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Fungal Ecology of Whitebark Pine Phyllospheres in the Southern Cascades

by

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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Ehren Moler find it satisfactory and recommend that it be accepted.

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ABSTRACT

Fungal endophytism and a molecular approach for studying the system are described through an introductory literature review. Two chapters are devoted to a description of molecular analyses of microfungal communities in fascicles of whitebark pine (*Pinus albicaulis*). Through controlled and natural studies, foliar endophytes were assessed for: 1) persistence in plant-hosts after artificial introduction to host mycobiomes, 2) variation in endophyte community structure across conspecific host-plant genotypes, and 3) trends of taxonomic occurrence across environmental and host-demographic variables. Information generated through these studies will be useful to forest pathologists seeking to identify and mitigate the effects of disease-causing organisms, and ecologists developing a predictive framework for understanding the distribution of fungal endophytes.

LITERATURE REVIEW AND PROJECT BACKGROUND

Whitebark pine (*Pinus albicaulis* Engelm.) is one of nine North American white-pine species taxonomically classified within subgenus *Strobus*, genus *Pinus*. Other *Strobus* pines native to North America include: limber pine; western, southwestern and eastern white pine; foxtail pine; sugar pine; and the Rocky Mountain and Great Basin bristlecone pines (Schwandt 2006; Geils, Hummer and Hunt 2010) . Further classification of whitebark pine as a member of subsection *Cembrae,* subsection *Strobus* identifies it as one of five "stone pine" species in the world, a distinction that describes the species' large seeds and indehiscent seed-cones. Its ability to colonize harsh timberline environments and identity as the only *Cembrae* pine in the new world sets *P. albicaulis* apart from all other white pines (Schwandt 2006; Kearns and Jacobi 2007; Zhang *et al.* 2010; Tomback and Achuff 2010; Geils, Hummer and Hunt 2010).

Multiple investigators describe the fire-adapted whitebark as a keystone species of subalpine habitats (Kendall and Arno 1990, Richardson *et al.* 2002 & 2007, Schwandt 2006**,** Tomback and Achuff 2010). Major ecosystem services provided by *P. albicaulis* include: increasing the duration of snowpack which moderates watershed pulses, provision of otherwise scarce nutrients for alpine animals, and enrichment of edaphic properties as a seral species in harsh or recently disturbed habitats (Tomback, Arno and Keane 2001; Schwandt 2006).

A corvid species, the Clark's nutcracker (*Nucifraga columbiana* Wilson), coevolved with whitebark pine and is solely responsible for extracting and

distributing whitebark pine seeds from their indehiscent cones (Tomback, Arno & Keane 2001, Richardson *et al.* 2002). *N. columbiana* caches seeds on recently burned sites at ranges of up to 20 km from the parent tree. The behavior of Clark's nutcracker contributes to the distinct population genetics of whitebark pines described below (Richardson *et al.* 2002).

Highly randomized, long-ranging seed distribution via *N. Columbiana* result in the high within-population genetic variation found in whitebark pine stands (Richardson *et al.* 2002). Richardson et al. (2002) investigated maternally-inherited mitochondrial (mt) DNA variation to describe seed dispersal patterns, and variation in paternally-inherited chloroplast (cp) DNA to determine the extent of pollen dispersal. Three distinct haplotypes of *P. albicaulis* mtDNA were revealed from the work of Richardson *et al.* (2002), which shows geographic clustering around: 1) the northern Sierras and Cascade mountains of Oregon and S. Washington, 2) the Cascades of northern Washington and Canada, and Northern Rockies; and 3) central and eastern Idaho and the Greater Yellowstone Ecosystem. Two important aspects of whitebark pine natural history elucidated by Richardson *et al.* (2002) include: 1) stretches of land over 100 km long that are unsuitable habitat for Clark's nutcrackers do not contain whitebark pine, and 2) as whitebark pine stands become increasingly geographically isolated, genetic diversity of wind-vectored pollen (paternal gene flow) decreases, resulting in increasingly genetically homozygous seed-stock.

North American forestry has a rich history of research and management of the commercially important western, eastern and sugar *Strobus* pines (*P. monticola, P.*

strobus and *P. lambertiana*, respectively). A U.S. Forest Service conference dedicated to *Strobus* species of the North American subalpine was held in 1990 to discuss the precipitous decline of *P. albicaulis* and *P. flexilis* (limber pine) populations across western U.S. and Canada (Schmidt and McDonald 1990). The white pine blister rust epidemic – which had commanded the attention of North American forest scientists since the early 21st century, thus shifted from a topic of economic importance in the timber industry to an issue of intense ecological concern regarding high-elevation species of white pine. These concerns have led to support for listing whitebark pine as an endangered species in the United States and Canada (Schwandt 2006, NRDC 2008, COSEWIC 2010).

White pine blister rust (*Cronartium ribicola* J.C. Fisch. in Rabh.) is an exotic fungal pathogen of *Strobus* pines that was accidentally introduced to North America from pine seedlings from Europe in the early 1900's (Kinloch 2003; Kinloch *et al.* 1998; Geils, Hummer and Hunt 2010). In addition to white pines, the fungus infects *Ribes* sp. in the family Rosaceae and *Castilleja* and *Pedicularis* sp. in the Orobanchaceae family (Van Arsdel *et al.* 2006, McDonald *et al.* 2006, Richardson *et al.* 2010 & 2007). Asia is the center of greatest genetic diversity of *C. ribicola*, where only local short-lived disease epidemics occur on Asian white pines, suggesting that *C. ribicola* originated in Asia (Kinloch 2003, Kim *et al.* 2010, Zhang *et al.* 2010, Geils and Vogler 2011). Order Pucciniales (subclass Teliomycetidae, class Basidiomycotina, phylum Basidiomycota) contain the economically important and

biologically complex plant pathogens known as the "rust" and "smut" fungi (Vogler and Bruns 1998, Vogler 2000, Mauseth 2009) to which *C. ribicola* belongs.

The life-cycles of rust fungi are distinct among the fungal kingdom, and differ greatly within genera (Schumann and D'Arcy 2009, Geils and Vogler 2011). Two major factors that differ among rust species include: 1) the number of plant-hosts they infect, and 2) the number of spore stages exhibited during a complete life-cycle. Autoecious rusts are able to produce all spore stages on one plant-host, while heteroecious rusts require two unrelated hosts to complete their life-cycle. Some heteroecious rusts, such as *Puccinia graminis* (causal agent of stem rust of wheat) infect alternate hosts within one Division (Magnoliophyta). The number of spore stages exhibited by a rust species is either macrocyclic (five stages), demicyclic (four stages), or microcyclic (two stages).

Cronartium ribicola is a heteroecious, macrocyclic rust that alternates between hosts from different taxonomic Divisions: Conferophyta and Magnoliophyta. Two of the five spore stages, both asexual, are completed on white pines. The three remaining spore stages are completed on a species of *Ribes* (gooseberry or currant). *C. ribicola* is a parasite of its hosts during all life-cycle stages (Mauseth 2009).

A description of the white pine blister rust life-cycle follows. Genetic terms are described in greater detail in the following section. After overwintering on pine, haploid binucleate (dikaryotic) aeciospores are carried up to 1200 km by wind to infect an alternative host (including: *Ribes* spp., *Pedicularis* spp. and *Castilleja* spp.) in the spring or early summer (Kinloch 2003; McDonald *et al.* 2006; Richardson *et al.* 2007; Geils, Hummer and Hunt 2010; Zambino 2010). Aeciospores germinate on

the alternative host, enter by growing a hypha through an open stoma, and develop into dikaryotic urediniospores which can travel hundreds of kilometers and cause polycyclic infections other *Ribes* sp. (Mauseth 2009; Geils, Hummer and Hunt 2010). Telia and teliospores are eventually produced on the underside of *Ribes* leaves, where karyogamy and meiosis occurs to produce haploid basidiospores. Only basidiospores can infect pine. Pine infection typically occurs between June and November and is accomplished by basidiospores traveling much shorter distances than that traveled by urediniospores or aeciospores – only up to about 300 meters. Basidiospores that successfully land on needles of white pines then germinate to grow hyphae through stomata, which continue growth through the apoplast until hyphae arrive in woody tissue (Lachmund 1933; Hoff 1992; Geils, Hummer and Hunt 2010). Up to three years may lapse before bark lesions, called cankers, develop in woody tissue and produce the first of two spore stages on the primary host. A canker erupts to reveal spermagonia, which occur in two mating types, each of which holds spores, called spermatia, of a given mating type (Hunt 1985, Mauseth 2009). Various species of flies, mostly of the *Megaselia* and *Paracacoxenus* genera, actively seek out spermagonia and fertilize receptive spermagonial hyphae of each mating type with spermatia of the opposite mating type (Hunt 1985). Without fertilization, the monokaryotic spermagonial stage may repeat for six years without proceeding to the aecial stage (Hunt 1985). Dikaryotic hyphae growing from fertilized spermagonia form aecia, which release dikaryotic haploid aeciospores, thus ending the aecial stage on the primary host (Kinloch 2003; Schumann and D'Arcy 2006; Mauseth 2009; Geils, Hummer and Hunt 2010).

Rust infections elicit the formation of red and yellow spots on penetrated needles, increased host-cell division results in bark swelling, and the production of traumatic resin ducts (Geils, Hummer and Hunt 2010). Depending on the location and severity of infection, the genetic susceptibility of the host, and the virulence of the rust strain, infected trees may exhibit canker suppression, branch dieback, reproductive failure and/or mortality (Kinloch 2003; Tomback, Arno, and Keane 2001; Tomback and Achuff 2010; Geils, Hummer and Hunt 2010; Zambino 2010; Schwandt *et al.* 2010).

Prior to the invasion of white pine blister rust, white pines defined the principal forest type and timber species in the Inland Northwest (Tomback, Arno, and Keane 2001; Tomback and Achuff 2010). Lumber of western white pine (*P. monticola*) was reportedly of the very highest commercial value before blister rust and lesser biotic and abiotic constraints led to abandonment of large-scale *P. monticola* silviculture, though production continues in some areas at about 5% of its historical productivity (Tomback, Arno, and Keane 2001; Kinloch 2003; Tomback and Achuff 2010).

Controlled trials have shown all nine species of North American white pine to be susceptible to white pine blister rust, though infection has never been observed in natural stands of *Pinus longaeva*, the long-lived great-basin bristlecone pine (Kinloch 2003, Tomback and Achuff 2010). In contrast, whitebark pine populations exhibit infection frequencies ranging from 70-100% across the species' northern range (Schwandt 2006, Kearns and Jacobi 2007). *P. albicaulis* is considered the most susceptible to the effects of white pine blister rust (Tomback, Arno, and Keane 2001; Tomback and Achuff 2010; Geils, Hummer and Hunt 2010).

Fungal genetic principles unique to the Fungal Kingdom, and rust fungi (Pucciniales) in particular, are described below. In addition to the "deuteromycota," the "higher fungi" (Ascomycota and Basidiomycota) may diversify their genetic code apart from karyogamy through *plasmogamy* (merging of the cytoplasm of two parent cells without nuclear fusion), (Raper 1966, Mauseth 2009). *Karyogamy* is the fusion of two gamete nuclei to create a diploid cell, and a *heterokaryon* is a cell containing nuclei of 2 or more mating types, which may eventually undergo karyogamy to produce a diploid zygote (James and Vilgalys 2008, Mauseth 2009). Many fungal hyphae are haploid and vegetative for the majority, if not all, of their life-cycle, even when different hyphae undergo plasmogamy to produce a heterokaryon (Anderson and Kohn 2007). Plasmogamy enables hyphae to retain both haploid nuclei separately, employ both nuclear genomes, and undergo further plasmogamy prior to potential karyogamy. The dikaryotic state confers a high degree of phenotypic plasticity to a hypha, arguably more-so than that of a diploid hypha (Raper 1966, Burnett 2003, Anderson and Kohn 2007, Mauseth 2009).

The highly dimorphic vegetative and sexual forms of fungi have routinely led taxonomists to mistakenly name the same species differently based on the presence or absence of sexual structures – which develop under very specific environmental conditions (Kinloch 2003, Frankland 1998, O'Brien *et al.* 2005, James and Vilgalys 2008) Though sexual and asexual reproductive strategies are thought to be equally common among filamentous fungi, molecular methods are revealing that for many species, only the asexual morphs have been discovered (James and Vilgalys 2008). Dikaryotization (the inclusion of two genetically distinct nuclei in one hyphal cell)

occurs through plasmogamy when compatible hyphae of differing mating types anastomose (Burnett 2003, Anderson and Kohn 2007, Mauseth 2009).

Parasexuality describes the phenomenon of nuclear fusion for compatible nuclei in asexual dikaryotic cells, yielding a diploid state that may proceed to meiosis and crossing over (Anderson and Kohn 2007, James and Vilgalys 2008, Mauseth 2009). Dikaryosis is an appropriate mode of enhancing phenotypic plasticity in filamentous fungi, a thallus of which may bear extensions that simultaneously pass through differing life cycles and colonize disparate substrates with unique resource limitations (Burnett 2003, James and Vilgalys 2008). Once dikaryotization occurs, it may persist through successive sexually-reproductive fruiting events, undergoing meiosis and nuclear conjugation without disrupting the dikaryotic state, or may be disrupted by nuclear migration that returns the hypha to a monokaryotic state (Raper 1966, Burnett 2003, Mauseth 2009).

Despite speculation regarding the evolutionary utility of heterokaryosis, few studies have successfully explored the phenomenon empirically (Anderson and Kohn 2007). One study recalled by Raper (1966) tested the growth rate of haploid dikaryons evolved *in vitro* for over 13,000 generations against uni-nucleate haploid fungi (monokaryons) of the same species. The dikaryons demonstrated a greater growth rate than the monokaryotic cultures, which resulted from mutations in the dikaryotic cultures. It is interesting to note that if syngamy had occurred between the two nuclei of the dikaryon, recombination may have terminated this beneficial mutation. This study also found that a deleterious mutation in a dikaryotic culture

was eliminated, or compensated for, by the presence of a non-mutated gene that the dikaryon transmitted asexually (Raper 1966).

Fungal heterokaryosis gives rise to emergent phenotypic properties resulting in diverse genetic mosaics dependent upon the random encounter of monokaryons and dikaryons (Raper 1966). Another example of such emergent properties resulting from repeated dikaryotization of conspecific hyphae is illustrated through the 1961 study of *Coprinus lagopus* by Swiezynski, as discussed by Raper (1966). Swiezynski mixed two auxotrophic dikaryons in culture, which formed recombinant dikaryons that were then able to utilize the substrate, which was previously limiting to both dikaryons independently. Upon addition to the culture, the vegetative dikaryons underwent plasmogamy and became able to sporulate (Raper 1966).

The utility of heterokaryosis may have predisposed fungi to be among the first organisms to colonize primordial terrestrial Earth (Redecker, Kodner and Graham 2000). Early terrestrial Earth would have exerted considerable selective pressures on the metabolic and environmental tolerances of early colonizers, thus favoring the adaptability of parasexuality and heterokaryosis.

Global fungal diversity and phyllosphere microfungi studies are entangled such that investigation of the latter leads to revision of the former (Hawksworth and Rossman 1997). Fungus-plant symbioses are ubiquitous, and include associations in aboveground foliage (phyllosphere), bark and xylem tissues as well as the hypogeous arbuscular-mycorrhizae, ectomycorrhizae, and dark-septate endophytes of plant roots (Carroll 1988; Hawksworth and Rossman 1997; Stone, Sherwood and

Carroll 1996 & 2004; Rodriguez *et al.* 2008). Whether vascular or nonvascular, angiosperm or gymnosperm, and irrespective of growth form - every plant intensively studied for fungal symbionts has been found to host endophytic fungi (Isaac 1992, Sieber 1997, Sun and Guo 2012).

About 98,000 of the estimated 1.5 million fungal species are currently described (Hawksworth and Rossman 1997, Hawksworth 2001, Moore *et al.* 2011, Porras-Alfaro and Bayman 2011). This often-cited estimate was calculated, albeit in 1997, by extrapolating the average of 5.3 unique fungal species discovered per plant-host inspected to the known minimum of 270,000 extant plant species, and reducing the value to account for likely multiple names attributed to polymorphs (Hawksworth and Rossman 1997, Moore *et al.* 2011). Additionally, many insect species similarly host unique fungal associates both internally and externally, and the 1 million species currently ascribed to Class Insecta are estimated to represent only an eightieth of the extant members of that speciose Class (Hawksworth and Rossman 1997, Foottit and Adler 2009). More recent estimates of global fungal species richness report figures closer to 5 million (Peay 2014). Additionally, Mauseth (2009) asserts that speciation in the Fungal Kingdom exceeds the rate of species discovery. Thus our proportional knowledge of fungal biodiversity is only likely to decrease over time.

Phyllosphere microfungi are categorized into the following broad groups based on their location upon or within plant tissues, and not on the nature of the symbiotic relationship shared with their plant-host (Stone, Sherwood and Carroll 1996; Porras-Alfaro and Bayman 2011). Included are foliar endophytes, which complete

most of their life cycle within the apoplasts of plant leaves, foliar epiphytes which attach to leaf surfaces and may invade leaf tissue, epiphytes and endophytes of bark, and xylem endophytes (Carroll 1988; Stone, Sherwood and Carroll 1996; Stone, Polishook and White 2004; Arnold and Engelbrecht 2007; Rodriguez *et al.* 2008).

Rodriguez et al. (2008) refine endophyte classification by splitting the group into four classes based on characteristics such as: modes of host-colonization and transmission, *in planta* biodiversity, fitness benefits conferred to hosts and ecological function.

1) Class 1endophytes are *Clavicipitaceous* fungi (Family Clavicipitaceae) that grow almost exclusively within cool and warm-season grasses, exhibit high host specificity, and are often mutualistic (Saikkonen *et al.* 1998; Saikkonen, Ion and Gyllenberg 2002; Rodriguez *et al.* 2008; Cheplick and Faeth 2009).

Classes 2–4 represent strictly *Nonclavicipitaceous* fungi:

2) Class 2 endophytes colonize host plant shoots, leaves, roots and rhizomes, display relatively low biodiversity *in planta*, and are primarily vertically transmitted through host generations via colonization of seeds and other propagules (Ganley and Newcombe 2006, Rodriguez *et al.* 2008). Though this class of endophytes may increase host plant fitness by an increase in biomass and tolerance to biotic and abiotic stresses - such as disease, drought, salinity, heat and desiccation – the degree to which a given symbiosis is mutualistic or parasitic is environmentally dependent (Saikkonen *et al.* 1998, Sieber 2007, Rodriguez *et al.* 2008). The extent to which an endophyte parasitizes hosts or confers habitat-specific benefits to hosts also covaries

with endophyte genotype (Smith and Goodman 1999, Rodriguez *et al.* 2008, Parrent *et al.* 2010).

3) Class 3 endophytes vary from those of Class 2 in three important ways: i) they are restricted to aboveground plant tissues, ii) they display high species richness in leaves where numerous, independent, localized intra and intercellular infections are formed near photosynthetic and herbaceous cells, and iii) they predominate in coniferous hosts (Saikkonen *et al.* 1998, Jumpponen and Jones 2009, Rodriguez *et al.* 2008). Once successfully established in the host, the largely inactive Class 3 endophytes enable the formation of highly diverse communities across small spatial scales. Studies have reported isolation rates of one fungal species per $2mm^2$ of leaf tissue, dozens of species per leaf, and dozens to hundreds of species per plant (Sieber 2007, Rodriguez *et al.* 2008). Unlike Class 2 endophytes, those of Class 3 are only rarely detected in plant propagules, and are known to produce spores on senescent or abscised host plant tissue (Carroll 1988; Stone, Sherwood and Carroll 1996; Ganley and Newcombe 2006; Rodriguez *et al.* 2008; Porras-Alfaro and Bayman 2011). Spores are then horizontally disseminated by adiabatic cooling, wind, rain, or insects (Saikkonen *et al.* 1998, Rodriguez *et al.* 2008, Zambino 2010). Although drought abatement is a noted benefit of endophytes conferred to host plants (Rodriguez *et al.* 2008), Arnold and Engelbrecht (2007) showed that circumstances of severe drought may shift the symbiotic relationship from mutualistic to parasitic;

plants inoculated with endophytes and then exposed to severe drought lost water faster than non-inoculated plants.

