

THESIS

A SURVEY OF FOLIAR FUNGAL ENDOPHYTE COMMUNITIES OF ROCKY
MOUNTAIN BRISTLECONE PINE POPULATIONS IN THE COLORADO ROCKY
MOUNTAINS

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Alyssa Albertson

Department of Biology

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Master's Committee:

Advisor: Patricia Bedinger

Co-Advisor: C. Kenneth Kassenbrock

Jane E. Stewart

Anna W. Schoettle

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ABSTRACT

A SURVEY OF FOLIAR FUNGAL ENDOPHYTE COMMUNITIES OF ROCKY MOUNTAIN BRISTLECONE PINE POPULATIONS IN THE COLORADO ROCKY MOUNTAINS

Rocky Mountain bristlecone pine (*Pinus aristata*) is an exceptionally long-lived charismatic tree species found at high elevations in the southern Rocky Mountains of Colorado, New Mexico, and Arizona (Fryer, 2004). This species has recently come under threat from the disease white pine blister rust (WPBR).

White pine blister rust is caused by the pathogenic fungus *Cronartium ribicola*, which was inadvertently introduced into North America from Europe in the early 1900's, and has since spread widely with devastating impacts (Burns et al., 2008). In North America, WPBR is largely lethal to five-needle pine species.

In Colorado, WPBR has been found in stands of Rocky Mountain bristlecone pine and limber pine (*Pinus flexilis*), and efforts have been made to identify trees with increased resistance to the disease. The USDA Forest Service Rocky Mountain Research Station has identified specific trees that harbor some level of heritable resistance to WPBR, versus those appearing fully susceptible (Schoettle, 2004; Schoettle et al., 2012; Schoettle et al., 2014).

Essentially all plants in the wild harbor endophytic bacteria and fungi, which are defined as co-existing in plant tissues without causing evidence of disease, and it is increasingly appreciated that endophytes can alter plant responses to both biotic and abiotic stresses

(Rodriguez et al., 2008). It has been reported that fungal endophytes can enhance resistance to blister rust in western white pine (*Pinus monticola*) (Ganley et al., 2008).

The endophytic fungi of Rocky Mountain bristlecone pines have not been previously studied. We used two techniques to survey the endophytic fungal communities present in wild populations of resistant and susceptible bristlecone pines. The first technique was to isolate endophytic fungi by culturing surface-sterilized pine needles. The second technique was to extract DNA from the pine needles, and use PCR amplification of fungal-specific sequences, followed by high-throughput Next Generation sequencing (NGS) to identify and quantify fungi present, regardless of whether or not they could be cultured.

By culture we recovered 259 fungal isolates that were placed into 81 morphological groups, whereas the NGS returned 42,003 useable DNA sequences that were grouped into 791 operational taxonomic units (OTUs). The two techniques used in this study had significant overlap; most of the cultured fungi were also identified in the NGS data set. The high throughput sequencing data also revealed differences between the endophyte populations of trees previously inferred to have or lack resistance to white pine blister rust at each location. Further research will be needed to understand whether endophytes may modify, slow, or even prevent infection by the pathogen *C. ribicola*

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CHAPTER 1: INTRODUCTION

1.1 – Bristlecone pines

Bristlecone pines have been described as the oldest living organisms on earth. The Great Basin bristlecone, *Pinus longaeva*, occurs in California and Nevada, and includes individuals documented to be over 4,000 years old. Its close relative, the Rocky Mountain Bristlecone, *Pinus aristata*, is found in the southern Rocky Mountains of Colorado, New Mexico, and Arizona (Baker, 1992). While Rocky Mountain bristlecones do not reach the extreme ages of the Great Basin species, they regularly exceed 1000 years, and the oldest known specimen has been identified to be at least 2,435 years old (Brunstein & Yamaguchi, 1992).

These trees typically grow at high elevations near treeline (between 2,498-3,676 meters), and are found in upper montane and subalpine environments, on dry, steep slopes usually with south or west facing aspects. Most of these communities are found on the eastern side of the Continental Divide of Colorado (Krebs, 1973; Baker, 1992; Schoettle and Coop, 2017). Depending on their age and the habitat they grow in, bristlecone pine form can vary. Rocky Mountain bristlecone pines found at tree line typically form krummholz (Barrick & Schoettle, 1996), which are stunted windblown trees that only grow at or near the tree line on mountains. Older bristlecones can display exposed wood, which can lead to partial cambial mortality (Schauer et al., 2001). This partial cambial mortality gives older bristlecone pines an irregular crown, with multiple dead and/or dying limbs. When younger, or when found at lower elevations, their trunks tend to be more intact, with a less windblown appearance, and have more densely spreading limbs, with living crowns (Flora of North America Association, 2009).

Rocky Mountain bristlecone pine stands consist of mixed ages (Brunstein & Yamaguchi, 1992). Seedling establishment is somewhat rare, and occurs less than 100 hundred meters away from the parent tree. For seed germination to occur, bare mineral soil is necessary as well as open areas, as Rocky Mountain bristlecone pines are shade intolerant (Crane, 1982). Within Colorado, bristlecone regeneration occurred best in areas with bare soil, and experienced abundant cone production (Schoettle and Coop, 2017).

Overall, Rocky Mountain bristlecone pines are a long-lived species that regenerates well after fires, and grows in sites poorly suited for other species (Baker, 1992; Schoettle, 2004). These trees are extremely stress tolerant, persevering on dry sites, and often promote the establishment of late successional species (Schoettle, 2004).

1.2 – Endophytes

Endophytes are bacteria or fungi that live within plant tissues without causing apparent disease symptoms or evidence of harm. Essentially all plants in the wild harbor endophytic microorganisms (Petrini, 1991). Plant-endophyte relationships are complex and have varying biological and ecological importance (Stone et al., 2000).

Interactions between fungal endophytes and plant hosts are both variable and dynamic. Endophytes can be commensalists, mutualists, or latent pathogens, and these relationships can change over time. A mutualist may become a saprotroph as plant tissues senesce, and latent pathogens may emerge from their latency to cause overt disease (Schulz & Boyle, 2005). Because of this complexity, the plant endophyte relationship has been described as “balanced antagonism” (Schulz et al., 1999).

1.2.1– Types of endophytes

Endophytes vary widely among the hosts they infect, the tissues they reside within, the level of colonization and the amount of diversity present. The majority of endophytic fungi belong to the phylum Ascomycota (Schultz & Boyle, 2005). Ascomycetes are the largest group of fungi, containing approximately 60,000 described species, and are characterized by their sexual spores, which are produced within a “sac-like” structure called an ascus (FIGURE 1). Ascomycota also contains many fungi for which sexual reproduction has not (yet) been observed. Clonally reproducing fungi can be assigned to Ascomycota based on ultra-structural findings such as the presence of Woronin bodies or by DNA sequence similarity (Kimbrough, 1981; Schoch et al., 2012).

Recently Rodriguez et al. (2009) have proposed a classification system that divides all endophytes into four groups. The first group are Clavicipitaceous endophytic fungi (class 1 or C-endophytes), which only infect some grasses. C-endophytes infect plant tissues intracellularly, and are widely distributed throughout above ground tissues. All C-endophytes are primarily vertically transmitted, with parent plants passing these fungi to their progeny through seed infections, rather than acquired from the environment (horizontally transferred) (FIGURE 2) (Rodriguez et al., 2009; Saikkonen et al., 2002).

The remaining three groups are Non-Clavicipitaceous endophytes (NC-endophytes), which are found in plants other than grasses, and are much more heterogeneous. Class 2 NC-endophytes are found in both above and below ground tissues, can occupy large amounts of tissue, are both vertically and horizontally transferred, and typically have a low level of biodiversity. Class 3 NC-endophytes are found only in above ground tissue, stay fairly concentrated in one area within plant tissues, are only horizontally transferred, and have a high

level of biodiversity. Class 4 NC-endophytes are found only in roots, can occupy large amounts of tissue, are only horizontally transferred, and have an unknown level of biodiversity (Saikkonen et al., 2002; Rodriguez et al., 2009).

In the case of horizontal transmission, acquisition of or colonization by endophytes typically begins in the seedling stage, but can continue throughout the life of the plant. As a result, species-richness of endophytic fungi tends to increase with the age of the plant or tissue (Petrini & Carroll, 1981; Espinosa-Garcia & Langenheim, 1990).

1.2.2 – *Plant-endophyte interactions*

At first glance, the plant-endophyte relationship would appear to favor endophytes, because endophytes receive both food and shelter/habitat from the plant host, while possible plant benefits are less obvious. However, the situation is balanced and dynamic. Although fungal endophytes have a protected habitat within their host plant and receive photosynthates, growth is restricted by both passive and active plant defenses (described further below). This growth restriction may be lifted as tissues senesce, and endophytes are well positioned to act as saprobes in dead or dying tissue. Similarly, latent pathogens may emerge from latency and begin causing disease (Rodriguez et al., 2009).

Clear examples of mutualism have also been described. Some endophytes promote plant growth through the production of hormones. Fungal endophytes have been demonstrated to produce both indole acetic acid (auxin) and gibberellins (Waqas et al., 2012). Recently, Carrell and Frank (2014) described bacterial endophyte populations in the needles of *Pinus flexilis* (limber pine) and *Picea engelmannii* (Engelmann spruce). They found that these communities were dominated by species in the *Acetobacteraceae* related to species known to fix nitrogen, and

they suggest that endophyte-mediated nitrogen fixation may be important for conifer growth, particularly in severely nitrogen-limited soils.

Endophytes can also mediate increased resistance to both biotic and abiotic stresses. Clavicipitaceous endophytes occupy the above ground tissues of some grasses such as tall fescue (*Festuca arundinacea*) or perennial ryegrass (*Lolium perenne*) and deter herbivory through the production of a variety of fungal toxins (Clay, 1996). Redman et al. (2002) have shown that the ability of panic grass (*Dichanthelium lanuginosum*) to survive at elevated temperatures in geothermally heated soils depends upon the presence of a *Curvularia* sp. as a fungal endophyte.

1.2.3 – Endophytes and plant defenses

Plants employ a variety of defense mechanisms to protect against potentially harmful organisms such as pathogenic bacteria, fungi, or insects (Yang et al., 1997). Endophytes must evade or overcome these systems to establish residency, without compromising the ability of the plant to defend against disease (Saikkonen et al., 2004).

Plants have both passive and active defenses. Passive defenses are traits that are constitutive, or always present in the plant, such as the waxy cuticle or a thick cell wall. Active defenses are triggered or induced by an attack by a pathogen. When a plant is attacked by a pathogen, the plant must first sense the attack, via a variety of mechanisms, and then respond. As an example, the expression of PR proteins is induced following infection as an effort to limit or prevent disease (Loon, 1985). Another early defense response as a pathogen attempts to penetrate the plant cell wall is for the plant to start creating and accumulating lignin and callose at the site, forming papillae, which can block the invasion. A third example of active plant defense is the production of toxic secondary metabolites. There are many different types of chemical compounds plants can produce to ward off pathogens, insects, and/or herbivores,

including glucosinolates, terpenoids, phenolics, and phytoalexins (Wink, 1988; Wink, 2003; Grayer & Kokubun, 2000). Such compounds can be expressed throughout the plant and may be toxic to, or otherwise deter a variety of pathogens (Schumann & D'Arcy, 2013; Neilson et al., 2013; Grayer & Kokubun, 2000).

1.2.4 – Endophytes as plant pathogen modifiers

Endophytes can also affect the ability of microbial pathogens to cause disease in plants, either by promoting or by inhibiting disease progression. Antagonism of plant disease can occur by at least two mechanisms: endophytes can directly compete with pathogens for resources, or endophytes can induce plant defense responses (Busby et al., 2016). Fungi, as well as plants, create toxic secondary metabolites to defend themselves against pathogens, insects, or herbivores. For example, as mentioned above, mycotoxins produced by Clavicipitaceous endophytes can confer resistance to herbivory to their grass hosts (Bultman & Murphy, 2000).

When plants are infected with fungal endophytes, occasionally these endophytes are able to deter other fungi. This can occur when mycelia of genetically distinct fungi come into contact with one another and they become antagonistic. This antagonism can sometimes be seen in wood decay fungi, where opposing fungi create zone lines between them, distinguishing individual colonies (Rayner & Todd, 1980). In these areas of confrontation, it has been found that fungi have a shift in their metabolic processes and begin producing secondary metabolites that can inhibit growth of competitors. This change affects the activity of fungi and bacteria that are competing for the same space, making it more difficult for certain fungi and bacteria to move into an already infected area (Glauser et al., 2009).

Several studies have examined endophytes for their potential to be deliberately introduced by humans into plants to increase resistance to pathogens. Findings have shown that

endophytes could potentially be used as a biological control in susceptible populations (Arnold et al., 2003). This approach has been utilized in sustainable agriculture, where spraying susceptible plants with salicylic acid (a known inducer of disease resistance in plants) triggers plant defense responses, increasing resistance to pathogens (Terry & Joyce, 2004). But before endophytes can be employed in this manner, specific fungal endophytes need to be identified and the biological responses of the plant need to be determined. As an example, the foliar endophytes found in Japanese knotweed were shown to have a variety of different effects on the pathogen *Puccinia polygoniamphibii*, which causes a leaf rust on the host (Kurose et al., 2012). Some endophytes facilitated the pathogen, some endophytes antagonized it, and a third group of endophytes had no effect. A *Phomopsis* sp. was the most useful as an antagonist to *Puccinia polygoniamphibii* (Kurose et al., 2012).

A similar array of endophyte effects have been seen in the black cottonwood (*Populus trichocarpa*) found in the Pacific Northwest. The foliar endophytes identified either facilitated, antagonized, or had no effect on the leaf rust disease caused by the pathogen *Melampsora columbiana* (Busby et al., 2016; Raghavendra & Newcombe, 2013). These studies show endophytes are able to modify a host's response to pathogens, either by benefiting the host or the pathogen, or having no effect at all.

Ganley et al. (2006) cultured endophytic fungi from *Pinus monticola* seeds and needles. They cultured several thousand isolates from surface sterilized needles, but only 16 from surface sterilized seeds. From their population of isolated endophytes, 90% were in the *Rhytismataceae* fungal family; this allowed Ganley et al. (2008) to determine which fungal endophyte genera to use in subsequent inoculation experiments of seedlings. They compared eight different fungal endophyte treatments in five different experiments to determine whether endophytes influenced

seedling response to *C. ribicola* infection. The first set of experiments involved six different combinations of endophyte treatments and non-inoculated control seedlings. A second set of experiments compared two different combinations of endophyte treatments with non-inoculated control seedlings. These fungal endophytes were given 2-3 weeks for colonization to sufficiently occur before the seedlings were challenged with *C. ribicola* (Ganley et al., 2008).

This study (Ganley et al., 2008) demonstrated that *P. monticola* seedlings inoculated with any one of the endophyte combinations had an increased survival rate of 3-16% after inoculation with *C. ribicola* compared to non-inoculated controls. Treatment 1 of experiment 1, had the best results, with the seedlings surviving 611 days in comparison to 479 days for the control. This treatment consisted of a combination of *Cladosporium* sp., *Geopyxis* sp., *Hormonema* sp., *Zalerion* sp. and *Rhizophraera* sp. There appeared to be a trend toward increased survival when seedlings were inoculated at 1 year of age versus two years of age, though this effect did not reach statistical significance. Since each treatment involved a mix of fungal endophytes, it is difficult to determine which were responsible for influencing the seedlings resistance to white pine blister rust (Ganley et al. 2008). More research is needed to observe the fungal endophytes on an individual basis and determine which may have more activity as pathogen antagonists.

