fumonisin contamination, though two isolates in this study were isolated from soybean-corn feed mixture. This indicates that soybeans may also be at risk of fumonisin contamination due to colonization by *F. verticillioides*, though the isolations most likely originated in the corn portion of the feed mixture.

Fusarium thapsinum is known to rarely have the ability to produce fumonisins, but reports of production do exist (Klittich *et al*., 1997; Leslie *et al*., 1996). Two isolates were included in the fumonisin production experimentand only one produced FB_1

(category 1 to 3), as well as FB_3, FC_2, FC_3, and FC_4. The isolate found o produce fumonisins was originally isolated from soil, though other isolates of this species have been recovered from sorghum. This suggests that this species may potentially be a threat to sorghum production through contamination with fumonisins.

Fusarium nygamai is known to produce fumonisins (Thiel *et al*., 1991). Two isolates of this species were included in the fumonisin production survey and both were category 3 producers of FB_1 and FB_2. One of these isolates was originally cultured from sorghum, and the other from soil. This observation indicates that this species may also be a threat to sorghum production via fumonisin contamination.

Fusarium phyllophilum was here represented by four isolates originally recovered from *Dracaena* and *Sansaveria* species. This species is known to be a fumonisin producer (Fotso *et al.*, 2002). Our data indicate two of the four isolates analyzed are category 4 producers of fumonisins. This species does appear to be associated with food crops, and so the danger posed by its production of fumonisin is potentially limited. There does exist one isolate of this species recovered from pig feed in New York, however. Further work should address the potential host range of this species, as its high toxin production and unclear substrate preference warrants attention.

An unnamed lineage was evident in relation to *F. pseudocircinatum*, *F. nygamai*, and *F. thapsinum*. This lineage composed isolates M-1521, M-1995, M-1906, NRRL 25221 (=M-8656), and M-1986. Many isolates in this lineage were fumonisin producers. These isolates were originally from corn, soil, and soil near sorghum. The combination of high fumonisin production in this group and an association with food crops indicate that

this lineage may be a threat due to fumonisin contamination. Future studies should investigate the potential impact this lineage may have.

C. Fumonisin Production in the Asian Clade of the GFC

A monophyletic group of species and unnamed lineages was evident in our analyses (and in previous works) that has been suggested to have biogeographical association with Asia. The most notable toxigenic species included in this clade is *F. proliferatum*, which is well known to produce large amounts of fumonisins from previous work. This species represents a major producer of fumonisins, and hence is a significant source of this class of toxins in the food supply of both humans and animals. Isolates from this clade included in our analyses were originally isolated from food crops such as corn, sorghum, rice, and sugar cane, among other substrates. The presence of *F. proliferatum*, as well as other producers of fumonisins in the Asian clade of the GFC makes this group of organisms a concern for human exposure to these toxins.

The Asian clade was found to be monophyletic in our analysis, with the exception of *F. sacchari*, which resolved nested within the African clade. *Fusarium sacchari* is highly divergent at the sequence level from the rest of the isolates in the Asian clade of the GFC, and this anomalous placement outside of the Asian clade has been witnessed before when only single-gene analyses are performed. As it was not our goal to indicate specific phylogenetic placements for the species investigated here, this was not deemed to be an issue. This clade contained the following species: *F. fujikuroi*, *F. proliferatum*, *F. globosum*, *F. concentricum*, and two unnamed lineages.

The species that were determined to not represent a threat to agricultural crops included *F. concentricum* and two unnamed lineages. *Fusarium concentricum* has not been the subject of extensive survey for fumonisin production, but the data that do exist indicate it is a non-producer (Fotso *et al*., 2002). Both isolates of *F. concentricum* analyzed for fumonisin production (M-8659, from rice, and M-8658, from wheat) were non-producers of all fumonisin analogues analyzed for here. This species does not appear to be a threat to agriculture via fumonisin contamination.

Two undescribed lineages were identified within the Asian clade, basal to *F. proliferatum* and *F. fujikuroi*. One of these lineages seems to be associated with mangoes, and was represented here by isolates M-6202 (previously identified as *F. concentricum*), NRRL 25226 (=M-8657), and NRRL 26427 (=M-8663). These isolates were originally recovered from mango and soil, and were found to be non-producers of fumonisins in the LC-MS study. Analysis of additional isolates in this lineage will be needed to confirm the potential toxin production this lineage, though it appears to be a non-producer.

Another undescribed lineage in close relation to the above-mentioned mango lineage was also evident in our phylogeny. This lineage was comprised of NRRL 26794 (=M-8665, previously identified as *F. fractiflexum*, from *Cymbidium*), and 28852 (=M-8667, previously identified as *F. fractiflexum*, of unknown origen). Neither of these isolates was determined to be fumonisin producers, though further analysis of other isolates in this lineage will be needed to confirm this observation for the entire lineage. The lack of production and association with food crops would indicate this species as a non-threat due to fumonisin contamination.

Our data support the following species as significant threats to agriculture via potential fumonisin contamination of food crops: *F. fujikuroi*, *F. proliferatum*, *F. globosum. Fusarium fujikuroi* is closely related to *F. globosum* and *F. proliferatum*. *Fusarium fujikuroi* isolates are often found to be fumonisin non-producers, though there have been reports of low-level production from this species (Proctor *et al*., 2004; Desjardins *et al*., 2000a). Our data support this concept, in that ten of the fourteen isolates analyzed here were either non-producers (M-5088, M-6884, M-8497, M-8512, M-8521, M-1137, M-1250) or category 1 producers (M-2455, M-0947, M-0927). Only four isolates were found to be high producers (M-1732, M-0965, M1724, M-3682), and these were mainly producing B-series fumonisins. This is a new observation with regards to the potential of this species to be a high producer of fumonsins, and, hence, a significant threat to food crops. Isolates of this species used here originated from rice, sorghum, pine seeds, soil, hay, *Gladiolus*, and even a weevil, though the majority came from rice isolations. Future work should re-evaluate the potential for high production by isolates of this species, and hence, potential contamination of rice and sorghum, among other crops, with fumonisins.

Fusarium proliferatum was found to constitute a monophyletic group that is sister to *F. globosum* and closely related to *F. fujikuroi*. This species is one of the most prolific of fumonisin producers known (Leslie *et al*., 2004). Our data show most of the isolates analyzed as category 4 producers of FB_1 and category 3 producers of FB_2 (see Table 4.2). Isolates of this species used here were originally obtained from soil, sorghum, corn, pecan, alfalfa, and asparagus. As is known, this species represents a significant threat to agriculture, and our data supports this concept.

Fusarium globosum is known to produce fumonisins from previous studies (Proctor *et al.,* 2004). Two isolates were included in our toxin analysis, M-7534 and M-8688. These isolates were not analyzed phylogenetically, and were determined to be *F. globosum* as per previous identifications. One isolate, M-7534, evidenced category 4 production of FB_1 and production of FB_3, FC_2, FC_3, and FC_4. Isolate M-7534 was originally isolated from corn kernels. Isolate M-8688, originally isolated from wheat, was a non-producer of all fumonisins analyzed for here. The fact that isolates of this species are capable of high-level production of fumonisins, and that they have association with food crops, indicates that this species is a threat to humans via exposure through foodstuffs.

Our data show the following species in the Asian clade of the GFC to be fumonisin producers: *F. proliferatum*, *F. globosum*, and *F. fujikuroi.* Within this clade, only *F. concentricum* and two unnamed lineages were found to be fumonisin non-producers. With the exception of *F. proliferatum*, this clade does not seem to be a major threat to food crops via fumonisin contamination, though it may represent a minor source of food contamination. The other two species, *F. globosum* and *F. fujikuroi*, may represent significant threats under certain conditions, but for the most part appear to be minor threats.

D. Fumonisin Production in the American Clade of the GFC

The American clade was found to be monophyletic in our analysis. This clade contained the following species: *F. subglutinans*, *F. succisae, F. anthophilum*, *F.*

bactridioides, *F. bulbicola*, *F. begoniae*, *F. circinatum*, *F. guttiforme*, and six undefined lineages. In the American clade, only *F. subglutinans* and *F. anthophilum* are known to be fumonisin producers, and these are usually at low levels. This clade does not represent species or lineages that are significant threats to food safety via production of fumonisins.

Fusarium subglutinans was evident in the American clade of the GFC. This species is known to include both non-producing and low-level fumonisin producers, though the prior appear to be predominant in nature (Desjardins *et al*., 2000b; Leslie *et al*. 1992). The one isolate of this species analyzed here, M-1294, isolated from moldy corn, was negative for toxin production.

Fusarium succisae was evident as a sister group to *F. subglutinans*. This species is not known as a fumonisin producer, and our results support this. Two isolates were investigated, M-3699 from corn, and M-7792, from *Teosinte*, and both were negative for all fumonisin analogues searched for.

Fusarium anthophilum strains have been shown to have the ability to produce fumonisins, though the levels found have not been very high (Nelson *et al*., 1992). One isolate previously identified as *F. anthophilum* was included in the toxin analysis, but was not included in the phylogenetic study (M-3829, from soil). This isolate was negative for fumonisin production.

An unnamed lineage was evident as a sister group to *Fusarium circinatum*. This lineage was represented here by isolates NRRL 29123 (=M-8598) and 29124 (=M-8599). Neither of these isolates showed evidence of fumonisin production. Both of these lineages were isolated from *Bidens*, a Midwestern U.S. wetland plant.

An unnamed lineage of *Fusarium* was also witnessed falling between *Fusarium begoniae* and *Fusarium guttiforme.* This lineage was only represented in our phylogeny by isolate NRRL 25204 (=M-8590, no information on original isolation available). This isolate was negative for all fumonisin analogues analyzed for here.

A lineage comprising isolates NRRL 25346, NRRL 26756 (=M-8596, from ornamental grass), and NRRL 26757 (=M-8597, no information on original isolation) was also evident in the basal region of the American clade of the GFC. Only isolates M-8597 and M-8597 were investigated for fumonisin production. M-8596 was negative for all fumonisin analogues tested, and M-8597 was a category 4 producer of FB_1 and a category 2 producer of FB_2. Further investigation into this lineage will be required to gauge the threat it may pose with regard to food contamination, though it appears to be a minor threat, if any, due to an apparent lack of association with food crops.

Yet another lineage was evident near the basal portion of the American clade. This lineage was represented by isolates M-0063, NRRL 25195 (=M-8655), NRRL 25623 and NRRL 25807 (=M-8595). All isolates tested from this lineage (M-0063, from *Arucaria*, M-8655, from wood, and M-8595, no information about original isolation) were negative for fumonisin production. This lineage does not appear to be a threat to food production as all isoltes investigated here were not found in association with food crops.

The most basal branch of the American clade was comprised of two undescribed lineages. The first of these was represented by M-3260, from corn, and NRRL 25622 (=M-8592, no information on original isolation). Both isolates were found to be negative fumonisin production. The second lineage in this basal group was represented by isolate

M-6864 (no information on original isolation). This isolate was also negative for fumonisin production.

The only significant fumonisin producer from the American clade was M-8597 (no information on original isolation), an unnamed isolate that was evident in an unnamed lineage in this clade. All other isolates analyzed here were negative or low-level fumonisin producers. This lineage does not appear to be a threat to foods and feeds through fumonisin production. Based upon the collective toxin data presented above, and the overall trend towards a lack of association of these species with food crops, the American clade is a non-threat, or, at most, a minor threat, to agricultural crops.

E. Other Isolates Analyzed

Two final, previously undescribed lineages were revealed in the phylogenetic analysis of the GFC. These two lineages were actually basal to the entire species complex, and it is unclear whether these should actually be considered part of the complex, or just outside of it. They are comprised of the following isolates: 1) M-7470, M-7471, M-7472, and M-7473; 2) M-7424, all isolated in India from *Java citronella* plants. All four isolates were negative for fumonisin production. The remaining lineage represented by M-7424 was was negative for fumonisin production as well.

Fusarium oxysporum isolates have previously been reported to produce B and C-series fumonisins (Abbas *et al*., 1995; Seo *et al*. 1996; Proctor *et al.*, 2004). Isolates O-1890 and O-2137 were shown to produce C-series fumonisins previously, and so were included here (Seo *et al*. 1996; Proctor *et al.*, 2004). Other isolates of *F. oxysporum* were

also investigated for fumonisin production, including isolates O-2284, O-2285, O-1956, and O-2283. Only isolates O-1890 and O-2137 evidenced any detectable fumonisins in our survey. These isolates showed category 2 production of B-series and production of C-series fumonisins, the first evidence that B-series are produced by these isolates. Isolate O-1890 was a producer of FB_1, FB_2, and FB_3, and a high producer of FC_1, FC_2, FC_3, and FC_4. Isolate O-2137 was a producer of FB_1, FB_2, and FB_3 (in one of two replicates), and a high producer of FC_1, FC_2, FC_3, and FC_4.

Numerous other isolates were included in the fumonisin production analysis to serve as negative controls. These included isolates of *Fusarium solani* (S-1710, S-1711, and S-1712), *F. avenaceum* (R-9495, and R-9490), *F. boothii* (R-7775), *F. asiaticum* (R-5469), *F. austroamericanum* (R-9632). All of these were negative for fumonisin production, as expected, with one exception. One isolate of *F. solani* (S-1710) showed low level production of FB_1 and FB_2. These results are surprising, as they are in disagreement with all previous observations regarding the non-production of fumonisins by this species. Contamination of cultures can never be ruled out in this type of analysis, and this seems the most likely explanation. Carryover of fumonisins in the LC-MS system, while unlikely given all of the steps taken to eliminate this issue from the data analysis, may also be the cause of this observation. Further work is being performed to confirm or deny the observation of fumonisin production by these isolates.

Fourteen other isolates were also analyzed for fumonisin production. These isolates appear at the bottom of Table 4.2 and represent the following species (as they were previously identified) *F. proliferatum*, *F. andiyazi*, *F. fujikuroi*, *F. nisikadoi*, *F. nygamai ssp. amizimbae*, *F.lateritium*, and *F. moniliforme*. The only producers witnessed

in this remaining group were isolates identified previously as *F. proliferatum* and *F. moniliforme*. None of these results are surprising, as these data are in agreement with previous observations.