4) Class 4 is solely comprised of root endophytes known as the "dark septate endophytes" (Rodriguez *et al.* 2008).

Fungal communities are highly diverse and abundant in conifer phyllospheres (Carroll and Carroll 1978, Stone *et al.* 2004, Sieber 2007), and compete *in planta* for space and carbohydrates (Isaac 1992, Saikkonen *et al.* 1998, Cheplick and Faeth 2009). Like in all natural habitats, endophytic fungal populations form various guilds within unit communities (Isaac 1992, Atlas and Bartha 1997). Most endophytes are primarily saprobes that remain metabolically inactive until host tissue is disturbed or becomes senescent, others are able to absorb nutrients from host cells via haustoria, while others are known entomopathogens and mycotrophs (Stone, Sherwood and Carroll 1996; Stone *et al.* 2004; Rodriguez *et al.* 2008). Resource specialization is a common trait among fungal pathogens, and is exhibited by many endophytic species which appear to be latent plant pathogens (Carroll 1988; Isaac 1992; Stone, Sherwood and Carroll 1996).

Depending on the species, endophytes may exhibit host-specificity at the level of genotype (Saikkonen *et al.* 1998, Lamit *et al.* 2015), species or taxonomic family (Rodriguez *et al.* 2008), or they may by facultatively free-living (Stone *et al.* 2004). Endophytes are often host-specific, however, and many derive from plant-pathogenic strains that have undergone a lengthy co-evolution with their hosts (Isaac 1992, Saikkonen *et al.* 1998, Stone *et al.* 2004, Geils and Vogler 2011). To

illustrate the duration of this co-evolutionary relationship, Sieber (2007) explained that fungi in the class Helotioles, which occur overwhelmingly in gymnosperms, diverged from the Diaporthalean fungi, which dominate phyllospheres of angiosperms, at the same time that those plant phyla diverged (300 Mya).

Fungal biological control efforts have mostly focused on the control of plant pests and fungal diseases of agricultural crops (Boyette, Walker and Abbas 2002), though the approach has also been applied to tree fruit crops (Gees and Coffey 1989; Pandey, Arora and Dubey 1993; Arnold *et al.* 2003) and forestry concerns involving white pines and *C. ribicola* (Wicker 1981; Ganley, Sniezko and Newcombe 2008). Results from a growing number of studies suggest that endophytic fungal communities may guard against pathogenic infection of their host plants (Sieber 2007). Recent work has shown that endosymbiotic microfungi in needle tissue of western white pine may inhibit or slow the onset of white pine blister rust infection (Ganley, Sniezko and Newcombe 2008). Arnold *et al.* (2003) inoculated *Theobroma cacao* with native foliar microfungi, which resulted in significantly reduced leaf mortality compared to un-inoculated trees after introducing a virulent pathogen to the experimental trees.

The Hypocrealean fungal genus, *Myrothecium*, has been investigated for activity against a wide range of pest and disease organisms, including various fungi and bacteria *in vivo* (Turhan and Grossman 1994), water hyacinth (Ponnappa 1970), an oomycete pathogen of avocado trees (Gees and Coffey 1989), kudzu (Boyette,

Walker and Abbas 2002), guava trees (Pandey, Arora and Dubey 1993), and whitebark pine (this study).

Technological advances in species detection and identification necessarily follow from advances in how species are defined. Mycologists have, and still, rely heavily upon morphological species concepts to identify and discover new species. Generations of mycologists have worked within the discipline only by way of an encyclopedic knowledge of often cryptic growth habits and knowledge of organisms' diagnostic sexual structures. Classical botany presents a similar scenario, and a good botanist can often be reasonably certain of the taxonomy of the organism under study unless the plant has not produced sexual organs and bears largely indistinct morphological features, as is the case for many grasses. Unlike plants, however, many fungi are highly polymorphic and do not undergo any discernible sexual stage (i.e. the Deuteromycota). One endophyte of pines, *Sphaeropsis sapinea*, exhibits more than five different morphotypes in culture (Porras-Alfaro and Bayman 2011). This presents obvious dilemmas in conducting fungal ecology research that depends upon species delimitation. The biological species concept – which defines species based on reproductive isolation – also requires investigators to first grow fungal isolates in culture and induce sporulation in order to ascertain different mating types *in vitro*. This approach assumes that the organism under study has a sexual stage, which is not the case for about 20% of the 98,000 known fungi (Moore *et al.* 2011). Additionally, many fungi that are known to exhibit a sexual stage *in vivo* fail to do so *in vitro*, precluding the feasibility of controlled mating studies. The

ecological and physiological species concepts are prone to the same obstacles as those aforementioned approaches (Moore *et al.* 2011).

Culture-independent molecular methods used to detect and measure fungal biodiversity, as opposed to the culture-dependent methods described above, enable the rapid identification of large numbers of sequences from an environment, many of which may belong to taxa that would not grow in culture and thus remain undiscovered (Arnold *et al*. 2007; Sieber 2007; Jumponnen and Jones 2009; Unterseher *et al.* 2011; Moore *et al.* 2011; Aly, Debbab and Proksch 2011; Cordier *et al.* 2012; Lindahl *et al.* 2013; Peay 2014). Genetic fingerprinting techniques are only one class of myriad molecular tools employed in fungal ecology. Some commonly used techniques for separating nucleic acids based on size and composition include: denatured gradient gel electrophoresis (DGGE), terminal-restriction and amplified fragment length polymorphism analysis (T-RFLP, AFLP), (Atlas and Bartha 1997). New high-throughput nucleic acid pyrosequencing technologies have enabled researchers to sample microbial communities at a greater depth and speed than ever before (Monard *et al.* 2012, Kozich *et al.* 2013, Lindahl *et al.* 2013, Peay 2014).

Bioinformatics software has had to develop apace with sequencing tools to make use of the large number of sequences generated by high-throughput sequencing platforms, such as the Illumina Miseq® used in the present study. Currently, the two most commonly cited applications for the interrogation of next-generation sequence data include an all-encompassing, open-access program called "mothur" (Schloss *et al.* 2009), and a package that binds together disparate

programs to provide a flexible analytical tool "QIIME" (Lozupone and Knight 2005). McFrederick, Mueller and James (2014) used both programs to analyze a dataset consisting of bacterial and fungal DNA sequences, and showed that the use of similar workflows in both applications led to different outcomes. Some investigators report that estimates of fungal molecular richness (i.e. molecular "species" or *operational taxonomic units*, OTUs) generated in *mothur* are more conservative than estimates generated using QIIME (McFrederick, Mueller and James 2014). A major difference between the two programs lies in their respective approaches for reducing the number of erroneously-synthesized DNA fragments, which is of considerable importance when working with samples (i.e. sequences) that number in the millions or, in the case of the Illumina® HiSeq, billions (Zhou *et al.* 2011, Kozich *et al.* 2013).

Quality control procedures are essential for generating reliable sequence data, whether downstream applications include OTU or phylogenetic descriptions. Some method of sequence-quality screening, for instance using so-called Q–scores that accompany Illumina® output metadata, is essential for eliminating erroneous reads. The *mothur* platform implements a "consensus screening" approach for sequences generated by the Illumina MiSeq®, which instead of referring to a single Q-score for a unidirectional read, relies on the Q-scores of both forward (5'->3') and reverse (3'->5') reads as they are paired to form one contiguous read (a.k.a. "contig"). The Q-scores for forward and reverse reads are compared for each nucleotide, and if they differ and are above a given Q-score threshold, the higher nucleotide is retained. If they differ and have the same Q-score, an IUPAC ambiguous base term replaces the

ambiguous nucleotide (Kozich *et al.* 2013). The investigator can then dictate to what degree sequences with mismatches or ambiguities are allowed.

The analysis of microbial sequence data is typically accomplished through one of two approaches: 1) phylogenetically-based "phylotyping" of sequences that resemble sequences of described taxa, and 2) non-taxa-based methods of clustering sequence fragments into groups that share a common percent-dissimilarity across phylogenetically-relevant sections of DNA to form a molecular operational taxonomic unit (Monard, Gantner and Stenlid 2012). Given that only 2-6% of extant fungal species have been described and sequenced (Hawksworth and Rossman 1997, Peay 2014), and that pyrosequencing provides the opportunity to describe microbiomes to greater depths than previously possible, phylotyping necessarily limits the purview of a sequencing effort, and as a result is often used when the computational resources required for clustering sequences into OTUs are not available (Herr J., Per Com).

Although many studies use amplicon-based high-throughput sequence data quantitatively, as in the description of the number of occurrences of a given organism, some authors argue that such data are not only non-quantitative, but also non-replicable (Zhou *et al.* 2011). The authors confess, however, that their lack of ability to reproduce the findings of their study was probably due to issues related to random sampling of the sample-environment. Other particularly contentious issues regarding the use of this relatively new technology include: 1) the size of DNA fragment to sequence, and the size to which synthesized sequences should be chopped for bioinformatics processing and eventual assignment to a species (Porter

and Golding 2011; Monard, Gantner and Stenlid 2012), 2) whether to include or remove OTUs that occur only once in a dataset (singletons) (Huse *et al.* 2010, Paulson *et al.* 2013), 3) how many times a given sample should be subjected to PCR before being combined in equimolar proportion to one aliquot for sequencing (to reduce PCR-related bias) (Smith and Peay 2014), and 4) whether, and to what extent, sequence libraries should be normalized, or standardized, before comparing libraries (de Cárcer *et al.* 2011, Schloss MiSeq® SOP 2015).

In all cases, the present study sought to use the most widely-published, well-reasoned, and most affordable solution to the bioinformatic considerations listed above. In particular, I used a novel touchdown-PCR approach in my nested PCR to address the reasoning behind pooling multiple PCR products, but without the associated cost. I followed the advice of an in-house microbial ecologist regarding library-normalization, which entails randomly sampling all sample-libraries to the size of the smallest library prior to clustering sequences into groups. This step aims to retain the most representative sequences in each sample without over-representing any one sample. Following the reasoning of Paulson *et al.* (2013) and Huse *et al.* (2010), who maintain that "singleton OTUs frequently represent valid rare phylotypes in diverse environmental samples," I did not discard singletons from my project library. Finally, I assessed perhaps the least-understood contention of these, regarding the sequence length-standard that must be chosen in order to process sequences, by graphing the effect of various minimum sequence length thresholds on the number of unique sequences retained in my dataset. Fig. 1 illustrates that the majority of unique paired-sequences in my project library were

around 250 nucleotides long (the average length is actually 265.4 nt), and that the Illumina® MiSeq® chemistry appears to be optimal for sequencing DNA fragments in the range of 175-225 nucleotides, which is about 2/3 of what the manufacturer advertises, but plenty long for the present study. Taking this into account, and the methods of recent publications, I constrained my contig-lengths to 225 nts (Bell *et al.* 2014; McFrederick, Mueller and James 2014; Arif *et al.* 2014).

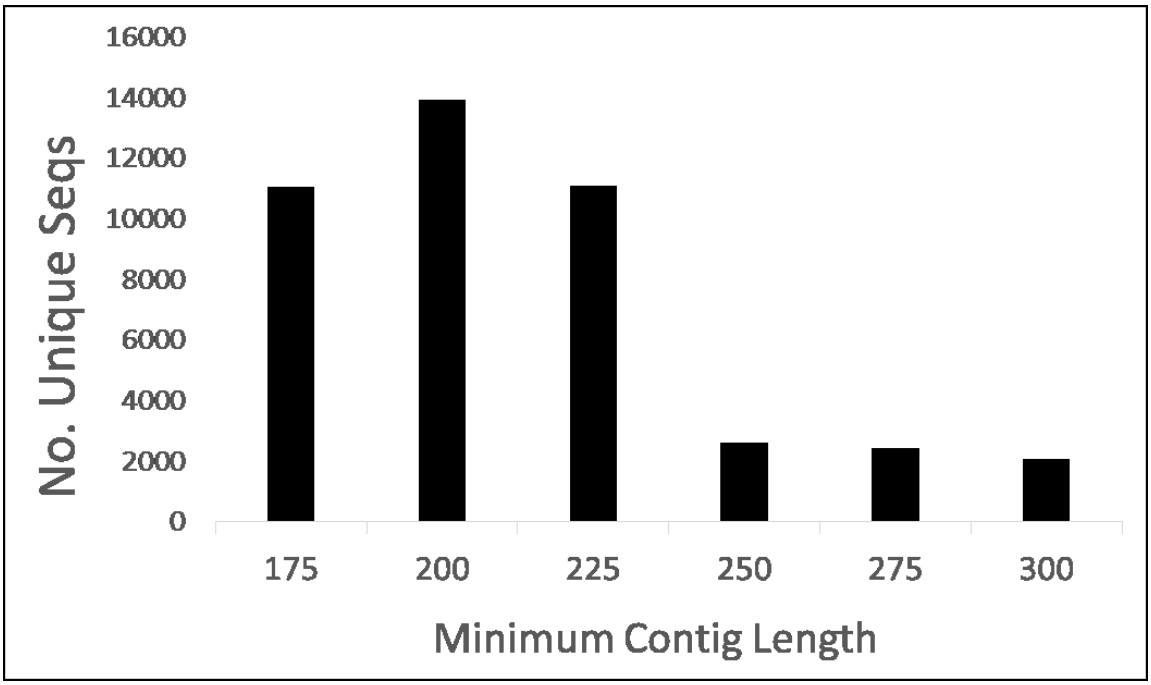

Fig 1 The number of unique sequences retained at various minimum contig lengths.

Chapter 1

HOST GENOTYPE, NOT INOCULATION, AFFECTS ENDOPHYTIC FUNGAL COMMUNITIES OF WHITEBARK PINE

Summary

- We investigated foliar fungal communities of a threatened pine species through barcoded amplicon high-throughput sequencing. Phyllosphere communities from five replicated host genetic-families were sampled four years after controlled inoculation with a potentially beneficial fungal symbiont. Fungal community amplicons of the nuclear ribosomal DNA internal transcribed spacer (ITS1) region were pyrosequenced to detect fine-scale changes in phyllosphere mycobiomes across host-genotypes and inoculation treatment. Sequence libraries were assessed through taxonomic and OTU-based community metrics using univariate and multivariate statistical approaches.
- Differences in phyllosphere mycobiome dynamics were significantly correlated with host genetics across five half-sib genetic families and explained 31%, 20% and 19% of the variation in endophyte community structure ($F_{4,74}$=1.43, p=0.021), richness ($F_{4,74}$=4.65, p=0.002) and diversity ($F_{4,74}$=4.34, p=0.003) after controlling for spatial autocorrelation and relevant environmental and physiological host-plant variables.

- Whitebark pine mycobiomes exhibited no difference in univariate or multivariate community metrics when examined four years after inoculation with a native, suspected mutualistic fungal endophyte. Inoculation did, however, result in species-level differences in community composition.

Introduction

Fungal communities within aboveground plant biomass alter host phenotypes and often covary with host genotype (Smith and Goodman 1999, Sthultz *et al.* 2009, Arnold *et al.* 2010, Lamit *et al.* 2015). Endosymbiotic fungi that asymptomatically inhabit plants for a substantial portion of their life cycle are known as endophytes (Carroll and Carroll 1978, Stone *et al.* 1996 & 2004). Fossil and molecular evidence suggests that fungal endophytes have co-evolved with plants for ca. 400 mya, which is reflected by the ubiquity of endophytism across all lineages of *Plantae* (Hawksworth and Rossman 1997, Sieber 2007, Rodriguez *et al.* 2008, Porras-Alfaro and Bayman 2011). Fungal endophytes of woody plants evolved from plant pathogens, and subsist at basal metabolic rates *in planta* until they begin to decompose senescent or stressed host tissues (Stone *et al.* 2004, Porras-Alfaro and Bayman 2011). All aboveground plant structures constitute the *phyllosphere*, which collectively increase the habitable surface area of terrestrial Earth more than five-fold (Bailey *et al.* 2006). When considered alongside the hyperdiverse polyphyletic fungal endophytes, the phyllosphere remains one of the most under-studied biotic systems in proportion to its size, habitat diversity, and species richness (Arnold and Lutzoni 2007, Zimmerman and Vitousek 2012).

The composition and structure of plant-associated microbial communities may alter host phenotypes in ways that affect the survival of the symbiosis, including: abiotic or biotic stress-tolerance (Whitham *et al.* 2003, Rodriguez *et al.* 2008, Cheplick and Faeth 2009, Sthultz 2009, Giauque and Hawkes 2013), increased resistance to disease (Arnold *et al.* 2003, Ganley and Newcombe 2008), or increased susceptibility to disease (Stone *et al.* 2004, Cheplick and Faeth 2009). Less well-known is how intraspecific genotypic variation of plant hosts shapes their internal microbiomes (Whitham *et al.* 2006). The term *community phenotype* was used by Whitham *et al.* (2006) to describe the influence of genes, especially those of keystone species, on the fitness of associated species. If an individual phenotype shapes the environment of other species, the underlying genotype indirectly affects the fitness of other species that form a community with the keystone organism (Whitham *et al.* 2003 & 2006, Shuster *et al.* 2006). The community phenotype theory builds upon Dawkins' concept of the *extended phenotype* (1982) to consider the effect of phenotypes on the larger community in which they occur (Whitham *et al.* 2003 & 2006), i.e. to investigate the effect of genes above the level of populations.

The field of community genetics seeks to elucidate the genetic foundation of interactions *among* species and their environment, which yields community phenotypes (Whitham *et al.* 2006). Community genetics studies have primarily centered on the effects of intraspecific genetic variation in keystone or dominant organisms on their associated communities because they have inordinately large effects on their environments, and are likely to express extended phenotypes (Whitham *et al.* 2003 & 2006). Research in community genetics has shown that host

genotype strongly shapes the composition and structure of associated communities of phyllosphere fungi (Whitham *et al.* 1999 & 2003, Lamit *et al.* 2008 & 2015), arthropods (Dungey, Potts and Whitham 2000; Johnson and Agrawal 2005; Crutsinger *et al.* 2006; Lamit *et al*. 2015), rhizosphere microbes (Schweitzer *et al*. 2011), and birds (Whitham *et al.* 1999).

Almost the entire life cycle of all fungal endophytes occurs within the physical and biological environment of their host plant. This intimate relationship suggests that host phenotype may strongly affect endophyte community structure, which in turn should vary among intraspecific hosts. Yet despite the extraordinarily large number of fungal endophyte species (Hawksworth and Rossman 1997, Stone *et al.* 2004), and the significant impact that endophytes may have on plant functions (Arnold and Lutzoni 2007, Zimmerman and Vitousek 2012, Saikkonen, Mikola and Helander 2015), the extent to which host-genetics shape fungal endophyte communities has yet to be intensively explored in natural ecosystems.

Data suggest that some phyllosphere fungi guard against pathogenic infection of their host plants (Wicker 1981, Arnold *et al.* 2003, Sieber 2007). Fungal endophytes may mitigate pathogenesis by physical or chemical exclusion of pathogenic fungal species (Isaac 1992, Stone et al. 2004), thus influencing fungal community assemblage (Frankland 1998, Dickie *et al*. 2012, Hiscox *et al*. 2015). Also, the sequence of taxonomic recruitment into host mycobiomes (i.e. the history of endophyte community assembly termed *priority effects*) may impact fungal community assemblage and preclude infection by plant pathogens through

competitive exclusion (Connell and Slatyer 1977, Fukami and Morin 2003, Hiscox *et al*. 2015).