Busby et al. (2016) analyzed recent literature on fungal endophyte disease modification. Their research found that many fungal endophyte taxa can antagonize pathogen growth. Many of these antagonists are plant pathogens themselves, with differing levels of virulence. The most commonly identified pathogen antagonists included *Trichoderma*, *Aureobasidium*, *Fusarium*, *Penicillium*, *Chaetomium*, *Bionectria*, and two yeasts *Pichia* and *Candida*, which are found within five different fungal orders within the *Ascomycota* phylum and include *Hypocreales* spp. (*Trichoderma*, *Fusarium* and *Bionectria*), *Eurotiales* sp. (*Penicillium*), *Sordariales* sp.

(*Chaetomium*), *Dothideales* spp. (*Aureobasidium* and *Penicillium*), and *Saccaromycetales* spp. (*Pichia* and *Candida*). This small number of orders suggests disease-modifying endophytes might be phylogenetically restricted.

1.3 – DNA barcoding

“DNA barcoding” is employed for identification of fungal isolates and molecular operational taxonomic units. The concept of DNA barcoding was introduced by Hebert et al. (2003) as an alternative to morphological approaches, which suffer from several significant problems including “phenotypic plasticity and genetic variability in the characters employed”, “morphologically cryptic taxa”, and problems with morphologic keys which may be “effective only for a particular life stage or gender” and often require “a high level of expertise” in their use (Hebert et al. 2003). Hebert et al. argued that DNA sequence diversity among organisms essentially provides genetic ‘barcodes’ in every cell and proposed the mitochondrial cytochrome c oxidase I gene (COI) as an appropriate target sequence for the DNA barcoding of animals. Ideally, the region chosen should have sufficient diversity to distinguish between taxa, but sufficient conservation such that members of a single species can be recognized. Although COI has proven useful for animals, it is not ideal for all organisms. Schoch et al. (2012) evaluated six different DNA regions for their potential as barcodes for fungi, and concluded that the internal transcribed spacer region (ITS) of nuclear ribosomal genes was the most useful for the fungal kingdom.

The DNA barcoding approach used for species identification has only been partially successful. In many cases the best sequence matches have not allowed identification at the species level but only higher levels such as genus, family, order, or even “uncultured fungus”.

This deficiency reflects insufficient species level information currently available in DNA sequence databases.

1.4 – *Cronartium ribicola* and white pine blister rust

Cronartium ribicola is a pathogenic fungus in the phylum Basidiomycota that causes white pine blister rust (WPBR) in five-needle pine species. *Cronartium ribicola* was inadvertently introduced into North America from Europe in the early 1900's and has since spread widely with devastating impacts among North American white pine species, which have little innate resistance to blister rust (Burns et al., 2008). There are nine white pine species native to the United States: western white pine, eastern white pine Sugar pine, whitebark pine, limber pine, southwestern white pine, foxtail pine, Rocky Mountain bristlecone pine, and Great Basin bristlecone pine (Schwandt et al., 2010; Samman et al., 2003). The discovery of white pine blister rust in Colorado was first found on limber pine in 1998 (Johnson & Jacobi, 2000). In 2003 blister rust was found for the first time on Rocky Mountain bristlecone pines (Blodgett & Sullivan, 2004).

White pine blister rust is native to Asia but was unintentionally introduced to Europe, and subsequently into North America from Europe. While the white pines of Asia have been coevolving with *C. ribicola* since the Miocene epoch (5.3 million years ago), the white pines of Europe and North America have only been exposed to WPBR within the last several hundred years (Hummer, 2000), and therefore have not evolved resistance to this pathogen, which is rapidly spreading. Understanding this pathogen's life cycle is integral to controlling white pine blister rust in our nation's forests.

Cronartium ribicola has an elaborate life cycle that is composed of five different spore stages (FIGURE 3), and alternates between two types of plant hosts. The alternate hosts are

white pines, with the other host being either *Ribes* spp. (Kinloch, 2003), *Pedicularis racemosa* (Parrot's beak) or *Castilleja miniata* (giant red Indian paintbrush) (Zambino et al., 2007). In the United States gooseberries and currants are the *Ribes* spp. more commonly found as the alternate host for white pine blister rust to complete its life cycle. Of the five spore stages, two occur on pine, and the remaining three take place on currants or gooseberries (Malloy, 1997). Pine needles are infected by basidiospores, which germinate into monokaryotic fungal hyphae and penetrate the needles through their stomata. The infection spreads from the needle into the twigs and branches of the tree, and subsequently spermatia develop on infected bark. Spermatia look like small amounts of sap on the tree, and these small pitch-like pustules are filled with spermatia, haploid monokaryotic spores that fertilize receptive hyphae of the growing *C. ribicola* fungus. Fertilization forms dikaryotic mycelium that initiates aecial development. Aecia emerge in the same spots formerly occupied by spermatia. The aecia are covered with a peridium or outer skin; this outer skin will later break releasing huge quantities of dikaryotic aeciospores. These aeciospores land on a currant or gooseberry host to continue the life cycle. Once the gooseberry or currant plant is infected with the aeciospores, uredinial swellings, which appear as orange-yellow masses, will develop on the underside of the leaves. These swollen orange-yellow spots will produce dikaryotic urediniospores until the leaves die. Within the same swollen uredinial spots, telia columns develop. These telia columns are made up of dikaryotic teliospores. The teliospores remain in place within the orange-yellow uredinial spots. The teliospores are not dispersed but instead germinate inside the uredinial spots, undergo karyogamy and meiosis, and turn into four haploid basidiospores, starting the life cycle over again (Malloy, 1997).

Significant efforts have been made to attempt to combat the spread of *C. ribicola* (Malloy, 1997). Since *C. ribicola* needs two hosts to complete its life cycle, if one host is

removed near the other host then the life cycle cannot be completed and the fungus no longer can reproduce and spread (Kinloch, 2003; Schumann & D'Arcy, 2013). Organized control of white pine blister rust began in 1909, with efforts to eradicate *Ribes* spp. in the vicinity of white pine species. Black currant was commonly planted and cultivated in North America, and known to be highly susceptible to white pine blister rust (Malloy, 1997). Black currant was eradicated from the east within ten years (Arthur, 1934), and from the west by 1927 (Mielke, 1943). However, the eradication of wild *Ribes* was a much more difficult task, especially in the west. Many different methods of wild *Ribes* eradication were utilized and tested (Filler, 1919), but by 1950 genetic and antibiotic projects were gaining more interest and eradication efforts deteriorated (Malloy, 1997). More recently it has been determined *Ribes* spp. are not the only alternate hosts for *C. ribicola*. Studies conducted in 2004 and 2005 identified *Pedicularis racemosa* and *Castilleja miniata* as alternate hosts for *C. ribicola* (Zambino et al., 2007).

Since *C. ribicola* initially infects white pines through the needles, endophytic fungi may have a higher likelihood of altering/enhancing host responses in this location, versus other tissues such as bark or roots. Given this biology, scientists have begun looking into whether endophytes may alter the resistance of white pine trees to infection with white pine blister rust. Ganley et al. (2006) cultured endophytes from seeds and needles of eastern white pines; then used these endophytes in inoculation experiments (2008). As discussed earlier, seedlings were inoculated with different combinations of cultured endophytes, allowed 2-3 weeks for sufficient fungal colonization, then inoculated with *C. ribicola* (Ganley et al., 2008). This research showed fungal endophytes do indeed enhance seedling resistance to infection by *C. ribicola*. The objectives of this study were to survey and identify the fungal endophyte populations of wild Colorado *P. aristata* in trees previously identified as sensitive or resistant to WPBR. Identification of fungal

endophytes in *P. aristata* may suggest candidate endophytes to be tested directly for any role in WPBR resistance.

CHAPTER 2: DESCRIPTION OF STUDY

Prior to this work, nothing was known about endophytic fungi in Rocky Mountain bristlecone pines (*Pinus aristata*). Here we have surveyed needle endophyte communities within *P. aristata* in Colorado using two methods: first, we used traditional culture of fungi from surface-sterilized pine needles, and PCR amplification of fungal-specific sequences from extracted DNA followed by Sanger DNA sequencing; second, extraction of total DNA from needles, PCR amplification of fungal sequence, followed by high-throughput Next Generation Sequencing (NGS). This research had three goals: first, to survey and identify fungal endophytes present in wild populations of *P. aristata* in Colorado, to determine whether or not the constitutive fungal endophyte populations vary in susceptible trees versus those with some resistance to the disease white pine blister rust, and to determine if variation exists among endophyte populations at different geographic locations.

2.1 – Materials and Methods

2.1.1 – *Host species and study site*

Schoettle and colleagues have established multiple locations in Colorado where cones were collected from *P. aristata* (Schoettle and Coop 2017), and their progeny were assayed for susceptibility to white pine blister rust (TABLE 1) (Schoettle et al., 2011). In this way, specific trees were identified that harbor some level of heritable resistance to WPBR, compared to others that appear to be fully susceptible (Schoettle, 2004; Schoettle et al., 2012).

Field sampling was conducted during June and July of 2016. We chose three sites for which bristlecone pine trees have been assessed for susceptibility to WPBR. At the time of sampling there was no evidence of invasion by *C. ribicola* at any of the three sites. The sites

include: site 7 (St. Mary's), site 11 (Lookout Mountain), and site 4H (Trinchera) (FIGURE 4). These 3 sites were chosen to include differences in geographic variability (northern, central, and southern Colorado). At each site, 6 trees were sampled: 3 trees previously identified as resistant, and 3 trees identified as highly susceptible to WPBR, which were tagged and georeferenced (TABLE 3). From each tree, twigs were collected from asymptomatic branches between 3-6 meters off the forest floor, and from 4 sides (north, south, east, and west) of the tree. These collected twigs were placed in individually labeled freezer bags, then stored in coolers, and moved to a 4°C refrigerator within 12-48 hours.

2.1.2 – Fungal Endophyte Isolations

For each tree, 12 asymptomatic needles (2-8 years of age) from each cardinal direction, were surface sterilized. The fascicles were pulled from the twig, the needles were removed, and surface sterilized by immersion in 95% ethanol (30 seconds), 0.5% NaOCl (2 minutes), and 70% ethanol (2 minutes) as described by Arnold et al., 2007. After surface sterilization, needles were aseptically cut at a diagonal angle into 4 pieces and plated on potato-dextrose agar (PDA). Each PDA plate contained 4 needles, consisting of 16 needle segments per plate. Once the needles were placed on PDA, they were no longer separately identified as to which cardinal direction they originated, an additional variable deemed unnecessary for this research. Plates were sealed with Parafilm (Parafilm M®, Bemis NA, Neenah WI), incubated at room temperature, and checked weekly for fungal growth for up to 8 months. Any endophytic fungi growing from the needles were subcultured onto a fresh PDA plate. Subcultured endophytic fungi were grown at room temperature until they were large enough for morphological grouping and removal of tissue for DNA isolation and barcoding.

2.1.3 – Fungal DNA extraction, PCR, and Sanger sequencing

Within the first 6 months after plating the surface sterilized needles on PDA media, 259 fungal isolates were recovered by culture and were organized into 81 morphological groups. Morphological groups were characterized by macroscopic appearances of the cultured fungi on the PDA plates. These macroscopic appearances were size, opacity (transparent, opaque, or translucent), surface (smooth, glistening, rough, wrinkled, or dull), color (black, grey, blue, green, pink, etc.), form (circular, filamentous, rhizoid, or irregular), elevation (raised, convex, flat, umbonate, or crateriform), and margin (entire, undulate, filiform, curled, or lobate). All morphological groups were identified by DNA barcoding, with PCR amplification of the internal transcribed spacer regions (ITS1, 5.8s and ITS2) using fungal specific primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). A small amount of tissue, 2-20 mg, was taken from each isolate and homogenized with a micropestle in 0.5 mL of a lysis buffer called “Shorty buffer” in a 1.5 mL microcentrifuge tube. The lysis buffer consists of 0.2M Tris HCl pH9.0, 0.4M LiCl, 25 mM EDTA pH8.0, 1%SDS, in sterile double deionized water. After homogenization, samples were centrifuged 5 minutes at 15K x g to pellet insoluble debris and 350 µL of supernatant was removed to a clean 1.5 mL microcentrifuge tube; 350 µL of isopropanol was added to the supernatant, mixed by vortexing, and the tubes centrifuged as above to pellet the DNA. The supernatant was poured off, and then 500 µL of 70% ethanol was added to the microcentrifuge tube and centrifuged for 5 minutes. The ethanol was poured off and the microcentrifuge tube was inverted on a paper towel and left to dry. Once dry, 100 µL of 10mM Tris HCl pH8 was added to the microcentrifuge tube and vortexed to dissolve the DNA pellet. This crude genomic DNA was used in PCR reactions, 2 µL per 50 µL reaction. The DNA was amplified with ITS1-F and ITS4 primers at 0.4 µM for 35

cycles with an annealing step of 55°C and an extension time of 1 minute. A portion of each reaction product was analyzed by electrophoresis on a 1.5% agarose gel, and the remainder of each successfully amplified product was purified using Zymo Research DNA Clean & Concentrator (catalog number D4013, California) according to the manufacturer's directions. The purified DNA was then sent to Quintara Biosciences (Albany, CA) for sequencing. These sequences were then analyzed by BLAST against the NCBI Genbank database to identify the closest match for each sequence; for a successful match the expect value had to be less than $1e-5$.

2.1.4 – Analysis of Cultured endophytes

After morphogrouping cultured fungi into 81 groups, and DNA barcoding of isolated endophytic fungi from each morphological group, an excel spreadsheet was created to organize all 259 of them. Most of the isolates fell into the single phylum *Ascomycota* and subphylum *Pezizomycotina*, but some could only be identified as “Fungal sp.” (TABLE 1). Within *Pezizomycotina*, OTUs were partitioned into six different taxonomic classes and eleven orders, with many of the cultured fungi identified at the genus and specie levels. TABLE 1 shows each identified taxon (shown as groups 1-33) with the number of fungal isolates cultured, and how many OTUs resulted from DNA sequencing. The group numbers assigned to each fungal taxon (TABLE 1) were used to create three simple excel spreadsheets. The first spreadsheet contained a row with all 33 group names, a column with the tree identities, and the number of fungi cultured per group for each tree. The second spreadsheet contained a contained a row with all 33 group names, a column with resistance level, and the number of fungi cultured per group for either resistant of susceptible trees. The third spreadsheet also contained a row with all 33 group names, a column with the three sites, and the number of fungi cultured per group for each site.

The three files mentioned above were used in Rstudio to analyze the isolated endophytic fungi. Using a script created for analysis in Rstudio, the program read each file (.csv) to check all the data for any errors. When no errors were found the program then analyzed the fungal taxa identified, and looked at their relative abundance. The first excel file was used to create a stacked bar graph that showed the relative abundance of each cultured fungal group per tree. The second file was used to create another stacked bar graph that looked at the relative abundance of cultured fungal groups between resistant and susceptible trees. The third file was used to create a stacked bar graph that showed the relative abundance of fungal groups cultured per site.

