STUDIES IN MOLECULAR PHYLOGENETICS OF FUSARIUM SPECIES

A Thesis in

Plant Pathology

by

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Species of *Fusarium* cause disease on plants, animals and humans, and many produce mycotoxins. Three different studies involving molecular phylogenetics of *Fusarium* species were carried out. *Fusarium avenaceum* has been associated with severe outbreaks of crown and stem rot of lisianthus, and *Fusarium solani* with caladium tuber rot outbreaks in Florida. In the first study, we sequenced parts of two genes, translation elongation factor 1-alpha (*tef*) and beta-tubulin (*benA*), in *F. avenaceum*. Phylogenetic analyses showed *F. avenaceum* isolates to be monophyletic with strong bootstrap support and genealogical concordance. Isolates from lisianthus were scattered within the *F. avenaceum* clade. The low degree of phylogenetic divergence contrasted with a high degree of vegetative incompatibility structure among isolates from lisianthus. Pathogenicity tests of several *F. avenaceum* isolates including those from other hosts showed the ability to cause disease on lisianthus suggesting that any *F. avenaceum* is potentially a pathogen of lisianthus.

In the second study, we investigated the evolutionary origins of *F. solani* isolates from caladium. Portions of the *benA* and *tef* genes were sequenced in 57 isolates and a subset of 20 isolates were chosen to sequence the internal transcribed spacer regions (ITS) and nuclear large sub unit (NLSU) of the rRNA gene. The individual and combined analysis of the genes showed two new clades associated with caladium within clade 3 of the *F. solani* species complex (FSSC). There were significant differences in growth rate and pigmentation between clades suggesting that these were two different
species that cause disease on caladium. These species appear to be closely related to opportunistic human pathogens commonly found in our environment.

In the third study, *F. solani* isolates were obtained from soil and from perithecia found on bark and fruit in primary forests of Sri Lanka and Australia. Portions of the *tef*, ITS and NLSU genes were sequenced in over 100 isolates. All soil-derived isolates from Sri Lanka were in Clade 3 and most ascospore isolates in Clade 2 respectively, while all isolates from Australia were in Clade 3. These results shed light on the possible taxonomic revision and epitypification of *F. solani*. 
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DEDICATION

To my husband, Anil,

for his patience and encouragement not only through the writing of this dissertation but also through the rigors of research and parenting, when I might have quit.

It is with great sadness that I add another name to this page.

I dedicate this dissertation also to the memory of Dr. Ishrat Mustafa, 64, who passed away on March 27, 2004, a few weeks before my graduation.

Ishrat Mustafa had a doctorate in education and worked tirelessly to promote leadership and education, irrespective of color, creed, or gender. She was a community leader and civil rights activist, a wife and mother, her home--a haven to many; she will be missed by all whose lives she touched.
Chapter 1

Introduction

*Fusarium* is a large ubiquitous genus of fungi containing some of the most important plant pathogens (Marasas, Nelson, and Toussoun 1984; Nelson, Toussoun, and Cook 1981). Species of *Fusarium* cause a wide range of diseases on many plant species (Farr et al. 1989) while some species can infect humans, animals (Nelson, Dignani, and Anaissie 1994) and many produce mycotoxins (Marasas, Nelson, and Toussoun 1984), (Aoki and O'Donnell 1999), (Booth 1971). *Fusarium* taxonomy has been a challenge with many conflicting morphological species definitions (Gams and Nirenberg 1989; O'Donnell 1996). Molecular tools are now being used for a practical molecular taxonomy of *Fusarium* (Geiser 2004) based on a phylogenetics species concept. A phylogenetic species can be stated as the smallest recognizable group of individuals sharing a recent common ancestor (Cracraft 1983).

**Morphology and traditional taxonomy**

The traditional species concept and the most dominant in identifying fungi is the Morphological Species Concept (MSC), where species are defined as a group of isolates that share a set of morphological characters and are distinguished from other groups. The defining morphological characteristic of the genus *Fusarium* is the production of distinct septate, sickle-shaped macroconidia. The morphology of microscopic characteristics,
particularly the shape and dimensions of macroconidia, the production of microconidia, chlamydospores, sclerotia, sexual stages and pigmentation, are the primary means used to identify and define *Fusarium* species. However, all of these morphological characteristics can be subjective and can vary depending on the environmental and cultural conditions.

Table 1-1: A summary of the history of *Fusarium* systematics

<table>
<thead>
<tr>
<th>Year</th>
<th>Taxonomic system</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1809-</td>
<td>Link’s description of genus</td>
<td>Unorganized species description</td>
</tr>
<tr>
<td>1809-1935</td>
<td>Unorganized species description</td>
<td>Over 1000 species</td>
</tr>
<tr>
<td>1936-</td>
<td>Wollenweber and Reinking</td>
<td>65 species, 16 Sections</td>
</tr>
<tr>
<td>1940-45</td>
<td>Snyder and Hansen</td>
<td>9 species</td>
</tr>
<tr>
<td>1971-</td>
<td>Booth</td>
<td>44 species</td>
</tr>
<tr>
<td>1982-</td>
<td>Gerlach and Nirenberg,</td>
<td>78 species (G&amp;N),</td>
</tr>
<tr>
<td>1983-</td>
<td>Nelson <em>et al.</em></td>
<td>30 species (N, T &amp; M)</td>
</tr>
<tr>
<td>1990’s-</td>
<td>Molecular phylogenetics</td>
<td>Several hundreds of species</td>
</tr>
</tbody>
</table>

The taxonomy of *Fusarium* began with the description of the genus by Link in 1809 (Link 1809) (Table 1-1). In the following century there were at least a thousand named *Fusarium* species with many described more than once for each host. In 1935, Wollenweber and Reinking published a detailed monograph of *Fusarium* where they took this large number of species of *Fusarium* and organized them into sixteen sections containing 65 species, 55 varieties and 22 forms (Wollenweber 1931; Wollenweber and Reinking 1935). Wollenweber and Reinking brought some order to the previous state of chaos by organizing these species into sections (Table 1-2) and by recognizing synonyms. They used differences in spore length, width, and septation, presence or
absence of sporodochia and sclerotia, and other characters to differentiate the species. Although later systems did not corroborate the Wollenweber & Reinking scheme, most systems for *Fusarium* taxonomy that followed were based on this work. Based on phylogenetics we know that the species within these sections are often not monophyletic.

In 1940, Snyder and Hansen lumped all of Wollenweber and Reinking’s species into 9 species (Snyder and Hansen 1940, 1941, 1945). They found that differences in morphological traits that Wollenweber et al. used to differentiate the species, varieties and forms were not necessarily stable characteristics but subject to mutation. They also discovered that single-spored isolates from a single culture could be classified into different Wollenweber & Reinking (1935) Sections, due either to mixed cultures or mutation. The streamlined Snyder and Hansen system resulted in tremendous morphological variation within a “species” with loss of information and difficulty in interpreting species boundaries. For example, according to the nine species system of Snyder and Hansen, “*F. roseum*” replaced 4 of the Wollenweber & Reinking sections: Roseum, Arthrosporiella, Gibbosum, and Discolor. Scientists in the USA mostly followed the nine-species system of Snyder and Hansen throughout the twentieth century, and its influence remains today. Also, the monographs by Booth (Booth 1971), and then Gerlach and Nirenberg (Gerlach and Nirenberg 1982), were widely accepted. In “The Genus *Fusarium*”, Booth recognized 44 species and 7 varieties, paying extra attention to the production of microconidia as a distinguishing characteristic. Gerlach and Nirenberg (1982) recognized 73 species and 26 varieties of *Fusarium* in a monograph that was really a revision of Wollenweber and Reinkings. The Nelson, Toussoun and Marasas system (1983) was a simple expansion of Snyder and Hansen’s, to 30 species (Nelson,
Tousson, and Marasas 1983). The systems of Gerlach and Nirenberg, and Nelson et al. both require considerable care and expertise in that single spore isolates should be grown on particular media under specific conditions to be correctly identified.

Table 1-2: Wollenweber Sections and Snyder and Hansen’s regrouping of all *Fusarium* species

<table>
<thead>
<tr>
<th>Wollenweber and Reinking’s 16 Sections</th>
<th>Snyder and Hansen’s 9 species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eupionnotes</td>
<td><em>episphaeria</em></td>
</tr>
<tr>
<td>Macroconia</td>
<td><em>episphaeria</em></td>
</tr>
<tr>
<td>Spicarioioides</td>
<td><em>rigidiuscula</em></td>
</tr>
<tr>
<td>Submicrocera</td>
<td>none</td>
</tr>
<tr>
<td>Pseudomicrocera</td>
<td>none</td>
</tr>
<tr>
<td>Arachnites</td>
<td><em>nivale</em></td>
</tr>
<tr>
<td>Sporotrichiella</td>
<td><em>tricinctum</em></td>
</tr>
<tr>
<td>Roseum</td>
<td><em>roseum</em></td>
</tr>
<tr>
<td>Arthrosporiella</td>
<td><em>roseum</em></td>
</tr>
<tr>
<td>Gibbosum</td>
<td><em>roseum</em></td>
</tr>
<tr>
<td>Discolor</td>
<td><em>roseum</em></td>
</tr>
<tr>
<td>Lateritium</td>
<td><em>lateritium</em></td>
</tr>
<tr>
<td>Liseola</td>
<td><em>moniliforme</em></td>
</tr>
<tr>
<td>Elegans</td>
<td><em>oxysporum</em></td>
</tr>
<tr>
<td>Martiella</td>
<td><em>solani</em></td>
</tr>
<tr>
<td>Ventricosum</td>
<td><em>solani</em></td>
</tr>
</tbody>
</table>

Morphology-based taxonomic systems have recognized as many as 73 species (Gerlach and Nirenberg 1982), and as few as nine (Snyder and Hansen 1940, 1941, 1945), but molecular phylogenetic analyses has revealed a far more complex picture of species recognition. While our picture of *Fusarium* phylogenetics is far from complete, it is clear that 1) there is often weak correlation between previously defined morphological Sections and monophyletic groups, 2) single morphological species may harbor dozens of distinguishable phylogenetic species, 3) once phylogenetic species boundaries are identified, we can usually go back and identify morphological and biological characters
that correlate with these boundaries, and 4) previously defined sub specific categories, including sub-species, varieties and *formae speciales*, usually satisfy phylogenetic criteria for species recognition.

**Phylogenetic species concept (PSC)**

Phylogenetic species have been defined as the smallest diagnosable cluster of individual organisms sharing a common ancestry (Cracraft 1983), besides other definitions (Baum and Donoghue 1995; Mayden 1997). To determine species limits using the PSC, the concordance of more than one gene genealogy is necessary. Dykhuizen and Green (Dykhuizen and Green 1991) first used multiple gene genealogies to study *Echerichia coli* and other bacteria, and applied to Eukarya by Cohan (Cohan 1994). The Geneological Concordance Concept (Avise and Ball 1990; Baum and Shaw 1995; Mayden 1997) was used by Taylor et al. to identify species limits in fungi (Taylor et al. 2000).

**Using multiple gene genealogies**

Many techniques, including those that compare nucleic acids, have been used to study the phylogenetics of fungal groups (Bruns, White, and Taylor 1991). The use of molecular techniques to study phylogenetic relationships has helped us to characterize morphologically similar but unrelated fungi (Geiser 2004). Molecular data is especially useful when morphological characters alone are insufficient for identifying clear
taxonomic groups. Unlike the use of morphology, the use of DNA sequences for phylogenetic studies provides large numbers of characters for comparing organisms. Results between laboratories can be compared, and often retrieving published sequences from electronic databases (GENBank, EMBL) reduces the need to repeat experiments. With automated sequencing machines, large numbers of individuals can be quickly compared.

The evolution of one genic region may not accurately represent the evolution of the entire genome of an organism, particularly at the species level where recombination reassorts unlinked genes. Therefore, the construction of gene trees using single gene sequences may not allow one to infer the correct phylogenetic relationships between the taxonomic units being studied (Li and Graur 2000). Additional independent genes in an organism should be analyzed and the gene genealogies compared to look for congruency. If the gene trees are not in conflict with each other there is greater confidence in the phylogenetic inference. Genealogical Concordance Phylogenetic Species Recognition (GCPSR) was a term used by Taylor et al. to define the process of comparing more than one gene genealogy to recognize phylogenetic species in fungi (Taylor et al. 2000). The common partitions across multiple gene genealogies define species and occur due to lack of actual reproduction between species.

In several species complexes of the genus *Fusarium*, the concordance of several nuclear and mitochondrial gene genealogies has provided a robust and reliable means for phylogenetic species recognition (Fig. 1-1). In the first such study, O’Donnell et al. (O'Donnell, Cigelnik, and Nirenberg 1998; O'Donnell, Nirenberg et al. 2000) used beta-tubulin (*benA*), ITS and nuclear large subunit sequences to define species boundaries in
the *Gibberella fujikuroi* species complex, that includes species causing ear rot of corn, bakanae disease of rice, pitch canker of pine, producing the mycotoxin fumonisin. This group, corresponding roughly to Section *Liseola* and recognized as the single species *F. moniliforme* by Snyder and Hansen (Snyder and Hansen 1945), was known to comprise at least seven biological species (Leslie 1995) and a few others with no known sexual stage. Concordance of three gene genealogies revealed at least 45 phylogenetic species in the complex, divided among three clades, more than doubling the recognized species diversity. Based on the degree of apparent biogeography signal, O'Donnell et al. (1998) termed these clades “American”, “Asian” and “African”. Later work on this species complex incorporated sequences from the translation elongation factor 1-alpha (*tef*) and calmodulin (*cmdA*) genes, and further supported the high level of phylogenetic species diversity in this group (O'Donnell 2000).
The genus is composed of two major clades, one associated with dark purple *Gibberella* teleomorphs, the other with orange/red *Nectria*-like teleomorphs. Within both groups are several well-characterized species complexes, as well as large numbers of species that have not yet been characterized phylogenetically. Toxins associated with each of the major groups, and their approximate correspondence with traditional morphological sections are listed. (O’Donnell and Geiser, unpublished).

In the example of *F. oxysporum*, an important cause of soil-borne wilt diseases of plants and human infections, many *formae speciales* subdivided into pathogenic races are recognized. Wollenweber (Wollenweber 1931; Wollenweber and Reinking 1935) placed *F. oxysporum* in Section Elegans together with six other taxa. Snyder and Hansen considered all of these taxa as synonymous with *F. oxysporum* based on their
morphological similarity (Snyder and Hansen 1945). However, using multiple gene

genealogies, O’Donnell et al. (1998) showed that *F. oxysporum* alone comprises dozens

of phylogenetically distinguishable species spread among 3 well-supported clades. Other

species in Section *Elegans* such as *F. redolens*, was recognized as a variety of *F.

*oxysporum* by Snyder and Hansen and Nelson *et al.*, and is now recognized as a species

quite distinct from the many lineages of *F. oxysporum* (Baayen et al. 2001). After

phylogenetic species are defined it is likely that unique morphological characteristics, as

had been described by Wollenweber and Reinking, can be recognized (Geiser *et al.*

2001).


<table>
<thead>
<tr>
<th>Genes</th>
<th>*Fusarium species complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta tubulin (<em>benA</em>)</td>
<td><em>G. fujikuroi</em>, <em>G. zeae</em>, <em>F. redolens</em></td>
</tr>
<tr>
<td>Calmodulin (<em>cmdA</em>)</td>
<td><em>F. solani</em>, <em>G. fujikuroi</em></td>
</tr>
<tr>
<td>Intergenic Spacer of rRNA (IGS)</td>
<td><em>G. fujikuroi</em>, <em>F solani</em></td>
</tr>
<tr>
<td>Internal Transcribed Spacer rRNA (ITS)</td>
<td><em>F. solani</em></td>
</tr>
<tr>
<td>Mitochondrial small subunit rDNA</td>
<td><em>F. redolens</em>, <em>F. oxysporum</em></td>
</tr>
<tr>
<td>Nitrate reductase, Phosphate permease</td>
<td><em>G. zeae</em></td>
</tr>
<tr>
<td>Large sub-unit nuclear rRNA (NLSU)</td>
<td><em>F. solani</em></td>
</tr>
<tr>
<td>Translation elongation factor 1-alpha (<em>tef</em>)</td>
<td><em>G. fujikuroi</em>, <em>F. redolens</em>, <em>G. hostae</em>, <em>F.oxysporum</em>, <em>G. zeae</em>, <em>F. solani</em></td>
</tr>
</tbody>
</table>

Table 1-3: Genes that have been used for phylogenetic analysis of *Fusarium* species

**Genes used for sequencing**

**Ribosomal RNA genes:** Ribosomal RNA (rRNA) genes (rDNA) are the most commonly

used markers in molecular phylogenetics. Several universal primers are available for

amplifying regions of the rDNA of a wide range of taxa (White *et al.* 1990). They are
tandemly arranged repeats on one or more chromosomes maintained uniform through concerted evolution. In eukaryotes, each rDNA gene repeat unit is made up of a transcriptional unit that codes for three rRNAs: a small subunit rRNA (~18S), a 5.8S rRNA, and a large subunit rRNA (~28S). The three genes are separated by two internal transcribed spaces, the ITS1 and ITS2. Each repeat unit is also separated by a non-transcribed spacer, the intergenic spacer (IGS). In the Basidiomycota and in basal groups of the Ascomycota, a 5S rRNA gene is situated in the IGS region – this is not the case in the genus *Fusarium*.