Forest diseases that occur across large, environmentally-heterogeneous areas provide effective model systems for the investigation of pathogenic and non-pathogenic fungal-plant symbioses. The white pine blister rust disease system, involving multiple dominant and keystone host-species, presents an ideal opportunity for studying the community phenotype hypothesis (Tomback and Achuff 2010, Geils and Vogler 2011). White pine blister rust is caused by the heteroecious exotic Pucciniomycete fungus, *Cronartium ribicola,* which infects all but one of the nine new-world species of white pine (subgenus *Strobus*) across western (*Pinus albicaulis, P. aristata, P. balfouriana, P. flexilis, P. lambertiana, P. monticola,* and the *P. strobiformis* complex) and eastern (*P. strobus*) North America (Kinloch 2003, Tomback and Achuff 2010). In addition to white pines, the fungus infects *Ribes* spp. in the Rosaceae family, and *Castilleja* and *Pedicularis* spp. in the Orobanchaceae family (McDonald *et al*. 2006, Richardson *et al*. 2007 & 2010, Van Arsdel and Geils 2011). The obligate biotrophic, macrocyclic *C. ribicola* is of course itself a keystone species and intimately co-evolved plant-symbiont (Geils and Vogler 2011).

Whitebark pine (*P. albicaulis*) is a seral species of subalpine habitats, and exhibits frequencies of severe infection by white pine blister rust ranging from 70-100% across the species' northern range (Tomback *et al.* 2001, Schwandt 2006, Kearns and Jacobi 2007). Depending on the location and severity of infection, the genetic susceptibility of the infected tree, the virulence of the rust strain, and the

environmental context, infected trees may exhibit canker suppression, branch dieback, reproductive failure and/or mortality (Tomback, Arno and Keane 2001; Kinloch 2003; Tomback and Achuff 2010; Geils, Hummer and Hunt 2010; Zambino 2010; Schwandt *et al.* 2010). As a consequence of the invasive blister rust disease, increasingly severe infestation by the native mountain pine beetle (*Dendroctonus ponderosae*), and fire suppression limiting whitebark pine regeneration, the species is under consideration for protection as an endangered and threatened species in the United States and Canada (Tomback *et al.* 2001, NRDC 2008, COSEWIC 2010).

Efforts to mitigate devastation of *Strobus* pines by *C. ribicola* have led to effective approaches to enhance their resistance to blister rust by exploiting major and minor (polygenic) resistance genes of the host genome (Kinloch and Byler 1980, Schoettle and Sniezko 2007, Richardson *et al.* 2010). Antagonistic interspecific interactions, through a variety of mechanisms, are the basis of biological control approaches to pathogen suppression, and may complement or provide an alternative approach to enhancing genetic resistance against pathogenic infections (Pandey, Arora and Dubey 1993; Rubini *et al*. 2005). For instance, Ganley and Newcombe (2008) found that controlled inoculations of western white pine (*P. monticola*) with fungal endophytes from their native range slowed and reduced the effects of blister rust infection.

As a first attempt to control white pine blister rust of whitebark pine with an endophytic fungus, seedlings at Crater Lake National Park, Oregon, USA, were inoculated with a local isolate of *Myrothecium roridum*, Order Hypocreales, in 2009 (Worapong, Sun and Newcombe 2009). Controlling for leaf phytochemistry, spatial

autocorrelation, and microsite environmental features, we describe fungal phyllosphere endophyte communities in a common garden of 79 whitebark pines four years after inoculation with *M. roridum*. The present study satisfies three objectives. We evaluate: i) the persistence of *M. roridum* in the mycobiomes of inoculated whitebark pines; ii) alterations of fungal community dynamics that may have resulted from the inoculation treatment, which may account for differential resistance to blister rust; and iii) variation in endophyte community dynamics attributed to intraspecific genetic variation among whitebark pines.

We address our objectives through deep barcoded sequencing of whitebark pine endophyte communities to test two hypotheses:

1) Whitebark pines inoculated with *Myrothecium roridum* host significantly different endophyte mycobiomes than control trees of the same age.
2) Fungal endophyte community structure, diversity and richness vary significantly across genetic families of whitebark pine, as predicted by the community phenotype hypothesis.

Methods

Germination and Inoculation

Over 200 whitebark pine seeds, representing five half-sib genetic families (from five known maternal-parents), were collected at Crater Lake N.P. by National Park Service staff in 2005, and germinated in 2007 at the U.S. Forest Service Dorena Genetic Resource Center (DGRC), Orchard Grove, OR (R. Sniezko, Per Com). The

genetic families included in this study were determined to exhibit a range of susceptibility to the white pine blister rust disease. Half of the seedlings from each genetic family were inoculated with fungal spores and hyphae suspended in sterile water at DGRC by one overnight mist-dispersal of the inoculant upon seedling roots and shoots (G. Newcombe, Per Com). The inoculant consisted of fungal tissue from *Myrothecium roridum* cultures isolated from mature whitebark pine needles at Crater Lake N.P. as described in Worapong, Sun and Newcombe (2009). 192 seedlings from the five genetic families, half inoculated and half control, were outplanted across < 5000m^2 on the south slope of Crater Lake N.P. in 2009 by Crater Lake Park Service personnel. Seedlings ranged from 14-22 cm in height at the time of outplanting.

Data & Tissue Collection and Storage

Trees were sampled in August 2013 from each genetic family by the following method: each labeled tree was assigned an integer, and using a calculator to randomly generate a sequence of numbers we determined which trees to sample per group (n ≤ 10 per gene-family x treatment combination). Each sampled tree was spaced ≥ 1 m from other trees. The five genetic families were labeled as: *11p*, *21p*, *22p*, *24p*, *25p*, and were present on plots to varying degrees due to past mortality, yielding respective sample sizes of: n=18, n=16, n=17, n=8, n=20. Three asymptomatic needle fascicles, each bearing roughly 5 needles, were collected from evenly-spaced regions of the lower canopy of each tree following Ganley and Newcombe (2006). Grouped fascicles from each tree were placed in separate plastic

bags and frozen at -20^{0} C within 12 hours of collection following Lindahl *et al.* (2013). For each tree we recorded: latitudinal and longitudinal coordinates (Trimble® GeoXH™ handheld GPS device), average ground-slope, ground-surface aspect and evident microsite features (level, concave or convex ground surface around tree), estimated percent of dripline-to-bole ground-surface covered with needle litter, tree height, and visible disturbances. Canopy cover was measured at each plot-center with a spherical densitometer.

Surface Sterilization and DNA Extraction

Sterilization procedures followed Deng *et al.* (2011). Briefly, needles were immersed and stirred for 1 min. in sterile DI water to remove loosely-adhering dust and epiphytes that might not contribute to the endophyte consortia. The same was repeated for 1 min. in EtOH, followed by 3 min. in NaOCl (4% available Cl), 30 sec. in EtOH and 30 sec. in sterile DI water to remove salts. Samples were then air-dried in a sterile flow-hood. Dried needles were wrapped in sterile paper towels, placed in a plastic bag, and stored at -20^{0} C until DNA extraction.

Total genomic DNA was extracted from pine needle tissue by first grinding the full mass of each sample to a homogenous fine grain through four rounds of grinding under liquid nitrogen in a sterilized mortar and pestle. Samples were further homogenized by stirring with a sterile spatula (Lindahl *et al.* 2013). For each sample, 50 mg of needle tissue was added to a bead-tube provided in the MoBio PowerPlant® Pro kit (Carslbad, CA, USA). The MoBio phenolic separation solution was used, and two rounds of bead-beating were applied for two minutes each,

punctuated by two minutes on ice.. DNA concentrations were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific™).

PCR Amplification

Extracted genomic DNA was amplified following a nested PCR approach using two different combinations of primers specific to the ITS region of fungal rDNA. The internal primer set was chosen to selectively amplify PCR products within the gene target of the initial, external primers. The external and internal primer pairs included the ITS1-F forward primer originally developed by Gardes and Bruns (1993) to detect rust and mycorrhizal fungi. ITS4 was used as the external reverse primer and ITS2 was used as the internal reverse primer, both of which were developed by White *et al.* (1990). ITS1-F and ITS2 primers contained Illumina MiSeq® overhanging adapters, and an additional guanine base for T_m optimization of the ITS1-F primer, to generate the following working primers: **ITS1-F**: 5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATGTTAGA GGAAGTAA-3', **ITS2**: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGAT GC-3'.

Touchdown annealing was used in the nested reaction to amplify a broader range of taxa than might be obtained by a constant annealing temperature (Schmidt *et al*. 2013). Also, the use of such hierarchical gene-targeting methods enhances primer-specificity when target DNA templates are contaminated with plant DNA (Ekman 1999, Hamelin 2006). Hot-start *taq* was used in the nested PCR to further enhance primer specificity for amplification of fungal DNA, and to increase PCR

sensitivity and efficiency (Ekman 1999). Internal amplicons were generated directly after completion of the external PCR, and nested PCR products were used for all downstream applications directly after internal amplification. The external PCR reaction solution contained 12.5 uL of 2x Dream Taq (Thermo Scientific™), 25 uM ITS1-F primer (+ adapter), 25 uM ITS4 primer, 10 ng template DNA, and molecular-grade nanopure H_2O to 25 uL. The external thermocycle included an initial denaturation step of 85 sec. at 95^{O}C; 10 cycles of: 35 sec. at 95^{O}C, 55 sec. at 57.5^{O}C, 45 sec. at 72^{O}C; 10 cycles of: 35 sec. at 95^{O}C, 55 sec. at 57.5^{O}C, 120 sec. at 72^{O}C; and a final 72^{O}C elongation step of 10 min.

The internal (nested) PCR reaction solution consisted of 5 u/uL HotStar Taq (Qiagen©), 2.5 uM ITS1-F primer (+ adapter), 2.5 uM ITS2 primer (+ adapter), 25 mM $MgCl_2$ (Qiagen©), 10 mM dNTPs (Qiagen©), 2.5 uL of 10x PCR buffer (Qiagen©), 10% diluted external PCR product, and nanopure H_2O to 25 mL. The internal thermocycle included an initial hot-start *Taq* activation step of 15 min at 95^{O}C followed by an additional 3 min. of denaturation at 94^{O}C; 13 *touchdown* cycles of: 60 sec. at 94^{O}C, 60 sec. starting at 67^{O}C and decreasing 1^{O}C over each of the final 12 steps of the *touchdown* cycle, 120 sec. at 72^{O}C; 12 cycles of: 60 sec. at 94^{O}C, 60 sec. at 55^{O}C, 120 sec. at 72^{O}C; and a final elongation of 10 min. at 72^{O}C.

Diagnostic Cloning & Pyrosequencing

The efficacy of our PCR protocol was confirmed by isolating and identifying an individual sequence from mixed-template genomic DNA extracted from whitebark pine needles. Internal PCR products 300 bp in length were gel-extracted

from agarose with the Gel & PCR DNA Fragment Extraction Kit from Phenix© Research Products (USA). Overhanging-ended (TA) cloning was conducted on purified gel extracts with the pGEM-T® Vector kit from Promega© (USA) and competent *E. coli* cells courtesy of the Cretekos Lab (Idaho State University) following the pGEM-T® protocol. Blue-white screening of incubated cells aided the selection of successfully transformed cell colonies. The MoBio Ultraclean® Standard Mini Plasmid Prep Kit was used to isolate bacterial plasmids containing inserts. To evaluate the specificity of PCR primers, chemistry and thermocycle protocols prior to parallel pyrosequencing, Sanger dideoxy sequencing of isolated plasmids was performed at the Molecular Research Core Facility (MRCF), Idaho State University, Pocatello, ID, USA.

Megablast was used to align the isolate to a putative taxonomic match in the NCBI database (blast.ncbi.nlm.nih.gov/Blast.cgi). The isolate matched an uncultured clone of *Phialocephala* sp. with 100% similarity and a highly significant expectation (E-value) of $2e^{-109}$. This strengthened our conviction in the homology of the putative taxon and the cloned isolate, and confirmed the adequacy of our PCR method.

Directly following the nested PCR reaction, amplicons were delivered to the MRCF, where amplicons were cleaned with the Agencourt AMPure® XP-PCR Purification kit (Beckman Coulter™) and barcoded per tree-sample using the Nextera® XT DNA indexing kit (Illumina®). High-throughput barcoded amplicon pyrosequencing was conducted using the Illumina MiSeq® (V3-600 cycle) to generate libraries of 250-300 nt paired-end reads. The 83 samples of this study were pyrosequenced alongside 13 additional samples from a separate study.

Bioinformatics

Reads generated by Illumina MiSeq® were automatically de-multiplexed during sequence synthesis. Sequence processing was performed in *mothur* (v.1.35.1) by adapting protocols from: the MiSeq® SOP (accessed 04/2015, see Kozich *et al*. 2013), Bell et al. (2014) and McFrederick, Mueller and James (2014). Briefly, bi-directional reads were assembled into contigs using the consensus screening quality-control described by Kozich *et al.* (2013). Any reads that failed to join were discarded. Contigs not meeting the following three criteria were also discarded: 1) minimum length of 225nt, 2) no ambiguous bases, 3) ≤ 8 homopolymers. See additional procedural details in the supplied bioinformatics workflow.

Sequence length has been shown to impact the results obtained from sequence-based studies (Porter and Golding 2011; Monard, Gantner and Stenlid 2012). The average contig length prior to standardizing was 265nt. Contig length was limited to 225nt based on empirical analyses of the effect of contig length on the number of unique sequences identified and the effect of contig length on sequence classification. Our 225nt heuristic yielded 21.5% fewer unique sequences than a length of 200nt, but 72% more unique sequences than 250nt contigs. Our approach also draws from empirical studies that have reported average ITS1 region lengths for Eumycota between 200 and 250nt (Porter and Golding 2011, Toju *et al.* 2013). Additionally, we drew the same conclusions when restricting our contig lengths to either 200nt or 225nt. For simplicity, all results shown here are from analyses of 225nt contigs.

After length-trimming, contigs were pre-clustered following Huse *et al*. (2010), screened for chimeras using the *de novo* UCHIME detection algorithm (Edgar 2010), normalized to the smallest sample-library, classified against the published UNITE *mothur*-formatted *dynamic* database (v. 7), and clustered to OTUs at ≥ 97% sequence similarity using the Needleman-Wunsch algorithm (Kunin *et al*. 2010, Smith and Peay 2014, Talbot *et al.* 2014). A representative sequence from each classifiable OTU cluster was assigned to a putative taxonomic rank, with a minimum threshold of 80% confidence for species-level assignments. Eighty sequences were chosen at random from the library and cross-checked against the NCBI database using the megablast Basic Local Assignment Search Tool to compare taxonomic summaries from the *mothur* output with those in the FASTA file.

Phytochemistry

Leaves photosynthetically fix carbon from CO_2 as non-structural carbohydrates (White 1973). The disaccharide sucrose and monosaccharides glucose and fructose are among these reserves (Mauseth 2009). Although most endophytic fungi persist at basal metabolic rates *in planta*, it is no coincidence that they are abundant at sites of gluconeogenesis (Cheplick and Faeth 2009). Some endophytes induce carbon translocation in their hosts, and this has been related to plant osmotic adjustment that may enhance plant water-uptake (Rodriguez, Redman and Henson 2004; Nagabhyru *et al.* 2013). For a balanced subset of the homogenized pine needle tissue remaining after DNA extraction, a Roche® Yellow-Line Glucose/Fructose and Sucrose assay kit was used to determine the mass and

proportion of simple sugars present in pine needles following the manufacturer's protocol. Values were calculated for grams of glucose, fructose or sucrose per gram of leaf sample, and relative proportions of glucose, fructose and sucrose in leaf samples.

We used isotope-ratio mass-spectrometry to detect significant variation in isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ in a subset of the homogenized pine needle tissue. These measures were used to detect variation in plant drought stress integrated across the growth season (Farquhar, Ehleringer and Hubick 1989; Stewart *et al.* 1995; Warren, McGrath and Adams 2001; Craine *et al.* 2009). Four milligrams of needle tissue from a balanced subset of the experimental trees were packed into 4 x 6 mm tins and analyzed at the Center for Archaeology, Materials and Applied Spectroscopy at Idaho State University using a Costech ECS 4010 (Elemental Combustion System 4010) interfaced to a Delta V advantage mass spectrometer through the ConFlo IV system. The elemental analysis is done using evolutionary flash-combustion & chromatographic separation techniques with a furnace temperature of 1000° C and reduction oven temperature of 650° C. Gases generated from sample combustion are carried in a helium stream into a GC column held at 60° C before being separated and then diluted in the ConFlo IV prior to analysis in the mass spectrometer. δ13C values are reported as ‰ values relative to the VPDB scale. δ15N values are reported as ‰ values relative to air-N_2. Four in-house standards (ISU Peptone, Costech Acetanilide, Glycine and DORM-3), directly calibrated against international standards (IAEA-N-1, IAEA-N-2, USGS-25, USGS-40, USGS-41, USGS-24, IAEA-600), were used to create a two-point calibration curve to

correct raw isotope data. ISU Peptone and Costech Acetanilide are used to establish a two-point calibration line. DORM-3 and Glycine are used to monitor data accuracy (M. Hailemichael, Per Com).

Statistical Analyses

We tested the hypotheses that host genotype and priority effects shape multivariate community structure and univariate Shannon-Weiner diversity and observed OTU richness. Community structure provides insight into variation in the presence and abundance of communal populations that may not affect univariate measures of community diversity or richness (Anderson and Walsh 2013, Gotelli and Ellison 2013). Bray-Curtis (Steinhaus/Sorenson) dissimilarity describes differences among group resemblance-measure centroids, and is useful for detecting change in community assemblage across categorical and continuous variables (Anderson and Walsh 2013).

To allow multivariate tests and summaries, we collapsed the molecular community matrix into multivariate Bray-Curtis dissimilarities (Bray and Curtis 1957), which served as the multivariate response in hypothesis testing. We conducted null hypothesis tests of no community difference with permutational multivariate analyses of variance (PERMANOVA; Anderson 2005) using the function *adonis* from the **R**-package *vegan* (Oksanen *et al.* 2015). We constructed PERMANOVA models to approximate a type II SS approach, in which the sequence of predictors in the model does not impact the outcome of a test, unlike type I SS (Aho 2013). Specifically, reference models were created that included all predictors, but

only the *F* statistic and *p*-value were recorded for the last predictor in a given model. Distinct models were generated for each predictor with the predictor of interest placed last in each model. Predictors included: host-genetics, phytochemistry (leaf $\delta^{13}C$ and $\delta^{15}N$), tree height, and positional UTMs (latitude & longitude) to control for spatial autocorrelation. Canopy cover was omitted from models due to collinearity with latitude, which resulted from spatially blocking seedlings by genetic family. In a separate study in which canopy cover and latitude were independent of one another (n=108), we found a non-significant correlation of canopy cover to endophyte community dynamics (Moler, Reinhardt and Aho – in preparation). Isotopic carbon and nitrogen ratios of needle tissue were completed for 53 of the 79 sampled trees. Constrained PERMANOVA models (n=53) were constructed as above with the addition of $\delta^{13}C$ and $\delta^{15}N$ values both as covariates and focal variables. Carbohydrate assays were completed for 30 of the 79 sampled trees. Total and proportional carbohydrate values were considered independently as response variables in a constrained model (n=30) testing the null hypothesis of no difference among genetic families using 1-way ANOVA.

Non-metric multidimensional scaling (NMDS; Kruskal 1964) was used to express the site by species matrix in ordination space using Bray-Curtis dissimilarities as resemblance scores. Bray-Curtis dissimilarity was calculated as the lowest score obtained twice from 20 random starting configurations to prevent convergence to local minima. The ordination resulting in the lowest Kruskal stress was obtained with a 3-dimensional solution (stress = 0.1996). Procrustes rotations comparing of NMDS to PCA were significantly correlated (p = 0.001, 1000

permutations; correlation = 0.713) and supported our NMDS model. Vector fitting was used to fit predictor variables to data centroids in ordination space using the multiple regression function *envfit* from the **R** package *vegan* (Oksanen *et al.* 2015). *envfit* was used to calculate univariate coefficients of determination for each environmental and host-related predictor, which are calculated for one predictor at a time. Ordination values for each dimension of communities in species-space were used as predictors of variation in environmental or host-related measures in a multiple regression model. Needles from maternal parents of the experimental progeny seedlings were sampled and sequenced in the same way as the progeny-seedlings and assessed through NMDS ordination to investigate how ontogenetic effects impact endophyte communities within known genetic families

Univariate general linear models were also generated to test the effect of genetic family and treatment on the univariate community measures of Shannon-Weiner diversity and observed species (OTU) richness. We performed indicator species analyses (Dufrene and Legendre 1997) for genetic family using the **R** function *indval* from the package *labdsv* (Roberts 2007), and used the *mothur* implementation of Metastats (White, Nagarajan and Pop 2009) to assess species assemblage differences resulting from the inoculation trial. We used adjusted *p*-values from the Metastats output to control for false discovery rate (White, Nagarajan and Pop 2009) and *p*-values from the output from *indval* because *indval* was found to be more conservative than Metastats. Indicator species and Metastats results are only given here for species' sequences with greater than 90% confidence of taxonomic assignment.