2.1.5 – Pine needle DNA extraction and PCR

To find fungal species that were not culturable, total DNA was extracted from pine needles after surface sterilization following the Arnold et al., (2007) protocol stated earlier. For each tree, 8 microcentrifuge tubes were each filled with 2-5 needles (50-60 mg of tissue) and one 5mm stainless steel bead. DNA extraction from the pine needles was done using Qiagen DNeasy Plant Mini kits (catalog number 69104, Germany). Needles were frozen at -80°C for 2-24 hours then homogenized using the Qiagen TissueLyser II (catalog number 85300, Germany) with the TissueLyser Adapter Set 2 x 24 (catalog number 69982, Germany) and settings of 30 shakes/second for one minute. Homogenization was better when the needles were frozen longer; better homogenization resulted in better DNA yields. Each preparation typically yielded 2-18µg of DNA (200µl of 10-90ng/µl).

Equal amounts of DNA from four tubes were then pooled, to yield two independent DNA samples per tree. The DNA samples (2 samples/tree x 18 trees = 36 total) were sent to the University of Minnesota Genomics Center (UMGC) for fungal ITS amplification, library formation, and sequencing. The fungal ITS1 spacer region was amplified using the following

primers: ITS1_Nextera_Forward (UMGC): 5' CTT GGT CAT TTA GAG GAA G*T AA 3' and ITS1_Nextera_Reverse (UMGC): 5' GCT GCG TTC TTC ATC GA* TGC 3'. These primers are similar to ITS1-F and ITS2 (Gardes & Bruns, 1993). The DNA samples were sequenced at the UMGC on an Illumina MiSEQ Platform with 275 bp paired-end reads.

2.1.6 – Analysis of ITS1 Next-Gen data contained within 36 paired end sequence files

The ITS1 data contained 36 paired end sequence files. Using the web-based platform Galaxy and the tool Trimmomatic, the FASTQ Illumina data was trimmed to remove adapters (Bolger et al., 2014). Once the data was cleaned, it was downloaded onto Dr. Zaid Abdo's lab server. Next a text wrangler file was created which incorporated tree identification, and included all 36 forward and 36 reverse reads.

Using the program Mothur, sequences in the Text Wrangler file were converted to contigs using the `make.contigs` command; this command returned 43,166 sequences. Next using the `screen.seqs` command, these contigs were screened to remove any sequences with uncertain bases or any sequences shorter than 32 basepairs, and/or longer than 275 basepairs; this step returned 42,003 useable DNA sequences. After being screened, the data was scanned for unique sequences using the `unique.seqs` command. This step returns and counts only the unique sequences, while grouping the identical sequences, making processing easier (Kozich et al., 2016).

In order to determine the fungal species associated with each unique sequence a workflow created by Dr. Zaid Abdo was utilized. The first command `cluster_fast` implemented as part of VSEARCH (Rognes et al., 2016), used the output file from Mothur `screen.seqs` command, to sort the sequences using a centroid-based algorithm based on their length. This command then clusters these sequences into operation taxonomic units (OTUs) based on a 0.97

identity cutoff and identifies a representative per cluster. The resulting output file is then processed through the command `uchime_denovo`, also implemented within VSEARCH (Rognes et al, 2016) This command detects and removes chimeras in those clusters or OTUs; these OTUs were moved into their own new file (Rognes et al., 2016). Chimeric sequences are DNA sequences that form from two or more originally separate DNA sequences joining together. It has been suggested that majority of chimeras form during extension, when a partially extended strand of DNA binds to a template obtained from a different sequence. This template acts as a primer, leading to the formation of a chimeric sequence (Smyth et al., 2010).

Using a perl script, a table with columns containing OTUs and rows containing the sample identifiers to each sample tree was created. In the table each cell contained a count representing the putative abundance of each OTU per sampled tree. This table used the `make.contigs` output file as a key to recognize the tree identification that each OTU was associated with. The table contained 821 different OTUs, and was saved as a `.csv` file, which can be opened in excel and used in R studio (RStudio Team, 2015).

Another perl script was then used to remove the chimeric OTUs identified using `uchime_denovo` from the OTU table obtained above, 30 chimeric OTUs were removed. The end result was an excel spreadsheet (a `.csv` file) containing all identified chimeria-free OTU's, 791 in total, and which tree they were found in. Several additional columns were inserted into this file including: site, level of resistance, and finally a column pooling the two tree samples for each individual tree together. These columns were added in order to look at all the variables within the samples.

Using the OTU-representative sequences associated with the chimera-free file created earlier with the VSEARCH pipeline, `blastn` was run to identify the best matched fungal species

from the National Center for Biotechnology Information (NCBI) Genbank Database for each OTU identified. The most similar fungal taxon given by the NCBI Genbank Database (with an expect value equal to or less than $1e-5$) was used to identify each OTU. The output file from this command gave a single column with one identified fungal taxon for each OTU. This column was transposed to become a row, and inserted it into the OTU.csv file replacing all the OTU numbers with a fungal taxon name; which in most cases was not identified at the species level. This excel file was used in Rstudio analysis to identify what fungal endophytes are present in the foliar tissue of bristlecone pine, and examine differences between identified resistant and susceptible trees.

With the aforementioned file, a script written specifically for analysis in R (using RStudio) was used. This script read the OTU.csv file to check all my samples and look for any errors. Any OTUs that were too small, in which they either contained no reads, or less than or equal to 100 reads were removed. This depth of coverage (only 100 reads per sample) will not provide a quality picture of which fungal taxa were present in my sample or their abundance. Possible error sequences were then removed by eliminating two reads per OTU; this was conservative and based on previous experience. After the removal of samples with few sequences and possible error sequences, a rarefaction curve was created to examine how well the DNA sequences were sampled. A rarefaction curve examines species diversity (richness) for each sample (tree), indicating how well each individual sample's population of endophytes was sequenced (Drive5 bioinformatics and software devices, 2017). Using the script, OTUs were analyzed and their relative abundance examined by creating a stacked bar graph format showing the top 11 identified fungal taxa from 17 out of the 18 samples. The depth of coverage was too low for tree 1110 so it was not included in any of the remaining analysis. Diversity was

measured using the Shannon and Inverse Simpson indices for diversity. Diversity indices measure the species diversity in a community using evenness and richness. Richness is the number of species per sample, and evenness is a measure of the relative abundance of different species making up the richness of an area (Magurran, 2004; Levin, 2009). Levin (2009) states “Simpson diversity is less sensitive to richness, and more sensitive to evenness than Shannon diversity, which, in turn, is more sensitive to evenness.”

Lastly, ordination plots were created; tree 1110 with the low depth of coverage was not included in the ordination plot analysis. An ordination plot positions similar objects so they are close to one another, while dissimilar objects are far apart (Clark, 2017). The ordination plots look at differences between sites, resistant and susceptible trees, as well as resistant and susceptible endophyte populations per site.

2.2 – Results

2.2.1 – Cultured fungi

In total, 259 fungal endophytes were cultured, resulting in 81 morphological groups. For each morphological group at least one member was sequenced; for larger groups multiple members were chosen resulting in a total of 184 ITS sequences. Of the 259 cultured fungi, representatives of each morphological group (184 cultured fungi) were identified through DNA barcoding to 33 different fungal taxa. Upon closer examination of the 33 identified fungal taxa, it was apparent that some fungal taxa contained multiple OTUs. For example, when comparing the 19 sequences identified as the taxon *Sordariomycetes*, it was evident that they were not all the same OTU. Some of the sequences were less than 97% identical; meaning they are not similar enough to be considered the same species. Sequences were compared to determine the number of OTU’s within each fungal taxon, resulting in 69 different OTUs in total. All of the cultured

fungus taxa are shown in TABLE 1, with a taxonomic group number, the number of isolates cultured, the number of isolates in each taxonomic group that were sequenced, the number of OTU's in each taxonomic group, the Genbank accession number of the best match used to identify each OTU, the expect value, and the accession number for each cultured fungus that was sequenced and submitted to Genbank. FIGURES 5-7 show photos of each taxonomic group from TABLE 1. FIGURE 8 shows the variety of morphological types seen in Group 33, the unknown fungi. It should be noted three morphological groups would consistently not amplify with the fungal ITS primers. These groups, each with a single isolate, were confirmed to be bacteria by PCR using the 16S primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Frank et al., 2008), and were identified as *Pseudonocardia*, *Nocardia*, and *Sphingomonas* sp. These were not analyzed further.

The stacked bar graph showing the relative abundance of the top thirteen fungal groups cultured per tree (FIGURE 9) indicates that fungal Group 22, the endophyte *Cenangium ferruginosum* was recovered from 13 out of 18 trees (not cultured in trees 4H11, 4H12, 4H2, 71, 714). Group 11, a *Xenomeris* sp., was also recovered from a different subset of trees, 13 out of 18 trees (not cultured in trees 1113, 1120, 113, 4H12 and 4H6). *Cenangium ferruginosum* is a fungus commonly found on pine, spruce, and fir species that causes a needle cast disease (Cornell University, 2015), while *Xenomeris* spp. is a common pathogenic fungus (Mulvey, 2016). Group 33, which are the unknown fungi, were recovered from 14 out of the 18 trees sampled (not cultured in trees 114, 4H12, 71, and 78).

Differences between the abundance of resistant and susceptible trees cultured endophyte populations were compared in FIGURE 10. Nine out of the eleven Group 3 spp. (*Dothideomycete* spp.) were cultured from resistant trees, while 15 out of the 22 cultured from

Group 25 (*Sordariomycete* spp.), 8 out of the 12 cultured from Group 8 (*Hormonema* sp.), and 27 out of the 33 cultured fungi from Group 33 (unknown fungi) were from resistant trees. When looking at FIGURE 10 it appears susceptible trees have more *Helotiales* sp. (Group 21) and *Cenangium ferruginosum* (Group 22) isolates than resistant trees. But 33 out of the 60 cultured isolates from Group 21 (*Helotiales* spp.) and 28 out of the 54 cultured isolates from Group 22 (*Cenangium ferruginosum*) came from trees inferred as resistant. As for the susceptible tree fungal endophyte populations, 12 out of the 22 isolated cultured from the *Xenomeris* sp. were from susceptible trees.

FIGURE 10 shows Group 33 (the unknown fungi), Group 25 (*Sordariomycete* spp), and Group 3 (*Dothideomycete* sp) were more commonly cultured from resistant than susceptible trees. Lastly FIGURE 11 compares cultured fungal endophyte populations between sites. This figure suggests geographical differences could influence the endophytic populations, however, more research is needed to fully understand these differences between sites.

2.2.2 Next Generation Illumina data from pine needle DNA

To identify fungal endophytes that were not cultured, total DNA from Rocky Mountain bristlecone pine needles was analyzed by NGS. Two independent samples per tree (samples A and B) were compared to determine how much overlap there was between them. FIGURE 12 shows a Venn diagram for each individual tree. For each tree the number of unique OTUs found only in Sample A, unique OTUs found only in Sample B, and OTUs found in both Samples A and B are identified. The percentage of overlap between the two samples is identified below each Venn diagram and varies from 4-21%. This low amount of overlap suggests our NGS data may be insufficient to fully represent the endophytic populations of *P. aristata* needles.

Data from the A and B pools for each tree were combined for further analysis. The analysis of the next generation Illumina data (one sample of pooled DNA per tree) initially produced 43,166 sequences. The sequences were screened to remove any sequences with uncertain bases or any sequences shorter than 32 bp, and/or longer than 275 bp; resulting in 42,003 usable sequences for analysis. These usable sequences were clustered into 791 unique OTUs. It should be noted that when OTUs with sequence lengths shorter than 75bp were removed this number collapsed to 545 OTUs. The analysis presented here reflects the 791 original OTUs.

The OTUs unique to resistant and susceptible trees per site were compared in FIGURE 13. This figure shows there are more unique fungal endophytic OTUs found in the resistant trees than the susceptible trees per site. There was only 16-17% overlap between the fungal endophytic populations of resistant and susceptible trees, suggesting they could harbor distinct fungal populations.

Only samples from 17 out of the 18 trees sampled produced sufficient sequence information to be used in the remaining analysis. The depth of coverage was too low for tree 1110 (Tree 10 from the Lookout Mountain site) so it was not included in the remaining analysis. It must also be noted here that tree 4H9 (tree 9 from the Trinchera site) returned a significant number of reads identified as *Malazessia globosa*. This fungus is commonly found on human skin and likely reflects contamination. As such, this fungus was removed from further analysis. In contrast, *Pyrenochaeta unigis-hominis* was identified from multiple trees, and while this fungus has also been isolated from human skin, recent genome sequencing reveals the presence of a “wide array of plant cell wall degrading enzymes” in the genome of this organism (Toh et

al., 2016). Therefore, we believe our data likely reflects its presence as an endophyte of bristlecone pines.

Although 791 unique OTUs were identified overall, only 41-132 unique fungal endophytic OTUs were identified in each of the 17 trees (FIGURE 12). The rarefaction curves for the NGS data (FIGURE 14) are a function of the relative abundance of OTUs and species. Tree samples that plateau suggest we were able to sequence the majority of the fungal endophytes present in those DNA samples (trees 71, 78, 717, 715, 1120, 114, 4H2, 4H9, 4H11, 4H12, 4H15). While some of the tree samples are heading in an upward trajectory (trees 75, 714, 113, 1113, 1115, 4H6), this suggests only a portion of the fungal endophytes present in those DNA samples were sequenced. These curves also show some of the trees had very small endophytic populations with minimal diversity (trees 75, 114 and 4H2) or a small population with more diversity (tree 78). Other trees have more diversity and moderately sized populations (trees 1113, 1120, 4H15, 1115, 717, 71, 113 and 4H12). While some trees show more diversity within larger endophytic populations (trees 4H6, 4H9, 715 and 714) or minimal diversity but a large population (tree 4H11). The rarefaction curves indicate we managed to sequence a large portion of the endophytic species present in the DNA samples, a smaller portion of the endophytic species present in the tree needles, and obtain an estimate of their relative population sizes within our samples.

FIGURE 15 is a stacked bar graph showing the next generation Illumina data, with the top 12 identified fungal taxa and their proportion in each tree. This figure indicates that *Pezizomycotina* sp. 40 was by far the most abundantly identified endophytic fungus for half of the trees sampled (Trees 1115, 1120, 113, 114, 4H12, 4H15, 717, 75, 78). *Pezizomycotina* sp. 40 is the 40th OTU identified out of 129 total *Pezizomycotina* OTUs identified. There are four

different uncultured fungi that were also commonly found throughout the samples.

Unfortunately, species identity for these fungi is unknown, except that other researchers have previously encountered them and submitted sequence data.

Diversity of the next-generation data was analyzed using Inverse Simpson and Shannon indices for diversity (FIGURE 16 A & D). FIGURE 16 C shows richness for each individual tree, representing how many species are present in each tree, and FIGURE 16 B shows the observed diversity of sequenced fungal communities per tree. However, between these figures five trees (1120, 113, 4H6, 4H9, and 715) showed differing diversity levels; these five trees showed much lower diversity in the Shannon index figure than the InvSimpson figure. Further examination of these individual trees showed they had higher numbers of rare OTU's (these OTU's were only found in a single tree); 1120 had 36 rare OTU's, 113 had 21, 4H6 had 60, 4H9 had 84, and 715 had 60 rare OTU's. These differences indicate the InvSimpson diversity index is influenced more heavily by abundance of species, or evenness, and the Shannon diversity index is influenced more by richness (DeJong, 1975).