The discontinuous and somewhat difficult to predict production of fumonisins by isolates in the GFC poses a problem for food producers worldwide. Many of the species present in the GFC are commonly associated with grain and other food crops, such as corn, sorghum, rice, and millet, to name a few. A more comprehensive picture of the species and isolates capable of contaminating foodstuffs with fumonisins needs to be developed. This work is a step in that direction, by providing a semi-comprehensive survey of the GFC both phylogenetically and toxicologically. Much more work needs to be done to clarify the extent and location (phylogentically) of fumonisin production in this extremely diverse group of fusaria, and to determine the overall threat that they pose due to contamination of food crops. Overall, however, it appears that the threat of fumonisin contamination in food crops extends beyond the current focus on corn and corn products. Given that many high producers investigated here were isolated from a variety of food crops other than corn indicate that other foodstuffs may also be potentially contaminated, and that these may be going mostly unobserved in the foodsystem.

Table 4.2: Strains Within the GFC Analyzed for Toxin Production

Identified Previously As	Isolate	FB1	FB2	FB3	FC1 peak 1	FC1 peak 2	FC2 or FC3 peak 1	FC2 or FC3 peak 2	FC4
African Clade									
			F. andiyazi[a]						
F. andiyazi	M-6909a	-	-						
	M-6909b	-	-						
F. andiyazi	M-6912a	-	-						
	M-6912b	-	-						
F. andiyazi	M-6903a	-	-						
	M-6903b	-	-						
F. andiyazi	M-6914a	-	-						
	M-6914b	-	-						
F. andiyazi	M-6905a	-	+						
	M-6905b	-	-						
F. andiyazi	M-8360a	-	-						
	M-8360b	-	-	+					
F. moniliforme	M-6258a	-	-						
	M-6258b	-	-						
F. andiyazi	M-5192a	-	-						
	M-5192b	-	-						
F. andiyazi	M-5194a	-	-						
	M-5194b	-	-						

F. moniliforme	M-6236a	-	-							
	M-6236b	-	+							
F. moniliforme	M-5716a	-	-							
	M-5716b	-	-							
F. andiyazi	M-5245a	-	-							
	M-5245b	-	+							
F. andiyazi	M-5761a	-	+							
	M-5761b	-	-							
F. moniliforme	M-3675a	-	-			+	+			
	M-3675b	-	-							+
F. moniliforme	M-5727a	-	-							
	M-5727b	-	-							
F. andiyazi	M-5210a	-	+							
	M-5210b	-	-							
F. andiyazi	M-5820a	+	+							
F. andiyazi	M-3162a	++	+	+	+					+
	M-3162b	+	+	+	+					+
F. andiyazi relatives Unnamed Lineage 1										
F. moniliforme	M-5254a	-	-							
	M-5254b	-	-							
F. moniliforme	M-7205a	-	-							
	M-7205b	-	-							
F. moniliforme	M-7239a	+	+	+	+					

	M-7239b	+	+	+	+
F. moniliforme	M-7207a	-	-		
	M-7207b	-	+		
F. andiyazi relatives Unnamed Lineage 2					
F. moniliforme	M-6176a	-	-		
	M-6176b	-	-		
F. moniliforme	M-6534a	-	-		
	M-6534b	-	-		
F. andiyazi relatives Unnamed Lineage 3					
F. moniliforme	M-3673a	-	-		
	M-3673b	-	-		
F. moniliforme	M-5077a	-	-		
	M-5077b	-	-		
F. moniliforme	M-5078a	-	-		
	M-5078b	-	+		
F. moniliforme	M-5742a	-	-		
	M-5742b	-	-		
F. moniliforme	M-5735a	-	-		
	M-5735b	-	-		
F. moniliforme	M-6572a	-	-		
	M-6572b	-	-		
F. moniliforme	M-8351a	-	-		

	M-8351b	-	-
F. andiyazi relatives Unnamed Lineage 4			
F. moniliforme	M-7456a	-	-
	M-7456b	-	-
F. pseudonygamai			
F. pseudonygamai	M-5759a	-	-
	M-5759b	-	-
F. pseudonygamai	M-6209a	-	-
	M-6209b	-	-
F. pseudonygamai	M-6201a	-	-
	M-6201b	-	-
F. pseudonygamai	M-6206a	-	+
	M-6206b	-	-
F. pseudonygamai	M-7143a	-	-
	M-7143b	-	-
F. pseudonygamai	M-6340a	-	-
	M-6340b	-	-
Unnamed Lineage			
F. antholphilum	M-3210a	+	+
	M-3210b	-	-

		F. verticillioides						
F. verticillioides	M-5500a	-	-					
	M-5500b	-	-					
F. verticillioides	M-7072a	+++	-	+		+	+	+
	M-7072b	+++	+	+		+	+	+
F. verticillioides	M-3703a	+++	++	+		+		+
	M-3703b	+++	-			+	+	+
F. verticillioides	M-3125a	+	+	+	+			
	M-3125b	+	+	+	+		+	+
F. verticillioides	M-6715a	+	+					
	M-6715b	-	-					
F. verticillioides	M-2973a	+++	+++	++				+
	M-2973b	+++	+++	++		+		+
F. verticillioides	M-8082a	+++	+++	+		+	+	+
	M-8082b	+++	+++	+		+	+	+
F. verticillioides	M-1325a	-	+++	++		+	+	+
	M-1325b	+++	+++	++		+		+
F. verticillioides	M-1331a	+++	-	+		+	+	+
	M-1331b	+++	+++	+		+	+	+
F. verticillioides	M-1332a	+++	+	+				+
	M-1332b	+++	+++	+		+	+	+
F. verticillioides	M-1890a	+++	+++	++		+		+
	M-1890b	+++	+++	++		+		+

F. verticillioides	M-1544a	+++	+++	+			+	+	+
	M-1544b	+++	+++	++			+		+
F. verticillioides	M-1552a	-	+	+			+		
	M-1552b	-	-				+		
F. verticillioides	M-7040a	+++	+++	++	++		+		
	M-7040b	+++	+++	++					+
				Unnamed Lineage					
F. sp. 26793	M-8664a	-	+++	++	++		+		
	M-8664b	+	+++	++	++				
				Unnamed Lineage					
F. moniliforme	M-3072a	-	-				+		
	M-3072b	-	-						
F. moniliforme	M-3074a	-	-						
	M-3074b	-	-						
F. moniliforme	M-3073a	-	-						
	M-3073b	-	-						
F. sp. 25615	M-8660a	-	-						
	M-8660b	-	-						
				Unnamed Lineage					
F. moniliforme	M-1521a	+++	+++	++					
	M-1521b	+++	+++	++			+		

F. moniliforme	M-1995a	+	++	+					
	M-1995b	+	+	+					
F. moniliforme	M-1906a	-	+						
	M-1906b	+	+						
F. sp. 25221	M-8656a	-	+						
	M-8656b	-	-						
F. moniliforme	M-1986a	+++	+++	+					+
	M-1986b	+++	+++	+			+		+
				F. thapsinum					
F. thapsinum	M-8352a	-	-						
	M-8352b	-	-						
F. thapsinum	M-1313a	+++	-	+			+	+	+
	M-1313b	+	+	+	+		+	+	+
				F. nygamai					
F. nygamai	M-1412a	+	+	+					
	M-1412b	+++	+++	+					
F. nygamai	M-1455a	+++	+++	+			+		+
	M-1455b	+++	+++				+		+
				Unnamed Lineage					
F. moniliforme	M-1755a	-	-						
	M-1755b	-	-						

Asian Clade								
		F. fujikuroi						
F. fujikuroi	M-5088a	-	-					
	M-5088b	-	-					
F. fujikuroi	M-6884a	-	-					
	M-6884b	-	-					
F. fujikuroi	M-8497a	-	-					
	M-8497b	-	-					
F. fujikuroi	M-8512a	-	-					
	M-8512b	-	-					
F. fujikuroi	M-8521a	-	-					
	M-8521b	-	+		+	+		+
F. fujikuroi	M-2455a	+	-	+				
	M-2455b	+	+	+				
F. fujikuroi	M-1732a	+++	+++	++		+		
	M-1732b	+++	+++	++	+	+		
F. fujikuroi	M-0947a	-	+			+		
	M-0947b	-	-	+		+		
F. fujikuroi	M-0965a	+++	+++	++		+		
	M-0965b	+++	+++	++				
F. fujikuroi	M-1137a	-	+					
	M-1137b	-	-					
F. fujikuroi	M-0927a	+	+	+				

	M-0927b	+	+	+					
F. fujikuroi	M-1724a	+++	+	+	+				+
	M-1724b	+	+	+					
F. fujikuroi	M-1250a	+	-	+					
	M-1250b	-	-						
F. fujikuroi	M-3682a	+	+++				+		
	M-3682b	-	+++				+		
				F. proliferatum					
F. fujikuroi	M-1288a	+++	++	++					
	M-1288b	+++	-	++					
F. proliferatum	M-3117a	+++	++	+					
	M-3117b	+++	++	+					
F. proliferatum	M-6708a	+++	+++						
	M-6708b	+++	+++	+					
F. proliferatum	M-0730a	+	+	+					
	M-0730b	+	+	+					
F. proliferatum	M-6740a	+++	+++	++			+		
	M-6740b	+++	+++	++			+		
F. proliferatum	M-3089a	+++	-	+			+	+	+
	M-3089b	+++	-	+			+	+	+
F. proliferatum	M-0519a	+++	+	+					
	M-0519b	+++	++	+					
F. proliferatum	M-6205a	+++	-	+				+	

		M-6205b	+++	+++				+	
					F. globosum				
F. globosum		M-8688a	-	-					
		M-8688b	-	-					
F. globosum		M-7534a	+++	-	+		+	+	+
		M-7534b	+++	-	+		+	+	+
					Unnamed Lineage				
F. fractiflexum		M-8665a	-	-					
	NRRL 26794	M-8665b	-	-					
F. fractiflexum		M-8667a	-	-					
	NRRL 28852	M-8667b	-	-					
					Unnamed Lineage				
F. concentricum		M-6202a	-	-					
		M-6202b	-	-					
F. sp. 25226		M-8657a	-	-					
		M-8657b	-	+					
F. sp. 26427		M-8663a	-	-					
		M-8663b	-	-					
					F. concentricum				

F. concentricum		M-8659a	-	+					
	NRRL 25309	M-8659b	-	-					
F. concentricum		M-8658a	-	+					
	NRRL 25303	M-8658b	-	-					
African Clade									
			F. udum						
F. sp. 26064		M-8666a	-	-					
		M-8666b	-	-					
			F. phyllophilum						
F. phyllophilum		M-0061a	-	-			+		
		M-0061b	-	-			+		
F. phyllophilum		M-0062a	+++	+++	++		+		
		M-0062b	+++	+++	++				
F. phyllophilum		M-0915a	+	+	+				
		M-0915b	+	-	+				+
F. phyllophilum		M-0919a	+++	+++	++		+		
		M-0919b	+++	+++	++				
American Clade									
			F. subglutinans						
F. proliferatum		M-6959a	-	-					

	M-6959b	-	-
F.subglutinans	M-1294a	-	-
	M-1294b	-	-
		F. succisae	
F. moniliforme	M-3699a	-	+
	M-3699b	-	-
F. moniliforme	M-7792a	-	-
	M-7792b	-	-
		F. anthophilum	
F. anthophilum	M-3829a	-	-
	M-3829b	-	-
		Unnamed Lineage	
F. sp. 29123	M-8598a	-	-
	M-8598b	-	-
F. sp. 29124	M-8599a	-	-
	M-8599b	-	-
		Unnamed Lineage	
F. sp. 25204	M-8590a	-	-
	M-8590b	-	-

		Unnamed Lineage		
F. sp. 26756	M-8596a	-	-	
	M-8596b	-	-	
F. sp. 26757	M-8597a	+++	+	+
	M-8597b	+++	+	+

		Unnamed Lineage		
F. konzum	M-0063a	-	-	
	M-0063b	-	-	
F. sp. 25195	M-8655a	-	-	
	M-8655b	-	-	
F. sp. 25807	M-8595a	-	-	
	M-8595b	-	-	

		Unnamed Lineage		
F. moniliforme	M-3260a	-	-	
	M-3260b	-	-	
F. sp. 25622	M-8592a	-	-	
	M-8592b	-	-	

		Unnamed Lineage		
F. subglutinans	M-6864a	-	-	

	M-6864b	-	-	
African Clade				
Unnamed Lineage				
New Species	L-229a	-	-	
	L-229b	-	-	
New Species	L-232a	-	-	
	L-232b	-	-	
New Species	L-231a	-	-	
	L-231b	-	-	
New Species	L-230a	-	-	
	L-230b	-	-	
F. dlaminii				
F. moniliforme	M-1987a	+++	+	+
	M-1987b	+	+	+
F. moniliforme	M-1990a	-	-	
	M-1990b	-	-	
F. moniliforme	M-1992a	+	+	
	M-1992b	-	-	
F. moniliforme	M-1994a	+	+	
	M-1994b	-	-	
F. sp. 26152	M-8662a	-	-	
	M-8662b	-	-	

F. sp. 26061	M-8661a	-	-
	M-8661b	-	-
F. moniliforme	M-1991a	-	-
	M-1991b	-	-
		Unnamed Lineage	
F. moniliforme	M-7470a	-	-
	M-7470b	-	-
F. moniliforme	M-7473a	-	-
	M-7473b	-	-
F. moniliforme	M-7472a	-	-
	M-7472b	-	-
F. moniliforme	M-7471a	-	-
	M-7471b	-	-
		Unnamed Lineage	
F. moniliforme	M-7424a	-	+
	M-7424b	-	-
		F. solani	
F. solani	S-1710a	+	+
	S-1710b	+	+