Results

Sequence Library Summary

Almost half of the 80 randomly-sampled sequences searched with megablast (NCBI) were found to match the second and third most abundant sequences classified at the level of species using *mothur*. *Lophophacidium dooksii* sequences were 91% similar ($E = 3e^{-61}$) to *L. dooksii* reads in NCBI, and *Lachnellula calyciformis* was 99% similar ($E = 4e^{-109}$). Another highly abundant representative of the full project library, *Phaeomoniella* sp., that was only classifiable to the level of genus using the UNITE database via *mothur,* was assigned to a species in NCBI with 96% similarity ($E = 4e^{-99}$).

Sequence filtering and screening retained 209,034 sequences that grouped into 637 OTUs, of which 148 were classified as taxonomic species within 123 genera, 75 families, 44 orders, 18 classes and four phyla. As expected, the dominant phylum was represented by Ascomycota, comprising 98.4% of the sequences. Basidiomycota accounted for 1.6% of the sequences, and only a few representatives were detected from Chytridiomycota and Zygomycota.

Leotiomycetes was the most abundant fungal Class, accounting for 77% of sequences classified to or below that taxonomic level (Fig. 1). Leotiomycetes contains the Order that occurred in greatest abundance in our sequence library, which is typically the most abundant Order in gymnosperm foliage, Leotiales – i.e. the apothecial discomycetes (Sieber 2007; Fig. 2). The second most abundant Order represents 12.4% of classified sequences, and is placed in the holding group:

Ascomycota *incertae sedis*, which demonstrates the large number of presently unidentified taxa uncovered in this study. The most abundant Orders found are known to contain plant-associated, largely saprotrophic fungal taxa, such as the Leotiales and Rhytismatales. However, sequences aligning with ≥80% confidence to the Chytridiales, containing many plant pathogens; Teloschistales, containing mostly lichenized species; and even Diaporthales, which are mostly found on angiosperms, were well-represented.

The most abundant sequences classifiable to at least the level of genus belong to class Leotiomycetes, order Leotiales or Rhytismatales. *Hyaloscypha* sp. wasthe most abundant classifiable taxon, accounting for 19% of the total reads. The genus has been observed on decaying snags and, like most endophytes, is likely a latent saprotroph (Kaarik 1975). *Coccomyces multangularis* accounted for the second most abundant species (12.6% of all reads), and has been reported as a saprophyte on fallen leaves in Japan (Li *et al*. 2014). *Lophophacidium dooksii*, the causal agent of Dooks needle blight in eastern North America, accounted for the third most abundant species at 9.3% of the library (megablast= 91% similarity, $3e^{-61}$), (Mclaughlin *et al.* 2012). *Gyoerffyella entomobryoides*, a known saprophyte of woody tissue (Makela 1986), accounted for 9.1% of the 209,034 total reads.

We found a basic agreement of statistical signals obtained using either OTUs or taxonomic ranking at the level of Order as predictors of genetic community structure (Fig. 3). Figure 3 raises two important points: 1) the UNITE database appears to be most reliable for taxonomic assignment at levels other than family, probably reflecting the development of our understanding of fungal phylogenetics,

and 2) the comparable signal strength detected using OTU-level and Order-level group-comparisons confirms the biological relevance of an OTU-based approach to hypothesis testing in this study.

Endophyte Community Dynamics

Our data led us to support the community phenotype hypothesis by rejecting the null hypothesis of no effect of host gene family on endophyte community structure, diversity and richness. Host genetic family gave the strongest signal across all models, and explained 34.8% of the variability in community structure ($F_{4,72}$=2.04, p=0.001) in the 79-tree model (Fig. 5). Latitude, the only other strong predictor of endophyte community structure, explained 23% of structure variation ($F_{1,72}$=1.80, p=0.039). F and p-values of all moderately strong predictors of community structure are displayed in Table 1. Including the covariates of $\delta^{13}C$ and $\delta^{15}N$ in the constrained model (n=52), only genetic family explained a significant proportion of variation in community structure ($F_{4,44}$ = 1.81, p = 0.002, $R^2_{partial}$ = 0.137), while northing and all other covariates showed p-values ≥ 0.2 and r^2 values ≤ 0.03. Community diversity was significantly impacted by both host gene family ($F_{4,74}$=4.34, p=0.003, R^2=0.19; Fig. 6). Figure 7 shows host gene family also differentially and significantly affecting OTU richness ($F_{4,74}$=4.65, p=0.002, R^2=0.20).

Figure 5 shows seedlings colored by genetic family in NMDS ordination-space, and the explanatory power of latitude related to endophyte community structure is illustrated by the length of the vector overlain upon the ordination. Neither longitude nor $\delta^{13}C$ or $\delta^{15}N$ were included in the ordination because only

latitude correlated significantly to community structure when controlling for genetic family in the unconstrained model (Table 1). The latitude vector is roughly coextensive with the gradient of endophyte community dissimilarity explained by genetic family (Fig. 5).

Variation of all non-structural carbohydrate values was non-significant across gene-families (g. glucose/gram sample: $F_{4,24} = 0.78$, $p > 0.5$; proportion of NSC present at glucose: $F_{4,24} = 2.15$, $p > 0.1$; total grams of carbohydrates: $F_{4,24} = 0.331$, $p > 0.8$). Average phytochemical values and ranges are shown in Table 2.

Indicator species analysis revealed species-level assemblage differences among host gene families (Table 4). Sequences present in significantly greater abundance in genetic family 25p include: *Fulvoflamma eucalypti*, a Rhytismataceous fungus known from litter of Mediterranean tree leaves (Crous, Verkley and Groenewald 2006); *Pseudeurotium hygrophilum*, a Pseudeurotiaceae fungus thought to occupy caves and previously isolated from Antarctic regolith (Minnis and Lindner 2013); and *Alatospora flagellate*, an aquatic Leotiaceous hyphomycete. Gene family 24p harbored significantly higher abundances of *Phialocephala lagerbergii*, a known saprobe of woody plant tissues belonging to the Family Vibrisseaceae (Wang *et al*. 2009); and *Cistella* sp., a genus of Family Hyaloscyphaceae noted for parasitic canker-forming strain *C. japonica* (Suto 1997). Gene family 22p differentially expressed *Dascycyphella* sp. – which has been isolated from wood in Japan; *Caloplaca lenae*, a saxicolous lichen previously described on limestone and calciferous schist in Mongolia; and *Phaeophyscia ciliata*, a foliose lichen described on rocks in North America and the western Himalayas (Gupta *et al.* 2014). Considering

that the seedling study site is positioned near the area of the 11p seed source, it is interesting to note that gene-family 11p does not host differentially abundant endophytic species (Table 4).

Inoculation Trial

The controlled inoculation of whitebark seedlings did not significantly alter the fungal endophyte multivariate community structure ($F_{1,78}$=0.75, p=0.74), diversity ($F_{1,78}$=1.39, p=0.241) or richness ($F_{1,77}$=1.10, p=0.30). Indicator species analyses, however, revealed potential species-level assemblage differences between inoculated and non-inoculated trees (Table 5). Most notably, sequences aligning to *Lophophacidium dooksii* were differentially abundant in control seedlings. In contrast, reads matching to *Collophora hispanica*, a putative necrotrophic pathogen recently discovered in woody tissue of diseased almond trees (Granaje *et al*. 2012) were differentially abundant in leaves of inoculated trees.

An indicator species analysis of inoculated vs. control tree mycobiomes using the *indval* function in **R** did not reveal any significant differences between the groups. The Metastats algorithm implemented in *mothur* does not adequately correct for false-positives or zero-counts (Paulson *et al.* 2013), so species-level analyses implemented through Metastats are presented merely as descriptive tools. The disagreement between results from Metastats and the indicator species analysis provoked inquiry into potential inoculation effects at higher taxonomic levels. Figures 8 and 9 show a lack of difference in the abundance of Orders containing species with differential abundance between treatment and control trees.

Discussion

This study satisfied three objectives. We evaluated: i) the persistence of *Myrothecium roridum* in the mycobiomes of whitebark pines inoculated with the fungal biocontrol; ii) changes in fungal community dynamics resulting from an inoculation treatment; and iii) whether whitebark pines exhibit a community phenotype, i.e. effects of host genetics on the structure of fungal endophyte communities.

Community Genetics

Present-day plant-endophyte symbioses reflect an ancient co-evolutionary relationship that expresses propensities for mutualism, commensalism and parasitism (Sieber 2007, Rodriguez *et al.* 2008). Considering the coevolved nature of endophyte symbioses, evolutionary theory suggests that selection for traits derived from either a photobiont or a mycobiont will cause reciprocal changes in both (Arnold *et al.* 2010, Giaque and Hawkes 2013). Natural selection operates on the differential fitness of individuals due to phenotypic variation for traits, and closely coevolved systems raise important considerations for understanding constraints on selection and predicting the heritability of traits (Darwin 1859, Rice and Mack 1991a, Whitham *et al.* 2003).

Our model including seedling location and genetic-family as predictors explained 82% of the total variation observed in endophyte community structure. Host genetic family (p=0.001) and latitude (p=0.034) were statistically significant

predictors of community structure. Using Shannon-Weiner diversity indices as the response in the same model explained a total of 22.6% variation in endophyte community diversity, where genetic family was the only significant predictor. A constrained model of smaller sample size but including isotopic ratios of leaf carbon and nitrogen as covariates explained a total of 69.2% of variation in endophyte community structure. Again, only genetic family correlated significantly with variation in community structure in the constrained model ($F_{4,44} = 1.75$, $p = 0.002$, $r^2_{partial} = 0.137$). The reduction in multiple-r^2 values calculated from univariate test shows that community structure is a more robust indicator of fungal community variation than measures of diversity. Meanwhile, latitude becomes a non-significant predictor of community structure when phytochemical variables are included as covariates, which suggests that latitude represents environmental heterogeneity that were not measured in this study.

Half-sib seedlings in each genetic-family were naturally wind-pollinated and we can confidently state that they share contributions from the maternal genome. Studies of community phenotypes expressed by arthropod (Shuster *et al.* 2006) and rhizosphere microbe community composition (Schweitzer *et al.* 2011) associated with replicated full-sib cottonwood trees (*Populus* spp.) found that 56-63% and 70%, respectively, of the variation in community phenotype was explained by host genetics. The young age of our seedlings and lack of similarity of the paternal genome within one gene-family provides reasoning for our lower $r^2_{partial}$ for variation in community structure explained by genetic-family, which remained

significant even when controlling for host phytochemisty, microenvironment, and spatial autocorrelation.

Resistance screening trials have shown gene family 22p to have moderate resistance to *C. ribicola* infection, whereas gene family 25p is highly susceptible (R. Sniezko, Per Com). Interestingly, figures 6 & 7 illustrate that these most and least resistant genetic families have significantly similar values of fungal endophyte community diversity and richness. This suggests that univariate endophytic community properties may not relate to factors of host-resistance in general, or this may be an artifact of studying young organisms whose endosymbiotic communities may have yet to reach a state of successional equilibrium. In contrast, non-trivial differences in species assemblages were observed among genetic families and inoculated vs. non-inoculated seedlings. Assemblage differences are expected to either attenuate or amplify as a host ages and endophyte community succession occurs, and multiple researchers suggest a positive correlation between endophyte density and the age of host tissue (Sieber 2007), though it is not clear what density might imply for species richness or evenness. Table 3 shows mean richness and diversity values for all gene families.

The NMDS shown in Fig. 11 indicates that substantial variation in endophyte community structure remains unexplained by predictors that are significant among hosts of the same age. Variation in community structure is introduced by comparing endophyte communities from hosts occupying various positions on the landscape, and representing different ages and sizes (Fig. 11). This analysis suggests that elevation and host-height become increasingly important with increasing

geographic and demographic differences among hosts & endophyte communities. Differences between seedling and parent tree height reflect large differences among host ages. Constitutive defenses against infection and herbivory change within conifer leaves as trees age, with some species expressing greater defenses at older ages (Boege and Marquis 2005). Fungal community succession *in planta* also certainly results in community-level changes as living leaves age, though that dynamic remains undescribed in the literature. The wide divergence of community structure between progeny and parent tree endophytes shown in Fig. 10 suggests that while knowledge of host-microbiome and genetics is important for future approaches to enhance whitebark pine resistance against white pine blister rust, these mechanisms must be understood in the context of the high physiographic variability of whitebark pine habitat.

Differences in water-related stress may change the qualitative symbiotic relationship between hosts and endophytes. Except for $\delta^{13}C$, measures of leaf phytochemistry did not vary significantly among genetic families in univariate models. Heterogeneity of $\delta^{13}C$ among trees implies a potentially significant difference in drought stress among hosts (Fig. 4), though we found a lack of correlation of $\delta^{13}C$ to endophyte community structure when controlling for other relevant covariates. Also, the lack of significant difference of total non-structural carbohydrates across gene-families suggests that, in this system, endophytic communities did not differentially induce osmotic adjustment in hosts across genetic families.

Inoculation Effects and Taxonomic Analyses

Myrothecium spp., order Hypocreales, are noted for their high production of antibiotic compounds such as macrocyclic trichothecenes (type B mycotoxin), which have been investigated for activity against myriad pest and disease organisms (Gees and Coffey 1989; Worapong, Sun and Newcombe 2009). *Myrothecium roridum* has been studied for the biological control of soil-borne plant pathogenic fungi (Turhan and Grossman 1994), water hyacinth (Ponnappa 1970), an Oomycete pathogen of avocado trees (Gees and Coffey 1989), the invasive vine *kudzu* (Boyette, Walker and Abbas 2002), pathogenic epiphytes of guava trees (Pandey, Arora and Dubey 1993), and the causal agent of white pine blister rust (this study).

Kmer and blast search methods were employed to align the known inoculant-ITS gene against our sequence library prior to library normalization. We also searched classified sequences via taxonomic terms. The *M. roridum* inoculant used in this study was not recovered from environmental DNA of pine needle mycobiomes. We did detect multiple reads of a Hypocrealean species in one of our 83 samples, which suggests that our amplification protocol would have detected *M. roridum* if it were present in the sampled needles. Although the ITS region of *M. roridum* is regarded as conducive to amplification and Sanger sequencing (G. Newcombe, Per. Com.), it is possible that it was present in our samples at very low concentrations and eluded our detection. Annealing temperature can dramatically change the outcome of molecular-based biodiversity surveys due to the differential specificity of primers under different temperatures (Schmidt *et al.* 2013). However, we used nested PCR to enhance the specificity and sensitivity of PCR reactions involving plant-

contaminated DNA templates (Ekman 1999, Jaeger *et al.* 2000), and touchdown PCR methods to both increase primer specificity and to minimize erroneous PCR products (Don *et al.* 1991). Combining both procedures should optimize amplification for detecting a high degree of genetic diversity while reducing the number of chimeric sequences produced (Ekman 1999). Due to the detection of a Hypocrealean species and our amplification approach, we conclude that *M. roridum* was eliminated from inoculated trees. Future research should address modes of competitive exclusion and elimination of endophytes during community succession.

Indicator species analysis of the inoculation trial detected differential abundance of *Lophophacidium dooksii*, the causal agent of Dook's Needle Blight of pine trees in eastern North America, in non-inoculated trees. McLaughlin *et al.* (2012) reported western white pine (*P. monticola*) as a new host for the pathogen, and *Lophophacidium pini* was recently detected on white pines in Oregon (L. Bullington, 2014, Per Com). To our knowledge, this is the first report of *L. dooksii* in western North America, and specifically on *P. albicaulis*. Our detection of a high proportional abundance of sequences aligning to *L. dooksii* in whitebark pines may become important as climate change-related abiotic stresses place forests at risk of intensified disease epidemics (Desprez-Loustau *et al.* 2006).

Some studies have concluded that traditional culturing methods are *complementary* to older pyrosequencing technologies for surveys of endophyte communities. Newer tools for amplicon-based barcoded pyrosequencing provide ever-greater coverage of microbial communities at lower costs than ever, enabling more comprehensive surveys of a larger sample populations, thus increasing the

statistical power of null hypothesis testing (Unterseher *et al.* 2011, Toju *et al*. 2012, Schmidt *et al.* 2013, Smith & Peay 2014, U'Ren *et al.* 2014). While no approach for studying fungal biodiversity is without limitations, non-culture based surveys avoid the inherent limitations of culture-based approaches, which rely on speculation of the nutritional and physicochemical demands of unknown organisms. We chose to use a DNA barcoding approach and the ITS1 rRNA gene for this study because this combination currently enables the most comprehensive insight into differences among fungal communities down to the level of species.

With the advantages of next-generation sequencing technologies come new challenges. In particular for molecular-based studies of pine phyllospheres, fungal DNA amplification can be altogether inhibited by the presence of plant terpenoids and resins in DNA extracts. DNA extraction and amplification must be optimized for reducing contaminants in order to obtain targeted amplicons from pine material. Even then, the vast majority of DNA fragments amplified and sequenced in barcoded studies have yet to be described taxonomically, and often can only be classified as a member of a given class or order.

Studies that seek to detect emergent properties of endophyte communities by comparing OTUs across sites are not limited by the accuracy or comprehensiveness of taxonomic databases. However, an end-goal is to understand how species assemblage affects hosts, and how biotic and abiotic forces shape the composition of endophytic communities. This level of understanding requires morphological and physiological species delineation that can only be accomplished through traditional culture-based studies. When molecular taxonomic databases reflect information

gained from culture-based studies, DNA-barcoding will enable the study of endophyte community function. Meanwhile, DNA barcoding provides valuable insights into the vastness of fungal biodiversity, and the intricate genetic relationships between endosymbionts and their hosts.