Most molecular phylogenetic studies have utilized ribosomal RNA genes (rRNA) because they are conserved regions of the genome and have been well characterized (Hibbett et al. 1997). It is also easy to amplify this region using PCR. Since the different regions of rDNA evolve at variable rates, these regions have proven very useful for investigating relationships between fungi at all taxonomic levels including genus and species (Bruns, White, and Taylor 1991). However, with the exception of the IGS region, rDNA regions do not give as much phylogenetic information to infer close relationships between species as do some protein coding regions like the translation elongation factor 1-alpha (*tef*) gene (Geiser 2004). Orthologous protein coding genes may have no variation in their amino acid sequences but show levels of divergence in their third codon positions and introns appropriate for distinguishing closely related species. Ribosomal DNA has also proven problematic on occasion in *Fusarium*, where non-orthologous ITS2 regions were found in the *Gibberella fujikuroi* complex (O'Donnell and Cigelnik 1997). This is a problem if a single gene phylogeny is used in describing species because the ITS phylogeny based on two non-orthologous sequence
types can be misleading. Other than the existence of an apparent *benA* paralog in one of the major clades of the *Fusarium solani* species complex (not the focus of work in this thesis), no such problems have yet been identified in coding genes in *Fusarium*, although we must remain vigilant in identifying them where they may exist.

**Protein coding genes:** Intron-rich protein coding genes are more useful than ribosomal genes for species-level phylogenetic studies in the fungi because they have conserved exon regions that are easy to align and intron sequences that provide more variable characters than the ITS region (Bruns, White, and Taylor 1991; Geiser 2004). The beta-tubulin (*benA*) gene and the translation elongation factor 1-alpha (*tef*) genes are two proven markers, having been used in previous phylogenetic studies of several fungi including *Fusarium* species (Table 1-3). These genes encode highly conserved proteins involved in the cytoskeleton and protein translation, respectively, harbor several introns with high nucleotide substitution rates. Primers have been developed some specific for the genus *Fusarium* and others with broader taxonomic utility that allow easy PCR and sequencing of portions of these genes which contain three or more intron sequences (Fig. 1-2)
Biological species concepts (BSC)

In fungi, a single morphological species generally corresponds to many biological species and phylogenetic species (Taylor et al. 2000). Biological species or mating populations have been defined as groups of cross-fertile isolates. In nature, different mating populations could arise due to separation of life cycles in time and/or space. A biological species classification has been developed for species within the Gibberella fujikuroi species complex (Leslie 1995) and the F. solani species complex (Matuo and Snyder 1973). In a study of the F. solani species complex, mating populations corresponded to phylogenetic species (O'Donnell 2000). However, there are several reasons why the application of a biological species concept to Fusarium is a problem. Purely asexual reproduction, homothallic species, and a lack of “female” fertile tester
isolates in heterothallic species, are some of them. For example, in heterothallic species like *F. solani*, successful crosses are determined by the ability of different isolates to act as “female fertile” (ability to produce receptive hyphae and produce viable perithecia) or “male fertile” (ability to produce fertilizing spermatia) parents. This factor, which is under the control of many genes, is independent of the single-locus mating-type system that determines sexual compatibility. Therefore, two isolates may have compatible mating-types, but fail to reproduce sexually because both are dominant as females, or dominant as males. In many cases, sexual crosses of *F. solani* have not been very successful in the laboratory because of individual tendencies of particular isolates toward “femaleness” or “maleness”. It is also possible that the environmental conditions may not be right for mating to occur, as in the example of *Gibberella circinata*, where temperature is very important for a successful cross (Covert et al. 1999). With the availability of PCR primers for identification of the mating type specific alleles, MAT1-1 and MAT1-2, it is easier to identify compatible mates in the laboratory (Covert et al. 1999). Crosses can be made only with representatives of the opposite mating type, thus greatly reducing the number of crosses that need to be made.

**Fusarium diseases of potted ornamentals**

*Fusarium* species cause severe diseases in many potted ornamentals (Daughtrey, Wick, and Peterson 1995; Knauss 1975). The ornamental industry in the United States has suffered increasing losses in the production of lisianthus plants, cut flowers, and caladium plants due to *Fusarium* diseases (McGovern and Harbaugh 1997; McGovern,
Harbaugh, and Polston 1997). In lisianthus (*Eustoma grandiflorum*), at least 3 different species of *Fusarium* can cause disease: *Fusarium avenaceum* (crown and stem rot), *Fusarium oxysporum* (wilt) and *Fusarium solani* (root and crown rot) (Raabe 1985). In caladium seed tubers, both *F. solani* and *F. oxysporum* have been shown to cause disease.

In spite of the importance of these diseases very little research on *Fusarium* diseases of ornamentals has been carried out since they were first described (Knauss 1975), (Raabe 1985). A consistent problem in studying diseases caused by *Fusarium* is incorrect species identification of the disease-causing agent, as well as poor species concepts assigned to them. For example, the cause of a recently identified disease of container-grown hostas, *Fusarium* crown rot was initially identified as *Fusarium oxysporum* using the Nelson et al. (1983) morphological system. Molecular phylogenetic methods using the *benA* and *tef* gene regions, however, identified the causative agent as a new species, *F. hostae*, related to *F. redolens* (Geiser et al. 2001). *Fusarium redolens*, synonymized with *F. oxysporum* under the Nelson et al. (1983) system, turns out to be a fairly distant relative to the *F. oxysporum* complex. In the first two chapters of this thesis, I apply similar methodologies to two important diseases of ornamental crops, lisianthus and caladium to determine the precise phylogenetic identification of these pathogens, and to define reasonable species boundaries around them. The first disease, *Fusarium* crown and stem rot of lisianthus, has been previously associated with the species *F. avenaceum*, and received very little previous phylogenetic attention. The second disease, tuber rot of caladium, has been associated with *F. solani*, which we know to represent an extremely diverse species complex. By looking at different genic regions we inferred the evolutionary history of these *Fusarium* species contributing towards the
ongoing taxonomic revision of the genus *Fusarium* and also made predictions about the biology of the pathogen.

**Fusarium avenaceum (Fr.) Sacc.**

*Fusarium avenaceum* (Fr.) Sacc. is one of the many *Fusarium* spp. that causes disease on lisianthus, (Raabe 1985). Recent severe outbreaks of crown and stem rot of lisianthus have been attributed to *F. avenaceum* (McGovern, Harbaugh, and Polston 1997). In spite of its importance, very little research has been carried out in recent years to study this disease. In this study, we explored the relationship between host and evolutionary origin in *F. avenaceum*.

*Fusarium avenaceum* possesses some distinctive morphological characteristics that make it fairly easy to identify (Nelson, Tousson, and Marasas 1983). It produces distinctive long, thin macroconidia and no chlamydospores (Fig. 1-3). Microconidia are generally scarce, and produced from branched and unbranched monophialides with rare phialides producing two conidiogenous openings. A *Gibberella avenacea* perfect stage was observed on wheat (Cook 1967; Cook 1968), but is rarely encountered in nature. Cultural variation in *F. avenaceum* can be quite problematic for correct identification, as pionnotal mutants occur with high frequency (Nelson, Toussoun, and Cook 1981).
Fusarium avenaceum is globally distributed among a wide range of plant hosts (Farr et al. 1989) where it can exist as a parasite or as a saprophyte. It is also commonly isolated from soil. It is responsible for root and stem rots of cereals, legumes, and vegetables and is commonly associated with F. culmorum and F. graminearum (Nelson, Toussoun, and Cook 1981). Although F. avenaceum causes disease on a wide range of hosts, we do not know if there is host specificity, where certain strains are capable of causing disease only on a single or limited number of host species. Furthermore, it is unknown whether this species corresponds to a species complex, as has been observed in so many other morphological species of Fusarium.

Crown and stem rot of lisianthus (Fig. 1-4) became widespread in Florida and California in 1995, with crop losses of up to 70%. Fusarium avenaceum may be spread by air, reuse of transplant trays infested with F. avenaceum, and by fungal gnats that help spread the disease. Control is by sanitation, elimination of the gnats, and fungicide application (McGovern, Harbaugh, and Polston 1997) resulting in dramatic decrease in disease incidence. To determine if isolates from other hosts could cause disease on lisianthus, collaborators performed pathogenicity tests with strains not originally isolated

Fig. 1-3: Macroconidia (200x) of Fusarium avenaceum
from lisianthus, and also carried out vegetative compatibility tests (Elmer and McGovern 1997).

Fig. 1-4: Non-infected plants of lisianthus (*Eustoma grandiflora*)

**Systematics of *F. avenaceum* (Fr.:Fr.) Sacc.**

Wollenweber and Reinking listed 77 synonyms for *F. avenaceum* in their monograph “Die Fusarien” (Wollenweber 1931; Wollenweber and Reinking 1935). But, according to the nine species system of Snyder and Hansen, “*F. roseum*” replaced all of the species from four Sections: Roseum (which included *F. avenaceum*), Arthrosporiella, Gibbosum, and Discolor. As this “lumping” was criticized by others (Gordon 1959), Snyder, Hansen and Oswald (Snyder, Hansen, and Oswald 1957) added the notion of “cultivar” to their species and described *F. roseum* as having cultivars, *Culmorum, Avenaceum, Graminearum, Sambucinum, Equiseti,* and *Acuminatum*. *F. roseum* cultivar *avenaceum* was then changed to *F. roseum* var. *avenaceum* by Messiaen and Cassini (Nelson, Toussoun, and Cook 1981). This variety represented those cultures with very long and very thin macroconidia and no chlamydospores. However, mutations in *F. roseum* var. *gibbosum* (loss of chlamydospores) and *F. roseum* var. *graminearum* (mutants with long spores) have reportedly resulted in *avenaceum*-like cultures (Snyder
Gerlach and Nirenberg (Gerlach and Nirenberg 1982) and Nelson et al. (Nelson, Tousson, and Marasas 1983) both applied similar morphological species concepts to *F. avenaceum*. The *F. avenaceum* isolates analyzed in this study were first identified using the criteria described in Nelson et al. (1983), before the application of molecular phylogenetics.

The objectives of the first study were to identify phylogenetic structure within *F. avenaceum* with respect to origins from lisianthus versus other hosts as well as geographic origin, and to generate a database of *F. avenaceum* sequences useful for purposes of taxonomy, management and control. We sequenced portions of two protein coding genes, translation elongation factor 1-alpha (*tef*) and beta-tubulin (*benA*) and carried out a phylogenetic analysis. Other studies using these protein coding genes have shown that they are variable between closely related species even when no apparent morphological differences are known (Geiser, Frisvad, and Taylor 1998; O'Donnell 1996). Even though previous phylogenetic studies of *Fusarium* species have shown that a single morphological species could actually be several phylogenetic species, the morpho-species *F. avenaceum* appears to consist of a single phylogenetic species (Nalim et al. 2001).

*Fusarium solani* (Mart.) Sacc.

The morphological species *Fusarium solani* is one of the most common species of *Fusarium* found ubiquitously in soil and plant debris. The sexual stage (teleomorph) for the fungi known as *F. solani* (anamorph) is usually known as *Nectria haematococca*
Berk. & Br. (Berkeley and Broome 1873; Berkeley and Broome 1967). *F. solani* can be identified morphologically by the presence of chlamydospores, long unbranched monophialides, predominantly cream mycelia that can vary in pigmentation, and the shape and size of microconidia and macroconidia (Gerlach and Nirenberg 1982).

**Systematics of *F. solani* (Mart.) Sacc.**

Wollenweber and Reinking placed three species, seven varieties and three forms of *Fusarium* into the section Martiella (Wollenweber 1931; Wollenweber and Reinking 1935) including *F. solani*. In addition, two species of *Hypomyces* (the name incorrectly given to the sexual stage of *Fusarium*) with their three varieties and one form were also included in this section. Snyder and Hansen combined all the fungi in sections Martiella (Snyder and Hansen 1941) and Ventricosum (Snyder and Hansen 1945) and used the name *F. solani* to describe them as a single species sharing the teleomorph as *Hypomyces solani* (Snyder and Hansen 1941). There is currently a controversy as to whether the teleomorph should be placed in the genera *Nectria, Haematonectria*, or *Neocosmospora*.

Five pathogenic isolates were classified as *formae specialis* of this species on the basis of pathogenicity alone (Snyder and Hansen 1941). Therefore, the species names were based on morphology alone and the forms were based on pathogenicity alone. These forms were less specialized than the *formae specialis* of *F. oxysporum* in that they can sometimes attack a whole species or several genera of host plants. The 10 *formae specialis* of *F. solani* to date are *phaseoli, pisi, cucurbitae, batatas, radicicola, robiniae, mori, piperis, eumartii* and *xanthoxyli* (Snyder and Hansen 1941; Suga et al. 2000). Snyder and Hansen also renamed as *F. oxysporum*, all of the 10 species in section
Elegans (Snyder and Hansen 1940). Booth (Booth 1971, 1981) recognized 4 species within section Martiella while Gerlach and Nirenberg recognized 6 species (Gerlach and Nirenberg 1982). Matuo and Snyder recognized seven mating populations (MP I-MPVII) within the *F. solani* polytypic species based on sexual crosses (Matuo and Snyder 1973). An initial phylogenetic analysis of isolates in the *F. solani* species complex was carried out by using rDNA sequence data and Random Amplified Polymorphic DNA (RAPDs) (O'Donnell and Gray 1995), suggesting a correspondence between biological and phylogenetic species within the *F. solani* species-complex.

In 1996, O'Donnell et al. identified at least 50 phylogenetic species within the *F. solani* species complex (O'Donnell 1996). In a more recent and complete study, phylogenetic relationships and biogeography of the *Fusarium solani* species complex were inferred by sequencing parts of the nuclear large subunit 28S rDNA, the nuclear ribosomal internal transcribed spacer (ITS) region, and translation elongation factor (*tef*) genes (O'Donnell 2000). Maximum parsimony analysis of the combined dataset identified 26 phylogenetic species. The isolates in his study included 15 heterothallic, 7 homothallic and 4 that were known only to reproduce asexually. Also, 9 biological species corresponding to known formae speciales were all resolved as phylogenetically distinct species (O'Donnell 2000). O'Donnell identified three major clades in the *Fusarium solani* species complex, termed clades 1, 2 and 3. Clade 1 consisted of two species known only from New Zealand. Clade 2 included a number of different lineages, and showed a geographical connection with South America. Clade 3 included most of the commonly encountered *F. solani* isolates associated with plant diseases and soil, and showed a cosmopolitan distribution (O'Donnell 2000).
**Fusarium solani on caladium**

Caladium (*Caladium x hortulanum* Birdsey), a plant with attractive multicolored foliage, is native to the Amazon basin. In the US, it is used primarily as an indoor potted plant and for home landscaping. Caladium is propagated using tubers. Caladium production was once a $2-million industry in Florida, producing approximately 95% of the world’s commercial crop (Holms et al. 1965; Sheehan 1967). Over the past 10 years, there has been a great decline in caladium tuber production due to tuber rot caused by *F. solani*. A number of commercial varieties were abandoned due to susceptibility. Root and stem rot of caladium begins as water soaked areas that later turn brown to black. Infection reduces the stand, growth and yield of infected plants. *Fusarium solani* can survive on infected tubers and in the soil in association with dead organic matter. In the greenhouse, heat treatment of tubers with fungicide soaks is used as a method of control. It is almost impossible to obtain disease-free tubers for propagation. The initial description of the disease and its control was published in 1975. There has been no large-scale study of this disease to determine the origins of the causative agent, or to determine whether it is caused by a single phylogenetic species or by a number of different species within the *F. solani* complex.

The objectives of this study were to carry out a phylogenetic analysis of *F. solani* from caladium based on 4 genic regions: ITS1 and ITS2, the large subunit rRNA gene sequences (NLSU) and 2 protein coding gene sequences, beta tubulin (*benA*) and translation elongation factor (*tef*). We compared our results with those obtained by O’Donnell *et al.* (2000) in his study of the *F. solani* species complex and placed our
isolates into a broader phylogenetic and biogeographical framework based on all of the available data sets of *F. solani* DNA sequences (Nalim et al. 2002). We attempted to identify mating types and populations to compare biological species boundaries with phylogenetic species boundaries.

**Characterization of Clade 2 of the *Fusarium solani* species complex**

Today’s continents are fragmented pieces of previous larger landmasses called “super-continents”. The theory of continental drift by Alfred L. Wegener, and Alexander Du Toit, states that the super-continent Pangaea began to break up about 200 million years ago into two large landmasses, Laurasia in the Northern Hemisphere and Gondwanaland in the southern hemisphere (Strickberger 2000). Laurasia and Gondwanaland began to then break up into smaller continents that we have today. Gondwana broke into two: South America/Africa, and India/Antarctica/Australia. The American and African landmasses that once fit together then broke away. Sri Lanka and India were part of Gondwanaland until Sri Lanka broke away from the Indian Peninsula during the Precambrian age (Balakrishnan 1997; Cooray, Abeysinghe, and Prame 1987). In accordance with the plate tectonic model (Weijermars 1989), India was surrounded on the North by the Tethys sea before it collided with Tibet and joined the Laurasian land mass forming the Himalayas (Balakrishnan 1997). The two land masses, the Tibetan mass and the Indian mass, have very different thickness of the earth’s crust (Balakrishnan 1997). Geologically, the island of Sri Lanka is an extension of the Indian Peninsula and forms part of the Indian Shield (plate) (Balakrishnan 1997; Cooray, Abeysinghe, and
Prame 1987). Plant and animal fossils that are identical have been found on the coastlines of South America and Africa. The discovery of fossils of tropical plants and ferns on Antarctica supports the conclusion that this land mass must have been situated closer to the equator.

The previous phylogenetic work of O'Donnell (O'Donnell, Cigelnik, and Nirenberg 1998; O'Donnell et al. 1998), (O'Donnell, Nirenberg et al. 2000; O'Donnell, Kistler et al. 2000), identified a high degree of biogeographic structure within species complexes of *Fusarium*. Presumably, this structure reflects the ancient origins of these complexes, with divergence among them occurring after the separation of the continents. Such structure was identified in the *Fusarium solani* species complex with three major clades, two corresponding to New Zealand (1) and South America (2), with a third cosmopolitan clade (3) (O'Donnell 2000). Some degree of geographic structure was identifiable within Clade 3, although it was far from complete. The author of this study concluded a probable Gondwanan origin of the complex, with further divergence among the three clades and within Clade 3 following the separation of the continents.