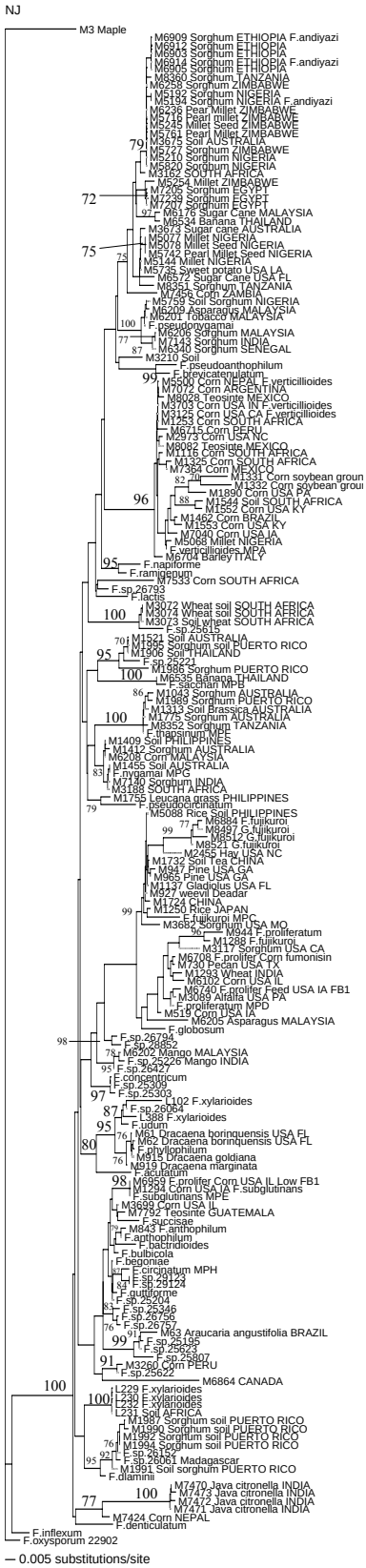
F. solani	S-1711a	-	+						
	S-1711b	-	-						
F. solani	S-1712a	-	-						
	S-1712b	-	-						
				F. oxysporum					
F. oxysporum	O-2284a	-	-						
	O-2284b	-	-						
F. oxysporum	O-1890a	+	+	+	++	++	++	++	++
	O-1890b	+	+	+	++	++	++	++	++
F. oxysporum	O-2285a	-	-						
	O-2285b	-	-						
F. oxysporum	O-2137a	+	+	+	++	++	++	++	++
	O-2137b	+	+		++	++	++	++	++
F. oxysporum	O-1956a	-	-						
	O-1956b	-	-						
F. oxysporum	O-2283a	-	-						
	O-2283b	-	-						
				Trichothecene clade fusaria					
F. avenaceum	R-9495a	-	-						
	R-9495b	-	-						
F. boothii	R-7775a	-	-						
	R-7775b	-	-						

F. avenaceum	R-9490a	-	-	
	R-9490b	-	-	
F. asiaticum	R-5469a	-	-	
	R-5469b	-	-	
F. austroamericanum	R-9632a	-	-	
	R-9632b	-	+	
		Other isolates analyzed		
F. proliferatum	M-8669a	+++	+	+
	M-8669b	+++	+	+
F. proliferatum	M-6183a	-	-	
	M-6183b	-	-	
F. proliferatum	M-6184a	-	-	
	M-6184b	-	-	
F. andiyazi	M-8413a	-	-	
	M-8413b	-	-	
F. fujikuroi	M-1269a	-	+	
	M-1269b	-	-	
F. fujikuroi	M-1268a	-	-	
	M-1268b	-	-	
F. nisikadoi	M-8430a	-	-	
	M-8430b	-	-	
F. nygamai ssp. amazimbae	M-8411a	-	+	
	M-8411b	-	-	

F. nygamai ssp. amazimbae	M-8415a	-	-						
	M-8415b	-	+						
F. nygamai ssp. amazimbae	M-8412a	-	-						
	M-8412b	-	-						
F. nygamai ssp. amazimbae	M-8414a	-	-						
	M-8414b	+	-						
F. lateritium	L-286a	-	-						
	L-286b	-	-						
F. moniliforme	M-8335a	-	++	++	+	+	++	+	++
	M-8335b	+	+	++					
F. moniliforme	M-0003a	-	-						
	M-0003b	-	-						

[a]names in header bars indicate name as per phylogenetic placement in the analysis of translation elongation factor 1-alpha
- less than 5μg/g rice fumonisin, + 5 to 50μg/g rice fumonisin, ++ 50-1500μg/g rice fumonisin, +++ above 1500μg/g rice fumonisin
For FB_3 and C-series fumonisins, + indicates approximately 5 to 1500μg/g rice fumonisin, ++ indicates over 1500μg/g rice fumonisin

Fig. 4.2: Phylogenetic Tree Created by Neighbor Joining Analysis of EF-1α



CHAPTER 5: Conclusion

In 1984, in the preface to the pivotal work *Toxigenic* Fusarium *Species*, Marasas, Nelson and Tousson eloquently elaborate on the tremendous difficulties that must be addressed in order to broach the field of *Fusarium* mycotoxicology. Of high significance in this discussion are the numerous problems inherent in trying to link a particular species with production of a particular toxin:

> The taxonomy of the genus is further complicated by the extreme variability of *Fusarium* species in culture and the fact that they mutate and degenerate rapidly, particularly under conditions of repeated subculturing on common laboratory media. This situation has led to great confusion in the extensive literature on *Fusarium* mycotoxicologybecause the same fungus is known under a variety of different names, because different fungi are lumped together under names such as *F. tricinctum* Corda emend. Snyd. & Hans. and *F. roseum* Lk. emend. Snyd. & Hans., because several *Fusarium* toxins have been named for mis-identified producing species, because elaborate chemical and pathological studies have been reported in the literature and attributed to incorrectly named species, and because many toxigenic *Fusarium* strains have become degenerate and lost their toxigenic ability due to maltreatment in laboratories that do not specialize in the maintenance of *Fusarium* cultures.

The work presented here has advanced the field of mycotoxicology by placing the production of specific mycotoxins, particularly the trichothecenes, zearalenone, and fumonisins, in association with the organisms that produce them within robust phylogenetic frameworks. This procedure allows phylogenetically defined lineages to be linked inexoriably to toxin production. By doing this, the name of a species does not hinder the process of linkage of toxin to fungus, as the name is not what is used to place the organism in its place phylogenetically; the very sequence of its DNA does that much more succinctly.

The three parts of the study presented here each address a different group of mycotoxins. The first study (Chapter 2) thoroughly investigates the trichothecene-producing potential of members of the genus *Fusarium* within a robust, multigene phylogeny developed to encompass the diversity of isolates known to produce trichothecenes. In the process of this analysis, it was revealed that many species within this phylogeny have a much higher diversity of toxin-producing capabilities than had previously been noted. Particularly, the type B trichothecenes were found to be much more prevalent throughout the entire group than was previously known.

The second study reported on here (Chapter 3) endeavors to locate the lineages within the trichothecene-producing fusaria that have the ability to produce another toxin, zearalenone. A secondary goal of this work was to address the frequent reports in the literature of temperature effects on zearalenone production by *Fusarium* species. The results of this work indicate that Clade 2 of the trichothecene-producing fusaria (that clade containing *Gibberella zeae* and its relatives) is the group most uniformly associated with zearalenone production, though other species outside of this group also evidenced production. The results of the investigation into temperature effects indicate that there is no one temperature treatment regime that will be optimal for zearalenone production for all species. Individual species, and even strains, have individual responses to environmental treatments, and toxin production is no different.

The third study reported on here (Chapter 4) addresses the production of fumonisins within the phylogenetic framework of the *Gibberella fujikuroi* species complex (GFC). In this case, a robust phylogenetic framework was first established and this was used to direct the search for fumonisin production. The production of this group

of toxins was found to be discontinuous within the GFC, in some cases strongly associated with a particular species, in others, discontinuous even at the species level.

The salience of this type of research, that of linking toxin production to individual phylogenetic taxa, is that a more permanent concept of toxin association may be developed. For instance, instead of reporting that the species *Fusarium sporotrichioides* produces neosolaniol, it may be more correctly reported that isolate NRRL 13440, previously identified as *F. sporotrichioides*, and in a phylogenetically distinct group along with isolate NRRL 3299 with 100% bootstrap support within clade 1 of the trichothecene-producing fusaria, produces neosolaniol. While this may seem tedious, it is essentially leaving a roadmap to toxigenic species, one that may be followed much more easily than the previously established system of landmarks that names represent.

Ultimately, the goal of this type of research is to be able to identify the exact threats to agricultural commodities. As many of the species examined here have been isolated from food crops such as grains, the potential for human exposure to fumonisins as a result of fusarial contamination is high. By understanding the mycotoxicology of a given isolate or species, one has a much better chance of addressing and even preventing the threats to food safety that mycotoxigenic fungi and mycotoxins pose. This research is one step in that direction.

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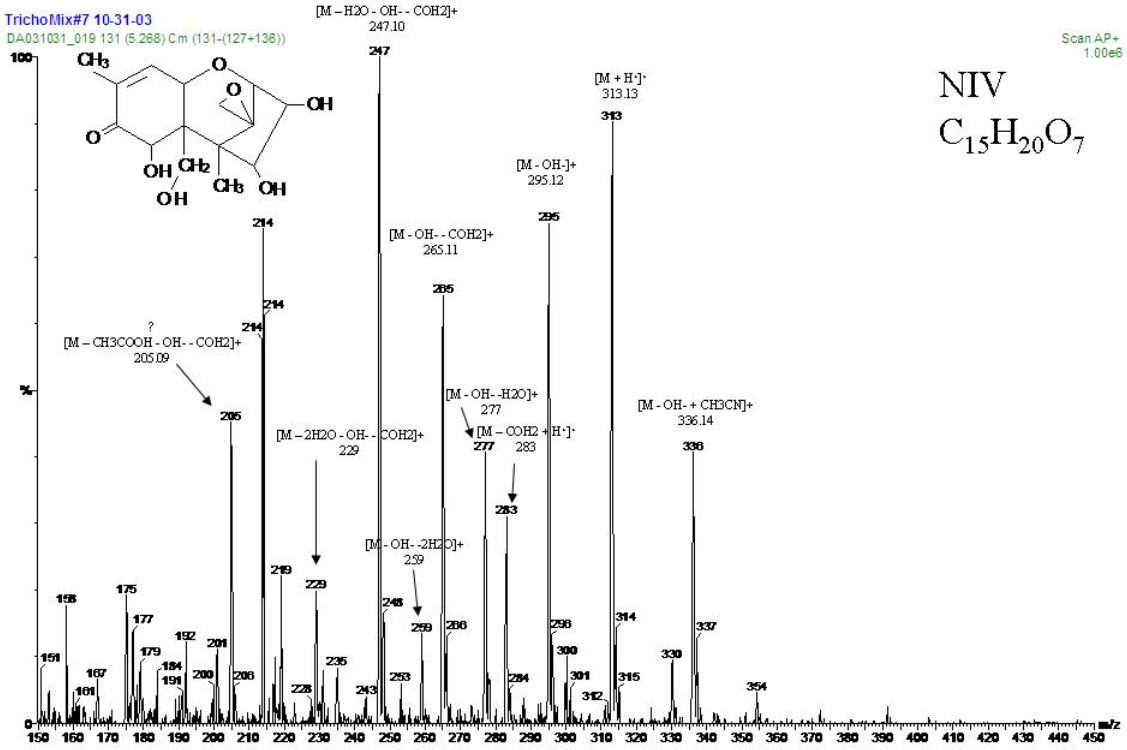
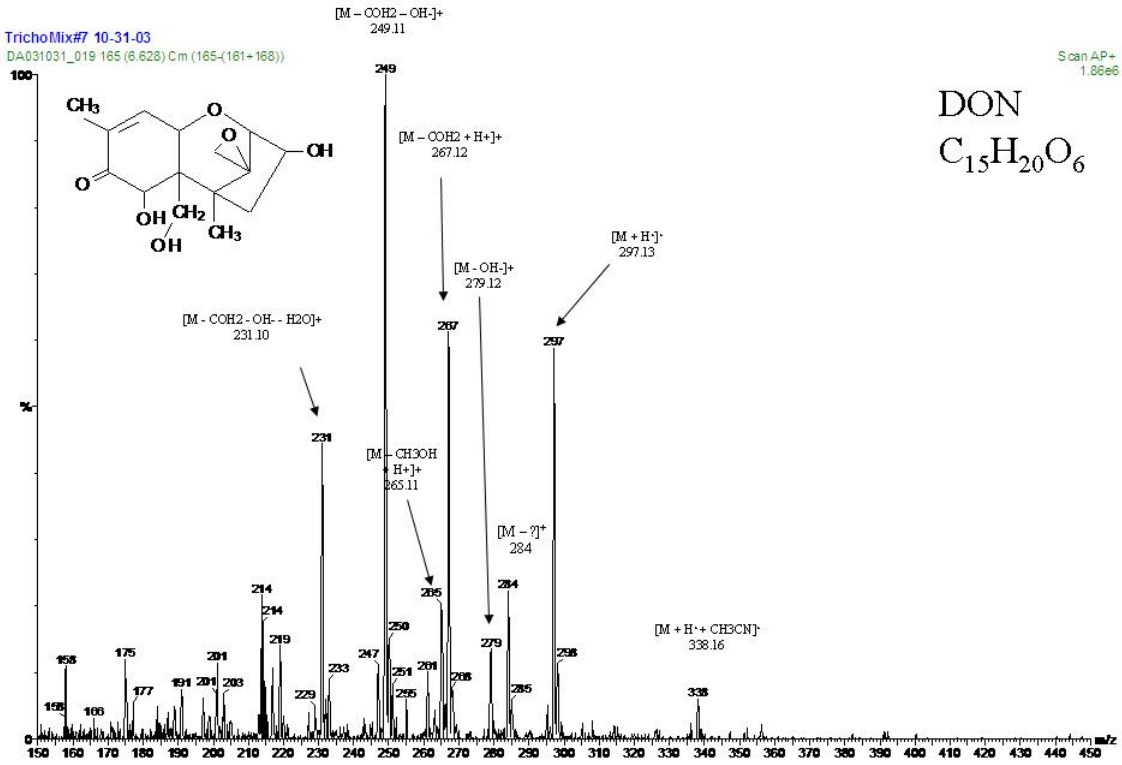
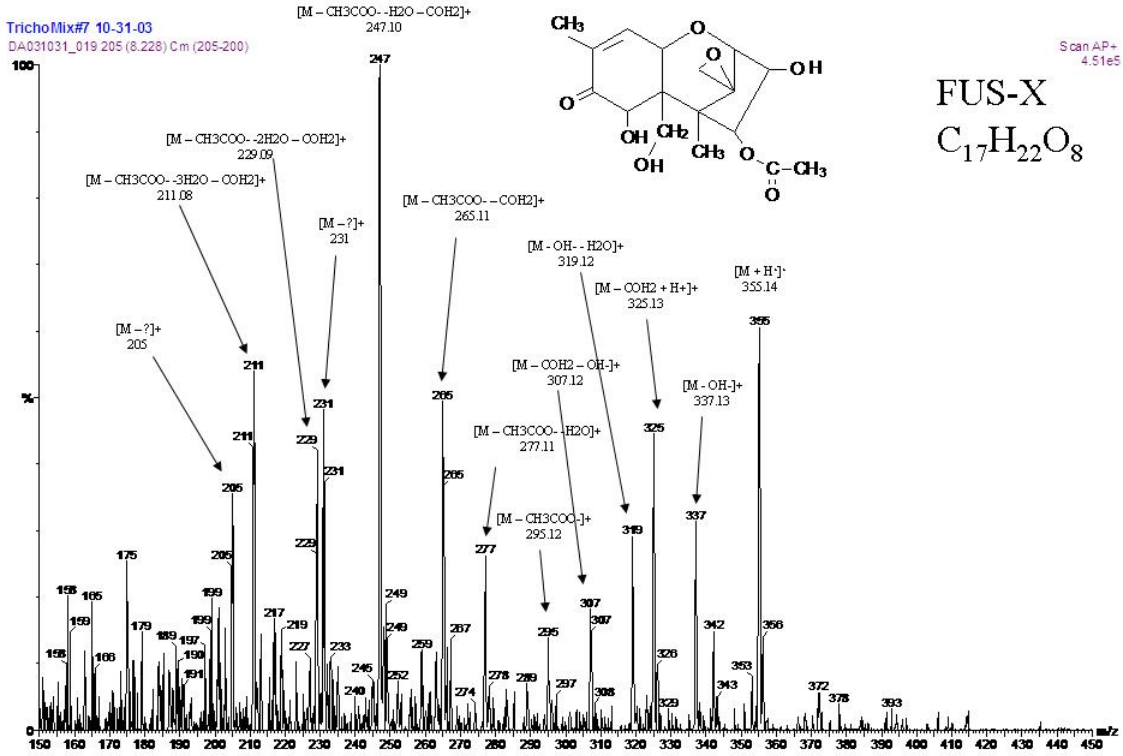
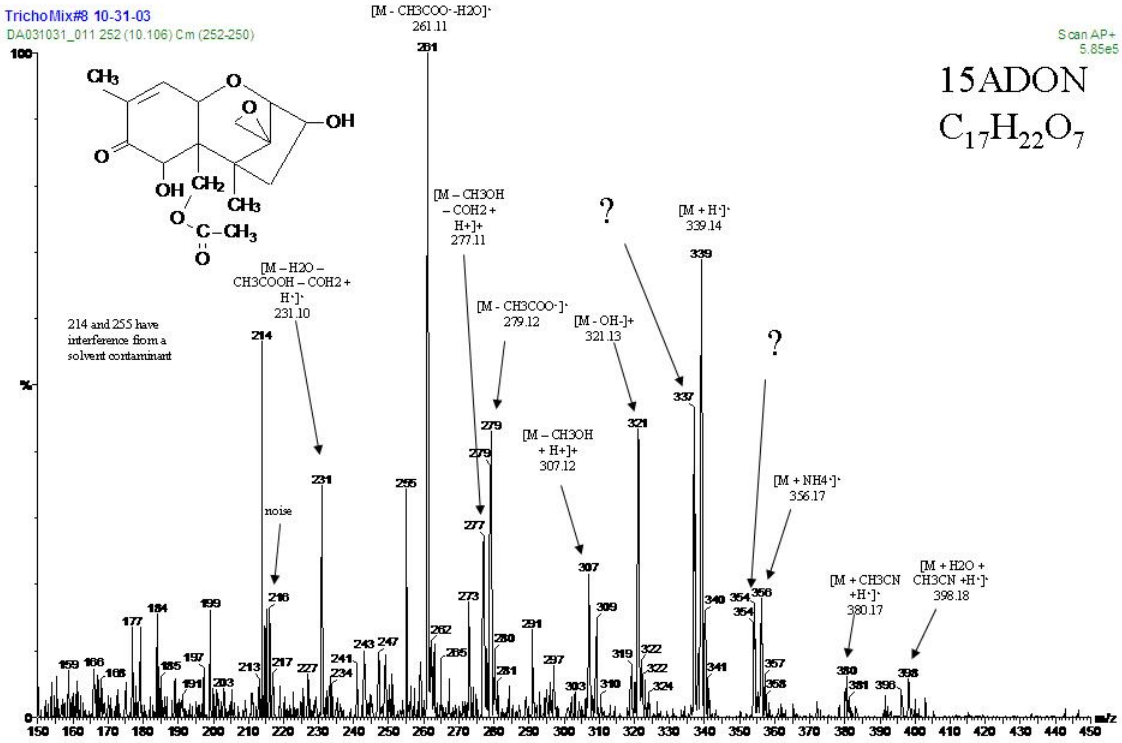
APPENDICES