Table 1. Variables in multivariate model explaining endophyte community structure (top rectangle - PERMANOVA).

response	predictor	num. df	den. df	*F* (*p*-val)	partial *r*^2
community structure	gene family	4	72	2.04 (0.001)	0.1
community structure	northing UTM	1	72	1.80(0.039)	0.024
community structure	easting UTM	1	72	1.38 (0.166)	0.019
community structure	δ13C	1	44	1.28 (0.191)	0.028
community structure	δ15N	1	44	0.66 (0.82)	0.015

Table 2. Univariate models explaining phytochemical differences across gene families (ANOVA).

response	predictor	num. df	den. df	*F* (*p*-val)	partial *R*^2
glu.(g)/g sample	gene family	4	24	0.78 (0.548)	0.115
proportion glu.	gene family	4	24	2.15 (0.106)	0.264
total carbohydrate (g)	gene family	4	24	0.331 (0.854)	0.052

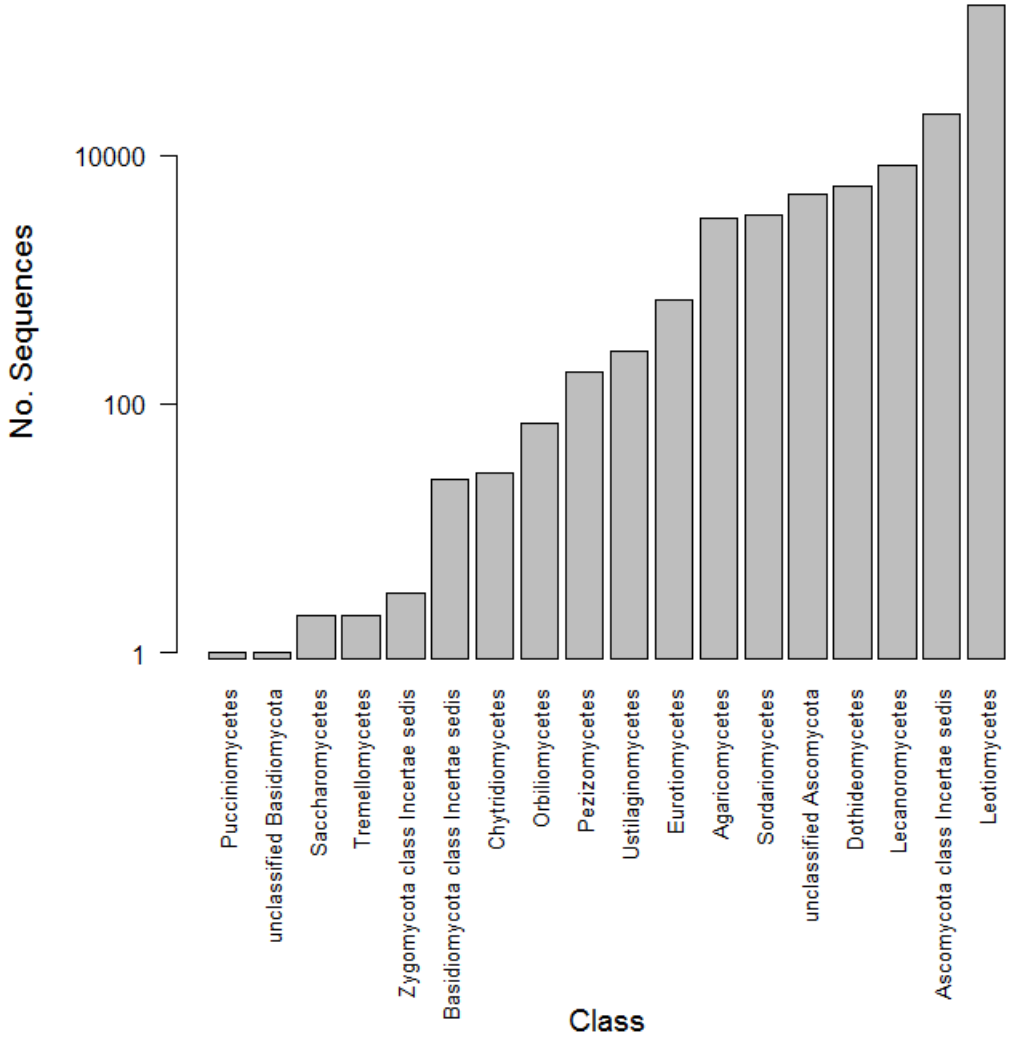

Fig. 1. Log_{10} transformed frequency of sequences aligning to a given fungal taxonomic Class.

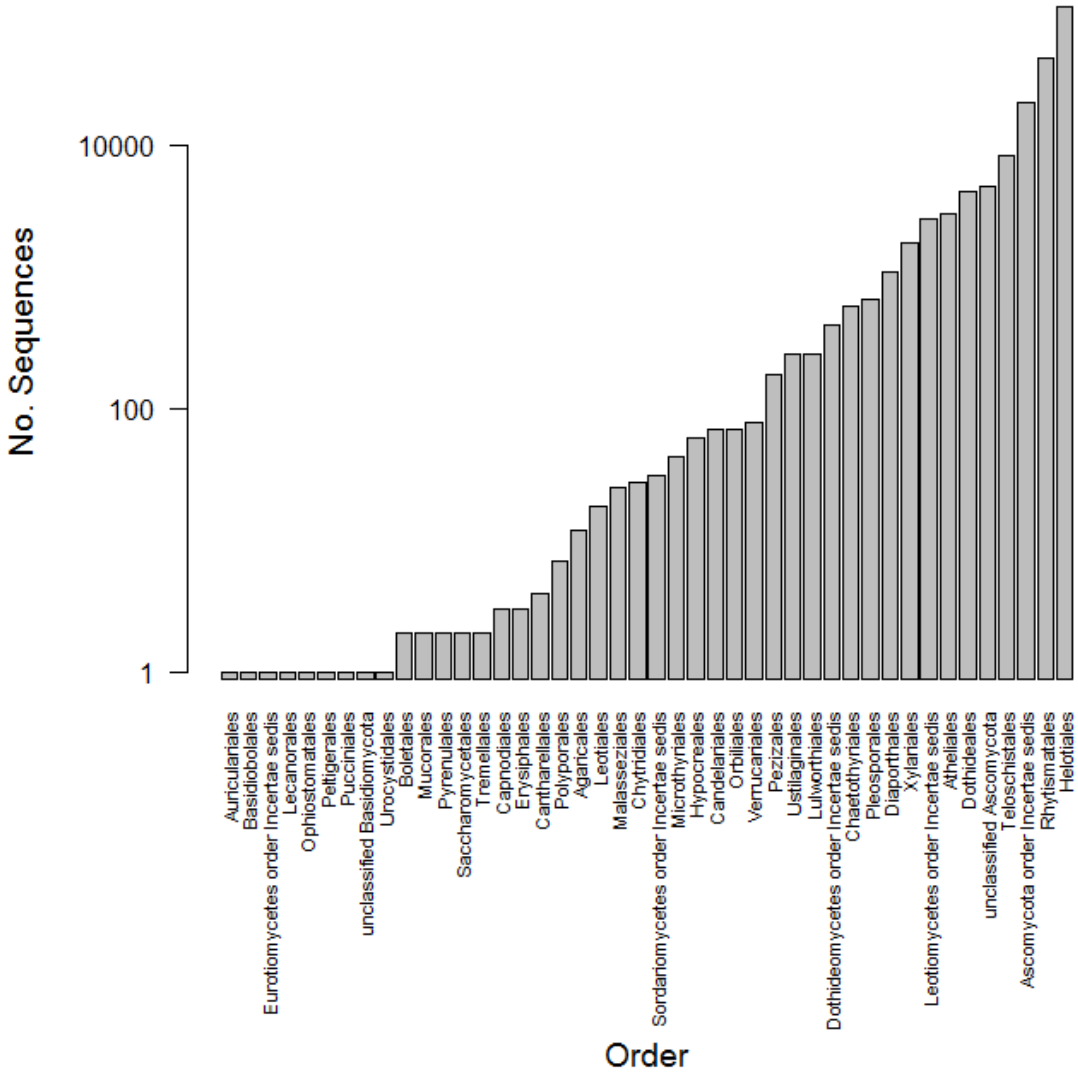

Fig. 2. Log_{10} transformed frequency of sequences aligning to a given fungal taxonomic Order

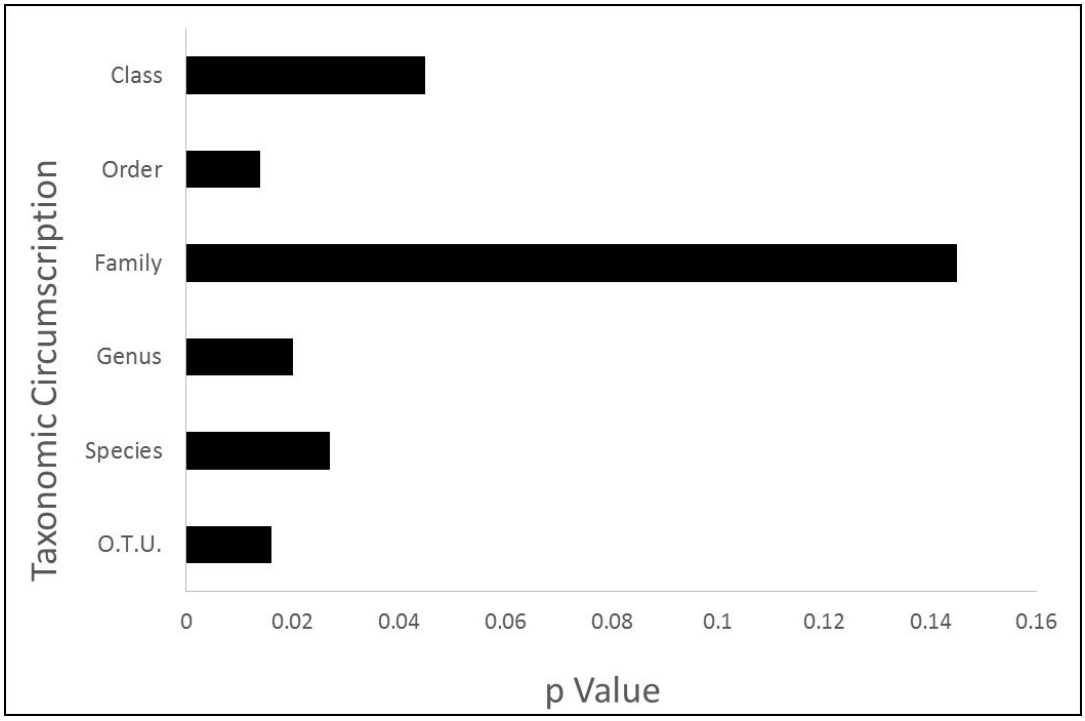

Fig. 3. The effect of taxonomic ranking on experimental response signal

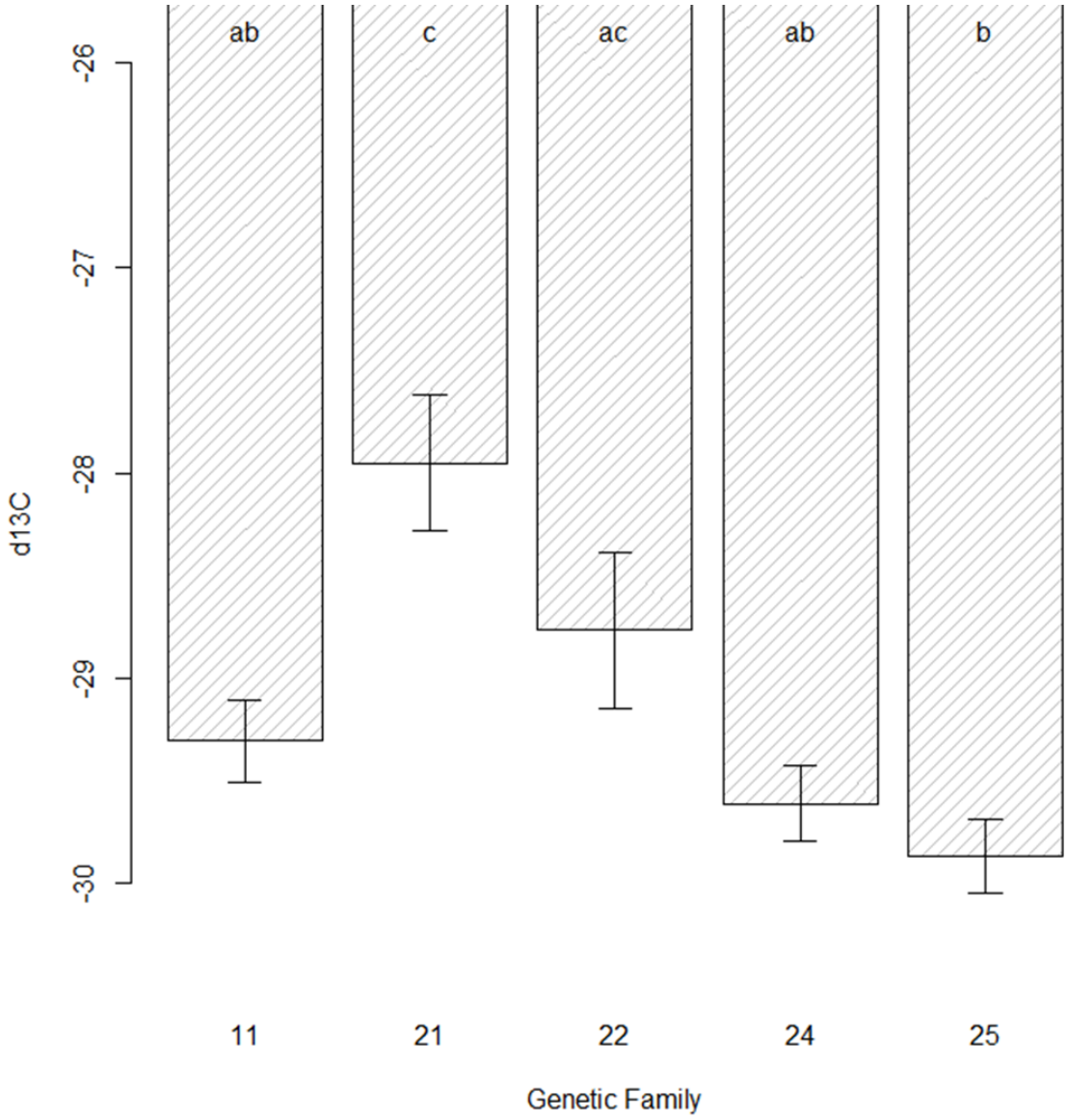

Fig. 4. $\delta^{13}C$ values vary among host genetic families ($F_{4,48} = 7.037$, $p < 0.0002$)

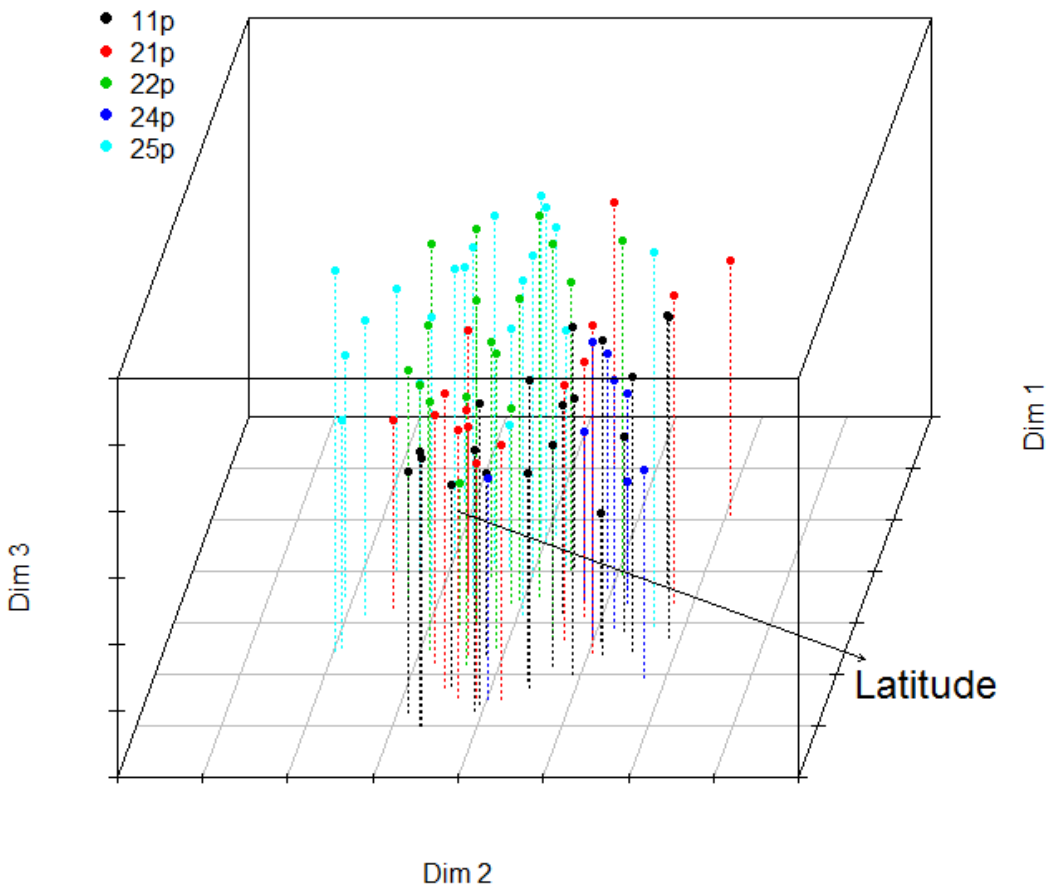

Fig. 5. NMDS ordination of endophytic communities colored according genetic family. Each circle represents the fungal community of a host-tree in species-space. The vector shows the direction of the greatest gradient of change in community due to latitude.

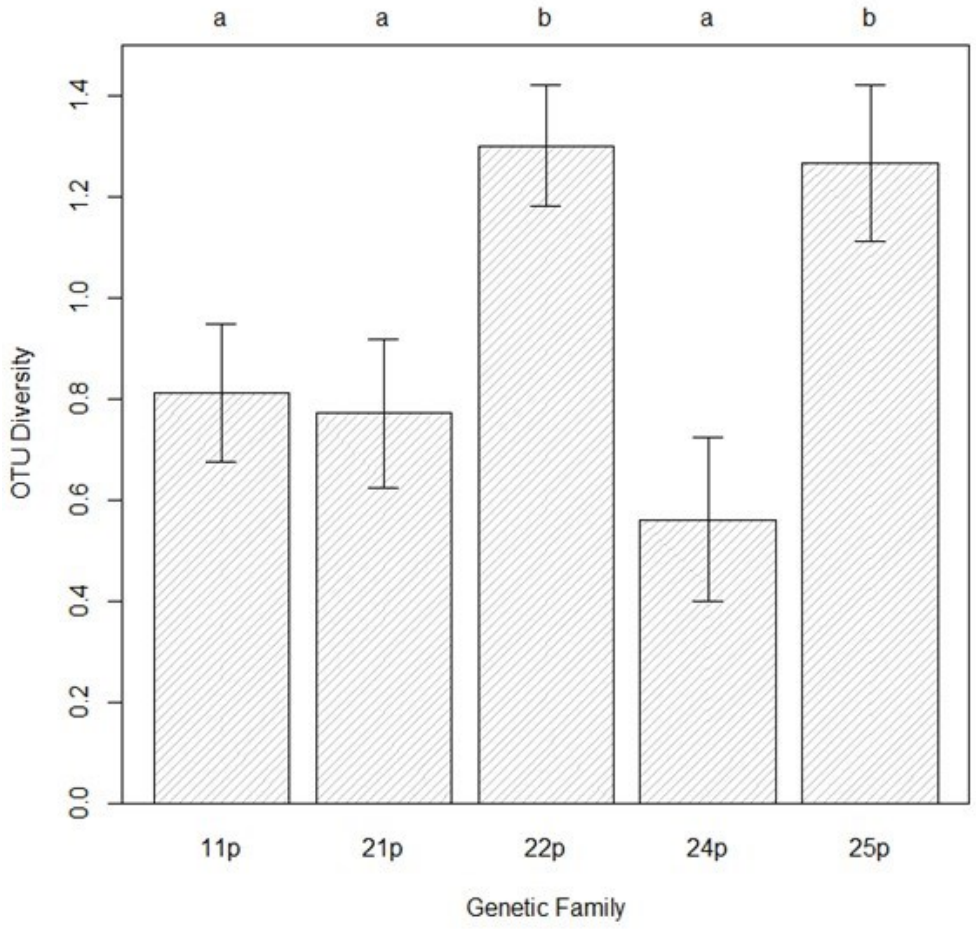

Fig. 6. Shaded bars represent mean observed community OTU richness per gene-family, error-bars show standard errors. Tukey's post-hoc testing represents significant differences in community richness among genetic families with different letters at figure head ($F_{4,69} = 4.308$, $p = 0.0036$).