Confounding conclusions in his study were two important factors, a general lack of isolates derived from the tropics and Gondwanan regions, with particularly poor representation of isolates from non-agricultural sources in the Old World tropics, and lack of material associated with the original descriptions of the *F. solani* anamorph and *N. haematococca* teleomorph.

Fungi are some of the most important but least studied organisms in the biologically diverse tropics. Accurate assessment of the diversity and species richness of tropical microfungi and their specialized habitats is very important in understanding
aspects of their evolution, biology, pathogenicity, management and control of fungal pathogens and non-pathogens. Ascomycete fungi in the order Hypocreales are very diverse. Saprophytic forms decompose organic matter and can be commonly found in soil, wood, and plant debris. Hypocrealean microfungi such as *Fusarium* have small, almost microscopic sexual reproductive structures that can be found on fallen leaf litter and parts of dead trees that are lying on the soil surface. These structures known as perithecia release ascospores, the sexual spores, that then gives rise to the vegetative part of the life cycle of the fungus. Fungal spores and mycelia can also be extracted from soil by plating the soil on selective media in the laboratory.

Our goal for this project was to increase our knowledge about the species diversity of fungi in the *Fusarium solani* species complex by adding collections, describing new taxa, and defining species limits. This is part of a larger study of under-represented areas, like Australia, and Africa, that may have Gondwanic origins. We were particularly interested in *F. solani* in Sri Lanka, because the taxonomists Berkeley and Broome described the *Nectria haematococca* teleomorph from Sri Lankan collections in the mid to late 19th century. We collected ‘*N. haematococca*’ perithecia from recently killed trees and other woody substrates throughout the country. We isolated *F. solani* from soil samples taken from natural, old-growth forest areas, where the native fungal flora presumably have not been subjected to a high degree of human impact. We sampled several diverse locations covering as many different forest types as possible (Nalim et al. 2003). This collection and phylogenetic species identification will help place fungi in this order into a broader phylogenetic and bio-geographical framework based on a large data set of DNA sequences and shed light on the possible taxonomic
revision of the anamorph and teleomorph names of *F. solani*, both have been very controversial.

The results of this work showed that isolates derived from field-collected perithecia belonged to a number of new and diverse phylogenetic lineages in Clade 2, which O’Donnell (2000) had previously associated with South America. This finding has led us to reinterpret biogeographical associations with Clade 2, and opened up the possibility that it is pan-tropical or perhaps even cosmopolitan. Since the name *Nectria haematococca* is associated with perithecial collections from Sri Lanka, and our collections are morphologically similar to those in Berkeley and Broome’s descriptions, we conclude that there is a strong connection between the *haematococca* epithet and Clade 2. On the other hand, the name *F. solani* is based on the description of a fungus on potato collected in Germany, and other results suggest that this is probably a member of Clade 3. Thus, our results suggest that the *F. solani* anamorph and *N. haematococca* teleomorph do not share a strong phylogenetic connection.

**Significance**

*Fusarium* is an economically important fungus that is easy to study and its taxonomic history has been filled with controversy. As such, it is a very important fungus to focus molecular evolutionary studies, to revise the taxonomy of the genus. Phylogenetic species identification should help us to understand the biology of these pathogens to effectively control *Fusarium* diseases. Correct species identification is very important for determining the ecological roles of *Fusarium* species, for diagnosing
disease, studying host resistance, for understanding epidemiology, and for plant pest quarantine. By understanding speciation on different continents and the biogeographic structure and phylogenetic origins of species in the *Fusarium solani* species complex, we can gather information on the persistence and global spread of these pathogens.

**Questions and Objectives:**

**Diseases of potted ornamentals caused by *Fusarium* species:**

**F. avenaceum on lisianthus**

**Questions:** At least 3 different species of *Fusarium* can cause disease on lisianthus. Is our current morphological concept of *F. avenaceum* accurate and reliable? Is *F. avenaceum* a single phylogenetic species or a species-complex? Is there a correlation between phylogenetic species and the host and location of isolates of *F. avenaceum*? Is it likely that *F. avenaceum* from other hosts and locations can cause disease on lisianthus?

**Objective 1:** To carry out a phylogenetic analysis of *F. avenaceum* from lisianthus based on 2 protein coding gene sequences, b-tubulin (*benA*) and translation elongation factor (*tef*)

**Objective 2:** To compare *F. avenaceum* from lisianthus with *F. avenaceum* from other hosts and locations.

**F. solani on caladium**
Questions: *F. solani*, previously a single morpho-species, has been shown to be a species-complex. The *F. solani* isolates from caladium show variation in morphology. Are the isolates from caladium a single phylogenetic species? Are they new species in the *F. solani* species complex? Do they correspond to one or more already known phylogenetic species? A large number of *F. solani* isolates from a single host have not been analyzed so far.

Objective 3. To carry out a phylogenetic analysis of *F. solani* from caladium based on 4 genic regions: ITS1 and ITS2, the large subunit rRNA gene sequences (NLSU) and 2 protein-coding gene sequences, $\beta$-tubulin (*benA*) and translation elongation factor (*tef*).

Objective 4. To compare our sequences with those from previous studies to increase our understanding of species boundaries based on phylogenetics, morphology and biology.

The *Fusarium solani* Species Complex

Questions: If *F. solani* from several Gondwanic regions are obtained and analyzed, will these isolates show a biogeographic pattern, will previous biogeographic hypotheses be supported (O’Donnell 2000)? How will the isolates from Sri Lanka compare with isolates that are from other places of Gondwanic origin (example, Australia, Africa)? Is there a connection between isolates collected from teleomorph material and from soil in the same forest locations?

Objective 5: To collect and determine the multilocus phylogenetics of *F. solani* isolates from non-agricultural soil samples from forests in Sri Lanka, as well as isolates derived from perithecia collected in the same locations.
Objective 6: To better define major clades of this important complex and their biogeographic associations, and to clear up taxonomic problems associated with the anamorph and teleomorph names.

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Chapter 2

Low levels of phylogenetic structure in *Fusarium avenaceum* from lisianthus and other hosts

Abstract


*Fusarium avenaceum* (Fr.) Sacc. is globally distributed among a wide range of plant hosts. Recent severe outbreaks of crown and stem rot of lisianthus (*Eustoma grandiflorum*) have been attributed to *F. avenaceum*. We sequenced parts of two protein coding genes, translation elongation factor 1-alpha and beta-tubulin, in 34 *F. avenaceum* isolates from lisianthus and several other hosts and generated a database of *F. avenaceum* sequences useful for taxonomy, management and control. Isolates of *F. heterosporum*, *F. polyphialidicum* and *F. graminum* were included as the outgroup. Phylogenetic analyses of both genes showed *F. avenaceum* isolates to be monophyletic with strong bootstrap support and no significant incongruence among gene genealogies. Isolates from lisianthus were scattered within the clade and did not form a distinct group. The low degree of phylogenetic divergence contrasted with a high degree of vegetative incompatibility structure among isolates from lisianthus. Pathogenicity tests of several *F. avenaceum* isolates from other hosts showed the ability to cause crown and stem rot on
lisianthus suggesting that any isolate of *F. avenaceum* may be pathogenic on lisianthus regardless of its phylogenetic origin.

Additional keywords: beta-tubulin, crown rot, DNA sequencing, *Eustoma grandiflorum*, stem rot, translation elongation factor 1-alpha

**Introduction**

*Fusarium* is a large, ubiquitous genus that includes many important plant pathogens and mycotoxin producers (Nelson, Dignani, and Anaissie 1994), as well as saprophytes and endophytes. Correct species identification is very important for determining the ecological roles of *Fusarium* species and for diagnosing disease. The morphology of microscopic characteristics, particularly the shape and dimensions of macroconidia, is the primary means used to identify and define *Fusarium* species. Morphology-based taxonomic systems have recognized as many as 78 *Fusarium* species (Gerlach and Nirenberg 1982), and as few as nine (Snyder and Hansen 1940, 1941, 1945), but molecular phylogenetic approaches using genealogical concordance to determine species boundaries (Taylor et al. 2000) identify far more (O'Donnell 2000; O'Donnell, Cigelnik, and Nirenberg 1998; O'Donnell et al. 1998). For example, *Fusarium oxysporum*, an ubiquitous soil species, is successful both as a saprophyte and as a pathogen with a wide host range (Gordon and Martyn 1997) and many formae speciales of *F. oxysporum* are recognized within this species based on host specificity. Molecular phylogenetic analyses of *F. oxysporum* have demonstrated that this ‘species’ is actually an extremely diverse complex of lineages that show continental biogeographic
structure (O'Donnell et al. 1998). Molecular phylogenetic analyses have also demonstrated multiple, independent origins of most *formae speciales* in this complex, indicating that this is not a useful taxonomic category, and that common pathogenicity is often the result of convergent evolution (Baayen et al. 2000; O'Donnell et al. 1998; Skovgaard et al. 2001). Another possible explanation is the horizontal transfer of genes that confer pathogenicity.

In this study, we explored the relationship between host and evolutionary origin in *F. avenaceum*. *Fusarium avenaceum* is globally distributed among a wide range of plant hosts (Booth 1971) where it can exist as a parasite or as a saprophyte. It is also commonly isolated from soil. It is responsible for root and stem rots of cereals, legumes, and vegetables and is commonly associated ecologically with *F. culmorum* and *F. graminearum* (Cook 1968). Although *F. avenaceum* causes disease on a wide range of hosts, we do not know if there is host specificity, where certain strains may be capable of causing disease only on a single or limited number of host species.

Many potted ornamentals have diseases caused by several *Fusarium* spp. (Knauss 1975). *Fusarium avenaceum* (Fr.) Sacc. is one of the many *Fusarium* spp. that cause disease on lisanthus (Raabe 1985). Recent severe outbreaks of crown and stem rot of lisanthus have been attributed to *F. avenaceum* (McGovern, Harbaugh, and Polston 1997). Crown and stem rot of lisanthus became widespread in Florida and California in 1995, with crop losses of up to 70%. *Fusarium avenaceum* may be spread by air, the reuse of transplant trays infested with *F. avenaceum* and by fungus gnats that help spread the disease (McGovern and Harbaugh 1997). In addition to good sanitation practices, fungicides are the main agents used to control *F. avenaceum*. In spite of its importance,
very little research has been carried out in recent years to study this disease (McGovern and Harbaugh 1997; McGovern, Harbaugh, and Polston 1997).

Wollenweber and Reinking listed 77 synonyms for *F. avenaceum* in their monograph *Die Fusarien* (Wollenweber 1931; Wollenweber and Reinking 1935). But, according to the nine species system of Snyder and Hansen, all of the species from 4 *Fusarium* Sections *sensu* Wollenweber & Reinking (Roseum (which included *F. avenaceum*), Arthrosporiella, Gibbosum, and Discolor) were synonymized under the name *F. roseum*. Gerlach and Nirenberg (Gerlach and Nirenberg 1982) and Nelson et al. (Nelson, Tousson, and Marasas 1983) both applied similar morphological species concepts to *F. avenaceum*. The *F. avenaceum* isolates analyzed in this study were preliminarily identified using the morphological criteria described in Nelson et al. (Nelson, Tousson, and Marasas 1983).

*Fusarium avenaceum* is fairly easy to identify in culture (Gerlach and Nirenberg 1982; Nelson, Tousson, and Marasas 1983). It produces distinctively long, thin macroconidia and no chlamydospores. Microconidia are generally scarce, and produced from branched and unbranched monophialides with rare phialides producing two conidiogenous openings. A sexual state for this fungus has been observed and described as *Gibberella avenacea*, although it is rarely encountered in nature (Cook 1967). Cultural variation in *F. avenaceum* can be problematic for correct identification, as pionnotal mutants occur with high frequency (Nelson, Tousson, and Marasas 1983).

The cosmopolitan nature of *F. avenaceum*, and its diverse host range, suggest that this morphologically defined species may comprise multiple phylogenetic species. The objectives of this study were to identify phylogenetic structure within *Fusarium*
avenaceum with respect to origins from lisianthus versus other hosts, as well as geographic origin, and to generate a database of F. avenaceum sequences useful for purposes of taxonomy, management and control. We sequenced parts of two protein-coding genes, translation elongation factor 1-alpha (tef) and beta-tubulin (benA) and carried out a phylogenetic analysis. Some studies using these protein-coding genes have shown that they are variable between closely related species even when no apparent morphological differences are apparent (Geiser et al. 2001; O'Donnell et al. 1998; O'Donnell, Nirenberg et al. 2000; Yan and Dickman 1996). To determine if isolates from other hosts could cause disease on lisianthus, pathogenicity tests were performed with strains not originally isolated from lisianthus. Vegetative incompatibility was tested among isolates from lisianthus to determine genetically similar groupings.

**Materials and Methods**

**Collection of isolates:**

A total of 42 Fusarium isolates were analyzed in this study. Fifteen F. avenaceum from diseased lisianthus plants collected in Florida, California, and Connecticut, and 19 F. avenaceum chosen from the Fusarium Research Center (FRC) culture collection to represent a wide host and geographic diversity (Table 2-1). Isolates of F. heterosporum, F. polyphialidicum and F. graminum were used as the outgroup. All isolates were cultured on carnation leaf agar (CLA; 2% water agar amended with pieces of sterilized carnation leaves) from single germinated macroconidia, lyophilized, and stored at the FRC at −4°C.
Table 2-1: *Fusarium avenaceum* isolates from lisianthus and other hosts. *Fusarium species* used as the outgroup are included.

The host substrate\(^a\). Identification based on morphology\(^b\). Geographic origin\(^c\). Vegetative Compatibility Group (VCG)\(^d\). Pathogenicity test denoted by a “-” if not pathogenic, and a “+” if it caused disease on lisianthus\(^e\).

<table>
<thead>
<tr>
<th>Host(^a)</th>
<th>Species(^b)</th>
<th>Origin(^c)</th>
<th>FRC (^d)</th>
<th>VCG(^d)</th>
<th>Pathogenicity(^e)</th>
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<td>Fpy7</td>
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</table>

PA = Pennsylvania, MO = Missouri, DE = Delaware, AL = Alabama, CT = Connecticut, FL = Florida, CA = California, ND = North Dakota

**DNA extraction and Polymerase Chain Reaction (PCR)**

Isolates taken from the culture collection were grown on CLA for 24 h and several ~10mm² plugs of agar containing fresh growth of mycelia were transferred to test tubes containing 10 ml of sterile potato dextrose broth (PDB; Difco laboratories, Detroit, Michigan). The test tubes were placed on a shaker (35rpm) for 4 days at 22-24 C and mycelia were filtered and frozen in 0.5ml eppendorf tubes. The samples were lyophilized for 24 h before DNA was extracted using a modified CTAB method as described previously (Geiser et al. 2001)

A ~690 bp fragment of the translation elongation factor 1-alpha (*tef*) gene was amplified using primers ef1 and ef2 (O'Donnell et al. 1998) in all isolates using the
polymerase chain reaction (PCR) as previously described (Geiser et al. 2001). Also a ~1100 bp fragment of the beta tubulin gene (*benA*) was amplified using the primer pair benat1 and benat22 (O’Donnell and Gray 1995). QIAquick PCR purification kit (QIAGEN Inc, Valencia, CA), and quantified by comparison with a known DNA standard using gel electrophoresis.

**Direct Sequencing:**

Sequencing of the amplification product of the *tef* gene (~690 bp) was carried out using primers ef1 and ef2 (O'Donnell et al. 1998). A ~600 bp portion of the *benA* T1/T22 PCR product was sequenced in both directions using the primer pair benat1 and benat2. Sequences were generated using an Applied Biosystems Big Dye Terminator sequencing kit according to manufacturer’s recommendations. Sequences were generated on an automated DNA Sequencer (Applied Biosystems 377; PE Biosystems, Foster City, California), at the Penn State Plant Pathology Core Instrumentation facility.

**Phylogenetic Analysis:**

Sequences were edited using Sequencher 3.1.1. (Gene Codes Corporation, Ann Arbor, Michigan) and initially aligned using Clustal X Multiple Sequence Alignment Program (v.1.81, Julie Thompson and Francois Jeanmougin), followed by visual adjustment using Se-Al v.2.Oa3 (University of Oxford, Oxford, UK). Phylogenetic Analysis Using Parsimony (PAUP4.0b10) was used for generating phylogenetic trees (Swofford 2001). Modeltest version 3.06 (Posada and Crandall 1998) was used to determine the nucleotide substitution model best suited to the dataset. Phylogenetic and
molecular evolutionary analyses using Jukes-Cantor, Kimura-2-Parameter, and Tajima-
Nei models to generate UPGMA (not shown) and Neighbor-Joining phylogenetic trees
were also conducted using MEGA version 2.1 (Kumar et al. 2001). Gaps in the
alignment were considered missing data. Isolates of \textit{F. heterosporum} and \textit{F. graminum}
were used as outgroups. Bootstrap tests were performed with 1000 replications. A
Bayesian analysis was carried out using MrBayes v3.0b4 (Huelsenbeck, J.P and Ronquist,
F., University of California, San Diego).

\textit{Fusarium avenaceum tef} sequences were compared with those from a database of
158 diverse \textit{tef} sequences (kindly provided by Kerry O’Donnell), using a stand-alone
version of BLAST (Altshul et al. 1990; Madden, Tatusov, and Zhang 1996).