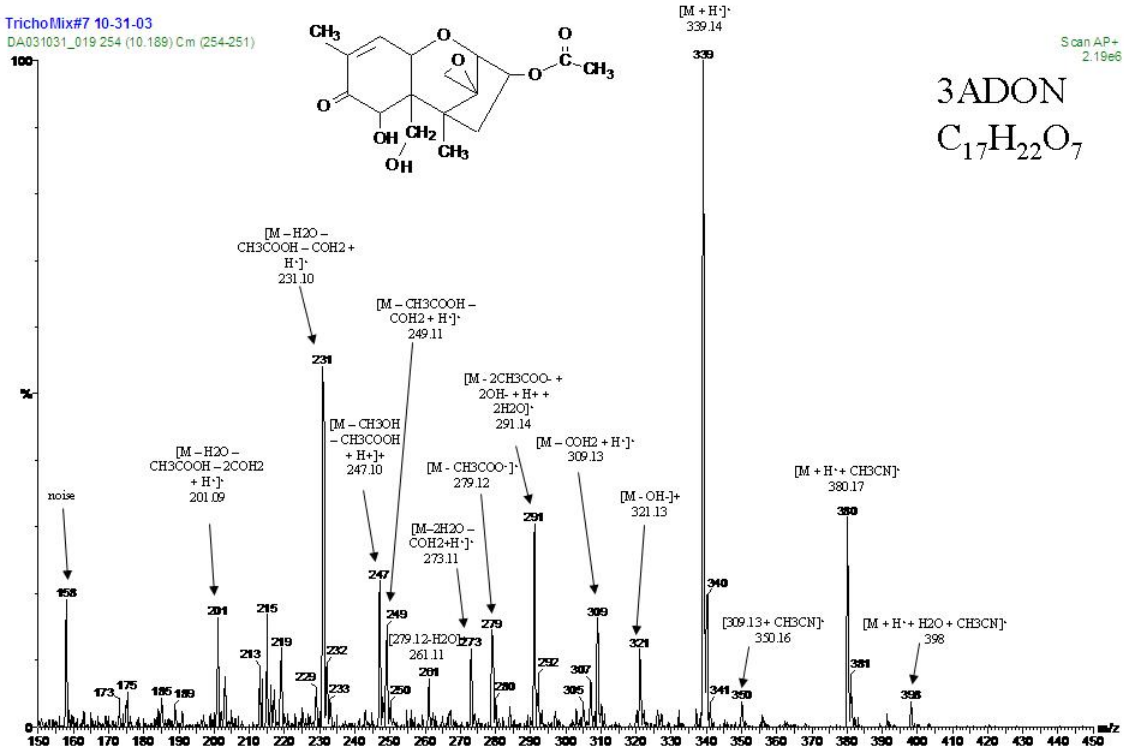
Appendix A: Example Mass Spectra of Trichothecenes and Hydrocortisone

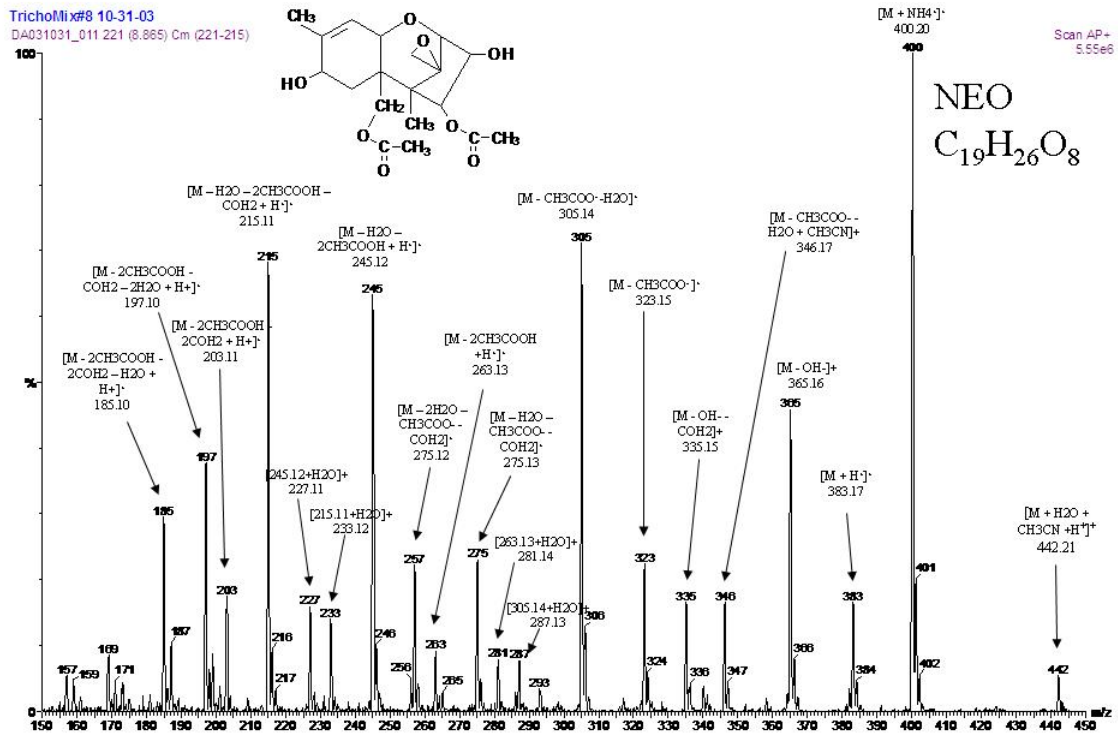


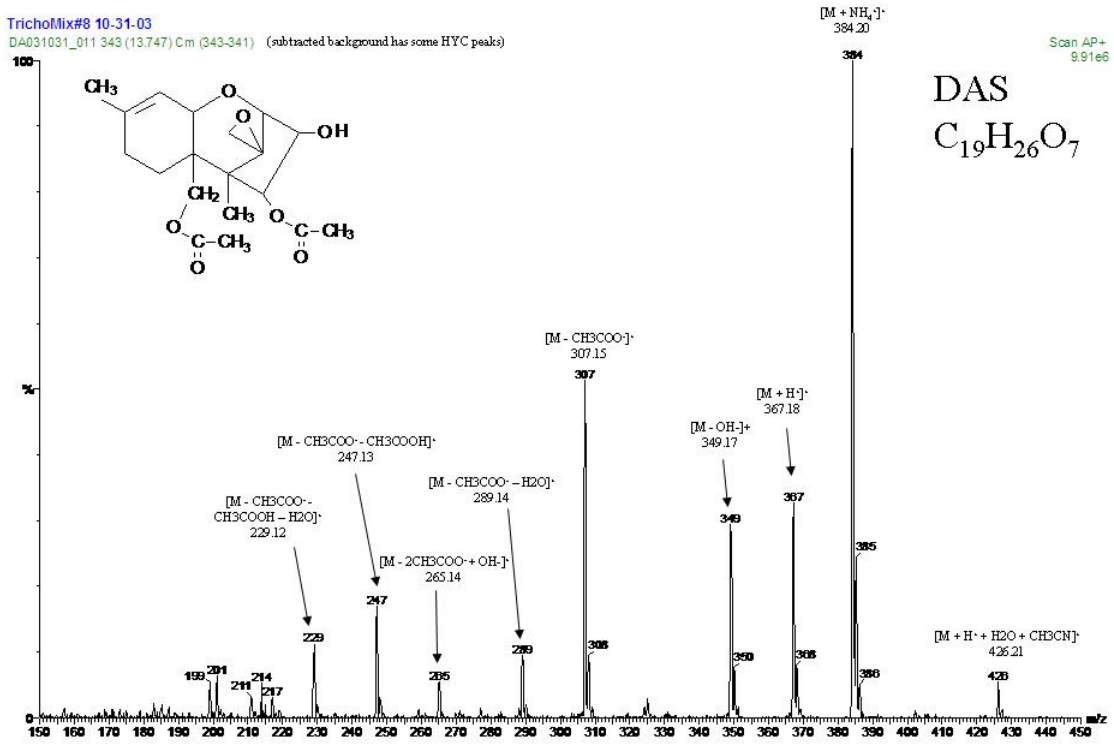

TrichoMix#7 10-31-03
DA031031_019 165 (6.628) Cm (165-(161+168))
Scan AP+
1.86e6
DON
$C_{15}H_{20}O_6$
[M – COH2 – OH-]+
249.11
[M – COH2 + H+]+
267.12
[M - OH-]+
279.12
[M + H·]·
297.13
[M - COH2 - OH- - H2O]+
231.10
[M – CH3OH + H+]+
265.11
[M – ?]+
284
[M + H· + CH3CN]·
338.16
100
%
0
m/z
150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450

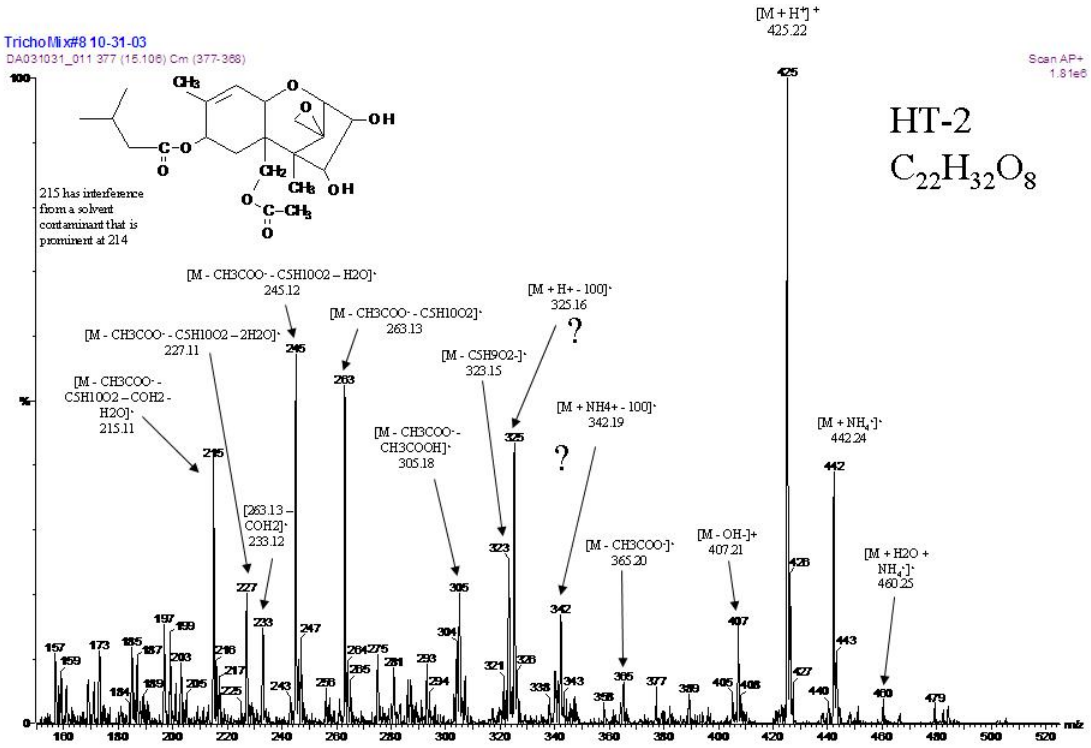

TrichoMix#7 10-31-03
DA031031_019 205 (8.228) Cm (205-200)
Scan AP+
4.51e5
[M – CH3COO- -H2O – COH2]+
247.10
[M – CH3COO- -2H2O – COH2]+
229.09
[M – CH3COO- -3H2O – COH2]+
211.08
[M – ?]+
231
[M – CH3COO- – COH2]+
265.11
[M - OH- - H2O]+
319.12
[M – COH2 + H+]+
325.13
[M + H·]·
355.14
[M – ?]+
205
[M – COH2 – OH-]+
307.12
[M - OH-]+
337.13
[M – CH3COO- -H2O]+
277.11
[M – CH3COO-]+
295.12
%
m/z
FUS-X
C17H22O8

