USE OF TRADITIONAL AND METAGENOMIC METHODS TO STUDY FUNGAL DIVERSITY IN DOGWOOD AND SWITCHGRASS.

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ABSTRACT OF THE DISSERTATION

USE OF TRADITIONAL AND METAGENOMIC METHODS TO STUDY FUNGAL DIVERSITY IN DOGWOOD AND SWITCHGRASS

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Fungi are the second largest kingdom of eukaryotic life, composed of diverse and ecologically important organisms with pivotal roles and functions, such as decomposers, pathogens, and mutualistic symbionts. Fungal endophyte studies have increased rapidly over the past decade, using traditional culturing or by utilizing Next Generation Sequencing (NGS) to recover fastidious or rare taxa. Despite increasing interest in fungal endophytes, there is still an enormous amount of ecological diversity that remains poorly understood. In this dissertation, I explore the fungal endophyte biodiversity associated within two plant hosts (*Cornus* L. species) and (*Panicum virgatum* L.), create a NGS pipeline, facilitating comparison between traditional culturing method and culture-independent metagenomic method.

The diversity and functions of fungal endophytes inhabiting leaves of woody plants in the temperate region are not well understood. I explored the fungal biodiversity in native *Cornus* species of North American and Japan using traditional culturing

techniques. Samples were collected from regions with similar climate and comparison of fungi was done using two years of collection data.

To evaluate the use of metagenomic analysis in assessing fungal diversity from enviromental samples, I first developed a pipeline to analyze Illumina metagenomic data for fungi. I created a mock fungal community in triplicate and ran it on an Illumina MiSeq. I also compared the results from Illumina metagenomic analysis with those from culture methods for switchgrass root samples. I found the developed pipeline yielded high reproducibility among the three mock communities and a high correlation with the traditional culture method for the environmental samples. These results suggest that the developed pipeline is suitable for fungal metagenomic analysis and can capture more diversity than the culture-based methods. However, there still are software limitations and problems in taxonomy that need further improvement.

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Chapter 1

Introduction, Literature Review, Goals, Rationale and Significance

Abstract

An introduction and overview on the current understanding of fungal endophyte biodiversity and ecology is followed by a rationale for utlizing dogwood (*Cornus*) and switchgrass (*Panicum virgatum*) systems. Special attention will be given to literature that focuses on fungal endophytes from class 3 (non-clavicipitacious, horizontally transmitted) and class 4 (Dark Septate Endophytes) of fungal endophyte systems, relative to their biodiversity and ecology. I discuss relevant literature on host specificity, community assemblage in regards to geographic distance, and the complex ecological roles of symbionts. I briefly discuss limitations throughtout, and summarize these problems in the metagenomics overview. I briefly go over the the problems of metagenomics software and analysis in fungi. Following, is the significance and rationale of the study systems *Cornus* and switchgrass. To date there has been little research on the fungal endophytes and their ecology associated with *Cornus* species in their native habitats. In parallel, little research on switchgrass, an important biofuel crop, fungal biodiversity and ecology has been done. Much of the research in switchgrass focuses on breeding to create optimal biomass production.

Introduction

Fungal Endophyte Overview

Fungi are the second largest kingdom of eukaryotic life (Schoch et al. 2012) and are hypothesized to have an estimated 1.5 (Hawksworth 1991) to 5.1 million species on Earth (Blackwell 2011). Although the estimates of the true scale of diversity are debated (Hawksworth 1991; Hawksworth and Rossman 1997; O'Brien et al. 2005), studies have shown fungi to be ubiquitous in nature, playing vital roles in the environment as mutualistic symbionts, pathogens, and decomposers with significant impacts on all ecosystems (Alexopoulos et al. 1996). Despite their pervasive nature and pivotal roles in the environment, only ca. 100,000 (10%) of fungi have been described (Hawksworth 1991; Blackwell 2011; Mora et al. 2011). So where are all the undescribed species of fungi?

The leading hypothesis is that the major reservoir of novel biodiversity in fungi can be found in association with plants (Hawksworth and Rossman 1997). Most studies on plant/fungus relationships focus either on endophytic or mycorrhizal fungi. Ancient mycorrhizae, strongly similar to modern arbuscular mycorrhizae, played a central role in facilitating plant invasion on dry, nutrient poor land during the Devonian Period, 416-360 million years ago (Simon et al. 1993; Redecker et al. 2000). Consquently, a majority of vascular plants (95%) are associated with mycorrhizae that are phylogenetically diverse, and which play a crucial role in plant colonization and distribution (Redecker et al 2000; Rinaldi et al 2008). Mycorrhizae continue to play a pivotal role in the environment today, conferring benefits on plants such as the transfer of water uptake and drought resistance (Safir and Boyer 1971; Sylvia and Williams 1992), nutrient (e.g., phosphorous and

nitrogen) uptake (Abbot and Robson 1984; George et al. 1995), protection against rhizosphere pathogens (Linderman and Hendrix 1982) and giving competitive advantage to host plants, when compared to non-mycotrophic plants (Clark and Zeto 2000; Turnau and Haselwandter 2002). At the ecosystem scale, mycorrhizae promote soil stabilization and influence the dynamics and structure of plant communities (Grime et al. 1987; Smith and Read 1997).

Alongside mycorhizzal studies, there is an increase in studies of endophytic fungi associated with plant roots (Hyde and Soytong 2008; Sanchez Marquez et al 2010). Fungal endophytes are a diverse group of fungi that live asymptomatically in various plant organs, without causing disease symptoms (Petrini 1991; Wilson 1995; Tao et al. 2008). Unlike mycorrhizal fungi, fungal endophytes occur entirely within plant tissue such as roots, stems, and/or leaves, emerging to sporulate at plant/host tissue senescence (Stone et al. 2004). Plants harbor a diverse community of fungal endophyte classes, broadly defined as Clavicipitaceous and Nonclavicipitaceous, which can be further defined by modes of transmission (i.e., vertical vs horizontal transmission), tissue colonized (roots vs shoots), and fitness benefits to the host (Rodriguez et al. 2009).

Clavicipitaceous (Class 1) fungal endophytes are a well studied class of endophytic fungi. Plants that harbor clavicipitaceous endophytes harbor one to a few fungal isolates/genotypes, and are characterized by having low overall biodiversity. The classic model system of clavicipitaceous endophyte association is between a cool season C_3 grass and its fungal symbionts *Epichloë* (Fr.) Tul. & C. Tul. and *Neotyphodium* Glenn, C.W. Bacon & Hanlin species (White 1988; Clay & Shardl 2002). These fungi are vertically transmitted, and can be found in the rhizomes and above ground tissues of the

host, enhancing host survival. Class 1 endophytes have been extensively studied, due to their mutualistic symbiosis (Stone et al. 2004; Seiber 2007; Rodriguez et al. 2009). Their impact on plant hosts can be so substantial that their presence is required for sustainable production in certain parts of the world (Evans 2006; Milne 2006). Class1 endophytes enhance survival by increasing drought and heat tolerance, and by producing an array of pest-deterring secondary metabolites such as alkaloids, but they can sometimes be toxic to livestock (Powell & Petroski 1992; Roberts et al 2005). Yet, Class 1 endophytes represent a unique and specialized category of fungal endophytes.

The majority of terrestrial plants are inhabited by Class 3 endophytes (hereafter, fungal endophytes or endophytic fungi). They are defined by having a broad host range, forming highly localized infections in the leaves, sapwood (twigs and stems), bark, flowers and fruits (Rodriguez et al. 2009), and are accumulated by horizontal transmission (Arnold and Lutzoni 2007). Endophytic fungi are restricted to one or a few cells, rather than showing system-wide infection (Arnold & Herre 2003; Stone et al 2004). In tropical leaves, endophytic fungi are reported to have a higher fungal diversity (Hawksworth and Rossman 1997; Arnold and Lutzoni 2007), with ~ one fungal endophytic isolate per 2 mm^2 of leaf tissue (Arnold et al, 2000; Gamboa et al. 2002). Each leaf therefore represents a densely packed mosaic of fungal endophytes. These densely packed endophytes emerge only to reproduce through sexual or asexual spores, or hyphal fragmentation on dead or dying host tissue (Sinclair and Cerkauskas 1996; Herre et al. 2005), and can be dispersed by environmental vectors such as wind or water (Kirk et al. 2001; Guyot et al 2005), passively through herbivores frass (Monk & Samuels 1990), or actively through direct penetration, wounds and natural openings such as

stomata, much like fungal pathogens (Siebert 2007, Slippers & Wingfield 2007). Few studies have attempted to assess efficiency of spore dispersal of endophytic species, or to determine how dispersal of spores plays a role in endophytic assemblage (see Zambell and White 2014).

Many studies suggest that endophytic fungal diversity in woody tissues or stems is less than or equal to that found in foliar tissue (Barengo et al. 2000; Arnold 2007; Verma et al. 2007) though there are exceptions where woody tissues contain more diversity than leaves (Santamaría and Diez 2005). Gazis and Chaverri (2009) discovered more endophytes in the sapwood than in the leaves of tropical rubber trees. Less is known about how fungal endophytes found in the sapwood colonize their host. Commonly found fungal endophytes such as *Fusarium, Trichoderma* and *Penicillium* are also abundant in the soil (Evans et al. 2003; Hanada et al. 2008; Mejia et al. 2008). It is hypothesized that fungi in the soil attack a plant's root system and move to their niche inside the plant. Alternatively, fungal endophytes colonize from the crown into the stem or trunk through the plant's vascular system (Bailey et al. 2008).

The vast diversity of class 3 endophytes is not limited to tropical regions. Geographically, studies on fungal endophytes cataloguing species in angiosperms and conifers (Arnold and Herre 2003; Carroll and Carroll 1978; Petrini 1991; Kriel et al. 2000; Osono 2004, 2005, 2007) in a range of ecosystems other than the tropics, such as temperate zones (Espinosa-Garcia and Langenheim 1990), alpine zones (Fisher et al. 1995) and artic zones (Arnold and Lutzoni 2007), collectively harboring a tremendous array of fungal endophyte diversity. It is clear that class 3 fungal endophytes are ubiquitous and phylogenetically hyper-diverse (Arnold and Lutzoni 2007; Hoffman and

Arnold 2008; Jumpponen and Jones 2009; Rodriguez et al. 2009). The majority of the endophytes found in class 3 fungi belong to the Dikaryomycota (Ascomycota or Basidiomycota), with a majority belonging to Ascomycota. Commonly, members within the Pezizomycotina, a subdivision of Ascomycota, are well represented from all the non-lichenized classes such as: Sordariomycetes, Dothidiomycetes, Pezizomycetes, Eurotiomycetes and Leotiomycetes, and less frequently the Saccharomycotina (Arnold et al. 2009; Rodriquez et al. 2009).

Similar to Class 3 endophytes, the Class 4 endophytes also are characterized by exhibiting a broad host range and horizontal transmission, except that they colonize plant roots and show extensive plant colonization (Rodriguez et al. 2009). Historically, the Dark Septate Endophytes have been variously classified as Pseudomycorrhizal (Melin 1923), Rhizoctonia-like (Peyronel 1924), casual mycorrhizae (Burges 1936), weakly pathogenic (Wang & Wilco 1985), and even more recently as Dark Septate Endophytes. These root associated are capable of colonizing living plant organs without apparent overly negative effects (Haselwandter and Read 1982; Hirsch and Braun 1992; Jumpponen and Trappe 1998). Hereafter all class 4 endophytes will be referred to ask Dark Septate Endophytes (DSE) (Jumpponen and Trappe 1998; Rodriguez et al. 2009). DSE contain a broad and phylogenetically diverse group of fungi that are still poorly resolved. In practice, DSE are identified by dark pigmentation from melanin and septate hyphae (Stoyke and Currah 1991; Jumpponen and Trappe 1998).The most commonly studied group of DSE is the *Phialocephala fortinii-Acephala applanata* (PAC) group, in the Helotiales of Leotiomycetes (Wang et al. 2006).

Due to the ubiquitous nature of Class 3 foliar endophytes and DSE, individual leaves and roots can harbor vast amount of fungal isolates and numerous different species, even in temperate regions. The leaves and roots produce and store many secondary metabolites involved in plant defense against herbivores, pests, and pathogens (Wink 1988; Zhou et al. 1997), and can be subjected to vast temperature fluctuations and damage from pest and pathogens. The high diversity of fungal endophytes and their potential influence on plant fitness or pathogen resistance is an exciting area of research. Yet, with fungal endophyte studies cataloguing the diversity increasing over the years, the ambiguity regarding their ecology remains considerable.

Fungal Endophyte Host Specificity and Assemblage

Despite the increasing attention devoted to fungal endophytes, many questions remain about their taxonomic composition, spatial heterogeneity, host specificity and plant-host interactions; they remain the most debated and poorly understood group of fungi (Hyde et al. 2007; Yuan et al. 2010). The high phylogenetic diversity in fungal endophytes has made host specificity difficult to assess. Traditionally, a host-specific fungus was thought to inhabit a single host or a group of closely related hosts, but to date, there has been very few of inoculation studies that empirically assess host specificity for fungal endophytes. May (1988, 1991) has hypothesized that host specificity among plant-dependent organisms is less common in the tropics, where diversity is high but density is low. Host-specificity is limited by the probability that finding an appropriate host declines rapidly, due to the non-contiguous distribution. The general pattern in endophytic fungi, ectomycorrhizal fungi (Diediou et al. 2010; Tedersoo et al. 2010), arbuscular mycorrhizal fungi (Zhao et al. 2002), as well as other organisms like insects (Basset et al. 1996), exhibit low host specificity in the tropics. However, most studies still

rely on the presence/absence and frequency of isolates to estimate host specificity. These types of cultural studies typically present a log-normal pattern in which few endophytes are present in high frequency and rare species with low frequency are prevelant. Additionally, sample sizes are generally insufficient to assess the full diversity of endophytes within a host (Petrini and Muller 1979; Halmschlager et al. 1993; Higgins et al. 2007), making any hypothesis on their host selectivity difficult to assess, so that we often equate rarity with selectivity.

Conconmitantly, the host specificity of DSE are less understood than Class 3 fungal endophytes in terms of host specificity. DSE have been reported on ca. 600 species, with little to no host specificity, over a range of climatic zones, from polar zones, to alpine and subalpine zones, to temperate and tropical zones (Jumponnen and Trappe 1998; Jumpponen 2001). Jumpponen and Trappe (1998b) mounted a molecular study on fungi associated with the roots capable of colonizing seedlings of *Pinus contorta* Douglas, finding clear evidence of multiple species, which was compatible with an earlier study using similar methods but with longer rRNA sequences (LoBuglio et al. 1996). A recent study of five DSE species that have been described (*Chloridium paucisporum*, *Leptodontidium orchidicola*, *Phialocephala dimorphosphora*, *Philocephala fortinii* and *Phialophora finlandia*) in natural populations, as well as through inoculation studies in pots, further support DSE having a large host range and a lack of host specificity (Jumpponen 2001; Rodriguez et al. 2009).

In contrast to the lack of studies on fungal endophyte host specialization, many studies have found a strong effect of geographic location on endophytic fungal community composition and diversity. Diversity is typically high in the tropics, but tends

to decrease, the farther one moves towards the poles (Arnold and Lutzoni 2007). In terms of endophytic community composition, similarity decreases with increasing geographic distance, at both large and small scales (Arnold et al. 2003; Davis and Shaw 2008). For example, Hoffman and Arnold (2008) found that endophytes from the same host (but different locations) showed different composition than endophyte communities from the same host and location. Suryanarayanan et al. (2011) found a similar trend in endophytes recovered from various trees, along a rainfall gradient, in the Western Ghats of southern India. An ordination analysis of the endophyte composition in 5 conspecific trees growing along the gradient showed little similarity in fungal endophyte communites among the hosst. By contrast, evidence in temperate zones among endophytic and endolichenic communities across a continental scale in North America shows that at higher levels of taxonomy, endophytic fungal composition is similar despite geographic distance (U'Ren et al. 2012). However, they noted that at lower taxonomic levels, differences are related to environmental factors such as annual rainfall and temperature (see also Zimmerman and Vitosek 2012).

Ecological Specificity of Fungal Endophyte Symbiosis

Notably, some fungal endophytes share the same lineages with pathogens and several aspects of their biology (i.e., horizontal transmission, high diversity within host, and morphological similarity) have much similarity (Ganley et al. 2004; Lutzoni et al. 2004; James et al. 2006). It has been hypothesized that these endophytes represent inactive pathogens that are virulent on other hosts, or latent pathogens that are activated only under permissive biotic conditions (Carroll 1999; Shulz and Boyle 2005). Botryosphaeriaceae contains many species of endophytes and latent pathogens that can

infect natural openings of healthy plants (Slippers and Wingfield 2007). A classic example of an endophyte converting to a pathogen within Botryosphaeriaceae is *Diplodia mutila* in mature plants of *Iriartea deltoidea* (Alvarez-Loayzaet al 2011). Exposure of seedlings to high light triggered the endophyte to become pathogenic, wheras seedlings grown in shady conditions favored endosymbiotic development. Similarly, the amount of light on *Agonis flexuosa* can cause an endosybiotic switch in *Neofuicoccum austral*. Many tree cankers and pathogens are favored by low light and high moisture (Dakin et al. 2011), while latent pathogens or secondary pathogens are favored by high light and low moisture, and when these conditions are present, the endophytic fungi are able to overcome the host defense, resulting in canker formation (Dakin et al. 2011). Other mechanisms besides the level of light and moisture have been proposed for the fungal endophyte to pathogen or vice versa switch. For example, some pathogens are reliant on endohyphal bacteria producing toxins to become pathogenic (Partida-Martinez and Hertweck 2005; Marquez et al. 2007), and pathogens have also been reported to lose virulence and become endophytic after a single gene mutation within the fungus (Freeman and Rodriquez 1993; Redman et al. 2001).

Furthermore, fungal endophytes have been reported to have a negative effect on plant growth (Shultz et al. 1998; Shultz et al 1999). Studies have reported that some seedlings have double minimum leaf conductance after maximal stomatal closure, when infected with endophytes during a drought (Arnold and Engelbrecht 2007; Ren and Clay 2009). Other studies have shown that endophytes can also lead to a reduction in photosynthetic capacity in banana (Costa Pinto et al. 2000). However, other studies indicate that fungal endophytes can be beneficial to the host plant, producing novel

compounds (Tejesvi et al. 2007; Huang et al. 2008). Several studies have shown that endophytic fungi can increase biomass of plant structures, both above and below ground (Tan and Zou 2001, Hamayun et al. 2010; Bailey et al. 2011). Biomass is increased for various reasons, with fungal endophytes producing phytohormones such as indole-3-acetic acid (IAA), cytokines, and other growth promoting chemicals; or they can facilitate the host's uptake of macro- and micro-nutrients such as nitrogen and phosphorus (Lue et al. 2000; Mucciarelli et al. 2003; Yuan et al. 2010).

In parallel to the taxonomic similarity of endophytes and pathogens, some endophytic fungi are closely related to saprotrophic fungi. Many studies have shown that the saprotrophic fungi commonly isolated from decomposing leaf litter are isolated from plant tissues such as leaves and sapwood (Fröhlich and Hyde 1999; Promputtha et al. 2007; Gazis and Chaverri 2011; Zambell and White 2014). Such observations have fueled the hypothesis that endophytic symbiotrophs have a transient endophytic lifestyle, becoming active as primary decomposers only after host tissues senesce (Petrini et al. 1990; Girlanda et al. 1997; Schulz and Boyle 2005). To date, there have only been a few studies demonstrating fungal endophytic importance in litter-associated fungi. Voříšková and Bladrian (2013) showed that endophytic community assemblage rapidly changes at leaf senescence. *Mycosphaerella* species, which comprise of pathogenic and saprotrophic species, were reported as OTUs in live oak leaves. At the initial stage of leaf senescence, they reported a rapid increase in abundance of OTUs at the initial time of leaf senescence, showing that fungal endophytes can be efficient early saprotrophs. *Lophodermium piceae*, a main endophyte of Norway spruce, caused needles to lose significant mass *in*

vitro and was still the predominant fungus in the leaf litter after 2 years (Korkama-Rajala et al. 2008).

DSE fungi and their plant host are often compared to mycorrhizal fungi (Lewis 1987; Treu et al. 1996; Rodriguez et al. 2009), but mycorrhizal symbiosis is often assumed to be mutualistic in nature, where both the plant and the fungus benefit. DSE, on the other hand, do not form structures similar to those of mycorrhizal fungi, nor do they always provide benefits to the host plant. Trappe made the initial distinction between mycorrhizal fungi and DSE, suggesting that DSE do not form a mutualistic relationship and are not mycorrhizal (Trappe 1996). However, this appears to be an oversimplification, as mycorrhizal fungi can also have neutral and negative effects on the host (Johnson et al 1997; Smith and Smith 1990), and some DSE are known to confer benefits on the plants.

The best studied group of DSE is the *Phialocephala fortinii- Acephala applanata* complex (PAC), an asexual group of ascomycetes in the Helotiales of Leotiomycetes (Wang et al. 2006). In particular, *Phialocephala fortinii*, has been well documented to have a wide range of effects on plants, ranging from positive, to neutral, to negative, but each study had a different experimental system, making comparisons difficult (Jumpponen et al. 2001). Some studies have shown *P. fortinii* to have a clear pathogenic association with the plant host, increasing its mortality (Wilcox and Wang 1987; Stoyke and Currah 1993). Stoyke and Currah explained that the effect of mortality in their study may be due to lack of competition from other fungi in the experiments. Another study showed that various levels of nitrogen within *Pinus contorta* could change growth response caused by *P. fortinii* to switch from neutral to positive (Jumpponen et al. 1998).

More recently, there are several reports that the PAC complex is critical in nutrient limited environments and may play a role in aiding nutrient uptake (Addy et al 2005; Mandyam and Jumpponen 2005; Grünig et al. 2008; Knapp et al. 2012; Walsh et al 2014).

Fungal Endophytes as Bio-Control Agents

The use of fungal endophytes as bio-control agents has recently gained popularity (Bailey et al. 2008; Paparu et al. 2009), and studies have shown them to protect plants against pathogens (Arnold and Herre 2003; Evans 2003; Hanada et al. 2008; Gazis and Chaverri 2010). The ubiquity and sheer diversity of fungal endophytes, and their associations with plants since land colonization, suggest that endophytes and plants share a long co-evolutionary history (Evans 2006; Seiber 2007; Krings et al 2012). Traditional bio-control aims to restore ecological balance by introducing coevolved natural enemies, from the evolutionary origin of the invasive pest/pathogen (DeBach and rosen 1991; Evans 2002; Thomas et al. 2008). These 'natural enemies' are a critical resource for successful biocontrol, and are currently being implemented into integrated pest management (IPM) strategies (Evans 2007; Gentz et al. 2010). Any species used to suppress a target plant pathogen is called an "antagonist" in biological control. The ecological principle behind this type of biological control is that many populations are limited in their native habitat by upper trophic level organisms or by competitors at the same trophic level, which is the case for many bio-controls against plant pathogens (DeBach and Rosen 1991; Polis et al. 2000; Denno et al. 2008). Antagonists may use many types of mechanisms, and the action may fit under more than one label (Pal and McSpadden 2006; Tjamos et al. 2010).

For example, several studies have shown fungal endophytes to be antagonists of plant pests and pathogens. In a study of fungal endophytes in the center of genetic diversity for *Theobroma gileri*, a relative to *Theobroma cacao*, Evans and colleagues (2003) found several fungal endophytes that were naturally parasitic on frosty pod rot of cacao. Arnold et al. (2005) infected endophyte free seedlings of *Theobroma cacao*, with naturally occurring endophytes found in culture. They reported a significant decrease in leaf necrosis and mortality when inoculated plants were subjected to *Phytopthora*, a common pathogen of *T. cacao*. Hanada et al. (2008) have also shown that *Trichoderma martiale*, a fungal endophyte found in the sapwood of *Theobroma cacao*, is a biological control agent that limits the causal agent of black pod disease, *Phytophthora palmivora*.

Many studies have reported that fungal that endophytes provide bio-control of phylogenetically related pathogens (Rubini et al. 2005; Gazis and Chaverri 2010; Zhang et al. 2014). Gazis and Chaverri (2010) identified species of *Colletotrichum* and *Pestaliotopsis* as potential biocontrols in rubber trees. Both of these genera contain well known species of pathogens that typically cause leaf spots in plant hosts. However, numerous studies have shown these two genera produce a number of bioactive substances that have antifungal growth properties (Inacio et al. 2006; Tejesvi et al. 2007; Li et al. 2008). *Fusarium oxysporum* is another fungal endophyte that has been identified as providing potential biocontrol of closely related congeners known to cause plant diseases.

Studies have also shown that endophytic fungi aid in protecting the plant host against predators (Carroll 1988, 1995; Saikkonen et al. 1999; Wagner and Lewis 2000; Newcombe et al. 2009). For example, Webber (1981) has shown that *Phomopsis oblonga* in 75% of healthy Elm trees reduced the number of galleries and larval development of

elm bark beetles, the usual vector of Dutch elm disease. Diamandis (1981) reported herbivore resistance on pine needles infected by a latent endophyte *Elydroderma torres-juanii*, believed to be an endophyte under more favorable conditions. Cubit (1974) showed that marine green algae, infected by an ascomycete endophyte, were significantly less grazed upon (0-20%) than algae that were not infected (100%). Lopez et al. (2014) showed that two fungal endophytes, *Beauveria bassiana* and *Purpureocillium lilacinum*, protected inoculated cotton plant seeds against aphids. In greenhouse studies and field trials, the populations of aphids on cotton were significantly lower than on plants that were not inoculated with the endophytes after 7 and 14 days. *Beauveria bassiana* has also been reported to protect numerous other plant species such as banana (Akello et al. 2008), tomato (Powell et al. 2009), and sorghum (Reddy et al. 2009) aganst insects.

Relevance of Metagenomics

Interest and enthusiasm for discovering and understanding endophyte diversity has vastly expanded among mycological researchers. Despite the increase in attention in fungal endophyte ecology, many questions about their host specificity, community assemblage, and symbiotic roles remain poorly understood. Challenges associated with hypothesizing ecological roles are due to the limitations of typical culture-dependent methodology and are proven to be inadequate for description and comparison of environmental communities (Arnold and Hoffman 2007; Shulz and Boyle 2007). For example, a major problem with culture-dependent methods is that the fastidious or slow growing species, often the majority of the microbial community, are typically omitted so that diversity is not adequately assessed(Guo et al. 2001; Duong et al. 2007; van Wyk et al. 2007; Seena et al. 2008; Tao et al. 2008; Zhu et al. 2008). This is evident in unsaturated accumulation

curves from these cultural studies (Petrini and Muller 1979; Halmschlager et al. 1993; Higgins et al. 2007).

Additionally, culturing studies can be costly, requiring tremendous amounts of time and commitment to acquire and describe specimen-based information. Hibbett et al. (2011) calculated that at the current discovery rate, we will need 1,170 years to describe Hawksworth's (2001) estimate of the global fungal inventory. At that same rate, O'Brien's and Blackwell's estimate of 3.5 to 5.1 fungal species would require 2840-4170 years. Numerology aside, the amount of work needed for traditional morphology-based taxonomy is sobering. The number of active taxonomists needed to accomplish either a goal would be prohibitive, and the number of active taxonomists may be decreasing (Lucking 2008). To circumvent these problems, it is necessary to augment classic culturing studies with culture-independent methods (O'Brien et al. 2005).

The concept of directly sequencing rRNA from environmental samples was first introduced by Pace et al. (1985). Schmidt et al. (1991) were the first to implement the idea, constructing a phage library from a sample of seawater, and screening it for 16S rRNA genes. With the application of PCR and the design of new primers that could be used to amplify the ITS regions, incredible amounts of diversity from environmental samples became evident (Giovannoni et al. 1990; Eden et al. 1991) and the term "metagenome" was coined (Handelsman et al 1998). Metagenomics is the study of genetic material recovered directly from environmental samples (Handelsman et al. 1998; Hyde and Soytong 2007). However, these early techniques were technically challenging, focused heavily on prokaryotes, and relied on direct sequencing of RNA or reverse transcription of DNA copies from clones (Medlin et al. 1988; White et al. 1990).

Advancements in the chemistry and technology, as seen in Next Generation Sequencing (NGS) technologies have led to an astonishing reduction of cost and an increasing number of sequences. The development of (NGS) pyrosequencing with 454 and sequence tags (Kysela et al. 2005; Neufeld et al 2008; Bartram 2011) for metagenomics sparked new interest in the diversity and composition of both bacterial and fungal communities (Sogin et al 2006; Huber et al. 2007; Andersson et al 2008; Buee et al. 2009; Jumponnen 2009, 2010). Although these studies revealed greater depth and diversity of rare microbiota, with considerable scaled-up throughput, the rarefaction curves were still not saturated (Sogin 2006; Pedros-Ali 2007; Buee et al. 2009). While pyrosequencing is a tremendous advance beyond previous methods, it is still cost-constrained (Shendure and Hamlee 2008) and prone to relatively high (per-read) error rates associated with A- and T- homopolymers (Margulies et al. 2005; Quince et al. 2009).

New technology continues to develop and continues to offer new levels of insight into biological phenomena. Illumina NGS sequencing has been used to generate data sets of over an order of magnitude larger numbers of sequences (Neufeld and Mohn 2005; Gloor et al. 2010; Caporaso et al. 2011), at a much lower cost per sequence than 454 (Shendure and Hanlee 2008). This increase provides far superior depth in sequencing, and coupled with the use of barcoding, allows for numerous samples to be analyzed in a single run, with each sample having unprecedented coverage (Caporaso et al. 2010; Batram et al 2011; Gloor et al. 2011).

Implementing Illumina into ecological studies can be a powerful tool. Recent developmentss suggest that Illumina is more suitable for answering questions on fungal

community ecology, due to the high read numbers that provide for thorough replication (Schmidt et al. 2013). However, many of the currently established pipelines developed for Illumina are tailored to analyizing data on prokaryotes. Although, some do offer limited ability to accomadate fungal data (e.g., QIIME), the fungal barcode region used in metagenomics studies contains both hypervariable regions and highly conserved regions that are phylogenetically noninformative and that can affect BLAST results of the operational taxonomic units (OTUs). Adapting existing pipelines requires understanding of the sequencing platform and considerable computer programming. For this dissertation, I have put together a set of custom scripts and available software to assess the fungal community accurately, and then have used these data on fungal endophyte communities.

Significance of Dogwood (*Cornus*)

As noted above, many plant host interactions in species ranging from tropical, to boreal ecosystems have been studied. However, there are few studies that have been conducted in *Cornus* (Osono and Mori 2004, 2005; Osono 2007). *Cornus* species (dogwoods) are widely distributed understory trees in natural forests of the northern hemisphere (Figure 1-1), providing calcium for birds and nutrient recycling through leaf litter (Lay 1961; Thomas 1969; Rossell et al. 2001). The genus consists of approximately 58 species of shrubs and small trees, distributed mostly in temperate and subtropical regions of North America and Asia, with a few taxa being found in eastern tropical Africa and one in the high mountains of South America (Wangerin 1910; Edye 1988). Though the monophyly of *Cornus* has not been debated, the taxonomic composition, ranking and relationships within the genus have been controversial for a century (Eyde 1987; Murrell

1993; Murrrell 1996). Species such as *C. florida* L.(flowering dogwood) and *C. kousa* F. Buerger ex Hance (Japanese dogwood) are valued ornamentals; these two taxa are closely related species native to North America and Eastern Asia, respectively, both members of the big bract morphological group, along with *C. nuttallii* Audubon ex Torr. & A. Gray (Xiang et al. 2006). *Cornus florida, C. kousa,* and *C. nuttalli* are small deciduous trees reaching approximately 10 m to 25 m in height. *Cornus florida* is found along the eastern coast of the United States, while *C. nuttallii* is located along the western coast of the United States (Fig. 1-1). *Cornus kousa* is a closely related species that is native Korea, China, Japan, Bhutan, and the Ryukyu Isalnds. *Cornus kousa* and *C. florida* are both popular landscape trees, accounting for $30,901,000 in total sales for 2007 in the United States (USDA 2009).

However, a disease threatens the ecological integrity of forest ecosystems and massive economic losses for the nursery industry (Anderson et al. 1990). In North America, several native *Cornus* species including *C. florida* and *C. nuttallii* have been plagued with the dogwood anthracnose fungus (*Discula destructiva* Redlin) since the 1970's (Redlin 1991), whereas *C. kousa* appears to be naturally resistant to the disease. The disease was first noted in the west coast of the United States on *C. nuttalli* in 1979 (Byther and Davidson 1979), and was soon after reported on the east coast (Pirone 1980). Redlin (1991) concluded that isolates from both the east and west coast were morphologically indistinguishable and caused by the same pathogen, *D. destructiva*, and similar studies have concluded that it is not similar to any other N. American *Discula* species (Brasier 1995; Trigiano et al. 1995; Caetano-Anolles et al. 1996, 2001). Symptoms included bract necrosis, leaf spot, leaf blight, twig dieback, and trunk canker,

which usually start to develop in the spring and early summer (Figure 1-2). A helpful characteristic in identifying *D. destructiva* is that the leaf spots have a tan center, surrounded by a purple 'halo' (Fig. 1-3). Another distinguishing feature is the acervulus in the epidermis of the leaf associate with the leaf trichrome and forces the trichomes to stand up in a "Y" shape (Fig. 1-4). Infected trees live for 1 to 3 years and exhibit symptoms of varying severity, depending on environmental conditions (Daughtrey and Hibben 1994). Since first being discovered and described, the disease has quickly spread through native North American dogwood populations. Infections from British Columbia to northern California on the west coast of North America and Vermont to Georgia and Alabama have been reported (Daughtrey et al. 1996). Within the U.S., *D. destructiva* has resulted in mortality rates as high as 89% in some forests (Schneeberger and Jackson 1989, Sherald et al. 1996). The disease had not been reported outside North America until *D. destructiva* was reported on *C. florida* in Germany, Italy and Switzerland (Stinzing and Lang 2003; Tantardini et al. 2004).