\textbf{Vegetative Compatibility Groups (VCG’s):}

VCG tests were carried out in Connecticut (Elmer, personal communication).
Thirty-nine isolates of \textit{Fusarium avenaceum} (Table 2-1) were assayed for vegetative
compatibility (all isolates not shown) as described previously for \textit{F. oxysporum} (Correll,
Klittich, and Leslie 1987). Spontaneous chlorate mutants were selected on minimal
media amended with asparagine and potassium chlorate. Pairings that resulted in dense
heterokaryotic growth between \textit{nit} mutants were considered to be vegetatively
compatible. However, the failure to observe heterokaryotic growth was not considered as
evidence of vegetative incompatibility unless both phenotypes of the nitrate non-utilizing
mutants, Nit M and Nit, had been produced and paired with each other.
**Pathogenicity tests:**

Pathogenicity tests were conducted to determine whether isolates from non-lisianthus hosts were capable of causing stem and crown rot of lisianthus. Forty-one isolates from lisianthus, as well as 24 isolates from other hosts were tested on lisianthus in Florida and Connecticut. Four-week old lisianthus seedlings (cultivar Echo Blue), were provided by Ball Seed Company in 384 cell plug trays. Seedlings were transferred to 36 cell plug trays for 3 wk and inoculated with the *F. avenaceum* test isolates. Spore inoculum was produced by growing the cultures on carnation leaf agar plates for 10 days under cool white light at 20 C night/ 25 C day temperature. Spores were washed from the plates with distilled water and spore concentration adjusted to $10^6$ spores/ml. Twenty ml of inoculum were poured around the base of the seedlings. Four seedlings per isolate were inoculated and border cells on both sides of inoculated plants were left untreated. Control plants were treated with distilled water.

**Results**

**Phylogenetic analysis:**

The length of the *benA* sequence was 688 bp and the *tef* sequence was 776 bp. The first 150 b of the five prime and the last 200 b of the three prime regions of the alignment were excluded from the combined analysis of 1464 base alignment. The overall divergences of the sequences were less than 2 percent. Modeltest successfully calculated 3 of the 56 models it can test before terminating the run for this dataset. The
three models were the JC, JC+I and JC+G models of nucleotide substitution. Distance analysis using several distance correction models applied to both data sets indicated all *F. avenaceum* isolates to be a monophyletic group with 100% bootstrap support (Fig 1-1). Within this clade, isolates from lisianthus were scattered with no evidence of phylogenetic structure with respect to host. Bootstrap analysis of both data sets showed the *F. avenaceum* clade to have a 100% bootstrap support, with no strongly supported branches identified within the *F. avenaceum* clade. Because of the agreement between the two data sets, the data were combined and analyzed using PAUP. The results of the combined analysis showed the same topology as the two separate data sets with *F. avenaceum* isolates forming a monophyletic clade (Fig. 1-2), clearly distinct from the two outgroup clades. The results of the Baysian analysis, with gamma rates, on the combined dataset also supported this conclusion. Representative *tef* sequences from three *F. avenaceum* isolates were compared to a database of *tef* sequences from 158 diverse fusaria using BLAST. All three sequences were most closely related to a single *F. avenaceum* sequence in the database.

As additional evidence that the data do not contradict monophyly of the lisianthus isolates, the Kishino Hasegawa test in likelihood (Kishino and Hasegawa 1989), comparison of constraint trees in parsimony (Swofford 2001), or parametric bootstrapping could be done. These tests were not attempted due to the huge amounts of computer time and memory involved in parsimony or likelihood analysis of this data set.
beta-tubulin
~590 bp
NJ

F. avenaceum

F. heterosporum
and F. graminum

"F. polyphialidicum"
Fig. 2-1: Neighbor-joining trees inferred from translation elongation factor 1-alpha (*tef*) and beta tubulin (*benA*) sequences of *Fusarium avenaceum*.

A. The neighbor-joining tree inferred from the *benA* sequences. B. The neighbor-joining tree inferred from *tef* gene. Trees were rooted using *Fusarium graminum* and *F. heterosporum* as outgroups. Bootstrap frequencies from 1000 replicates are indicated. The *Fusarium avenaceum* clade is indicated.
**Vegetative Compatibility Tests:**

Ten VCGs were found among 20 isolates with half of these VCGs representing single member groups (Table 2-1, all isolates tested not shown). Nineteen isolates could not be assigned to a VCG because the Nit M phenotypes could not be recovered and their Nit 1 phenotypes did not complement other Nit M mutants (Elmer, personal).
communication). Most multi-member VCGs were composed of groups of 2 to 6 isolates that could be traced to one locale and were likely clonal lineages of a common progenitor that spread short distances. On the other hand, VCG 4 had two isolates, one from Florida and one from California.

**Pathogenicity Tests:**

Three months after inoculation seedlings were rated for the number of symptomatic plants. Isolations from the sporodochia produced on diseased plants confirmed the recovery of the pathogen. Forty-nine out of 65 isolates were pathogenic on lisianthus including isolates of *F. avenaceum* that were originally recovered from other host plants (Table 2-1) (Elmer and McGovern, personal communication). Symptoms did not develop until six weeks after inoculation. However, once the plant began to exhibit symptoms it died within a week. Disease assessment was based on presence/absence of dead plants. Bright orange sporodochia frequently appeared in the axils of the leaves. All control plants and border plants were asymptomatic. Thirty-seven of 41 isolates from lisianthus were found to be pathogenic, while 12 out of 24 from other hosts caused disease on lisianthus.

**Discussion**

Molecular data clearly indicated that *F. avenaceum* isolated from lisianthus shares a phylogenetic origin with *F. avenaceum* isolates from diverse localities and hosts. There
was no evidence that isolates from lisianthus formed a distinct group within the clade. There was no evident pattern of grouping within the clade based either on geographic origin or host substrate. Based on this lack of phylogenetic pattern, we predict that any isolate belonging to this clade can potentially cause disease on lisianthus.

Our results indicate that *F. avenaceum* isolates share enough synapomorphic characters in both the beta-tubulin and translation elongation factor 1-alpha sequences that molecular identification using these markers should be reliable. Simple PCR-RFLP methods could be developed from these markers that do not involve sequencing. This may prove particularly useful in identifying mutated isolates that lack key morphological characters.

Gerlach and Nirenberg (1982) and Nelson et al. (1983) both applied similar morphological species concepts to *F. avenaceum*, and isolates analyzed in this study were identified using the criteria described in Nelson et al. (1983). This correlation between the morphological and phylogenetic species concepts contrasts with other *Fusarium* morpho-species that have broad host ranges, including *F. solani*, *F. oxysporum* and *F. subglutinans*, corresponding to multiple phylogenetic species (O'Donnell, Cigelnik, and Nirenberg 1998; O'Donnell et al. 1998). Two isolates initially included in this study as *F. avenaceum* were found to possess sequences highly divergent from the other *F. avenaceum* isolates analyzed (not shown). BLAST searches from these isolates coupled with morphological re-analysis demonstrated clearly that these isolates were actually *F. solani* and *F. verticillioides*, and were probably contaminants.

The lack of phylogenetic structure observed in *F. avenaceum* could be the result of inadequate resolution from the molecular markers employed. However, these same
markers have demonstrated moderate to high levels of phylogenetic structure in other morphologically defined species of *Fusarium*, including *F. oxysporum* (O'Donnell et al. 1998), *F. solani* (O'Donnell 2000), *F. graminearum* (O'Donnell, Kistler et al. 2000), and both *F. hostae* and *F. redolens* (Baayen et al. 2001; Geiser et al. 2001). Still, more highly variable markers such as microsatellites may uncover phylogenetic structure not evident in the beta-tubulin and elongation factor 1-alpha gene trees.

Given the amount of interstate movement of lisianthus plugs to and from these states, it is not surprising that isolates were recovered from the same VCG in different places. Based on the fact that the *F. avenaceum* isolates form a monophyletic group, we would expect these *F. avenaceum* isolates to belong to one or a few vegetative compatibility groups. However, the low degree of phylogenetic divergence contrasted with a high degree of vegetative incompatibility structure among isolates from lisianthus. No pattern could be deduced between host, pathogenicity and locale using vegetative compatibility. Because of the lack of quantitative data and due to sampling bias involved in the collection of isolates from diseased plant hosts, statistical analysis of the pathogenicity data was not attempted and no quantitative conclusions were made about pathogenicity. The lack of phylogenetic structure within *F. avenaceum* and the results of the pathogenicity tests suggest that any isolate of *F. avenaceum* is potentially capable of causing disease on lisianthus. Therefore, the development of resistant cultivars should involve a wide variety of *F. avenaceum* isolates, and not just those taken from lisianthus. Most importantly, control methods would have to take into account the possible spread of *F. avenaceum* from other hosts in the same locality, whether it is in a large scale nursery
producing potted ornamentals or in any other hosts of *F. avenaceum* that could be a source of inoculum.

**Bibliography**


Chapter 3

New clades of the *Fusarium solani* species complex associated with tuber rot of caladium

Abstract


Tuber rot of caladium is the cause of serious decline in caladium tuber production. The disease is caused by fungi defined morphologically as *Fusarium solani*, known to represent a complex of highly diverse species lineages referred to as the *Fusarium solani* species complex (FSSC). To determine whether a single or multiple phylogenetic species in the FSSC is associated with the disease, we investigated the evolutionary origins of isolates associated with caladium tuber rot outbreaks in Florida by using multi-locus phylogenetics. A portion of the beta-tubulin (*benA*) and translation elongation factor 1-alpha (*tef*) genes were sequenced in 57 isolates and a subset of 20 isolates were chosen to sequence the internal transcribed spacer regions (ITS) of the rRNA gene. A combined analysis showed two new phylogenetic species associated with caladium within Clade 3 of the FSSC *sensu* O’Donnell. Both mating-type idiomorphs were identified in both
phylogenetic species. Measurements of growth rate and pigmentation showed a strong positive correlation with phylogenetic lineage.

**Introduction**

*Caladium (Caladium x hortulanum Birdsey)*, a plant with attractive multicolored foliage is used primarily as an indoor pot plant and for home landscaping and is propagated using tubers. The plant is native to the Amazon Basin of South America. Over the past 10 years, there has been a great decline in caladium tuber production due to a high incidence and severity of tuber rot caused by *Fusarium solani*, forcing the abandonment of a number of commercial varieties due to high susceptibility (Knauss 1975). Caladium production was previously a $2 million industry in Florida where approximately 95% of the world’s commercial crop was produced (Holms et al. 1965; Sheehan 1967). Fewer cultivars are grown in Florida today due to susceptibility to *Fusarium*.

Root and stem rot of caladium begins as water soaked areas that later turn brown to black (Knauss 1975). Infection reduces the stand, growth and yield of infected plants. *Fusarium solani* can survive on infected tubers and in the soil in association with dead organic matter. Airborne spores, contaminated equipment, water and soil have been suggested as potential means by which plants come in contact with the pathogen (Knauss 1975). Attempts to generate pathogen-free tubers for propagation have been
unsuccessful. In the greenhouse, heat and fungicide treatment of tubers are used as methods of control.

The morphological species *Fusarium solani* (Mart.) Sacc is one of the most common species of *Fusarium*, found ubiquitously as a saprophyte in soil and plant debris causing disease on plants. The sexual stage (teleomorph) associated with *F. solani* (anamorph) is most commonly referred to as *Nectria haematococca* Berk. & Br., although it is clear that the FSSC does not show a common evolutionary origin with the type species of that genus (O'Donnell 2000). The validity of *Haematonectria* Samuels & Nirenberg, a new teleomorph genus described to correspond with the FSSC, is controversial because it is paraphyletic with respect to the genus *Neocosmospora* E.F. Smith (O'Donnell 2000). The morphological concept of *F. solani* in culture is characterized by the presence of resistant chlamydospores, long unbranched monophialides, predominantly cream-colored mycelia that can vary in pigmentation, and the shape and size of microconidia and macroconidia (Gerlach and Nirenberg 1982).

An initial phylogenetic analysis of *F. solani* isolates was carried out by using rDNA sequence data and Random Amplified Polymorphic DNA (RAPDs) (O'Donnell and Gray 1995), suggesting a correspondence between biological and phylogenetic species within the *F. solani* species-complex. In 1996, O'Donnell et al. identified approximately 50 phylogenetic species within the FSSC (O'Donnell 1996). In a more recent study, phylogenetic relationships and biogeography of the FSSC were inferred by sequencing parts of the nuclear large subunit rDNA (NLSU), the nuclear ribosomal internal transcribed spacer (ITS) region, and translation elongation factor (*tef*) genes (O'Donnell 2000). Maximum parsimony analysis of the combined dataset identified 26
phylogenetic species. The isolates in this study included 15 heterothallic, seven homothallic and four species known only to reproduce asexually. The seven known biological species (mating populations I-VII; (Matuo and Snyder 1973)) known to exist within the morpho-species *F. solani* were all resolved as phylogenetically distinct species (O'Donnell 2000). These mating populations were initially associated with host specific groups, or *formae speciales* within *F. solani* (Matuo and Snyder 1973), but some were later found to be capable of attacking a wide range of hosts (VanEtten and Kistler 1988). At present, species identification and taxonomic revision within the species complex remains difficult due to overlapping morphological characteristics and a lack of detailed descriptive studies.

The twenty-six species lineages currently known within the FSSC were divided among three major clades (O’Donnell 2000). Clade 1 comprised two phylogenetic species known only from New Zealand. Clade 2 comprised six species, and showed a biogeographic connection with South America. This clade contains the two species associated with Sudden Death Syndrome of soybean, formerly known as *F. solani* f. sp. *glycines*, and recently described as *F. tucumaniae* and *F. virguliforme* (Aoki et al. 2003). Clade 3 was the most diverse clade, with 18 phylogenetic species comprising most isolates known under the concept of *F. solani*, including the seven previously identified mating populations. Clade 3 is cosmopolitan, and some continental biogeographic structure was noted within it. Interestingly, fungi in the sexual genus *Neocosmospora* were found to reside in Clade 3, a surprise because the teleomorph and anamorph of these fungi are morphologically distinctive, whereas the remainder of the clade appears fairly morphologically homogeneous.
The questions we set out to answer in this study were as follows: 1) do isolates of the FSSC associated with tuber rot of caladium belong to one or more phylogenetic species?; 2) do isolates associated with caladium tuber rot represent new or previously known phylogenetic species based on the concept of multiple gene genealogy and genealogical concordance?; 3) are isolates associated with this disease members of Clade 2, sharing a biogeographic origin with caladium?; and 4) can morphological traits like pigmentation and other characters like growth rate and pathogenicity be associated with the molecular phylogeny? To address these questions we carried out a phylogenetic analysis based on DNA sequences from four regions: ITS1 and ITS2 (ITS), nuclear large subunit (NLSU) rRNA gene sequences and 2 protein coding gene sequences, beta-tubulin (\textit{benA}) and translation elongation factor (\textit{tef}).

**Materials and Methods**

**Fungal isolates:** A total of 57 \textit{Fusarium solani} isolates from diseased caladium plants collected in Florida were analyzed in this study. All isolates were cultured on carnation leaf agar (CLA: 2% water agar amended with pieces of sterilized carnation leaves) from single germinated macroconidia, lyophilized and stored at the FRC at -4°C.

**DNA extraction and Polymerase Chain Reaction (PCR):** Isolates were grown on CLA for 24 h and several ~10mm$^2$ plugs of agar containing fresh growth of mycelia were transferred to test tubes containing 10 ml of sterile potato dextrose broth (PDB; Difco
laboratories, Detroit, Michigan). The test tubes were placed on a shaker (35 rpm) for 4 d at 22-24C and mycelia were filtered and frozen in 0.5ml eppendorf tubes. The samples were lyophilized for 24 h before DNA was extracted using a modified CTAB method as described previously (Geiser et al. 2001).

A ~690 bp fragment of the translation elongation factor 1-alpha (*tef*) gene was amplified using primers ef1 and ef2 (O'Donnell et al. 1998) in all isolates using the polymerase chain reaction (PCR) as previously described (Geiser et al. 2001) and a ~1100 bp fragment of the *benA* gene using the primer pair benat1 and benat22 were amplified (O' Donnell and Gray 1995) in 57 of the isolates. The ITS 1 and ITS 2 regions including the 5.8S rDNA were amplified using primers ITS 4 and ITS 5 (White et al. 1990), and the NLSU region was amplified using primers ITS5 and NL4 (White et al. 1990), in a subset of 20 isolates. All PCR products were confirmed by separation on 3% agarose gels, and purified using a QIAquick PCR purification kit (QIAGEN Inc, Valencia, CA) and quantified by comparing with known DNA standards.

**Direct Sequencing:** PCR products were sequenced in both directions using an Applied Biosystems Big Dye Terminator sequencing kit according to the manufacturer’s recommendations (and concentrations of components), except that 8 µl reaction volumes were used. The same primers were used for sequencing as in the initial PCR reactions, with the exception of benat2 (O' Donnell and Gray 1995) as the reverse primer using the benat1/benat22 PCR product as template, and NL1 as the forward primer for the ITS5/NL4 NLSU region (White et al. 1990). An automated DNA Sequencer (Applied
Biosystems 377) was used (PE Biosystems, Foster City, California), at the Penn State Plant Pathology Core Instrumentation facility.

**Phylogenetic analysis:** Fifty seven sequences each of the *tef* and *benA* genes of *F. solani* were generated. Sequences were edited using Sequencher 3.1.1. (Gene Codes Corporation, Ann Arbor, Michigan) and initially aligned using Clustal X Multiple Sequence Alignment Program (v.1.81, Julie Thompson and Francois Jeanmougin), followed by visual adjustment using Se-Al v.2.Oa3 (University of Oxford, Oxford, UK). Phylogenetic Analysis Using Parsimony (PAUP4.0b10) was used for phylogenetic analyses (Swofford 2001). Gaps in the alignment were considered missing data. Parsimony and Neighbor-Joining methods were used to generate phylogenetic trees. Modeltest version 3.06 (Posada and Crandall 1998) was used to determine the nucleotide substitution model best suited to the dataset. Likelihood settings from the best-fit model were (F81+G) selected by hLRT in Modeltest for distance analysis. Several other substitution models, (JC, K2P, F81, and AIC), were also used in the distance analysis of the combined dataset. Random sequence addition with 10 replications, and heuristic searches with branch swapping (TBR) were used in parsimony analyses of the individual and combined dataset. *Nectria atrofusca* (anamorph *F. staphyleae*) was used as the outgroup (O'Donnell 2000). Bootstrap tests were performed with 1000 replications.