Box plots were then created (FIGURES 17) using the Shannon index for diversity to examine the differences between resistant and susceptible trees at each site. St. Mary's (northern site) susceptible trees have a more diverse endophytic fungal population than the resistant trees. But the overlap of the box plots for resistant and susceptible endophyte populations suggests there may not be a difference between the two. Lookout Mountain's (central site) resistant trees had a more diverse endophytic population than the susceptible trees. The two box plots have no overlap, which suggests there is a significant difference in diversity between the fungal endophyte populations of resistant and susceptible trees. There is significant overlap between the

boxplots of the Trinchera (southern site) resistant and susceptible endophyte populations, suggesting there is no difference in diversity between these endophyte populations.

Ordination plots were created to examine the fungal community data, in which similar communities are plotted near each other, and dissimilar communities are placed farther apart from one another (Clark, 2017). The first ordination plot examined differences of the fungal endophytic diversity among all three sites (FIGURE 18). This figure suggests geography could influence the fungal endophytic populations, but more research is needed to address this question more thoroughly.

FIGURE 19 presents an ordination plot with fungal endophytic populations of resistant and susceptible trees across all sites. It combined resistant tree endophytic populations from all three locations to make one group of endophytes found in resistant trees, and combined susceptible fungal populations from all three locations to make one group of endophytes found in susceptible trees. While there is extensive overlap between the resistant and susceptible trees, some separation is also evident, indicating there are fungal taxa that may vary between the susceptible and resistant trees.

Differences between endophyte populations of resistant and susceptible trees are more evident when the data is also resolved by geographic location, as seen in FIGURE 20. When the data is examined by site, clear differences become apparent. Separation is more evident with the Lookout Mountain site, where resistant and susceptible trees appear completely separated, without overlap. The St. Mary's trees also appear notably separated, and they do not overlap with the other two sites, as was seen in FIGURE 18. Trees from Trinchera showed the most overlap, yet differences are still apparent. Again, this figure suggests geography influences the fungal endophytic populations, but more research is needed to address this question more accurately.

The OTU excel spreadsheet was examined to identify OTUs that were only found in resistant trees, or more commonly found in resistant trees than susceptible. All three sites had multiple “unknown fungi” found only in resistant trees, or more commonly found in resistant trees. All three sites also had *Cenangium ferruginosum* (*Helotiales* sp., a needle cast disease), and *Orbilia dorsalia* (*Orbiliales* sp., saprobic fungi) that were more commonly identified in resistant trees than susceptible. The most southern site (Trinchera), had several types of fungi that were more commonly found in resistant trees than susceptible. These fungi include: *Pezizomycotina* spp., *Fimentariella rabenhorstii* (*Sordariales* sp., saprobic fungi), *Pseudopeziza nigrella* (*Pezizales* sp., saprobic fungi), and *Preussia* spp. (*Pleosporales* spp., saprobic fungi). The most northern site’s (St. Mary’s) resistant trees had several fungi that were more commonly recovered, including: *Phaeoconiella* spp. (pathogenic fungi), *Fimentariella rabenhorstii* (*Sordariales* sp., a saprobic fungus), *Aureobasidium pullulans* (*Dothideales* sp., common epiphyte and/or endophyte), *Helotiales* spp. (mostly saprobes, some plant pathogens), *Preussia* spp. (*Pleosporales* spp., but a saprobic fungi), and *Pyrenochaeta unguis-hominis* (*Pleosporales* sp., common endophyte). The central site’s (Lookout Mountain) resistant trees had one fungus that was more frequently found in resistant trees than susceptible, and this was *Lachnellula arida* (*Helotiales* sp., saprobic). The only fungi more commonly identified in endophyte populations of susceptible trees included multiple “uncultured fungus clones”, several “fungal sp.,” and multiple “unknown fungus.”

The 33 fungal taxa identified through DNA barcoding were compared to the next-generation Illumina data to identify any taxa that were cultured but not captured within the Illumina data. The *Dothideomycete* fungi *Celosporium larixicola*, a *Hormonema* sp., *Cladosporium herbarum*, and *Preussia minimoides* were cultured but not captured in the

Illumina data. Although the species *C. herbarum* was not identified in the Illumina data multiple other *Cladosporium* spp. were found. Similarly, *Preussia minimoides* was not captured in the Illumina data, but multiple other *Preussia* spp. were identified. The *Sordariomycete* fungi *Valsa sordida*, and *Chaetomium globosum* were cultured but not captured in the Illumina data.

Although the species *C. globosum* was not identified, *Chaetomium convolutum* was captured multiple times within the Illumina data. Although *V. sordida* was not captured in the Illumina data, several *Cytospora* spp. (anamorph to *Valsa* spp.) were recovered. *Nemania* (*Ascomycete*), *Hypoxyton macrocarpum* (*Pyrenomycete*), *Aspergillus niger* (*Eurotiomycete*), and *Sarea* spp. (*Lecanoromycete*) were cultured but not captured in the Illumina data. Although *A. niger* was not recovered, multiple *Aspergillus* spp. were captured within the Illumina data. Similarly, *H. macrocarpum* was not recovered, but multiple *Hypoxyton* spp. were captured.

2.3 – Discussion

A major finding from these studies is that *P. aristata* hosts a diverse community of fungal endophytes. We recovered 69 OTUs by culture and 791 OTUs by NGS analysis. The larger number of OTUs identified by next generation sequencing was expected, since this technique is both more sensitive and reveals fungi that may not be culturable. Surprisingly, some of the fungi recovered by culture were not among the 791 OTUs identified by NGS. Two factors may be responsible for this. For cultured fungi, the entire ITS region (ITS1, 5.8S, ITS2) was used for fungal identification, whereas our NGS data only used ITS1 due to the technical limitations of the method employed (the Illumina sequencing platform can only provide short read lengths). Thus, some of the cultured fungi identified to species might only be identified to genus (for example) in the NGS data. Limiting the cultured sequence data to the ITS1 region would be expected to lead to more complete overlap. The second factor is depth of sampling. To gain

insight into how well each tree was sampled, we prepared two independent DNA pools for each tree, extracted from different needles. Comparing the OTUs identified from the two independent DNA pools for each tree yielded an overlap that varied from 4-21%. This low overlap indicates that out depth of sampling was insufficient to fully capture the diversity of endophytes present in the trees we sampled, and that additional sampling would recover more taxa.

Given this limitation in sampling depth, our ability to make strong conclusions regarding other variables, such as possible geographic variation, is also limited. Nonetheless, the available data, though only suggestive, are intriguing.

The relative proportions of endophytic fungal taxa cultured varied between resistant and susceptible populations, among sites, and individual trees. *Dothideomycete* sp., *Sordariomycete* spp., *Hormonema* spp., unknown fungi, *Helotiales* sp., and *Cenangium ferruginosum* were commonly cultured from resistant trees. In contrast, *Xenomeris* sp. were more commonly cultured from susceptible trees. The cultured fungi showed there was variation between the fungal endophytes found in resistant trees versus susceptible trees. These differences do not appear large with this data set, but could become more significant if the sample size were larger. There appeared to be differences in the endophytic populations per site, but more research is necessary to determine if these differences are authentic. If this portion of the research were to be repeated it would be ideal to visit more locations, and sample more trees at each location.

There were 11 fungal taxa identified as abundant in the next generation Illumina data, but *Cenangium ferruginosum* (*Helotiales* sp.) and *Orbilialia dorsalia* (*Orbiliales* sp.) were more often identified in all the resistant trees sampled than compared to the susceptible tree endophyte populations. Looking at each individual site there were more specific differences, suggesting each location had their own endophyte populations; but as mentioned earlier more research is

needed to identify if geography truly influences these fungal endophytic populations. When comparing the fungi of resistant trees identified in the Illumina data and the cultured fungi, *Cenangium ferruginosum* was one of the most regularly identified fungi from both techniques.

Comparing our data to other research reveals both commonalities and differences. One noticeable difference was the number of cultured fungi recovered from white pines. Studies conducted by Ganley et al. (2004, 2006) isolated over 2000 cultured endophytes from western white pines, whereas this research only recovered 259 isolated fungi. These differences could reflect either sampling depth or perhaps physiological differences between the two white pine species. It is known that Rocky Mountain bristlecone pines are highly resinous, for example, (Miller, 2005), and this or other factors could possibly impact the number and/or the culturability of the fungal endophytes.

Kurose et al. (2012) identified five fungal taxa that antagonize the rust pathogen *Puccinia polygami-amphibii* var. *tovariae*. Of their five antagonistic fungal taxa, three were *Sordariomycete* spp. (*Colletotrichum*, *Pestalotiopsis*, and *Phomopsis*) and two were *Dothideomycete* spp. (*Phoma* and *Alternaria*). We also commonly identified *Sordariomycete* spp. and *Dothideomycete* spp. in this research, and it appeared these taxa may be more common in resistant trees. Although there are multiple fungal taxa within these classes, there is evidence that a wide array of fungal endophytes can impact white pine seedling survival rates. Ganley et al. (2008) showed that white pine (*Pinus monticola*) seedlings pretreated with different combinations of endophytes had increased survival rates of 3-16%, when challenged by WPBR.

Our data suggest that WPBR resistant trees may harbor a different and/or more diverse community of endophytes than sensitive trees, as the overlap in OTUs between resistant and susceptible trees at each site was only 16-17%, and more OTUs in total were recovered from

resistant trees at each site. The observed differences in endophyte populations between WPBR resistant and susceptible trees could reflect genetic differences between the trees, possibly in general resistance genes, or other genetic and/or physiological differences.

While this first survey of bristlecone endophytes hints at intriguing differences in endophyte populations at different geographical sites and between resistant and sensitive trees, additional sampling would be required to make these findings definitive.

2.4 – Conclusions

This work surveyed fungal endophyte communities in wild populations of Rocky Mountain bristlecone pines trees for the first time. Trees previously identified as either susceptible or partially resistant to white pine blister rust appeared to harbor different endophyte communities. In addition, endophyte communities were observed to vary geographically. The collection of cultured endophytes isolated in this work may provide a resource for future seedling inoculation experiments to test whether such treatments may be useful in combating white pine blister rust. It is hoped that this work may be of some benefit towards preserving these majestic and charismatic bristlecone pines for future generations.

2.5 – Tables and Figures

TABLE 1 – Fungal isolate ITS1 sequences analyzed by BLAST against the Genbank database and organized by their taxonomic identification. All except “Fungal spp.” are in Phylum *Ascomycota* and subphylum *Pezizomycotina* unless otherwise indicated.

^a Division/ Subdivision/ Class/ Subclass/ Order/ Family/ Genus and Specie)

^b The number of fungi isolated from culture of each identified taxonomic group

^c The number of cultured fungi for each taxonomic group that were identified through DNA barcoding

^d Different OTU’s (each varying by less than 3%) within each taxonomic group

^e GenBank accession numbers for endophyte taxa with unique ITS sequences (number of isolates with the same accession number)

^f Expect value (E value) of 0.0 means <1e-179

^g GenBank accession numbers for the sequenced cultured fungi

^h(The OTU each isolate was associated with) The accession number assigned to each sequenced isolated fungi

Group # ^a	Taxonomic Identification ^b	#Isolates ^c	#Sequenced ^d	OTU’s ^e	Best Match Accession # (# isolates) ^f	E value ^g	(Associated OTU) Isolate Accession # ^h
1	<i>Ascomycota</i>	1	1	-1	HM239727.1	0.0	MF347875
2	<i>Pezizomycotina</i> sp.	3	3	-1 -2 -3	GQ153153.1 GQ153221.1 GQ153004.1	0.0 0.0 0.0	(-1) MF347765 (-2) MF347766 (-3) MF347770
3	<i>Dothidomycete</i> spp.	11	9	-1 -2 -3 -4 -5	KU059942.1 KP990991.1 (2x) KT264663.1 (2x) KT264672.1 (2x) KX909227.1 (2x)	0.0 0.0 0.0 0.0 0.0	(-1) MF347800 (-2) MF347779 (-2) MF347869 (-3) MF347773 (-3) MF347813 (-4) MF347771 (-4) MF347816 (-5) MF347806 (-5) MF347810
4	<i>Dothidomycete/ Dothideomycetidae/ Incertae sedis/ Incertae sedis/ Cladosporium larixicola</i>	1	1	-1	FJ997287.1	0.0	MF347918
5	<i>Dothidomycete/ Dothideomycetidae/Capnodiales/ Davidiellaceae/ Cladosporium</i> sp.	1	1	-1	KX788169.1	0.0	MF347880
6	<i>Dothidomycete/ Dothideomycetidae/Capnodiales/ Davidiellaceae/ Cladosporium herbarum</i>	1	1	-1	KY039306.1	0.0	MF347854
7	<i>Dothidomycete/ Dothideomycetidae/</i>	1	1	-1	KP193122.1	0.0	MF347756

	<i>Dothideales/ Dothioraceae/ Sydowia polyspora</i>						
8	<i>Dothidomycete/ Dothideomycetidae/ Dothideales/ Dothioraceae/ Hormonema sp.</i>	12	12	-1	KT264708.1	0.0	MF347753 MF347759 MF347760 MF347761 MF347764 MF347772 MF347790 MF347797 MF347809 MF347811 MF347828 MF347890
9	<i>Dothidomycete/ Pleosporomycetidae/ Pleosporales/</i>	2	2	-1	GU910721.1 GU910854.1	0.0 0.0	MF347820 MF347796
10	<i>Dothidomycete/ Pleosporomycetidae/ Pleosporales/ Sporormiaceae/ Preussia minimoides</i>	1	1	-1	KP101211.1	0.0	MF347847
11	<i>Dothidomycete/ Pleosporomycetidae/ Pleosporales/ Venturiaceae/ Xenomeris spp.</i>	22	19	-1	KT264669.1	0.0	MF347919 MF347907 MF347921 MF347923 MF347922 MF347924 MF347758 MF347925 MF347873 MF347876 MF347894 MF347914 MF347895 MF347927 MF347928 MF347897 MF347929 MF347916 MF347917
12	<i>Eurotiomycete sp.</i>	1	1	-1	KP992004.1	4.00E-145	MF347793