The origin of *D. destructiva* is unknown. The current hypothesis is that *D. destructiva* was an introduced pathogen. Introduction events may involve a population bottleneck, due to a few initial colonists (Nei et al. 1975). The sudden appearance of the disease near several US ports (Daughtrey and Hibben 1994), low genetic diversity within the population, and previous work on its infectivity on Japanese dogwood (*C. kousa*), which demonstrates a natural resistance to the pathogen, supports the hypothesis that *D. destructiva* was first introduced, probably from the *C. kousa* host (Daughtrey et al. 1996; Triagano et al. 1995). *Discula destructiva* is likely to be an endophyte or latent pathogen that does not typically cause anthracnose type symptoms to its host plant in its

native range. However, this conjecture has not been tested, due to a lack of fast and efficient tools, as well as a lack of research into the biodiversity of *C. kousa* in its native range, where the pathogen is hypothesized to have originated.

As noted before, *D. destructiva* identification relies on identification of culture morphology and disease symptoms (Zhang et al. 2011). The species is fastidious in culture, growing slowly and taking at least two weeks, before accurate identification is possible. Furthermore, it does not sporulate readily on the conventional media used in fungal endophyte studies, making morphological identification challenging (Daughtrey and Hibben 1994). Diseases symptoms caused by *D. destructiva* presents similar symptoms of leaf spot and twig dieback from other pathogens such as *Colletrichum acutatum*, and specialized skills are needed to accurately diagnose the fungal pathogen (Farr 1991; Redlin 1991), making it difficult to find the origin. However, a real-time PCR protocol has been developed to detect *D. destructiva*, and can be used to accurately test for the presence of the fungus bypassing the need for culturing.

Although *Cornus* species are of great ecological and economical value, little is known about their fungal endophytes, or the origin of the disease currently devastating *Cornus* in their native environment. Previously, phyllosphere fungi associated with *C. stolonifera* and *C. controversa* were catalogued based on morphological identification (Osono and Mori 2004, 2005; Osono 2007), but offer little insite into the biodiversity in other *Cornus* species, particularly those infected with *D. destructiva*. To date there has not been an attempt to search for the origin of *D. destructiva* in the native environment of *C. kousa*, nor have there been any fungal endophyte biodiversity and comparisons between *C. florida* and *C. kousa*. As noted earlier, co-evolved endophytes can confer

beneficial effects such as disease resistance. It is critical to attempt to find the origin of the pahtogen, but we also need to build a baseline study on the fungal endophyte communities in our search for bio-control agents, as pesticides are not an economically benefical means of control in wild environments.

Significance of Switchgrass

As noted above, C_3 grasses are well studied for their interactions with clavicipitaceous (Class 1) endophytes. By contrast, there have been few studies of warm season C_4 grasses and their associated endophytes, which usually contain DSE complex. Switchgrass (*Panicum virgatum* L.) is a C_4 perennial grass that is native to North America (Keshwani & Chen 2009), occurring primarily east of the Rocky Mountains, and from Mexico to southern Canada. Switchgrass has adapted to a wide range of ecosystems, including nutrient poor habitats, high drought and high heat environments enabling the plant to inhabit regions throughout North America (Casler et al. 2007), and has long been used as a forage crop. Switchgrass can reach up to 3 m tall and has rhizomatous roots that reach 3 m in depth. Switchgrass has a perennial lifecycle, reproduces via rhizome, as well as by seeds, and an established stand can last indefinitely (McLaughlin et al. 1999). It has also been used since the 1940s for conservation purposes in the Great Plains and Midwestern states, because of its adaptations to poor soil types and lengthy perennial lifestyle (Vogel 2004).

More recently, switchgrass has been identified as a potential bioenergy crop by the US Department of Energy (DOE) (McLaughlin 1993). Broad adaptability, high biomass production, perennial growth, low water requirements and the ability to grow on marginal sites have made switchgrass an excellent candidate for a biofuel source

(Lewandowski et al. 2003; Vogel et al. 2002). Among the reasons switchgrass was selected for such development by the DOE, was the expected positive environmental impact cited from the conservation reserve program (CRP). This program noted that planting switchgrass remediated the negative effects of row-crop production, significantly reduced soil erosion (Sanderson et al. 1996), and significantly reduced pesticide use (Hohenstein and Wright 1994). However, the (DOE) has issued a demanding goal of replacing 30% of petroleum based fuels with biofuels by 2030. Switchgrass responds favorably to conventional fertilizers and pesticides (Parrish and Fikke 2005), but using fossil soil amendments would reduce the net energy balance of biofuel crops, increases environmental pollution (Tilman et al. 2002) and greenhouse gas emmissions (Robertson et al. 2000; Adler et al. 2007). It is important to increase biomass sustainably, but without pesticide or fertilizer use.

One potential way of increasing biomass is with DSE, but few studies have studied the DSE associated with switchgrass and focus on nutrient rich areas in the Midwest (Ghimire et al. 2011; Kleczewski et al. 2012). No research in nutrient poor areas such as the Pine Barrens in NJ has been conducted. A cultivar of lowland switchgrass can be commonly found in open, disturbed areas of the Pine Barrens in NJ. The Pine Barrens is an ecosystem that is characterized by its dry, acidic, nutrient-poor soil and is prone to fires (Forman 1998; Tedrow 1952). Early settlers from Europe named the region "Barrens" because of the infertile soil (Forman 1998). The 57,000 km^2 Pine Barrens located in southern New Jersey is the largest and most uniform Pine Barrens in the U.S., and is similar to Pine Barrens elsewhere around the world, in terms of soil infertility (Forman 1998). In the New Jersey Pine Barrens, pines (*Pinus*) and oaks (*Quercus*) are the

dominant tree species, while understory and open areas are mainly composed of grasses (Poaceae), sedges (Cyperaceae), orchids (Orchidaceae), blueberries and other members of the Ericaceae family.

Much remains unknown regarding the fungal diversityin the pine barrens ecosystem (Forman 1998; Tuininga and Dighton 2004). Recently, Luo et al. (2014) compared the tropical rainforest in China and the New Jersey Pine Barrens. As noted above, many class 3 fungal endophyte-plant relationships show that tropical rainforests represent fungal bio-diversity hot spots. Although the tropical rainforest represented higher fungal diversity in that study, 47% of the species recovered in culture from the Pine Barrens had not been previously described. Recently, a few novel fungal lineages associated with Poaceae roots have been described from this region. Two new DSE generea, which are closely related to PAC, have been described, *Acidomelania* and *Pseudophilaphora*, containing 10 newly described species (Walsh et al. 2014). It is clear that the Pine Barrens contain many species of DSE, some of which may increase bio-mass production in switchgrass without resorting to contra-indicated fertilizers and pesticides.

By contrast with the Pine Barrens, the farms at the Rutgers University Plant Biology Research and Extension Farm at Adelphia have sandy loam soil (pH ~ 6.0) (Bonos et al. 2009). They also represent a managed field setting that is richer in macro and micro-nutrients than the Pine Barrens. Many types of switchgrass cultivars, which can be broken into lowland and highland ecotypes, are grown in the field. Lowland ecotypes are tall, thick-stemmed and adapted to high moisture, whereas upland ecotypes are shorter, thinner-stemmed and adapted to drier climates (Porter 1966; Gunter et al.

1996). Both types are grown at Adelphia, but the Pine Barrens are dominated by a lowland cultivar; low-land cultivars at Adelphia Farm provide a useful comparison.

It is clear that taxonomic identification of DSE and their plant hosts is the first step toward elucidating their diversity and ecological roles. As demonstrated above, switchgrass in the Pine Barrens is a location rich in undescribed species with little knowledge of the true biodiversity. Comparison of switchgrass endophytes in the Pine Barrens and at Adelphia provides a unique opportunity to explore the diversity and ecological roles of these mysterious DSE; it will also provide baseline for the indentification of potentially biofuel-productive fungal endophytes.

Conclusion, Goals and Importance of the Study

Fungal endophytes are a diverse group of fungi that are phylogenetically rich and occur in virtually all major lineages of plant species from a wide range of ecosystems. Due to their phylogenetically rich diversity and the number of fungi that can inhabit a plant host with a complex ecological set of interactions, uncovering their ecology is a daunting task. In order to contribute to the overall understanding of fungal endophyte biodiversity and ecology, dogwood (*Cornus*) species and switchgrass (*Panicum*) were chosen as plant systems, due to their economic and ecological values. The first objective, aimed at improving our understanding of fungal biodiversity and ecology, is to: (a) characterize and compare fungal endophyte communities of *Cornus* species in their native range of North America and Japan, and to find the origin of the *Discula destructiva* pathogen. I will use *Cornus* species from their native region will use molecular tools to search for the origin of the disease, which is believed to have been introduced from *C. kousa*, where it may be either an endophyte or latent pathogen; but I

will also catalogue fungal species of *Cornus* in two geographically separate regions that are similar in climate over two years. This study will serve as a baseline for future research into understanding the endophyte ecology and provide cultures of potential bio-controls of the disease. (b) The second objective is to create a user-friendly metagenomics pipeline standard, specifically geared towards uncovering fungal biodiversity, using Illumina NGS. At this time, the established pipelines are catered to analyzing bacteria 16S rRNA. In order to obtain useful information on fungal biodiversity and ecology, it is imperative that a pipeline be created that is specifically geared to fungi and the uniqure biology fungi possess. (c) The final objective is to gauge the diversity of DSE and their ecological roles and potential to increase biomass in a biofuel candidate, switchgrass, by utilizing metagenomics to provide a baseline on potential DSE for further research.

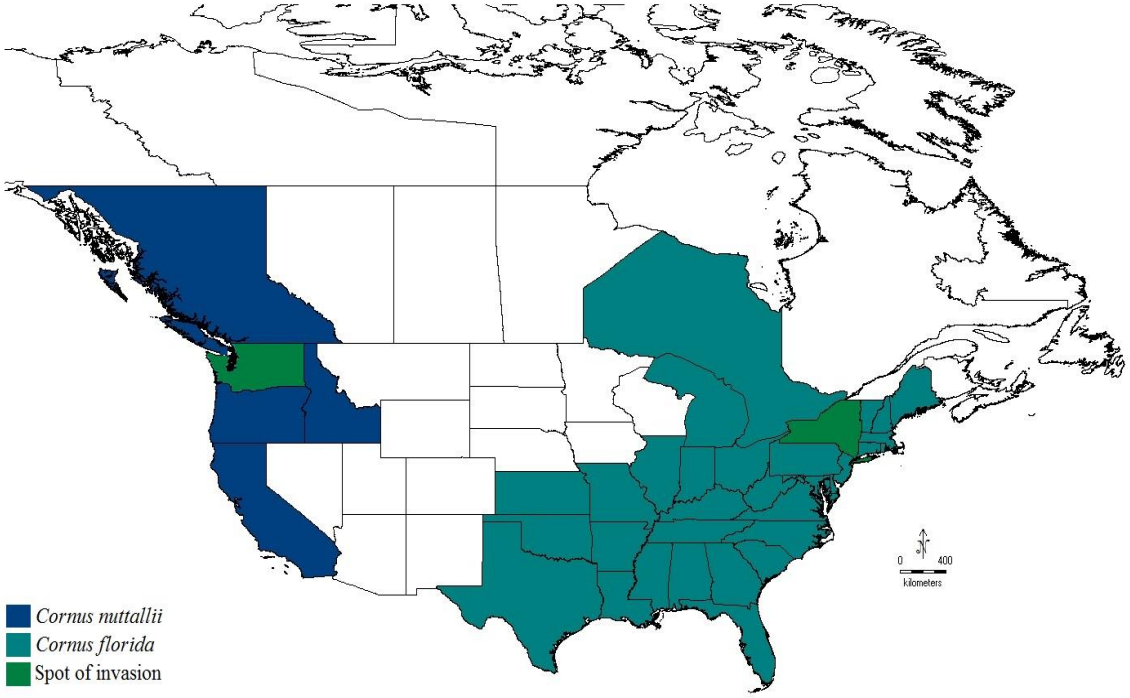

Fig. 1-1. Distribution of native North American species *Cornus nuttalli* and *Cornus florida* adapted from USDA.gov in DIVAGIS.

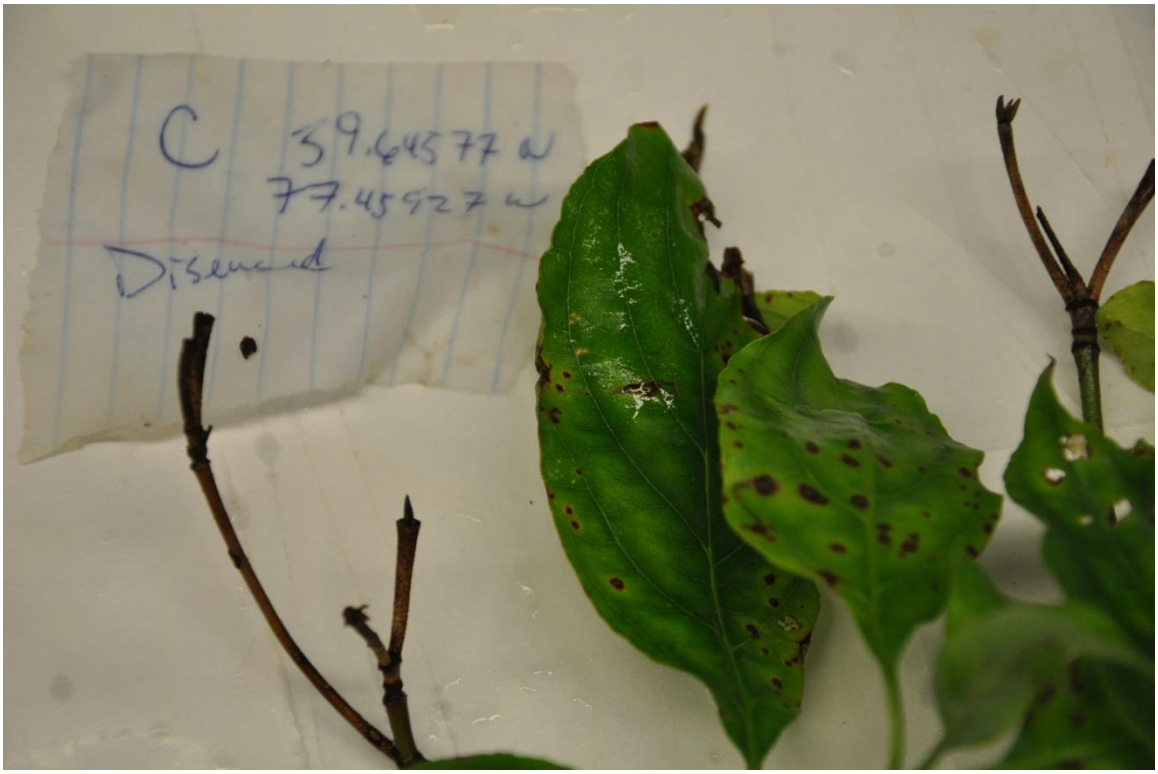

Figure 1-2. Photo showing leaf spots and twig dieback caused by *Discula destructiva*.

Figure 1-3. *Discula destructiva* causing leaf spot *Cornus*. Note the characteristic of a purple 'halo' surrounding a tan center indicative of *D. destructiva* shown by the arrow.

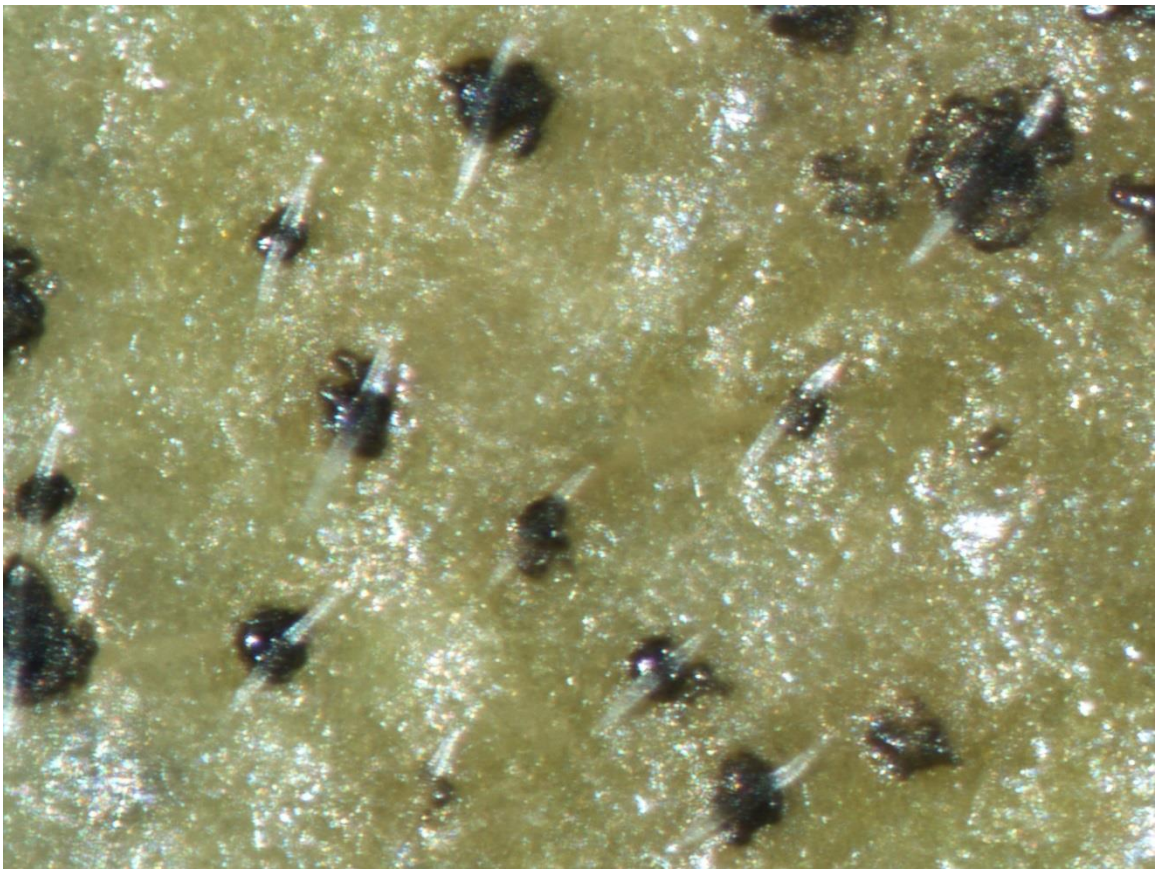

Figure 1-4. Magnification (6X) of *Discula destructiva* acervuli. Note the "Y" shape of the leaf trichomes.

Chapter 2

Comparison of fungal endophytes in *Cornus florida* and *Cornus kousa* in the United States and Japan and the origin of dogwood anthracnose.

Abstract

Cornus species (dogwoods) are popular ornamental trees and important understory plants in natural forests of northern hemisphere. One of the major diseases affecting *Cornus* is caused by the fungal pathogen *Discula destructiva*. The origin of this pathogen is not known, but it is hypthosized that it was imported with the Japanese dogwood *Cornus kousa* from Asia. In this study, I compared the fungal endophyte communities associated with leaves of the native flowering dogwood *Cornus florida* in the United States and the native Japanese dogwood *Cornus kousa* from Japan collected in 2010 and 2012. I used real-time PCR on herbarium and fresh samples, to detect *Discula destructiva*. A total of 371 fungal cultures were isolated, 99% of which belong to Ascomycota. For the Japanese samples, fungal diversity increased in 2012, while no significant change in fungal diversity was detected in the United States during the two years. *Pestalotiopsis* was the most dominant genus for the United States samples (2010 and 2012) and for the 2010 Japan samples; but it was replaced by *Colletotrichum* in the 2012 Japan samples. Real-time PCR results of both *Cornus* species sampled from the U.S. and Japan in 2010 were detected, but not from the 2012 samples. Several Japanese and Chinese herbarium samples were also positive for the pathogen, including samples that were collected before the hypothesized introduction of the pathogen to North America. Our findings further support the conclusion that *D. destructiva* is an introduced species from Asia, which shows complex interactions with its *Cornus* host species.

Introduction

Fungal endophytes colonize asymptomatically within the leaves or stems of living plants (Petrini 1991; Wilson 1995; Tao et al. 2008; Gazis and Chaverri 2010). Traditionally, fungal endophyte studies have focused primarily on the vertically transmitted clavicipitaceous fungi, recently categorized as Class 1 endophytes, associated with grasses (Rodriguez et al. 2009), yet these represent a unique group of fungal endophytes. The majority of terrestrial plants are inhabited by endophytes that accumulate by horizontal transmission (Arnold and Lutzoni 2007). These types of endophytes are both ubiquitous and phylogenetically diverse (Arnold and Lutzoni 2007; Hoffman and Arnold 2008; Rodriguez et al. 2009). Despite being associated with almost all living plant tissues examined to date (Shulz et al. 2002; Li et al. 2007; Tao et al. 2008), they remain one of the most poorly understood groups of fungi, seldom sampled and even more poorly characterized (Hyde et al. 2007). Studies on fungal endophytes have concentrated on cataloguing species in angiosperms and conifers (Carroll and Carroll 1978; Petrini 1991; Kriel et al. 2000; Arnold and Herre 2003; Osono and Mori 2004, 2005; Osono 2007) from a range of ecosystems, ranging from temperate zones (Espinosa-Garcia and Langenheim 1990), to artic (Arnold and Lutzoni 2007) and tropical zones (Suryanarayanan et al. 2002). Though still poorly understood (Arnold 2007; Saikkonen 2007), endophyte studies are beginning to move from simple cataloguing of diversity to elucidation of ecosystem roles, showing how fungal endophytes provide plants with heat tolerance (Redman et al. 2002), protection from plant pathogen attacks (Arnold et al. 2003), increasing drought and salinity tolerance (Rodriguez et al. 2004; Redman et al. 2005), providing defense against insect damage (Wagner and Lewis 2000). Studies have begun on medicial planet endophytes, their roles in the production of novel

compounds (Huang et al. 2008, Tejesvi et al. 2007), and their utility for biological control (Arnold and Herre 2003; Evans 2003; Hanada et al. 2008; Gazis and Chaverri 2010). Sampling and characterizing endophytes from new environments often leads to novel species and compounds (Wiyakrutta et al 2004; Huang et al 2009), and sheds light on their ecosystem roles, as well as improving the robustness of the phylogeny of this group (Arnold 2007).

This study focuses on cataloguing the endophytic fungal community associated with *Cornus* species (dogwoods) in their native wild environments, in particular *C. florida* L. and *C. kousa* F. Berger ex Hance, as well as searching for the origin of *D. destructiva* itself. *Cornus florida* and *C. kousa* are closely related species native to North America and Eastern Asia, respectively (Xiang et al. 2006). They are both are widely distributed understory trees in natural forests of the northern hemisphere, providing calcium for birds and nutrient recycling through leaf litter (Lay 1961, Thomas 1969, Rossell et al. 2001). Both *C. florida* (flowering dogwood) and *C. kousa* (Japanese dogwood), are also valued ornamentals. *Cornus florida* is one of the most popular landscape trees in the United States with $30,901,000 in total sales for 2007 (USDA 2009). Despite the fact that native *Cornus* species are of great ecological and economical value, little is known about their fungal endophytes. Only a few studies have cataloged the fungal endophytes of *Cornus* species (*C. controversa* Hemsl. ex Prain and *C. sericea* L.), and the fungal identifications were based on morphological features (Osono and Mori 2004, 2005; Osono 2007).

In North America, both native *Cornus* species have been plagued by the dogwood anthracnose fungus *Discula destructiva* Redlin since the 1970's (Redlin 1991). This has

resulted in mortality rates as high as 89% in some forests (Schneeberger and Jackson 1989, Sherald et al. 1996). The pathogen was dispersed to Italy and Switzerland in the 1990's but has not been reported in Asia. The origin of this disease organism is unknown, but *D. destructiva* is hypothesized to be an introduced pathogen, similar to the chestnut blight pathogen (Daughtrey et al. 1996; Zhang and Blackwell 2002). The sudden appearance of the pathogen near several US ports, its low genetic variation within the population (Zhang and Blackwell 2001), and the natural resistance of the native Asian species (*C. kousa*) suggests that *D. destructiva* was introduced, likely from *C. kousa*. Furthermore, studies show that *D. destructiva* is not similar to any other North American *Discula* species (Trigiano et al. 1995, Caetano-Anolles et al. 1996, 2001, Brasier 1995). It is likely that *D. destructiva* is an endophyte or latent pathogen and does not typically cause disease on its native host plant. However, testing for the presence of the pathogen's origin has never been performed.

The objectives of this study were: 1) to catalogue fungal endophytes associated with leaves of *C. florida* and *C. kousa*, 2) to provided a baseline of fungal endophytes for the future study on the identification of potential bio-control and 3) to find the origin of *Discula destructiva*. Culturing methods are utilized in this study in order to lay a ground work for future studies with increased sampling efforts and next generation sequencing. Molecular sequence data are used to provide further accuracy of fungal identification. Real-time PCR is used on herbarium samples as well as our environmental samples to test for the presences of *D. destructiva*. Fungal endophytes isolated in this study will also provide future resources for identification of potential biological control agents for management of dogwood anthracnose or other diseases.

Materials and Methods

Study sites and sampling strategy.

Samples were collected in late May to early June of 2010 and 2012 from temperate deciduous forests in the United States and Japan. Mature leaf samples of wild *C. florida* were collected from the same five trees (13-30 cm diam.; 5-10 m. in height) each year at Hutchinson Memorial Forest in Somerset, New Jersey, USA (40°30′01″N 74°34′02″W), within the native range of *C. florida*. The forest is a nature preserve, one of the few uncut forests in New Jersey (National Natural Landmark Summary 2004, Davis and Shaw2008). One branch each from the north, west and east-facing sides of the tree were collected from the top of the (Toofanee and Dulymamode 2002). Samples were kept below 4˚ C until fungal endophyte isolation. Three leaves from each branch were randomly selected for fungal isolation within three days of sample collection. Following the same sampling strategy, leaf samples of wild *C. kousa* (13-30 cm diam.; 5-10 m. in height) were collected from a natural forest in the Ibaraki Prefecture in Japan (36°14′60″N 140°5′20″E), with a similar climate to the United States sampling site. Five trees were sampled in each site, each year giving a total of 20 tree samples.

Fungal isolation and morphological identification.

In the laboratory, three leaves from each branch sampled from eac tree were cut along various sections of the leaf (lamina tip, lamina margin to midrib, and lamina base) into multiple 0.5 cm segments that were surface-sterilized through sequential immersion in 0.5% sodium hypochlorite, 70% (v/v) ethanol, and rinsed three times in sterilized distilled water (Arnold et al. 2001, 2003). Leaf segments were air dried and placed on Petri plates containing 2% acidified MEA (AMEA). One liter AMEA contains 20 g malt

extract (BD Biosciences, Sparks, MD), 20 g agar (BD Biosciences, Sparks, MD) and 1 ml of 85% lactic acid (Sigma-Aldrich, St. Louis, MO, U.S.A). Five leaf segments from each sample were placed on a control AMEA plate for 30 seconds and then removed (Arnold et al. 2001, 2003, Shultz et al. 1998). Control plates, with AMEA, also had leaf samples placed on them and removed, were monitored for epiphytic fungal growth. Plates were incubated for six months under room temperature (22-24˚C).

Emerging colonies were sub-cultured to obtain pure fungal isolates. Some frequently isolated cultures were identified on the original plates (based on morphology) without subculturing. After a week, subcultures growing in AMEA were grouped into morphotaxa (Arnold and Herre 2003, Guo et al. 2001, Lacap et al. 2003) based on spore morphology (if present), as well as on colony characteristics such as shape, color, texture, aerial hyphae, and margin. If more than three isolates were present in a morphotaxon, three representative isolates were selected for sequencing. If there were less than three morphotaxa present, all isolates were sequenced.

DNA isolation, PCR and Sequencing of morphospecies

DNA isolation and sequencing were conducted for representatives of each morphotaxon. Isolates were grown on AMEA at room temperature for four days to two weeks depending on growth rate. Genomic DNA was extracted from mycelium with Qiagen DNeasy Plant Mini kit (Qiagen, Germany) following the manufacturer's protocol. The internal transcribed spacer (ITS) rRNA genes were amplified with ITS1 and ITS4 primers (White et al. 1990). ITS1F was used with ITS4 if no PCR product was found with ITS1 and ITS4. PCR reaction mixture (25 µl) consisted of 5 µl of 5X GoTaq Flexi Buffer (Promega, WI, USA), 1.5 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs mix, 1 µl of

10 mM forward primer and 1 µl of 10 mM reverse primer, 0.125 µl (5U/µl) of GoTaq DNA polymerase (Promega, WI, USA), and a maximum of 25 ng/µl of genomic DNA. The PCR cycling conditions were as follows: 94˚C for 5 minutes, followed by 32 cycles of denaturation at 95˚C for 1 minute, annealing at 55˚C for 1 minute, and primer extension at 72˚C for 1.5 minutes, followed by a final extension at 72˚C for 5 minutes. PCR products were verified using gel electrophoresis and purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the manufacturer's instructions. Purified PCR products were sequenced by GeneWiz, Inc. (South Plainfield, NJ, USA) using primers ITS1, ITS4, ITS1F and LR5 (White et al. 1990).

Morphospecies analysis

Fungal isolates were identified to morphospecies, based on such characters as spore production, spore characteristics, colony diameter, colony color, aerial hyphae, emerged hyphae, and colony margins. Representatives of different morphospecies were chosen for sequencing. The ITS sequences were assigned to phylotypes by using 97% similarity using USEARCH v7.0 (Edgar, 2010). ITS sequences were aligned with ClustalX (Larkin et al. 2007) and later refined manually in BioEdit v7 (Hall 1999). The ITS regions (ca. 500 bp) were compared against a curated GenBank database using their Mega BLAST program on a local server. The top subject BLAST sequences that matched 97% similarity were considered belonging to the same operational taxonomic unit (OTU). This percentage was based on previous studies which have used 98% identity for the variation in ascomycetes (Nilsson et al. 2008) and 95% in ITS similarity to delimit species (Arnold & Lutzoni 2007; U'ren et al. 2009). The phylotype sequences were performed for three major groups (Sordariomycetes and Leotiomyctes, Dothideomycetes

and Eurotiomycetes, and Basidiomycetes). Maximum Parsimony analyses were performed using MEGA version 5 (Tamura et al. 2011) with the following settings: the number of bootstrap replications was set at 500 with the nucleotide substitution model selected, gaps/missing data treatment was set to partial deletion, with a site coverage cutoff at 95%, Tree-Bisection-Reconnection (TBR), with the number of initial trees set to 10, the search level to 1, and the max number of trees to retain at 100, with bootstrap values above 70% retained on the branch nodes. The phylotype ITS regions (ca. 500 bp) were compared against a curated GenBank database, using the Mega BLAST program on a local server. The output trees generated by MEGA5, along with numbers of fungal endophytes, are shown in Figure 2-4 and Figure 2-5. Sequences that did not exhibit 97% similarity were assigned to the closest taxonomic rank that matched 97% identity. Fungi collected in this study were persevered in the Zhang lab at Rutgers University, USA. DNA sequences obtained in this study are deposited in GenBank under accession numbers (KJ921855-KJ921969).

Endophyte diversity analyses

Species evenness and richness estimates, Shannon-Wiener, and Simpson's indices were calculated for collections of individual samples in each location and year. Shared species indices of Chao-1, Chao-2, Fisher's α, Bray-Curtis dissimilarity index, Morsitia-Horn, Sørenson and Jaccard's similarity indices were calculated across geographic locations and years (Table 2-2). However, only Bray-Curtis and Morisita-Horn analyses will be interpreted for discussion. The classical Sørenson and Jaccard's similarity indices do not perform well when samples contain many rare, i.e. singleton, species. All calculations were performed with EstimateSWin820, version 8.20 (Colwell 2005) and

verified manually. An isolate accumulation curve was also developed and graphed using the VEGAN package (Oksanen et al. 2010) for R (R Development Core Team, http://www.r-project.org/). One-way ANOVA was performed to compare the four communities, each has five samples (trees), using SPSS (IBM Corp. Inc. 2012).

Herbarium specimen preparation

Herbarium samples from a variety of *Cornus* species that were collected from the U.S., Canada, Mexico, China and Japan 4-140 years ago were obtained from the New York Botanical Garden (NYB) and the Harvard Herbarium (HH) (Table 2-4). All necessary permissions for the described plant and specimen sampling were obtained from the respective curators.