**Relative growth rates:** To determine growth rates, 23 isolates were grown on water agar and used as a source of inoculum. For each isolate, 3 replicate plates of PDA were inoculated each with a ~10 mm sq. plug of mycelia and incubated under a 12 h dark/light
cycle. Experiments were performed both at 25 C and 30 C. The average of two measurements of the diameter of growth, perpendicular to each other, was recorded for each plate after 6 days. For each isolate, the average of all three replicate plates was recorded. To answer the question, are growth rates significantly different for isolates in Clade A and B, the data were subjected to analysis of variance (ANOVA) and multiple comparisons of the mean values of the isolates in both Clades A and B using the Duncan’s Multiple Range Test (P\(\leq 0.05\)). The mean growth rate of isolates in the two clades were also compared using the students T-test for comparison of two means to see if the averages of the means were significantly different between Clades A and B.

**Mating Type.** Before crosses were set up between isolates, PCR tests of mating type were carried out. The mating type, MAT1-1 or MAT1-2, was determined using degenerate primer sets, M1F-1 and M1R-2 for MAT1-1, and M2F-1 and M2R-4 for MAT1-2, (Covert et al. 1999) and those specifically designed for FSSC (Ning Zhang, unpublished, MAT1-S1F: ATGGCTTTCCGCAGTAAGGA, MAT1-S1R: CATGATAGGGCAGCAAAGAG, MAT2-S1F: GGGATCTGAGAAAGATACGTA, MAT2-S1R: CGGTACTGTAGTCGGGAT) wherever possible. Cycling parameters differed from those described by Covert et al. (1999) in that the annealing temperature was 57 C instead of 45 C. The mating type assay was carried out with both sets of primers in a single reaction tube as well as in separate reaction tubes if the result needed to be verified a second time. The different amplification products were distinguished by their sizes on a 2% agarose gel, ~350 bp for MAT1-1 and ~850 bp for MAT1-2.
Based on the PCR test for mating type and the different clades to which they belonged, isolates were selected for the mating experiments. Sexual compatibilities of isolates within clades A and B and between clades were tested by co-inoculating V8 plates (Hawthorne, Rees-George, and Broadhurst 1992) with mycelial plugs from the two cultures, placed side by side. The experiment was duplicated using carrot agar as described previously (Leslie 1995). Inoculated plates were placed at room temperature (23-25 C) under a 12 h dark/light cycle and observed for 2-4 weeks for the appearance of perithecia/protoperithecia.

Results

**Phylogenetic analysis:** A ~690 base fragment of the *tef* gene, and a ~600 base fragment of the *benA* gene were generated by PCR and sequenced in 57 isolates of *F. solani* from caladium. An unrooted neighbor-joining analysis of the *tef* and *benA* sequences from isolates associated with caladium showed that the isolates fell into two distinctive groups, sharing a common partition (Fig. 3-1). To determine the relationships of these two groups to the rest of the FSSC, ten members of each group were chosen for additional sequencing of the ITS and NLSU regions, for comparison to the entire *Fusarium solani* species complex (FSSC) (Table 3-1).
Table 3-1: The *F. solani* isolates from *Caladium* in Clades A and B: mean growth rates, pigmentation, and mating types.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Color on PDA</th>
<th>Mating type</th>
<th>Clade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>@25°C</td>
<td>@30°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1607</td>
<td>48.0 i</td>
<td>66.7 j, k</td>
<td>Nonpigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1609</td>
<td>51.5 h</td>
<td>68.3 h, i, j</td>
<td>Nonpigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1614</td>
<td>44.1 k</td>
<td>64.7 l</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1624</td>
<td>56.8 e, d</td>
<td>76.83 c, d</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1626</td>
<td>59.1 b, c</td>
<td>72.2 g</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1627</td>
<td>45.2 j, k</td>
<td>63.8 l</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1628</td>
<td>54.5 f</td>
<td>73.2 f, g</td>
<td>Pigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1631</td>
<td>46.6 i, j</td>
<td>66.5 k</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1640</td>
<td>60.8 a, b</td>
<td>87.0 a</td>
<td>Pigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1644</td>
<td>51.3 h</td>
<td>69.0 i, h</td>
<td>Pigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1645</td>
<td>55.2 e, f</td>
<td>75.0 e</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1647</td>
<td>48.0 i</td>
<td>64.2 l</td>
<td>Nonpigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1649</td>
<td>61.2 a, b</td>
<td>77.6 c</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1652</td>
<td>46.5 i, j</td>
<td>68.5 i, h</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1659</td>
<td>56.2 e, f</td>
<td>74.6 e, f</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1660</td>
<td>52.2 g, h</td>
<td>73.0 g</td>
<td>Pigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1661</td>
<td>54.2 f, g</td>
<td>75.5 d, e</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1663</td>
<td>46.8 i, j</td>
<td>67.3 i, j, k</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
<tr>
<td>S1665</td>
<td>54.0 f, g</td>
<td>77.7 c</td>
<td>Pigmented</td>
<td>Mat1-1</td>
</tr>
<tr>
<td>S1672</td>
<td>45.5 j, k</td>
<td>63.3 l</td>
<td>Nonpigmented</td>
<td>Mat1-2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Growth rates (mean of three replications) within each column followed by different letters are significantly different (P≤0.05) according to the Duncan’s Multiple Range Test for Averages.

<sup>b</sup>Incubation temperature at 25°C and 30°C
Parsimony and Neighbor-joining methods gave the same results with all four data sets, individually (benA, data not shown) and combined (benA data was excluded in the combined analysis). Using the AIC model, F81, F81+G, JC and K2P models for distance correction applied to the combined analysis indicated the *F. solani* isolates from caladium formed the same two new groups of isolates with strong bootstrap support within Clade 3 of the FSSC (Fig. 3-2) (Fig. 3-3) (Fig. 3-4). Although the two groups of caladium isolates were always the same, the position of the two new clades within Clade 3 of the FSSC was slightly different for tef, ITS and NLSU gene trees with no strong bootstrap support for the differences.

Fig. 3-1: An unrooted Neighbor-Joining analysis of the tef and benA sequences showing the 57 isolates in two distinctive groups shown in green and red, sharing a common partition (indicated by a black arrow).
Fig. 3-2: Neighbor-joining analysis of *tef* gene sequences showing the phylogenetic position of isolates in Clade A and Clade B relative to the O’Donnell sequences. Bootstrap values > 50% are indicated at the nodes.
Fig. 3-3: Neighbor-joining analysis of ITS gene sequences showing the phylogenetic position of isolates in Clade A and Clade B relative to the O'Donnell sequences. Bootstrap values >50% are indicated at the nodes.
Because of the agreement between the three data sets, with the caladium isolates belonging to two new groups within Clade 3 of the FSSC, and the lack of strong bootstrap support for any minor differences in the position of these two clades within Clade 3, the data were combined and analyzed using maximum parsimony methods. The results of the combined analysis showed the same two groups of isolates from caladium, Fig. 3-4: Neighbor-joining analysis of NLSU gene sequences showing the phylogenetic position of isolates in Clade A and Clade B relative to the O’Donnell sequences. Bootstrap values >50% are indicated at the nodes.
referred to henceforth as Clades A and B, which received 84% and 100% bootstrap support, respectively (Fig. 3-5).

**Relative growth rates**: The results of the growth rate experiment are summarized in Table 3-1. Isolates belonging to the two clades A and B showed differences in growth rates and pigmentation. In growth rate experiments, the presence of pigmentation and no
pigmentation was clearly correlated with isolates from Clade A and Clade B respectively (Table 3-1). Isolates from Clade A consistently showed a dark colony reverse on PDA plates in comparison to Clade B isolates that formed no pigmentation at 6 days of growth (Fig. 3-6). After 6 days, isolates that were pigmented averaged 53.59 +/- 2.00 mm of radial growth, whereas isolates that were nonpigmented averaged 45.90 +/- 1.48 mm of radial growth at 25 C. Similarly, pigmented isolates averaged 73.88 +/- 2.84 mm and nonpigmented isolates averaged 64.90 +/- 2.17 mm of radial growth at 30 C.

The results of the analysis of variance (ANOVA) and multiple comparisons of the mean growth rates using the Duncan’s Multiple Range Test (P≥0.05) indicated a significant difference between the means of most isolates in Clade A versus Clade B. The statistical analysis was conducted using the Statistical Analysis System (SAS) version 8.02 (SAS Institute, Cary, NC). According to the Duncan test, means followed by the same letter within a column are not significantly different. Means followed by different letters within a column are significantly different.

At 25C, the growth rates of isolates S1649, S1640, and S1626 are not significantly different and belong to Clade A. Similarly, S1607, S1631, S1647, S1652, S1663 are not significantly different from each other and belong to Clade B. Also S1614, S1627, S1672 are not significantly different from each other and belong to Clade B. However, S1624 and S1626 belong to Clade A and have significantly different growth rates. Although there are some exceptions, growth rates within a clade are not significantly different from each other but are significantly different between clades. The Duncan’s test may not be the best comparison test of means for this type of data. Other tests that
can be used on this data to compare means are the Student-Newman-Keul’s test or the Tukey’s test.

The average growth rates of pigmented isolates and nonpigmented isolates were compared using the T-test for comparison of two means. The mean growth rate for the pigmented isolates was greater than and significantly different (P<0.001) from the mean growth rate for the non-pigmented isolates (see appendix B).

Fig. 3-6: Caladium isolates showing differences in colony diameter and pigmentation after 6 d. Top row: Nonpigmented cultures. Bottom row: Pigmented cultures.

**Mating type/sexual crosses:** Within Clade A, six isolates were found to be of mating-type MAT1-1 and four isolates were found to be MAT1-2. In Clade B three isolates out of ten were found to be of mating-type MAT1-2. To determine whether the two groups were interfertile, we paired 4 isolates from Clade A with 4 isolates from Clade B in a total of 12 crosses and looked for the production of fertile perithecia. A single inter-clade cross, (S1631 X S1665), produced four protoperithecia, none of which matured. In addition, 2 MAT1-1 and 3 MAT1-2 isolates from Clade B were paired, which also produced no fertile perithecia.
Discussion

Isolates of *Fusarium solani* causing tuber rot disease on caladium in Florida were found to correspond to two new phylogenetic species in Clade 3 of the FSSC. The two clades were identifiable in the genealogies of four different genes, and appeared to satisfy the criteria of genealogical concordance phylogenetic species recognition (GCPSR; (Taylor et al. 2000)). Isolates from these two clades showed significant differences in growth rate in culture, and also showed a characteristic difference in pigmentation. Members of both isolates fall within the traditional morphological concepts applied to *F. solani* (Gerlach and Nirenberg 1982; Nelson, Tousson, and Marasas 1983), and lack obvious micromorphological differences, but it remains to be determined whether they can be distinguished from other phylogenetic species in Clade 3 using precise measurements of macroconidial dimensions or other micrometric means (Aoki et al. 2003).

The two phylogenetic species associated with caladium tuber rot did not correspond to any of the 26 clades that had previously been identified by O’Donnell (2000). Other work from our laboratory indicates that there are far more undescribed clades in the FSSC, and that these species associated with caladium correspond to only two of them. Preliminary data indicates that these 20 isolates from caladium are closely related to other isolates in the FSSC that are commonly found in the environment as saprophytes or opportunistic human pathogens. The association of the caladium pathogen with Clade 3 does not rule out a South American origin of the pathogen, since isolates from Clade 3 have been previously identified from South America (O’Donnell...
We also cannot determine whether the pathogen may have found its way onto caladium from another biogeographic source, including Florida where the isolates were collected.

Although these isolates were collected from diseased caladium tubers, and some cause disease on caladium in inoculation experiments (data not shown), there is no evidence supporting any degree of host specificity. However, efforts to breed for resistant cultivars of caladium, need to consider the fact that at least two phylogenetic species are responsible for the disease, and utilize members of both Clade A and B in inoculation experiments. In addition, further studies should be undertaken to determine differences in virulence among isolates from the two clades.

Clade A and Clade B contained a mixture of the two mating-types MAT1-1 and MAT1-2. To investigate a possible connection between phylogenetic species and mating populations, isolates of opposite mating type both within clades and between clades were paired to induce sexual reproduction. Four protoperithecia were observed only in a single cross between two isolates in different clades and did not give rise to any visible ascospore formation. This cannot be considered a successful cross. This failure to induce sexual crosses in culture both within and between clades is only a negative result that does not preclude sexual interfertility, however, so these negative results are subject to limited interpretation. It is possible that these clades represent groups of related asexual clones that have lost the ability to undergo sexual reproduction.
Bibliography


Chapter 4

New Sri Lankan and Australian species of the *Fusarium solani* species complex derived from field collected perithecia and soil

Abstract


The *Fusarium solani* species complex (FSSC) is a highly diverse, cosmopolitan group of fungi that occur in soil and on living and dead plant tissue, and cause both human and plant infections. This monophyletic group was previously divided into three clades with some biogeographic structure, termed Clades 1, 2 and 3. The sexual stage associated with the FSSC is commonly known as *Nectria haematococca*, and was originally described based on material collected in Sri Lanka. To identify new species in the FSSC and to better understand its biogeography, we investigated the phylogenetics of *F. solani* isolates obtained from soil and perithecia found on bark and fruit from primary tropical forests in Sri Lanka and North Queensland, Australia. Portions of the translation elongation factor 1-alpha (*tef*) gene, the nuclear large subunit (NLSU), and internal transcribed spacer regions (ITS) of the nuclear ribosomal RNA genes were sequenced in over 100 of the isolates. All soil isolates from Sri Lanka and Australia were found to be members of Clade 3. Perithecial isolates from Australia, all collected from fallen fruit, were found to represent new phylogenetic species in Clade 3. All perithecial isolates but
one from Sri Lanka were identified as members of Clade 2, previously a South American clade. All Sri Lankan isolates were collected from recently dead trees and fallen wood of recently killed trees. Isolates from perithecia and those cultured from soil were almost always members of different species lineages, even when derived from proximal locations. The results of this study demonstrate a great deal of undiscovered diversity in the FSSC from Gondwanan locations, and motivates the hypothesis that Clade 2 of the FSSC is at least pan-Gondwanan.

**Introduction**

*Fusarium solani* (Mart.) Sacc. is found ubiquitously in soil and plant debris and other plant and animal substrates and can be a very serious plant and human pathogen (Booth 1971). Snyder and Hansen considered *F. solani* to be a single species (*F. solani*) a combination of the seven species, twelve varieties and six forms described in Sections *Martiella* and *Ventricosum* by Wollenweber and Reinking (Wollenweber and Reinking 1935). The system of Nelson *et al.* (Nelson, Tousson, and Marasas 1983) followed that of Snyder and Hansen (1941), although mating studies in the interim determined that *F. solani* comprised at least seven biological species (mating populations I-VII; (Matuo and Snyder 1973)) that correlated somewhat with host ranges. The morphological concept of *F. solani* proposed by Snyder and Hansen (1941) and Nelson *et al.* (1983) comprises the production of slightly curved, usually 3-septate macroconidia with a blunt apical cell and foot-shaped apical cell from usually cream-colored but sometimes green, blue or red sporodochia, abundant production of resistant
chlamyspores reflecting its common soil habitat, production of 1-2 celled microconidia that vary in shape, and long monophialides that have a distinct collarette. While this morphological concept contains a great deal of variation, it is generally very easy to distinguish members of the morpho-species *F. solani* from other *Fusaria*.

Phylogenetic analysis based on DNA sequences showed that *Fusarium solani* is not a single species but a species complex composed of at least twenty-six phylogenetic species distributed among three major clades, Clades 1, 2 and 3 (Guadet et al. 1989; O'Donnell 1996, 2000). Clade 1 of the *F. solani* species complex (FSSC) had only two members, both known only from New Zealand. Clade 2 contained 6 phylogenetic species, including the important Soybean Sudden Death Syndrome pathogens *F. viguliforme* and *F. tucumaniae* (Aoki et al. 2003), and showed a strong biogeographic connection with South America (O'Donnell 2000). Clade 3 was the largest, most common and most diverse group, containing 18 lineages and all seven of the mating populations previously defined by Matuo and Snyder (1973). All mating populations, or biological species, were found to correspond to diagnosable phylogenetic species, giving credence to the validity of both concepts. Clade 3 showed some degree of biogeographic substructure, itself containing clades with possible connections to South America, Asia and Africa. O'Donnell (2000) postulated that the complex historical biogeography of the FSSC reflected vicariant events associated with the fragmentation of Gondwanaland, both among the three major clades and perhaps also within Clade 3.

Nested within Clade 3 are fungi from the genus *Neocosmospora*, a sexual genus that is distinctive compared to the teleomorph typically associated with *F. solani*, known usually as *Nectria haematococca* Berk. & Broome (Berkeley and Broome 1873; Berkeley
and Broome 1967; Booth 1971). Type material for *N. haematococca* was collected in Sri Lanka in 1873 (Berkeley and Broome 1873; Berkeley and Broome 1967). Because of its lack of phylogenetic connection to the type of the genus *Nectria*, the teleomorph genus associated with the FSSC has recently been redescribed as *Haematonectria* (Rossman 1999). However, since *Neocosmospora* renders *Haematonectria* paraphyletic, the validity of the new genus has been questioned (O'Donnell 2000). The FSSC contains both heterothallic and homothallic isolates, as well as isolates that have no known sexual stage. Perithecia are frequently encountered in the field in tropical locations, particularly on falled rain forest fruits, recently killed trees, and on leaf litter (Rossman 1999). When members of the FSSC are isolated from soil and other substrates, no teleomorph is usually observed, except in rare instances where homothallic isolates are encountered.