13	<i>Eurotiomycete/ Chaetothyriomycetidae</i> sp.	2	2	-1	KP202995.1 KP202996.1	0.0 0.0	MF347778 MF347859
14	<i>Eurotiomycete/ Chaetothyriomycetidae/ Chaetothyriales/ Herpotrichiellaceae/ Phaeomoniella</i> sp.	1	1	-1	KT264593.1	0.0	MF347870
15	<i>Eurotiomycete/ Eurotiomycetidae/ Eurotiales/ Trichocomaceae/ Penicillium</i> sp.	1	1	-1	HM535390.1	0.0	MF347768
16	<i>Eurotiomycete/ Eurotiomycetidae/ Eurotiales/ Trichocomaceae/ Penicillium chrysogenum</i>	1	1	-1	KY617054.1	0.0	MF347780
17	<i>Eurotiomycete/ Eurotiomycetidae/ Eurotiales/ Trichocomaceae/ Aspergillus niger</i>	1	1	-1	KY318505.1	0.0	MF347834
18	<i>Lecanoromycetes/ Ostropomycetidae/ Agryiales/ Agryiaceae/ Sarea</i> sp.	1	1	-1	DQ317349.1	0.0	MF347844
19	<i>Lecanoromycetes/ Ostropomycetidae/ Agryiales/ Agryiaceae/ Sarea resinae</i>	1	1	-1	JF440615.1	0.0	MF347866
20	<i>Leotiomycetes</i> sp.	3	3	-1 -2 -3	GQ153028.1 KX909185 KX909200.1	0.0 0.0, 2.00E-152	(-1) MF347784 (-2) MF347902 (-3) MF347807
21	<i>Leotiomycetes/ Leotiomycetidae/ Helotiales</i> sp.	60	26	-1 -2 -3	HQ845747.1 (23x) HQ845748.1 (2x) KM246498.1	0.0 0.0 0.0	(-1) MF347906 (-1) MF347882 (-1) MF347883 (-1) MF347881 (-1) MF347891 (-1) MF347887 (-1) MF347864 (-1) MF347910 (-1) MF347868 (-1) MF347867 (-1) MF347912 (-1) MF347871 (-1) MF347872 (-1) MF347874 (-1) MF347926

							(-1) MF347913 (-1) MF347915 (-1) MF347850 (-1) MF347855 (-1) MF347858 (-1) MF347857 (-1) MF34786 (-1) MF347900 (-2) MF347879 (-2) MF347889 (-3) MF347886
22	<i>Leotiomyces/ Leotiomycetidae/ Helotiales/ Helotiaceae/ Cenangium ferruginosum</i>	54	17	-1	KY462796.1 (17x)	0.0	MF347839 MF347920 MF347865 MF347877 MF347878 MF347908 MF347849 MF347863 MF347852 MF347853 MF347856 MF347862 MF347861 MF347840 MF347841 MF347843 MF347848
23	<i>Leotiomyces/ Leotiomycetidae/ Helotiales/ Helotiaceae/ Lachunellula arida</i>	1	1	-1	KC464635.1	0.0	MF347763
24	<i>Pezizomycete</i> sp.	5	5	-1 -2 -3 -4	KP991515.1 GQ153137.1 KX909079.1 KX909147.1 (2x)	0.0 0.0 0.0 0.0	(-1) MF347823 (-2) MF347766 (-3) MF347808 (-4) MF347782 (-4) MF347755
25	<i>Sordariomycete/</i>	22	19	-1 -2 -3 -4	KP992032.1 (12x) KP991990.1 GQ153162.1 (2x) GQ153238 (2x)	0.0 0.0 0.0 0.0	(-1) MF347805 (-1) MF347884 (-1) MF347888 (-1) MF347787

				-5	GQ153014.1 (2x)	0.0	(-1) MF347814 (-1) MF347835 (-1) MF347893 (-1) MF347898 (-1) MF347791 (-1) MF347821 (-1) MF347901 (-1) MF347903 (-2) MF347783 (-3) MF347798 (-3) MF347788 (-4) MF347829 (-4) MF347838 (-5) MF347911 (-5) MF347904
26	<i>Sordariomycete/ Sordariomycetidae/ Coniochaetales/</i>	4	4	-1	GU910453.1 (4x)	0.0	MF347885 MF347833 MF347785 MF347846
27	<i>Sordariomycete/ Sordariomycetidae/Diaportheales/ Valsaceae/ Valsa sordida</i>	2	2	-1	KU143710.1 (2x)	0.0	MF347767 MF347754
28	<i>Sordariomycete/ Sordariomycetidae/ Sordariales/ Chaetomiaceae/ Chaetomium globusum</i>	2	2	-1	KX013209.1 (2x)	0.0	MF347892 MF347851
29	<i>Sordariomycete/ Sordariomycetidae/ Sordariales/Lasiopaeriaceae/Fimen tariella rabenhorstii</i>	1	1	-1	KP050669.1	0.0	MF347781
30	<i>Sordariomycete/ Xylariomycetidae/ Xylariales/ Xylariaceae/ Nemania sp.</i>	2	2	-1	HM123573.1 (2x)	0.0	MF347804 MF347822
31	<i>Sordariomycete/ Xylariomycetidae/ Xylariales/ Xylariaceae/Hypoxylon macrocarpum</i>	3	2	-1	HM192912.1 (2x)	0.0	MF347817 MF347789
32	<i>Sordariomycete/ Xylariomycetidae/Trichosporiales/ Incertae sedis/ Nigrospora oryzae</i>	1	1	-1	EU821485.1	0.0	MF347896
33	Fungal spp.	34	34	-1	EF419929.1 (3x)	0.0	(-1) MF347769

				-2	KP335596.1	0.0	(-1) MF347799
				-3	KP335599.1	0.0	(-1) MF347825
				-4	KY944997.1	0.0	(-2) MF347803
				-5	HM123152.1 (2x)	0.0	(-3) MF347845
				-6	HM123159.1	0.0	(-4) MF347930
				-7	HM123603.1	0.0	(-5) MF347812
				-8	HM123668.1 (2x)	0.0	(-5) MF347831
				-9	KF752649.1	0.0	(-6) MF347905
				-10	KF752688.1	0.0	(-7) MF347802
				-11	JX978247.1	0.0	(-8) MF347826
				-12	HQ535887.1 (3x)	0.0	(-8) MF347909
				-13	HQ535937.1	0.0	(-9) MF347775
				-14	HQ540687.1 (4x)	0.0	(-10) MF347794
				-15	HQ535867.1 (4x)	0.0	(-11) MF347818
				-16	JX136324.1	0.0	(-12) MF347827
				-17	JF300529.1 (2x)	0.0	(-12) MF347786
				-18	KC965928.1	0.0	(-12) MF347842
				-19	KF617854.1	0.0	(-13) MF347830
				-20	KT965065.1	0.0	(-14) MF347801
				-21	JX136080.1	0.0	(-14) MF347757
							(-14) MF347836
							(-14) MF347819
							(-15) MF347762
							(-15) MF347832
							(-15) MF347815
							(-15) MF347792
							(-16) MF347776
							(-17) MF347777
							(-17) MF347774
							(-18) MF347837
							(-19) MF347899
							(-20) MF347824
							(-21) MF347795

TABLE 2 – Table showing the coordinates, elevation, site identification number, seedtree identification number, and the susceptibility (S) or partial resistance (R) of the tree to white pine blister rust based on seedling survival after artificial inoculation with *C. ribicola* in a nursery setting.

Site Name	Site Lat. (WGS84)	Site Long. (WGS84)	Site Elev. (m)	Site ID	Seedtree ID	Inferred relative resistance phenotype within the site
St Marys	39.82993	-105.65501	3400	7	1	S
				7	8	S
				7	5	S
				7	14	R
				7	15	R
				7	17	R
				7	17	R
Lookout Mountain	37.92809	-106.45804	2870	11	13	S
				11	4	S
				11	17	S
				11	20	R
				11	3	R
				11	15	R
Trinchera	37.33838	-105.15704	3560	4H	2	S
				4H	12	S
				4H	15	S
				4H	11	R
				4H	6	R
				4H	9	R



FIGURE 1 – Picture of isolate B11.3 (the second isolated fungi from site 11, Lookout Mountain, tree 3) (*Sordariomycete* sp.) under the microscope, showing the ascus with four multiseptate ascospores (taken by Alyssa Albertson)

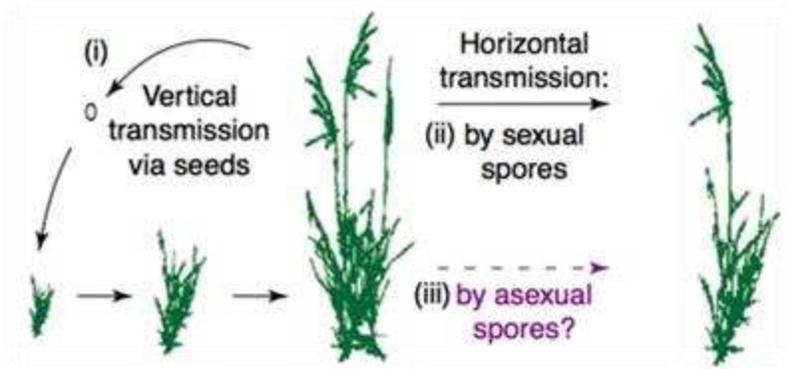


FIGURE 2 – Showing how endophytes can be transmitted between parent and progeny; vertical or horizontal transmission (from Saikkonen et al. 2004)



FIGURE 3 –Life cycle of *Cronartium ribicola* (White Pine blister rust) (from Burns et al. 2008).



FIGURE 4 – Map of the three locations sampled. Image obtained from <http://www.freeworldmaps.net/united-states/colorado/map.html>

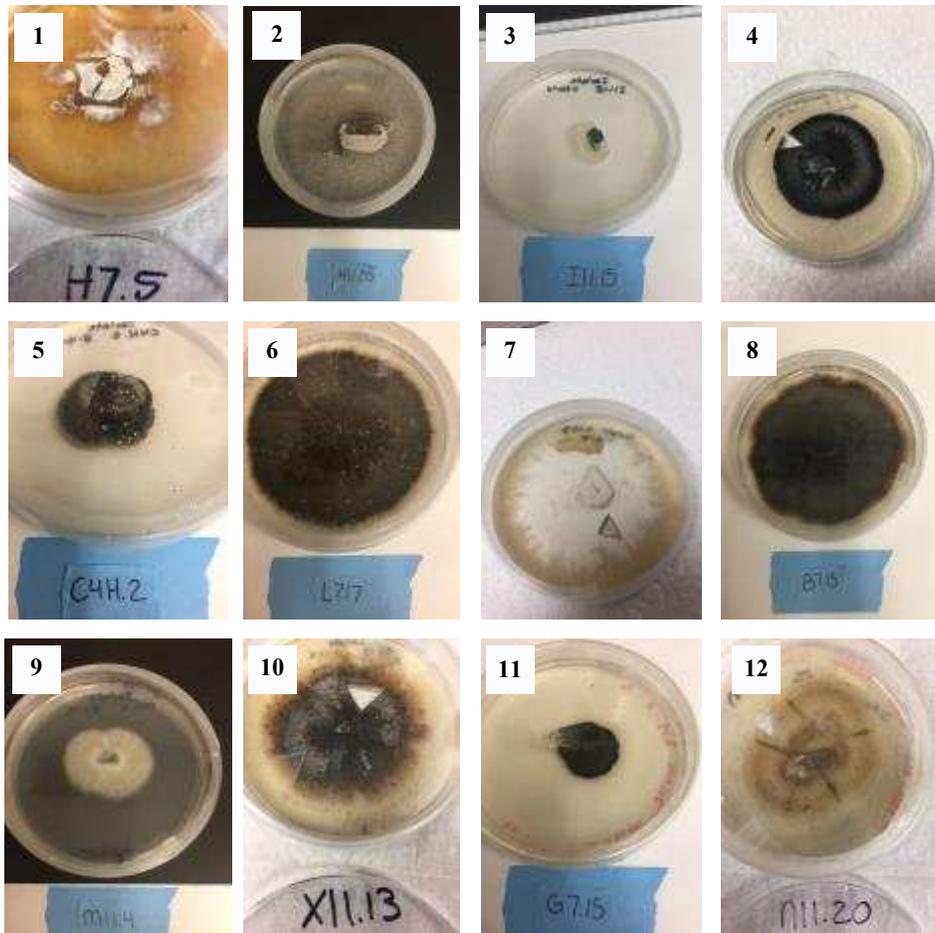


FIGURE 5 – Picture identification of cultured groups 1-12. The group number is located in the upper left hand corner. These pictures are associated with the group numbers in TABLE 1.

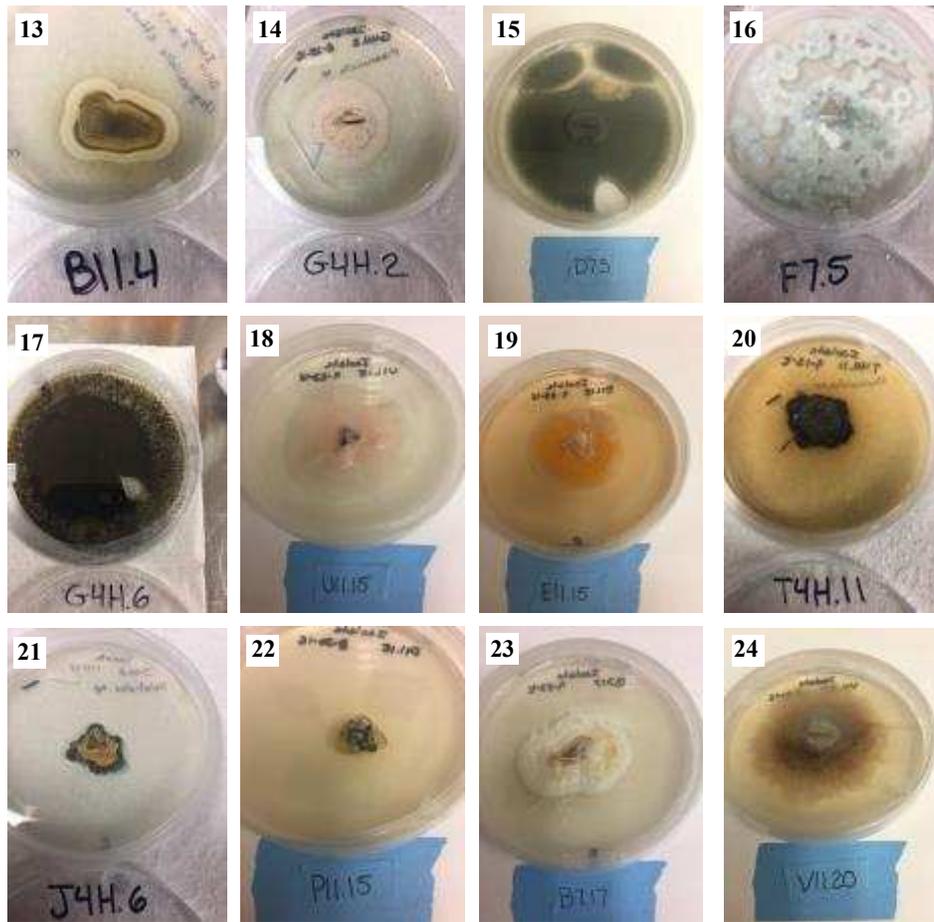


FIGURE 6 – Picture identification of cultured groups 13-24. The group number is located in the upper left hand corner. These pictures are associated with the group numbers in TABLE 1.