Real-time PCR DNA extraction, amplification, and sequencing

Real-time PCR detection of our fresh samples collected in 2010 and 2012 were conducted for each location and year. Leaves from one branch each from the north, west and east-facing sides of the tree were collected from the top of the canopy. Isolates from individual trees were pooled together and genomic DNA was isolated using the same protocol as listed above, for a total of 20 samples (i.e., 5 samples each from the US 2010 and 2012, and 5 samples each for Japan 2010 and 2012). DNA from the herbarium samples (Table 2-4) and from U.S. (2010 and 2012) and Japan (2010 and 2012) leaf samples, were extracted with the Qiagen DNeasy Plant Mini kit (Qiagen, Germany), following the manufacturer's protocol. All real-time PCR reactions were performed on the StepOnePlus real-time PCR system (Applied Biosystems, CA, USA) following the procedures of Zhang et al. (2011). Primers used for the detection of *D. destructiva* were DdITS_F1 and DdITS_R1, along with the probe DdITS_Probe1 (Zhang et al. 2011). The

following conditions were used to carry out the real-time PCR reaction: 3 min of 95˚C, followed by 45 cycles of 15 s at 95˚C, and 40 s at 60˚C. The amplifications specific to the *D. destructiva* binding site consisted of 10 µl iTaq Supermix with ROX (Bio-Rad, CA, USA), 250 nM probe, 500 nM of each primer, and 4 µl template DNA for a total volume of 20 µl. A standard curve was also contructed using genomic DNA from the type species of *D. destructiva* isolate MD235. A Ct value of less than 32 was counted as positive detection of *D. destructiva*. Each sample was tested in triplicate. All samples under a CT value were further subjected to purification using QIAquick PCR purification kit (Qiagen, CA), following the manufaturer's protocol. The purified amplicons were sequenced with primers DdITS_F1 and DdITS_R1 to confirm the expected amplicon length of 191 bp.

Results

Cornus endophyte diversity

A total of 371 fungal isolates were obtained from 1825 leaf segments collected from 20 *Cornus* trees, and a total of 121 representative isolates were sequenced (48 species, 26 genera, 13 orders). There was no fungal growth present on the control plates after surface sterilization of the leaf samples. Some of the leaf segments had more than one endophyte. However, the number of fungal isolates is low, due to rapidly growing endophytes over-growing onto other leaf segments. Furthermore, U'Ren et al. (2012) collected endophytic fungi from 20 phylogenetically diverse plant species, spread along a continental gradient that ranged from subtropical to temperate to subalpine eco-systesms and recovered endophytic fungi from 23% of the tissue segements. A total of 48 OTUs were identified from the collection of (121) isolates. As is typical of many culture-based

studies similar to this one, the frequency spectrum of identifiable isolates was log-normally distributed (Magurran 1988), with just a few common taxa and many rare taxa (Figure 2-1; by individual year Figure 2-6 to Figure 2-9). *Discula destructiva* was found in low abundance in the US samples in both 2010 and 2012, but not found in the Japanese samples. A list of the identified OTUs and their frequencies is provided in Table 1.

Ascomycota represented 99% of the fungal endophyte isolates in the sampled *Cornus* leaves, with Basidiomycota representing the remaining 1% of the community. Within Ascomycota, *Pestalotiopsis* was the most frequently isolated genus in the (28% of 2010, 17% of 2012) U.S. samples and (31% of 2010) Japan samples, *Colletotrichum* was the dominant genus in the (27% of 2012) Japan samples, with *Pestalotiopsis* as the second most frequently isolated genus (14% of 2012) Japan sample. Other genera in high abundance were *Elsinoë* (10% in 2010, 8% in 2012) among U.S. samples, *Pleuroceras* (9% in U.S. 2010) samples, *Trichoderma* (13% in 2010, 10% in 2012) Japan samples and (7% in 2010, 13% in 2012) U.S. samples, and *Fusarium* (12% in 2010, 8% in 2012) Japan samples. Taxa found less commonly include *Verticillium*, *Neonectria*, *Discula*, *Tubakia*, *Xylaria*, *Cryptosportiopsis, Neofabrea, Alternaria*, *Lophiostoma*, *Phoma*, *Botryshaeria*, *Cladosporium, Aureobasidium*, *Aspergillus*, *Penicillum* and Dothideomycetes sp. and Xylariales species. Comparing the four fungal endophyte communities, *Lophiostoma*, *Neofabrea*, *Pleuroceras* and *Verticillium* were only found in the 2010 U. S. community. Xylariales sp., and Dothideomycetes sp. were only found in the 2012 U. S. community. *Neonectria* was unique to the 2010 Japan community. *Meira* was a unique Basidiomycetes in the 2012 Japan community. Discula was found only in the 2010 and 2012 U.S. communities, and not in the 2010 and 2012 Japan communities.

Cornus endophyte ecology and statistics

There was no significant difference in the species evenness among the four communities, which ranged from 0.912 to 0.921 (Table 2-2). The average Shannon-Wiener diversity index (H') of the five samples in each region ranged from 1.77 to 2.29 (Table 1). The Shannon entropy is usually between 1.5 and 3.5. The average Simpson index values were (US 2010; 0.142), (Japan 2010: 0.201), (US 2012: 0.141) and (Japan 2012: 0.118), respectively. The average species evenness for the combined samples were (US 2010: 0.912), (Japan 2010: 0.921), (US 2012: 0.917) and (Japan 2012: 0.914), respectively. All the indices suggest a relatively high diversity in the fungal endophyte community, which is further supported by the accumulation curves, which did not asymptote.

Chao-1 is an estimator that calculates the total species richness based on the numbers of singletons and doubletons. Chao-2 does not rely on species abundance (singletons and doubletons), but instead uses presence and absence data. Because the abundance distribution appears to be log-normal (with few common taxa and many infrequent tax), common in endophyte studies, Chao-2 was considered to be the most informative sample coverage fraction. The Chao-1 and Chao-2 measures suggest that none of the communities were fully sampled (Table 2-2), a suggestion also supported by the rarefaction curves (Fig. 2). The Chao 1 and Chao 2 estimates suggest that the number of missing species in the samples range from 16 to 115 species.

The average Shannon entropy of five samples from each year and location sample were also calculated. The average Shannon entropy (H') for was (US 2010: 2.12), (Japan 2010: 1.77), (US 2012: 2.20) and (Japan 2012: 2.29), respectively. A one-way ANOVA

was used to test for any significant difference among the communities. The results indicate that the (Japan 2010) collection contained significantly less diversity than did the other three collections (Fig. 2-3). Other diversity metrics (Simpson, Fisher's α) also suggest the (Japan 2010) collection had the least internal diversity.

Bray-Curtis is bound between 0 and 1, where 0 is completely similar and 1 means the communities do not share any of the same species. In terms of species sharing the most similar collections were (US 2010) and (Japan 2012), with a Bray-Curtis index of 0.148 (Table 2-3). This contrast also had the lowest Morisitia-Horn index of 0.131. Morisitia-Horn is a similarity index which is bound between 0 and 1, where 0 indicates species and 1 indicates complete sharing of species). The highest dissimilarity between collections is represented with intermediate Bray-Curtis of 0.525 between 2010 (US 2010) and (Japan 2010), with (US 2012) and (Japan2010) collections with a similar Bray-Curtis index of 0.449. These results are further supported by the ANOVA analysis of Shannon Entropy, which found the (Japan 2010) collection to be the only collection with significantly different diversity.

Real-time PCR results

The CT value of 33 for the herbarium samples H6, H7, H13, H14, H16 and H48 were below the cutoff CT value. A CT valule under the cutoff value represents a positive reading of *D. destructiva*. All values were shown to occur before the negative control, which was run in a similar manner as above, but replacing the sample DNA with distilled water. Samples H6, H7, H13, H14, and H16 were from various *Cornus* species located in Japan and China. H48 was collected in the US in 2011 (Table 2-4). All samples tested were verified to have a 191 bp amplicon specific to *D. destructiva*. Samples from the

individual trees in which the endophyte biodiversity was assessed showed positive detection in the US (2010) and the Japan (2010) samples (Table 2-5). There was, however, no detection from the 2012 Japan and US samples were all inconclusive. This is likely attributed to contamination from the DNA extraction. Although, there was no culture recovered in the Japanese samples, the real time PCR found that *D. destructiva* was present in the 2010 Japanese samples.

Discussion

Diversity of Cornus *endophytes*

For the four fungal endophytic communities analyzed, none of the rarefaction curves were saturated. This indicates that the fungal endophyte diversity was not exhaustively sampled, and would require more sampling to obtain an accurate picture of the endophytic communities associated with *Cornus*. The indices of the Chao-1 and Chao-2 deduce an estimated level of species richness in the community, based on the concept that rare species, i.e. singletons, carry the most information about the number of missing diversity (Colwell and Coddington 1994). Chao-1 and Chao-2 estimated that 16 to 76 species were missing from the US (2010) endophytic community, 11 to 104 species in the US (2012) community, 21 to 52 species from the Japan (2010) community and 44 to 115 in the Japan (2012) community. As a rough approximation, Chao-1 and Chao 2 suggest that the communities of potential species may be double what we has already detected from culture-based methods of detection.

Under-sampling is typical in culture-dependent studies (Arnold et al. 2001, Arnold and Herre 2003, Arnold and Lutzoni 2007, Gazis and Chaverri 2010). Recent discoveries of vast fungal biodiversity within the plant phyllosphere, conducted via

metagenomic pyrosequencing, reflect our lingering ignorance of microbial communities (Shulz and Boyle 2007; Jumpponen and Jones 2009; Amend et al. 2010, Caporaso et al. 2010). A major problem with culture-dependent methods is the fastidious or slow growing species, which often constitute the majority of the fungal community, will not be detected (Guo et al. 2001; Duong et al. 2006; van Wyk et al. 2007; Seena et al. 2008; Tao et al. 2008; Zhu et al. 2008). To compound the difficulties, the instability and/or overlap of phenotypic traits among different taxa render identification unreliable at lower taxonomic levels (Photita et al. 2005). Further indication that we still have not collected the full arrary of diversity in the samples is the persistant failure of all diversity indices to reach a plateau. It is thus necessary to augment with culture-independent methods in order to assess the true biodiversity of fungi in nature (O'Brien et al. 2005).

It should also be noted that the ITS region exhibits a high level of interspecific similarity in many groups of fungi (e.g., *Colletotrichum*, Hypocreales, Botryosphaeriales) so that we cannot identify cryptic species without the use of multiple genes (Chaverri and Samuels 2003, Saldanha et al. 2007, Crouch et al 2009). The addition of genes with faster rates of evolution to our assay battery can only aid our efforts to detect more species within phylogenetically cryptic genera.

Community composition and assemblage

Fungal endophyte spatial heterogeneity is still in debate, even in large scale studies, often due to the large fraction of singleton species (Arnold et al. 2001; Gilbert et al. 2002; Arnold and Lutzoni 2007). In this study, we tested the community assemblage in *Cornus*-associated endophytic communities in two separate years and two widely geographically separated localities. Our study shows typical log-normal patterns (Figure

1-1), where few a few species dominant and many species are rare (only isolated once or twice) as is common for these types of studies. Ascomycota was the predominant phylum in the *Cornus*-associated fungal endophytic communities sampled here, with a modest fraction of Basidiomycota, but a total of 48 species and 26 genera in 13 different orders were identified. Such composition and diversity are typical of similar culture-based fungal endophyte studies (Stone et al. 2004; Rubini et al. 2005; Crozier et al. 2006; Neubert et al. 2006; Stone et al. 2004; Gazis and Chaverri 2010).

For the U.S. site, there was no significant difference in species abundance and diversity between the 2010 and 2012 samples. Previous studies have found a strong effect on geographic location on fungal endophytic communities (Hoffman and Arnold 2008; Suryanarayanan et al. 2011). Yet other studies show that fungal endophyte assembly reflects climate changes more strongly than geographic distance alone (U'ren et al. 2012). Here we show that the endophyte community of Japan significantly increased in both abundance and diversity between 2010 and 2012. This is further supported in the increase in the estimated number of species missing, estimated by Chao-1 and Chao-2 methods. Furthermore, endophyte frequencies of specific OTUs also changed more dramatically in Japan. For example, *Pestalotiopsis* was the most frequently isolated genus in the U. S. (2010 and 2012) collections, and in the Japan (2010) community, but not in the Japan (2012) collections. The similar compositions of both US samples and (Japan 2012), however, the change in Japan's endophyte community between 2010 and 2012, is probably not attributable to sampling error or a seasonal shift. Other factors that could have altered the endophytic community of Japanese dogwood species are micro-climatic patterns, inter-annual variation, or radionuclide contamination (discussed below), many

of which has been demonstrated to all have influence in fungal endophytic community assembly (Davis and Shaw 2008, U'Ren et al. 2012, Ruiz and Arnold 2013). It is unlikely that micro-climatic patterns or inter-annual variation is the cause of the differences between the U.S. and Japan, due to the similarity between years in the U.S. (2010 and 2012).

Many of the genera found in this study (e.g. *Alternaria, Colletotrichum, Fusarium,* and *Pestalotiopsis*) coincide with the genera identified in other studies (Cannon et al. 2002; Osono and Mori 2004, 2007; Santamaria and Bayman 2005) Both *Pestalotiopsis* and *Colletotrichum* have been found associated with other *Cornus* species, sometimes as the dominant genera (Osono and Mori 2004, 2007). Several undescribed species were also found here that are as phylogenetically diverse such as Doithedeomycetes and a Xylariales. Several other genera of fungi, not commonly reported, include species from the genera: *Lophiostoma, Trichoderma,* and *Tubakia*. Interestingly, *Trichoderma* species have been reported in plant genera such as *Theobroma cacao* L. and *Theobroma gileri* Cuatrec., which were reported as sapwood isolates, rather then from the phyllosphere of the leaves. Further research and larger sample sizes are needed to elucidate year-to-year dynamics of the endophytic community, as well as the functional roles of each endophytic fungal species.

Radionuclide contamination and fungi

The accident at Fukushima Daiichi nuclear power plant in 2011 has released large amounts of radionuclide pollution into the environment (Morino et al. 2011), potentially now playing a role in causing changes in the endophyte community of *Cornus* species in Japan. There has been no fungal endophyte study that demonstrates an effect on diversity

and composition from radionuclide contamination, but fungi play a significant role in radionuclide transport and fate in terrestrial ecosystems, by virture of accumulating radionuclides such as ^{137}Cs in mycelia (Grueter 1964, 1967, Steinera et al. 2002). Fungal community composition has also been shown to be affected by long-term exposure to ionizing radiation in soil samples (Dighton et al. 2008). According to the deposition maps (Yasunari et al. 2011), our sampling site in Japan had a daily deposition of ^{137}Cs of 2500 MBq/km^2 on March 21, 2011. The half-life of ^{137}Cs is 30.1 years and it may influence the environment for decades. Due to limited sampling and the complexity of the natural environment, we cannot make a direct connection between the observed change in the fungal endophytic community and the Fukushima accident, but the data provide baseline information for future investigation on whether radionuclide pollution may or may not have an impact on fungal endophyte diversity in Japanese *Cornus*.

Resources for biocontrol agents against dogwood anthracnose and other Cornus diseases

Co-evolved fungal endophytes may provide beneficial effects such as pathogen resistance on their plant hosts (Evans 2002). Interest in using fungal endophytes as bio-controls is increasing (Bailey et al. 2008, Hanada et al. 2008). Results from this study indicate that *Cornus* species harbor a phylogenetically diverse array of endophytes, and many species in this study are similar to phylogenetically related species known to be plant pathogens, saprobes, and potential mutualists. Despite the presence of a phylogenetically rich assembly, further sampling is needed to fully understand the community assemblage.

A few of the endophytes identified in the community may be tested for bio-controls for pathogens. For example, *Trichoderma*, *Pestalotiopsis* and *Colletotrichum* are among the most abundant fungi discovered from the *Cornus* samples in this study. *Trichoderma* species have long been used in fighting various plant diseases (Elad et al. 1980, McLean et al. 2012, El-Hassan et al. 2013). More recently, *Pestalotiopsis* and *Colletotrichum* species have also been reported to have antagonistic effects on pathogenic fungi (Rubini et al. 2005; Boyette et al. 2007, 2012; Ding et al. 2008). At least four *Penecillium* species have been isolated as well, and are known to produce a wide range of chemicals and have antibiotic activity. Endophytic fungal cultures produced from this study provide biological resources for future studies on identifying potential biocontrol agents against dogwood anthracnose.

Real-time PCR detection of D. destructiva

One of the major challenges in searching for *D. destructiva* is identification of the disease. *D. destructiva* is a slow growing culture and can take up to two weeks to identify, requiring specialized media to sporulate. When assessing the fungal endophyte community, many of the fast growing cultures will out-compete *D. destructiva*. In the culture analysis, we were only able to detect *D. destructiva* from samples in the US, and only at low frequency. Development of real-time PCR previously by Zhang et al. (2011), allowed for rapid testing both fresh environmental and herbarium samples. The results here present an early attempt to utilize the new assay method on herbarium samples, as well as and from extracted DNA from fresh leaves of *Cornus* in their native habitats. The results from the herbarium samples collected in Japan confirmed the presence of *D. destructiva* in *Cornus* species, prior to the disease being introduced to the United States.

However, none of the *C. kousa* herbarium samples were confirmed for the pathogen. The results also show that at least one of our field samples from Japan (2010) was positive for *D. destructiva*, and we confirmed the presence of *D. destructiva* in our U.S. (2010) samples.

The results from the culture study and the real-time PCR further support the hypothesis of *D. destructiva* being an introduced pathogen from Asia. However, positive DNA assays from Chinese herbarium samples also showed the presence of *D. destructiva*. This study nevertheless provides a baseline for further investigation of both the endophyte community and the origin of *D. destructiva*. Larger sampling efforts are needed to identify the pathogen reliably and to be able to culture it.

Perspectives

Most of our sampling has been based solely on cultured isolates, so it is probable that we are still under-estimating the diversity, species richness, and geographic assemblage within environmental communities. Having acknowledged that, culture-independent methods, such as metagenomics with 454 pyrosequencing can recover far greater fungal diversity by including fastidious fungi that require specialized media for growth, not typically used in culturing studies (Guo et al. 2001; Duong et al. 2007; van Wyk et al. 2007; Seena et al. 2008; Tao et al. 2008; Zhu et al. 2008; Jumpponen and Jones 2009).

However, culture-independent studies are not free from sampling errors. Higgins et al. (2011) found that not all endophytic clades reported from culturing were also captured by culture-independent methods. Despite the limitations of culture-independent methods, the addition of the results should enhance our pursuit of fungal endophyte

biodiversity and their ecology as well. The real-time PCR results further support (but do not prove) the hypothesis of *D. destructiva* origin being located in Japan. Culture-independent methods would allow for a larger sampling effort on an efficient time scale. In turn, the culture study presented here represents a baseline to enhance furture studies that will shed light on fungal community associated with geography, climate and bio-controls.

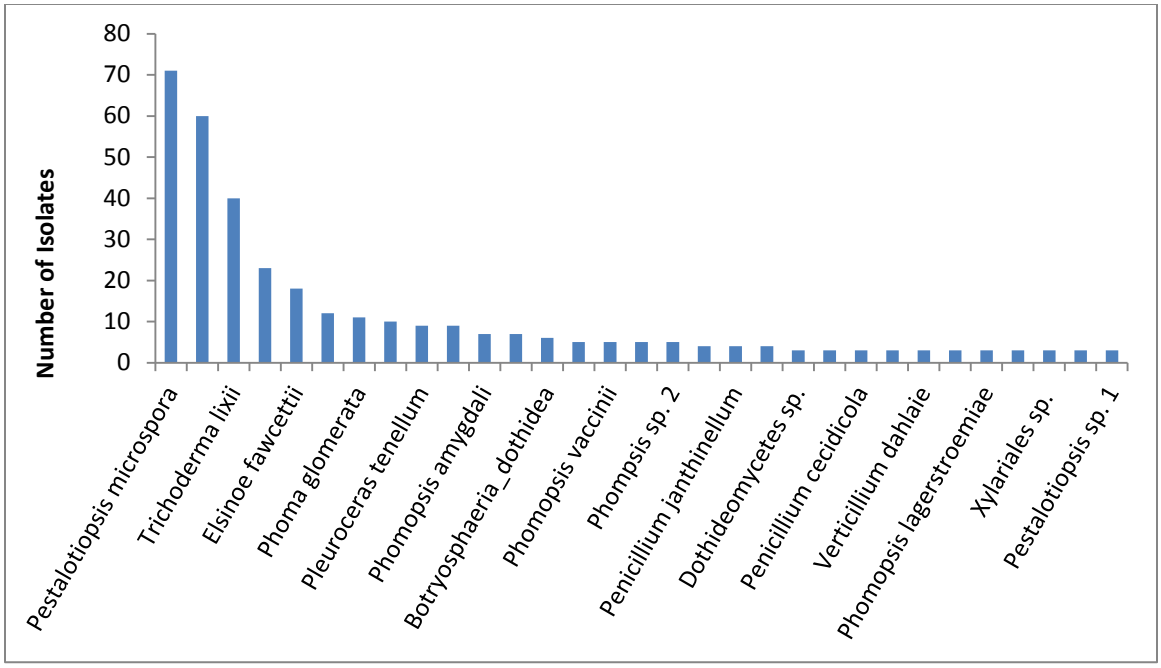

Fig. 2-1. Frequency of OTUs in relation to abundance showing a log-normal pattern.

Only OTUs with 3 or more species have been included.

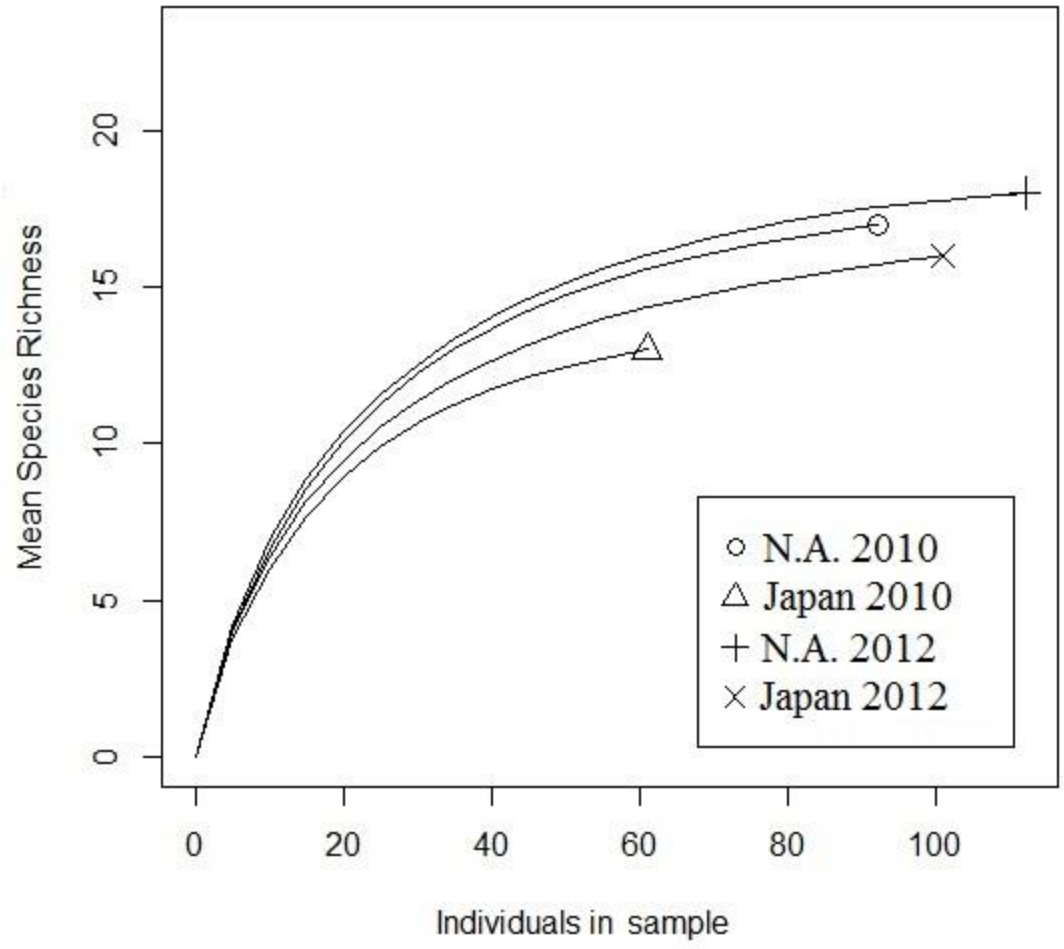

Figure 2-2. Cumulative number of fungal endophyte OTUS as function of the number of collections.

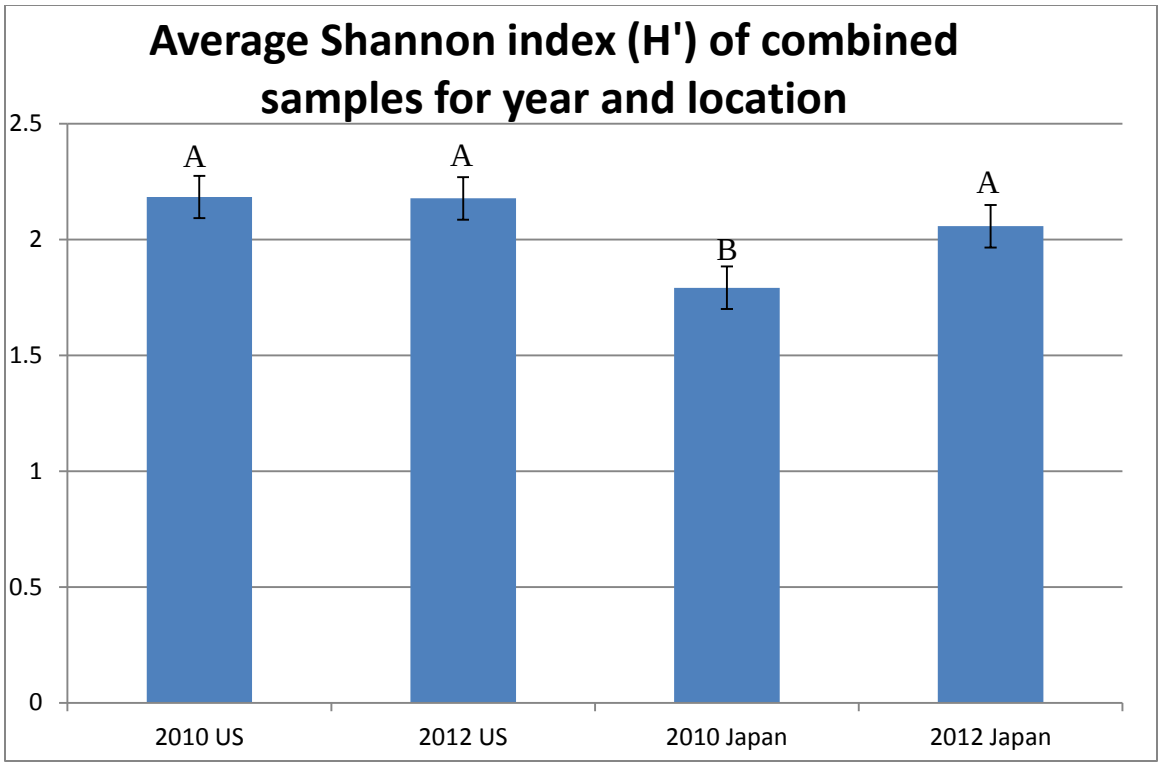

Figure 2-3. Average Shannon index (H') of combined samples for year and location. ANOVA analysis reported 2010 Japan was significantly different from all other communities ($p<0.01$).

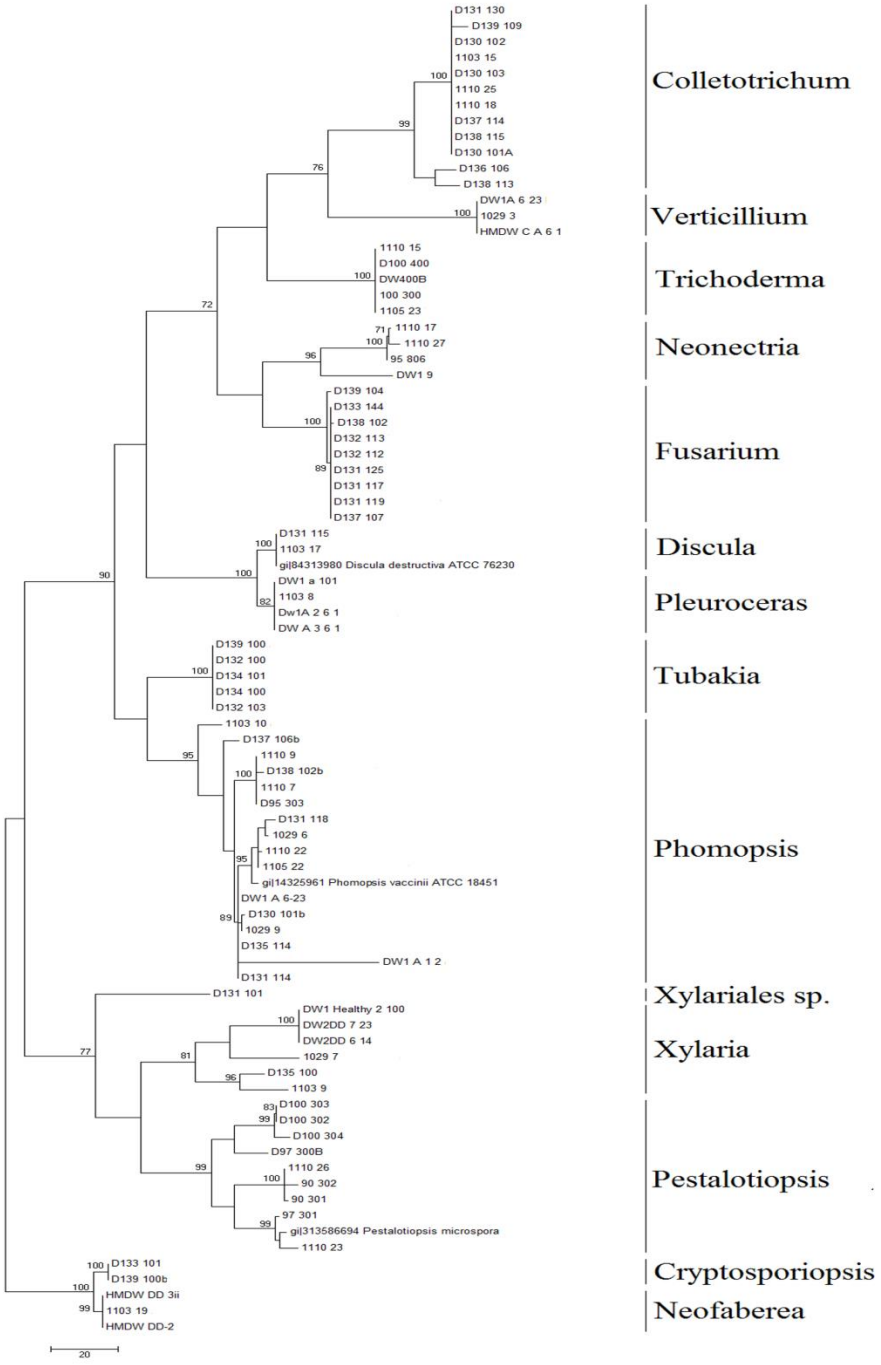

Figure 2-4 Phylogenetic relationship of 81 representative fungal endophyte isolates of Sordariomycetes and Leotiomycetes obtained in culture from foliage of *Cornus* species. The tree was produced by Maximum Parsimony (MP) method based on the ITS region. Tree Length = 821. Bootstrap values >70% are shown on nodes. CI = 0.438, RI=0.884