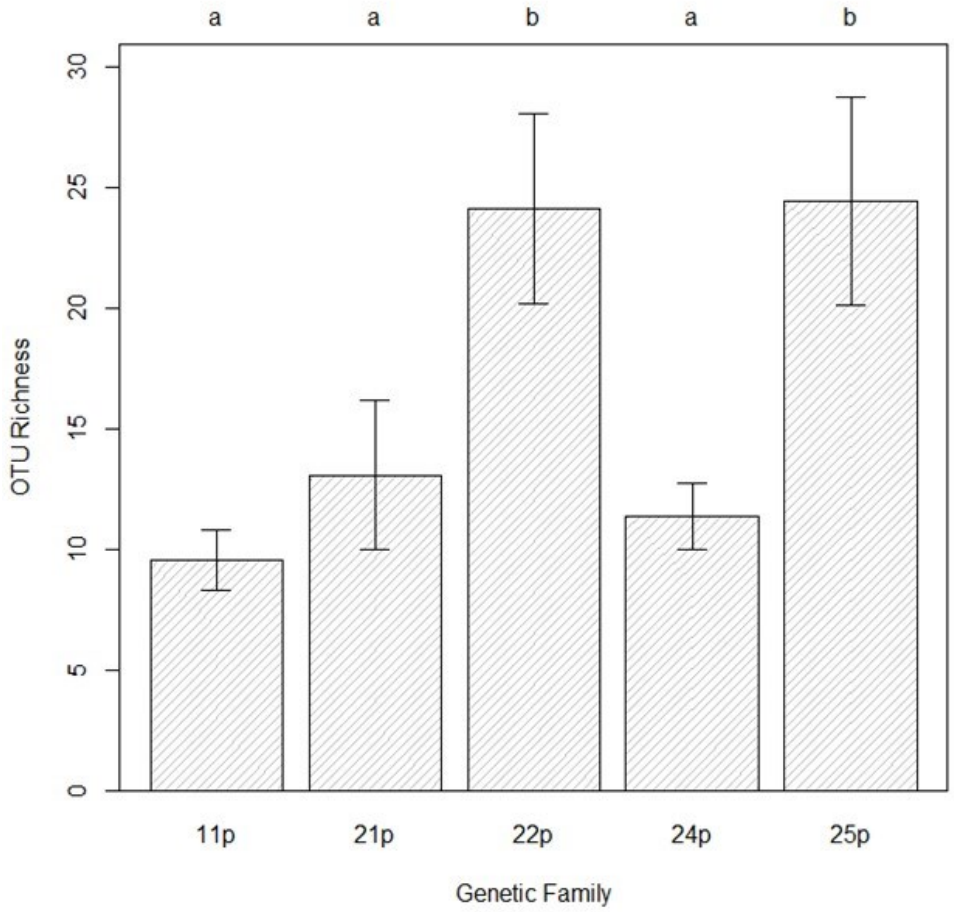

Fig. 7. Shaded bars represent mean observed community OTU richness per gene-family, error-bars show standard errors. Tukey's post-hoc testing represents significant differences in community richness among genetic families with different letters at figure head ($F_{4,69}$ = 4.48, p = 0.0028).

Table 3. Mean richness and diversity of experimental groups

Group	Mean Obs. OTU Richness	Mean OTU Shannon-Weiner Diversity
CRLA total progeny	17.30	1.00
Family 11p	9.53	0.81
Family 21p	13.07	0.77
Family 22p	24.12	1.30
Family 24 p	11.38	0.56
Family 25p	24.45	1.27
CRLA inoculated	19.05	1.08
CRLA control	15.51	0.91

Table 4. Average Phytochemical values of whitebark pine needles

δC13 avg.	δN15 Avg.	g glu./g. sample	g. fru./g. sample	g. su./g. sample
-29.0281	-0.7862	0.0083	0.0096	0.0106
δC13 range	**δN15 range**	**glu. range**	**fru. range**	**su. range**
-30.8 -> -25.7	-2.94 -> 2.19	0.0009-> 0.0501	0 -> 0.0443	0 -> 0.0788

Table 5. Species varying significantly by abundance between inoculated vs. control trees ($q \leq 0.05$).

Inoculated	Control
Lachnellula calyciformis	*Malassezia restricta* (*Malasseziales)
Scleropezicula sp.	*Delfinachytrium mesopotamicum* (*Chytridiales)
Coccomyces dentatus	*Coccomyces multangularis*
Collophora hispanica	*Lophophacidium dooksii*
Lirula exigua	
Dasyscyphella sp.	
Chaetothyriales sp.	
Hypocreales sp. (*Hypocreales)	
Savoryella longispora (*Sordariomycetes incertae sedis)	
Lachnum sp.	
Fibulorhizoctonia sp.	
Chalara piceae-abietis	
* indicates orders present in </= 5 trees	

Table 6. Species varying significantly among genetic families.

Genetic Family	Species
21	*Hyalopeziza* sp. (p =0.044)
21	uncultured Sordariomycete (p=0.022)
22	*Caloplaca lenae* (p=0.044)
22	*Dasyscyphella* sp. (p =0.003)
22	*Phaeophyscia ciliata* (p =0.039)
24	*Cistella* sp. (p =0.007)
24	*Gyoerffyella entomobryoides* (p =0.001)
24	*Phialocephala lagerbergii* (p =0.042)
25	*Pseudeurotium hygrophilum* (p =0.019)
25	*Fulvoflamma eucalypti* (p =0.046)
25	*Alatospora flagellata* (p =0.006)

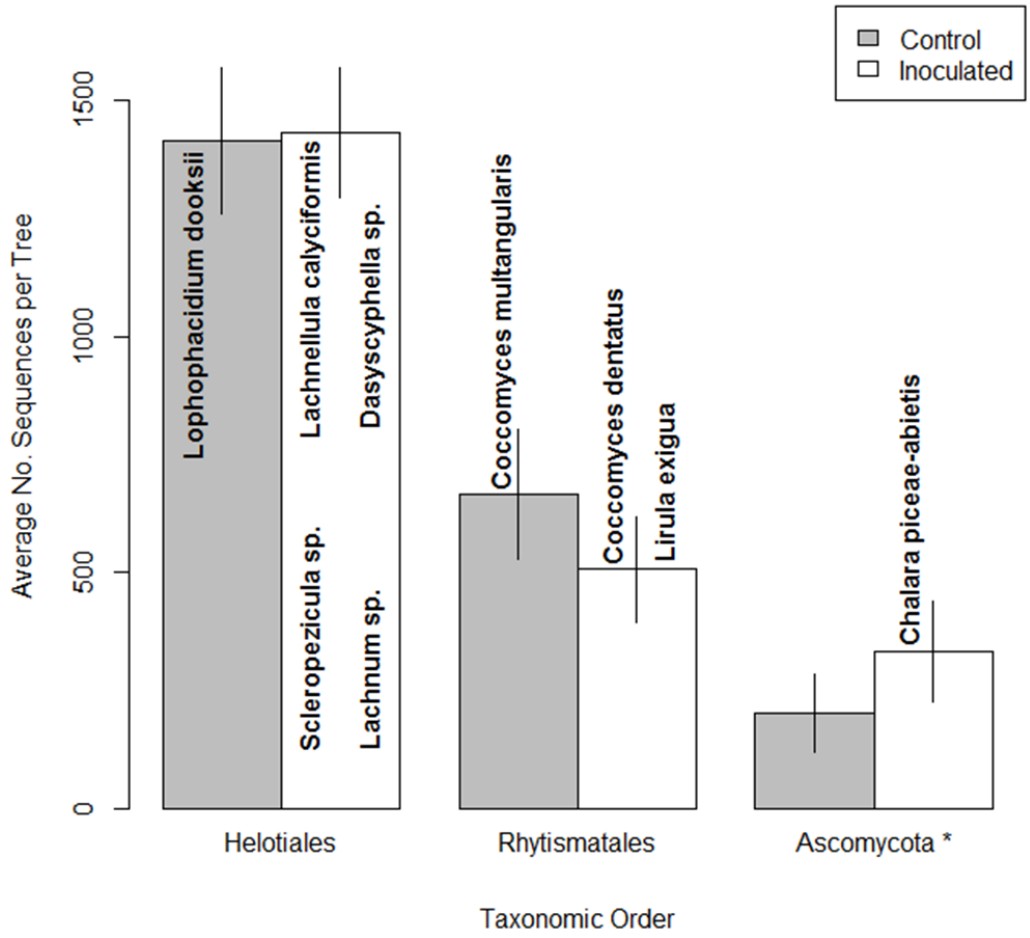

Fig. 8. Mean abundance of taxonomic order containing species that occur significantly more often in a treatment (species name placement designates treatment affiliation, $q \leq 0.05$). Line segments represent SE. * incertae sedis

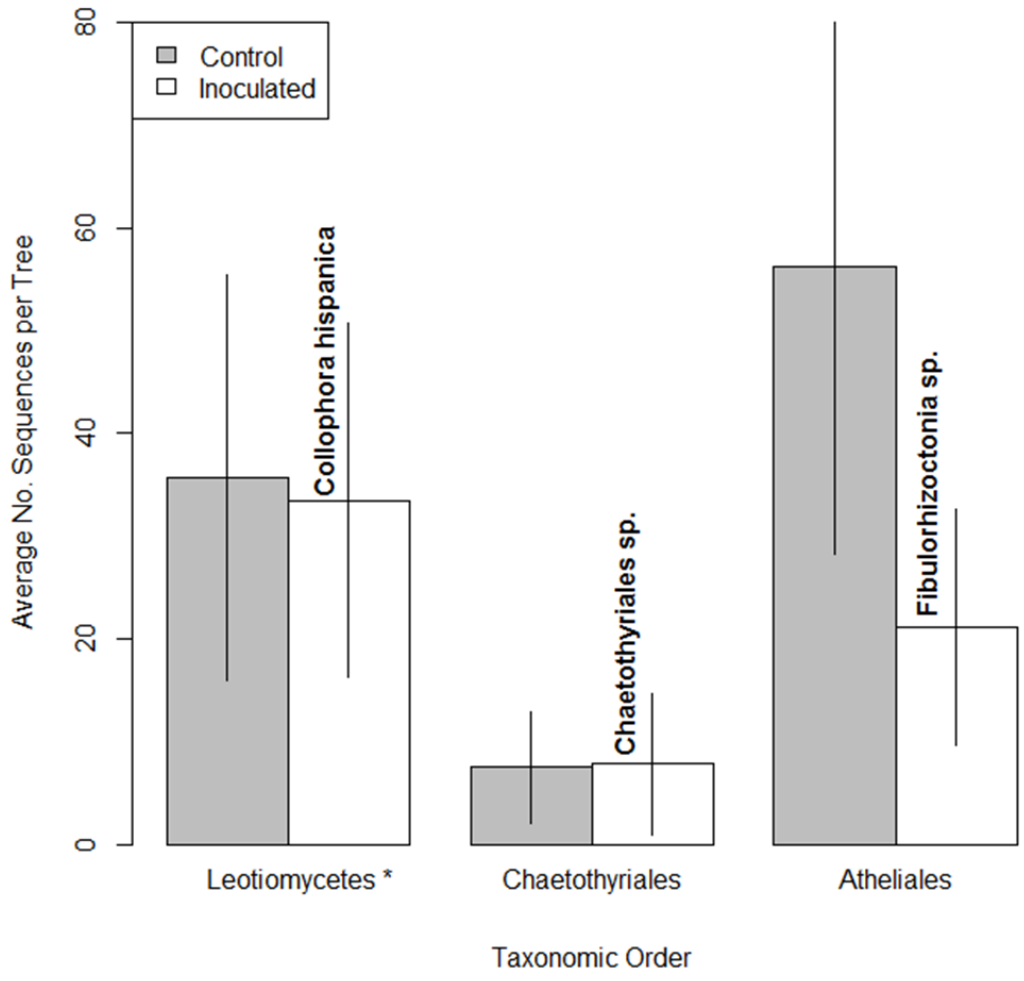

Fig. 9. Mean abundance of taxonomic order containing species that occur significantly more often in a treatment (species name placement designates treatment affiliation, $q \leq 0.05$). Line segments represent SE. * incertae sedis

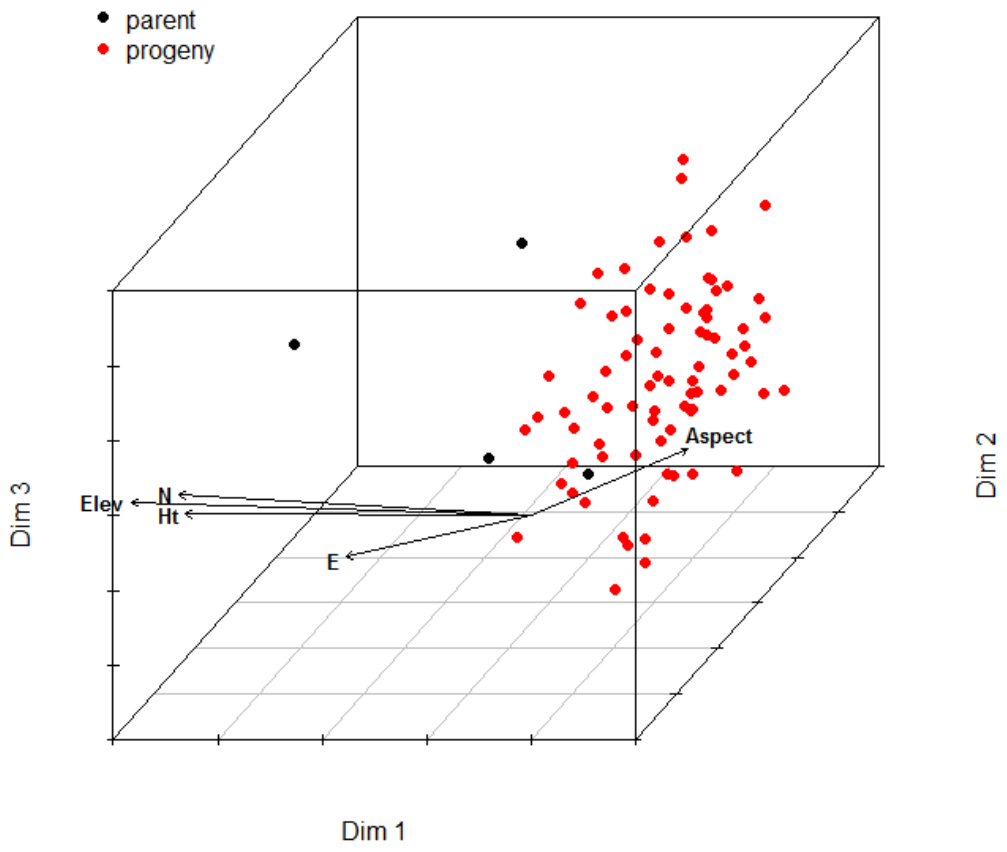

Fig. 10. NMDS ordination of communities in species-space. Endophyte communities in progeny trees as red or black circles. Predictive environmental and demographic variables point toward the direction of the greatest gradient change for each given variable.

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Chapter 2

ENVIRONMENTAL CORRELATES OF FUNGAL ENDOPHYTE COMMUNITY DYNAMICS IN WHITEBARK PINE FORESTS

Abstract

At this time, we lack fundamental knowledge of the variables that most influence fungal endophyte distribution. Studies that do not consider predictive variables that shape fungal endophyte community measures will miss opportunities to enhance understanding of the functional significance of fungal taxa *in planta*. Here we apply concepts from the discipline of forest pathology to the study of fungi that occur in fascicles of whitebark pine trees in the southern Cascade Range of Oregon, USA.

Introduction

Forest pathologists rate the *hazard* of a site or region to predict the likelihood that environmental parameters will favor the incidence of a given disease (Van Arsdel, Geils and Zambino 2006). While the term may have originated from the outdated concept from microbial ecology that "everything is everywhere, the environment selects," insolation, wind patterns, moisture and temperature are indeed robust predictors of plant-associated microbial distribution (Van Arsdel 1965a; Atlas and Bartha 1997; Van Arsdel, Geils and Zambino 2006). For instance, the likelihood of a widespread fungal disease occurring at a given site within its range was predicted with 89% accuracy by considering site-level insolation and topography (Van Arsdel 1972).

Notably, pathogenesis is a rather exceptional outcome of microbial plant-infections, and not the rule (Hamelin 2006, Bonello 2006, Porras-Alfaro and Bayman

2011). Non-pathogenic plant-infection is a common niche especially among fungi, which have coevolved with plants at least since their mutual migration onto land 400 mya, and have been recovered from every plant species examined to date (Isaac 1992, Stone *et al.* 2004, Sieber 2007, Unterseher *et al.* 2011, Sun and Guo 2012). Plant-fungal symbioses include associations within (endophytes) and upon (epiphytes) aboveground plant foliage and bark - comprising the phyllosphere, in xylem, and within and surrounding plant roots (arbuscular-mycorrhizae and dark-septate endophytes, and ectomycorrhizae, respectively) (Stone *et al.* 1996 and 2004, Porras-Alfaro and Bayman 2011). Though most nonclavicipitaceous endophytes are not mutualistic plant-symbionts, there are often benefits conferred to hosts by fungal endophytes, including increased water and nutrient acquisition, reduced herbivory, enhanced growth, and resistance to pathogens (Arnold 2003; Bonello 2006; Rodriguez *et al.* 2008; Ganley, Sniezko and Newcombe 2008).

Within a decade, non-culture-dependent molecular approaches and discovery of high levels of endophytic diversity have prompted estimates of global fungal richness to skyrocket from 1.5 million to over 5 million species (Peay, Bidartondo and Arnold 2010; Blackwell 2011; Toju *et al.* 2012; Peay 2014). High-throughput DNA barcoding coupled with studies of molecular dissimilarity enables surveying of both described and undiscovered taxa, allowing comprehensive investigation of habitats as speciose as plant phyllospheres (Arnold and Lutzoni 2007, Sieber 2007, Jumpponen and Jones 2009, Zimmerman and Vitousek 2012). Although it is now possible to investigate hyperdiverse nonclavicipitaceous endophyte communities more thoroughly than ever before, there is a paucity of work regarding how these

communities are organized in their environment and affect host-plant performance (Parrent *et al*. 2010, Peay 2014).

Decreases in the diversity of plant and animal microbiomes are known to affect the stability of microbial communities, and may lead to disease of host organisms (Atlas and Bartha 1997). Microbial community destabilization can promote disease in hosts through disturbance of the community's various metabolic pathways and subsequent microbe-microbe competition through secondary metabolites (Isaac 1992, Atlas and Bartha 1997, Marsh 2003), promoting the proliferation of virulence factors (Marsh 2003, Liu *et al.* 2012). Community destabilization may be prompted by abiotic or biotic factors that cause changes in the proportional abundance of populations within a community when diversity is low (Atlas and Bartha 1997). For instance, some foliar fungal endophytes are latent pathogens that are stimulated to proliferate when their host is affected by drought or cold-stress (Stone, Sherwood and Carroll 1996; Hamelin 2006; Rodriguez *et al.* 2008).

Multivariate measures of community structure may vary across environmental variables even when univariate community diversities or richness appear stable (Anderson and Walsh 2013, Gotelli and Ellison 2013). The variance-covariance matrix of species interactions summarized by community structure provides a tool for detecting variation in the presence and abundance of communal populations (Gotelli and Ellison 2013). Dissimilarity indices, describing differences among group resemblance-measure centroids, may be used to detect and describe important environmental effects on community assemblage (Anderson and Walsh 2013).

The *hazard rating* concept from forest pathology may be applied to investigations of foliar endophyte community dynamics across biophysical gradients to better understand endophyte-ecology. Approaching the study of endophyte communities within the context of a foliar fungal pathosystem allows investigation of whether hazard ratings, already known to predict the incidence of certain foliar fungi, also impact endophytic community dynamics (Van Arsdel, Geils and Zambino 2006). This approach addresses whether endophyte community dynamics relate to host-plant health, as evidence suggests (Arnold 2003; Ganley, Sniezko and Newcombe 2008).

Few pathosystems that affect forests on a continental scale are as extensively described as the white pine blister rust disease. Severe infection of trees by the white pine blister rust fungus ultimately precludes seed production and is the predominant cause of mortality of North American white pines (subgenus *Strobus*). *Cronartium ribicola*, the causal agent of white pine blister rust, is a basidiomycetous biotroph of white pines and plants in the family Rosaceae (genus *Ribes*) and Orobanchaceae (genus *Pedicularis* and *Castilleja*) (Kearns and Jacobi 2007, Zambino 2010). The destructive effects of *C. ribicola* on white pines was discovered shortly after the fungus was inadvertently introduced to western North America from Europe around 1910, and have led to a half-century of intensive yet ineffective management efforts to stop its spread (Schumann and D'Arcy 2006). Accordingly, the diverse abiotic environmental requirements of all five stages of the *C. ribicola* life-cycle, on both alternate and primary obligate hosts, are well-characterized, as

are its complex patterns of wind-blown transport and temporal infectious cycles (Van Arsdel, Geils and Zambino 2006; Frank *et al.* 2008b).