Previous molecular phylogenetic studies of the FSSC emphasized materials available in culture collections, which were somewhat biased toward agricultural sources (O'Donnell 2000). To the extent where isolates derived from undisturbed forests were included, a great deal of phylogenetic diversity was observed, but sampling from Gondwanan regions of the world were somewhat limited, with a bias toward New World sources. In this study, we expanded the Gondwanan sample of isolates to include new collections from undisturbed forests in Sri Lanka and North Queensland, Australia. We added sequences from three genes from these isolates, a ~690 bp portion of the translation elongation factor 1-alpha gene (tef), a ~580 bp portion of the nuclear large ribosomal subunit gene (NLSU), and a ~550 bp region including the ribosomal internal transcribed spacer regions and 5.8S nuclear ribosomal subunit genes (ITS). These sequences were added to pre-existing alignments of sequences from the known diversity
of the FSSC and subjected to phylogenetic analyses to identify new lineages and better understand the biogeography of this important complex.

**Materials and Methods**

**Collection of Isolates.** Groups of perithecia (Fig. 4-1) were found on fallen fruits (Australia), on standing dead trees (Sri Lanka) and on fallen wood of recently dead trees that were lying on the soil surface (Sri Lanka) in primary forests. Locations for collections are listed (Table 4-1). Collections were made during the rainy season months of November (Sri Lanka) and January (Australia). Efforts were made to collect from a diversity of forest types and climatic zones. Substrata containing perithecia were either air dried for subsequent isolation of ascospores up to two months later, or specimens with perithecia were divided into two parts. One small part was air-dried and was used for isolation of ascospores up to 2 months later; the larger part was preserved by immediately drying with heat. Materials were taken back to the laboratory, and cultures were obtained by isolating single ascospores from perithecial squashes with the aid of a micromanipulator. Ascospores typically germinated within 6 hours on cornmeal dextrose agar (Difco cornmeal agar + 2% dextrose) at ca. 20 C. Single-spore cultures that were found to produce perithecia on SNA with filter paper (Nirenberg 1976) were deemed homothallic.
Soil isolates. Soil and/or leaf litter were collected at each site, from a few inches below the soil surface, and placed in Eppendorf tubes or small plastic bags. Soils were kept at room temperature after collection and air-dried overnight. Cultures were derived from soil by sprinkling ~1g of soil onto Nash-Snyder medium (Nash and Snyder 1962), amended with 12 ml/L of a 1:100 Neomycin solution, and incubation under 12 h

Table 4-1: List of isolates collected in Australia and Sri Lanka from soil and perithecia

<table>
<thead>
<tr>
<th>Collection#</th>
<th>Location</th>
<th>Substrate</th>
<th>Reproductive mode</th>
<th>Mating type</th>
<th>Morphology on PDA</th>
<th>FRC#</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.J.S 02-89</td>
<td>Kurunegala, Arankale Sinharaja, SL</td>
<td>Recently dead tree</td>
<td>No perithecia</td>
<td></td>
<td>dark brown</td>
<td>S1831</td>
</tr>
<tr>
<td>G.J.S 02-90</td>
<td>Sl</td>
<td>Dying tree</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td>pale brown, spreading on CMD</td>
<td>S1832</td>
</tr>
<tr>
<td>G.J.S 02-94</td>
<td>Kurunegala, SL</td>
<td>Recently felled tree, garden bark</td>
<td>Homothallic</td>
<td></td>
<td>reddish brown, spreading on CMD</td>
<td>S1833</td>
</tr>
<tr>
<td>G.J.S 02-95</td>
<td>Kurunegala, Arankale Giritale, SL</td>
<td>Bark</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td>pale creamy brown, on CMD</td>
<td>S1834</td>
</tr>
<tr>
<td>G.J.S 02-102</td>
<td>SL</td>
<td>Dead branch on tree</td>
<td>One ascospore</td>
<td></td>
<td>pale brown, cottony on SNA</td>
<td>S1847</td>
</tr>
<tr>
<td>G.J.S 02-105</td>
<td>Giritale, SL</td>
<td></td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy brown, on CMD</td>
<td>S1845</td>
</tr>
<tr>
<td>G.J.S 02-109</td>
<td>Minneriya</td>
<td>Trunk of standing dead tree</td>
<td>No perithecia</td>
<td></td>
<td>brown restricted on SNA</td>
<td>S1835</td>
</tr>
<tr>
<td>G.J.S 02-113</td>
<td>Giritale, SL</td>
<td>Hanging dead branch</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy brown, spreading on SNA</td>
<td>S1836</td>
</tr>
<tr>
<td>G.J.S 02-114</td>
<td>Giritale, SL</td>
<td>Branch of dead tree</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy brown, spreading on SNA</td>
<td>S1837</td>
</tr>
<tr>
<td>G.J.S 02-121</td>
<td>Minneriya, SL</td>
<td>Branch of recently fallen tree</td>
<td>Heterothallic</td>
<td></td>
<td>slightly brown, restricted on SNA</td>
<td>S1838</td>
</tr>
<tr>
<td>Collection#</td>
<td>Location</td>
<td>Substrate</td>
<td>Reproductive mode</td>
<td>Mating type</td>
<td>Morphology on PDA</td>
<td>FRC#</td>
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<tr>
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</tr>
<tr>
<td>G.J.S 02-122</td>
<td>Minneriya, SL</td>
<td>Trunk of fallen live tree</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy, slightly brown</td>
<td>S1839</td>
</tr>
<tr>
<td>G.J.S 02-123</td>
<td>Minneriya, SL</td>
<td>Rotting wood</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy almost brown, spreading on SNA pale brown</td>
<td>S1846</td>
</tr>
<tr>
<td>G.J.S 02-124</td>
<td>Minneriya, SL</td>
<td></td>
<td>Homothallic?</td>
<td></td>
<td></td>
<td>S1840</td>
</tr>
<tr>
<td>G.J.S 02-126</td>
<td>Yala, SL</td>
<td>Twigs of recently dead tree</td>
<td>Homothallic</td>
<td>Brown</td>
<td></td>
<td>S1841</td>
</tr>
<tr>
<td>G.J.S 02-127</td>
<td>Yala, SL</td>
<td>Twigs of recently dead tree</td>
<td>Homothallic</td>
<td>MAT1-2</td>
<td>very dark brown</td>
<td>S1842</td>
</tr>
<tr>
<td>G.J.S 02-129</td>
<td>Yala, SL</td>
<td>Dead branch on tree</td>
<td>No perithecia</td>
<td>dark brown</td>
<td></td>
<td>S1843</td>
</tr>
<tr>
<td>G.J.S 03-01-5</td>
<td>DG80, Australia</td>
<td>Seed</td>
<td>Homothallic</td>
<td>Both</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.J.S 03-04-1</td>
<td>DG45, Australia</td>
<td>Seed</td>
<td>No perithecia</td>
<td>MAT1-1</td>
<td></td>
<td>S1925</td>
</tr>
<tr>
<td>G.J.S 03-05-1</td>
<td>DG32, Australia</td>
<td>Seed</td>
<td>No perithecia</td>
<td>MAT1-2</td>
<td></td>
<td>S1930</td>
</tr>
<tr>
<td>G.J.S 03-12-1</td>
<td>Schroers Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td></td>
<td>S1933</td>
</tr>
<tr>
<td>G.J.S 03-14-1</td>
<td>Schroers Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td></td>
<td>S1936</td>
</tr>
<tr>
<td>G.J.S 03-21-1</td>
<td>Schroers Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT1-2</td>
<td></td>
<td>S1940</td>
</tr>
<tr>
<td>AU1-1-1</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-1</td>
<td>pink slightly fluffy, creamy, white clear zonation</td>
<td>S1945</td>
<td></td>
</tr>
<tr>
<td>AU1-1-3</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-2</td>
<td></td>
<td>S1946</td>
<td></td>
</tr>
<tr>
<td>AU1-1-4</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-1</td>
<td>white, fluffy, dark reverse, creamy, white zonation</td>
<td>S1947</td>
<td></td>
</tr>
<tr>
<td>AU1-1-5</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-2</td>
<td></td>
<td>S1948</td>
<td></td>
</tr>
<tr>
<td>AU1-1-6</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-1</td>
<td>Had trichoderma creamy white</td>
<td>S1949</td>
<td></td>
</tr>
<tr>
<td>AU4-1-1</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-2</td>
<td></td>
<td>S1950</td>
<td></td>
</tr>
<tr>
<td>AU4-1-5</td>
<td>DC Australia</td>
<td>Soil</td>
<td>MAT1-2</td>
<td>Creamy white</td>
<td>S1951</td>
<td></td>
</tr>
<tr>
<td>Collection#</td>
<td>Location</td>
<td>Substrate</td>
<td>Reproductive mode</td>
<td>Mating type</td>
<td>Morphology on PDA</td>
<td>FRC#</td>
</tr>
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<td>------</td>
</tr>
<tr>
<td>AU4-1-6</td>
<td>DC Australia</td>
<td>Soil</td>
<td><em>i.d as F. oxysporum</em></td>
<td>MAT1-2</td>
<td>white, fluffy, pink reverse</td>
<td>S1952</td>
</tr>
<tr>
<td>G-1-2</td>
<td>Giritale, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1953</td>
</tr>
<tr>
<td>G-5-1</td>
<td>Giritale, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1956</td>
</tr>
<tr>
<td>G-13-1</td>
<td>Giritale, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1957</td>
</tr>
<tr>
<td>G-16-1</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1958</td>
</tr>
<tr>
<td>K-8-1</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1959</td>
</tr>
<tr>
<td>K-12-2</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1960</td>
</tr>
<tr>
<td>M-2-1</td>
<td>Minneriya, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1961</td>
</tr>
<tr>
<td>M-9-1</td>
<td>Minneriya, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1965</td>
</tr>
<tr>
<td>Y-1-1</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1966</td>
</tr>
<tr>
<td>Y-15-1</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1967</td>
</tr>
<tr>
<td>Y2-5-1</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1968</td>
</tr>
<tr>
<td>Y3-5-4</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1973</td>
</tr>
<tr>
<td>Y4-1-2</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1974</td>
</tr>
<tr>
<td>Y4-1-4</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1975</td>
</tr>
<tr>
<td>Y4-1-5</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1976</td>
</tr>
<tr>
<td>Y4-5-2</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1977</td>
</tr>
<tr>
<td>Y6-1-1</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1978</td>
</tr>
<tr>
<td>Y6-1-4</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1979</td>
</tr>
<tr>
<td>Y6-5-4</td>
<td>Yala, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1980</td>
</tr>
<tr>
<td>Samuels9-3-6</td>
<td>Cameroon Thampana, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1744</td>
</tr>
<tr>
<td>3-1-2</td>
<td>Thampana, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1754</td>
</tr>
<tr>
<td>12-4-1</td>
<td>Thampana, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td>S1763</td>
</tr>
<tr>
<td>Code</td>
<td>Origin</td>
<td>Isolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>7-1-2</td>
<td>Thampana, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3-2</td>
<td>Thampana, SL</td>
<td>Soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Davies Creek in Australia (DC), Sri Lanka (SL)*

Alternating white/black light and dark. Colonies emerging from soil that resembled *Fusarium* were transferred to potato dextrose agar (PDA; Difco laboratories, Detroit, Michigan) slants. Single macroconidia were transferred onto Carnation Leaf Agar (1.5% water agar amended with a few pieces of dried, radiation-sterilized carnation leaves), from isolates judged to be members of the FSSC based on colony appearance, from which they were lyophilized and stored at -40°C. All isolates were accessioned into the Fusarium Research Center (FRC) culture collection and are available upon request.

**DNA manipulation.** For DNA extraction, isolates were grown on CLA for 24 h and several 10 mm² plugs of agar containing fresh growth of mycelia were transferred to test tubes containing 10 ml of sterile potato dextrose broth (PDB; Difco laboratories, Detroit, Michigan). The test tubes were placed on a shaker at 35 rpm for ~4 d at 25°C and mycelia

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**Fig. 4-1:** *Nectria haematococca* (200x) perithecia on bark, Sri Lanka
were collected and frozen in 0.5ml eppendorf tubes. The samples were lyophilized for 24 h before DNA extractions using a modified CTAB method (O'Donnell 1996) or by using a DNeasy kit (QIAGEN Inc., Valencia, CA). A portion of the tef gene was amplified using primers ef1 and ef2 (O'Donnell, Cigelnik, and Nirenberg 1998), in all 129 isolates using PCR as previously described (Geiser et al. 2001). The ITS 1 and ITS 2 regions including the 5.8S rDNA were amplified using primers ITS 4 and ITS 5 (White et al. 1990), and the NLSU region was amplified using primers ITS5 and NL4 (White et al. 1990) in all isolates. All PCR products were confirmed by separation on 3% agarose gels, and purified using a QIAquick PCR purification kit (QIAGEN Inc, Valencia, CA) and quantified by comparing with known DNA standards.

**Direct sequencing:** PCR products were sequenced in both directions using an ABI automated DNA sequencer. ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) using the same primers as used for amplification, with the exceptions of NL1 as the forward primer for the ITS5/NL4 NLSU region (White et al. 1990). Sequences were edited using Sequencher v3.1.1. (Gene Codes Corporation, Ann Arbor, Michigan) and initially aligned using Clustal X (CLUSTAL X Multiple Sequence Alignment Program version 1.81, June 2000, Julie Thompson and Francois Jeanmougin), followed by visual adjustment. Phylogenetic Analysis Using Parsimony (PAUP4.0b10) was used for generating trees (Swofford 2001) using Neighbor Joining methods specifying the Jukes Cantor model of DNA substitution (Jukes and Cantor 1969) and other models (K2P, F81, HKY85). Maximum likelihood analysis of the combined dataset (tef, ITS, NLSU) was carried out using PAUP. Gaps in the
alignment were considered missing data. Bootstrap tests were performed with 1000 replications. Sequences from *Fusarium staphyleae* (teleomorph, *Nectria atrofusc*ca) were kept as the outgroup, based on previous studies (O'Donnell 2000).

**Mating Experiments:** Single-spore isolates found to produce perithecia on V-8 agar (Hawthorne, Rees-George, and Broadhurst 1992) or CLA were labeled as homothallic, while those that did not were deemed potentially heterothallic. Before crosses were set up between putative heterothallic isolates, PCR tests of mating type were carried out. A portion of the mating type locus, MAT1-1 or MAT1-2 was amplified using degenerate primer sets (Covert et al. 1999) and those specifically designed for *F. solani*, MAT1-S1F and MAT1-S1R for MAT1-1, MAT2-S1F and MAT2-S1R for MAT1-2 (Ning Zhang, unpublished, MAT1-S1F: ATGGCTTTCCGAGTAAGGA, MAT1-S1R: CATGATAGGGCAGCAAAGAG, MAT2-S1F: GGGATCTGAGAAAGATACGTA, MAT2-S1R: CCGTACTGTTAGTCGGGAT) wherever possible. Cycling parameters differed from those described by Covert et al. (1999) in that the annealing temperature was 57°C instead of 45°C. The mating type assay was carried out with both sets of primers (M1F-1 and M1R-2 for MAT1-1, and M2F-1 and M2R-4 for MAT1-2) in a single reaction tube as well as in separate reaction tubes if the result needed to be verified a second time. The different amplification products were distinguished by their sizes, ~350 bp for MAT1-1 and ~850 bp for MAT1-2 on a 2% agarose gel. Isolates for the mating experiments were selected based on the clades they belonged to in the combined phylogenetic tree and the mating type where known. Sexual compatibilities of some of the single ascospore cultures within clades and between clades were tested by co-
inoculating V8 agar plates with mycelial plugs from the two cultures, placed side by side. The experiment was duplicated using carrot agar as described previously (Leslie 1995). Inoculated plates were placed at room temperature under a 12 hr dark/light cycle and watched for 2-4 weeks for the appearance of perithecia/protoperithecia. Perithecia produced were opened and observed for the production of fully formed ascospores in some of the crosses.

**Results**

**Phylogenetics:** Sequences for the *tef* (Fig. 4-2), ITS (Fig. 4-3) and NLSU (Fig. 4-4) genes were generated for all Sri Lankan and Australian isolates and their phylogeny was inferred using the Neighbor-Joining method as individual and combined datasets with Jukes Cantor as the specified model. Neighbor-joining analysis specifying the Jukes Cantor model of DNA substitution (Jukes and Cantor 1969) and other models (K2P, F81, HKY85) gave trees with similar topology. To make the data set more manageable, 36 isolates from well represented lineages were removed, so that a total of 93 representative isolates were analysed: Twenty-five were from Sri Lankan soil, seven from Australian soil, 13 from Sri Lankan perithecia and six from Australian perithecia. The remaining forty-one isolates were those from the previous study of O’Donnell (2000). The Maximum likelihood tree of the combined dataset (*tef*, ITS, NLSU) was also congruent with the NJ tree of the combined data.
Fig. 4-2: Neighbor-Joining tree inferred from *tef* sequence data. Bootstrap values >50% are indicated. *Fusarium staphyleae* was the outgroup.
Fig. 4-3: The Neighbor-Joining tree inferred from ITS sequence data. The bootstrap values > 50% are indicated. *Fusarium staphylectae* was the outgroup.
With all three data sets, the isolates from soil were in clade 3: the Australian soil isolates were a distinct lineage with the single Sri Lankan soil isolate S1754 included in this group, and the same Sri Lankan soil isolates formed another clade. With all three
data sets K12-2, a soil isolate from Sri Lanka, did not share its origins with other Sri Lankan soil isolates. There was no disagreement between the data sets in the placement of Sri Lankan perithecial isolates (except for G.J.S.0295) in Clade 2, and all of the Australian perithecial isolates in Clade 3. The position of the Sri Lankan perithecial isolate G.J.S.0290 was not resolved. With all of the datasets, the Australian perithecial isolate G.J.S.03015 was not part of the Australian perithecial clade. Differences in the tree topologies did not have strong bootstrap support. Because of the agreement between the three data sets, the data were combined and analyzed using Neighbor-Joining methods with Jukes-Cantor as the evolutionary model (Fig. 4-5). As in the individual trees, the soil isolates from Australia and Sri Lanka were all in Clade 3, forming separate new lineages in the FSSC, while all but one perithecial isolate from Sri Lanka were in Clade 2. The Sri Lankan perithecial isolates formed 5 different groups in Clade 3, with G.J.S.0290 also in Clade 2, although its position in the individual datasets had been unresolved. A single Sri Lankan perithecial isolate G.J.S.0295 was in Clade 3 and part of the same lineage as two other Sri Lankan isolates obtained previously from agricultural crops. The perithecial isolates from Australia except for G.J.S.03015 formed a distinct clade in Clade 3. All known mating populations are also in Clade 3 (O'Donnell 2000). Crosses have not been performed between known mating populations and the Australian perithecial isolates. The single soil isolate from Cameroon, S1744, is closely related to the Australian perithecial isolates.
The red arrows point to isolates in red (five new clades in Clade 2) and a single isolate in Clade 3 that are from perithecia collected in Sri Lanka. The isolates in green are from perithecia collected in Australia. The soil isolates are in blue (Australia) and yellow (Sri Lanka). Isolates in black are from previously published studies (O’Donnell, 2000), and belong to previously defined clades, Clade 1, Clade 2, and Clade 3.
The maximum likelihood analysis also gave the same results as the above NJ analysis of the combined dataset except that G.J.S.02-90 appears to be placed in Clade 3 (Fig. 4-6).