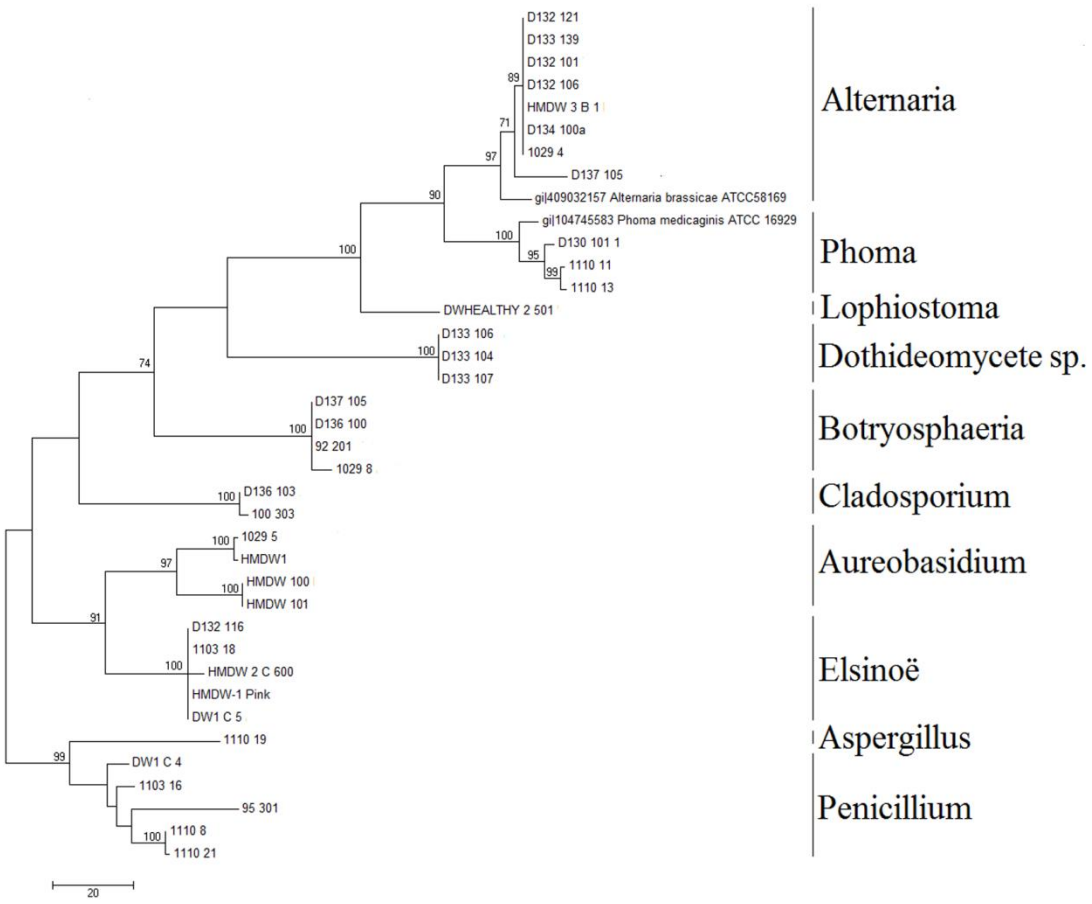

Figure 2-5 Phylogenetic relationship of 36 representative fungal endophyte isolates of Dothidiomycetes and Eurotiomycetes obtained in culture from foliage of *Cornus* species. The tree was produced by Maximum Parsimony (MP) method based on the ITS region. Tree Length = 573. Bootstrap values >70% are shown on nodes. CI = 0.619, RI=0.882

Table 2-1 List of Identified OUT and their abundance by Year and Location

	2010 NJ	2010 Japan	2012 NJ	2012 Japan
Alternaria alternata	4	3	4	1
Alternaria tenuissima	0	0	0	1
Phoma peniodella	0	0	2	0
Phoma glomerata	1	3	3	4
Lophiostoma sp.	1	0	0	0
Dothideomycetes sp.	0	0	3	0
Botryosphaeria_dothidea	0	2	0	4
Botryosphaeria_sp. 1	0	1	0	0
Cladosporium sp.	0	1	0	1
Aureobasidium pullulans	2	0	1	0
Aureobasidium sp. 2	3	0	1	0
Elsinoe fawcettii	10	0	8	0
Aspergillus sp.	2	1	1	1
Penicillium cecidicola	0	1	0	2
Penicillium chrysogenum	2	0	1	0
Penicillium janthinellum	2	0	2	0
Penicillium spinulosum	0	2	0	2
Colletotrichum acutatum	2	4	26	28
Colletotrichum sp. 1	0	0	0	2
Verticillium dahlaie	3	0	0	0
Trichoderma lixii	6	9	15	10
Neonectria discophora	0	2	0	0
Neonectria sp 1	0	1	0	0
Neonectria sp 2	1	0	0	0
Fusarium sp.1	0	0	0	1
fusarium vilior	4	3	9	7
Discula Destructiva	1	0	1	0
Pleuroceras tenellum	9	0	0	0
Tubakia sp.	0	0	3	6
Phomopsis endophytica	1	0	0	0
Phomopsis lagerstroemiae	0	1	0	2
Phomopsis amygdali	3	1	2	1
Phomopsis vaccinii	0	2	0	3
Phomopsis nobilis	1	1	1	2
Phomopsis sp. 1	1	1	1	0
Phompsis sp. 2	2	0	3	0
Xylariales sp.	0	0	3	0
Xylaria submonticulosum	2	1	3	1
Xylaria sp. 1	1	0	0	0

Xylaria sp. 2	2	2	3	3
Cyrptosporiopsis sp.	0	0	2	0
Neofabrea sp.	2	0	0	0
Pestalotiopsis mangiferae	1	0	2	0
Pestalotiopsis microspora	25	16	18	12
Pestalotiopsis monochaeta	0	1	0	0
Pestalotiopsis sp. 1	0	2	0	1
Pestalotiopsis sp. 2	0	1	0	1
Pestalotiopsis sp. 3	0	1	0	1

Table 2-2 Fungal endophyte diversity indices by location and year

Year	Location	Shannon H'	Chao-1	Chao-2	Species Evenness	Fishers	Simpsons
2010	US	2.12	16.66	76	0.912	5.65	0.142
2012	US	2.2	22.12	104.5	0.921	6.56	0.141
2010	Japan	1.77	21	52	0.917	5.01	0.201
2012	Japan	2.29	44.5	115	0.914	7.68	0.118

Table 2-3 Fungal Endophyte comparison of various similarity and dissimilarity indices of combined samples by year and geographical location

Bray-Curtis above / Morisita-Horn below

	J-2010	J-2012	U-2010	U-2012	Br-Cu
J-2010	---	0.375	0.525	0.449	J-2010
J-2012	0.316	---	0.148	0.386	J-2012
U-2010	0.788	0.131	---	0.472	U-2010
U-2012	0.653	0.583	0.613	---	U-2012
Mo-Ho	J-2010	J-2012	U-2010	U-2012	---

Sorensen above / Jaccard below

	J-2010	J-2012	U-2010	U-2012	Br-Cu
J-2010	---	0.375	0.525	0.449	J-2010
J-2012	0.316	---	0.148	0.386	J-2012
U-2010	0.788	0.131	---	0.472	U-2010
U-2012	0.653	0.583	0.613	---	U-2012
Mo-Ho	J-2010	J-2012	U-2010	U-2012	---

Table 2-4 List of real-time PCR on herbarium samples with host, location, and year.

Sample	Plant Species	Location	Year	qPCR mean CT value (Standard deviation)
h2	*C. capitata*	Nepal	1972	-
h3	*C. canadensis*	Japan	1952	-
H4	*C. canadensis*	Japan	1959	38.0 (2.07)
H6	*C. brachypoda*	Japan	1951	31.2 (0.46)
H7	*C. brachypoda*	Japan	1949	31.9 (0.62)
H8	*C. brachypoda*	Japan	1951	5.87 (1.02)
H9	*C. alba*	China	1997	38.0 (0.86)
H10	*C. capitata*	China	1946	38.2 (2.25)
H11	*C. capitata*	China	2000	38.6 (2.34)
H12	*C. paucinervis*	China	1974	40.2 (2.50)
H13	*C. walteri*	China	2007	31.2 (0.28)
H14	*C. walteri*	China	1976	31.1 (0.16)
H16	*C. controversa*	Japan	1956	31.6 (0.10)
H18	*C. controversa*	Japan	1952	-
H19	*C.controversa*	China	1975	41.1 (0.32)
H20	*C. controversa*	Japan	1953	-
H21	*C. controversa*	Japan	1953	-
H22	*C. controversa*	Japan	1955	-
H23	*C. chinesis*	China	1942	38.1 (0.30)
H24	*C. chinesis*	China	1938	35.3 (1.70)
H25	*C. sanguinea*	France	1959	34.2 (0.46)
H26	*C. amomum*	New York	1947	34.7 (0.65)
H27	*C. macrophylla*	China	1960	-
H28	*C. paucinervis*	China	1980	36.2 (1.11)
H29	*C. kousa*	Japan	1987	-
H30	*C. kousa*	Japan	1988	38.0 (0.57)
H31	*C. kousa*	Japan	1984	-
H32	*C. kousa*	Japan	1984	-
H33	*C. kousa*	Japan	1984	36.0 (0.12)
H34	*C. kousa*	Japan	1982	-
H35	*C. kousa*	Japan	1982	38.5 (1.82)
H36	*C. kousa*	Japan	1978	-
H37	*C. kousa*	Japan	1985	36.3 (1.13)
H38	*C. alba*		1909	34.0 (0.80)
H39	*C. kousa*	Japan	1935	-
H40	*C. kousa*	Japan	1988	36.1 (0.75)
H41	*C. kousa*	Japan	1968	36.7 (0.03)

H42	*C. alba*	CCCP	1929	36.0 (0.65)
H43	*C. kousa*	Japan	1965	-
H44	*C. kousa*	Japan	1978	35.6 (1.11)
H45	*C. kousa*	Japan	1978	-
H46	*C. kousa*	Japan	1977	41.3 (2.98)
H47	*C. kousa*	Japan	1965	34.1 (0.47)
H48	*C. florida*	USA	2011	26.4 (0.07)
H49	*C. kousa*	Japan	1976	38.8 (1.67)
H51	*C. kousa*	Korea	1987	35.6 (1.01)
H52	*C. kousa*	Japan	1961	36.3 (0.50)
H53	*C. kousa*	Japan	1960	36.3 (0.50)
H54	*C. kousa*	Japan	1968	36.7 (0.82)
H55	*C. kousa*	Japan	1982	35.0 (0.74)
H56	*C. japonica*	China	1979	-
H57	*C. kousa*	China	1960	36.3 (1.45)
H58	*C. stolonifera*	Mexico	1968	-
H59	*C. kousa*	Japan	1983	35.7 (0.13)
H60	*C. kousa*	Japan	1973	32.2 (0.42)
H61	*C. kousa*	Japan	1976	-
H62	*C. stolonifiera*	Mexico	1972	36.3 (1.44)
H63	*C. stolonifiera*	Mexico	1977	33.5 (0.38)
H64	*C. stolonifera*	Mexico	1960	34.35 (0.74)
H65	*C. kousa*	China	1980	34.67 (0.87)
H66	*C. kousa*	China	1975	33.5 (0.06)
n1	*C. nuttallii*	US	1987	35.4 (1.22)
n2	*C. nuttallii*	Canada	1963	-
n3	*C. nuttallii*	US	1976	-
n4	*C. nuttallii*	US	1975	-
n5	*C. florida*	US	1955	35.5 (0.63)
n6	*C. florida*	US	1971	33.0 (0.34)
n7	*C. florida*	US	1985	32.8 (0.37)
n8	*C. nuttallii*	US	1988	33.7 (0.63)
n9	*C. nuttallii*	US	1968	33.0 (0.24)

Table 2-5 Real-time PCR of individual samples from 2010 and 2012 U.S. and Japan leaf tissue.

Sample	Plant Species	Location	Year	qPCR mean CT value (Standard deviation)
US10-1	*C. florida*	US	2010	-
US10-2	*C. florida*	US	2010	-
US10-3	*C. florida*	US	2010	30.6 (1.90)
US10-4	*C. florida*	US	2010	34.9 (0.60)
US10-5	*C. florida*	US	2010	17.01 (0.43)
US12-1	*C. florida*	US	2012	-
US12-2	*C. florida*	US	2012	-
US12-3	*C. florida*	US	2012	-
US12-4	*C. florida*	US	2012	-
US12-5	*C. florida*	US	2012	-
JAP10-1	*C. kousa*	JAPAN	2010	23.9 (0.42)
JAP10-2	*C. kousa*	JAPAN	2010	-
JAP10-3	*C. kousa*	JAPAN	2010	-
JAP10-4	*C. kousa*	JAPAN	2010	-
JAP10-5	*C. kousa*	JAPAN	2010	38.3 (0.46)
JAP12-1	*C. kousa*	JAPAN	2012	38.2 (1.43)
JAP12-2	*C. kousa*	JAPAN	2012	34.8 (0.76)
JAP12-3	*C. kousa*	JAPAN	2012	35.0 (0.59)
JAP12-4	*C. kousa*	JAPAN	2012	34.1 (0.91)
JAP12-5	*C. kousa*	JAPAN	2012	34.8 (0.76)

Chapter 3

Metagenomic analysis of a known mock fungal ITS1 rRNA community with a multimillion sequence depth, obtained via Illumina Paired-End reads.

Abstract

High-throughput sequencing continues to evolve, even as it becomes more popular for elucidating fungal diversity. Sequencing platforms and bioinformatic tools are providing unprecedented amounts of data. However, as sequencing platforms evolve and become more complex, software tools need to be developed or utilized appropriately to capture results accurately and in user-friendly form. Current Illumina pipelines were specifically for bacterial 16S rRNA analysis. The pipeline developed here is specifically configured for the fungal barcoding region ITS rDNA. The software and customizable scripts can easily be adapted for various Illumina platforms, as well as for specific user requirements. This study utilizes environmental samples from a previous culture study and three mock communities (of known composition) to test the efficicy of the pipeline. We show excellent consistency in taxonomic recovery within the mock communities and diversity patterns from environmental data previously analyzed.

Introduction

There are estimated to be 1.5 to 5.1 M fungal species on Earth (Hawksworth 1991); although these estimates have been debated (Hawksworth and Rossman 1997; O'Brien et al. 2005; Taylor et al. 2012), but only about 100,000 taxa have (so far) been described (Blackwell 2011). Fungi can be found in above ground plant tissue in biomes that range from tropical to boreal and Arctic ecosystems (Carroll & Carroll 1978; Petrini 1986; Espinosa-Garcia and Langenheim 1990; Arnold and Lutzoni 2007; Hoffman and Arnold 2008). The staggering estimate of fungal diversity has led to exciting and challenging research on fungal associations and ecology.

Despite the increasing attention to fungal ecology, particularly in fungal endophytes and soil fungi, questions persist about fungal species composition, genetic/taxonomic diversity and spatial heterogeneity, all of which remain poorly understood. Culturing studies suggest that endophytic fungi, in particular, are hyper-diverse (Arnold et al. 2000) and play important roles as symbiots, pathogens, and decomposers. Nevertheless, culturing of environmental samples is known to be inadequate, as a means of describing / comparing environmental communities (Arnold and Hoffman 2007; Shulz and Boyle 2007). A major limitation of culture-dependent methods is that they fail to detect the vast majority of organisms that cannot (yet) be grown in culture (Guo et al. 2001; Duong et al. 2007; van Wyk et al. 2007; Seena et al. 2008; Tao et al. 2008; Zhu et al. 2008). Accumulation curves from culture-based studies do not plateau (Petrini and Muller 1979; Halmschlager et al. 1993; Higgins et al. 2007; Miller et al. 2013), strongly suggesting that we may well be missing many more taxa than we are recovering. It has became necessary to develop and deploy culture-independent

methods, capable of revealing a much larger fraction of the microbial world (O'Brien et al. 2005).

The genetic material present in environmental samples was coined "metagenome" and the sequencing and analysis of the genetic material directly was labeled as "metagenomics" (Handelsman et al 1998). The use of metagenomics allowed genetic material to be directly recovered from an environmental sample, bypassing the need for culturing (Hyde and Soytong 2007). Venter et al. (2004) were among the first to use a shotgun approach to metagenomics, using Sanger culture independent methods and identified 148 new bacterial phylotypes. However, Sanger culture-independent sequencing methods have offered low throughput at a relatively high cost (Tedersoo et al. 2010) and are known to be inadequate for description and comparison of environmental communities (Neufeld and Mohn 2005; Woodcock et al 2006.)

The development of 454 pyrosequencing and sequence tags (Kysela et al. 2005; Neufeld et al. 2008; Bartram 2011) for metagenomics of the microbial world offers higher throughput than Sanger sequencing, and has spurred interest in the diversity and composition of both bacterial and fungal communities (Sogin et al. 2006; Huse et al. 2007; Andersson et al. 2008; Buee et al. 2009; Jumponnen 2009, 2010). Although these efforts haved demonstrated considerably greater depth and diversity of rare microbiota with considerabley scaled-up throughput, the accumulation curves (to date) remain far from saturated (Sogin 2006; Pedros-Ali 2007; Buee et al. 2009).

Meanwhile, even newer high-throughput sequencing technologies such as Illumina, coupled with advances in the technology, have become powerful and cost effect

tools that offer even greater coverage at a lower cost (Shendure and Hanlee 2008; Lazarevic et al. 2009; Gloor et al. 2010; Caporaso et al. 2011).

Despite considerable advances in sequencing technology, one of the biggest bottlenecks in using next generation sequencing (NGS) is the poor bioinformatic tools available to analyze the data (Scholz et al. 2012). The number of reads has been increasing steadily, and is likely to continue to increase as the technology continus to evolve. There are a number of metagenomics pipelines available for Illumina metagenomics analysis, but most of the available bioinformatics tools, such as QIIME (Caporaso et al. 2010) and MOTHUR (Schloss et al. 2009) are configured explicitly for analysis of the rRNA 16S gene (Caporaso et al. 2010; Gloor et al. 2010; Werner et al. 2012; Bokulich et al. 2013). These tools are generally straightforward for use with prokaryotes, but offer little utility for eukaryotic fungal work, typically based on ITS rDNA region instead, although a few limited adaptions of QIIME are available for fungi (Caporaso et al. 2010).

Standard pipelines are unsuitable for the unique characteristics of the ITS region typically used in molecular fungal analyses. The ITS1 and ITS2 regions are highly variable and are flanked by highly conserved but uninformative regions that typically distort BLAST searches, and which are recommended for removal (Nilsson et al. 2010). There are suitable programs such as PLUTOF (Abarenkov et al. 2010) and CLOTU (Kumar et al. 2011) are available to analyze fungal metagenomic data, but these platforms are specifically tailored to 454 platforms and are web-based programs that can limit the user's ability to analyze the data or adapt it for different platforms.

It is necessary to create a pipeline that is appropriate for analysis of Illumina metagenomic fungal data from environmental samples. Creating a pipeline is uniquely challenging and requires excellent programming skills, usually in a variety of different languages, i.e. perl, Python, BioPerl. It is necessary to quantify defined "control" samples in order to validate the analysis of newly developed pipelines, allowing assessment of errors and effectiveness (Caporaso et al. 2010). Here, we present a novel pipeline composed of custom perl scripts and common bio-informatic software for analyzing paired end Illumina files of environmental samples of fungi. The goal was to create and test custom scripts in parallel with currently available free bio-informatic tools on a mock community to delimit species of a known fungal community. This study was performed on the Illumina MiSeq, and represents a user friendly method for ecologists who wish to utilize Illumina for their studies.

Materials and Methods

Sample Preparation

The "mock community" was generated using DNA isolated from pure fungal cultures which can be found in a previous culture study of dogwood (Chapter 2). *Pestalotiopsis* (30%), *Colletotrichum* (20%), *Trichoderma* (10%), *Fusarium* (5%), *Pleuroceras* (3.5%), *Verticllium* (3.5%), *Phomopsis* (3.5%), *Botrosphareia* (3.5%), *Cladosporium* (3.5%), *Discula destructiva* (3.5%), *Alternaria* (3.5%), *Phoma* (3.5%), and *Rhizoctonia* (3.5%) were used as representative mock communities. Three mock communities were created from the genomic DNA isolated from the 14 different fungal strains and normalized using a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences, USA). The fungal genomic DNA (gDNA) was pooled together at various ratios to mimic the community differences found in the culture study. A table of the combined

fungi and relative abundance percentages, prior to PCR, is presented in Table 3-1. All fungi used for the mock community were individually tested with Illumina primers, as described below and verified by gel electrophoresis.

Environmental samples of switchgrass (*Panicum virgatum*) were also collected at Wharton State Forest (WSF), NJ (N39^{O}46', W74^{O}40') in 2013. At that location, 10 seemingly healthy root samples were randomly collected, but at least 5 m apart from each other. Individual samples were placed in separated sterile zip-lock bags in the iced cooler and transported to the laboratory for processing within 24 h for culturing. Root cuttings 10 cm in length were randomly selected from the 10 original individuals and were pooled together. Root fragments were surface sterilized under the following conditions in sequential order: 75% ethanol for 5 min., 0.6% sodium hypochlorite for 5 min, and rinsed in distilled water two times. One hundred roots were allowed to air dry before being plated on malt extract agar (MEA) with 0.07% lactic acid. All root samples were sequenced with ITS5, which is similar to ITS1, and ITS4 primers (White et al. 1990). Sequences and spore morphology and characteristics were used to identify the species. A list of cultures and frequencies found in culture at WSF can be found in Table 3-2. The rest of the samples were kept in the freezer at -80 ºC, until used for metagenomic analysis. The samples used for the metagenomic analysis were removed from the freezer and processed the same way the original culture samples had been treated. The mock communities and the environmental sample were processed exactly the same way in the pipeline.

Primers, DNA Sequencing and Purification

Primers ITS1 and ITS4 were adapted for the metagenomics study, because they represent the fungal barcode (Schoch et al. 2012) and are commonly used in phylogenetic studies (White et al. 1990). The ITS region was amplified using modified ITS1 and ITS4 primers, as seen in Table 3-3 (Bartram et al. 2011). These primers not only amplify the ITS region, but are complementary to Illumina forward, reverse and multiplex sequencing primers that contain a 6 bp index to allow for multiplexing. All primers were created and purified by SDS-PAGE by IDT (Coralville, IA). PCR polymerases typically have high rate of errors (10^5 to 10^6) per nucleotide base, and a high fidelity polymerase was used to limit PCR sequencing errors (Cline et al. 1996), so a high fidelity DNA polymerase was utilized to reduce sequencing errors. PCR reaction mixture (25 µl) consisted of 5 µl of 5X Fusion GC Buffer Buffer (New England BioRad, MA, USA), 0.5 µl of 10 mM dNTPs mix, 1.25 µl of 10 µM forward primer and 1.25 µl of 10 µM reverse primer, 0.25 µl of Phusion DNA Polymerase, 1 µl of DNA from the mock community, and 15.75 µl of DNase, RNase free water (Signma CO, US). Each mock community was prepared in triplicate, in order to run at different annealing temperatures, and was then pooled after PCR amplification. Previous studies had shown that lower annealing temperatures aid in recovering taxa by alleviating primer mismatch; but higher annealing temperatures also aid in recovering taxa, due to enhanced primer binding (Fonseca et al. 2012; Schmidt et al. 2013). The PCR cycling conditions were as follows: 98˚ C for 30 s, followed by 25 cycles of denaturation at 98 ˚ C for 10 s, annealing at either 52˚ C, or 55˚ C, or 58˚ C for 30 s, and primer extension at 72˚ C for 30 s, followed by a final extension at 72˚ C for 5 minutes. PCR cycling conditions can lead to sources of error by saturating PCR amplification and introducing bias (Suzuki and Giovannoni 1996; Schmidt et al.

2013). Allowing only 25 cycles for PCR amplification, we prevent the sample from reaching PCR saturation. Three annealing temperatures of 52˚ C, 55˚ C and 58˚ C were chosen to account for the stochasticity of PCR reactions (Schmidt et al. 2013). PCR amplifications were verified, using gel electrophoresis, purified with Qiagen's QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany), and again visualized on a gel to ensure primer dimers were removed. The purified PCR products with unique barcodes were combined in equal nanogram quantity, quantified on a NanoVue Plus Spectrophotometer, and run on Illumina MiSeq, located at Rutgers University, generating paired end reads of 2 x 300 bp. Due to low complexity of the sample, Phi-X was added as a control. After sequencing was complete, sequences were de-multiplexed using Illumina Basespace web-based cloud service (basespace.illumina.com).

Quality Filtering

Sequences were de-multiplexed using Illumina Basespace web-based cloud service (basespace.illumina.com). Phi-X was used as a control, due to the low complexity of the sample. Phi-X sequences were removed by the sequencing facility, as well as trimming the adaptors. The pipeline utilizes custom made perl scripts that require perl v5.7 or greater. Other dependencies for the quality filtering and primer checking require tre- agrep v0.8.0 or higher (https://github.com/laurikari/tre/), as well as Python 2.7.4. Custom scripts can be found at and downloaded from (http://plantbiopath.rutgers.edu/faculty/zhang/zhang.htm), along with command lines and options available.

Clustering and taxonomic assignment

The pipeline (http://microbiology.se/software/itsx/) utilizes ITSx v1.0.10 or higher to separate the ITS1 and ITS2 region from the conserved flanking regions. UPARSE is needed for the dereplication and 'greedy' clustering, which is part of USEARCH v7.0 or higher (http://drive5.com/uparse/). In order to obtain the fungal abundances, we used the python scripts supplied from USEARCH v7. The python scripts label OTU-representatives according to the pattern OTU_1, OTU_2, etc. before they are clustered. The samples are specified by the barcode label from the Illumina forward and reverse reads, and also have the number of ISUs that were collapsed by the previous de-replication step. A custom Python script was used to create a fungal database that eliminates "uncultured", "unknown" and "environmental" sequences and created using BLAST+ v2.2.28 or higher, available from (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST). Finally, taxonomic assignment requires MEGAN v5.0 or later, available at (http://ab.inf.uni-tuebingen.de/software/megan5/). MEGAN LCA parameters were set as follows: Min Score 120, Top Percent 1.0, Min Support: 1, LCA Percent 100, and Use Minimal Coverage Heuristic checked. A conceptual workflow can be seen in Figure 3-1.

Results

Quality Filtering and Extracting Sequences

As is the case with other sequencing technologies, the unprecedented sequencing depth poses computational problems (Caporaso et al. 2010; Gloor 2010). We recovered 40,883,522 paired end reads that were exactly 305 nt long, from each end of the PCR amplified region. Because there is little literature describing or discussing quality-filtering of sequences for fungi, strategies for the community analysis were adapted from

16S rRNA analysis. For example, traditionally, a collection of known 16S rRNA gene fragments, known as mock communities, have been sequenced to test and measure error rates. Kunin et al. (2010) created a mock community by sequencing a strain of *E. coli* and expected to find only 1 OTU (operational taxonomic unit), when clustering at 97%, which represents the 3% cutoff for bacterial species. Instead they found 16 OTU's. In order to get to the expected OTU of 1, they implemented the software LUCY developed by Chou and Homes (2001), which can identify low quality scores based on the Phred score, and remove poor basepairs from high quality basepairs in the sequence. A Phred score is quality value that is assigned for each base call. The formula for how quality (*Q*) and probability (*P*) of base call error is defined as:

$$Q = -10 \times \log_{10} P$$

For example a $Q = 30$ corresponds to the probability of $P = 0.001$ error rate. The probability of error is based on a number of factors such as: peak shape, spacing between peaks, signal strength and background noise (Ewing and Green 1998; Ewing et al. 1998). These quality scores are converted into ASCII characters (see Table 3-4 for conversion of Phred scores to ASCII in various Illumina Platforms), which allow for storing the output of the sequence and the quality of the basepairs in an easily parsed FASTQ format (Cock et al. 2010). However, the number of reads from Illumina prevents applications of the 454 pyrosequencing data analysis software, such as LUCY, from being utilized because the tremendous amount of reads from Illumina is computationally demanding, and specifically modeled for 454 errors (Quince et al. 2009; Reeder and Knight 2010; Quince et al. 2011). I designed a set of custom perl-scripts developed in house to extract and trim

sequences, which can be customized by the user, based on relevant literature for filtering NGS sequences.

First, a custom perl-script which utilizes tre-agrep was used to find any sequences in which 1 or more errors in the primer were found, while also orienting reads in the correct 5' to 3' direction. A 454 study by Huse et al. (2007) showed that removing sequences with mismatches in primers sequences reduced the error rate. Unlike QIIME, the primer is kept attached for analysis downstream. Following the initial primer check and orientation, another perl script was developed to truncate reads where two or more base pairs had a consecutive low quality score (#) and truncated the sequence at the first low quality scored nt and removed any sequence which contained one or more 'N' characters in the sequence. This is similar to LUCY (as noted above) and other 454 filtering software, but adapted to Illumina and requires little computational power. After sequences are truncated or removed, the script removes sequences smaller than 150 base pairs from further analysis. A total of 21,253,143 (of the original 40,883,522) sequences were retained, after the quality filtering step (Table 3-5).

Combining Sequences

Following quality filtering, both files will not be the same size. This is a requirement of for a majority of sequence mergers. Python scripts were developed to resynchronize the forward and the reverse reads. However, a majority of the reads which passed the initial quality filtering step were from the forward reads. The majority of the reverse reads failed to pass quality filtering, due to poor quality scores in the reverse reads. For two of the mock communities (MC-1, MC-3) only 1% of the reverse reads

were usable, and for MC-2, roughly 10%. The reverse reads were therefore discarded from the rest of the analysis.

However, studies utilizing 454 sequencing typically focus on either ITS1 or ITS2 (Buee et al. 2009; Jumpponen et al. 2010; Yu et al 2012; Balint et al 2013). Previous observations have shown that ITS2 is more variable and recovers more molecular diversity in 454 studies (Jumpponen and Jones 2009; Mello et al. 2011; Bazzicalupo et al. 2013; Blaalid et al. 2013), but in ecological context, the two regions show similar community structures (Mello et al. 2011; Bazzicalupo et al 2013; Blaalid et al. 2013), though they were difficult to compare, due to variations in data treatment. Mello et al. (2011) did not remove low-frequency clusters to remove PCR and sequencing artifacts, and the singletons were also retained. Although small cluster sequences may represent rare microbiota, most pyrosequencing singletons are (in fact) chimeric artifacts instead (Tedersoo et al. 2010), and were removed from this study. Ideally, both ITS1 and ITS2 would be used in a phylogenetic study, but due to chemistry and poor sequencing quality of our Illumina reverse reads, our analysis utilizes ITS1, but could easily accommodate ITS2 or both ITS1 and ITS2, separately. ITS1 is also an ideal candidate, due to recent database advances that utilize ITS1, such as ITSoneDB (Santamaria et al. 2012), and is better suited for species discrimination (Chen et al. 2001; Hinrikson et al. 2005).

ITSx and Sequence Clustering

As noted, the ITS region is designated as the formal barcode for fungi, but it does have its shortcomings. One of the inherhit difficulties of using the ITS region in metagenomics analysis is the compostition of the region itself. The ITS region is composed of two highly variable spacers, identified as ITS1 and ITS2, which flank the

highly conserved 5.8S gene (Hillis and Dixon 1991). Immediately upstream of ITS1 is the small subunit (SSU) and immediately downstream of ITS2 is the large subunit (LSU), which are also highly conserved. The ITS1 and ITS2 region are quite variable, and as noted above, they can be used separately or together to identify species. When using the conserved and variable regions in a similarity search like BLAST (Altschul et al. 1997), the output is not necessarily the intended or correct target. The conserved region will likely find a match in the database regardless of whether the variable part does as well. Nilsson et al. (2009) investigated the BLAST results of 86,000 BLAST searches and found that 11% produced a different result that were non-synonymous species names when conserved regions were present in the search. It is necessary to remove the ITS1 and/or the ITS2 region from the conserved regions, which are where the primers are located.

Extracting the fungal ITS1 and ITS2 regions from the conserved region is not a simple task. Nilsson et al. (2009) developed a fungal ITS Extractor, later improved upon in 2013 in ITSx. ITSx uses HMMR version 3 (Edy 2011), which is centered on profile hidden Markov Models (HMMs) on phylogenetically diverse alignments throughtout the kingdom of fungi, for the 3’ end of SSU, the 5’ and 3’ ends of 5.8S, and th e5’ end of LSU. The profile HMMS allow for statistical models to represent variations at specific locations, without storing the full alignment, and can account for a small proportion of the sequences that may contain a “T” instead of an “A” in any given position (Durbin et al. 1998). The program requires that primers be located on the sequence, hence they are only removed from the filtering if they represent a poor match and seququence. QIIME typically removes primers in the early filtering steps. This is a free program that is

available on UNIX-type operating systems, and is necessary to alleviate bias in the BLAST results. However, one of the major bottlenecks in sequencing fungal species is extracting fungal sequences from ITSx, which can require a lot of computational power and time. Of the 20,192,785 forward sequences that passed quality filtering, 3,434,508 ITS1 sequences were extracted. ITSx also detects chimeric sequences and found 117 of the sequences to be chimeric (>1%). Chimeric sequences are hybrid products between multiple parent sequences which form during PCR amplification, and often inflate the estimated diversity. The frequency of chimeric sequences can range from (5% to 45%) after PCR amplification (Haas et al. 2011). However, sequences should still be investigated for chimeras in the downstream analysis, as ITSx is not designed to catch chimeric sequences specifically.

Clustering large numbers of sequences with the free version of USEARCH is not practical, and it limits the computational capacity available for the clustering process (4GB). In order to reduce the computational time and memory required, sequence numbers must be reduced. Researchers have typically grouped identical sequences into individual sequence units (ISUs) by clustering sequences between the primers at 100% identity (Figure 3-2; Gloor et al. 2012). An ISU is a representative sequence for a group of sequences that are identical in sequence, but vary in length, while retaining the largest sequence of the combined matches and the number of sequences that are aligned to the representative ISU. USEARCH v7 offers a method to dereplicate identical sequences, so only one copy of the largest sequence is reported. The file is arranged so that ISUs are organized into a file that is arranged by the ISU with the largest number of sequences that are assigned to the representative sequence to the ISU with the lowest amount of

sequencing. The ISUs with the largest sequences assigned to them are the sequences that are most abundant in the sample. Parameters here were set to remove any ISU that did not have 4 or more sequences, which removes all singletons. As noted earlier, small cluster sequences (> 3) may represent rare microbiota, however, most pyrosequencing singletons are (in fact) chimeric artifacts instead (Tedersoo et al. 2010), and were removed. This also dramatically reduces the necessary computation power for clustering into OTUs by reducing the number of sequences considered. After the dereplication into ISUs, 61,681 sequences were recorded, and were then clustered (at 97%) into OTUs, using USEARCH. Out of the 61,681 ISUs that were clustered at 97%, 290 OTUs were recorded.