Implicit in the concept of *hazard rating* is the epidemiological concept of a disease triangle consisting of a host, its pathogen and the environment in which they co-occur (Schumann and D'Arcy 2006). In particular, climatological factors (Van Arsdel, Geils and Zambino 2006; Frank *et al.* 2008b) and host genetics (Whitham *et al*. 2003, Smith and Goodman 2009, Richardson *et al*. 2010, Lamit *et al*. 2015) impose strong constraints on fungal distribution and pathogenicity. Although varying levels of genetic resistance occur in white pines, trees are much more likely to elude infection by *C. ribicola* because of the microclimate they occupy than because of their genetic resistance (Van Arsdel 1972). The durations of exposure to various temperatures required for the three infectious stages of *C. ribicola* are critical factors in predicting infection, as are the macro and microclimatic features that precipitate these conditions on natural landscapes (Van Arsdel, Geils and Zambino 2006). Site-level features may generate favorable temperatures and the formation of dew on white pine needles that favor the germination of *C. ribicola* basidiospores required for host infection (Van Arsdel 1965a & 1972). For example, small openings in forest canopies lack convective insulation, and create nighttime and morning conditions wherein needles are cooler than the surrounding air, causing moisture to condense on the needle surface. In addition, small canopy openings (diameter < ½ the height of surrounding trees) do not receive the higher levels of insolation of larger openings, which could kill light-sensitive basidiospores within ~5 hrs (Van Arsdel 1972). As a result, tree needles in smaller canopy

openings do not reach temperatures that are lethal to foliar mycelia (>35^{0}C), and are not prohibitively cold at night, allowing mycelia to grow and develop spore-bearing structures (Van Arsdel 1972; Van Arsdel, Geils and Zambino 2006). Recent empirical studies have reported a strong positive relationship between white pine blister rust infection frequency and tree bole diameter, while summer precipitation and elevation have been positively and negatively correlated to infection frequency, respectively (Smith and Hoffman 2001, Kearns and Jacobi 2007).

Whitebark pine (*Pinus albicaulis* Engelm.) is one of nine North American white-pine species, and is the most susceptible member of *Strobus* to severe infection by *C. ribicola* (Tomback, Arno and Keane 2001; Tomback and Achuff 2010; Geils, Hummer and Hunt 2010). *P. albicaulis* populations occur at the highest latitudes and elevations of all North American white pine species populations, and exhibit high infection and mortality rates due to *C. ribicola* (Schwandt 2006, Kearns and Jacobi 2007). An average of 52% of whitebark pines surveyed in 1998 along the Pacific Crest Trail just north of Crater Lake National Park, U.S.A. were infected with white pine blister rust, while a survey of whitebark pines within park boundaries in 2000 found an average of 8% infection across the park (Murray and Rasmussen 2000 & 2003). White pine blister rust is found throughout the entire range of whitebark pine, and infection rates of 70-100% are common in populations occupying the highest latitudes at which the species occurs (Tomback, Arno and Keane 2001; Kendall 2010).

The primary seed-disperser of whitebark pine is Clark's nutcracker (Corvidae: *Nucifraga columbiana*). Clark's nutcracker preferentially caches seeds on

unoccupied, recently burned sites (Tomback and Achuff 2010). The random and wide-ranging (up to 20 km) caching behavior of Clark's nutcracker results in a high level of genetic heterogeneity within populations of whitebark pine (Richardson *et al.* 2010). As a seral species on harsh, bare mountain slopes, *P. albicaulis* colonization results in changes in edaphic properties essential for plant community succession (Tomback and Achuff 2010). Additionally, *P. albicaulis* is integral to high-altitude ecosystems by moderating the rate of snowmelt, providing cover for understory flora and nutrients for myriad alpine animals, and supporting rich communities of arboreal lichens (Tomback, Arno & Keane 2001; Murray and Rasmussen 2003).

In this study, we studied macroscopic and endosymbiotic features of whitebark pine forests in the southern Cascades to address three questions:

1) Do environmental predictors of white pine blister rust infection affect the structure of native endophytic fungal communities?
2) Do endophytic fungal community assemblages vary between north and south-facing mountain slopes?
3) Does endophyte community structure or composition differ between trees with and without blister rust cankers, and are similar patterns observed between trees with and without *inactive* cankers?

Methods

Field Site, Plot & Sample Selection

Four study sites were selected in the southern Cascades range of Oregon, USA based on four criteria: 1) site elevation had to be ≥ 2500m, 2) sites had to occupy a distinct latitude but a similar longitudinal position, 3) whitebark pines had to be present on both north-facing and south-facing slopes, and 4) sites had to be ≤ a one-day hike from a freezer for tissue-storage. Listed from south to north, sites were: 1) the north end of Munson Ridge at Crater Lake National Park (42.905^{o}N/122.138^{o}W), 2) Mt. Bailey (43.154^{o}N/122.219^{o}W), 3) Tipsoo Peak (43.219^{o}N/122.035^{o}W), and Cowhorn Mtn. (43.399^{o}N/122.054^{o}W). Sites 2, 3 & 4 are mountainous locations in the Umpqua National Forest (sites 2 and 3) and the Deschutes National Forest (site 4; see map insert). Sixteen 50 m. × 50 m. plots were randomly established and sampled at each mountain-site (sites 2, 3 & 4). Half of the plots at each site were selected from predominantly north-facing slopes and half from predominantly south-facing slopes. Each plot was ≥ 100 m from the nearest trail, and was selected randomly from a larger group of designated plots spanning the breadth of each site aspect along the slope contour using a handheld random number generator. All size-classes of whitebark pines were counted in each plot, and one eighth of the trees present were sampled using a random number generator and random walk procedure as follows. Trees were counted as plots were crossed laterally in transects 5 meters wide. Trees were sampled if the sequence in which they were encountered matched a randomly generated number. Each plot was sampled in this manner starting at the uphill plot dimension and ending at the

downhill dimension. The Crater Lake site was not sampled as heavily as the mountainous sites (n=13, vs. n≥29 for sites 2-4) and only one 50 m. × 50 m. plot was established per aspect there, where trees were selected via a random walk procedure as above.

Field Measurements

Measurements recorded for each selected tree included demographic measures (i.e., tree height using a clinometer, or length using measuring tape for prostrate or krummholz forms, diameter at breast height for trees ≥ 1.37m in length, estimate of percent living, visible disturbances), environmental metrics [estimated slope position (toe, mid, shoulder, top), adjusted degrees from north (magnetic aspect ranging from 0-180^{o}), degree of slope using a Brunton® compass, estimated percent needle litter cover in understory, microsite features (concave, convex or level ground within tree dripline), and estimated proportion of tree canopy directly overtopped by over-story foliage (0-25%, open; 26-75%, broken; 76-100%, closed canopy)], infection symptoms (presence/absence of active and apparently inactive cankers, number of cankers, size of most severe canker), and latitudinal and longitudinal coordinates (Trimble® GeoXH™ handheld GPS device). Cankers displaying aecial sacs, sap discharge, orange discoloration accompanied by fusiform swelling, or recent canker removal by chewing activity were defined as active (Kearns and Jacobi 2007). Inactive, dead cankers were identified by the presence of darkened swollen bark-eruptions lacking aecial sacs and sap discharge, and signs of aged girdling (Hoff 1986). Only trees bearing live needles were sampled and

recorded. Asymptomatic needle fascicles, each typically containing 5 needles, were collected from evenly-spaced regions of the lower canopy of each tree following Ganley and Newcombe (2006). The number of fascicles collected per sampled tree was determined by tree length (or height). Specifically, four fascicles were harvested from trees 0-3 m tall, five fascicles from 3-6 m tall trees, and six fascicles from >6 m tall trees. All fascicles from one sampled tree were grouped in a single plastic bag and stored in the shade prior to freezing at -20^{0} C within 60 hours of collection.

Alternate host presence (*Ribes* spp. and *Castilleja* spp.) was recorded at each plot by calculating a random azimuth at plot-center and following that course for 50 meters. Host species and abundance were recorded along each 50 m transect. GPS coordinates (UTMs) were recorded for the host nearest to plot-center.

Surface Sterilization and DNA Extraction

Sterilization procedures followed Deng *et al.* (2011). Briefly, needles were immersed and stirred for 1 min. in sterile DI water to remove loosely-adhering dust and epiphytes that might not contribute to the endophyte consortia. Needles were then immersed and stirred for 1 min. in EtOH, followed by 3 min. in NaOCl (4% available Cl), and 30 sec. in EtOH and 30 sec. in sterile DI water to remove salts. Samples were then air-dried in a sterile flow-hood. Dried needles were wrapped in sterile paper towels, placed in a plastic bag, and stored at -20^{0} C until DNA extraction.

Total genomic DNA was extracted from pine needle tissue by first grinding the full mass of each sample to a homogenous powder over four rounds of grinding under liquid nitrogen using a sterilized mortar and pestle. Samples were further homogenized by stirring with a sterile spatula (Lindahl *et al.* 2013). For each sample, 50 mg of needle tissue was added to a bead-tube provided in the MoBio PowerPlant® Pro kit (Carslbad, CA, USA). The MoBio phenolic separation solution was used, and two rounds of bead-beating were applied for two minutes each, punctuated by two minutes on ice. Extracted DNA concentrations were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific™).

PCR Amplification

Extracted genomic DNA was amplified following a nested PCR approach using two different combinations of primers specific to the ITS region of fungal rDNA. The internal primer set was chosen to selectively amplify PCR products within the gene target of the initial, external primers. The external and internal primer pairs included the ITS1-F forward primer originally developed by Gardes and Bruns (1993) to detect rust and mycorrhizal fungi. ITS4 was used as the external reverse primer and ITS2 was used as the internal reverse primer, both of which were developed by White *et al.* (1990). ITS1-F and ITS2 primers contained Illumina® MiSeq® overhanging adapters, and an additional guanine for T_m optimization of the ITS1-F primer, to generate the following working primers: **ITS1-F**: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATGTTAGAGGAAGTAA-3',

ITS2: 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC-3'.

Touchdown annealing was used in the nested reaction to amplify a more broad range of taxa than might be obtained by a constant annealing temperature (Schmidt *et al.* 2013). The use of hierarchical gene-targeting methods, employed here, also enhances primer-specificity when target DNA templates are contaminated with plant DNA (Ekman 1999, Hamelin 2006). Hot-start *taq* was used in the nested PCR to further enhance primer specificity for amplification of fungal DNA, and to increase PCR sensitivity and efficiency (Ekman 1999). Internal amplicons were generated directly after completion of the external PCR, and nested PCR products were used for all downstream applications directly after internal amplification. The external PCR reaction solution contained 12.5 uL of 2x Dream Taq™ DNA polymerase (Thermo Scientific™), 25 uM ITS1-F primer + adapter, 25 uM ITS4 primer, 10 ng template DNA, and molecular-grade nanopure H_2O to 25 uL. The external thermocycle included an initial denaturation step of 85 sec. at 95^{O}C; 10 cycles of: 35 sec. at 95^{O}C, 55 sec. at 57.5^{O}C, 45 sec. at 72^{O}C; 10 cycles of: 35 sec. at 95^{O}C, 55 sec. at 57.5^{O}C, 120 sec. at 72^{O}C; and a final 72^{O}C elongation step of 10 min.

The internal (nested) PCR reaction solution consisted of 5 u/uL HotStarTaq (Qiagen©), 2.5 uM ITS1-F primer + adapter, 2.5 uM ITS2 primer + adapter, 25 mM $MgCl_2$ (Qiagen©), 10 mM dNTPs (Qiagen©), 2.5 uL of 10x PCR buffer (Qiagen©), 10% diluted external PCR product, and nanopure H_2O to 25 mL. The internal thermocycle included an initial hot-start *Taq* activation step of 15 min at 95^{O}C followed by an additional 3 min. of denaturation at 94^{O}C; 13 *touchdown* cycles of: 60 sec. at 94^{O}C,

60 sec. starting at 67^{O}C and decreasing 1^{O}C over each of the final 12 steps of the *touchdown* cycle, 120 sec. at 72^{O}C; 12 cycles of: 60 sec. at 94^{O}C, 60 sec. at 55^{O}C, 120 sec. at 72^{O}C; and a final elongation of 10 min. at 72^{O}C.

Diagnostic Cloning & Pyrosequencing

We confirmed the efficacy of our PCR protocol by isolating and identifying an individual sequence from mixed-template genomic DNA extracted from whitebark pine needles. Internal PCR products 300 bp in length were gel-extracted from agarose with the Gel/PCR DNA Fragment Extraction Kit from PHENIX© Research Products (USA). Overhanging-ended (TA) cloning was conducted on purified gel extracts with the pGEM®-T Easy Vector System I kit from Promega© (USA) and competent *E. coli* cells courtesy of the Cretekos Lab (Idaho State University) following the pGEM®-T protocol (Promega©, USA). Blue/white screening of incubated cells aided the selection of successfully transformed cell colonies. The MoBio Ultraclean® Standard Mini Plasmid Prep Kit was used to isolate bacterial plasmids containing inserts. Sanger dideoxy sequencing of isolated plasmids was performed at the Molecular Research Core Facility, Idaho State University, to evaluate the specificity of PCR primers, chemistry and thermocycle protocols prior to parallel pyrosequencing.

Megablast was used to align the isolate to a putative taxonomic match in the NCBI database (blast.ncbi.nlm.nih.gov/Blast.cgi) The isolate matched an uncultured clone of *Phialocephala* sp. with 100% similarity and a highly significant expectation

(E-value) of $2e^{-109}$. This strengthened our conviction in the homology of the putative taxon and the cloned isolate, and confirmed the adequacy of our PCR method.

Directly following the nested reaction, PCR amplicons were delivered to the Molecular Research Core Facility (MRCF) at Idaho State University (Pocatello, ID, USA), where amplicons were cleaned with the Agencourt AMPure XP-PCR Purification kit (Beckman Coulter®) and barcoded per tree-sample using the Nextera® XT DNA indexing kit (Illumina®). High-throughput barcoded amplicon pyrosequencing was conducted using the Illumina MiSeq® (V3-600 cycle) to generate libraries of 250-300 nt paired-end reads.

Bioinformatics

Reads were de-multiplexed by MRCF personnel using software from Illumina®. All further sequence processing was performed in *mothur* (v.1.35.1; Schloss *et al*. 2009) loosely following the protocol described by the MiSeq® SOP (accessed 04/2015, see Kozich *et al.* 2013), Bell *et al.* (2014) and McFrederick, Mueller and James (2014). Briefly, bi-directional reads were assembled into contigs using the consensus screening quality-control described by Kozich *et al.* (2013). Any reads that failed to join were discarded. Contigs not meeting the following three criteria were also discarded: 1) minimum length of 225nt, 2) no ambiguous bases, 3) ≤ 8 homopolymers. See additional procedural details in the supplied bioinformatics workflow.

Contig length has been shown to impact the results obtained from molecular studies of microbial ecology (Porter and Golding 2011; Monard, Gantner and Stenlid

2012).The average contig length prior to standardizing was 265 nt. Contig length was limited to 225nt based on empirical analyses of the effect of sequence length on the number of unique sequences identified. Our 225nt heuristic yielded 21.5% less unique sequences than a length of 200nt, but 72% more uniques than 250nt contigs. Our approach also draws from empirical studies that have reported average ITS1 region lengths for Eumycota between 200 and 250nt (Porter and Golding 2011, Toju *et al.* 2013).

Contigs were then pre-clustered following Huse *et al*. (2010), screened for chimeras using the *de novo* UCHIME detection algorithm (Edgar 2010), normalized to the smallest sample-library, classified against the published *dynamic* UNITE database formatted for *mothur* (v. 7), and clustered to OTUs at ≥ 97% sequence similarity using the Needleman-Wunsch algorithm (Kunin *et al.* 2010, Smith and Peay 2014, Talbot *et al.* 2014). A representative sequence from each classifiable OTU cluster was assigned to a putative taxonomic rank, with a minimum threshold of 80% confidence for species-level assignments. Eighty sequences were chosen at random and cross-checked against the NCBI database using the megablast Basic Local Assignment Search Tool.

Statistical Analyses

We used both multivariate and univariate analyses to investigate associations of environmental and host-demographic variables to endophyte community structure, Shannon-Weiner diversity and richness. Subsequent references to Shannon-Weiner community diversity are stated as "diversity." To

allow multivariate tests and summaries we collapsed the molecular community matrix into multivariate Bray-Curtis dissimilarities (Bray and Curtis 1957), which served as a multivariate response variable. We tested for community structural changes with respect to predictors with permutational multivariate analyses of variance (PERMANOVA; Anderson 2005), using the function *adonis* from the **R**-package *vegan* (Oksanen *et al.* 2015). *P*-values were calculated using 1000 permutations of predictor vectors. We constructed PERMANOVA models to approximate a type II SS approach, in which the sequence of predictors in the model does not impact the outcome of a test, unlike type I SS (Aho 2013). Specifically, reference models were created that included all predictors, but only the *F* statistic and *p*-value were recorded for the last predictor in a given model. Distinct models were generated for each predictor with the predictor of interest placed last in each model. The 14 predictors are listed in Table 1.

Non-metric multidimensional scaling (NMDS; Kruskal 1964) was used to express the site by species matrix in ordination space using Bray-Curtis dissimilarities as resemblance scores. Bray-Curtis dissimilarity was calculated as the lowest score obtained twice from 20 random starting configurations. An ordination with sufficiently small Kruskal stress was obtained with a 3-dimensional solution (stress= 0.223). Procrustes rotations comparing the NMDS solution to PCoA scores verified the robustness of our NMDS model (corr = 0.577, $p = 0.001$, 1000 permutations). Vector fitting was used to fit predictor variables to community locations in ordination space using the multiple regression function *envfit* from the **R** package *vegan* (Oksanen *et al.* 2015). *envfit* was used to calculate univariate

coefficients of determination for each environmental and host-related predictor, one predictor at a time. Ordination scores for each dimension of communities in species-space were used as predictors of variation in environmental or host-related variables in a multiple regression model.

Univariate general linear models were used to test for the individual effects of predictors on univariate summaries of diversity and observed molecular species richness. We performed indicator species analyses (Dufrene and Legendre 1997) using the **R** function *indval* from the package *labdsv* (Roberts 2007) to explore species-level differences in fungal communities between 1) trees with and without *active* blister rust cankers, 2) trees with and without *inactive* cankers, and 3) trees on predominantly north-facing slopes versus those on south-facing slopes. Indicator species analysis *p*-values were calculated by comparing observed indicator values (Dufrene and Legendre 1997) of species to groups, and indicator values obtained after randomly assigning host trees to defined groups 1000 times (Roberts 2007).

Results

Sequence Library Summary

Almost half of the 80 randomly-sampled sequences searched with megablast (NCBI) were found to match the second and third most abundant sequences classified at the level of species using *mothur*. *Lophophacidium dooksii* sequences were 91% similar ($E = 3e^{-61}$) to *L. dooksii* reads in NCBI, and *Lachnellula calyciformis* was 99% similar ($E = 4e^{-109}$). Another highly abundant representative of the full project library, *Phaeomoniella* sp., was only classifiable to the level of genus using

the UNITE database via *mothur,* but was assigned to a species in NCBI with 96% similarity and high confidence ($E = 4e^{-99}$).

Sequence filtering and screening retained 288,414 sequences that grouped into 2306 OTUs, 283 taxonomic species within 212 genera, 119 families, 61 orders, 18 classes and four fungal phyla. As expected, Ascomycota represented the most dominant phylum (96.2%), followed by Basidiomycota (3.79%), Chytridiomycota (<0.01) and Zygomycota (<0.001).