Fig. 4-6: Maximum likelihood tree inferred from the combined tef, ribosomal ITS and NLSU gene sequences, rooted using sequences of *F. staphyleae*. G.J.S.O2-90, shown in red does not group with the rest of the Sri Lankan perithecial isolates.
Morphological observations:

**Sri Lankan Collections:** Sri Lankan perithecial isolates G.J.S.02-89, G.J.S.02-90, G.J.S.02-94, G.J.S. 02-102, G.J.S.02-105, G.J.S.02-109, G.J.S.02-113, G.J.S.02-114, G.J.S.02-122, G.J.S.02-123, G.J.S. 02-124, G.J.S.02-127, and G.J.S.02-129 all produced ascospores averaging about 12 x 6 μm, matching the description of *N. haematococca* (Rossman 1999). They all looked much the same in their *Fusarium* anamorphs. None of these isolates produced microconidia. They all produced 5-7 septate macroconidia from pionnotes or from erect, acrewonium-like conidiophores. There was no dimorphism of the macroconidia between those produced from pionnotes and those produced from erect conidiophores, a feature that has been noted in undescribed South American collections from the FSSC (G.J.S., pers. obs.). Isolate G.J.S. 02-102, however produced abundant microconidia in the aerial mycelium, were unbranched, monophialidic or once-branched, each branch terminating in a single phialide and predominantly 6-7 septate macroconidia, some blue in color on the pionnotes formed on filter paper. The Sri Lankan perithecial isolate G.J.S.02-95, also produced abundant microconidia, and predominantly 5-6 septate macroconidia, with a wide range of 1-6 septations. Crosses between Sri Lankan perithecial isolates G.J.S.02-113 and G.J.S.02-114, G.J.S.02-113 and G.J.S.02-123, and G.J.S.02-114 and G.J.S.02-123 resulted in the production of abundant perithecia.

**Australian Collection:** The Australian isolates, G.J.S. 03-01, G.J.S. 03-04, G.J.S. 03-14, and G.J.S. 03-21, fell in the same clade within Clade 3, and all looked very similar with masses of unicellular microconidia and predominantly 3-5-septate macroconidia, 40-50 x 5-6 μm, although macroconidia were observed with 6-9 septa. There was no evidence of
macroconidial dimorphism and, unlike the Sri Lankan collection, no production of pionnotes. The conidia were shorter and narrower than those produced by Sri Lankan collections. The crosses between these four isolates were all intercompatible, although some crosses resulted in several protoperithecia that failed to mature and produce asci or spores. Spore abortion was observed in some crosses (such as 03-21 x 03-14), mostly between isolates in different sister clades. Additionally, crosses between isolates G.J.S.03-05 and G.J.S.03-12, and G.J.S.03-05 and G.J.S.03-21 produced fertile perithecia.

**Discussion**

The phylogenetics of a total of 32 isolates from soil and 19 isolates from perithecia from Sri Lanka and Australia were determined by comparing their sequences in three gene regions with 41 other isolates representing the known diversity of the FSSC. All soil isolates fell into Clade 3 of the complex, as defined by O’Donnell (2000), with most Sri Lankan soil isolates falling into separate, strongly supported clades in the combined genealogy. Two Sri Lankan soil isolates, S1754 and K-12-2, fell elsewhere in Clade 3, with S1754 grouping with the Australian soil isolates, and K-12-2 unresolved. Most perithecial isolates fell into distinct new clades as well. All but one Sri Lankan perithecial isolate, G.J.S. 02095, which fell into Clade 3, fell into five different species clades in Clade 2, while all Australian perithecial isolates fell into Clade 3. All but one Australian perithecial isolate (G.J.S.03015) formed a strongly supported clade in Clade 3.
The discovery of five new Sri Lankan perithecial clades in Clade 2 is counter to the proposal that this clade has a biogeographic connection to South America (O’Donnell 2000), motivating the new hypothesis that Clade 2 is a species complex with pan-Gondwanan distribution. While no tropical Australian isolates were found to be members of Clade 2, they were all found on substrates quite different from the Sri Lankan perithecial isolates. In Sri Lanka, ‘Nectria haematococca’-like perithecia were found exclusively on bark from recently killed woody plants, whereas all Australian isolates were derived from perithecia found on fallen fruits, mostly from members of Lauraceae. While collecting in Australia, the authors (DG and HJS) looked carefully at fallen woody substrates, and all red or orange perithecia collected were from members of different genera, particularly *Cosmospora*. This difference in substrate may reflect different ecologies between these two groups, which in turn is reflected in their very different phylogenetic origins. While no such collections were found in this study, it will be interesting to determine whether ‘*Nectria haematococca*’ isolates from fallen fruits collected in Sri Lanka and from woody substrates in Australia would group in Clades 2 and 3, respectively, if such fungi exist.

The high level of diversity in Clades 2 and 3 leads us to propose that these are distinct species complexes. The name *F. solani* Link is based on a description of a fungus growing on potato collected in Germany (Link 1809). We have identified an isolate in the *Fusarium* Research Center culture collection from potato collected in Germany, S-679 that resembles the original description morphologically. This isolate is clearly a member of Clade 3. The original description of *Nectria haematococca*, on the other hand, is based on material collected in Sri Lanka, and all but one Sri Lankan
perithecial isolate, G.J.S.02-95, are members of Clade 2. This isolate produces microconidia, a unique characteristic compared with the other Sri Lankan isolates in Clade 2.

While a high level of morphological variation is observed in the anamorphs across Clades 2 and 3, it will take an extensive micro-morphological analysis to identify traits that correlate with the molecular phylogeny. In this study, we observed that anamorphs in Clade 2 do not produce microconidia but tend to produce pionnotal as well as erect conidiophores on SNA-filter paper cultures, features that may hold up as distinguishing morphological features with further analysis. In terms of the sexual stage, no traits have been observed that distinguish the collections, even between Clades 2 and 3, despite the high degree of phylogenetic divergence observed. Since the name *F. solani* appears to be associated with Clade 3, and Clade 2 has a wide geographic distribution, we propose that Clade 2 be recognized as the *F. phaseoli* species complex to distinguish it from *F. solani*.

G.J.S.02-90 (MAT1-1) and G.J.S.02-95 (MAT1-1) were the only two perithecial isolates from Sri Lanka for which the MAT primers worked. G.J.S.02-95 is the only Sri Lankan perithecial isolate in Clade 3, while G.J.S.02-90 falls on a very divergent branch arbitrarily assigned to Clade 2 and not part of the other Sri Lankan lineages. This likely reflects a bias toward Clade 3 in the design of the MAT PCR primers. The primers worked for all Australian perithecial isolates and both mating types were seen. One isolate G.J.S.03-01-5 had both mating types, consistent with homothallism.

A limited number of attempts to cross isolates produced mature perithecia. Inducing sexual fruiting bodies depends on many different factors involving genetics and the environment. An unsuccessful cross that fails to produce fertile fruiting bodies is not
proof of different biological species, nor is the lack of fruiting body production proof of heterothallism. Often, protoperithecial bodies may be produced that are not the result of fertilization and do not go on to produce any ascospores. Once homothallism was confirmed for those isolates that produced perithecia, the rest were assumed to be heterothallic and used for setting up crosses based on the information we had regarding their phylogenetic position. A few crosses set up between Sri Lankan isolates in different lineages within clade 2 formed perithecia, suggesting that they were not different mating populations. The Australian perithecial isolates can be considered one mating population based on the fact that they were intercompatible, which is reflected in their mostly monophyletic origins. We do not know if these Australian perithecial isolates have any connection with the known mating populations which are all different phylogenetic species in Clade 3.

The soil isolates from Sri Lanka and Australia were all in clade 3. All but one of the soil isolates from diverse locations in Sri Lanka were very closely related, but not all from one clone. All soil isolates were phylogenetically distinct from perithecial isolates. This suggests that there is likely an ecological separation between soil and perithecial isolates that is reflected in their phylogeny, although it is apparently possible to isolate members of primarily perithecial groups from soil in forests.

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Summary

Understanding the evolutionary relationships of *Fusarium* species is very important. A plant pathologist diagnoses a *Fusarium* species and expects to be able to make inferences about its biology, pathogenicity, economic importance and methods of control, based on that identification. Correct species identification has important implications for the control of diseases worldwide. Our studies demonstrate the importance of phylogenetic species concepts to practical plant pathology. Phylogenetic analysis showed that *Fusarium avenaceum* isolates were monophyletic and that any *F. avenaceum* isolate is potentially a pathogen of lisianthus. Species limits need to be considered in breeding experiments to achieve resistance against specific groups of pathogens. Phylogenetics provides a much better basis for defining meaningful species boundaries than cryptic morphology for both the pathologist and the grower, as the presence of particular *Fusarium* species may be a matter of life or death.

Although most plant pathogenic *Fusarium* species have a broad host range, many species, including *F. solani*, were thought to cause disease only on a narrow range of plant hosts. Hence, the idea of “*formae specialis*” in *F. solani*, meant to indicate the ability of causing disease only on particular hosts. Previous studies refute the hypothesis of *formae specialis* monophyly. In our study of *F. solani* isolates collected from diseased caladium tubers, there was no evidence supporting any degree of host specificity. The caladium isolates appear to be closely related to other common inhabitants of soil and
water in our environment that exist as saprophytes or opportunistic pathogens. This explains why the use of fungicide to control disease is inevitable and why the caladium growers are not be able to get caladium free tubers for propagation. Efforts to breed for resistant cultivars of caladium, need to consider the fact that at least two phylogenetic species are responsible for the disease and need to look at wild relatives of caladium for sources of resistance. Similarly, *F. avenaceum* causes disease on a wide range of hosts, and our work on *F. avenaceum* indicates a lack of host specificity as isolates from other hosts besides lisianthus were capable of causing disease on lisianthus.

We have shown that *F. avenaceum* does not correspond to a species complex, as has been observed in so many other morphological species of *Fusarium*. The emerging phylogenetic framework of the *Fusarium solani* species complex developed in the last two studies provides important taxonomic information for additional phylogenetic studies and formal descriptions of members of the species complex. There is an urgent need to understand the species limits, biogeography, ecology and behavior of these common soilborne, human, mycotoxigenic, and economically important plant pathogens, in this extremely diverse complex of *Fusarium* pathogens that cause disease globally. Our results suggest that the *F. solani* anamorph and *N. haematococca* teleomorph do not share a strong phylogenetic connection. Sampling biogeographically interesting regions that are underrepresented will likely result in the discovery of many more new phylogenetic species. A database of *F. avenaceum* and *F. solani* sequences is now available providing an important framework for additional phylogenetic studies of this species causing disease on ornamental and other plant hosts.
Appendix A

Fusarium avenaceum from lisianthus

Method used for single sporing Fusarium cultures.
10ml sterile water in tubes
solid water agar plates. About 3 ml 1.5% water agar in 100mm x15 mm petri dishes. Let
excess moisture evaporate.
Lactic acid
Carnation Leaf plates (regular)
A beaker of alcohol (approx. 10 ml)

Procedure:
Place a drop of lactic acid in each tube of water (this prevents growth of bacteria if there
are any in the original culture. This may also delay germination of fungal spore.
Touch sporodochia with sterile needle
Prepare a spore suspension by placing a small drop of sporodochium in 10 ml sterile
water blank tube. Drop the needle into the tube of water. You will see a cloud of spores
go into suspension
Give the tube a shake before emptying spore suspension into the water agar plate
Let sit for 30 seconds and then strain off the excess water into a beaker of alcohol (to kill
left over spores).
Place the plate at an angle on the bench top overnight for 16-24 hrs, then pick a small
square of agar containing a single germinated spore, using a flat edge needle. Place on
carnation leaf agar to grow the single spores.

Fungal DNA extraction
Place mycelia in 1.5 ml eppendorf, freeze dry (lyophilize mycelia overnight)
Crush mycelia in the eppendorf tube
Add 600-800 µl of CTAB extraction buffer (100mM tris-Hcl pH 8.4, 1.4 M NaCl, 25mM
EDTA, 2% CTAB), mix well
Incubate at 65 °C for 30 minutes to overnight
Add equal volume of Chloroform, vortex, spin 10 mts at 12.3 kg
Invert aqueous layer to fresh tube, add 600 Ml IPA
Invert and mix gently, Spin 5 mts 12.3 kg
Discard supernatant, wash pellet using 70% EtOH (500µl)
Dry pellet, resuspend in 100 µl TE

Primers used for PCR and Sequencing
1990).
Nuclear large subunit (28S rDNA)
NL1 -GCATATCAATAAGCGGAGGAAAAG
NL4 -GGTCCGTGTTTCAAGACGG

Internal Transcribed Spacer (ITS)
ITS1 -TCCGTAGGTGAACCTGCGG
ITS2 -GCTGCGTTCTTCATCGATGC
ITS4 -TCCTCCGCTTATTGATATGC
ITS5 -GGAAGTAAAAGTCGTAACAAGG

Translation Elongation Factor (TEF1-a)
EF1 -ATGGGTAAGGA(A/G)GACAAGAC
EF2 -GGA(G/A)GTACCAGT(G/C)ATCATGTT

BetaTubulin (benA)
T1 -AACATGCGTGAGATTGTAAGT
T22 -TCTGGATGTTGTTGGGAATCC
T2 -TAGTGACCCTTGGAATCC

Cleaning up sequence products: (For cleaning up a 8 µl sequencing reaction)
In 1.5ml Epp. tube, Add to each: 2 µl of 3M NaOAc pH 4.6, 62 µl of 75.5 % EtOH
Spin sequencing products briefly
Transfer to each eppendorf tube containing the EtOH, vortex briefly
allow to sit at room temperature for ~ 15 minutes
Place hinge side outward to locate pellet after spin
Spin 20 minutes, 13K, immediately decant using a pipette tip (fresh tip)
Add 100µl of 70% EtOH to each tube, vortex briefly
Spin 15 minutes, 13K, immediately decant using pipette tip
Leave on bench top to air dry, store at -20C

Purifying PCR Products:
Add 200µl PB to sample (sequencing reaction)
Add sample to column (mark column with tube number)
Spin 1 minute at 13K
Add 750 µls of PE, Spin 1 minute at 13 K
Discard eluate, spin again to get rid of all PE
Place column in a clean 1.5 eppendorf tube
Label top of eppendorf with tube number
Add 30 µl of EB, let stand one minute
Spin 1 minute at 10k
Do a spot test to check concentration
DNA standards (ng)
1,2,3,4,5 = 1, 4.25, 8.5, 17, 34 ngs
**Appendix B**

*Fusarium solani* from caladium

**Farms from which isolates were obtained.**
Farms located in Lake Placid, Central Florida, the largest caladium producing location: Bates, D&L, Happiness, Parker Island, Lake Huntley
Farm located in Apollo Beach, West Central Florida: Elsberry

**Cultivars of caladium:**
Candidum, Frieda Hemple, Pink Beauty, Rosebud, Red Flash and White Christmas.

**Table of all *F. solani* isolates from caladium that have been sequenced.**

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<td>S1672</td>
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<td>Mat1-2</td>
<td>B</td>
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<td>Nonpigmented</td>
<td>Mat1-2</td>
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</table>

**List of abbreviations.**

- Beta tubulin gene (*benA*)
- Biological Species Concept (BSC)
- *Fusarum solani* species complex (FSSC)
- Gerlach and Nirenberg (G & N)
- Intergenic spacer (IGS)
- Intergenic Spacer region (IGS)
- Internal Transcribed Spacer region (ITS)
- Morphological Species Concept (MSC)
- Nelson, Tousson and Marassas (N, T & M)
- Nuclear large subunit ribosomal DNA (NLSU)
- Phylogenetic Species Concept (PSC)
- Small sub unit rRNA (18S)
- Translation elongation factor gene (*tef*)
- South Asian American Women’s Association (SAAWA)

**States abbreviations:**
- PA = Pennsylvania
- MO = Missouri
- DE = Delaware
- AL = Alabama
- CT = Connecticut
- FL = Florida
- CA = California
- ND = North Dakota
A plot of Growth rate of *F. solani* isolates from caladium after 6 days at 25 C. Brown bars are for pigmented isolates and white bars for non-pigmented isolates.

A plot of Growth rate of *F. solani* isolates from caladium after 6 days at 30 C. Brown bars are for pigmented isolates and white bars for non-pigmented isolate.
Appendix C

Fusarium solani / Nectria haematococca from Sri Lanka/Australia

Technique for collection of soil samples from forest soils:

Nash medium amended with 12 ml/liter of a 1:100 Neomycin solution. Use plates after 5 days so the surface of agar is dry.
100 ml of 0.1 % water agar in flasks.
9 ml water blanks in test tubes.
Scrape away top 2 cm of soil and any vegetation lying on it
Take a teaspoon of soil per location.
Place in a paper bag for about 3 days
Take part of this soil and place in small eppendorf tubes.
Write down location:
Name of forest, elevation, closest research station or other landmark, city, etc.
Weigh out 1 g of soil
Spread on surface of agar in each plate
Place plates at room temperature under white light for 2-3 days
Begin to pick colonies as soon as they appear before the edges touch each other
Transfer small plug of agar from growing culture to PDA slants
Identify morphologically by growing culture on PDA and on CLA

Soil plated on Nash’ medium showing growth of several fungal colonies including Fusarium.
**Collection of perithecia found on dead or dying trees.**  
A. Bark of recently dead tree covered with red perithecia.  
B. Perithecia  
C. Asci within perithecium  
D. Ascospores within asci

Map of Sri Lanka showing locations sampled (in red)

The Six different locations sampled in Sri Lanka, representing the different climactic regions based on average annual rainfall.