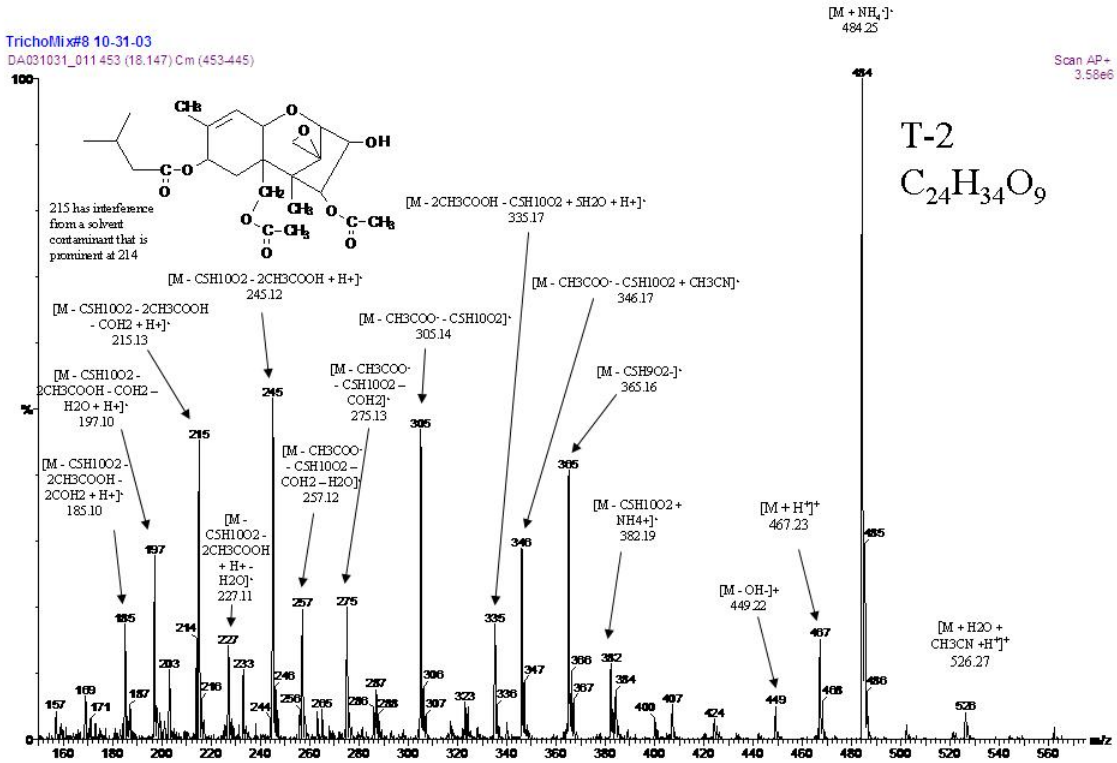

TrichoMix#8 10-31-03
DA031031_011 252 (10.106) Cm (252-250)
Scan AP+
5.85e5
15ADON
C17H22O7
[M - CH3COO-·-H2O]·+
261.11
[M – CH3OH – COH2 + H+]+
277.11
[M - CH3COO·]·+
279.12
[M – CH3OH + H+]+
307.12
[M - OH-]+
321.13
[M + H·]·+
339.14
[M + NH4·]·+
356.17
[M + CH3CN +H·]·+
380.17
[M + H2O + CH3CN +H·]·+
398.18
[M – H2O – CH3COOH – COH2 + H·]·+
231.10
noise
214 and 255 have interference from a solvent contaminant
?
?
%
m/z

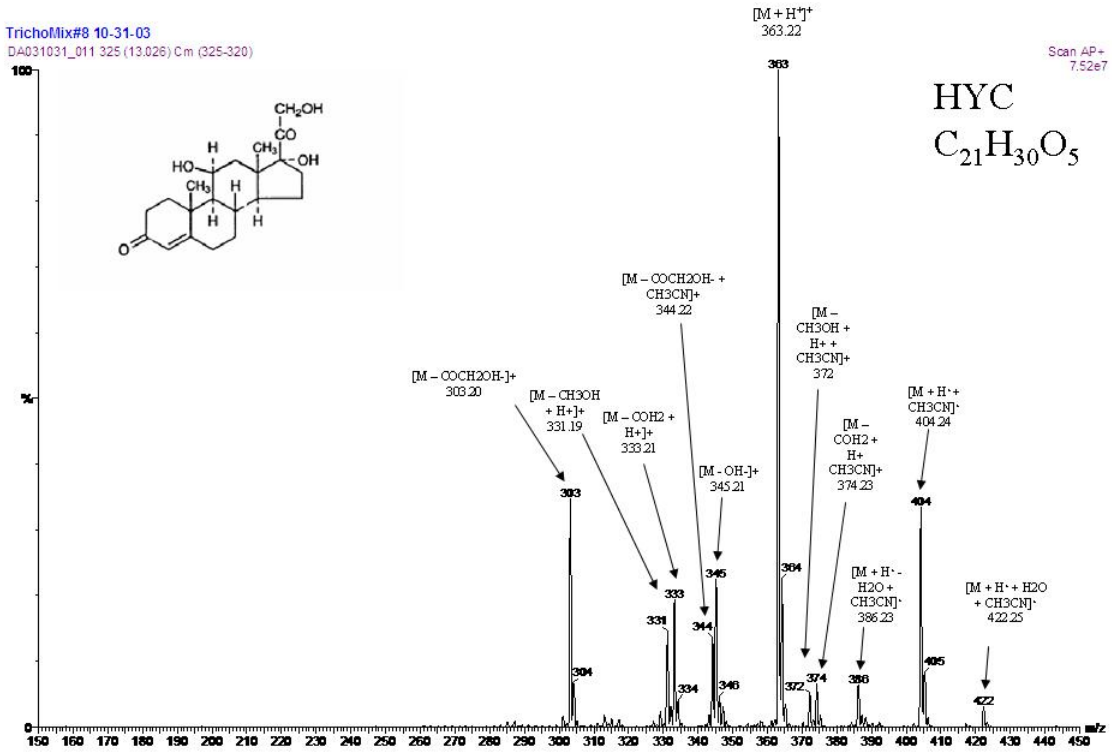

TrichoMix#7 10-31-03
DA031031_019 254 (10.189) Cm (254-251)
Scan AP+
2.19e6
3ADON
C17H22O7
[M + H·]·
339.14
[M – H2O – CH3COOH – COH2 + H·]·
231.10
[M – CH3COOH – COH2 + H·]·
249.11
[M - 2CH3COO- + 2OH- + H+ + 2H2O]·
291.14
[M – CH3OH – CH3COOH + H+]+
247.10
[M – COH2 + H·]·
309.13
[M - CH3COO·]·
279.12
[M + H·+ CH3CN]·
380.17
[M – H2O – CH3COOH – 2COH2 + H·]·
201.09
[M - OH-]+
321.13
noise
[M–2H2O – COH2+H·]·
273.11
[279.12-H2O]
261.11
[309.13+ CH3CN]·
350.16
[M + H·+ H2O + CH3CN]·
398
%
m/z


TrichoMix#8 10-31-03
DA031031_011 221 (8.865) Cm (221-215)
Scan AP+
5.55e6
NEO
$C_{19}H_{26}O_8$
[M + NH4·]·
400.20
[M – H2O – 2CH3COOH – COH2 + H·]·
215.11
[M – H2O – 2CH3COOH + H·]·
245.12
[M - CH3COO·-H2O]·
305.14
[M - CH3COO- - H2O + CH3CN]+
346.17
[M - 2CH3COOH - COH2 – 2H2O + H+]·
197.10
[M - 2CH3COOH - 2COH2 + H+]·
203.11
[M - CH3COO·]·
323.15
[M - 2CH3COOH - 2COH2 – H2O + H+]·
185.10
[M - 2CH3COOH +H·]·
263.13
[M - OH- - COH2]+
335.15
[M - OH-]+
365.16
[M – 2H2O – CH3COO- - COH2]·
275.12
[M – H2O – CH3COO- - COH2]·
275.13
[M + H·]·
383.17
[245.12+H2O]+
227.11
[215.11+H2O]+
233.12
[263.13+H2O]+
281.14
[M + H2O + CH3CN +H+]+
442.21
[305.14+H2O]+
287.13
%
m/z


TrichoMix#8 10-31-03
DA031031_011 343 (13.747) Cm (343-341) (subtracted background has some HYC peaks)
Scan AP+
9.91e6
DAS
C19H26O7
[M + NH4·]·
384.20
[M - CH3COO·]·
307.15
[M + H·]·
367.18
[M - OH-]+
349.17
[M - CH3COO· - CH3COOH]·
247.13
[M - CH3COO· - H2O]·
289.14
[M - CH3COO· - CH3COOH - H2O]·
229.12
[M - 2CH3COO· + OH-]·
265.14
[M + H· + H2O + CH3CN]·
426.21
%
m/z


TrichoMix#8 10-31-03
DA031031_011 377 (15.106) Cm (377-388)
Scan AP+
1.81e8
HT-2
$C_{22}H_{32}O_8$
215 has interference from a solvent contaminant that is prominent at 214
[M + H+] +
425.22
[M - CH3COO- - C5H10O2 – H2O]·
245.12
[M - CH3COO- - C5H10O2]·
263.13
[M + H+ - 100]·
325.16
?
[M - CH3COO- - C5H10O2 – 2H2O]·
227.11
[M - CSH9O2-]·
323.15
[M - CH3COO- - C5H10O2 – COH2 - H2O]·
215.11
[M + NH4+ - 100]·
342.19
?
[M - CH3COO- - CH3COOH]·
305.18
[M + NH4+]·
442.24
[263.13 – COH2]·
233.12
[M - CH3COO-]·
365.20
[M - OH-]+
407.21
[M + H2O + NH4+]·
460.25
%
m/z


TrichoMix#8 10-31-03
DA031031_011 453 (18.147) Cm (453-445)
Scan AP+
3.58e6
T-2
$C_{24}H_{34}O_9$
[M + NH4+]+
484.25
215 has interference from a solvent contaminant that is prominent at 214
[M - CSH10O2 - 2CH3COOH + H+]+
245.12
[M - CSH10O2 - 2CH3COOH - COH2 + H+]+
215.13
[M - CSH10O2 - 2CH3COOH - COH2 – H2O + H+]+
197.10
[M - CSH10O2 - 2CH3COOH - 2COH2 + H+]+
185.10
[M - CSH10O2 - 2CH3COOH + H+ - H2O]+
227.11
[M - CH3COO- - CSH10O2 – COH2]+
275.13
[M - CH3COO- - CSH10O2 – COH2 – H2O]+
257.12
[M - CH3COO- - CSH10O2]+
305.14
[M - 2CH3COOH - CSH10O2 + 5H2O + H+]+
335.17
[M - CH3COO- - CSH10O2 + CH3CN]+
346.17
[M - CSH9O2-]+
365.16
[M - CSH10O2 + NH4+]+
382.19
[M + H+]+
467.23
[M - OH-]+
449.22
[M + H2O + CH3CN +H+]+
526.27
%
m/z


TrichoMix#8 10-31-03
DA031031_011 325 (13.026) Cm (325-320)
Scan AP+
7.52e7
HYC
C21H30O5
[M + H•]+
363.22
[M – COCH2OH-]+
303.20
[M – CH3OH + H+]+
331.19
[M – COH2 + H+]+
333.21
[M – COCH2OH- + CH3CN]+
344.22
[M - OH-]+
345.21
[M – CH3OH + H+ + CH3CN]+
372
[M – COH2 + H+ CH3CN]+
374.23
[M + H• - H2O + CH3CN]•
386.23
[M + H•]+ CH3CN]•
404.24
[M + H• + H2O + CH3CN]•
422.25
%
m/z

Appendix B: Type A and B Trichothecene Production by Strain in Phylogenetic Order in µg/g Rice