Taxonomic assignment and Alpha Diversity Analysis of Mock Communities and Environmental samples

A custom BioPython script was developed to acquire sequences from the GenBank database. As have others (Caporaso 2010; Gloor 2010), we excluded "uncultured" and "environmental" samples from the database. Using BLAST+, the OTUs were blasted against the database for the closest sequence, based on its length and e-value, and the best match was chosen. Unlike previous pipelines, where sequences are essentially double-blasted to remove novel sequences (Caparoso 2010), the new pipeline retains novel sequences. BLAST data are then exported to MEGAN = (MetaGenome Analyzer), which assigns each sequence to the lowest common ancestor (LCA of the set of taxon hits). The "min support" parameter is set to 1 to include all assigned sequences. This retains the novel sequences, while those not matched to species or genus are assigned to higher taxonomic levels (Huson et al. 2007). By eliminating OTUs with less

than 4 sequences assigned and with the strict filtering, we are presumably limiting the numbers of sequences that appear novel, due to PCR or sequencing errors.

We applied our pipeline to three mock communities' samples, representing 14 fungal isolates pooled at various concentrations that represent similar abundances found in culture from *Cornus* sp. (see Chapter 2) as well as to an environmental sample which represents the fungal community associated with the roots of switchgrass located at WSF, NJ. The environmental sample is a recently analyzed sample that was obtained via culture-based identification. Ideally, metagenomics data will capture the community present in the culture data, as well as capturing data that are not easily obtained by traditional culturing means. It is necessary to quantify defined "control" samples in order to validate the analysis of newly developed pipelines (Caporaso et al. 2010). The analysis presented for the four samples were identical.

The consistency of the new pipeline between replicates was excellent. As with any NGS analysis, higher taxa are more accurately assigned (Figure 3-3). For 12 out of the 14 genera used in this analysis, the relative abundances were consistent between MC-1, MC-2 and MC-3, compared to the projected levels; for each of the 10 genera that were shown to be more or less abundant than projected, the projected results were consistent in all three mock communities. Bray-Curtis dissimilarity between mock communities shows no significant deviation at either class or genus level. Major departures of abundance from theoretical expectation were seen for *Verticillium*, *Trichoderma*, and *Pleuroceras*, and it is noteworthy that Kuschner et al. (2014) also found large differences in four of 16 genera, both in balance and unbalanced mock communities. With equimolar concentrations of pooled DNA, they found four of the 16 genera to be significantly

divergent from their projected abundances. In another unevenly distributed community, many of the projected abundances were significantly divergent. They attributed this to PCR bias and to different methods of preparing individual samples. Similar departures of abundances from expectation were seen in the analysis of the QIIME software (Caporaso et al. 2010), although not nearly as dramatic as those in the Kuschner et al. (2014) study. With QIIME, however, generic data were only accurately captured when OTUs with less than 10,000 sequences were eliminated. Applying high standards to OTUs in an unknown data set, of course, eliminates any rare microbiota that are present, and there is a tradeoff between discovering novel species and chasing phantom species.

The new metagenomics pipeline was then used to analyze the environmental fungal community of WSF. The same parameters used for the mock communities were used for the WSF sample. Consistency between culturing and metagenomics is excellent at the class level (Fig. 3). The largest portion of both the culture and metagenomic data are represented by Leotiomycetes (85.5% and 92.5%, respectively). The metagenomic data also detected similar patterns in the Sordariomycetes (6% for metagenomics vs 3.2% for culturing). Eurotiomycetes represented a small, but significant portion of the culture data (6.5%), compared with (0.5%) from metagenomic analysis. The metagenomic analysis did not detect any Zygomycetes, which represented 4.8% of the cultured species, but it did detect Basidiomycetes, which were not in the cultured collections.

At the generic level a large portion of the culture data were *Barrenia* (84%), which accounts for the large fraction of Leotiomycetes (Fig. 4). In the metagenomic data, the Leotiomycetes were composed of three dominant genera (*Acidomelenia* - 38%, *Barrenia* - 31% and *Lachnum* - 22%). Luo et al. (2014) found that switchgrass and other

grasses at WSF predominantly harbor various types of Leotiomycetes. Not only do they dominate managed ecosystems where switchgrass grows, but Leotiomycetes also play a role in increasing plant tolerance to abiotic stresses (e.g., low pH, low nutrients, and drought resistance) and contribute to improved establishment and persistence in acidic, poor quality soils. The genera *Penicillium*, *Mortierella*, and *Umbelopsis* (representing the Eurotiomycetes and Zygomycetes) were not identified by the metagenomic analysis. The largest difference between the culture and metagenomic datasets is the level of Sordariomycete diversity. Culture data are only capable of capturing fungal species of substantial abundance or rapid growth on media. The total diversity was significantly higher in the metagenomic dataset than in the cultured dataset. The metagenomic analysis was able to capture 15 genera, with 5 different species composing 84% of the Leotiomycetes. At the culture level, only 5 genera were identified with over 92% coming from 1 species in the class of Leotiomycetes.

Discussion

It is critical to use defined communities to validate the analysis by sequencing the "control" data set, as well as environmental samples that have been previously analyzed by alternative methods in order to provide insight into the distinction between sequencing error and differences among communities. It is important to note that all current analytical pipelines and variations for Illumina (Amend et al. 2010; Caporaso et al. 2010; Gloor et al 2010; Bartram et al. 2011; Caporaso et al. 2012) and 454 (Jumpponen and Jones 2009; Jumpponen et al 2010; Hang et al 2014) vary in taxon abundances, when compared to expectations for mock communities. Factors that may cause the observed

taxon abundances to differ include: sequencing error, PCR primer bias, limited clustering options, and incorrect taxonomic assignment (Fig. 2).

One other group has presented a pipeline similar to ours (Balint et al. 2014), but their pipeline differed in terms of tools and how the data were processed. Furthermore, they did not use a mock community or data from a previous metagenomic study. They sampled 96 soil samples, but only classified the sequences as Fungi or unassigned. In contrast, our study allows for strict quality filtering of individual reads before merging sequences, does not need both reads in order to merge sequences, and are adaptable across various Illumina platforms. For example, the scripts are set by default to utilize Illumina 1.8 quality scores, but can also detect and utilize Illumina 1.5 and lower FASTQ formats. This gives the researcher the ability to process paired end reads separately, due to the limitation of sequence length, or to combine the sequences, depending on the primers used.

Despite the success of the pipeline presented here, there are still computational and taxonomic problems specific to Illumina and to the fungal community. One of the major issues still needing attention is the public and independently curated databases. Currently, a large number of sequences in GenBank consist of "environmental" samples that are not incorporated into the taxonomic code. In order to facilitate deeper analysis of fungal biodiversity and ecology, there is a persistent need to develop a classification system based on environmental samples. Hypothetical examples have been presented, based on pure DNA extracts of a single fungus by Reynolds and Taylor (1991). Meanwhile, Hibbet et al. (2011) have proposed examples of a formal classification of environmental samples, but implementing latin binomials will entail both technical and

conceptural challenges, and not all mycologists agree on the appropriate strategy. Another problem is that the Illumina platform still allows analysis of either ITS1 or ITS2, but not both together. Using traditional primers of ITS1 and ITS4 (White et al. 1990) or newly developed primers ITS1-F_KYO1 (Toju et al. 2012) will leave a large gap between sequences. Our new pipeline is designed to enable joint use of primers such as ITS1 and ITS2 to sequence the ITS1 region, as illustrated here, or to use ITS1 and ITS4 to sequence both ends, without overlapping the reads in the 5.8S region. Although, 454 offers longer reads, most ITS studies utilize either ITS1 (Buee et al. 2009; Jumpponen and Jones 2009; Jumpponen et al. 2010; Yu et al. 2012; Balint et al. 2013) or ITS2 (Wallander et al. 2010; Davey et al. 2012).

Regardless of sequencing artifacts and computational challenges, advances in sequencing technology are generating large amounts of data in both microbial and fungal communities; and interest in elucidating novel fungal species and their ecology and evolution is steadily increasing.. These advances far outperform current culture-dependent methods, and the Illumina platform offers deeper read coverage, at a significantly lower cost, relative to 454 platforms, despite the shorter read lengths. Although culture-dependent methods can be sufficient to detect underlying patterns of fungal communities, larger data sets are required to understand more subtle responses to environmental factors and to capture the rare microbial data. The pipeline presented and demonstrated here represents an effective and free method for processing and analyzing paired-end reads, run on Illumina MiSeq. The advantage of this pipeline is that it represents up-to-date methods of checking primer sequences, strict quality filtering to eliminate erroneous sequences, the ability to process forward and reverse reads separately

or together, and the ability to capture and assign novel sequences. It also represents a current, transparent and user-friendly pipeline that can be used by researchers to answer questions about their particular plant host.

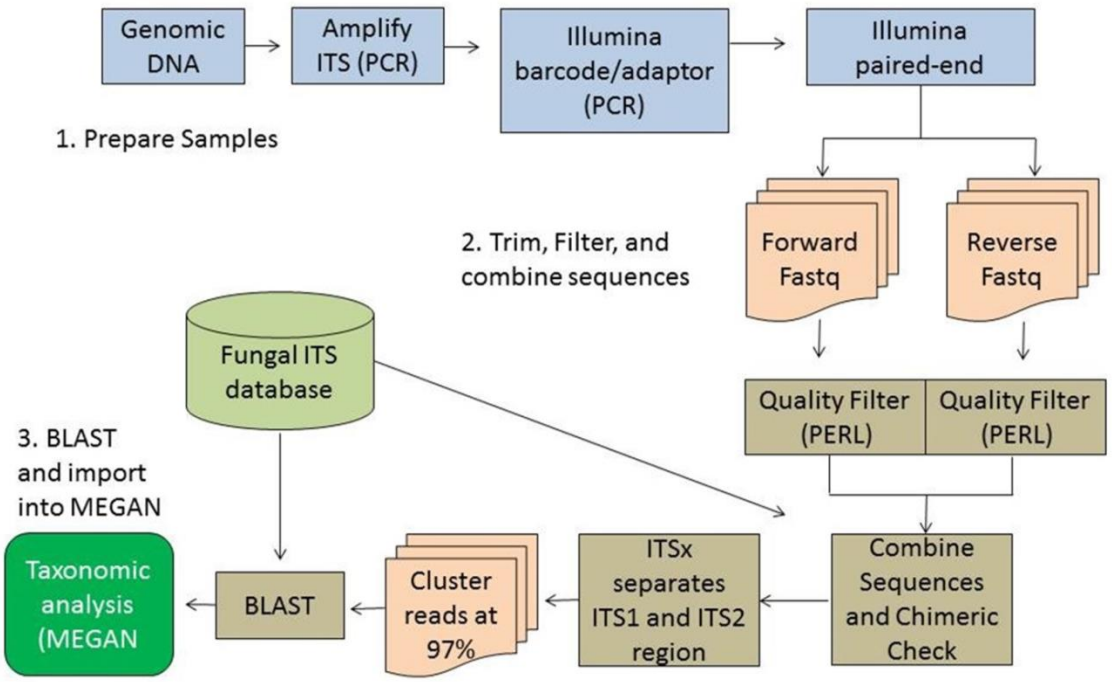

Fig 3-1. Conceptual workflow of data analysis. PCR products which amplify the ITS rRNA region of fungi were sequenced on a single paired-end Illumina run. Reads were filtered for quality, combined, extracted by ITSx and clustered according to the outline in the text.

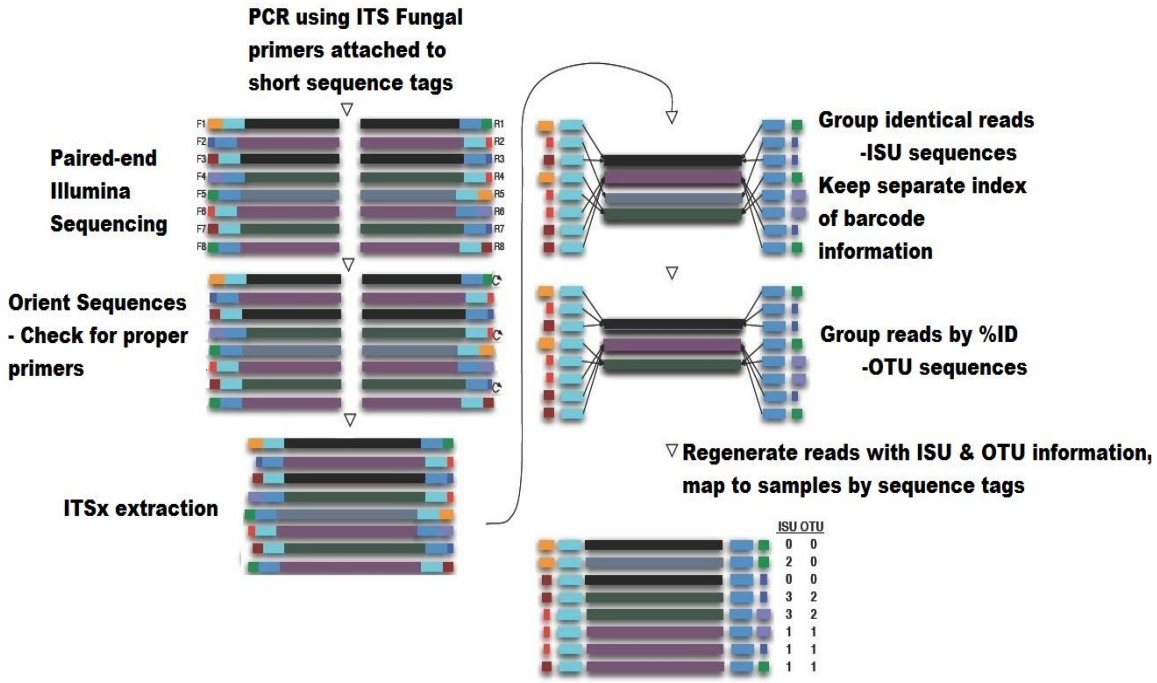

Figure 3-2: Overview showing how sequences are grouped into ISUs followed by OTUs. (Adapted from Gloor et al. 2012).

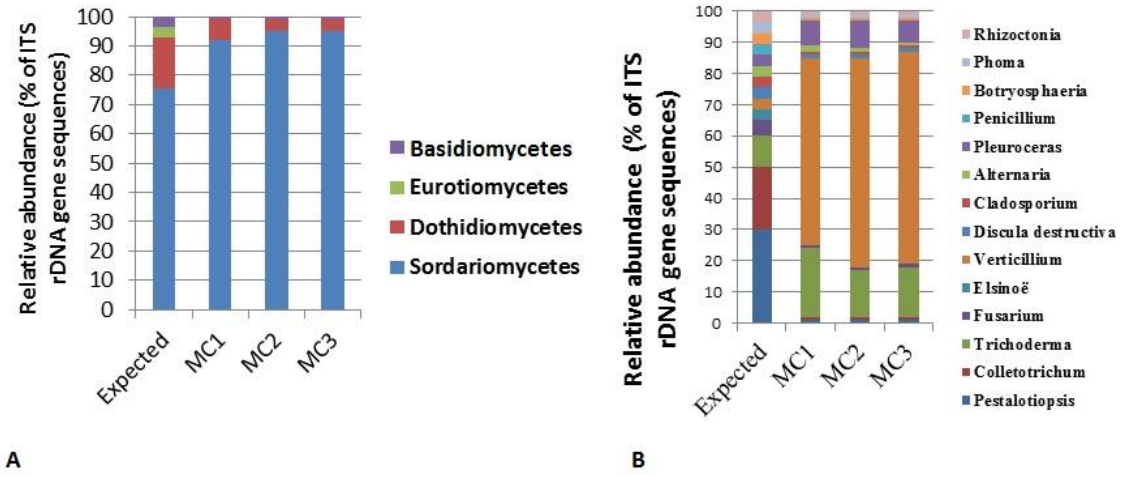

Fig. 3-3. Reproducibility of fungal taxon assignment at class level and genus level. Reproducibility at class level was excellent (A); reproducibility at the genus level (B) is extremely consistent within the ITS region.

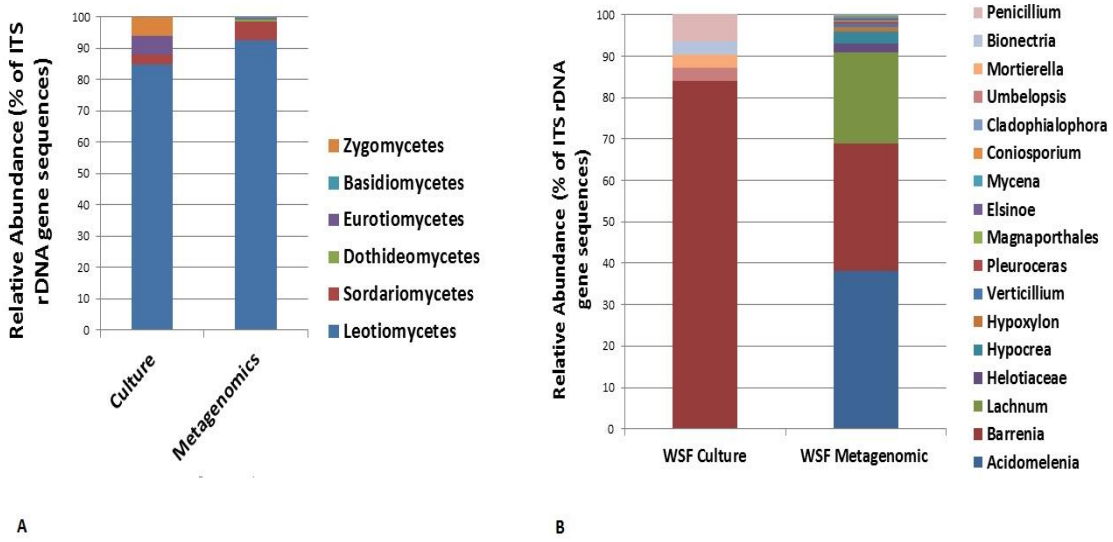

Figure 3-4. Reproducibility of fungal taxon assignment at class level and genus level. Reproducibility at order level was excellent (A); reproducibility at the genus level (B) is extremely consistent within the ITS region, both matching previously analyzed data.

Table 3-1. Relative abundance of gDNA used in 3 mock communities.

Mock community Composition	
Species	Relative Abundance Percentage
Pestalotiopsis	30
Colletotrichum	20
Fusarium	10
Trichoderma	5
Pleuroceras	3.5
Phomopsis	3.5
Botrosphareia	3.5
Cladosporium	3.5
Discula destructiva	3.5
Alternaria	3.5
Rhizoctonia	3.5
Verticllium	3.5

Table 3-2. List of cultures and their abundances found in Wharton State Forest Switchgrass Roots

WSF L1	
Species	Abundance
Barrenia panicica	53
Umbelopsis sp	1
Umbelopsis angularis	1
Mortierella sp.	2
Bionectria	2
Penicillium sp.	4

Forward Primers	Sequence(5' to 3')
ITS1_FOR	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctTCCGTAGGTGAACCTGCGG
Reverse Primers	
ITS4_1R	caagcagaagacggcatacgagat**CGTGAT**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_2R	caagcagaagacggcatacgagat**ACATCG**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_3R	caagcagaagacggcatacgagat**GCCTAA**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_4R	caagcagaagacggcatacgagat**TGGTCA**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_5R	caagcagaagacggcatacgagat**CACTGT**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_6R	caagcagaagacggcatacgagat**ATTGGC**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_7R	caagcagaagacggcatacgagat**GATCTG**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_8R	caagcagaagacggcatacgagat**TCAAGT**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_9R	caagcagaagacggcatacgagat**CTGATC**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_10R	caagcagaagacggcatacgagat**AAGCTA**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_11R	caagcagaagacggcatacgagat**GTAGCC**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC
ITS4_12R	caagcagaagacggcatacgagat**TACAAG**gtgactggagttcagacgtgtgctcttccgatctTCCTCCGCTTATTGATATGC

Table 3-3. Nucleotide sequences of primers used for Illumina sequencing. Lowercase letters denote adapter sequences to bind to flow cell, underlined lowercase are binding sites for Illumina primers, bold uppercase are the barcode seequences and regular uppercase are teh ITS1 (forward primer) and ITS2 (reverse primer).

Table 3-4 Quality scores and there conversion to ASCII (adapted from Illumina Manual).

Phred Score	Probability (P) Of Wrong Base	Illumina 1.3 to 1.7 "Q + 64" Shift	Illumina 1.3 to 1.7 "Q + 64" ASCII GLYPH	Illumina 1.8+ "Q + 33" Shift	Illumina 1.8+ "Q + 33" ASCII GLYPH
0	1	64	@	33	!
1	0.794328235	65	A	34	"
2	0.630957345	66	B	35	#
3	0.501187234	67	C	36	$
4	0.398107171	68	D	37	%
5	0.316227766	69	E	38	&
6	0.251188643	70	F	39	'
7	0.199526232	71	G	40	(
8	0.158489319	72	H	41	)
9	0.125892541	73	I	42	*
10	0.1	74	J	43	+
11	0.079432824	75	K	44	,
12	0.063095734	76	L	45	-
13	0.050118723	77	M	46	.
14	0.039810717	78	N	47	/
15	0.031622777	79	O	48	0
16	0.025118864	80	P	49	1
17	0.019952623	81	Q	50	2
18	0.015848932	82	R	51	3
19	0.012589254	83	S	52	4
20	0.01	84	T	53	5
21	0.007943282	85	U	54	6
22	0.006309573	86	V	55	7
23	0.005011872	87	W	56	8
24	0.003981072	88	X	57	9
25	0.003162278	89	Y	58	:
26	0.002511886	90	Z	59	;
27	0.001995262	91	[	60	<
28	0.001584893	92	\	61	=
29	0.001258925	93	]	62	>
30	0.001	94	^	63	?
31	0.000794328	95	_	64	@
32	0.000630957	96	`	65	A

33	0.000501187	97	a	66	B
34	0.000398107	98	b	67	C
35	0.000316228	99	c	68	D
36	0.000251189	100	d	69	E
37	0.000199526	101	e	70	F
38	0.000158489	102	f	71	G
39	0.000125893	103	g	72	H
40	0.0001	104	h	73	I
41	0.0000794328	105	i	74	J
42	0.0000630957	106	j	75	K
43	0.0000501187	107	k	76	L
44	0.0000398107	108	l	77	M
45	0.0000316228	109	m	78	N
46	0.0000251189	110	n	79	O
47	0.0000199526	111	o	80	P
48	0.0000158489	112	p	81	Q
49	0.0000125893	113	q	82	R
50	0.0000100000	114	r	83	S
51	0.0000079433	115	s	84	T
52	0.0000063096	116	t	85	U
53	0.0000050119	117	u	86	V
54	0.0000039811	118	v	87	W
55	0.0000031623	119	w	88	X
56	0.0000025119	120	x	89	Y
57	0.0000019953	121	y	90	Z
58	0.0000015849	122	z	91	[
59	0.0000012589	123	{	92	\
60	0.0000010000	124	|	93	]
61	0.0000007943	125	}	94	^
62	0.0000006310	126	~	95	_
63	0.0000005012			96	`
64	0.0000003981			97	a
65	0.0000003162			98	b
66	0.0000002512			99	c
67	0.0000001995			100	d
68	0.0000001585			101	e
69	0.0000001259			102	f
70	0.0000001000			103	g
71	0.0000000794			104	h
72	0.0000000631			105	i
73	0.0000000501			106	j

74	0.0000000398			107	k
75	0.0000000316			108	l
76	0.0000000251			109	m
77	0.0000000200			110	n
78	0.0000000158			111	o
79	0.0000000126			112	p
80	0.0000000100			113	q
81	0.0000000079			114	r
82	0.0000000063			115	s
83	0.0000000050			116	t
84	0.0000000040			117	u
85	0.0000000032			118	v
86	0.0000000025			119	w
87	0.0000000020			120	x
88	0.0000000016			121	y
89	0.0000000013			122	z
90	0.0000000010			123	{
91	0.0000000008			124	|
92	0.0000000006			125	}
93	0.0000000005			126	~

Table 3-4. Number of sequences left after trimming, extracting ITS1, collapsing, and clustering into OTUs.

		MC1-D	MC2-D	MC3-D	Total
Total Reads		9283842	16778874	14820806	40,883,522
Quality filtering	Forward	4584303	8287476	7321006	20,192,785
	Reverse	51070	919891	89397	1,060,358
	Total	4635373	9207367	7410403	21,253,143
ITSx	ITS1	1183828	1285639	965041	3,434,508
Chimeras (ITSX)		23	46	48	117
ISUs		1036005	11339691	1043328	13,419,024
ISUs > 3		10615	25606	25460	61,681
OTUs		78	126	86	290

Chapter 4

Comparison of root-colonizing fungi associated with switchgrass (*Panicum virgatum* L.) in native Pine Barrens and managed farms in New Jersey.

Abstract

Fungal endophytes are a phylogenetically diverse group of fungi that live asymptomatically in various plant organs. These fungi are broadly classified as Clavicipitaceous and Nonclavicipitaceous, which are further subdivided by modes of transmission. Clavicipitaceous fungi (Class 1) are the most well studied group of fungi. Class 2 and Class 3 endophytes also have an abundant research literature, though many ecological roles are still not fully elucidated. Class 4, or Dark Septate Endophytes (DSE) have been shown to be phylogenetically rich and can be beneficial to the plant host, but the group remains the most poorly understood class of endophytes. Here, I use metagenomics to characterize the fungal community associated with switchgrass from an unmanaged habitat in the nutrient-poor Pine Barrens, as well as from a managed, nutrient rich farm in New Jersey. I found that the fungal communities in Pine Barrens switchgrass differ significantly from those on farmland. Leotiomycetes were significantly more abundant in the Pine Barrens, whereas Sordariomycetes were more abundant at the farm. This study also compared metagenomic results with previously recorded culture results. There was considerable taxonomic similarity at higher taxonomic levels but larger differences at the species level.

Introduction

Fungi are the second largest kingdom of eukaryotic life (Alexopoulous et al. 1996) and are hypothesized to include an estimated 1.5 to 5.1 million species on Earth (Hawksworth 1991), but only about 100,000 species have been described to date (Blackwell 2011). Although the estimates of the true scale of diversity are debated (Hawksworth 1991; Hawksworth and Rossman 1997; O'Brien et al. 2005; Taylor et al. 2012), fungal species are known to be ubiquitous in nature, playing vital roles in the environment as symbionts, pathogens, and decomposers. The staggering estimate of fungal diversity has led to exciting and challenging research on fungal associations and ecology. Despite that increased attention, particularly for fungal endophytes and for soil organisms, many questions remain about community composition, true diversity and spatial heterogeneity.

So where are all the fungi? The leading hypothesis is that the major reservoir of novel biodiversity in fungi can be found in association with plants (Hawksworth and Rossman 1997). Most studies on plant-fungus relationships focus either on mycorrhizal or endophytic fungi. Mycorrhizal fungi colonize cortical tissues of plant roots, play a crucial role in the environment, and also a central role in facilitating plant invasion of dry, nutrient poor land (Blackwell 2000; Redecker et al. 2000) during the Devonian Period (416 to 360 mya). They continue to play a pivotal role in the environment, conferring benefits on plants such as water uptake and the transfer of drought resistance (Safir and Boyer 1971; Sylvia and Williams 1992), uptake of phosphorous and nitrogen (Abbot and Robson 1984; George et al. 1995), protection against rhizosphere pathogens (Linderman and Hendrix 1982), and competitive advantage – relative to non-mycotrophice plants (Clark and Zeto 2000; Turnau and Haselwandter 2002). At the

ecosystem scale, mycorrhizae promote soil stabilization and influence the dynamics and structure of plant communities (Grime et al. 1987; Smith and Read 1997). The majority of vascular plants (95%) are associated with a phylogenetically diverse array of mycorrhizal fungi (Redecker et al 2000; Rinaldi et al 2008), which play a crucial role in plant colonization and distribution.

More recently, there has been an increase in studies on endophytic fungi associated with plant roots (Hyde and Soytong 2008; Sanchez-Marquez et al. 2008). Fungal endophytes are a diverse group of fungi that live asymptomatically in various plant organs, without causing disease symptoms (Petrini 1991; Wilson 1995; Tao et al. 2008). Recent investigations on plant roots suggest that plant roots harbor fungal endophytes, as well as mycorhizal fungi (Vallino et al. 2008; Schmidt et al. 2008). The classic model system of clavicipitaceous endophyte association is between a cool season C_3 grass and the fungus *Neotyphodium* species (Clay & Shardl 2002). Conversely, there have been few studies on warm season C_4 grasses and their associated (non-clavicipitaceous) endophytes. Fungal endophytes of C_4 grasses are typically identified as dark septate endophytes (DSE), a group of ascomycetes that colonize plant roots and that produce melanized, septate hyphae (Stoyke and Currah 1991, Jumpponen and Trappe 1998). A few reports on the distribution of endophytes in plant roots suggest that they have a similar role in plant health, aiding in nutrient uptake in nutrient limited environments (Addy et al 2005; Mandyam and Jumpponen 2005; Grünig et al. 2008 Knapp et al. 2012; Walsh et al 2014). Despite the benefits conferred by plant-fungal symbiosis, little is known about their diversity, taxonomy, ubiquity or ecological functions (Luo et al. 2014).

Switchgrass (*Panicum virgatum* L.) is a C_4 perennial grass that is native to North America (Keshwani & Chen 2009). It has adapted to a wide range of ecosystems occupying nutrient-poor habitats, high drought and high heat environments, enabling the plant to inhabit various regions throughout North America (Casler et al. 2007). Switchgrass has long been used as a forage crop, but more recently, has emerged as a potential bioenergy crop for cellulosic ethanol production in the United States. Broad adaptability, high biomass production, perennial growth, and the ability to grow on marginal land have made switchgrass an excellent candidate for biofuel production. In order to meet the stringent demands and goals of the US DEP to replace 30% of petroleum based with bio-fuels by 2030, bioenergy crops would require substantial increases in yield and dedicated acreage (Bouton 2007). Switchgrass responds favorably to fertilizer and pesticides (Parrish & Fikke 2005), but fossil-based inputs would reduce their net energy output as biofuels (Hill et al. 2006), add to environmental pollution (Tilman et al. 2002) and would add to greenhouse gas emissions (Robertson et al. 2000; Adler et al. 2007). It would be highly valuable to find an alternative path to sustainably increasing switchgrass biomass. The hope is that endophytic fungal communities harbor a diverse array of fungi, some of which may provide alternative approaches to maximizing productivity.

Luo et al. (2014) conducted a survey of fungal mycorrhizae associated with Poaceae in two ecosystems, the Pine Barrens in New Jersey, USA and the tropical rainforests of Yunnan, China (2014). They found that the Pine Barrens are a major reservoir of novel species, with roughly 47% of their samples consisting of previously undescribed species. The Pine Barrens is an ecosystem characterized by dry, acidic,

nutrient-poor soils, prone to fire (Forman 1998; Tedrow 1952). The 57,000 km^2 Pine Barrens, located in southern New Jersey, is the largest and most uniform Pine Barrens in the U.S., and is similar to other Pine Barrens scattered around the world (Forman 1998). Pine Barrens are dominated by the presence of pines and oaks, while the canopy gaps or disturbed areas are mainly occupied by grasses (Poaceae), sedges (Cyperaceae), orchids (Orchidaceae), blueberries and other (Ericaceae). Recently, a few novel fungal lineages associated with Poaceae roots have been described from this region. Three new genera have been described: *Pseudophialophora* in Magnoporthales (Luo et al. 2014), *Acidomelania* a DSE, closely related to PAC in Leotiomycetes (Walsh et al. 2014), and *Barrenia*, also a DSE in Leotiomycetes (Walsh, unpublished). Both *Acidomelania* and *Barrenia* have also been shown to promote root hair growth in switchgrass. Despite the preliminary assessment of the Pine Barrens, we do not know how the fungal communities of switchgrass located in the Pine Barrens differ from switchgrass grown on nutrient rich lands.

For this study, we used culture-independent methods to compare fungal endophytes in switchgrass located at Wharton State Forest in the NJ Pine Barrens and within the Pigmy Pine Barrens located near Wharton State Forest, both sampled in 2012, and cultivated switchgrass fields at the Rutgers University Turfgrass Extension at Adelphia, NJ in 2011 and 2012. Our understanding of fungal endophyte communities in the region is limited, so the objects of the study were to identify fungal endophytes and to from surface- sterilized switchgrass root tissue. I compared the sampled roots of apparently healthy switchgrass plants from across the various habitats and used Illumina Next Generation Sequencing (NGS) for genetic characterization. The data were analyzed

and the fungal root endophytes identified with a custom-developed pipeline (see Chapter 3). Our specific objectives were to: (1) taxonomically identify root-associated fungal endophytes of switchgrass, (2) use metagenomics to compare and evaluate these endophyte communities between natural and cultivated landscapes, (3) compare recovery success with that from traditional culturing methods, and (4) identify endophytic species that could be potentially introduced into switchgrass cultivars, thus enhancing bioenergy productivity.