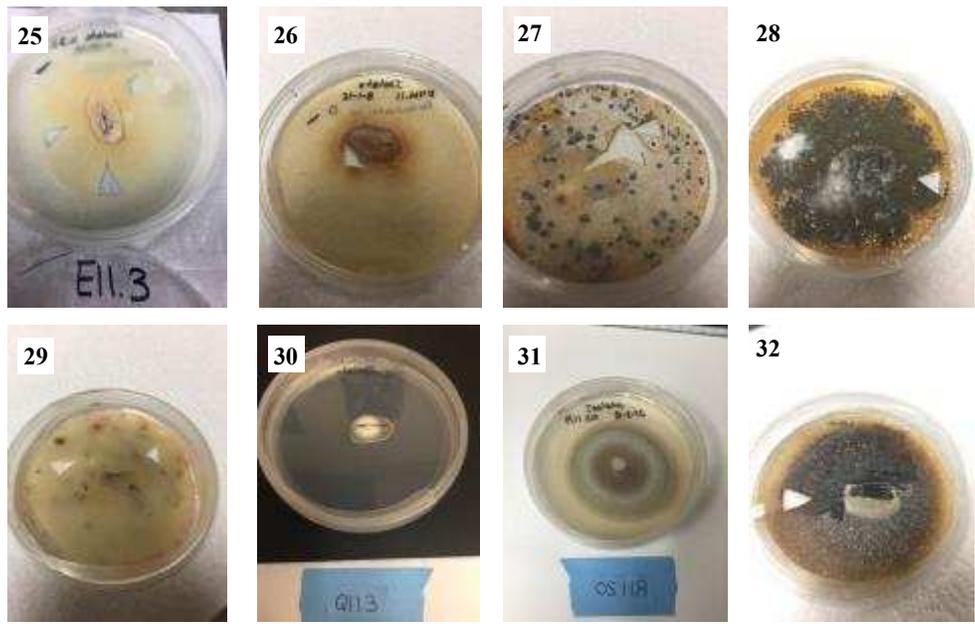


FIGURE 7 – Picture identification of cultured groups 25-32. The group number is located in the upper left hand corner. These pictures are associated with the group numbers in TABLE 1.

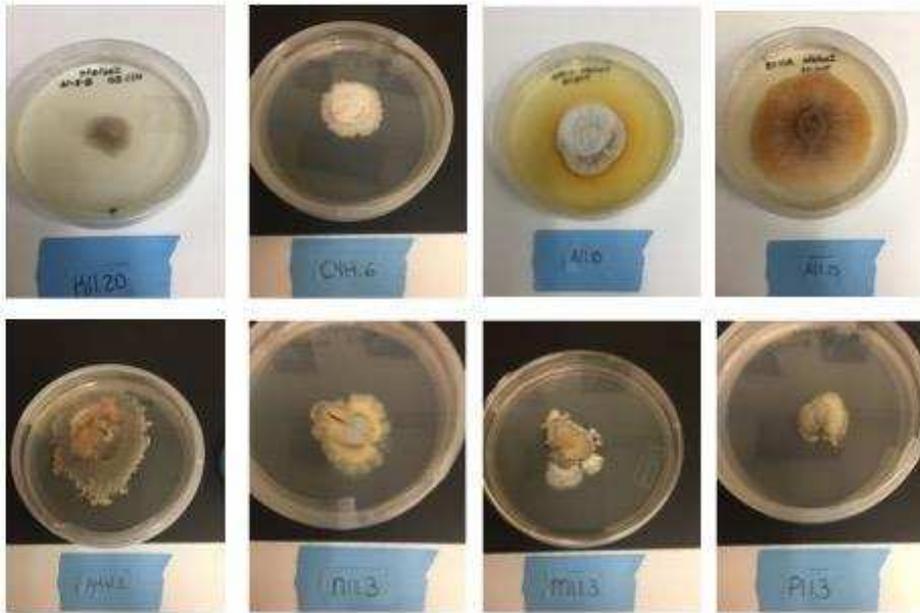


FIGURE 8 - Picture identification of the cultured group 33. These are just 8 of the 34 unknown fungi cultured from group 33, associated with TABLE 1.

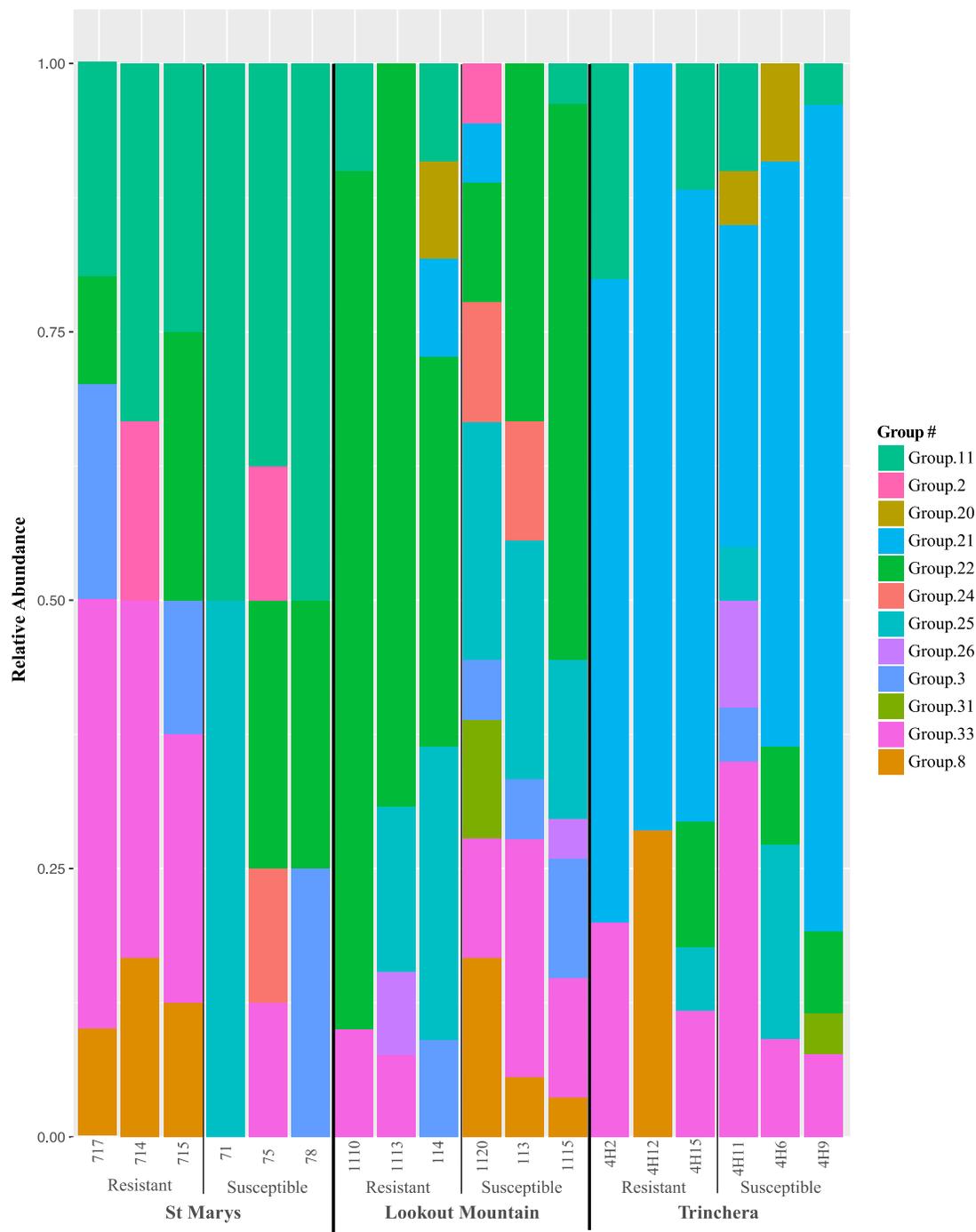


FIGURE 9 – This is a stacked bar graph showing the relative abundance of the top twelve fungal groups identified from culture per tree. Tree identification numbers are shown beneath each column. Inferred WPBR resistance phenotype is listed below tree ID, and site locations are at the bottom.

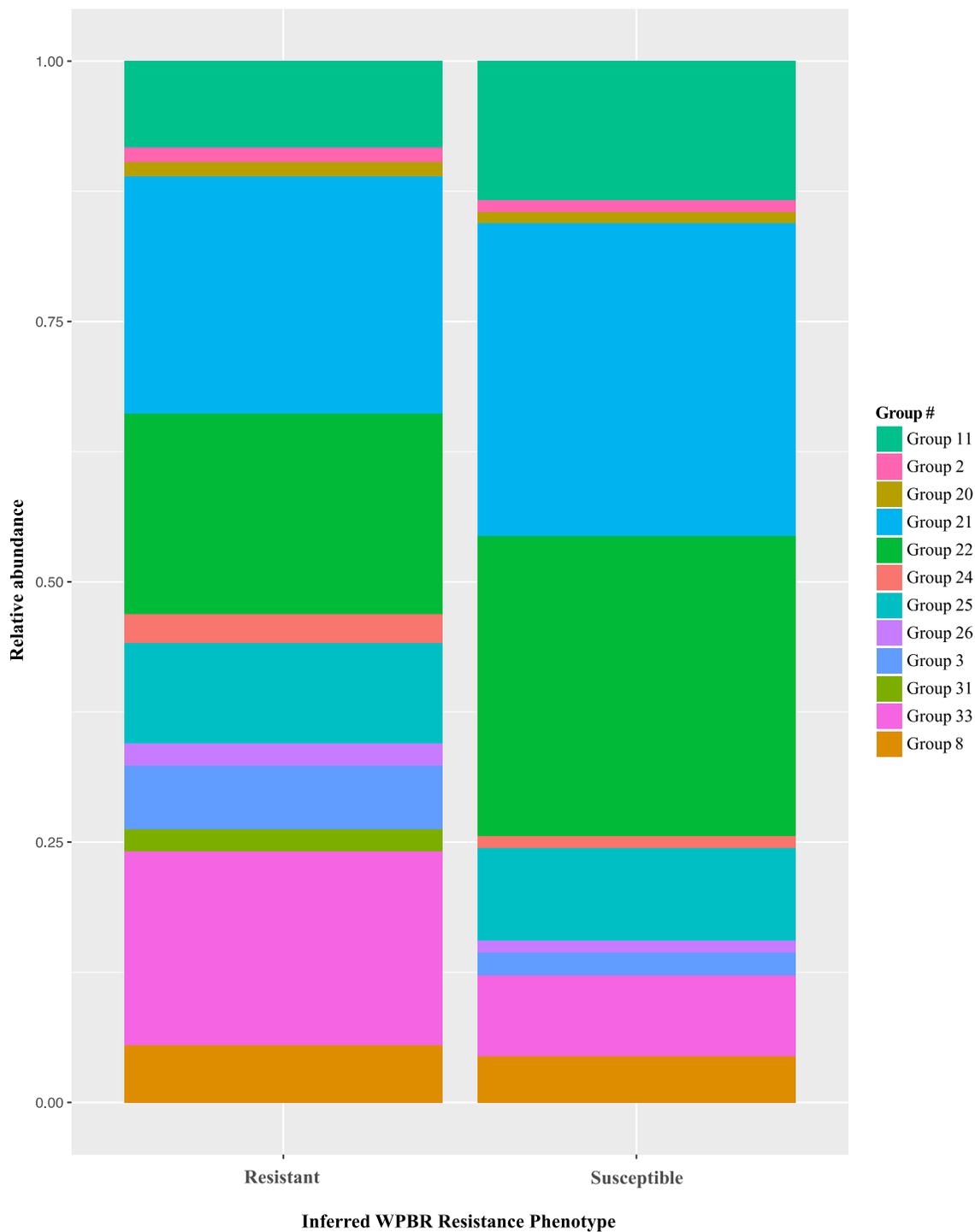


FIGURE 10 – This stacked bar graph shows the relative abundance of the top twelve fungal groups identified from culture for pooled resistant and susceptible trees across all three sites.

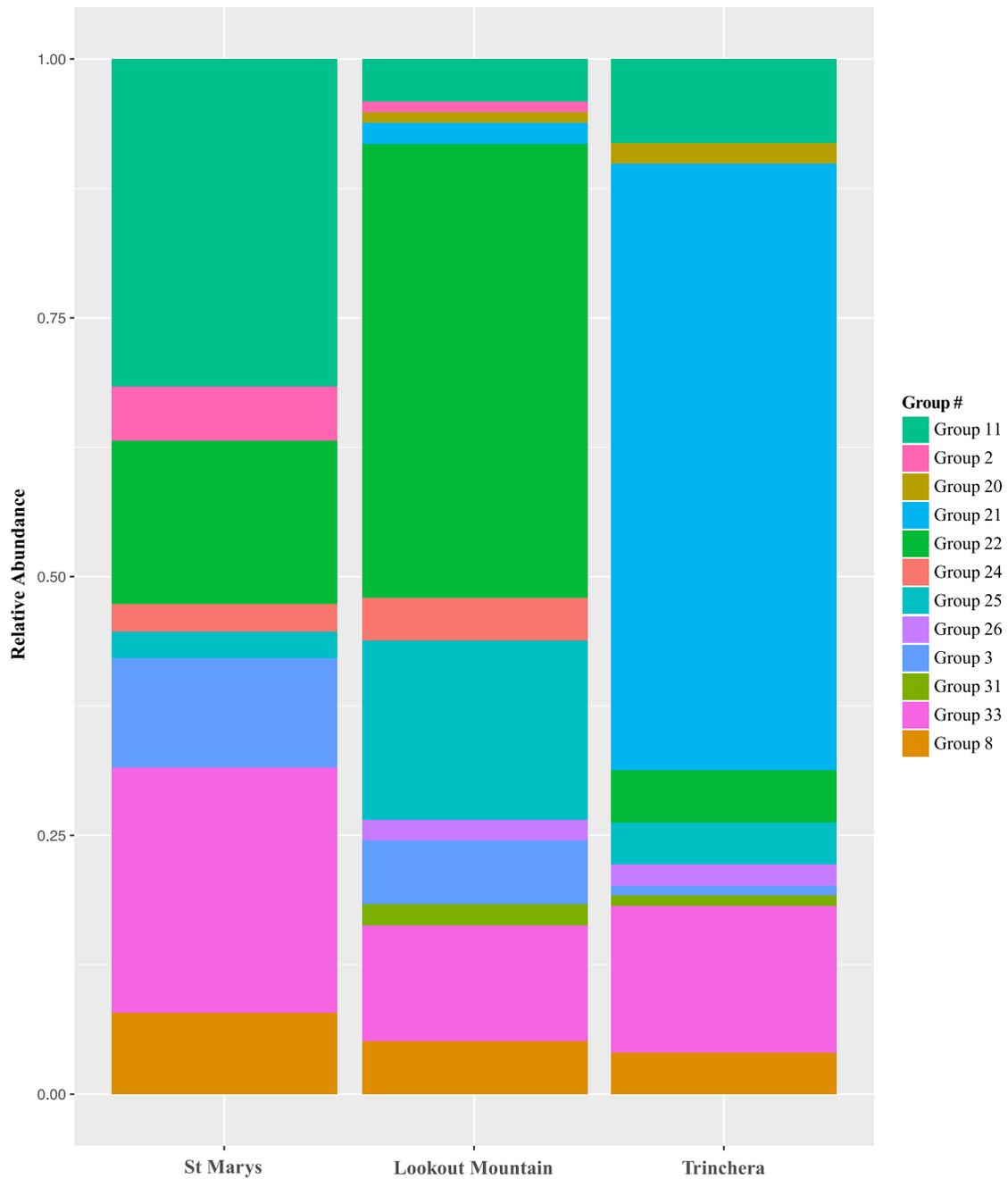


FIGURE 11 – This stacked bar graph shows the relative abundance of the top twelve fungal groups identified from culture per site.