NRRL #*	FRC #**	Previously Identified As	NIV 295	DON 249	FUS-X 355	3-A DON or 15-A DON 339	SUM of B's	TYPE B AVG's	NEO 305	DAS 307	HT2 425	T2 305	SUM of A's	TYPE A AVG's
CLADE 1														
3509	T-346	*F. kyushuense*	6.2	6.8	-	-	12.4	6500	-	-	-	1.6	1.6	110
3509	T-346	*F. kyushuense*	7.3	-	-	28	35		-	0.84	-	-	0.84	
3509	T-346	*F. kyushuense*	5100	150	9000	39	14000		47	23	-	-	70	
3509	T-346	*F. kyushuense*	4600	140	7300	14	12000		27	12	-	-	39	
6491	T-0565	*F. kyushuense*	370	250	-	730	1400	1700	10	7.9	-	-	18	74
6491	T-0565	*F. kyushuense*	390	390	-	1100	1900		96	34	-	3.5	130	
26204	T-988	*F. kyushuense*	21	48	-	-	69	49	5.3	2.1	-	-	7.4	13
26204	T-988	*F. kyushuense*	-	-	28	-	28		1.2	1.0	16	-	18	
20721	R-9843	*F. kyushuense*	-	-	-	-	0	70	2.8	-	-	-	2.8	44
20721	R-9843	*F. kyushuense*	6.2	14	200	34	250		2.6	8.4	-	-	11	
20721	R-9843	*F. kyushuense*	-	-	-	-	0		-	0.55	-	-	0.55	
20721	R-9843	*F. kyushuense*	6.7	-	-	23	30		-	-	160	-	160	
13392	R-5821	*F. robustum*	-	79	-	83	162	100	16	1.9	-	-	18	54
13392	R-5821	*F. robustum*	110	96	-	-	206		16	-	-	11	27	
13392	R-5821	*F. robustum*	-	4.9	-	-	4.9		4.4	1.2	-	-	5.6	
13392	R-5821	*F. robustum*	-	1.5	27	-	29		1.5	1.1	-	0.87	3.5	
22187	R-9177	*F. sp. cf. sambucinum*	11	15	-	79	110	110	-	260	-	-	260	190
22187	R-9177	*F. sp. cf. sambucinum*	17	12	-	85	110		-	120	-	-	120	
22203	R-5187	*F. sp. cf. sambucinum*	-	1300	330	130	1800	640	64	21000	200	-	21000	6900
22203	R-5187	*F. sp. cf. sambucinum*	150	520	-	-	670		11	13000	68	-	13000	
22203	R-5187	*F. sp. cf. sambucinum*	3.6	300	41	49	390		3.2	4400	500	-	4900	
22203	R-5187	*F. sp. cf. sambucinum*	-	500	61	190	751		35	1900	410	-	2300	
22203	R-5187	*F. sp. cf. sambucinum*	120	-	-	-	120		-	-	-	-	0	
22203	R-5187	*F. sp. cf. sambucinum*	94	-	-	-	94		-	-	-	-	0	
29130	R-9858	*F. sp. cf. sambucinum*	1.3	6.1	-	23	30	50	1.7	990	-	-	990	1300
29130	R-9858	*F. sp. cf. sambucinum*	-	12	22	36	70		2.9	1600	-	-	1600	
22240	R-9849	*F. tumidum*	10	37	9.2	-	56	90	1800	11	4.3	-	1800	1500

22240	R-9849	*F. tumidum*	11	5.1	4.3	-	21		6.0	1.9	-	-	7.9	
22240	R-9849	*F. tumidum*	10	-	63	32	110		540	1.6	-	-	540	
22240	R-9849	*F. tumidum*	15	85	-	67	170		2700	5.0	900	3.9	3600	
31965	R-9851	*F. tumidum*	14	-	53	92	160		140	0.87	-	-	140	
31965	R-9851	*F. tumidum*	-	340	-	-	340	220	170	4.1	-	-	170	170
31965	R-9851	*F. tumidum*	-	-	-	-	0		160	27	-	-	190	
31965	R-9851	*F. tumidum*	-	380	-	-	380		120	34	-	2.9	160	
31967	R-9853	*F. tumidum*	31	41	-	730	800	410	28	-	-	3.3	31	17
31967	R-9853	*F. tumidum*	10	15	-	-	25		2.2	-	-	-	2.2	
31969	R-9855	*F. tumidum*	-	-	-	-	0		1.5	-	-	-	1.5	
31969	R-9855	*F. tumidum*	21	21	-	160	200	81	-	-	-	-	0	170
31969	R-9855	*F. tumidum*	6.6	6.9	-	-	14		-	17	-	-	17	
31969	R-9855	*F. tumidum*	64	-	-	46	110		-	29	100	21	150	
31964	R-9850	*F. tumidum*	6.8	7.5	68	7.9	90	120	330	2.3	-	-	330	720
31964	R-9850	*F. tumidum*	-	26	120	-	150		430	18	610	5.9	1100	
31966	R-9852	*F. tumidum*	-	27	32	87	150	110	1100	15	-	0.79	1100	640
31966	R-9852	*F. tumidum*	16	8.1	9.5	30	64		180	2.2	-	0.28	180	
31968	R-9854	*F. tumidum*	11	37	-	-	48		1900	7.1	16	1.2	1900	
31968	R-9854	*F. tumidum*	46	53	61	-	160	72	1700	19	570	6.6	2300	2200
31968	R-9854	*F. tumidum*	75	-	-	-	75		2000	29	-	-	2000	
31968	R-9854	*F. tumidum*	4.8	-	-	-	4.8		2400	39	-	-	2400	
29296	R-9861	*F. sp.*	4.9	-	-	-	4.9		1000	-	-	-	1000	
29296	R-9861	*F. sp.*	-	-	-	-	0	28	480	-	-	-	480	1800
29296	R-9861	*F. sp.*	98	-	-	-	98		3100	170	-	-	3300	
29296	R-9861	*F. sp.*	7.3	-	-	-	7.3		2400	78	-	-	2500	
22189		*F. sp. cf. sambucinum*	11	3.2	-	-	14	760	72	1.1	-	-	73	540
22189		*F. sp. cf. sambucinum*	39	31	1200	240	1500		1000	-	-	-	1000	
22192	R-10045	*F. sp. cf. sambucinum*	5.9	5.5	-	20	31	86	3.5	-	-	2.4	5.9	120
22192	R-10045	*F. sp. cf. sambucinum*	-	48	95	-	140		12	2.7	210	-	225	
22196	R-9187	*F. venenatum*	8.8	4.7	4.7	2.0	20	59	1.6	83	-	-	85	140
22196	R-9187	*F. venenatum*	4.4	20	-	2.4	27		3.5	86	-	-	90	
22196	R-9187	*F. venenatum*	10	-	-	150	160		-	51	-	-	51	
22196	R-9187	*F. venenatum*	28	6.3	48	-	82		-	19	-	-	19	

22196	R-9187	*F. venenatum*	42	-	-	-	42		49	270	-	-	320	
22196	R-9187	*F. venenatum*	25	-	-	-	25		-	280	-	-	280	
22198	R-9847	*F. venenatum*	7.3	8.5	20	15	51	40	-	12	20	1.2	33	26
22198	R-9847	*F. venenatum*	7.4	22	-	-	29		0.90	17	-	-	18	
13714	T-0503	*F. poae*	-	7.4	-	-	7.4	22	-	0.71	-	-	0.71	43
13714	T-0503	*F. poae*	24	6.8	-	-	31		-	0.83	-	-	0.83	
13714	T-0503	*F. poae*	12	2.9	-	-	15		-	0.39	-	-	0.7	
13714	T-0503	*F. poae*	2.8	-	-	30	33		-	0.47	170	-	170	
25799	T-487	*F. poae*	2.7	4.6	-	-	7	17	-	0.51	-	-	0.51	220
25799	T-487	*F. poae*	7.6	-	-	-	7.6		-	1.1	540	1.4	540	
25799	T-487	*F. poae*	4.5	4.5	40	-	49		2.5	2.1	550	-	560	
25799	T-487	*F. poae*	9.8	-	-	-	9.8		-	-	-	-	0	
25799	T-487	*F. poae*	9.1	-	-	-	9.1		-	-	-	-	0	
28507	R-9400	*F. musarum*	170	220	81	840	1300	380	7400	9.4	180	60	7600	3000
28507	R-9400	*F. musarum*	87	-	-	71	160		300	0.90	120	81	500	
28507	R-9400	*F. musarum*	22	180	7.2	3.5	210		3700	2.1	130	240	4100	
28507	R-9400	*F. musarum*	9.8	82	4.8	10	110		2900	1.8	160	350	3400	
28507	R-9400	*F. musarum*	5.5	230	-	-	240		600	-	260	314	1200	
28507	R-9400	*F. musarum*	48	190	-	-	240		540	-	210	180	930	
CLADE 2														
13393	R-5822	*F. lunulosporum*	14	-	-	-	14	11	-	-	-	-	0	0.00
13393	R-5822	*F. lunulosporum*	7.2	-	-	-	7.2		-	-	-	-	0	
13721	R-9961	*F. cerealis*	28	-	-	-	28	26	-	-	-	-	0	0.00
13721	R-9961	*F. cerealis*	24	-	-	-	24		-	-	-	-	0	
25805	R-9626	*F. cerealis*	-	-	-	-	0	0.00	-	-	-	-	0	0.00
25805	R-9626	*F. cerealis*	-	-	-	-	0		-	-	-	-	0	
13818	R-5469	*F. asiaticum*	-	-	-	-	0	5	-	-	-	-	0	0.00
13818	R-5469	*F. asiaticum*	-	-	-	-	0		-	-	-	-	0	
13818	R-5469	*F. asiaticum*	-	-	-	-	0		-	-	-	-	0	
13818	R-5469	*F. asiaticum*	18	-	-	-	18		-	-	-	-	0	
28720	R-9402	*F. asiaticum*	-	-	-	-	0	14	-	-	-	-	0	0.00
28720	R-9402	*F. asiaticum*	-	27	-	-	27		-	-	-	-	0	
28723	R-9438	*F. meridionale*	52	-	-	-	52	44	-	-	-	-	0	0.00

28723	R-9438	*F. meridionale*	36	-	-	-	36		-	-	-	-	0	
28436	R-5329	*F. meridionale*	19	-	-	-	19	10	-	-	-	-	0	0.00
28436	R-5329	*F. meridionale*	-	-	-	-	0		-	-	-	-	0	
28436	R-5329	*F. meridionale*	3.7	-	-	-	3.7		-	-	-	-	0	
28436	R-5329	*F. meridionale*	19	-	-	-	19		-	-	-	-	0	
31238	R-9839	*F. brasilicum*	-	-	-	-	0	4	-	-	-	-	0	0.00
31238	R-9839	*F. brasilicum*	-	-	-	-	0		-	-	-	-	0	
31238	R-9839	*F. brasilicum*	-	-	-	-	0		-	-	-	-	0	
31238	R-9839	*F. brasilicum*	14	-	-	-	14		-	-	-	-	0	
31281	R-9838	*F. brasilicum*	6.2	-	-	40	46	170	-	-	-	-	0	0.00
31281	R-9838	*F. brasilicum*	-	-	-	48	48		-	-	-	-	0	
31281	R-9838	*F. brasilicum*	11	10	-	61	82		-	-	-	-	0	
31281	R-9838	*F. brasilicum*	-	-	-	490	490		-	-	-	-	0	
28585	R-9632	*F. austroamericanum*	67	-	-	-	67	57	-	-	-	-	0	0.00
28585	R-9632	*F. austroamericanum*	46	-	-	-	46		-	-	-	-	0	
28718	R-6964	*F. austroamericanum*	25	-	-	-	25	53	-	-	-	-	0	0.00
28718	R-6964	*F. austroamericanum*	-	-	-	92	92		-	-	-	-	0	
28718	R-6964	*F. austroamericanum*	-	-	-	55	55		-	-	-	-	0	
28718	R-6964	*F. austroamericanum*	-	-	-	40	40		-	-	-	-	0	
29297	R-9681	*F. cortaderiae*	7.9	-	-	-	7.9	2.0	-	-	-	-	0	0.00
29297	R-9681	*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
29297	R-9681	*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
29297	R-9681	*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
31185		*F. cortaderiae*	2.4	-	-	-	2.4	3.9	-	-	-	-	0	0.00
31185		*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
31185		*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
31185		*F. cortaderiae*	21	-	-	-	21		-	-	-	-	0	
31185		*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
31185		*F. cortaderiae*	-	-	-	-	0		-	-	-	-	0	
29148	R-4079	*F. mesoamericanum*	-	-	-	-	0	220	-	-	-	-	0	0.00
29148	R-4079	*F. mesoamericanum*	-	-	-	-	0		-	-	-	-	0	
29148	R-4079	*F. mesoamericanum*	53	35	-	-	88		-	-	-	-	0	
29148	R-4079	*F. mesoamericanum*	520	360	-	-	880		-	-	-	-	0	
26755	R-9630	*F. acaciae-mearnsii*	360	-	740	-	1100	880	-	-	-	-	0	0.00

26755	R-9630	*F. acaciae-mearnsii*	8.9	-	650	-	660		-	-	-	-	0	
26754	R-9629	*F. acaciae-mearnsii*	-	-	-	-	0	9.3	-	-	-	-	0	0.00
26754	R-9629	*F. acaciae-mearnsii*	-	-	-	-	0		-	-	-	-	0	
26754	R-9629	*F. acaciae-mearnsii*	21	-	-	-	21		-	-	-	-	0	
26754	R-9629	*F. acaciae-mearnsii*	16	-	-	-	16		-	-	-	-	0	
28063	R-6574	*F. graminearum*	8.5	180	37	29	260	200	-	-	-	-	0	0.00
28063	R-6574	*F. graminearum*	26	110	-	-	140		-	-	-	-	0	
29169	R-9635	*F. graminearum*	40	10	-	100	150	350	-	-	-	-	0	0.00
29169	R-9635	*F. graminearum*	62	1100	-	28	1200		-	-	-	-	0	
29169	R-9635	*F. graminearum*	15	-	-	-	15		-	-	-	-	0	
29169	R-9635	*F. graminearum*	-	-	-	23	23		-	-	-	-	0	
26916	R-9631	*F. boothii*	-	-	-	-	0	0.00	-	-	-	-	0	0.00
26916	R-9631	*F. boothii*	-	-	-	-	0						0	
26916	R-9631	*F. boothii*	-	-	-	-	0		-	-	-	-	0	
29020	R-7775	*F. boothii*	-	-	-	-	0	0.00	-	-	-	-	0	0.00
29020	R-7775	*F. boothii*	-	-	-	-	0		-	-	-	-	0	
29020	R-7775	*F. boothii*	-	-	-	-	0		-	-	-	-	0	
29020	R-7775	*F. boothii*	-	-	-	-	0		-	-	-	-	0	
25475	R-8504	*F. culmorum*	17	-	-	-	17	17	-	-	-	-	0	0.00
3288	R-5321	*F. culmorum*	4.6	-	-	-	4.6	4.4	-	-	-	-	0	0.00
3288	R-5321	*F. culmorum*	-	-	-	-	0		-	-	-	-	0	
3288	R-5321	*F. culmorum*	13	-	-	-	13		-	-	-	-	0	
3288	R-5321	*F. culmorum*	-	-	-	-	0		-	-	-	-	0	
28062	R-5921	*F. pseudograminearum*	4.5	-	-	7.0	12	3	-	-	-	-	0	0.00
28062	R-5921	*F. pseudograminearum*	-	-	-	-	0		-	-	-	-	0	
28062	R-5921	*F. pseudograminearum*	-	-	-	-	0		-	-	-	-	0	
28062	R-5921	*F. pseudograminearum*	-	-	-	-	0		-	-	-	-	0	
28065	R-6761	*F. pseudograminearum*	-	-	-	-	0	0.00	-	-	-	-	0	0.00
28065	R-6761	*F. pseudograminearum*	-	-	-	-	0		-	-	-	-	0	
29298	R-9962	*F. sp.*	17	-	92	-	110	290	-	-	-	-	0	0.00
29298	R-9962	*F. sp.*	24	-	47	-	71		-	-	-	-	0	
29298	R-9962	*F. sp.*	120	-	600	-	720		-	-	-	-	0	
29298	R-9962	*F. sp.*	29	-	240	-	270		-	-	-	-	0	