Materials and Methods

Sample Collection

Environmental samples of naturally growing switchgrass (*Panicum virgatum*) were collected at Wharton State Forest (WSF), NJ (N 39º 46', W 74º 40') in June 2011 and 2012 and two locations in the Pigmy Pines (PPL1-2 and PPL1-3), outside of Wharton State Forest (N 39˚ 42', W 74˚ 22'). Two switchgrass samples were collected from nutrient rich farms at Adelphia, NJ (SWK69111 and CA612) (N 40˚ 13', W 74˚ 14') in 2011 and 2012. In both locations, lowland cultivars were selected for comparison. 'Kanlow' is a lowland type switchgrass that was released as a cultivar in 1963 (USDA), and was found at both WSF and the PP locations. Neither WSF nor PP environments were enriched with either fertilizer or cuttings and both regions are nutrient poor. 'Carthage' is another lowland switchgrass cultivar, developed in 1957 (USDA), and was the current cultivar that was being grown at the nutrient rich farmland. Although the samples from the PP locations are near WSF, the two landscapes are slightly different. In location PPL1-2, switchgrass plants were located near a small path, with a landscape occupied by small oaks and laurels. At location PPL1-3, Pitch Pines formed a denser

canopy and understory species were sparse. Switchgrass plants were collected during reproductive stages in June. At each location, 10 seemingly healthy switchgrass root samples were randomly collected, at least 5 m apart, to avoid sampling clonal ramets (Hartnett 1993; Kleczewski et al. 2012). Individual samples were placed in separated sterile zip-lock bags, placed in an iced cooler and transported to the laboratory for processing. Samples not used for traditional culturing were immediately placed into a -80˚ C freezer.

Endophyte Isolation

For each location, 10 individuals were sampled. A set of 10 individual root cuttings (10 cm in length) were randomly selected from each of the 10 individual samples and pooled together as one sample, so each site represents root cuttings from 10 distinct clones. Root fragments were surface sterilized under the following conditions in sequential order: 75% ethanol for 5 min., 0.6% sodium hypochlorite for 5 min, and rinsed in distilled water two times. The one hundred root samples were allowed to air dry before being plated on malt extract agar (MEA) with 0.07% lactic acid. All root samples were sequenced with ITS5, which is similar to ITS1, and ITS4 (White et al. 1990) primers. Sequences and spore morphology and characteristics were used to identify fungal species. A list of cultures and frequencies found in culture at all the locations can be found in the Supplementary Table 1. The rest of the samples were stored at -80ºC, and kept in the freezer until used for metagenomic analysis. The samples used for the metagenomic analysis were removed from the freezer and surface sterilized in the same fashion as the original culture samples.

Fungal DNA extraction and amplification

The sterilized root samples from each location were pooled together and were extracted with an UltraClean Soil DNA Isolation Kit (MoBio, California), following the manufacturer's protocol, with one modification by Luo Jing. Liquid Nitrogen was used to homogenize the root samples, instead of using the bead tubes provided in the kit. The ITS region was amplified with modified ITS1 and ITS2 primers, as shown in Table 4-1 (White et al. 1990; Bartram et al. 2011). These primers not only amplify the ITS region, but are complementary to Illumina forward, reverse and multiplexing sequencing primers that contain a 6 bp index to allow for multiplexing. All primers were created and purified by SDS-PAGE by IDT (Coralville, IA). PCR reaction mixture (25 µl) consisted of 5 µl of 5X Fusion GC Buffer (New England BioRad, MA, USA), 0.5 µl of 10 mM dNTPs mix, 1.25 µl of 10 µM forward primer and 1.25 µl of 10 µM reverse primer, 0.25 µl of Phusion DNA Polymerase, 1 µl of DNA from the sample, and 15.75 µl of DNase, RNase free water (Signma CO, US). Each sample was prepared 3 times in order to attempt different annealing temperatures, and pooled together after PCR amplification. Previous studies had shown that lower annealing temperatures aid in recovering taxa by alleviating primer mismatch; but higher annealing temperatures also aid in recovering taxa, due to enhanced primer binding (Fonseca et al. 2012; Schmidt et al. 2013). The PCR cycling conditions were as follows: 98˚ C for 30 s, followed by 25 cycles of denaturation at 98 ˚ C for 10 s, annealing at either 52˚ C, or 55˚ C, or 58˚ C for 30 s, and primer extension at 72˚ C for 30 s, followed by a final extension at 72˚ C for 5 minutes. PCR amplifications were verified using gel electrophoresis and purified with Qiagen's QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) twice, and again visualized on a gel to ensure primer dimers were removed. The purified PCR products with unique barcodes

were combined in equal nanogram quantity and quantified on a Nanoview and run on Illumina MiSeq located at Rutgers University, generating paired end reads of 2 x 300 bp. The sample was of low complexity, so Phi-X was added as a control.

Quality Filtering and Taxonomic assignment

Sequences were de-multiplexed using Illumina Basespace web-based cloud service (basespace.illumina.com). The protocols for filtering, clustering and assigning taxonomy to the reads described in Chapter 3 were used here. OTUs were assigned using UPARSE with a threshold of 97% sequence identity. OTUs were classified taxonomically using a custom built fungal database, a modified database of sequences downloaded from Genbank that eliminates "uncultured", "unknown" and "environmental" sequences and was created on April 15, 2014 in BLAST+ v2.2.28 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST). Finally to assign taxonomy MEGAN v5.0 or later is needed (http://ab.inf.uni-tuebingen.de/software/megan5/). MEGAN LCA parameters were set as following: Min Score 120, Top Percent 1.0, Min Support: 1, LCA Percent 100, and Use Minimal Coverage Heuristic checked.

Endophyte Diversity Analyses

Fungal species were grouped according to their highest taxonomic level (ordinal) and at species level to be evaluated. OTU richness comparisons between sites were carried out with 50 randomizations of sample order in EstimateS 9.1, for the following similarity indices: Classical Jaccard, Sorensen, Adjusted Jaccard, Adjusted Sorensen, Morisita-Horn and Bray-Curtis (Colwell 2005) and were verified manually (Table 4-2 to Table 4-4). However, only Morisita-Horn estimates will be discussed in results and interpretation. Both Sorensen and Jaccard perform poorly when species are missing or when there are

only a few dominant species and many rare species. Bray-Curtis also performs poorly when sampling fractions are not equal (Chao et al. 2006). All other statistical tests such as two-tailed *t*-test and ANOVA were performed on SPSS (IBM Corp. Inc. 2012).

Results

Fungal Endophyte Diversity

The cultures grown from the locations in the PP, WSF, and Adelphia farms are the cultures recorded by Walsh et al. (unpublished). There were a total of seven fungal classes identified, but only one of them (Chaetothyriomycetes) was absent from the culture data. There were a total of 38 species (OTUs) in the culture data and a total of 80 different species-level OTUs in the metagenomics analysis. Only 16% of the species were shared by both assay procedures (Table 1): *Acidomelania panicicola*, *Alternaria longissima*, *Barrenia panicia*, *Fusarium oxysporum*, *Leptodontidium orchidicola*, and *Rhizoctonia solani*. All sites were not significantly different between the culture and metagenomic analyses at the class level (Table 4-2 to 4-4), but at the species level, sites SWK69111, CA612, PPL1-3 were significantly different ($p < 0.05$), while WSF and PPL1-2 were not significantly different.

Diversity of fungal endophyte communities at the class and species level

Endophytic isolates from WSF belonged to four of the severn fungal classes based on the culture data and five of the seven fungal classes in the metagenomic data, with the Leotiomycetes being the most dominant according to both culture and metagenomic analyses, at 53% and 85%, respectively (Fig. 1). Species in the class of Leotiomycetes were the most frequently recovered fungi, representing 85% and 92.5% of the fungal communities in the cultural and metagenomic analyses, respectively. The most dominant

species found in the culture data was *Barrenia panicica*, which accounts for the entire 85% of the Leotiomycetes species found in culture. According to the metagenomic analysis, *Acidomelania panicola* (38%), *Barrenia panicica* (31%), and *Lachnum pygmaeum* (22%) were the dominant species in the analysis, constituting 91% of the 92.5% of the Leotiomycetes species, respectively.

In contrast to WSF, for both locations SWK69111 and CA612 at Adelphia, Leotiomycetes composed only 14% and 5% in culture data, respectively, and 1% and 6% in the metagenomic analyses, respectively. Sordariomycetes were the dominant cclass in both the culture and metagenomic analyses, representing 67% and 72% of the cultured samples from SWK69111 and CA612, respectively, and 58% and 65% of the metagenomics samples, respectively. *Fusarium oxysporum* makes up 19% of the Sordadiomycetes in culture, and combined with *Fusarium fujikuroi*, *Fusarium proliferatum* and *Fusarium verticilliodes*, the genus *Fusarium* represents 33% of the Sordariomycetes species found in culture. In the metagenomic analysis, *Fusarium* represented less than 1% of the species. The main Sordariomycetes species present in the metagenomics sample was *Hypocrea* sp.-1, which accounts for 58% of the Sordariomycete specimens and 42% overall. The other Sordariomycetes (*Verticillium* sp. 1, *Hypoxylon submonticulosum*, and *Pleuroceras* sp. BPI 87723A) make up 41% of the Sordariomycetes and 30% of the overall species present. In CA612, *Fusarium* species represent the dominant Sordariomycetes in culture (81%), but no *Fusarium* species were found by metagenomic analysis. *Hypocrea* sp. 1 represents 53% of the Sordariomycetes and 34% overall, with *Verticillium* sp. 1, *Hypoxylon submonticulosum*, and *Pleuroceras* sp. BPI 87723A composing the rest of the Sordariomycetes.

In PPL1-2, Sordariomycetes were the dominate class in culture (61%) and consisted entirely of *Fusarium* species (*F. oxysporum, F. verticilliodes, F. fujikuroi*, *Fusarium* sp. 1). However, there was still a large portion of Leotiomycetes present (23%) which consisted of *Barrenia panicia* and *Phialocephala* sp. 1, which made up 92% and 7% of the Leotiomycetes species in the sample respectively. There were no Basidiomycetes found through culturing techniques. However, in the metagenomics analysis, Basidiomycetes were the dominant class (48%), followed by Leotiomycetes at 39% and Sordariomycetes at 10% of the total community. *Barrenia panicia* was 91% of the total Leotiomycetes found in the metagenomics analysis.

At location PPL1-3, 68% of the culture data consisted of Leotiomycetes, entirely *Barrenia panicia*. The rest of the cultured entries were Eurtiomycetes (16%), Sordariomycetes (12%), Dothideomycetes (3%) and Zygomycetes (1%). Again, there were no Basidiomycetes found through the culturing analysis. In the metagenomic analysis, Basidiomycetes were again the dominate class at 53%. Leotiomycetes made up 23% of the captured ITS sequences with *Barrenia panicia* making up 98% of the Leotiomycetes sequences. Sordariomycetes made up 21% of the community sequences as well.

Comparison of fungal endophyte composition between site locations

The greatest numbers of Sordariomycetes was found at the managed Adelphia site for both 2011 (SWK69111) and 2012 (CA612). This location also had the highest overlap similarity in the metagenomics level and one of the highest in the culture data as well (MH 0.990 and 0.920 respectively) (Table 3). Leotiomycetes were largely located at the unmanaged location WSF and PPL1-3 in culture, but the metagenomic analysis revealed

that Basdiomycetes were more dominant at locations PPL1-2 and PPL1-3. WSF showed the lowest amount of overlap between the managed sites SWK69111 and CA612 in both the culture and metagenomic analysis. These two locations differ in being unmanaged (WSF) and managed with fertilizers, pesticides and cutting (SWK69111 and CA612). The soils at these locations are significantly different in pH, Potassium (ppm), Magnesium (ppm) Total Nitrogen %, and Total Phosphorus %, with WSF being the lowest in available nutrients and pH (Table 4)

PPL1-2 and PPL1-3 have similar soil composition to WSF and are significantly different from the managed Adelphia sites. In culture, PPL1-2 was more closely related to Adelphia in both years than to WSF, and PPL1-3 was more closely related to WSF than to Adelphia in both years. However, in the metagenomic analysis, we see the reverse trend, where PPL1-2 is more closely related to WSF then to Adelphia, and PPL1-3 is more closely related to Adelphia in 2011, but not in 2012.

Discussion

Diversity of fungal endophytes between wild native habitats vs managed habitats

Switchgrass studies typically focus on mycorrhizal fungi (Cullings and Makhita 2001; Hempel et al. 2007; Appoloni et al 2008). Here we used a global approach with metagenomics to identify and quantify species present, demonstrating differences between unmanaged native habitats (WSF, PPL1-2 and PPL1-3) and managed habitats (SWK69111 and CA612). At the metagenomic level, we were able to identify seven classes and 80 OTUs across all habitats, an additional class and 41 more OTUs than by traditional culturing. Studies of fungal endophytes on all plant life are typically dominated by a few species with high frequencies and many species with low

frequencies, both with metagenomics and culture-based studies (Jumpponen and Jones 2009; Amend et al. 2010; Gazis and Chaverri 2010; Ghimire et al. 2011, Kleczewski et al. 2012). By comparing endophyte diversity between nutrient rich and nutrient poor samples, endophytic fungi associated with plant growth, persistence, and stress tolerance from "barren" environments are identified. It is anticipated that these fungal endophytes can be utilized to associate with switchgrass cultivars to maximize their potential as a biofuel crop.

Leotiomycetes were the dominant group in WSF, compared to the managed farms in Adelphia, where Sordariomycetes were dominant in both years. The diversity indices show that WSF and Adelphia had very little overlap in 2011 and 2012, at either the class or species levels. Adelphia had the highest similarity of any comparison (MH = 0.990) between 2011 and 2012, suggesting that fungal composition at a managed farm does not change much from year to year, though a larger sample size from year to year is needed to determine the variation of Adelphia from year to year. Despite similar soil composition in the Pigmy Pine locations and WSF, the metagenomics analysis revealed that Basidomycetes were dominant at PPL1-2 and PPL1-3. Both locations had higher levels of Leotiomycetes than Sordariomycetes. At the species level, *Acidomelania panicicola* and *Barrenia panicica* were the dominant Leotiomycetes found throughout all the sites. At the managed farm, *Hypocrea* sp. 1 was the dominant endophyte in the metagenomic analysis. The results reported at Adelphia are similar to other studies on Poaceae (e.g., *Dactylis glomerata*, *Festuca paniculata*, *Holcus lanatus*) in similar environments, including switchgrass (Marquez et al. 2008; Marquez etl al. 2010; Mouhamadou et al. 2011), in which species in the Sordariomycetes were the dominate endophytes recovered.

Metagenomic analysis compared to culture analysis

At the class level, metagenomic analysis showed no statistical differences from the culturing analysis, but at the species level, sites SWK69111, CA612 and PPL1-3 were significantly different ($p < 0.05$), while WSF and PPL1-2 were not significantly different. More important, only 6% of species were recovered by both analyses. Despite using the metagenomic pipeline from Chapter 3, metagenomics should be viewed as complementary to (rather than a substitute for) traditional culturing methods. Factors that may account for the observed taxonmic differences include sequencing error, PCR primer bias, limited clustering options, and incorrect taxonomic assignment. In the metagenomics analysis, for example, *Fusarium oxysporum* was the only *Fusarium* species to be identified, compared with eight different *Fusarium* species found by culturing. *Fusarium* species are cryptic, due to their low nucleotide divergence, relative to other clades. It is necessary to use multiple genes in order to decipher cryptic species (O'Donnell et al. 2008). Current bioinformatics software limits clustering to 97%. The species clustered into the *Fusarium* OTU cannot be identified with currently available software.

As mentioned above, Luo et al. (2014) found that 47% of cultured isolates of grass root colonizing fungi from the Pine Barrens were undescribed. It is critical that species be recovered, identified, and placed into a curated database. Further compounding the problem is the lack of available databases and a large number of sequences in GenBank that consist of "environmental" samples that have yet to be incorporated into the taxonomic code. Hibbett et al. (2011) estimates it will take up to 4,000 years to describe all species of Fungi, using the current specimen-based taxonomy. In order to

facilitate deeper analysis into fungal biodiversity and ecology, there is a strong imperative to develop a classification system based on environmental samples. Hypothetical examples have been presented based on pure DNA extracts of a single fungus by Reynolds and Taylor (1991), but the mycological community and experts on particular clades must establish a consensus on how to approach the larger problem.

Potential endophytes to increase plant growth.

Fungal endophytes may be used to increase the bio-energy output of switchgrass, without the addition of fossil fuel-based soil amendments. Leotiomycete species, in particular *Acidiomelania panicicola* and *Barrenia panicica*, would be excellent candidates for further exploration of their effects on switchgrass growth. *Acidomelania panicicola* is widespread around the globe (Walsh et al. 2014) and is usually associated with plants found in acidic or infertile growing conditions (Keddy 2007). Their detection at all our sample locations, as well as globally, is consistent with the observation that DSE are ubiquitous and widespread in plant roots (Jumpponen and Trappe 1998; Schadt et al. 2001; Walsh et al. 2014). Research has shown that DSE aid in plant protection and survival, typically for populations under abiotic stress. DSE are able to trap free radicals generated under stressful conditions and/or aid in nutrient uptake. The relatively high abundance of Leotiomyctes (*A. panicicola* and *B. panicica*) at WSF, which has significantly lower nutrients than the managed fields, suggests that these two species contribute to the growth of switchgrass. Further sampling and experiments utilizing one or a combination of these endophytes are warranted.

To our knowledge, this is one of the first attempts to use metagenomic analysis for this important bioenergy crop. Our approach has catalogued species from an

environment that is nutrient poor, in order to identify endophytes that may well play an important role in maximizing the sustainable growth of switchgrass, while minimizing the cost of growing the bioenergy crop. We also compared our metagenomic analysis to traditional culturing techniques (Walsh et al., unpublished). By comparing the fungal communities between nutrient poor and managed farms, I have shown that switchgrass may harbor fungal endohpytes that are beneficial to the plant biomass production that can aid in development of its utility as a biofuel along with its physical attributes.

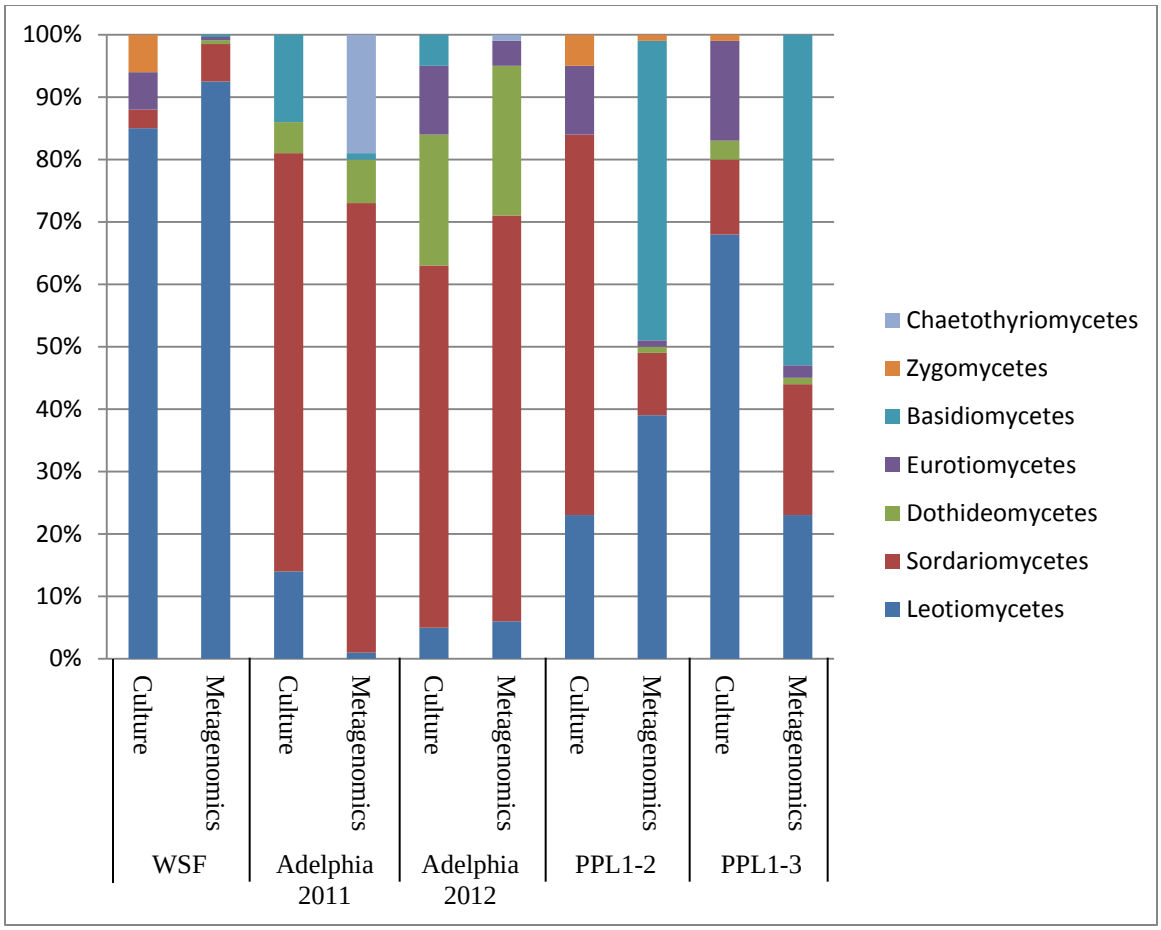

Figure 4-1. Comparison between culture and metagenomics relative abundances at class level.

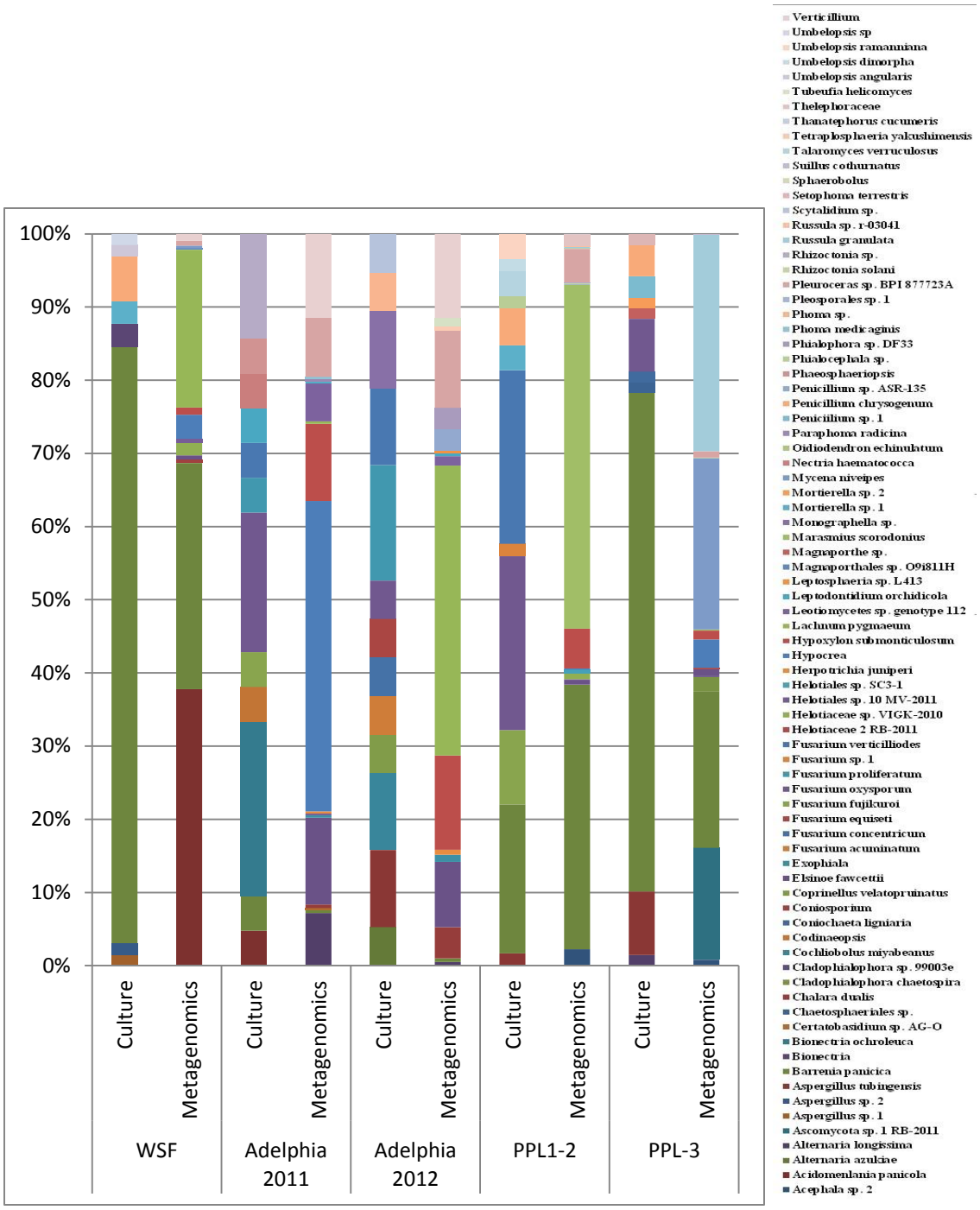

Figure 4-2 Comparison between culture and metagenomics relative abundance at species level. (*Acephala sp. 2, Acidomenlania panicola, Alternaria azukiae, Alternaria longissima, Ascomycota sp. 1 RB-2011, Aspergillus tubingensis, Barrenia panicica, Bionectria, Bionectria ochroleuca, Certatobasidium sp. AG-O, Chaetosphaeriales sp., Chalara dualis, Cladophialophora chaetospira, Cladophialophora sp. 99003e, Cochliobolus miyabeanus, Codinaeopsis, Coniochaeta ligniaria, Coniosporium, Coprinellus velatopruinatus, Elsinoe*

fawcettii, Exophiala, Fusarium acuminatum, Fusarium concentricum, Fusarium equiseti, Fusarium fujikuroi, Fusarium oxysporum, Fusarium proliferatum, Fusarium sp. 1, Fusarium verticilliodes, Helotiaceae 2 RB-2011, Helotiaceae sp. VIGK-2010, Helotiales sp. 10 MV-2011, Helotiales sp. SC3-1, Herpotrichia juniperi, Hypocrea, Hypoxylon submonticulosum, Lachnum pygmaeum, Leotiomycetes sp. genotype 112, Leptodontidium orchidicola, Leptosphaeria sp. L413, Magnaporthales sp. O9i811H, Magnaporthe sp., Marasmius scorodonius, Monographella sp., Mortierella sp. 1, Mortierella sp. 2, Mycena niveipes, Nectria haematococca, Oidiodendron echinulatum, Paraphoma radicina, Peniciilium sp. 1, Penicillium chrysogenum, Penicillium sp. ASR-135, Phaeosphaeriopsis, Phialocephala sp., Phialophora sp. DF33, Phoma medicaginis, Phoma sp., Pleosporales sp. 1, Pleuroceras sp. BPI 877723A, Rhizoctonia solani, Rhizoctonia sp., Russula granulata, Russula sp. r-03041, Scytalidium sp., Setophoma terrestris, Sphaerobolus, Suillus cothurnatus, Talaromyces verruculosus, Tetraplosphaeria yakushimensis, Thanatephorus cucumeris, Thelephoraceae, Tubeufia helicomyces, Umbelopsis angularis, Umbelopsis dimorpha, Umbelopsis ramanniana, Umbelopsis sp, Verticillium, Acephala sp. 2, Acidomenlania panicola, Alternaria azukiae, Alternaria longissima, Ascomycota sp. 1 RB-2011, Aspergillus tubingensis, Barrenia panicica, Bionectria, Bionectria ochroleuca, Certatobasidium sp. AG-O, Chaetosphaeriales sp., Chalara dualis, Cladophialophora chaetospira, Cladophialophora sp. 99003e, Cochliobolus miyabeanus, Codinaeopsis, Coniochaeta ligniaria, Coniosporium, Coprinellus velatopruinatus, Elsinoe fawcettii, Exophiala, Fusarium acuminatum, Fusarium concentricum, Fusarium equiseti, Fusarium fujikuroi, Fusarium oxysporum, Fusarium proliferatum, Fusarium sp. 1, Fusarium verticilliodes, Helotiaceae 2 RB-2011, Helotiaceae sp. VIGK-2010, Helotiales sp. 10 MV-2011, Helotiales sp. SC3-1, Herpotrichia juniperi, Hypocrea, Hypoxylon submonticulosum, Lachnum pygmaeum, Leotiomycetes sp. genotype 112, Leptodontidium orchidicola, Leptosphaeria sp. L413, Magnaporthales sp. O9i811H, Magnaporthe sp., Marasmius scorodonius, Monographella sp., Mortierella sp. 1, Mortierella sp. 2, Mycena niveipes, Nectria haematococca, Oidiodendron echinulatum, Paraphoma radicina, Peniciilium sp. 1, Penicillium chrysogenum, Penicillium sp. ASR-135, Phaeosphaeriopsis, Phialocephala sp., Phialophora sp. DF33, Phoma medicaginis, Phoma sp., Pleosporales sp. 1, Pleuroceras sp. BPI 877723A, Rhizoctonia solani, Rhizoctonia sp., Russula granulata, Russula sp. r-03041, Scytalidium sp., Setophoma terrestris, Sphaerobolus, Suillus cothurnatus, Talaromyces verruculosus, Tetraplosphaeria yakushimensis, Thanatephorus cucumeris, Thelephoraceae, Tubeufia helicomyces, Umbelopsis angularis, Umbelopsis dimorpha, Umbelopsis ramanniana, Umbelopsis sp, Verticillium).

Table 4-1. List of fungal OTUs from cultured and metagenomic sampling. An * denotes how each OTU was characterized.

Class	OTU	Culture	Metagenomic
Leotiomycetes	Acidomelania panicicola	*	*
Dothideomycetes	Alternaria azukiae	*	
Dothideomycetes	Alternaria longissima	*	*
Sordariomycetes	Ascomycota sp. 1 RB-2011		*
Eurotiomycetes	Aspergillus tubingensis	*	
Leotiomycetes	Barrenia panicia	*	*
Sordariomycetes	Bionectria	*	
Sordariomycetes	Bionectria ochroleuca	*	
Basdiomycetes	Certatobasidium sp. AG-O		*
Sordariomycetes	Chaetosphaeriales sp.	*	
Leotiomycetes	Chalara dualis		*
Eurotiomycetes	Cladophialophora chaetospira		*
Eurotiomycetes	Cladophialophora sp. 9003e		*
Dothideomycetes	Cochliobolus miyabeanus	*	
Sordariomycetes	Codinaeopsis	*	
Sordariomycetes	Coniochaeta ligniaria	*	
Eurotiomycetes	Coniosporium		*
Basdiomycetes	Coprinellus velatopruinatus	*	
Dothideomycetes	Elsinoe fawcettii		*
Chaetothyriomycetes	Exophiala		*
Sordariomycetes	Fusarium acuminatum	*	
Sordariomycetes	Fusarium concentricum	*	
Sordariomycetes	Fusarium equiseti	*	
Sordariomycetes	Fusarium fujikuroi	*	
Sordariomycetes	Fusarium oxysporum	*	*
Sordariomycetes	Fusarium sp. 1	*	
Sordariomycetes	Fusarium sp. 2	*	
Sordariomycetes	Fusarium verticilliodes	*	
Sordariomycetes	Gaeumannomces		*
Leotiomycetes	Helotiaceae 2 RB-2011		*
Leotiomycetes	Helotiaceae sp. VIGK-2010		*
Leotiomycetes	Helotiales sp. 10 MV-2011		*
Leotiomycetes	Helotiales sp. SC3-1		*
Dothideomycetes	Herpotrichia juniperi		*
Sordariomycetes	Hypocrea sp. 1		*
Sordariomycetes	Hypocreaceae		*
Sordariomycetes	Hypoxylon submonticulosum		*

Leotiomycetes	Lachnum pygmaeum		*
Leotiomycetes	Leotiomycetes sp. genotype 112		*
Leotiomycetes	Leptodontidium orchidicola	*	*
Dothideomycetes	Leptosphaeria sp. L413		*
Sordariomycetes	Magnaporthales sp. O9i811H		*
Sordariomycetes	Magnaporthe sp.	*	
Basdiomycetes	Marasmius scordonius		*
Sordariomycetes	Monographella sp.	*	
Zygomycetes	Mortierella sp. 1	*	
Zygomycetes	Mortierella sp. 2	*	
Basdiomycetes	Mycena sp. 1		*
Basdiomycetes	Mycena niveipes		*
Sordariomycetes	Nectria haematococca	*	
Leotiomycetes	Oidiodendron sp.1		*
Leotiomycetes	Oidiodendron echinulatum		*
Sordariomycetes	Paraphoma radicina		*
Eurotiomycetes	Peniciilium sp. 1	*	
Eurotiomycetes	Penicillium chrysogenum	*	
Eurotiomycetes	Penicillium sp. ASR-135		*
Dothideomycetes	Phaeosphaeriopsis	*	
Eurotiomycetes	Phialophora sp. DF33		*
Sordariomycetes	Phoma medicaginis		*
Dothideomycetes	Phoma sp.	*	
Sordariomycetes	Pleosporales		*
Sordariomycetes	Pleuroceras sp. BPI 87723A		*
Basdiomycetes	Rhizoctonia solani	*	*
Basdiomycetes	Russula granulata		*
Basdiomycetes	Russula sp. r-03041		*
Leotiomycetes	Scytalidium sp.	*	
Dothideomycetes	Setophoma terrestris	*	
Basdiomycetes	Sphaerobolus		*
Basdiomycetes	Suillus cothurnatus		*
Eurotiomycetes	Talaromyces verruculosus	*	
Dothideomycetes	Tetraplosphaeria yakushimensis		*
Basdiomycetes	Thanatephorus cucumeris		*
Basdiomycetes	Thelephoraceae		*
Dothideomycetes	Tubeufia helicomyces		*
Zygomycetes	Umbelopsis		*
Zygomycetes	Umbelopsis angularis	*	
Zygomycetes	Umbelopsis dimorpha	*	
Zygomycetes	Umbelopsis ramanniana	*	

Zygomycetes	Umbelopsis sp	*	
Sordariomycetes	Verticillium		*

Table 4-2: Comparison of Morsita Horn (Mo-Ho) and Bray Curtis (BC) in Traditonal vs Metagenomics at the species level, but, only Mo-Ho was considered statistically relevant. SWK6911, CA612 and PPL1-3 were significantly different ($p<0.05$) At the class level no statistical differences were noticed.