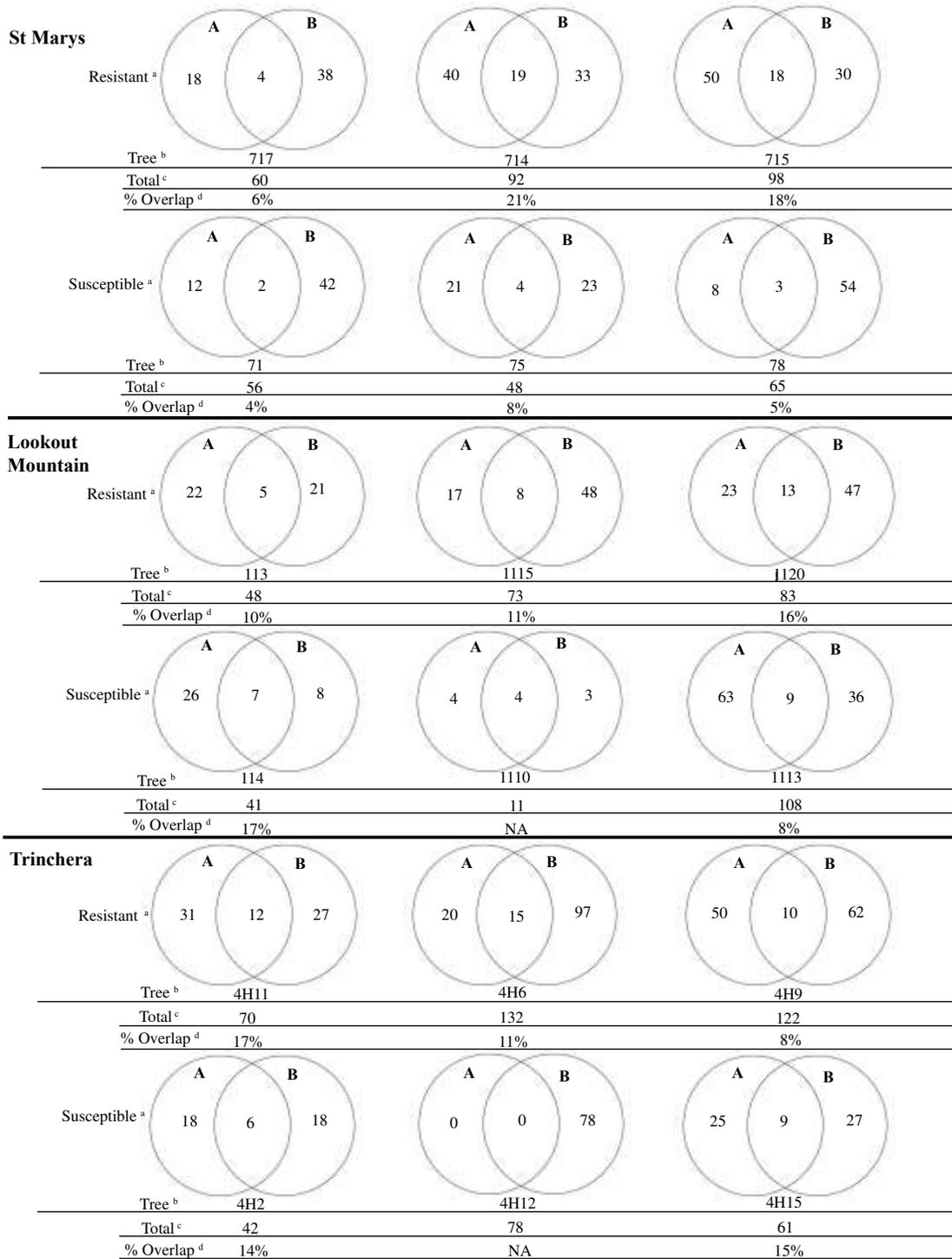


FIGURE 12 –Shows the overlap of unique OTUs between the two independent DNA samples (A or B) per tree and per site

^a Inferred level of resistance for each tree

^b Tree identification

^c Total number of unique OTUs for each tree

^d Percent overlap between sample A and B

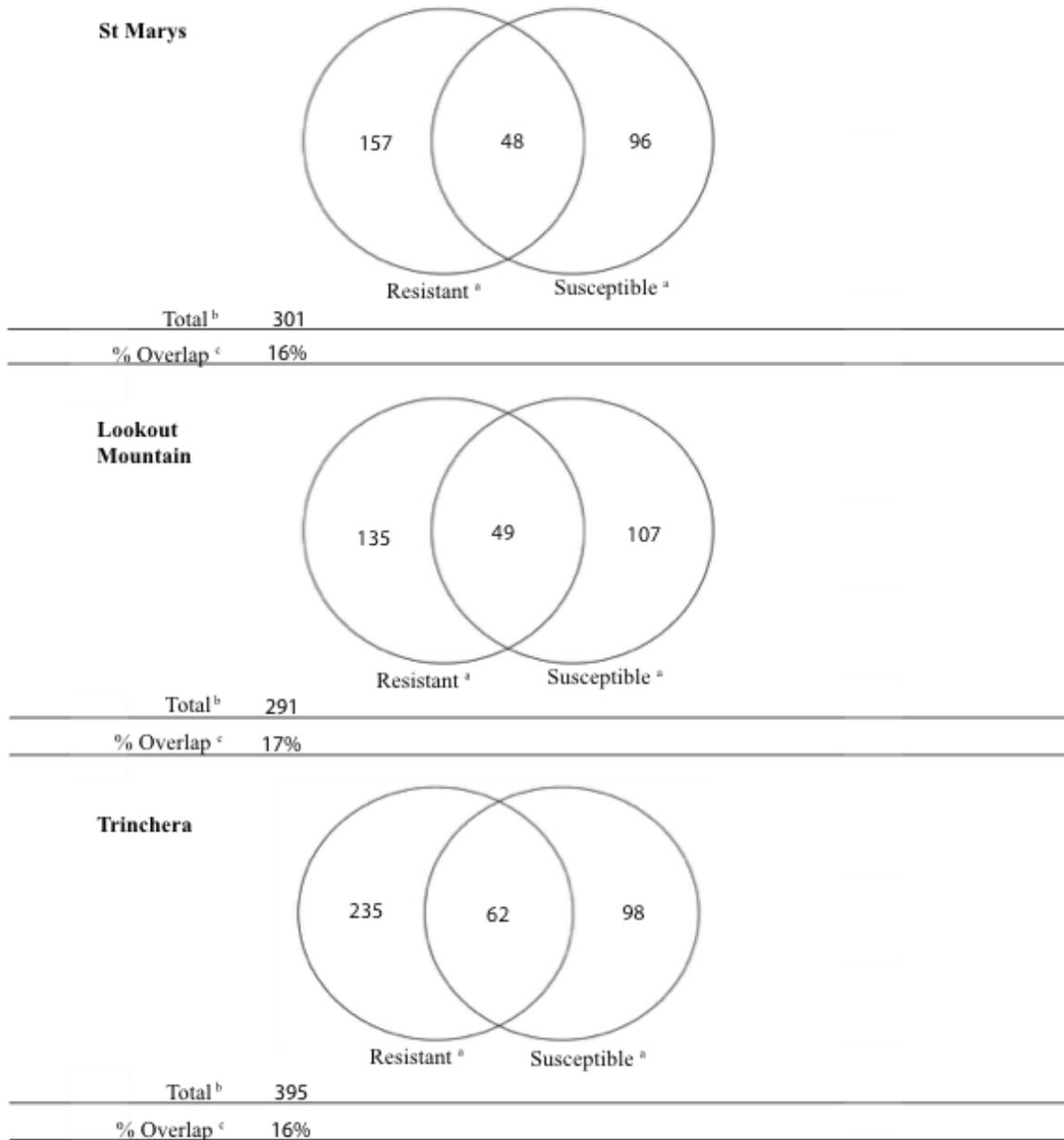


FIGURE 13 – Shows the overlap of unique OTUs between the resistant and susceptible tree’s fungal endophytic populations per site.

^a Inferred level of resistance for each tree

^b Total number of unique OTUs for each site

^c Percent overlap between resistant and susceptible tree’s fungal endophytic populations

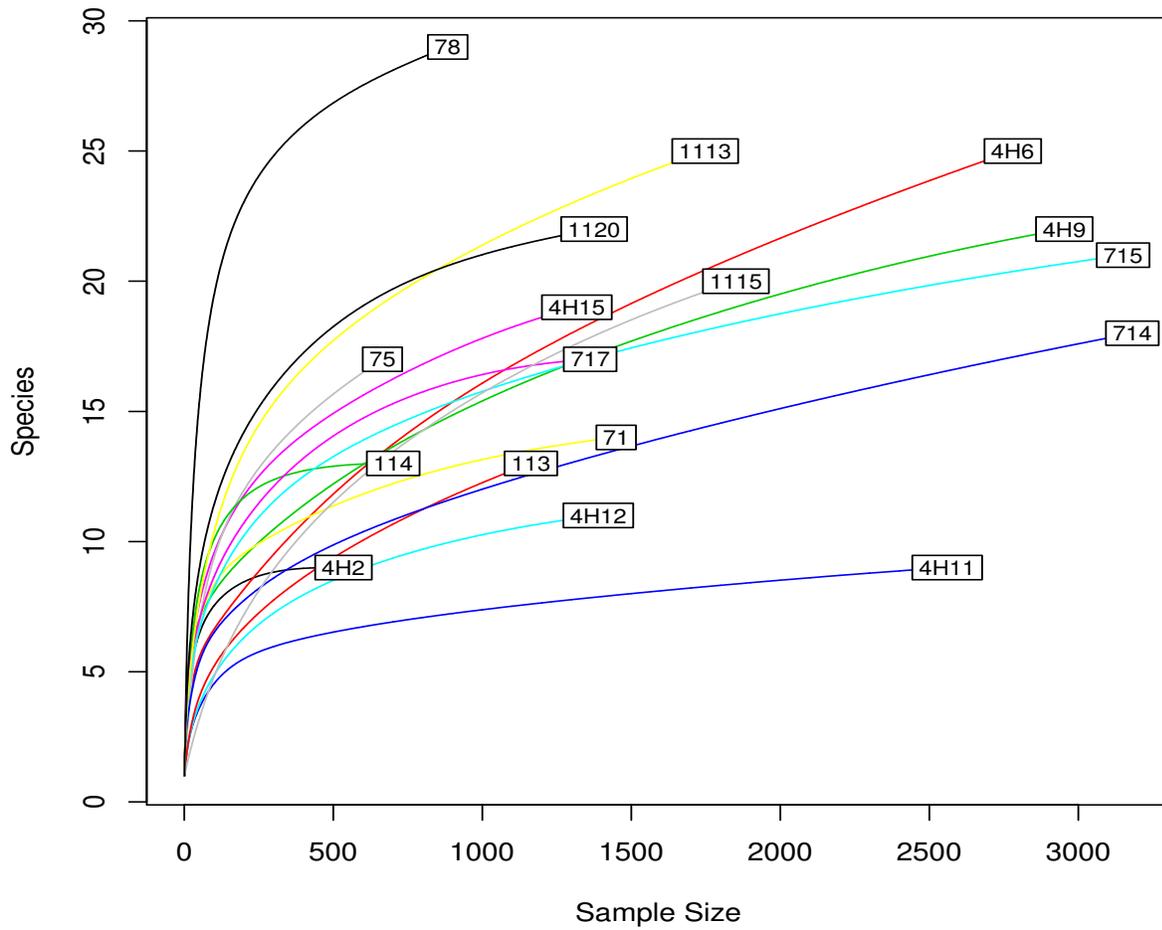


FIGURE 14 –Individual rarefaction curves for 17 of the 18 trees sampled by NGS. These curves are a function of the relative abundance of OTUs and species per sample.

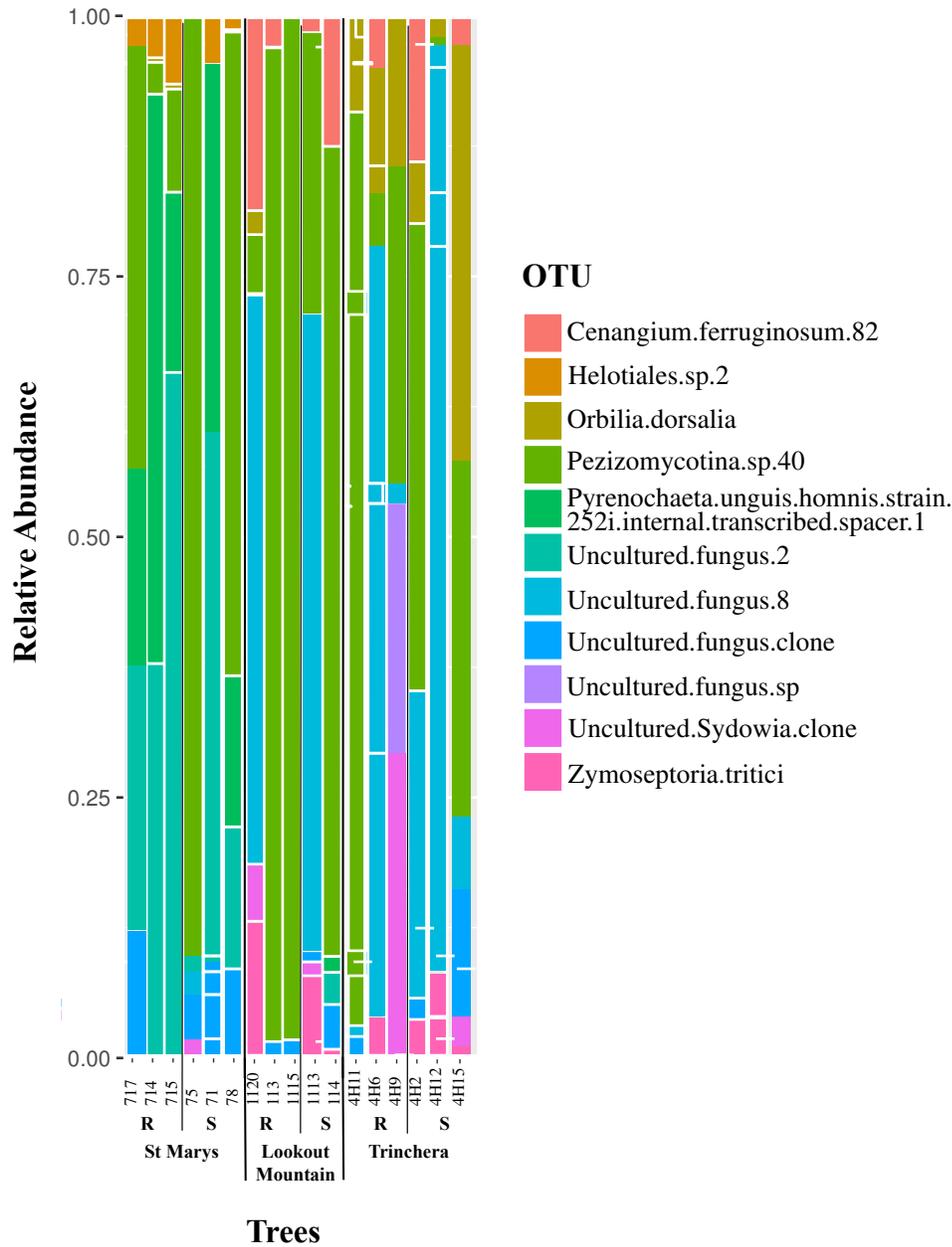


FIGURE 15 – This stacked bar graph shows the relative abundance of the top 11 identified OTUs from the NGS data for 17 out of the 18 trees sampled. Tree identification numbers are shown beneath each column. Previously inferred resistant and susceptible tree phenotypes to WPBR are below tree ID, and site locations are at the bottom.