29380	R-7593	*F. sp.*	-	-	93	400	490	450	2.7	-	-	-	2.7	2.6
29380	R-7593	*F. sp.*	48	-	360	-	410		2.4	-	-	-	2.4	
34498	R-9700	*F. sp*	-	-	-	-	0	3.3	-	-	-	-	0	0.00
34498	R-9700	*F. sp.*	6.6	-	-	-	6.6		-	-	-	-	0	
34197	R-4339	*F. sp.*	-	-	-	-	0	0.00	-	-	-	-	0	0.00
34197	R-4339	*F. sp.*	-	-	-	-	0		-	-	-	-	0	
34461	R-8601	*F. sp.*	6.5	-	-	-	6.5	3.3	-	-	-	-	0	0.00
34461	R-8601	*F. sp.*	-	-	-	-	0		-	-	-	-	0	
34502	R-9705	*F. sp.*	6.1	-	-	-	6.1	3.1	-	-	-	-	0	0.00
34502	R-9705	*F. sp.*					0						0	
CLADE 3														
3299	T-0348	*F. sporotrichioides*	13	77	-	-	90	91	3.2	-	-	-	3.2	15
3299	T-0348	*F. sporotrichioides*	100	130	-	-	230		15	1.9	38	-	55	
3299	T-0348	*F. sporotrichioides*	-	30	-	-	30		1.6	0.39	-	-	2.0	
3299	T-0348	*F. sporotrichioides*	-	15	-	-	15		-	0.51	-	-	0.51	
13440	T-0521	*F. sporotrichioides*	-	9.3	-	-	9.3	26	44	-	-	-	44	290
13440	T-0521	*F. sporotrichioides*	34	31	-	-	65		32	0.79	-	2.3	35	
13440	T-0521	*F. sporotrichioides*	-	6.4	-	-	6.4		370	4.6	-	29	400	
13440	T-0521	*F. sporotrichioides*	-	9.7	14	-	24		660	5.6	-	14	680	
25474	T-467	*F. sporotrichioides*	-	3.2	-	-	3.2	34	15	2.4	310	-	330	200
25474	T-467	*F. sporotrichioides*	-	-	-	64	64		61	2.1	-	-	63	
29131	T-983	*F. sporotrichioides*	59	-	-	-	59	30	430	100	-	16	550	500
29131	T-983	*F. sporotrichioides*	-	-	-	-	0		440	1.8	-	1.6	440	
25479	T-485	*F. sporotrichioides*	46	-	59	39	140	390	160	0.70	39	9.5	210	230
25479	T-485	*F. sporotrichioides*	210	150	100	180	640		200	1.3	-	51	252	
34176		*F. langsethii*	64	-	-	-	64	32	-	-	16	-	16	8.0
34176		*F. langsethii*	-	-	-	-	0		-	-	-	-	0	
29896	R-9673	*F. sp.*	-	36	85	55	180	150	3.5	5.6	-	-	9.1	100
29896	R-9673	*F. sp.*	21	11	-	-	32		-	3.2	-	-	3.2	
29896	R-9673	*F. sp.*	21	290	-	-	310		-	190	32	-	220	
29896	R-9673	*F. sp.*	110	100	-	-	210		1.7	180	-	4.5	190	
29896	R-9673	*F. sp.*	86	32	-	-	120		-	93	-	-	93	
29896	R-9673	*F. sp.*	15	-	-	-	15		-	90	-	-	90	

29897	R-9674	*F. sp.*	42	120	110	-	270	140	2100	64	-	270	2400	1000
29897	R-9674	*F. sp.*	-	59	70	160	290		1500	37	-	160	1700	
29897	R-9674	*F. sp.*	-	-	-	-	0		-	-	-	-	0	
29897	R-9674	*F. sp.*	-	-	-	-	0		-	-	-	-	0	
6227	R-5319	*F. armeniacum*	140	190	-	-	330	220	1400	-	20	-	1400	1700
6227	R-5319	*F. armeniacum*	37	140	-	-	180		260	-	-	-	260	
6227	R-5319	*F. armeniacum*	66	140	-	-	210		2600	-	60	35	2700	
6227	R-5319	*F. armeniacum*	120	51	-	-	170		2100	-	65	140	2300	
29133	R-9860	*F. armeniacum*	6.9	2.4	-	-	9.3	15	11	1.2	-	-	12	6.5
29133	R-9860	*F. armeniacum*	-	2.3	20	-	22		4.1	1.1	-	-	5.2	
29133	R-9860	*F. armeniacum*	-	-	14	-	14		1.4	0.83	-	-	2.2	
31970	R-1957	*F. armeniacum*	4.1	8.5	-	-	13	62	7.0	-	-	-	7.0	69
31970	R-1957	*F. armeniacum*	-	12	-	93	110		110	20	-	2.8	130	
CLADE 4														
13369	R-4422	*F. longipes*	-	-	-	60	60	13	-	0.67	-	-	0.67	0.80
13369	R-4422	*F. longipes*	-	-	-	-	0		-	-	-	-	0	
13369	R-4422	*F. longipes*	-	8.9	-	-	8.9		-	2.2	-	-	2.2	
13369	R-4422	*F. longipes*	-	4.6	-	-	4.6		-	1.9	-	-	1.9	
13369	R-4422	*F. longipes*	-	-	-	-	0		-	-	-	-	0	
13369	R-4422	*F. longipes*	6.3	-	-	-	6.3		-	-	-	-	0	
13368	R-4421	*F. longipes*	-	-	-	290	290	150	-	-	-	-	0	0.55
13368	R-4421	*F. longipes*	-	-	-	-	0		1.1	-	-	-	1.1	
13374	R-5128	*F. longipes*	-	-	-	-	0	9.2	-	5.3	-	7.7	13	4.8
13374	R-5128	*F. longipes*	17	-	-	-	17		-	1.9	-	-	1.9	
13374	R-5128	*F. longipes*	-	5.8	-	-	5.8		0.71	0.55	-	-	1.3	
13374	R-5128	*F. longipes*	6.4	7.5	-	-	14		1.2	1.7	-	-	2.9	
20694	R-9840	*F. longipes*	-	-	120	190	310	130	0.94	-	-	-	0.94	240
20694	R-9840	*F. longipes*	23	7.9	-	140	170		1.2	0.63	-	1.5	3.3	
20694	R-9840	*F. longipes*	21	7.7	-	-	29		2.4	0.28	-	-	2.7	
20694	R-9840	*F. longipes*	-	-	-	-	0		-	3.3	220	8.9	230	
20695	R-9841	*F. longipes*	8.9	16	-	130	150	88	1.5	-	-	-	1.5	0.75
20695	R-9841	*F. longipes*	26	-	-	-	26		-	-	-	-	0	
20696	R-9842	*F. longipes*	66	-	-	-	66	100	6.1	0.91	51	-	58	37

20696	R-9842	*F. longipes*	180	57	-	53	290		11	2.0	74	-	87	
20696	R-9842	*F. longipes*	-	-	40	-	40		0.59	0.83	-	-	1.4	
20696	R-9842	*F. longipes*	-	12	-	-	12		-	1.7	-	-	1.7	
20723	R-9844	*F. longipes*	32	15	-	-	47	33	-	0.43	-	-	0.43	0.14
20723	R-9844	*F. longipes*	35	-	-	-	35		-	-	-	-	0	
20723	R-9844	*F. longipes*	9.5	8.2	-	-	18		-	-	-	-	0	
6358	T-0502	*F. equiseti*	-	-	-	-	0	64	13	0.51	-	-	14	120
6358	T-0502	*F. equiseti*	-	15	-	170	190		14	4.2	140	-	160	
6358	T-0502	*F. equiseti*	-	15	-	-	15		11	22	-	-	33	
6358	T-0502	*F. equiseti*	28	23	-	-	51		60	190	-	5.6	260	
13829	R-6784	*F. equiseti*	-	11	-	-	11	51	8.3	2.1	-	-	10	13
13829	R-6784	*F. equiseti*	-	7.9	-	-	7.9		5.2	2.1	-	-	7.3	
13829	R-6784	*F. equiseti*	110	8.9	-	-	120		9.1	3.0	-	-	12	
13829	R-6784	*F. equiseti*	36	-	-	27	63		14	5.8	-	3.3	23	
28448	R-9859	*F. equiseti*	2.6	33	-	15	51	45	810	83	-	-	890	630
28448	R-9859	*F. equiseti*	-	18	18	-	36		460	53	-	-	510	
28448	R-9859	*F. equiseti*	7.8	15	24	-	47		470	26	-	-	500	
20954	R-9845	*F. equiseti*	5.8	-	-	-	5.8	24	0.75	0.67	-	-	1.4	1.9
20954	R-9845	*F. equiseti*	-	4.1	-	7.9	12		-	0.39	-	-	0.39	
20954	R-9845	*F. equiseti*	-	8.1	-	-	8.1		-	0.28	-	1.2	1.5	
20954	R-9845	*F. equiseti*	79	13	-	-	92		-	2.2	-	3.8	6.0	
20954	R-9845	*F. equiseti*	-	-	-	-	0		-	-	-	-	0	
28725	R-8755	*F. sp.*	5.4	-	-	-	5.4	5.4	1.0	-	-	-	1.0	15
28725	R-8755	*F. sp.*	-	5.3	-	-	5.3		0.31	3.3	-	25	29	
31008	R-9856	*F. brachygibbosum*	5.3	-	15	-	20	49	2.4	1.6	-	-	4.0	8.1
31008	R-9856	*F. brachygibbosum*	5.2	1.5	15	-	22		0.63	1.3	-	-	1.9	
31008	R-9856	*F. brachygibbosum*	13	-	-	-	13		4.5	2.9	-	-	7.4	
31008	R-9856	*F. brachygibbosum*	140	-	-	-	140		-	3.8	-	15	19	
32021	R-3023	*F. sp.*	4.9	-	-	-	4.9	19	-	0.51	-	-	0.51	45
32021	R-3023	*F. sp.*	-	-	-	-	0		-	1.9	140	-	140	
32021	R-3023	*F. sp.*	-	-	-	-	0		-	-	-	-	0	
32021	R-3023	*F. sp.*	70	-	-	-	70		13	6.2	-	21	40	
31009	R-9857	*F. brachygibbosum*	110	-	-	-	110	36	-	2.4	-	2.4	4.8	9.2
31009	R-9857	*F. brachygibbosum*	-	6.7	-	-	6.7		-	-	28	-	28	

31009	R-9857	*F. brachygibbosum*	5.9	12	-	-	18	-	1.9	-	-	1.9	
31009	R-9857	*F. brachygibbosum*	-	10	-	-	10	-	2.0	-	-	2.0	

*NNRL indicates strain number from the USDA National Regional Research Laboratory.

**FRC indicates strain number from the Fusarium Research Center.

NIV=nivalenol, DON=deoxynivalenol, FUS-X=fusarenon X, ADON=total 3/15ADON, NEO=neosolaniol, DAS=diacetoxyscirpenol, T2=T-2 toxin, HT-2=HT-2 toxin

Appendix C: Strain List for the Fumonisin Assays

FRC#	Species	Substrate	Geographic Origin
L-0102	*F. xylarioides*	*Coffea robusta*	Guinea
L-0230	*F. xylarioides*	Soil Dilution	South Africa
L-0231	*F. xylarioides*	Soil	South Africa
L-0232	*F. xylarioides*	Soil	South Africa
L-0229	*F. xylarioides*	Soil Dilution	South Africa
L-0286	*F. lateritium*	Seed Lot	Peru
L-0388	*F. xylarioides*	Coffee	Uganda
M-0003	*F. moniliforme*	Maple bark	
M-0061	*F. phyllophilum*	*Draecena borinquensis*	Florida
M-0062	*F. phyllophilum*	*Draecena borinquensis*	Florida
M-0063	*F. konzum*	*Arucaria angustifolia*	Brazil
M-0519	*F. proliferatum*	Corn	Idaho
M-0730	*F. proliferatum*	Pecan	USA, TX
M-0843	*F. anthophylum*	Amaryllis	Germany
M-0915	*F. phyllophilum*	*Draecena goldiana*	
M-0919	*F. phyllophilum*	*Sansevieria*	
M-0927	*F. fujikuroi*	Weevil	
M-0944	*F. proliferatum*	Sugar cane	India
M-0947	*F. fujikuroi*	Pine Slash Seed	USA, GA
M-0965	*F. fujikuroi*	Pine Slash Seed	USA, GA
M-1116	*F. verticillioides*	Corn	South Africa
M-1137	*F. fujikuroi*	Gladiolus Corm	Florida
M-1250	*F. fujikuroi*	Rice – Bakanae disease	Japan
M-1268	*F. fujikuroi*	*Oryza sativa*	Japan
M-1269	*F. fujikuroi*	Rice	Japan
M-1288	*F. fujikuroi*	Soil Coconut Grove	Philippines
M-1294	*F. moniliforme*	Moldy Corn	Iowa
M-1313	*F. thapsinum*	Soil *Brassica*	Australia
M-1325	*F. verticillioides*	Corn	South Africa

M-1331	*F. verticillioides*	Corn and Soybean	Pennsylvania
M-1332	*F. verticillioides*	Corn and Soybean	Pennsylvania
M-1412	*F. nygamai*	Sorghum	Australia
M-1455	*F. nygamai*	Soil	Australia
M-1521	*F. moniliforme*	Soil dilution	Australia
M-1544	*F. verticillioides*	Soil dilution	South Africa
M-1552	*F. verticillioides*	Corn	Kentucky
M-1724	*F. fujikuroi*		China
M-1732	*F. fujikuroi*	Soil	China
M-1755	*F. moniliforme*	*Leucana* and grass	Philippines
M-1890	*F. verticillioides*	Corn	Pennsylvania
M-1906	*F. moniliforme*	Pasture Soil	Thailand
M-1986	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1987	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1990	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1991	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1992	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1994	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-1995	*F. moniliforme*	Soil Sorghum	Puerto Rico
M-2455	*F. fujikuroi*	Hay	North Carolina
M-2973	*F. verticillioides*	Corn	North Carolina
M-3072	*F. moniliforme*	Wheat Soil	South Africa
M-3073	*F. moniliforme*	Soil Wheat	South Africa
M-3074	*F. moniliforme*	Wheat Soil	South Africa
M-3089	*F. proliferatum*	Alfalfa crown	USA, PA
M-3117	*F. proliferatum*	Sorghum	California
M-3125	*F. verticillioides*	Corn	California
M-3162	*F. andiyazi*		South Africa
M-3210	*F. anthophylum*	Soil Debris	
M-3260	*F. moniliforme*	Corn	Peru
M-3673	*F. moniliforme*	Sugar Cane	Australia