<table>
<thead>
<tr>
<th>Locations sampled</th>
<th>Climactic zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Arankale (Kurunegala)</td>
<td>Wet Zone/Dry Zone</td>
</tr>
<tr>
<td>2. Bodinagala, Horana</td>
<td>Riverine, Wet Zone</td>
</tr>
<tr>
<td>3. Giritale and Minneriya</td>
<td>Dry zone</td>
</tr>
<tr>
<td>4. Morning Side (Sinhara)</td>
<td>Wet Zone</td>
</tr>
<tr>
<td>5. Sinharaja Rain Forest</td>
<td>Hot/Wet Tropical</td>
</tr>
<tr>
<td>6. Yala</td>
<td>Arid Zone</td>
</tr>
</tbody>
</table>
Location Descriptions:

Sri Lanka is an island in the Indian Ocean at the southern most tip of India at 5.55-9.51’ Latitude and 79.41-81.54 East Longitude. It has an area of 66,000 sq. Km. with several climactic and vegetation zones: the dry zone, wet zone, and arid zone. The average annual rainfall is 37-288 in. There are two monsoon seasons, South West monsoon (May –September), and North East Monsoon (December-February). The collecting trip was in December when the rain was at its peak. At least 5 different climactic zones were sampled with 20-40 samples taken from each location of natural, old growth, preserved forestland. We collected small quantities of soils mixed with decayed plant material from which we obtained cultures of fungi. A total of 200 soil samples were collected. In addition, we collected perithecia formed on recently dead bark. Specimens were sent to the Beltsville laboratories for culturing and identification studies, and a portion of the specimens collected were deposited in a culture collection in Sri Lanka at the University of Colombo, with collecting data and subsequent descriptions.

1. Arankale forest in the Wayamba province and 45 minutes from Kurunegala city area at an elevation of 1060m.

2. Bodinagala a Riverine zone in the Western Province a half hour North of Colombo.

3. Giritale, in the North Central province at an elevation of 398m. The forest area was situated adjacent to the Wildlife Training Center. Minneriya National Park, also in the North Central province, in the area of the Yodha Ella river was sampled at an elevation of 268m. Minneriya, home of the giant irrigation reservoir, with a history of over 2500 years was declared as a sanctuary in 1938 and is an area that has dense forest, scrubland,
and grassland with many wild elephants. Found perithecia on “yakada marang” trees on
death branches.

4. Morningside Conservation Center forested areas on the eastern end of the Sinharaja
forest reserve and at an elevation of 1030m, getting there through Kalawana and
Rakwana.

5. Sinharaja Rain Forest: The largest area sampled. Situated in South Western Sri Lanka (6°
21’N, 80° 21’ E) is Sri Lanka’s last remaining and least disturbed hill rainforest with a mixed
dipterocarp type of vegetation and of significance as a relic of Gondwanan origin (Ashton and
Gunatilleke 1987). It belongs to the lowland wet zone and receives more than 2500 mm of
rainfall annually from both monsoons. The average annual temperature is 23 C. There is no dry
spell in the year. The vegetation is mainly tropical evergreen forest with ferns and epiphytes.
Moulawella ridge at 760 m and surrounding areas were sampled. Also followed Halmandiyadola
stream behind the Forest Department research station.

309 km. south of Colombo is the largest area of protected area in the country and belongs to the
dry zone where the average annual rainfall is 1281 mm (mainly from the north east monsoon)
and the average temperatures are around 27C. The vegetation ranges from open parkland to
dense jungle. Thorn scrub is interspersed with fairly dense forest. Water holes, small lakes,
lagoons and streams provide water for the animals and birds. Block I (Gonagala animal trails,
Parana Totupola, and Akasa Chaitya areas), and Block III (06deg25’N, 81deg 28’E, elev. 0-50 m,
along a newly cut road through forest from the army post to the bungalow).
Location and numbers of soil samples from Sri Lanka

<table>
<thead>
<tr>
<th>Location</th>
<th>Soil sample numbers</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodinagala</td>
<td>B-1 to B-12</td>
<td>12/05/02</td>
</tr>
<tr>
<td>Sinharaja</td>
<td>SR1-1 to SR1-37</td>
<td>12/07-12/08/02, around Martins Lodge</td>
</tr>
<tr>
<td>Sinharaja</td>
<td>SR2-1 to SR2-11</td>
<td>12/10/02, Morning Side, higher elevation</td>
</tr>
<tr>
<td>Kurunegala</td>
<td>K-1 to K-17</td>
<td>12/12/02, above Arankale monastery</td>
</tr>
<tr>
<td>Giritale</td>
<td>G-1 to G-23</td>
<td>12/13/02</td>
</tr>
<tr>
<td>Minneriya</td>
<td>M-1 to M-9</td>
<td>12/14/02, Minneriya Park</td>
</tr>
<tr>
<td>Yala</td>
<td>Y-1 to Y-17</td>
<td>day1, 12/18/02, Gonagala wewa animal trail</td>
</tr>
<tr>
<td>Yala</td>
<td>Y2-1 to Y2-5</td>
<td>Day2, location 1, 9A Dec 19, 2002</td>
</tr>
<tr>
<td>Yala</td>
<td>Y3-1 to Y3-5</td>
<td>Day2, location 2, 9B</td>
</tr>
<tr>
<td>Yala</td>
<td>Y4-1 to Y4-5</td>
<td>Day2, location 3, 9C</td>
</tr>
<tr>
<td>Yala</td>
<td>Y5-1 to Y5-5</td>
<td>Day2, location 4, 9D</td>
</tr>
<tr>
<td>Yala</td>
<td>Y6-1 to Y6-5</td>
<td>Day2, location 5, 9E</td>
</tr>
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<td>Kurunegala*</td>
<td>S1730 to S1737</td>
<td>Thampana</td>
</tr>
<tr>
<td>Kurunegala*</td>
<td>S1740 to S1744</td>
<td>Thampana</td>
</tr>
<tr>
<td>Kurunegala*</td>
<td>S1753 to S1759</td>
<td>Thampana</td>
</tr>
<tr>
<td>Kurunegala*</td>
<td>S1760 to S1769</td>
<td>Thampana</td>
</tr>
<tr>
<td>Kurunegala*</td>
<td>S1770</td>
<td>Thampana</td>
</tr>
</tbody>
</table>

*Forest area around Thampana reservoir

**Procedure for setting up crosses in Fusarium**

Grow female cultures on carrot agar. Does not need black light.
Grow males on carnation leaf tubes. (Few carnation leaves on water agar). Place under black light.

After one week: Need 300 ml double distilled water with 2 drops of Tween.
Pipette 2 mls sterile water to the male tubes. Make a slurry.
Pipette the “male” slurry onto female carrot agar plates. Mix well and tap mycelium flat on the female plate
Place under black light for 3 weeks and observe every few days.

<table>
<thead>
<tr>
<th>Sri Lankan Isolates</th>
<th>Carrot Agar</th>
<th>V8 agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.J.S.02-113-2 X G.J.S.02-114-2</td>
<td>abundant, one side</td>
<td>few good, middle</td>
</tr>
<tr>
<td>G.J.S.02-113-2 X G.J.S.02-123-1</td>
<td>sparse, both sides</td>
<td>No perithecia</td>
</tr>
<tr>
<td>G.J.S.02-113-2 X G.J.S.02-123-2</td>
<td>No perithecia</td>
<td>No perithecia</td>
</tr>
<tr>
<td>G.J.S.02-114-2 X G.J.S.02-123-1</td>
<td>sparse, one side</td>
<td>sparse, small, evenly distributed</td>
</tr>
<tr>
<td>G.J.S.02-114-2 X G.J.S.02-123-2</td>
<td>No perithecia</td>
<td>No perithecia</td>
</tr>
<tr>
<td>G.J.S.02-123-1 X G.J.S.02-123-2</td>
<td>No perithecia</td>
<td>No perithecia</td>
</tr>
<tr>
<td>Australian isolates</td>
<td>Carrot agar</td>
<td>V8 agar</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>G.J.S.03-05-2 (Mat1-2) X G.J.S.03-12-1(Mat1-1)</td>
<td>Perithecia all over the plate but larger and better developed on one side</td>
<td>sparse, one side</td>
</tr>
<tr>
<td>G.J.S.03-05-2 X G.J.S.03-14-1</td>
<td>well developed, one side/small other side</td>
<td>good one side; small, sparse other side</td>
</tr>
<tr>
<td>G.J.S.03-05-2 X G.J.S.03-21-1</td>
<td>very small, sparse</td>
<td>sparse, one side</td>
</tr>
<tr>
<td>G.J.S.03-12-1 X G.J.S.03-21-1</td>
<td>small, sparse both sides</td>
<td>2-3 small perithecia</td>
</tr>
<tr>
<td>G.J.S.03-14-1(Mat1-1)X G.J.S.03-21-1</td>
<td>All over but abundant, on one side</td>
<td>few on one piece of inoculum</td>
</tr>
</tbody>
</table>

### All soil and perithecial isolates from Sri Lanka (SL) and Australia.

<table>
<thead>
<tr>
<th>Original#</th>
<th>Location</th>
<th>Substrate</th>
<th>Reproductive mode</th>
<th>Mating type</th>
<th>Morphology on PDA slants</th>
<th>*FRC#</th>
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</thead>
<tbody>
<tr>
<td>G.J.S.02-89</td>
<td>Kurunegala, Arankale Sinharaja, SL</td>
<td>Recently dead tree Dying tree</td>
<td>No perithecia</td>
<td></td>
<td>dark brown</td>
<td>S1831</td>
</tr>
<tr>
<td>G.J.S 02-90</td>
<td>Sinharaja, SL</td>
<td>Dying tree</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td>slightly brown, spreading on CMD</td>
<td>S1832</td>
</tr>
<tr>
<td>G.J.S 02-94</td>
<td>Kurunegala, SL</td>
<td>Recently felled tree, private garden</td>
<td>Homothallic</td>
<td></td>
<td>reddish brown, spreading on CMD</td>
<td>S1833</td>
</tr>
<tr>
<td>G.J.S 02-95</td>
<td>Kurunegala, Arankale</td>
<td>Bark</td>
<td>Heterothallic</td>
<td>MAT1-1</td>
<td>pale creamy almost brown, pigmented on CMD</td>
<td>S1834</td>
</tr>
<tr>
<td>G.J.S 02-102</td>
<td>Giritalle, SL</td>
<td>Dead branch on tree</td>
<td>Only 1 ascuspor</td>
<td>Heterothallic</td>
<td></td>
<td>S1847</td>
</tr>
<tr>
<td>G.J.S 02-105</td>
<td>Giritalle, SL</td>
<td></td>
<td></td>
<td></td>
<td>pale creamy almost brown, spreading on CMD</td>
<td>S1845</td>
</tr>
<tr>
<td>G.J.S 02-109</td>
<td>Minneriya</td>
<td>Trunk of standing dead tree</td>
<td>No perithecia</td>
<td></td>
<td>brown pig, restricted on SNA</td>
<td>S1835</td>
</tr>
<tr>
<td>G.J.S 02-113</td>
<td>Giritalle, SL</td>
<td>Hanging dead branch</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy almost brown, spreading on SNA</td>
<td>S1836</td>
</tr>
<tr>
<td>G.J.S 02-114</td>
<td>Giritalle, SL</td>
<td>Branch of dead tree</td>
<td>Heterothallic</td>
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<td>pale creamy almost brown, spreading on SNA</td>
<td>S1837</td>
</tr>
<tr>
<td>G.J.S 02-121</td>
<td>Minneriya, SL</td>
<td>Branch of recently fallen tree</td>
<td>Heterothallic</td>
<td></td>
<td>slightly brown, restricted on SNA</td>
<td>S1838</td>
</tr>
<tr>
<td>G.J.S 02-122</td>
<td>Minneriya, SL</td>
<td>Trunk of fallen live tree</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy, slightly brown</td>
<td>S1839</td>
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<tr>
<td>G.J.S 02-123</td>
<td>Minneriya, SL</td>
<td>Rotting wood</td>
<td>Heterothallic</td>
<td></td>
<td>pale creamy almost brown, spreading on</td>
<td>S1846</td>
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<tr>
<td>Site Code</td>
<td>Location</td>
<td>Sample Type</td>
<td>Details</td>
<td>MAT Type 1</td>
<td>MAT Type 2</td>
<td>Notes</td>
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<tr>
<td>G.J.S 02-124</td>
<td>Minneriya, SL</td>
<td>Twigs of recently dead tree</td>
<td>No perithecia</td>
<td>Homothallic</td>
<td>Brown pigmentation</td>
<td>SNA very slightly brown</td>
</tr>
<tr>
<td>G.J.S 02-126</td>
<td>Yala, SL</td>
<td>Twigs of recently dead tree</td>
<td>Homothallic</td>
<td>Brown pigmentation</td>
<td>S1841</td>
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<tr>
<td>G.J.S 02-127</td>
<td>Yala, SL</td>
<td>Dead branch on tree</td>
<td>Homothallic</td>
<td>MAT-2</td>
<td>Very dark brown</td>
<td>S1842</td>
</tr>
<tr>
<td>G.J.S 02-129</td>
<td>Yala, SL</td>
<td>Dead branch on tree</td>
<td>No perithecia</td>
<td>Brown pigmentation</td>
<td>S1843</td>
<td></td>
</tr>
<tr>
<td>G.J.S 03-01-5</td>
<td>DG80, Australia</td>
<td>Seed</td>
<td>Homothallic</td>
<td>Both</td>
<td></td>
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<tr>
<td>G.J.S 03-04-1</td>
<td>DG45, Australia</td>
<td>Seed</td>
<td>No perithecia</td>
<td>MAT-1</td>
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<tr>
<td>G.J.S 03-05-1</td>
<td>DG32, Australia</td>
<td>Seed</td>
<td>No perithecia</td>
<td>MAT-2</td>
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<td>S1930</td>
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<tr>
<td>G.J.S 03-12-1</td>
<td>Schroers, Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT-1</td>
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<td>S1933</td>
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<tr>
<td>G.J.S 03-14-1</td>
<td>Schroers, Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT-1</td>
<td></td>
<td>S1936</td>
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<td>G.J.S 03-21-1</td>
<td>Schroers, Australia</td>
<td>Seed</td>
<td>Heterothallic</td>
<td>MAT-2</td>
<td></td>
<td>S1940</td>
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<tr>
<td>AU1-1-1</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-1</td>
<td>Pigmented pink, slightly fluffy growth</td>
<td></td>
<td>S1945</td>
</tr>
<tr>
<td>AU1-1-3</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-2</td>
<td>Creamy, non-pig, concentric zonation</td>
<td></td>
<td>S1946</td>
</tr>
<tr>
<td>AU1-1-4</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-1</td>
<td>White, fluffy, dark in the back</td>
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<td>S1947</td>
</tr>
<tr>
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<td>Soil</td>
<td>MAT-2</td>
<td>Creamy, non-pig, concentric zonation</td>
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</tr>
<tr>
<td>AU1-1-6</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-1</td>
<td>Contaminated with trichoderma, color?</td>
<td></td>
<td>S1949</td>
</tr>
<tr>
<td>AU4-1-1</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-2</td>
<td>Creamy, non-pig, Zonation not as distinct</td>
<td></td>
<td>S1950</td>
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<tr>
<td>AU4-1-5</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-2</td>
<td>Creamy, non-pig, Zonation not as distinct</td>
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<td>S1951</td>
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<tr>
<td>AU4-1-6</td>
<td>Davies Creek, Australia</td>
<td>Soil</td>
<td>MAT-2</td>
<td>White, fluffy, pink in the back</td>
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<td>S1952</td>
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<td>G-1-2</td>
<td>Giritale, SL</td>
<td>Soil</td>
<td></td>
<td></td>
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<td>S1953</td>
</tr>
<tr>
<td>G-5-1</td>
<td>Giritale, SL</td>
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</tr>
<tr>
<td>Code</td>
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<tr>
<td>G-16-1</td>
<td>Giritale, SL</td>
<td>Soil</td>
<td>S1957</td>
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<tr>
<td>K-8-1</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
<td>MAT1-2</td>
<td>S1958</td>
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<tr>
<td>K-12-2</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
<td>Both</td>
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<td>K-12-4</td>
<td>Kurunegala, SL</td>
<td>Soil</td>
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<tr>
<td>M-2-1</td>
<td>Minneriya, SL</td>
<td>Soil</td>
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Awards
The Henry W. Popp Award from Penn State University, for outstanding accomplishments as a Doctor of Philosophy Candidate in the Department of Plant Pathology, 2003-2004.
The Henry W. Popp Award from Penn State University, for outstanding accomplishments as a Doctor of Philosophy Candidate in the Department of Plant Pathology, 2002-2003.
Graduate Student Research Prize from The Mycological Society of America for best oral presentation, First Place, June 2002.
Graduate Exhibition Award. The Graduate School, Seventeenth Annual Graduate Exhibition, Penn State University. First Place, Health and Life Sciences. 2002.
Graduate Student Presentation Award from The American Phytopathology Society-North East Division (APS-NED) for the most outstanding research and presentation, First Place, Oct. 2001.
Research Fellowship (RCTF). Department of Plant Pathology, University of Kentucky, Lexington, KY 40546. 1997-2000

Publications & Presentations