Mo-Ho	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.230	0.126	0.381	0.964	WSF
SWK	0.148	---	0.920	0.949	0.355	SWK
CA-612	0.137	0.99	---	0.906	0.282	CA-612
PPL1-2	0.592	0.213	0.205	---	0.515	PPL1-2
PPL1-3	0.359	0.362	0.356	0.944	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	Mo-Ho

BC	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.196	0.123	0.366	0.829	WSF
SWK	0.133	---	0.666	0.435	0.266	SWK
CA-612	0.160	0.749	---	0.373	0.298	CA-612
PPL1-2	0.481	0.203	0.205	---	0.444	PPL1-2
PPL1-3	0.303	0.372	0.266	0.820	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	BC

Table 4-3: Comparison of Jaccard Classic (JC) and Sorenson (SC) in Traditonal vs Metagenomics at the species level. These measures are statistical unreliable due to reliance on presence/absence, but are reported for comparison.

JC	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.333	0.600	1	0.600	WSF
SWK	0.666	---	0.600	0.333	0.500	SWK
CA-612	0.666	0.666	---	0.600	0.800	CA-612
PPL1-2	0.481	0.571	0.571	---	0.515	PPL1-2
PPL1-3	0.999	0.666	0.666	0.833	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	JC

SC	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.500	0.750	1	0.750	WSF
SWK	0.800	---	0.750	0.500	0.666	SWK
CA-612	0.800	0.800	---	0.750	0.888	CA-612
PPL1-2	0.833	0.727	0.727	---	0.888	PPL1-2
PPL1-3	0.999	0	0.800	0.909	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	SC

Table 4-4: Comparison of Jaccard adjusted (JA) and Sorenson Adjusted (SA) in Traditonal vs Metagenomics at the species level. These measures are not discussed in depth but provided for comparison..

JA	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.724	0.738	1	0.91	WSF
SWK	0.992	---	0.774	0.702	0.726	SWK
CA-612	0.987	0.946	---	0.745	0.985	CA-612
PPL1-2	0.909	0.995	0.507	---	0.971	PPL1-2
PPL1-3	0.999	0.975	0.465	0.999	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	JA

SA	WSF	SWK	CA-612	PPL1-2	PPL1-3	Traditional
WSF	---	0.840	0.849	1	0.953	WSF
SWK	0.996	---	0.872	0.825	0.841	SWK
CA-612	0.993	0.972	---	0.854	0.992	CA-612
PPL1-2	0.999	0.997	0.672	---	0.985	PPL1-2
PPL1-3	0.999	0.987	0.635	0.999	---	PPL1-3
MetaGeno	WSF	SWK	CA-612	PPL1-2	PPL1-3	SA

Chapter 5

Summary and Future Directions

This dissertation sets out to establish the biodiversity and ecology of fungal endophytes and DSE in two econonomically and biologically model systems of native dogwoods and switchgrass. It has also set out to establish a metagenomics pipeline specifically catered to ascertaining fungi. As reported, despite the surge and enthuasiam in uncovering fungi endophyte biodiversity, their true diversity, ecological roles, and spatial heterogeneity are still unknown. Even though native species of *Cornus*, in particularly *C. florida* and *C. nuttallii*, are an important ecological understory tree and a significant economically valuable tree, and is severly threatened by the pathogen *D. destructiva*, it's fungal community has not been studied in detail. Based on current knowledge of the pathogen, it is highly likely that this species is an endophyte with latent pathogen biology. It is critical to to catalogue and the endophytic community, which could contain beneficial and mutualistic species to utilize as a bio-control agent. There have been only a few studies on species of *Cornus* however, these species are not genetically similar to the native North American species. Chapter 2 presents a preliminary assessment of the fungal communities present in the leaves of this tree. Collection sites were all in wild and in the case of NJ, Hutchinson Memorial forest is one of the oldest uncut forests. In this chapter I give a preliminary overview of diversity and abundance of fungal endophytes in the native North American species as well as a native Japanese tree. This illustrates the differences between these two hosts, and presents species that can be used in future bio-control research.

From chapter 2 it become clear that traditional techniques were not efficient in capture the true fungal biodiversity in plant hosts, and many studies the author calls for

the use of culture-independent assessments. It also became clear that many of the techniques used to analyze fungal biodiversity originated and were tailored for prokaryotes. Many studies from 454 and Illumina are not comparable, due to the differences in methodology. It is clear that a standard, user-friendly pipeline, specifically geared to fungi, will be critical to elucidate fungal diveresity. The main objective of Chapter 3 was to compile a methodology for fungal metagenomics and to validate a pipeline that can be used and updated for various types of fungal endophyte research. By testing a mock community as well as testing an environmental sample, I have compiled a customized pipeline that is efficient in capturing the diversity and abundance of fungal endophytes.

Chapter 4 utlizes this pipeline to uncover the biodiversity of DSE of switchgrass, which is an important biofuel crop. The roles of DSE are drastically understudied in terms of biodiversity and ecology compared with class 3 fungal endophytes. This dissertation represents an initial survey of the diversity of DSE. Previous work done in the lab on the DSE associated with switchgrass roots was done using traditional culturing work. Here, I used a metagenomic approach instead, and establish a preliminary baseline for this neglected class of endophytes. I find that metagenomic analysis captures more diversity than traditional culturing methods. I demonstate significant similarity between culture and metagenomics analysis at the class level, but identify important mutualistic species associated with the host, potentially useful for further biomass research for switchgrass.

In conclusion fungal endophyte diversity and ecology are exciting subjects for research. If dogwood species are to be relieved of the devastating pathogen *Discula*

destructiva, cataloging of the fungal biodiversity is only the first step. It is critical that future research utilizes the potential biological antagonists identified in this thesis, but also to utlize a metagenomic approach in general. Although the pipeline created in this thesis can be used as a standard research tool for fungi, similar to QIIME for bacteria, it will remain crucial to run cultural studies side by side. At the moment, the only way to describe new species is to have a cultural deposit as a type species. Also, given the intra- and inter-variation of ITS, it is critical to capture species through culture and use multiple gene sequences to identify cryptic species. Although metagenomics analysis captures more fungal diversity overall, many clades will remain under-represented. Either the chemisty must be changed to allow for multiple genes to be sequenced at once, or new clustering options must be developed. Finally, the biodiversity and ecology of DSE are clearly understudied. There is also a strong need to individually assess multiple different host species in numerous types of ecological studies in order to ascertain the true ecological roles fungal endophytes.

Literature Cited

Abarenkov, K., Tedersoo, L., Nilsson, R.H., Vellak, K., Saar, I., Veldre, V., Parmasto, E., Prous, M., Aan, A., Ots, M., Kurina, O., Ostonen, I., Joegeva, J., Halapuu, S., Poldmaa, K., Toots, M., Truu, J., Larsson, K.H. & Koljalg, U. 2010. PlutoF-a Web Based Workbench for Ecological and Taxonomic Research, with an Online Implementation for Fungal ITS sequences. Evolutionary Bioinformatics 6: 189-196.

Abbott LK, Robson AL (eds). 1984. The effect of VA mycorrhizae on plant growth. CRC, Boca Raton.

Addy HD, Piercey M, Currah RS. 2005. Microfungal endophytes in roots. Canandian Journal of Botany 83:1–13.

Adler PR, Del Grosso SJ, Parton WJ. 2007. Life-cycle assessment of net greenhouse-gas flux for bioenergy cropping systems. Ecological Applications 17: 675e691.

Akello J, Dubois T, Coyne D, Kyamanywa S. 2008. Endophytic *Beauveria bassiana* in banana (*Musa spp*.) reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. Crop Protection 27: 1437–1441. doi: 10.1016/j.cropro.2008.07.003.

Alexopoulos CJ, Mims CW, Blackweil M. 1996. Introduction Dry Mycology. New York. USA; John Wiley and Sons. Inc, 868 pp.

Allen MF. 1991. The ecology of mycorrhizae. New York, NY, USA: Cambridge University Press, 184pp.

Álvarez-Loayza P, White JF, Torres MS, Balslev H, Kristiansen T, J. Svenning J and Gil N. 2011. Light converts endosymbiotic fungus to pathogen, influencing seedling survival and niche-space filling of a common tropical tree, Iriartea deltoidea. PLoS One. 6: e16386.

Amend AS, Seifert KA, Samson R and Bruns TD. 2010. Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. *Proceedings of the National Academy of Sciences* 107(31): 13748-13753.

Anderson RL, Knighten JL, and Dowsett SE. 1990. Dogwood anthracnose: a Southeastern United States perspective. In 23rd annual Tennessee nursery short course Tennessee Nursery Industry, Nashville, TN, pp. 14-18.

Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nygren P and Engstrand L. 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE 3: e2836.

Arnold AE and Herre EA. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). Mycologia 95: 88–398.

Arnold AE and Lutzoni F. 2007. Diversity and host range of foliar fungal endophytes: are tropical trees biodiversity hot spots? Ecology 88: 541–549.

Arnold AE, Henk DA, Eells R, Lutzoni F, Vilgalys R. 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99: 185–206.

Arnold AE, Maynard Z, Gilbert GS, Coley PD and Kursar TA. 2000. Are tropical fungal endophytes hyperdiverse? Ecology Letters 3: 267–274.

Arnold AE, Maynard Z, Gilbert GS. 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycological Research 105: 1502–1507.

Arnold AE, Mejı´a LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America 100: 15649–15654.

Arnold AE, Miadlikowska J, Higgins KL, Sarvate SD, Gugger P, Way A, Hofstetter V, Kauff F, Lutzoni F. 2009. A phylogenetic estimation of trophic transition networks for ascomycetous fungi: Are lichens cradles of symbiotrophic fungal diversification? Systematic Biology 58(3): 283-297.

Arnold AE. 2005. Diversity and ecology of fungal endophytes in tropical forests. In: Biodiversity of Fungi: their role in human life. Deshmukh SK, Rai MK (Eds). Science Publishers Inc. Enfield (NH), pp. 50–68.

Arnold AE. 2007. Understanding the diversity of foliar fungal endophytes: progress, challenges, and frontiers. Fungal Biology Reviews 21: 51–66.

Arnold, AE, Engelbrecht BJM. 2007. Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species. Journal of Tropical Ecology 23: 369–372.

Bailey BA, Bae H, Melnick R, Crozier J. 2011. The Endophytic Trichoderma hamatum Isolate DIS 219b Enhances Seedling Growth and Delays the Onset of Drought Stress in Theobroma cacao. In: Endophytes of Forest Trees: Biology and Applications. Pirttilä AM, Frank AC (Eds.). Forestry Sciences, Springer Science, pp. 157-172.

Bailey BA, Bae H, Strem MD, Crozier J, Thomas SE, Samuels GJ, Vinyard BT, Holmes KA. 2008. Antibiosis, mycoparasitism, and colonization success for endophytic

Trichoderma isolates with biological control potential in Theobroma cacao. Biological Control 46: 24–35.

Bálint M, Schmidt PA, Sharma R, Thines M and Schmitt I. 2014. An Illumina metabarcoding pipeline for fungi. Ecology and Evolution 4(13): 2642-2653.

Bálint M, Tiffin P, Hallström B, O'Hara R, Olson MS, Fankhauser JD, Piepenbring M and Schmitt I. 2013. Host genotype shapes the foliar fungal microbiome of Balsam poplar. PLoS One 8: e53987.

Barengo N, Sieber TN and Holdenrieder O. 2000. Diversity of endophytic mycobiota in leaves and twigs of pubescent birch (Betula pubescens). Sydowia 52: 305–320.

Bartram AK, Lynch MDJ, Stearns JC, Moreno-Hagelsieb G and Neufeld JD. 2011. Generation of Multimillion-Sequence 16S rRNA Gene Libraries from Complex Microbial Communities by Assembling Paired-End Illumina Reads. *Applied and* Environmental Microbiology 77(11): 3846-3852.

Basset Y, Samuelson GA, Allison A, Miller SE. 1996. How many species of host-specific insects feed on a species of tropical tree? Biological Journal of the Linnean Society 59(2): 201-216.

Bazzicalupo AL, Bálint M. and Schmitt I. 2013. Comparison of ITS1 and ITS2 rDNA in 454 sequencing of hyperdiverse fungal communities. Fungal Ecology 6: 102-109.

Blaalid R, Kumar S, Nilsson RH, Abarenkov K, Kirk PM and Kauserud H. 2013. ITS1 versus ITS2 as DNA metabarcodes for fungi. Molecular Ecology 13(2): 218-224.

Blackwell M. 2011. The fungi: 1, 2, 3 … 5.1 million species? American Journal of Botany 98 (3): 426-438.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA and Caporaso JG. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nature Methods 10(1): 57-60.

Boyette CD, Hoagland RE, Weaver MA, Stetina K. 2012. Biological control potential of *Colletotrichum gloeosporioides* for coffee senna (*Cassia occidentatlis*). American Journal of Plant Sciences 3: 430–436.

Boyette CD, Hoagland RE, Weaver MA. 2007. Biocontrol efficacy of *Colletotrichum truncatum* for hemp sesbania (*Sesbania exaltata*) is enhanced with inrefined corn oil and surfactant. Weed Biology and Management 7: 70–76.

Brasier CM. 1995. Episodic selection as a force in fungal microevolution, with special reference to clonal speciation and hybrid introgression. Canadian Journal of Botany 73 (S1): 1213–1221.

Buée M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S and Martin F. 2009. 454 Pyrosequencing analyses of forest soils reeal an unexpectedly high fungal diversity. New Phytologist 184: 449–456.

Burges NA. 1936. On the significance of mycorrhiza. New Phytologist 35: 117-131.

Byther RS and Davidson RM Jr. 1979. Dogwood anthracnose. Ornamental NorthWest Newsletter 3:20-21.

Caetano-Anolles G, Trigiano RT, Windham WT. 1996. Sequence signatures from DNA amplification fingerprints reveal fine population structure of the dogwood pathogen *Discula destructiva.* Federation of European Microbiology Societies Microbiology Letters 145: 377–383.

Caetano-Anolles G, Trigiano RT, Windham WT. 2001. Patterns of evolution in *Discula* fungi and the origin of dogwood anthracnose in North American, studied using arbitrarily amplified and ribosomal DNA. Current Genetics 39: 346–354.

Cannon PF, Damm U, Johnston PR, Weir B. 2012. *Colletotrichum* – current status and future directions. Studies in Mycology 73: 181-213.

Caporaso JG, Kucynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koeing JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J and Knight R. 2010 QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7: 335-336.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Tumbaugh PJ, Fierer N, Knight R. 2010. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Acadamey of Sciences of the United States of America 108(Supp1): 4516–4522.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N and Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Science Unite States of America 108: 4516–4522.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D,Lozupone CA and Turnbaugh PJ. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108(Suppl 1): 4516–4522.

Carroll GC, Carroll FE. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. Canadian Journal of Botany 56: 3034–3043.

Carroll GC. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69: 2-9.

Carroll GC. 1995. Forest endophytes – pattern and process. Canadian Journal of Botany 73(1): 1316 – 1324.

Carroll GC. 1999. The foraging ascomycete. Abstracts, XVI International Botanical 912 Congress, St. Louis. 309.

Casler MD, Vogel KP, Taliaferro CM, Ehlke NJ, Berdahl JD, Brumme EC, Kallenbach RL, West CP, Mitchell RB. 2007. Latitudinal and longitudinal adaptation of switchgrass populations. Crop Science 47(6): 2249–2260.

Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, Lafe K, Bui U, Limaye AP and Cookson BT. 2001 Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. Journal of Clinical Microbiology 39: 4042–4051.

Clark RB, Zeto SK. 2000. Mineral acquisition by arbuscular mycorrhizal plants. Journal of Plant Nutrition 23:867–902.

Clay K, Schardl CL. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160: S99–S127.

Colwell RK, Coddington JA. 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society of Botany 345: 101–118.

Colwell RK. 2005. EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. Version8.2. User's Guide and application published at: http://purl.oclc.org/estimates (accessed 07.10.11).

Costa Pinto LS, Azeved JL, Pereira JO, Carneiro ML, Vieira A and Labate CA. (2000). Symptomless infection of banana and maize by endophytic fungi impairs 2264 photosynthetic efficiency. New Phytologist 147: 609–615.

Crozier J, Thomas SE, Aime MC, Evans HC, Holmes KA. 2006. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. Plant Pathology 55: 783–791.

Cubit JD. 1974. Interactions of seasonally changing physical factors and grazing affecting high intertidal communities on a rocky shore. Dissertation. University of Oregon, Eugene, Oregon.

Currah RS, Tsuneda A, Murakami S. 1993. Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum*. Canadian Journal of Botany 71: 1639-1644.

Dakin N, White D, Hardy GESJ, Burgess TI. 2011. The opportunistic pathogen, *Neofusicoccum australe*, is responsible for crown dieback of peppermint (*Agonis flexuosa*) in Western Australia. Australas Plant Pathology 39:202–206.

Daughtrey ML, Hibben CR, 1994. Dogwood anthracnose - a new disease threatens two native *Cornus* species. Annual Review of Phytopathology 32: 61–73.

Daughtrey ML, Hibben CR, Britton KO, Windham MT and Redlin SC. 1996. Dogwood anthracnose--Understanding a disease new to North America. Plant Disease 80: 349-358.

Davis CE, Shaw JA. 2008. Biogeographic and phylotgenetic patterns in diversity of liverwort-associated endophytes. American Journal of Botany 95(8): 914–924.

DeBach P and Rosen D. 1991. Biological control by natural enemies, 2nd edition. Cambridge University Press.

Denno RF, Gruner DS, Kaplan I. 2008. Potential for entomopathogenic nematodes in biological control: a meta-analytical synthesis and insights from trophic cascade theory. Journal of Nematology. 40(2): 61-72.

Diamandis S. 1981. Elytroderma torres-juanii Diamandis and Minter. A serious attack on Pinus brutia L. in Greece. Pages 9-12 in C. S. Millar, editor. Current research on conifer needle diseases. Aberdeen University Press, Aberdeen, Scotland.

Diédhiou AG, Selosse MA, Galiana A, Diabaté M, Dreyfus B, Bâ AM, De Faria SM, Béna G. 2010. Multi-host ectomycorrhizal fungi are predominant in a Guinean tropical rainforest and shared between canopy trees and seedlings. Environmental Microbiology. 12(8): 2219-2232.

Dighton J, Clint GM, Poskitt J. 1991. Uptake and accumulation of 137Cs by upland grassland soil fungi: a potential pool of Cs immobilization. Mycological Research 95(9): 1052–1056.

Dighton J, Tugay T, Zhdanova N. 2008. Fungi and ionizing radiation from radionuclides. Federation of European Microbiology Societies Microbiology Letters 281: 109–120.

Ding G, Shuchun L, Liangdong G, Yuguang Z, Yongsheng C, 2008. Antifungal metabolites from the plant endophytic fungus *Pestalotiopsis foedan*. Journal of Natural Products 71: 615–618.

Duong LM, Jeewon R, Lumyong S and Hyde KD. 2006. DGGE coupled with ribosomal DNA phylogenies reveal uncharacterized fungal phylotypes on living leaves of *Magnolia liliifera*. Fungal Diversity 23: 121-138.

Eden PA, Schmidt TM, Blakemore RP, Pace NR. 1991. Phylogenetic analysis of *Aquaspirillum magentotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. International Journal of Systemic Bacteriology 41(2): 324-325.

Elad Y, Chet I, Katan J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Disease Control and Pest Management 70(2): 119–121.

El-Hassan SA, Gowen SR, Pembroke B. 2013. Use of *Trichoderma hamatum* for Biocontrol of Lentil Vascular Wilt Disease: Efficacy, Mechanisms of Interaction And Future Prospects. Journal of Plant Protection Research 53(1): 12–16.

Espinosa-Garcia FJ and Langenheim JH. 1990. The endophytic fungal community in leaves of a coastal redwood population-diversity and spatial patterns. New Phytologist 116: 89–97.

Evans HC, Holmes KA, Thomas SE. 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycological Progress 2: 149–160.

Evans HC. 2002. Invasive Neotropical Pathogens of tree crops. In Watling R, Frankland JC, Ainsworth AM, Isaac S, Robinson CH, (Eds.). Tropical Mycology 2: Micromycetes. CABI Publishing, Wallingford, UK, pp. 135 - 152.

Evans HC. 2007. Cacao diseases - The trilogy revisited. Phytopathology 97:1640-1643.

Evans JW. 2006. Commercialization of AR1 in Australia. In: Popay AJ, Thom ER (eds) International symposium on fungal endophytes of grasses. New Zealand Grassland Association, Christchurch, pp 241–242.

Eyde RH. 1987. The case for keeping *Cornus* in the broad Linnaean sense. Systematic Botany. 12: 505-518.

Eyde RH. 1988. Comprehending *Cornus*– puzzles and progress in the systematics of the dogwoods. Botanical Review 54: 233–351.

Farr DF. 1991. Septoria species on Cornus. Mycologia 83: 611–623.

Fisher PJ, Graf F, Petrini LE, Sutton BC and Wookey PA. 1995. Fungal endophytes of Dryas octopetala from a high arctic Polar semidesert and from the Swiss Alps. 87(3): 319-323.

Fonseca VG, Nichols B, Lallias D, Quince C, Carvalho GR, Power DM and Creer S. 2012. Sample richness and genetic diversity as drivers of chimera formation in nSSU metagenetic analyses. Nucleic Acids Research 40: e66.

Forman RT. 1998. Pine Barrens: Ecosystem and Landscape: Academic Press.

Freeman, S. and R. J. Rodriquez. 1993. Genetic conversion of a fungal plant pathogen to 964 a nonpathogen, endophytic mutualist. Science 260: 75–78.

Fröhlich J and Hyde KD. 1999. Biodiversity of palm fungi in the tropics: are global 967 fungal diversity estimates realistic? Biodiversity and Conservation 8: 977–1004.

Gamboa MA and Bayman P. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (Guarea guidonia : Meliaceae). Biotropica 33: 352–360.

Ganley RJ, Brunsfeld SJ, and Newcombe G. 2004. A community of unknown, 2323 endophytic fungi in western white pine. Proceedings of the National Academy of 2324 Sciences of the United States of America 101: 10107–10112.

Gazis R, Chaverri P. 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3: 240–254.

Gentz MC, Murdoch G, King GF. 2010. Tandem use of selective insecticides and natural enemies for effective, reduced-risk pest management. Biological Control 52(3): 208–215.

George E, Marschner H, Jakobsen I. 1995. Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Critical Reviews in Biotechnology 15:257–270.

Ghimire SR, Charlton ND, Bell JD, Krishnamurthy YL, Craven KD. 2011. Biodiversity of fungal endophyte communities inhabiting switchgrass (*Panicum vrigatum* L.) growing in native tallgrass prairie of northern Oklahoma. Fungal Diversity 47:19-27.

Giovannoni SJ, Britschgi TB, Moyer CL and Field KG. 1990. Genetic diversity in Sargasso sea bacterioplankton. Nature. 345: 60-63.

Girlanda M, Isocrono D, Bianco C and Luppi-Mosca AM. 1997. Two foliose lichens 992 as microfungal ecological niches. Mycologia 89: 531–536.

Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, MacPhee R and Reid G. 2010. Microbiome Profiling by Illumina Sequencing of Combinatorial Sequence-Tagged PCR Products. PLoS ONE 5(10): e15406.

Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, MacPhee R, Reid G. 2010. Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 5: e15406.

Grime JP, Mackey JML, Hillier SH, Read DJ. 1987. Floristic diversity in a model system using experimental microcosms. Nature 328: 420–422.

Grueter H. 1964. Eine Selecktive Anreicherung des Spalkproduktes ^{137}Cs in Pilzen. Naturwissenchaften 51: 161-162.

Grueter H. 1971. Radioactive fission product ^{137}Cs in mushrooms in W. Germany during 1963- 1970. Health Physics 34: 713–715.

Grünig CR, Queloz V, Sieber TN, Holdenrieder O. 2008. Dark septate endophytes (DSE) of the Phialocephala fortinii s.l. - Acephala applanata species complex in tree roots: classification, population biology, and ecology. Botany- Botanique 86: 1355–1369.

Gunter LE, Tuskan GA and Wullschleger SD. 1996. Diversity among populations of switchgrass based on RAPD markers. Crop Science 36:1017–1022.

Guo LD, Hyde KD, Liew EC, 2001. Detection and taxonomic placement of endophytic fungi within frond tissues of *Livistona chinensis* based on rDNA sequences. Molecular Phylogenetics and Evolution 20: 1–13.

Guyot J, Omanda EM and Pinard F. 2005. Some epidemiological investigations on 995 Colletotrichum leaf disease on rubber tree. Crop Protection 24: 65–77.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program fpr Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Halmschlager E, Butin H and Donabauer E. 1993. Endophytic fungi in leaves and twig of *Quercus petrea*. European Journal of Forest Pathology 23(1):51–63.

Hamayun M, Khan SA, Khan AL, Tang DS, Hussain J, Ahmad B, Anwar Y, Lee I. 2010. Growth promotion of cucumber by pure cultures of gibberellinproducing Phoma sp. GAH7. World Journal of Microbiology and Biotechnology 26 (5): 889-894.

Hanada RE, de Jorge Souza T, Pomella AWV, Hebbar PK, Pereira JO, Ismaiel A, Samuels GJ. 2008. *Tricoderma martiale* sp. nov., a new endophye from sapwood of *Theobroma cacao* with a potential for biological control. Mycological Research 112: 1335–1343.

Handelsman J, Rondon MR, Brady SF, Clardy J and Goodman RM. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry & Biology 5(10): 245-249.

Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemical Biology 5: R245–49

Hang J, Desai V, Zavaljevski N, Yuang Y, Lin X, Satya RV, Mrtinez LJ, Blaylock JM, Jarman RG, Thomas SJ and Kuschner RA. 2014. 16S rRNA gene pyrosequencing of reference and clinical samples and investigation of the temperature stability of microbiome profiles. Microbiome 4(31): 1-15.

Haselwandter K, Read DJ. 1982. The significance of root-fungus association in two *Carex* species of high-alpine plant communities. Oecologia 53: 352-354.

Hawksworth DL, Rossman AY. 1997. Where are all the undescribed fungi? Phytopathology 87: 888-891.

Hawksworth DL. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycological Research. 95: 641-655.

Herre EA, Van Bael SA, Maynard Z, Robbins N, Bischoff J, Arnold AE, Rojas E et al. 2005. Tropical plants as chimera: some implications of foliar endophytic fungi for the study of host plant defense, physiology, and genetics. In D. F. R. P. Burslem, M. A. Pinard and S.E. Hartley [eds.] Biotic Interactions in the Tropics. 1011 Cambridge University Press, Cambridge, UK: 226-237.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT,Lutzon F, Matheny PB, Mclaughlin DJ, Powell MJ, Redad S, Schoch CL, Spatafora JW, Stalper JA, Vilgalys R, Aime CM, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Geiser DM, Griffith GWk Guidam C, Hawksworth DL, Hestmark G, Hoska K, Humber RA, Hyde KD, Ironside JE, Köljalg U,Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Monalvo JM, Mozley-standridge S, Oberwinkle F, Parmasto E, reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio, Schußler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N. 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111: 506-547.

Hibbett DS, Ohman A, Glotzer D, Nuhn M, Kirk P. and Nilsson RH. 2011. Progress in molecular and morphological taxon discovery in *Fungi* and options for formal classification of environmental sequences. Fungal Biology Reviews 25: 38-47.

Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD and Lutzoni F. 2007. Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Molecular Phylogenetics and Evolution 42: 543–555.

Hinrikson HP, Hurst SF and Lott TJ, Warnock DW and Morrison CJ. 2005. Assessment of ribosomal large-subunit D1-D2, internal transcribed region spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. Journal of Clinical Microbiology 43: 2092–103.

Hirsch G, Braun U. 1992. Communities of parasitic microfungi. In: Winterhoff W. ed. Handbook of Vegetation Science, Volume 19, Fungi in Vegetation Science. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 225-250.

Hoffman MT, Arnold AE. 2008. Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees. Mycological Research 112: 331–344.

Hohenstein WG, Wright LL 1994. Biomass energy-production in the United States—An overview. Biomass Bioenergy 6(3): 161–173.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M, 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity* 33: 61–75.

Huber JA, Welch DBM, Morrison HG, Huse SM, Neal PR, Butterfield DA, Sogin ML. 2007. Microbial population structures in the deep marine biosphere. Science 318: 97-100.

Huse SM, Huber JA, Morrison HG, Sogin ML and Mark Welch D. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biology 8: R143.

Huson DH, Mitra S, Ruscheweyh H-J, Weber N and Schuster SC. 2011. Integrative analysis of environmental sequences using MEGAN4. Genome Research 21: 1552-1560.

Hyde KD, Soytong K. 2008 Understanding microfungal diversity – a critique. Crptogamie Mycologie 28: 281-289.

Hyde KD, Bussaban B, Paulus B, Crous PW, Lee S, McKenzie EHC, Photita W, Lumyong S. 2007. Diversity of saprobic microfungi. Biodiversity and Conservation 16: 7–35.

IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.

Inacio ML, Silva GH, Teles HL, Trevisan HC,Cavalheiro AJ, Bolzani VS, Young MCM, Pfenning LH, Araujo AR. 2006. Antifungal metabolites from Colletotrichum gloeosporioides, an endophytic fungus in Cryptocarya mandioccana Nees (Lauraceae). Biochemical Systematics and Ecology 34: 822-824

James TY, Kauff F, Schoch C, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J *et al*. 2006. Reconstructing the early evolution of the fungi using a six gene phylogeny. Nature 443: 818–822.

Jeewon R, Liew ECY, Hyde KD. 2004. Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. Fungal Diversity 17: 39–55.

Johnson NC, Graham JH, Smith FA. 1997. Functioning of mycorrhizal associations along the mutualism±parasitism continuum. New Phytologist 135: 575-585.

Jumpponen A, Jones KL, Mattox D and Yaege C. 2010. Massively parallel 454-sequencing of fungal communities in *Quercus* spp. Ectomycorrhizas indicates seasonal dynamics in urban and rural sites. Molecular Ecology 19: 41-53.

Jumpponen A, Jones KL. 2009. Massively parallel 454-sequencing of *Quercus macrocarpa* phyllosphere fungal communities indicates reduced richness and diversity in urban environments. New Phytologist 184: 438–448.

Jumpponen A, Mattson KG, Trappe JM. 1998. Mycorrhizal functioning of *Phialocephala fortinii*: interactions with soil nitrogen and organic matter. Mycorrhiza 7: 261-265.

Jumpponen A, Mattson KG, Trappe JM. 1998b. Mycorrhizal functioning of *Phialocephala fortinii*: interactions with soil nitrogen and organic matter. Mycorrhiza **7**: 261-265.

Jumpponen A, Trappe JM. 1998. Dark septate endophytes: a review of facultative biotrophic root colonizing fungi. New Phytologist 140: 295–310.

Jumpponen A. 2001. Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* 11: 207–211.

Jumpponen AM, Trappe JM. 1996. Population structure of *Phialocephala fortinii* on a receding glacier forefront. In: Azcon-Aguilar C, Barea J-M, eds. *Mycorrhiza in Integrated Systems*. *From Genes to Plant Development*. Luxemburg: Commission of the European Union (Cost 8.21) pp. 128-130.

Keshwani DR and Cheng JJ. 2009. Switchgrass for Bioethanol and Other Value-Added Applications: A Review. Bioresource Technology 100(40): 1515-1523.

Kirk PM, Cannon PF, David JC, Stalpers JA. 2001. Ainsworth & Bisby's dictionary of the fungi. 9th edition eds. CABI, Wallingford, UK.