M-3675	*F. moniliforme*	Soil	Australia
M-3682	*F. fujikuroi*	Sorghum	Missouri
M-3699	*F. moniliforme*	Corn	Illinois
M-3703	*F. verticillioides*	Corn	Indiana
M-3829	*F. anthophylum*	Soil	South Carolina
M-5077	*F. moniliforme*	Millet	Nigeria
M-5078	*F. moniliforme*	Millet	Nigeria
M-5088	*F. fujikuroi*	Rice Debris	Phillippines
M-5144	*F. moniliforme*	Millet	Nigeria
M-5192	*F. andiyazi*	Sorghum	Nigeria
M-5194	*F. andiyazi*	Sorghum	Nigeria
M-5210	*F. andiyazi*	Sorghum seed stored	Nigeria
M-5245	*F. andiyazi*	Millet	Zimbabwe
M-5500	*F. verticillioides*	Corn	Nepal
M-5716	*F. moniliforme*	Pearl Millet	Zimbabwe
M-5727	*F. moniliforme*	Sorghum	Zimbabwe
M-5735	*F. moniliforme*	Sweet Potato	USA
M-5742	*F. moniliforme*	Pearl Millet	Nigeria
M-5759	*F. pseudonygamai*	Pearl Millet	Nigeria
M-5761	*F. andiyazi*	Pearl Millet	Zimbabwe
M-5820	*F. andiyazi*	Sorghum	Nigeria
M-6176	*F. moniliforme*	Sugar Cane	Malaysia
M-6183	*F. proliferatum*	Rice	Malaysia
M-6184	*F. proliferatum*	Rice	Malaysia
M-6201	*F. Pseudonygamai*	Tobacco	Malaysia
M-6202	*F. concentricum*	Mango	Malaysia
M-6205	*F. proliferatum*	Asparagus	Malaysia
M-6206	*F. pseudonygamai*	Sorghum	Malaysia
M-6209	*F. pseudonygamai*	Asparagus	Malaysia
M-6236	*F. moniliforme*	Pearl Millet	Zimbabwe
M-6258	*F. moniliforme*	Sorghum	Zimbabwe

M-6340	*F. pseudonygamai*	Sorghum	Senegal
M-6534	*F. moniliforme*	Banana	Thailand
M-6572	*F. moniliforme*	Sugar Cane	Florida
M-6708	*F. proliferatum*	Corn	
M-6715	*F. verticillioides*	Corn	Peru
M-6740	*F. proliferatum*	Feed	Iowa
M-6864	*F. subglutinans*		Canada
M-6884	*F. fujikuroi*		
M-6903	*F. andiyazi*	Sorghum	Ethiopia
M-6905	*F. andiyazi*	Sorghum	Ethiopia
M-6909	*F. andiyazi*	Sorghum	Ethiopia
M-6912	*F. andiyazi*	Sorghum	Ethiopia
M-6914	*F. andiyazi*	Sorghum	Ethiopia
M-6959	*F. proliferatum*	Corn	Illinois
M-7040	*F. verticillioides*	Corn	Iowa
M-7072	*F. verticillioides*	Corn	Argentina
M-7143	*F. pseudonygamai*	Sorghum	India
M-7205	*F. moniliforme*	Grain sorghum	Egypt Mallawi
M-7207	*F. moniliforme*	Sorghum	Egypt
M-7239	*F. moniliforme*	Sorghum	Egypt
M-7424	*F. moniliforme*	Corn	Nepal
M-7456	*F. moniliforme*	Maize	Zambia
M-7470	*F. moniliforme*	Java citronella	India
M-7471	*F. moniliforme*	Java citronella	India
M-7472	*F. moniliforme*	Java citronella	India
M-7473	*F. moniliforme*	Java citronella	India
M-7534	*F. globosum*	Corn	South Africa
M-7792	*F. moniliforme*	Teosinte	Guatemala
M-8082	*F. verticillioides*	Teosinte	Mexico
M-8335	*F. moniliforme*		
M-8351	*F. moniliforme*	Sorghum	Tanzania

M-8352	*F. thapsinum*	Sorghum	Tanzania
M-8360	*F. andiyazi*	Sorghum grain	Tanzania
M-8411	*F. nygamai ssp. amazimbae*	Sorghum	
M-8412	*F. nygamai ssp. amazimbae*	Sorghum	
M-8413	*F. andiyazi*	Sorghum	
M-8414	*F. nygamai ssp. amazimbae*	Sorghum	
M-8415	*F. nygamai ssp. amazimbae*	Sorghum	
M-8430	*F. nisikadoi*	*Phyllostachys nigra*	Japan
M-8497	*G. fujikuroi*	Rice	Nepal
M-8512	*G. fujikuroi*	Rice	Nepal
M-8521	*G. fujikuroi*	Rice	Nepal
M-8590	*F. sp.*		
M-8592	*F. sp.*		
M-8595	*F. sp.*		
M-8596	*F. sp.*	Ornamental grass	South Africa
M-8597	*F. sp.*		
M-8598	*F. sp*	*Bidens pilosa*	USA, FL
M-8599	*F. sp*	*Bidens pilosa*	USA, FL
M-8655	*F. sp.*	Wood	Venezuela
M-8656	*F. sp.*	*Zea mays*	Zimbabwe
M-8657	*F. sp.*	*Mangifera indica*	India
M-8658	*F. concentricum*	*Oryza sativa*	Japan
M-8659	*F. concentricum*	*Triticum aestivum*	Nigeria
M-8660	*F. sp.*	*Oryza sativa* seed	Nigeria
M-8661	*F. sp.*	*Striga hermonthica*	Madagascar
M-8662	*F. sp.*		Niger
M-8663	*F. sp.*	Rain Forest Soil	Papua New Guinea
M-8664	*F. sp.*	*Striga hermonthica*	Sudan
M-8665	*F. fractiflexum*	*Cymbidium sp.*	Japan
M-8666	*F. sp.*	*Sorghum bicolor* seed	Tanzania
M-8667	*F. fractiflexum*		

M-8669	*F. proliferatum*		
M-8688	*F. globosum*	*Triticum aestivum*	Japan
M-8689	*F. globosum*	*Triticum aestivum*	Japan
O-1890	*F. oxysporum*	Carnation	Korea
O-1956	*F. oxysporum* f. sp. *erythroxyli*	Erythroxylum coca	USA, HI
O-2137	*F. oxysporum*	Rice	Korea
O-2283	*F. oxysporum*	*Liseanthus*	Ecuador
O-2284	*F. oxysporum*	*Liseanthus*	Ecuador
O-2285	*F. oxysporum*	*Liseanthus*	Ecuador
R-5469	*F. asiaticum*	Barley Grain	Japan
R-7775	*F. boothii*	South Africa	
R-9490	*F. avenaceum*	*Liseanthus*	USA, FL
R-9495	*F. avenaceum*	*Liseanthus*	USA, CA
R-9632	*F. austroamericanum*	Herbaceous vine	Venezuela
S-1710	*F. solani*	*Liseanthus*	Ecuador
S-1711	*F. solani*	*Liseanthus*	Ecuador
S-1712	*F. solani*	*Liseanthus*	Ecuador

Appendix D: Strain List for the Trichothecene Analysis

FRC#	NRRL#	Species	Substrate	Geographic Origin
R-1957	31970	*F. armeniacum*	Soil	Australia
R-3023	32021	*F. sp.*		Australia
R-4079	29148	*F. mesoamericanum*	Grape ivy	
R-4339	34197	*F. sp.*	Soil	Australia
R-4421	13368	*F. longipes*	Soil	Australia
R-4422	13369	*F. longipes*	Soil	Australia
R-5128	13374	*F. longipes*	Soil Debris	New Guinea
R-5187	22203	*F. sambucinum*	*Pterocarya*	Iran
R-5319	6227	*F. armeniacum*	Hay Fescue	USA, MO
R-5321	3288	*F. culmorum*		Canada
R-5329	28436	*F. meridionale*	Sweet potato	New Caladonia
R-5469	13818	*F. asiaticum*	Barley grain	Japan
R-5821	13392	*F. robustum*	Soil	Germany, Berlin
R-5822	13393	*F. lunulosporum*		Germany, Berlin
R-5921	28062	*F. pseudograminearum*	Wheat crown	Australia
R-6574	28063	*F. graminearum*	Corn Stalk	USA, MI
R-6761	28065	*F. pseudograminearum*	Medics	South Africa
R-6784	13829	*F. equiseti*	River Soil	Japan
R-6964	28718	*F. austroamericanum*	Corn equine feed	Brazil
R-7593	29380	*F. sp.*	Grass Orchard	USA, Corvalis OR
R-7775	29020	*F. boothii*		South Africa
R-8504	25475	*F. culmorum*	Barley	Denmark
R-8506	25797	*F. mesoamericanum*	Banana fruit	Honduras
R-8601	34461	*F. sp.*	Soil Dilution	South Africa
R-8755	28725	*F. sp.*	Wheat	Argentina
R-9177	22187	*F. sambucinum*	*Solanum*	Great Britain
R-9187	22196	*F. venenatum*	Corn	Germany
R-9400	28507	*F. musarum*	Banana	Panama
R-9402	28720	*F. asiaticum*	Maize	Nepal

R-9438	28723	*F. meridionale*	Maize ear rot	Nepal
R-9626	25805	*F. cerealis*	Soil	Columbia
R-9629	26754	*F. acaciae-mearnsii*	*Acaciae mearnsii*	South Africa
R-9630	26755	*F. acaciae-mearnsii*	*Acaciae mearnsii*	South Africa
R-9631	26916	*F. boothii*	Corn	South Africa
R-9632	28585	*F. austroamericanum*	Herbaceous vine	Venezuela
R-9635	29169	*F. graminearum*	Wheat	USA, KS
R-9673	29896	*F. sp.*		
R-9674	29897	*F. sp.*		
R-9681	29297	*F. cortaderiae*	*Cordateria* sp.	New Zealand
R-9700	34498	*F. sp.*	Wheat	USA, Rock Springs PA
R-9705	34502	*F. sp.*	Wheat	USA, Rock Springs PA
R-9838	31281	*F. brasilicum*		
R-9839	31238	*F. brasilicum*		
R-9840	20694	*F. longipes*		
R-9841	20695	*F. longipes*		
R-9842	20696	*F. longipes*		
R-9843	20721	*F. kyushuense*		
R-9844	20723	*F. longipes*		
R-9845	20954	*F. equiseti*		
R-9847	22198	*F. venenatum*		
R-9849	22240	*F. tumidum*		
R-9850	31964	*F. tumidum*		
R-9851	31965	*F. tumidum*		
R-9852	31966	*F. tumidum*		
R-9853	31967	*F. tumidum*		
R-9854	31968	*F. tumidum*		
R-9855	31969	*F. tumidum*		
R-9856	31008	*F. brachygibbosum*		
R-9857	31009	*F. brachygibbosum*		
R-9858	29130	*F. heterosporum*		

R-9859	28448	*F. equiseti*		
R-9860	29133	*F. armeniacum*		
R-9861	29296	*F. sp.*		
R-9961	13721	*F. cerealis*		
R-9962	29298	*F. sp.*	*Dactylis glomerata*	New Zealand
R-10045	22192	*F. sambucinum*		
R-10047	22189	*F. sambucinum*		
T-0346	3509	*F. sporotrichioides*		
T-0348	3299	*F. sporotrichioides*		
T-0467	25474	*F. sporotrichioides*	Pasture	Michigan
T-0485	25479	*F. sporotrichioides*		
T-0487	25799	*F. poae*		
T-0502	6358	*F. equiseti*		
T-0503	13714	*F. poae*	Wheat	Canada
T-0521	13440	*F. sporotrichioides*	Grain Elevator	Wisconsin
T-0565	6491	*F. sporotrichioides*		Japan
T-0983	29131	*F. sporotrichioides*		
T-0988	26204	*F. kyushuense*	Wheat	Japan
T-0992	34176	*F. langsethii*		
	31185	*F. cortaderiae*		

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Education

The Pennsylvania State University, University Park Campus
Ph.D. Plant Pathology, December 2006
Thesis Title: "Mycotoxicology to Accompany Phylogenetic Revisions to the Genus Fusarium"

The University of Wisconsin – La Crosse
Master of Science in Biology, with concentration in Mycology, December 2003
Thesis Title: "Isolation and Identification of Antibiotics from Mushrooms"

The Pennsylvania State University, University Park Campus
Bachelor of Science in Biology, with concentration in Plant Biology, May 2001
Cumulative GPA: 3.45; major GPA: 3.77

The Pennsylvania State University, Abington Campus
Major in Plant Biology, May 1997 to May 1999

Professional Affiliations

Mycological Society of America (MSA), since 2000
American Phytopathological Society (APS), since 2003
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Selected Publications and Presentations

Zitomer, N. C., D. M. Geiser, D. D. Archibald, M. M. Jimenez-Gasco, K. O'Donnell, and G. A. Kuldau. 2005. The phylogenetics of fumonisin production in the Gibberella fujikuroi species complex. Phytopathology 95(6):S173.

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Zitomer, N. C., D. M. Geiser, D. D. Archibald, T. J. Ward, K. O'Donnell, A. D. Jones, M. M. Jimenez-Gasco, and G. A. Kuldau. 2004. HPLC-MS analysis of type-A trichothecene-producing Fusaria for correspondence between toxin profiles and molecular phylogenetic groups. Phytopathology 94:S117.

Zitomer, N. C., D. M. Geiser, D.D. Archibald, T. J. Ward, K. O'Donnell, A. D. Jones, M. M. Jimenez-Gasco, and G. A. Kuldau. "Correspondence Between Toxin Profiles and Phylogenetic Groups in Fusarium." Given at the 2005 national meeting of the Mycological Society of America, in Hilo, HI.

Zitomer, N. C., T. J. Volk, M. A. Rott. "Isolation and Characterization of Antimicrobial substances from Fruiting bodies of Macrofungi" given at the 2003 national meeting of the Mycological Society of America, in Asilomar, CA.