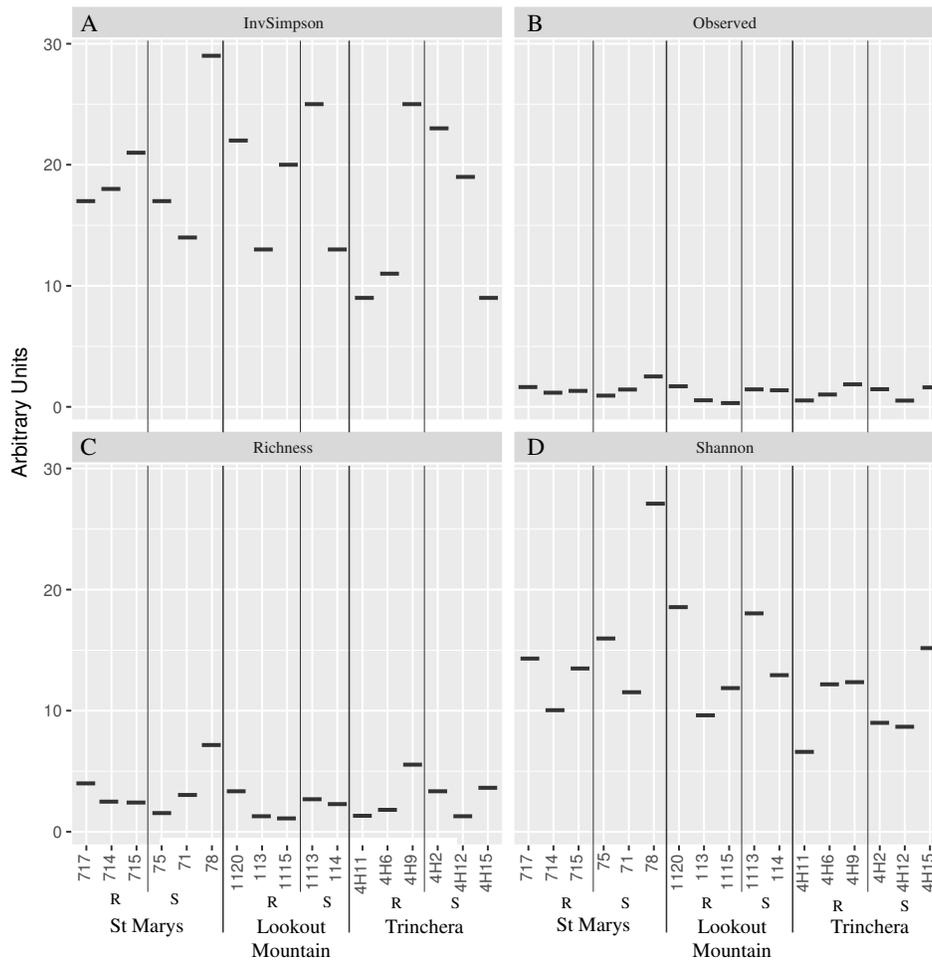


FIGURE 16 – This figure shows the level of endophytic biodiversity in 4 different ways for 17 out of the 18 trees sampled. This figure is using the NGS data. The dark, longer lines separate sites, while the light, shorter lines separate resistant (R) and susceptible (S) trees per site. The y axes represents: Inverse Simpson, Observed Richness, Expected Richness, and Shannon Index in arbitrary units.

Part A – Using Inverse Simpson to show endophytic diversity per tree.

Part B – Showing the observed diversity per tree.

Part C – Showing the richness of each tree.

Part D – Using Shannon Index to show diversity per tree.

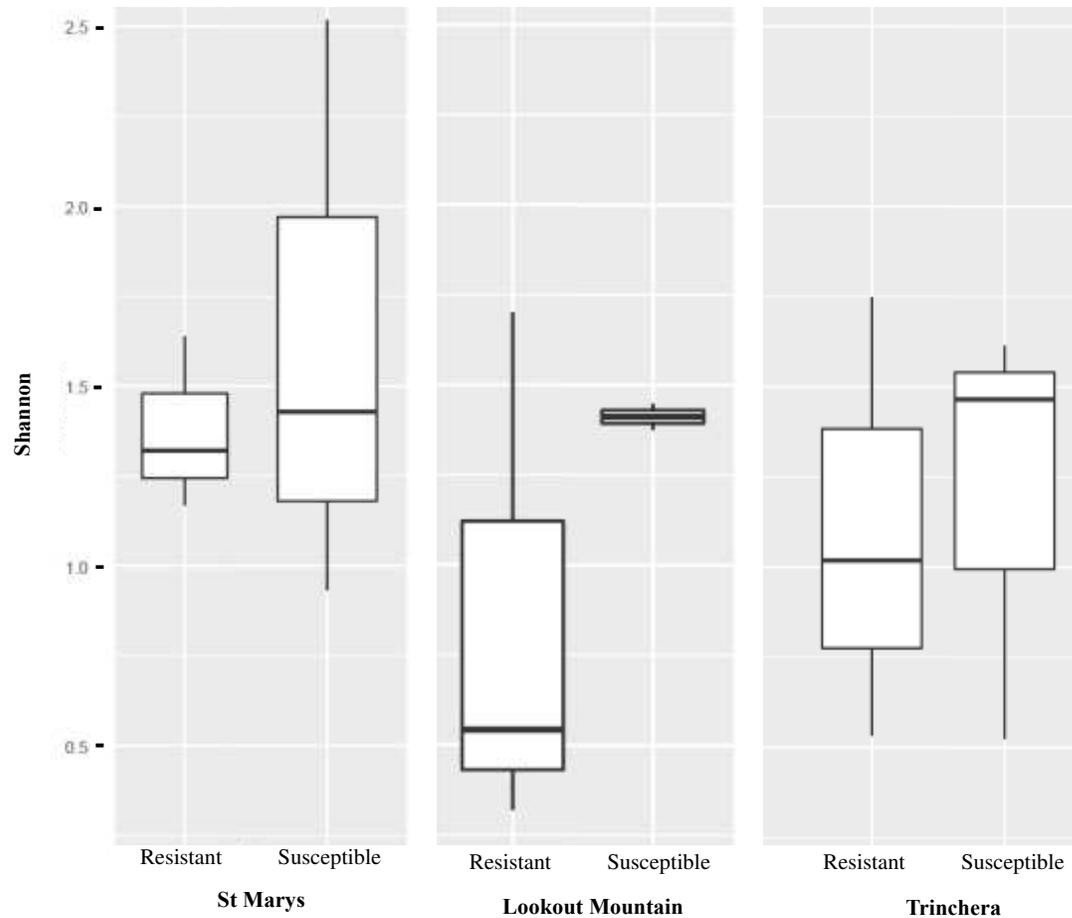


FIGURE 17 – Box plots using Shannon index for diversity to show the levels of fungal endophytic biodiversity for each site’s resistant and susceptible trees. The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles), while the middle hinge is the median. Lastly, the upper whisker extends to the largest value, and the lower whisker extends to the smallest value.

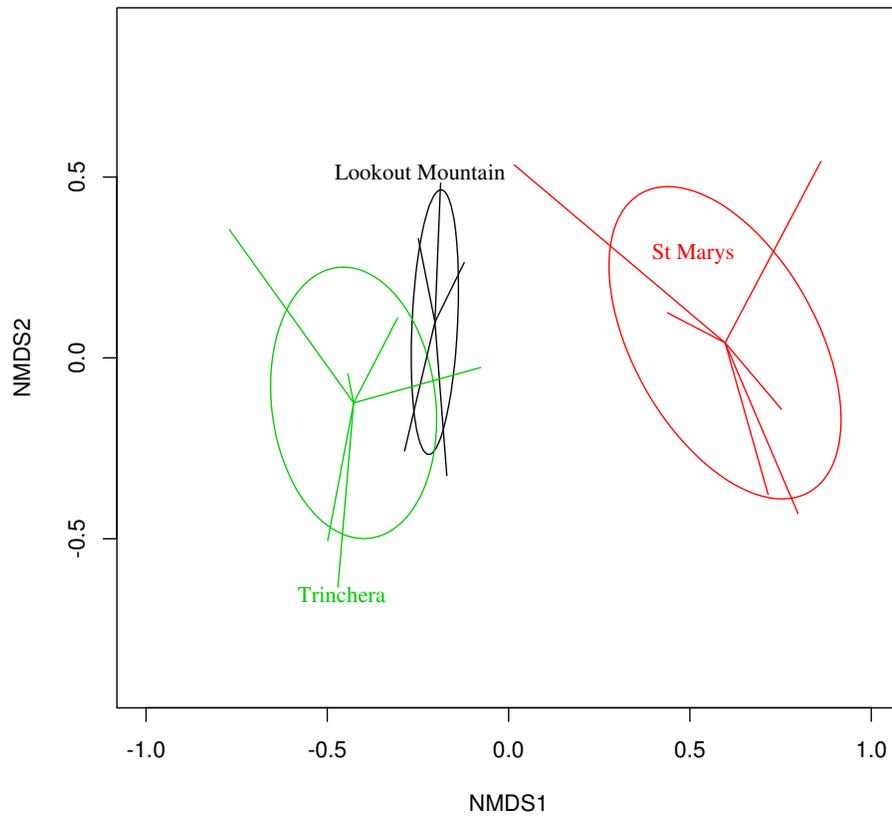


FIGURE 18 – Ordination plot showing the correlation between the NGS data of the fungal endophyte populations of each site, using non-metric multidimensional scaling (NMDS) to measure dissimilarity.

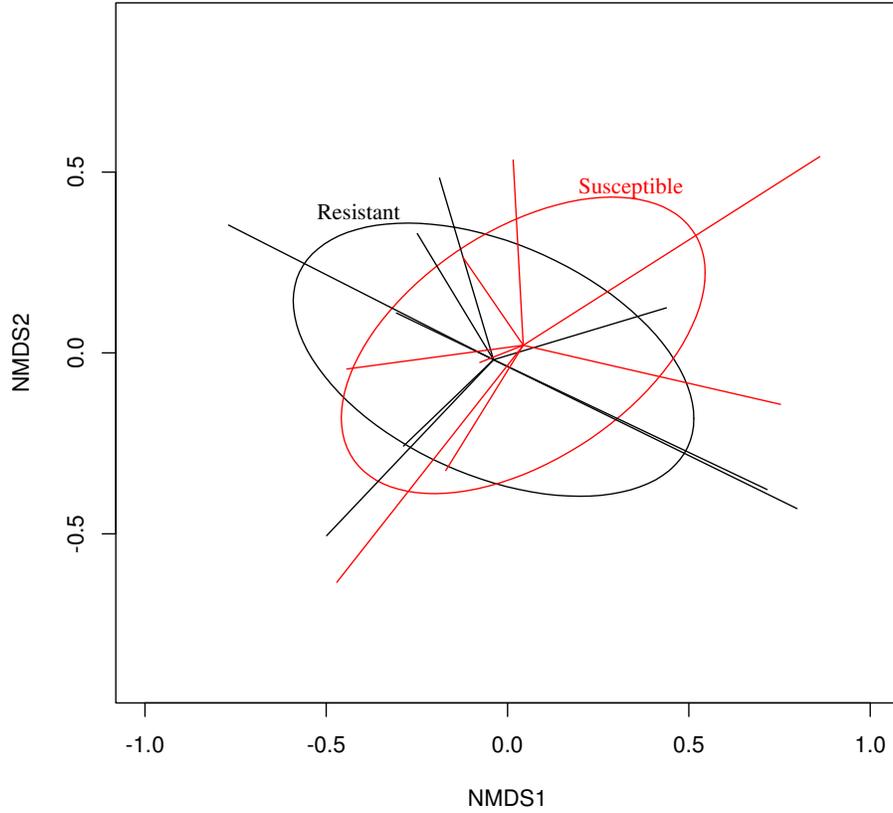


FIGURE 19 – Ordination plot showing the correlation between the NGS data of the combined fungal endophyte populations of resistant and susceptible trees from all three locations, using non-metric multidimensional scaling (NMDS) to measure dissimilarity.

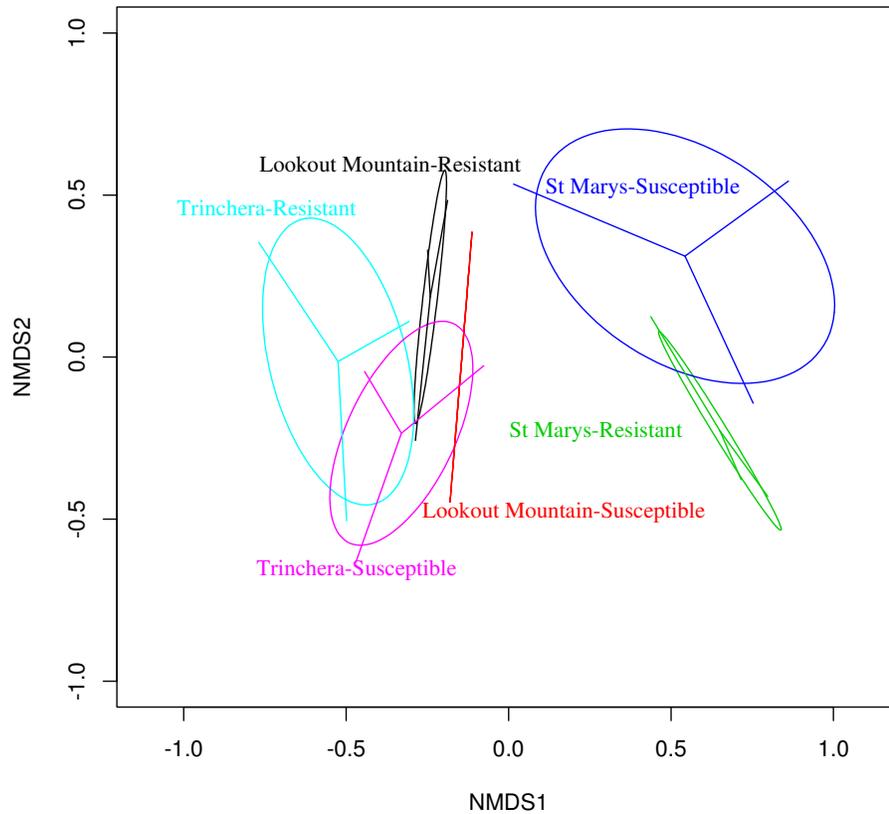


FIGURE 20 – Ordination plot showing the correlation between the NGS data of the susceptible and resistant trees populations of fungal endophytes per site, using non-metric multidimensional scaling (NMDS) to measure dissimilarity.

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