Kirk PM, Cannon PF, Minter DW and Stalpers JA. 2008. Dictionary of the Fungi, 10th ed. CABI, Wallingford, UK.

Kleczewski NM, Bauer JT, Bever K, Clay K, Reynolds HL. 2012. A survery of endophytic fungi of switchgrass (*Panicum virgatum*) in the Midwest, and their putative roles in plant growth. Fungal ecology 5: 521-529.

Knapp DG, Pintye A, Kovacs GM. 2012. The dark side is not fastidious – dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. Plos One 7(2): e32570.

Kõljalg U, Larsson K-H, Abarenkov K, Nilsson RH, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AF, Tedersoo L, Vrålstad T and Ursing BM. 2005. UNITE: a database providing web based methods for the molecular identifi cation of ectomycorrhizal fungi. New Phytologist 166: 1063-1068.

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Duenas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lucking R, Martin MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Poldmaa, Saag L, Saar I, Schussler A, Scott JA, Senes C, Smith ME, Suija A, Taylor DL, Teresa T, Weiss M and Larsson K-H. 2013. Towards a unified paradigm for sequence-based identification of Fungi. Molecular Ecology 22: 5271-5277.

Korkama-Rajala T,Müller MM and Pennanen T. 2008. Decomposition and fungi of 1041 needle litter from slow- and fast-growing Norway spruce. Microbial Ecology 56: 76–89.

Kriel WM, Swart WJ, Crous PW. 2000. Foliar endophytes and their interactions with host plants, with specific reference to the gymnospermae. Advances in Botanical Research 33: 1–34.

Krings M, Taylor TN, Hass H, Kerp H, Dotzler N, Hermsen EJ. 2007. Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. New Phytologist 174: 648–657.

Kumar S, Carlsen T, Mevik BH, Enger P, Blaadi R, Shalchian-Tabrizi K and Kauserud H. 2011. CLOTU: an online pipeline for processing and clustering of 454 amplicon reads into OTUs followed by taxonomic annotation. BMC Bioinformatics 12: 182.

Kysela DT,Palacios C, and Sogin ML. 2005. Serial analysis of V6 ribosomal sequence tags (SARST-V6): a method for efficient, high-throughput analysis of microbial community composition. Environmental Microbiology 7: 356–364.

Lacap DC, Hyde KD, Liew ECY, 2003. An evaluation of the fungal ‘morphotype’ concept based on ribosomal DNA sequences. Fungal Diversity 12: 53–66.

Lagomarcino AJ, Miller AI. 2012. The Relationship between genus richness and geographic area in Late Cretaceous marine biotas: epicontinental sea versus open-ocean-facing settings. PLoS ONE 7(8): e40472.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. 1985. Rapid determination of 16S ribiosomal RNA sequences for phylogenetic analyses. Proceeding of the National Academy of Science. U.S.A. 82(20): 6955-6999.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

Lay DW. 1961. Fruit production of some understory hardwoods. Proc. 15th Annual Meeting of Southeastern Game Fish Commissioners. Atlanta, GA. P.30–37.

Lazarevic V Whiteson K, Huse S, Hernandez D, Farinelli L, Osteras M, Schrenzel J, and Francois P. 2009. Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. Journal of Microbiology Methods 79: 266–271.

Lazerevic V, Whiteson K, Huse S, Hernandez D, Farinelli L, Osteras M, Schrenzel J and Francois P. 2009. Metagenoic study of the oral microbiota by Illumina high-throughput sequencing. Journal of Microbiology Methods 79: 266-271.

Lewandowski I, Scurlock JMO, Lindvall E, Christou M. 2003. The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass Bioenergy 25 (4): 335–361.

Lewis DH. 1987. Symbiosis and mutualism: crisp concepts and soggy semantics. In: Boucher DH, ed. The biology of mutualism. London, UK: Croom Helm Ltd pp. 29–39.

Li E, Jiang L, Guo L, Zhang H, Che Y. 2008. Pestalachlorides A-C, antifungal metabolites from plant endophytic fungus Pestalotiopsis adusta. Bioorganic & Medicinal Chemistry 16 (17): 7894–7899.

Li Y, Mmbaga MT, Windham AS, Windham MT, Trigiano RN. 2009. Powdery mildew of dogwoods: current status and future prospects. Plant Disease 93: 1084–1092.

Linderman RG, Hendrix JW (eds). 1982. Evaluation of plant response to colonization by vascular-arbuscular mycorrhizal fungi: a host variables. American Phytopathological Society, St. Paul

LoBuglio KF, Berbee ML, Taylor JW. 1996. Phylogenetic origins of the asexual mycorrhizal symbiont *Cenococcumgeophilum* Fr. and other mycorrhizal fungi among the ascomycetes. Molecular Phylogenetics and Evolution 6: 287-294.

Lopez DC, Zhu-Salzman K, Ek-Ramos MJ, Sword GA. 2014. The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS ONE 9(8): e103891.

Lue H, a, Zou WX, Meng JC, Hu J, Tan RX. 2000. New bioactive metabolites produced by Colletotrichum sp., an endophytic fungus in Artemisia annua. Plant Science 151(1): 67–73.

Lücking R. 2008. Taxonomy: a discipline on the brink of extinction. Are DNA barcode scanners the future of biodiversity research? Taxonomy 61: 75-88.

Luo J, Walsh E, Naik A, Zhuang W, Zhang K, Cai L, Zhang N. 2014. Temperate Pine Barrens and Tropical Rain Forests Are Both Rich In Undescribed Fungi. PLoS ONE. 9(7): e103753.

Lutzoni F, Kauff F, Cox CJ, McLaughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James TY, Baloch E et al. 2004. Assembling the fungal tree of life: progress,

classification and evolution of subcellular traits. American Journal of Botany 91: 1446-1480.

Magurran AE, 1988. *Ecological Diversity and its Measurement*. Princeton University Press, Princeton, U.S.A.

Mandyam K, Jumpponen A. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. Studies in Mycology 53:173–189.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380

Marquez LM, Redman RS, Rodriguez RJ and Roossinch MJ. 2007. A virus in a 1070 fungus in a plant: three-way symbiosis required for thermal tolerance. Science 315: 513–515.

May RM. 1988. How many species are there on Earth? Science 241: 1441-1449.

May RM. 1991. A fondness for fungi. Nature 352: 475-476.

McLaughlin SB, Bouton JH, Bransby DI, Conger BV, Ocumpaugh WR, Parrish D. et al. 1999 Developing switchgrass as a bioenergy crop. In: Janick, J. (Ed). Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA. 282-299.

McLaughlin SB. 1993. New switchgrass biofuels research program for the southeast. *In:* Proceedings 1992 Annual Automative Technology Development, pp. 111–115.

McLean KL, Hunt JS, Stewart A, Wite D, Porter IJ, Villalta O. 2012. Compatibility of a *Tricoderma atroviride* biocontrol agent with management practices of *Allium* crops. Crop Protection 33: 94–100.

Medlin L, Elwood HJ, Stickel S, Sogin ML. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71: 491-499.

Mejia LC, Rojas EI, Maynard Z, Van Bael S, Arnold AE, Hebbar P, Samuels G, Robbins N, Herre EA. 2008. Endophytic fungi as biocontrol agents of Theobroma cacao pathogens. Biological Control 46: 4-14

Melin E. 1923. Experimentelle Untersuchungen U $ ber die Konstitution und O $ kologie den Mykorrhizen von *Pinus silvestris* und *Picea abies*. *Mykol*

Mello A, Napoli C, Murat C, Morin E, Marceddu G and Bonfante P. 2011. ITS-1 versus ITS-2 pyrosequencing: a comparison of fungal populations in truffle grounds. Mycologia, 103: 1184-1193.

Milne GD. 2006. Technology transfer of novel ryegrass endophyte in New Zealand. In: Popay AJ, Thom ER (eds) Internation symposium on fungal endophytes of grasses. New Zealand Grassland Association, Christchurch, pp 237–239.

Mmbaga MT, Sauve RJ, Mrema FA. 2008. Identification of microorganisms for biological control of powdery mildew in *Cornus florida*. Biological Control 44: 67–72.

Monk KA, Samuels GJ. 1990. Mycophagy in grasshoppers (Orthoptera, Acrididae) in Indo-Malayan rain forest. Biotropica 22: 16–21.

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B. 2011. How many species are there on Earth and in the ocean? PLoS One 9(8): e1001127.

Morino Y, Ohara T, Nishizawa M. 2011. Atomospheric behavior, deposition, and budget of radioactive materials from the Fukushima Daiichi nuclear power plant in March 2011. *Geophysical research letters* 38: L00G11, 7PP.

Mucciarelli M, Scannerini S, Bertea C, Maffei M. 2003. In vitro and in vivo peppermint (Mentha piperita) growth promotion by nonmycorrhizal fungal colonization. New Phytologist 158: 579–591.

Murrell ZE. 1993. Phyloenetic relationships in *Cornus* (Cornaceae). Systematic Botany. 18: 469-495.

Murrell ZE. 1996. A new section of *Cornus* in South and Central America. Systematic Botany 21: 273-288.

National Natural Landmark Summary. NPS. Feb 5, 2004. Accessed 9.11.2013.

Nei M, Maruyama T, Chakraborty R. 1975. The bottleneck effect and genetic variability of populations. Evolution 29: 1–10.

Neubert K, Mendgen K, Brinkmann H, Wirsel SGR. 2006. Only a few fungal species dominate highly diverse mycofloras associated with common reed. Applied and Environmental Microbiology 72: 1118–1128.

Neufeld JD and Mohn WW. 2005. Flouroophore-labeled primers improve the sensitivity, versatility and normalization of denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 71(8): 4893-4896.

Neufeld JD, Li J and Mohn WW. 2008. Scratching the surface of the rare biosphere with ribosomal sequence tag primers. Federadion of Euopean Microbiological Socities Microbiology Letters 283, 146-153.

Newcombe G, Shipunov A, Eigenbrode SD, Raghavendra AKH, Ding H, Anderson CL, Menjivar R, Crawford M, Schwarzländer M. 2009. Endophytes influence protection and growth of an invasive plant. Communicative and Integrative Biology 2(1): 29–31.

Nilsson RH, Veldre V, Hartmann M, Unterseher M, Amend A, Bergsten J, Kristiansson E, Ryberg M, Jumpponen A and Abarenkov K. 2010. An open source software package for automated extraction of ITS1 and ITS2 from fungal *ITS* sequences for use in high-throughput community assays and molecular ecology. Fungal Ecology 3: 284-287.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R. 2005. Fungal Community Analysis by Large-Scale Sequencing of Environmental Samples. Applied and Environmental Microbiology 71 (9): 5544–5550.

Oksanen J, Guillaume Blanchet F, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2010. Vegan: Community ecology package. R package version 1.17-4. Available at http://CRAN.R-project.org/package=vegan.

Osono T and Mori A. 2004. Distributiono of Phyllosphere fungi within the canopy of Giant dogwood. Mycoscience 45: 161–168.

Osono T andMori A. 2005. Seasonal and leaf age-dependent changes in occurrence of phyllosphere fungi of giant dogwood. Mycoscience 46: 273–279.

Osono T. 2007. Endophytic and epiphytic phyllosphere fungi of red-osier dogwood (*Cornus stolonifera*) in British Columbia. Mycoscience 48: 47–52.

Pace NR, Stahl DA, Lane DJ, Olsen GJ. 198. Analyzing natural microbial populations by rRNA sequences. American Society of Microbiology News 51: 4-12.

Pal KK and McSpadden B. 2006. Biological Control of Plant Pathogens. The Plant Health Instructor.

Paparu P, Dubois T, Coyne D, Viljoen A. 2009. Dual inoculation of Fusarium oxysporum endophytes in banana: effect on plant colonization, growth and control of the root burrowing nematode and the banana weevil. Biocontrol Science and Technology 19(5-6): 639-655.

Parrish DH, Fike JH. 2005. The biology and agronomy of switchgrass for biofuels. Critical Reviews in Plant Sciences 24: 423–459.

Partida-Martinez, L. P. and C. Hertweck. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437: 884–888.

Pedro´s-Alio´ C. 2012. The rare bacterial biosphere. Annual Review of Marine Science 4: 449–466.

Petrini O and Muller E. 1979. Pilzliche Endophyten, am Beispiel von *Juniperus communis* L. Sydowia 32: 224-251.

Petrini O, Hake U and Dreyfuss MM. 1990. An analysis of fungal communities 1129 isolated from fruticose lichens. Mycologia 82: 444–451.

Petrini O. 1991. Fungal endophytes of tree leaves. In: Microbial ecology of the leaves (eds. N.J. Fokkema and 1. van den Heuvel). Cambridge University Press, Cambridge: 185–187.

Peyronel B. 1924. Prime ricerche sulla micorizae endotrofiche e sulla microflora radicola normalle della fanerograme. Revista Biologia 6: 17–53.

Pirone PP. 1980. Parasitic fungus affects region's dogwood. New York: New York Times, Feb. 24, pp. 34, 37.

Polis GA, Sears ALW, Huxel GR, Strong DR, Maron J. 2000. When is a trophic cascade a trophic cascade? Trends in Ecology and Evolution 15:473–475.

Porter CL. 1966. An analysis of variation between Upland and Lowland switchgrass, Panicum virgatum L., in central Oklahoma Ecology 47:980–992.

Powell RG, Petroski RJ. 1992. Alkaloid toxins in endophyteinfected grasses. Natural Toxins 1: 163-170.

Powell WA, Klingeman WE, Ownley BH, Gwinn KD. 2009. Evidence of Endophytic *Beauveria bassiana* in seed treated tomato plants acting as a systemic entomopathogen to larval *Helicoverpa zea* (Lepidoptera noctuidae) Journal of Entomology Science 44(4): 391–396.

Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD and Jeewon R. 2007. A phylogenetic evaluation of whether endophytes become saprotrophs at 1133 host senescence. Microbial Ecology 53: 579–590.

Quince C, Lanzen A, Curtis TP, Davenport RJ, Hall N, et al. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods 6: 639–641.

R Development Core Team. 2010. R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria).

Rappé MS and Giovannoni SJ. 2003. The uncultured microbial majority. Annual Review of Microbiology. 57:369-394.

Reddy N, Ali Khan AP, Devi UK, Sharma HC, Reineke A. 2009. Treatment of millet crop plant (*Sorghum bicolor*) with the entomopathogen fungus (*Beauveria bassiana*) to combat infestation by the stem borer, *Chilo partellus* Swinhoe (Lepidoptera:Pyralidae). Journal of Asia-Pacific Entomology 12: 221–226.

Redecker D, Kodner R, Graham LE. 2000. Glomalean fungi from the Ordovician. Science 289: 1920–1921.

Redlin SC. 1991. *Discula destructiva* sp. nov., cause of dogwood anthracnose. Mycologia 83 (5): 633–642.

Redman RS, Dunigan DD and Rodriquez RJ. 2001. Fungal symbiosis: from 1139 mutualism to parasitism, who controls the outcome, host or invader? New Phytologist 151: 705–716.

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM. 2002. Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. Science 298: 1581.

Ren A, Clay K. 2009. Impact of a Horizontally Transmitted Endophyte, Balansia henningsiana, on Growth and Drought Tolerance of Panicum rigidulum. International Journal of Plant Sciences 170 (5): 599-608.

Rinaldi AC, Comandini O, Kuyper TW. 2008. Ectomycorrhizal fungal diversity: separating the wheat from the chaff. Fungal Diversity 33:1–45.

Roberts CA, West CP, Spiers DE. 2005. Neotyphodium in Cool-Season Grasses:

Robertson GP, Paul EA, Harwood RR. 2000. Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forcing of the atmosphere. Science 289: 1922-1925.

Rodriguez RJ, White JF, Arnold AE and Redman RS. 2009. Fungal endophytes: diversity and functional roles. New Phytologist 182(2): 314–330.

Rossell IM, Rossell CR, Hining KJ, Anderson RL. 2001. Impacts of dogwood anthracnose (*Discula destructiva* Redlin) on the fruits of flowering dogwood

(*Cornus florida* L.): Implications for wildlife. American Midland Naturalist 146: 379–387.

Rubini MR, Silva-Ribeiro RT, Pomella AWV, Maki CS, Arau´ jo WL, Santos DRD, Azevedo JL. 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciossa*, causal agent of Witches' Broom Disease. International Journal of Biologial Sciences 1: 24–33.

Ruiz DO and Arnold AE. 2013. Interannual variation and host affiliations of endophytic fungi associated with ferns at La Selva, Coasta Rica. Mycologia 106(1): 8-21.

Safir GR, Boyer JS. 1971. Mycorrhizal enhancement of water transport in soybean. Science 172:581–583.

Saikkonen K. 2007. Forest structure and fungal endophytes. Fungal Biology Reviews 21: 67–74.

Sánchez Márquez S, Bills GF, Domínguez Acuña L, Zabalgogeazcoa I. 2010. Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Diversity 41: 115–123.

Sánchez Márquez S, Bills GF, Zabalgogeazcoa I. 2008. Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Diversity 33: 87-100.

Sanderson M A, Reed RL, McLaughlin SB, Wullschleger SD, Conger BV, Parrish DJ, Wolf DD, Taliaferro C, Hopkins AA, Ocumpaugh WR, Hussey MA, Read JC, Tischler CR. 1996. Switchgrass as a sustainable bioenergy crop. Bioresource Technology 56(1): 83–93.

Santamaría O and Diez JJ. 2005. Fungi in leaves, twigs, and stem bark of Populus 1152 tremula from northern Spain. Forest Pathology 35: 95–104.

Santamaria, M, Fosso B, Consiglio A, De Caro G, Grillo G, Licciulli F, Liuni S, Marzano M, Alonso-Alemany D, Valiente G and Pesole G. 2012. Reference databases for taxonomic assignment in metagenomics. Brief Bioinformatics 13(6): 682-695.

Saunders S, Glenn AE, Kohn LM. 2010. Exploring the evolutionary ecology of fungal endophytes in agricultural systems: using functional traits to reveal mechanisms in community processes. Evolutionary Applications 3(5-6): 525-537.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Shal JW, Stres B, Thallinger GG, Van Hordn DJ and Weber CF. 2009. Introducing mother: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied and Environmental Microbiology 75(23): 7537-7541.

Schmidt PA, Bálint M, Greshake B, Bandow C, Römbke J and Schmitt I. 2013. Illumina metabarcoding of a soil fungal community. Soil Biology & Biochemistry. 65: 128-132.

Schmidt TM, DeLong EF, Pace NR. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. Journal of Bacteriology 173: 4371–78.

Schneeberger NF, Jackson W. 1989. Impact of dogwood anthracnose on flowering dogwood at Catoctin Mt. Park. Plant Diagnosticians Quarterly 10: 30–43.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA and Chen W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences 109: 6241–6246.

Scholz MB, Lo C and Chain PSG. 2012 Next generation sequencing and bioinformatics bottlenecks: the current state of metagenomics data analysis. Current Opinion in Biotechnology 23: 9-15.

Schulz B, Boyle C. 2005. The endophytic continuum. Mycological Research. 109: 661–686.

Seena S, Wynberg N and Bärlocher F. 2008. Fungal diversity during leaf decomposition in a stream assessed through clone libraries. Fungal Diversity 30: 1-14.

Shendure J and Hanlee Ji. 2008. Next-generation DNA sequencing. Nature Biotechnology 26: 1135-1145.

Shendure J. and Hanlee J. 2008. Next-generation DNA sequencing. National Biotechnolology 26: 1135–1145.

Sherald JL, Stidham TM, Hadidian JM, Hoeldtke JE. 1996. Progression of the dogwood anthracnose epidemic and the status of flowering dogwood in Catoctin Mountain Park. Plant Disease 80: 310–312.

Shultz B, Guske S, Dammann U and Boyle C. 1998. Endophytes-host interactions II. Defining symbiosis of endophytes-host interaction. Symbiosis 25: 213–227.

Shultz B, Rommert AK, Dammann U, Aust A-H and Strack D. 1999. The endophyte-host interaction: a balanced antagonism? Mycological Research 10: 1175-1283.

Siebert TN. 2007. Endophytic fungi in forest trees: are they mutualists? Fungal Biology Reviews 21: 75-89.

Simon L, Bousquet J, Levesque RC and Lalonde M. 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363: 67–69.

Sinclair JB and Cerkauskas RF. 1996. Latent infection vs. endophytic colonization 1189 by fungi: In S. C. Redlin and L. M. Carris [eds.] Endophytic Fungi in Grasses and Woody 1190 Plants. APPS Press, St. Paul, Minnesota, USA: 3-30.

Slippers B, Wingfield MJ. 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact.Fungal Biology Reviews 21: 90–106.

Smith SE and Read DJ. 1997. Mycorrhizal symbiosis, 2nd edn. Academic, New York

Smith SE, Smith FA. 1990. Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytologist 114: 1-38.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM and Herndl GJ. 2006. Microbial diversity in the deep sea and the underexplored ‘‘rare biosphere’’. Proceedings of the National Academy of Sciences 103: 12115–12120.

Steinera M, Linkovb M, Yoshida S. 2002. The role of fungi in the transfer and cycling of radionuclides in forest ecosystems. Journal of Environmental Radioactivity 58: 217–241.

Stinzing A, and Lang K. 2003. Dogwood anthracnose. Erster fund von *Discula destructiva* an *Cornus florida* in Deutschland. Nachrichtenbl. Deut. Pflanzenschutzd 55: S1–S5.

Stone JK, Polishook JD, White JF. 2004. Endophytic fungi. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi, inventory and monitoring methods. Elsevier Academic Press, Burlington, pp 241– 270

Stone JK, Polishook JD, White JRJ. 2004. Endophytic fungi. In: Mueller, G, Bills, GF, Foster, MS(eds), Biodiversity of Fungi: Inventory and Monitoring Methods. Elsevier, Burlington, MA, USA, pp. 241–270.

Stoyke G and Currah RS. 1991. Endophytic fungi from the mycorrhizae of alpine ericoid plants. Canadian Journal of Botany 69: 347-352.

Stoyke G and Currah RS. 1993. Resynthesis in pure culture of a common subalpine fungus±root association using *Phialocephala fortinii* and *Menziesia ferruginea* (Ericaceae). Arctic and Alpine Research 25: 89-193.

Suryanarayanan TS, Murali TS, Thirunavukkarasu N, Govinda Rajulu M, Venkatesan G and Sukumar R. 2011. Endophytic fungal communities in woody perennials of three tropical forest types of the Western Ghats, southern India. Biodiversity Conservation 20(5): 913-928.

Suryanarayanan TS, Thirunavukkarasu N, Govindarajulu MB, Sasse F, Jansen R, Murali TS. 2009. Fungal Endophytes and Bioprospecting: An appeal for a concerted effort. Fungal Biology Reviews 23(1-2): 9-19.

Sylvia DM, Williams SE (Eds). 1992. Vesicular-arbuscular mycorrhizae and environmental stress. American Society of Agronomy, Madison, Wisconsin

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis usinig Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731–2739.

Tan RX and Zou WX. 2001. Endophytes: a rich source of functional metabolites. Natural Products Report 18: 448-459.

Tantardini A, Calvi M, Cavagna B, Zhang N and Geiser D. 2004. Primo rinvenimento in Italia di antracnosi causata da *Discula destructiva* su *Cornus florida* e *C. nuttallii*. Informatore Fitopatologico 12: 44–47.

Tao G, Liu ZY, Hyde KD and Yu ZN. 2008. Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (*Orchidaceae*). Fungal Diversity 33: 101-122.

Taylor DL, Herriott IC, Stone KE, McFarland JW, Booth MG, Leigh MB. 2010. Structure and resilience of fungal communities in Alaskan boreal forest soils. Canadian Journal of Forest Research 40: 1288–1301.

Tedersoo L, Nilsson RH, Abarenkov K, Jairus T, Sadam A, Saar I, Bahram M, Bechem E, Chuyong G and Kõljalg U. 2010. 454 pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. New Phytologist 188(1): 291–301.

Tedrow JCF. (1952) Soil conditions in the Pine Barrens of New Jersey. Bartonia 26: 28–35.

Tejesvi MV, Kini KR, Prakash HS, Ven S, Shetty HS. 2007. Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants. Fungal Diversity 24: 37–54.

Thomas SE, Crozier J, Aime MC, Evans HC, Holmes KA. 2008. Molecular characterization of fungal endophytic morphospecies associated with the indigenous forest tree, *Theobroma gileri*, in Ecuador. Mycological Research 112: 852–860.

Thomas SE, Crozier J, Aime MC, Evans HC, Holmes KA. 2008. Molecular characterization of fungal endophytic morphospecies associated with the indigenous forest tree, Theoborma gileri, in Ecuador. Mycological Research 112: 852-860.

Thomas WA. 1969. Accumulation and cycling of calcium by dogwood trees. Ecological Monograph 39: 101–120.

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. 2002. Agricultural sustainability and intensive production practices. Nature 418: 671-677.

Tjamos EC, Tjamos SE, Antoniou PP. 2010. Biological management of plant diseases: Highlights on research and application. Journal of Plant Pathology 92 (4):17-21.

Toju H, Tanabe AS, Yamamoto S and Sata H. 2012. High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLoS One 7(7): e40863.

Toofanee SB, Dulymamode R. 2002. Fungal endophytes associated with *Cordemoya intergrifolia*. Fungal Diversity 11: 169–175.

Trappe JM. 1996. What is a mycorrhiza? In: Azcon-Aguilar C, Barea J-M (eds) Mycorrhiza in integrated systems – from genes to plant development. Proceedings of Fourth European Symposium on Mycorrhiza. Commission of the European Union, Luxemburg, pp 3–6.

Treu R, Laursen GA, Stephenson SL, Landolt JC, Densmore R. 1996. Mycorrhizae from Denali National Park and Preserve, Alaska. Mycorrhiza 6:21–29.

Trigiano RN, Caetano-Anolles G, Bassam BJ, Windham WT. 1995. DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. Mycologia 87: 490–500.

Tuininga AR, Dighton J. 2004. Changes in ectomycorrhizal communities and nutrient availability following prescribed burns in two upland pine-oak forests in the New Jersey pine barrens. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 34: 1755–1765.

Turnau K, Haselwandter K. 2002. Arbuscular mycorrhizal fungi: an essential component of soil microflora in ecosystem restoration. In: Gianinazzi S, Schuepp H (eds) Mycorrhizal technology: from genes to bioproducts. Birkhauser, Basel, pp 137–149.

U.S. Department of Agriculture, 2009. 2007 Census of Agriculture, Washington, DC.

U'Ren JM, Lutzoni F, Miadilkowska J, Laetsch AD, Arnold AE. 2012. Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American Journal of Botany. 99(5): 898-914.

Van Wyk M, Al Adawil AO, Khan IA, Deadman ML, Al Jahwari AA, Wingfield BD, Ploetz R and Wingfield .MJ. 2007. *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. Fungal Diversity 27: 213-230.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Tillson HB, Pfannkock C, Rogers Y and Smith HO. 2004. Environmental Genome Shotgun Sequencing of the Sargasso Sea. Science 304(5667): 66-74.

Verma VC, Gond SK, Kumar A, Kharwar RN and Strobel G. 2007. The 1231 endophytic mycoflora of bark, leaf, and stem tissues of Azadirachta indica A. Juss 1232 (Neem) from Varanasi (India). Microbial Ecology 54: 119–125.

Vogel KP, Brejda JJ, Walters DT, Buxton DR. 2002. Switchgrass biomass production in the midwest USA: Harvest and nitrogen management. Agronomy Journal 94(3): 413–420.

Vogel KP. 2004. Switchgrass. *In:* Moser, L. E., Sollenberger, L. E., Burson, B. L. (Eds.), Warm-season (C4) Grasses. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, WI, pp. 561–588.

Voříšková J and Baldrian P. 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. International Society for Microbial Ecology Journal 7:477–486.

Wagner BL, Lewis LC. 2000. Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. Applied and Environmental Microbiology 66: 3468–3473.

Walsh E, Luo J and Zhang N. 2014. *Acidomelania paniciola* gen. et sp. nov. from swichgrass roots in acidic New Jersey Pine Barrens. Mycologia. 106(4): 856-864.

Wang CJK, Wilcox HE. 1985. New species of ectendomycorrhizal and pseudomycorrhizal fungi: *Phialophora* f*inlandia*, *Chloridium paucisporum*, and *Phialocephala fortinii*. Mycologia 77: 951-958.

Wang J, Ma QY, Du F. 2006. Flora diversity characteristics of seed plants of Dawei Mountain National Nature Reserve in Yunnan province, China. Scientia Silvae Sinicae 42: 7–15.

Wang Z, Binder M, Schoch CL, Johnston PR, Spatafora JW, Hibbett DS. 2006. Evolution of helotialean fungi (Leotiomycetes,Pezizomycotina): a nucluear rDNA phylogeny. Molecular Phylogenetic Evolution 41(2): 295-312.

Wangerin W. 1910. Cornaceae. In: Engler A (ed) Das Pflanzenreich, ser. IV, fam. 229 (Heft 41). W. Engelmann, Leipzig.

Webber J. 1981. A natural biological control of Dutch elm disease. Nature 292: 449-451.

Werner JJ, Zhou D, Caporaso JG, Knight R and Angenent LT. 2012. Comparison of Illumina paired-ed and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. The International Socieity of Microbial Ecology Journal 6: 1273-1276.

White JFJ. 1988. Endophyte-host associations in forage grasses. I. A proposal concerning origin and evolution. Mycologia 80: 442–446.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, ShinskyJJ, White TJ (eds), PCR Protocols a Guide to Methods and Applications. Academic Press, San Diego, California, pp. 315–322.

Wilcox HE and Wang CJK. 1987. Ectomycorrhizal and ectendomycorrhizal associations of *Phialophora ®nlandia* with *Pinus resinosa*, *Picea rubens*, and *Betula alleghaensis*. Canadian Journal of Forest Research 17: 976-990.

Wilson D. 1995. Fungal endophytes which invade insect galls-insect pathogens, benign saprophytes, or fungal inquilines. Oecologia 103(2): 255-260

Wink M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theorhetical Applied Genetics. 75: 225-233.

Woese CR, Stackebrandt E, Macke TJ, Fox GE. 1985. A phylogenetic definition of the major eubacterial taxa. Systematics of Applied Microbiology 6: 143–151.

Woodcock S, Curtis TP, Head IM, Lunn M and Sloan WT. 2006. Taxa-area relationships for microbes: the unsampled and the unseen. Ecology Letters 9(7): 805-812.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW. 2013. *Alternaria* redefined. Studies in Mycology 75: 171–212.

Xiang QYJ, Thomas DT, Zhang WH, Manchester SR, Murrell Z. 2006. Species level phylogeny of the genus *Cornus* (Cornaceae) based on molecular and morphological evidence - implications for taxonomy and Tertiary intercontinental migration. *Taxon* 55: 9–30.

Yasunari T, Stohl A, Hayano RS, Burkhart JF, Eckhardt S, Yasunari T, 2011. Cesium-137 deposition and contamination of Japanese soils due to the Fukushima nuclear

accident. Proceedings of the National Academy of Science of the United States of America 108: 19530–19534.

Yu L, Nicolaisen M, Larsen J and Ravnskov S. 201.2 Molecular characterization of root-associated fungal communities in relation to health status of *Pisum sativum* using barcoded pyrosequencing. Plant and Soil 357: 395-405.

Yuan ZL, Rao LB, Chen YC, Zhang CL and Wu YG. 2010. From pattern to process: species and functional diversity in fungal endophytes of Abies beshanzuensis. Fungal Biology 115: 197–213.

Zambell CB and White JF. 2014. In the forest vine *Smilax rotundifolia*, fungal epiphytes show site-wide spatial correlation, while endophytes show evidence of niche partitioning. Fungal Diversity. 6(4): 1-19.

Zhang J, Flouri KK and Stamatakis A. 2014. PEAR: a fast and accurate Illumina Paired-End read merger. Bioinformatics 30(5): 614-620.

Zhang N and Blackwell M. 2001. Molecular phylogeny of dogwood anthracnose fungus (*Discula destructiva*) and the Diaporthales. Mycologia 93: 355–365.

Zhang N and Blackwell M. 2002. Population structure of dogwood anthracnose fungus. Phytopathology 92: 1276-1283.

Zhang N, Tantardini A, Miller S, Eng A, Salvatore N. 2011. TaqMan real-time PCR method for detection of *Discula destructiva* that causes dogwood anthracnose in Europe and North America. European Journal of Plant Pathology 130: 551–558.

Zhao ZW, Wang GH, and Yang L. 2002. Biodiversity of arbuscular mycorrhizal fungi in a tropical forest of Xishuangbanna, southwest China. Fungal Biodiversity 13: 232-242.

Zhou Y, Hirotani M, Yoshikawa Y, and Furuya T. 1997. Flavonoids and phenylethanoids from hairy root cultures of Scutelaria baicalensis. Phytochemistry 44: 83–87.

Zhu GS, Yu ZN, Gui Y, Hyde KD and Lui ZY. 2008. A novel technique for isolating orchid mycorrihizal fungi. Fungal Diversity 33: 123-137.

Zimmerman NB and Vitousek PM. 2013. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. Proceedings of the National Acadamy of Science U.S.A. 109(32): 13022-13027.