Gymnosperm foliage is typically dominated by fungi in the class Leotiomycetes, which concurred with our findings (Sieber 2007). All other classes were represented by a small fraction of the abundance of the Leotiomycetes, as illustrated in Figure 1. Leotiales, previously Helotiales, appear as the most abundant order, followed by Rhytismatales (Fig. 2). These orders contain many plant-associated, saprotrophic fungal taxa, and also many important plant pathogens (Hou, Piepenbring and Oberwinkler 2004; Wang *et al*. 2006).

The four most abundant sequences classifiable to the level of genus belong to orders Rhytismatales, Leotiales and Chaetothyriales, and are described here to illustrate the broad functional diversity of fungi detected in needles of whitebark pine. *Coccomyces dentatus* was the most abundant sequence classifiable to the species-level, accounting for 13.1% of the total sequence library, and is a cosmopolitan Rhytismataceous saprobe mostly of angiosperm leaf litter (EOL 2015). *Lophophacidium dooksii*, the Rhytismataceous causal agent of Dooks needle blight in eastern North America, was the second most abundant species at 10.4% of sequences (megablast= 91% similar, $3e^{-61}$), (McLaughlin *et al.* 2012). The third most

abundant sequence aligned to *Lachnellula calyciformis* (Hyaloscyphaceae), which most often occurs as a saprobe of dead woody tissue on pines, but is also a noted necrotroph of *Pinus contorta* in Europe, and white pines *P. pumila* and *P. strobus* and *Abies* spp. in Japan (Minter 2005). *Phaeomoniella* sp., within family Herpotrichiellaceae, is the fourth most abundant read (8.9% of total reads) and was classifiable to the genus level. *Phaeomoniella* sp. was recently discovered and described as an epiphyte on pine needles in Korea (Lee 2006).

Environment and Host Effects

Table 1 shows the relationship of all predictor variables to endophyte community structure, and a subset of those tested for correlation to community diversity and richness. After controlling for spatial, physiographic and biotic influences, multivariate endophyte community structure was found to vary significantly with host-tree height ($F_{1,88} = 2.52$, $p = 0.003$, $r^2_{partial} = 0.028$), elevation ($F_{1,88} = 1.79$, $p = 0.015$, $r^2_{partial} = 0.02$) and degrees from north of host-tree occurrence ($F_{1,88} = 1.72$, $p = 0.031$, $r^2_{partial} = 0.019$). Presence or absence of visibly inactive cankers, a form of genetic resistance to white pine blister rust, explained 24% of variation in endophyte community structure ($F_{1,88} = 1.38$, $p = 0.118$).

The prominence of the prostrate *krummholz* growth habit in whitebark pines in general, and frequency of prostrate growth at our study sites in particular, warranted the measurement of height as a size and age-related variable instead of diameter at breast height (dbh). Larger (longer, taller and wider) trees tend to support more foliage and greater crown radii, providing greater surface areas for

windblown spores accumulation (Smith and Hoffman 2001), implying that tree height and dbh represent both host developmental and areal variables in this study. We were interested in the effects of this variable on endophyte community structure because multiple studies have reported a strong positive correlation between dbh and infection of high-elevation white pines by *C. ribicola* (Smith and Hoffman 2001, Kearns and Jacobi 2007). Dbh and tree height were significantly correlated (corr = 0.58, $t_{1,107} = 7.36$, $p < 0.001$). However, even when tested independently dbh did not explain variation in community structure significantly ($F_{1,107} = 1.33$, $p > 0.1$, r^2 =0.012). We found a significant correlation between tree height and crown radius (corr = 0.56, $t_{1,107} = 6.91$, $p < 0.001$), but only a weak effect of crown radius as an independent predictor of community structure ($F_{1,107} = 1.34$, $p > 0.1$). A weak positive relationship was found between tree height and endophyte community diversity ($F_{1,107} = 5.17$, $p = 0.025$, $R^2 = 0.0461$), but not for community richness ($F_{1,107}$ = 0.43, $p > 0.5$, $R^2 \leq 0.01$). Elevation has been noted as a significant negatively-correlated predictor of site hazard for white pine blister rust (Smith and Hoffman 2001, Kearns and Jacobi 2007). Univariate community diversity and richness did not change with elevation ($p \geq 0.3$, $R^2 \leq 0.01$).

Leaf senescence triggers some foliar endophytes to decay host tissue (Carroll 1988; Stone, Sherwood and Carroll 1996). We speculate that a larger population of latent foliar saprophytes in leaf tissue would likely result in more decaying needle litter beneath host trees. More importantly, needle litter in the organic soil-horizon may be expected to reduce evaporation of soil-water, and increase the extent of plant-available water. We tested this effect on endophyte community structure

while controlling for all other variables (Table 1). In this analysis, however, percent needle-litter cover did not explain an appreciable proportion of variation in community structure ($F_{1,88} = 1.16$, $p = 0.242$, $r^2_{\text{partial}} = 0.013$).

C. ribicola basidiospores that infect pine are wind-disseminated from alternate hosts up to several kilometers away (Van Arsdel, Geils & Zambino 2006). Additionally, all nonclavicipitaceous endophytes are thought to be transmitted horizontally by wind, and in some cases, insects (Rodriguez *et al*. 2008). Strong east-west winds at our study sites (WRCC 2015) prompted the establishment of sites at different latitudes to avoid spatial autocorrelation of endophyte communities with windblown *C. ribicola* basidiospores and spores of non-pathogenic fungi. The north-most site (Cowhorn Mtn.) is >30 latitudinal miles north of the south-most site (Crater Lake National Park), and the minimum latitudinal distance between sites is >4.5 miles. Controlling for all other variables, latitude accounted for only 1% of the variation in endophytic community structure ($F_{1,88} = 0.911$, $p = 0.56$).

Variables relating significantly to variation in endophyte community structure are displayed as vectors overlain on the three-dimensional NMDS ordination shown in Figure 4. Each point represents the trivariate NMDS community scores for one tree. Points are colored by mountain and positioned relative to their Bray-Curtis community dissimilarity to other points in NMDS ordination-space. Vector lengths illustrate the explanatory power of variables related to endophyte community structure (as multiple-coefficients of determination), whereas arrow direction indicates the trend of most rapid predictor change across endophyte communities (Fig. 3). To enhance clarity only variables with partial $r^2 \geq 0.10$ are

shown. Orthogonal effects on community structure are shown for the two strongest predictors of community structure, tree height and elevation.

Water-Limiting Physiographic Effects

At intermediate latitudes in the northern hemisphere, north-facing mountain slopes receive less direct shortwave radiation from the sun, are often less water-limited, and have biologically-driven soils that are deeper and more nutrient-rich than soils on south-facing slopes (Aho and Weaver 2010; Aho, Weaver & Eversman 2014). Aspect values (degrees) were adjusted to represent the degrees-from-north for a given site position. As a quantitative predictor, degrees-from-north related significantly to variation in community structure ($F_{1,88} = 1.72$, $p = 0.031$, $r^2_{partial} = 0.019$), but not diversity or richness ($p \geq 0.6$, $R^2 \leq 0.01$).

Indicator species analyses detected significant taxonomic differences between endophyte communities from trees on north vs. south-facing slopes. Compositional differences between communities on north and south slopes occurred up to the taxonomic level of Class (Table 2). The lack of knowledge available on the function of endophytes in conifers precludes interpretation of the observed patterns, though it is interesting to note that while community richness did not differ significantly between north and south-slope communities, Table 2 shows that many taxa, including multiple groups in the highly diverse Leotiales order, are significantly more abundant in trees on north-facing slopes. The only two lower-level taxa that were significantly more abundant in south-aspect trees are associated with plant disease. *Collophora hispanica* is a facultative necrotroph

recently discovered in woody tissue of diseased almond trees (Granaje *et al*. 2012), and *Phlogicylindrium* sp. has been collected from lesions on living eucalypt leaves in association with *Mycosphaerella* spp. (Crous *et al.* 2011).

White Pine Blister Rust Symptoms and Endophyte Communities

Trees with and without cankers supported statistically indistinguishable endophyte communities ($p > 0.48$, $r^2 = 0.012$). Nonetheless, indicator species analyses found three species differentially abundant in trees with active white pine blister rust cankers (Table 3). *Phialocephala virens* has been isolated from periderm tissue of living conifers, and has not been previously reported in association with plant disease as far as we are aware (Kowalski and Kehr 1995). *Schismatomma dirinellum* is a lichenized Ascomycete not known to be associated with plant disease. The only conspicuous associate of diseased whitebark pines is *Lachnellula calyciformis*, which was noted above as a necrotroph of *Pinus contorta* in Europe; and in Japan - *Pinus pumila*, *P. strobus*, and *Abies* spp. (Minter 2005).

Polygenic (quantitative) resistance against *C. ribicola* is fairly common in *Strobus* pines, including whitebark pine (Hoff 1986, Richardson *et al*. 2008). Genotypes with polygenic resistance may express a range of phenotypes that mitigate the effects of white pine blister rust, the most effective being a combination of slow-growing cankers and necrotic bark reactions that form a wound-periderm around *C. ribicola* mycelia growing in pine tissue (Hoff 1986, Hunt 2009). Bark reactions may starve-off mycelia and kill the fungus before cankers form, or they

may occur after cankers have developed and either cease or slow canker growth (Hoff 1986).

Evidence for the community phenotype hypothesis (Whitham *et al.* 2003) and endophyte-induced plant-pathogen resistance (Arnold 2003) shows fungal endophytes to: 1) vary by host genotype (Whitham et al. 2003, Lamit *et al.* 2015), and 2) confer resistance against pathogens to host plants (Ganley, Sniezko and Newcombe 2008; Eyles *et al*. 2009). The genetic heterogeneity of whitebark pine populations resulting from seed-caching behavior of Clark's nutcrackers ensures that any genetic resistance present in trees sampled in this study is highly randomized within and among sites. Presence or absence of inactive cankers was also not significantly correlated to any environmental or tree-demographic variables recorded. It is thus compelling to interpret correlations between canker inactivity and endophyte community structure as driven by tree genetics, ontogeny, and/or position on the landscape.

Table 4 shows one taxonomic family and four species that occurred in significantly greater abundance in trees with inactive cankers than in those without inactive cankers. One species was classifiable at only the Order level and another at the Family level. The two identified putative species were *Satchmopsis brasiliensis*, an endophytic species isolated from abscised leaves of *Eucalyptus* sp. in Colombia and Indonesia (Crous *et al.* 2006), and leaves of *Lagerstroemia* sp. in Thailand (Plaingam, Somrithipol and Jones 2003); and *Xenopolyscytalum* sp., isolated from bryophytes in Antarctica (Zhang *et al.* 2013). This analysis identified *Physcia magnussonii*, a lichenized fungus described as having a foliose growth habit, as the

only taxon found in significantly greater abundance in trees *without* inactive cankers (Lohtander *et al.* 2000).

Discussion

This study describes the first known isolation and amplification of environmental rDNA from foliar fungal communities of a high-elevation white pine critically threatened by the white pine blister rust disease. Using barcoded amplicon pyrosequencing, we investigated the effects of environment, host demographics, and symptoms of fungal pathogenesis on fungal endophyte communities of ecologically-important whitebark pine forests in the southern Cascades. Our results suggest that communities and assemblages of whitebark pine endophytes are strongly related to the size and position of host trees on the landscape. No significant differences were found between endophyte communities from trees with and without white pine blister rust cankers, or trees with and without inactive cankers, though both comparisons revealed potential indicator species of these conditions.

We interpret the large proportion of variance in endophyte community structure explained by host height as an ontogenetic effect for three reasons. 1) Needles were sampled only from the lower canopy of each tree ≤ 3 m from the ground regardless of a tree's height; 2) Endophytes of conifers are non-systemic and remain close to their point of host-infection, so we don't expect that our samples represent endophytes that penetrated hosts at other points in the phyllosphere (Rodriguez *et al.* 2008); 3) the radius of tree canopies did not correlate strongly to variation in community structure (Table 1). Together, these points support the view that the

increased surface area of larger, taller trees does not account for the correlation of tree height to endophyte community structure, but instead endophyte community structure changes significantly with host age as a result of community assembly processes (Frankland 1998). Notably, we also found that diversity, but not richness of endophyte communities, correlated to tree height. This indicates that changes in species evenness occur throughout host development that do not translate into changes in species richness. This may be a demonstration of equilibrium processes (MacArthur and Wilson 1963) in which all available endophytic niches are quickly filled, but replacement of individuals occurs as a result of competitive interactions and succession over time (Simberloff 1974).

Few studies have successfully isolated the effects of abiotic environmental gradients on endophyte community dynamics. One study of foliar endophytes in a Hawaiian tree species found a strong correlation between endophyte community structure and precipitation (Zimmerman and Vitousek 2012). White pines on north-facing slopes in the northern Rocky Mountains tend to support higher rates of white pine blister rust infections than trees on south-slopes (Smith *et al*. 2011). Higher infection rates on north-facing slopes likely result from more plant-available water on north vs. south slopes, which promotes the cool, moist conditions required for basidiospore germination and subsequent pine infection. We speculate that this trend is important not only for successful host infection by pathogenic fungi, but also for non-pathogenic infection of plants by myriad horizontally-transmitted fungi. This view was supported up the Class-level by our indicator species analyses of community assemblages across north and south-facing mountain-slopes of four

mountains with distinct aspect-effects. In particular, Table 2 shows that multiple taxa preferentially colonize trees on north-facing slopes of our study sites, while comparatively fewer taxa preferentially colonize trees on south-slopes.

The two leading explanations of endophyte-mediated plant defense are so-called *direct* and *indirect* effects (Arnold *et al.* 2003). Direct effects are exerted on potential pathogens or other secondary colonizers either by antifungal secondary metabolites produced by endophytes previously in the host, or by physical exclusion of additional organisms or resources from occupied plant interiors (*sensu* priority effects; Isaac 1992). Indirect effects are constraints exerted on potential pathogens by host defenses that have been primed by endophytes colonizing asymptomatic host tissues (Arnold 2003). Plant hormonal and anatomical modifications, such as organ abscission, have been related to indirect effects, and such "natural pruning" has been described as a function of some fungal endophytes (Kowalski and Kehr 1995, Bonello 2006). We suspected that host resistance mechanisms, such as bark reactions, might relate to indirect effects – expressed as a relationship between endophytic communities and the presence or absence of cankers on pine hosts. However, we did not find a correlation between community composition and the presence or absence of cankers. We also performed indicator species analyses to determine whether any endophytic species occurred in significantly greater abundance in hosts without *active* cankers, or with *inactivated* cankers. This result would suggest a *direct effect* of endophytes upon pathogenic species that results in mitigating host disease. We found support for this hypothesis through indicator species analyses (Table 3 & 4), though fungal endophytes preferentially colonizing

trees with *inactive* cankers were not found to also preferentially occupy trees lacking cankers.

To date, the only controlled inoculation study-system of endophyte-mediated effects on whitebark pines was implemented at Crater Lake National Park, Oregon, USA in 2009. It is not yet known whether inoculated whitebark seedlings in that study are differentially resistant to *C. ribicola* infection, though a comprehensive taxonomic survey of seedling mycobiomes did not detect the foliar inoculant four years after inoculation (Moler, Reinhardt & Aho – in preparation). Multigenic predisposition to different levels of constitutive defense against *C. ribicola* may be entirely independent of the presence of fungal endophytes (Hoff 1986, Bonello 2006), though we cannot yet say for certain.

Map Insert. Study sites in Oregon, USA: 1) Crater Lake, 2) Mt. Bailey, 3) Tipsoo Peak, 4) Cowhorn Mtn.

Table 1. Variables, statistical tests, and results for effects of environmental and host variables on endophyte community dynamics. Coefficient of determination fields with "community structure" as the response variable are multivariate partial r^2 values, while all others are univariate multiple R^2 values.

Response	Predictor	Test	Test Statistic	*p*-value	r^2 & R^2
community structure	elevation	PERMANOVA	F1,88=1.79	0.015	0.02
community structure	tree height	PERMANOVA	F1,88=2.52	0.003	0.028
community structure	deg. from N	PERMANOVA	F1,88=1.72	0.031	0.019
community structure	overstory class	PERMANOVA	F2,88=1.06	0.361	0.023
community structure	inactive canker presence/absence	PERMANOVA	F1,88=1.38	0.118	0.24
community structure	host presence on plot	PERMANOVA	F2,88=1.07	0.349	0.024
community structure	northing	PERMANOVA	F1,88=0.91060	0.56	0.01
community structure	easting	PERMANOVA	F1,88=0.8033	0.699	0.009
community structure	% duff groundcover	PERMANOVA	F1,88=1.16	0.242	0.013
community structure	slope	PERMANOVA	F1,88=0.63	0.92	0.007
community structure	slope position	PERMANOVA	F3,88=1.0346	0.385	0.034
community structure	canker presence/absence	PERMANOVA	F1,88=0.97420	0.485	0.011
community structure	% of crown living	PERMANOVA	F1,88=1.19	0.245	0.013
community structure	microsite surface	PERMANOVA	F3,88=1.07	0.326	0.035
diversity & richness	inactive canker presence/absence	type II SS ANOVA	-	>> 0.1	≤ 0.01
diversity & richness	canopy cover	type II SS ANOVA	-	>> 0.1	≤ 0.01
diversity	tree height	type II SS regression	F1,107=5.17	0.025	0.0461
richness	tree height	type II SS regression	F1,107=0.43	0.515	≤ 0.01
diversity	elevation	type II SS regression	F1,107=0.3034	0.58	≤ 0.01
diversity	elevation	type II SS regression	F1,107=0.7507	0.39	≤ 0.01
diversity	deg. from N	type II SS regression	F1,107=0.1491	0.7	≤ 0.01
diversity	deg. from N	type II SS regression	F1,107=0.1565	0.6932	≤ 0.01

Table 2. Taxa varying significantly among north and south-facing slopes.

Taxonomic Level	North Aspect	South Aspect
Class	Dothideomycetes (p =0.007)	-
Order	Diaporthales (p =0.016)	Dothideomycetes order *incertae sedis* (p =0.014)
	Dothideales (p =0.026)	Leotiomycetes order *incertae sedis* (p =0.013)
	Leotiales (p =0.05)	Xylariales (p =0.003)
Family	Bulgariaceae (p =0.039)	Leotiomycetes family *incertae sedis* (p =0.018)
	Dothioraceae (p =0.018)	Xylariaceae (p =0.028)
	Helvellaceae (p =0.026)	-
	Hyaloscyphaceae (p =0.001)	-
Genus / Species	*Pseudozyma parantarctica* (p =0.009)	*Collophora hispanica* (p =0.005)
	Gnomoniopsis idaeicola (p =0.025)	*Phlogicylindrium* sp. (p =0.008)
	Helicoma sp. (p =0.032)	-
	Phylloporus purpurellus (p =0.019)	-
	Lachnellula calyciformis (p =0.003)	-
	Monoblepharis hypogyna (p =0.022)	-
	Herpotrichiellaceae sp. (p =0.038)	-
	Dothioraceae sp. (p =0.012)	-

Classes, Orders and Families shown here represent taxa in the sequence library in addition to listed indicators

Table 3. Differentially abundant taxa in trees without white pine blister rust cankers.

Taxonomic Level	Cankers Present	Cankers Absent
Species	*Phialocephala virens* (p =0.033)	-
	Schismatomma dirinellum (p =0.031)	-
	Lachnellula calyciformis (p =0.047)	

Table 4. Taxa varying significantly between trees with and without inactive white pine blister rust cankers.

Taxonomic Level	Inactive Cankers Present	Inactive Cankers Absent
Family	Rutstroemiaceae (p =0.012)	-
Genus / Species	*Helotiales* sp. (p =0.045)	*Physcia magnussonii* (p =0.024)
	Rhytismataceae sp. (p =0.025)	-
	Satchmopsis brasiliensis (p =0.033)	-
	Xenopolyscytalum sp. (p =0.022)	-

Rutstroemiaceae represents taxa in the sequence library in addition to listed indicator species

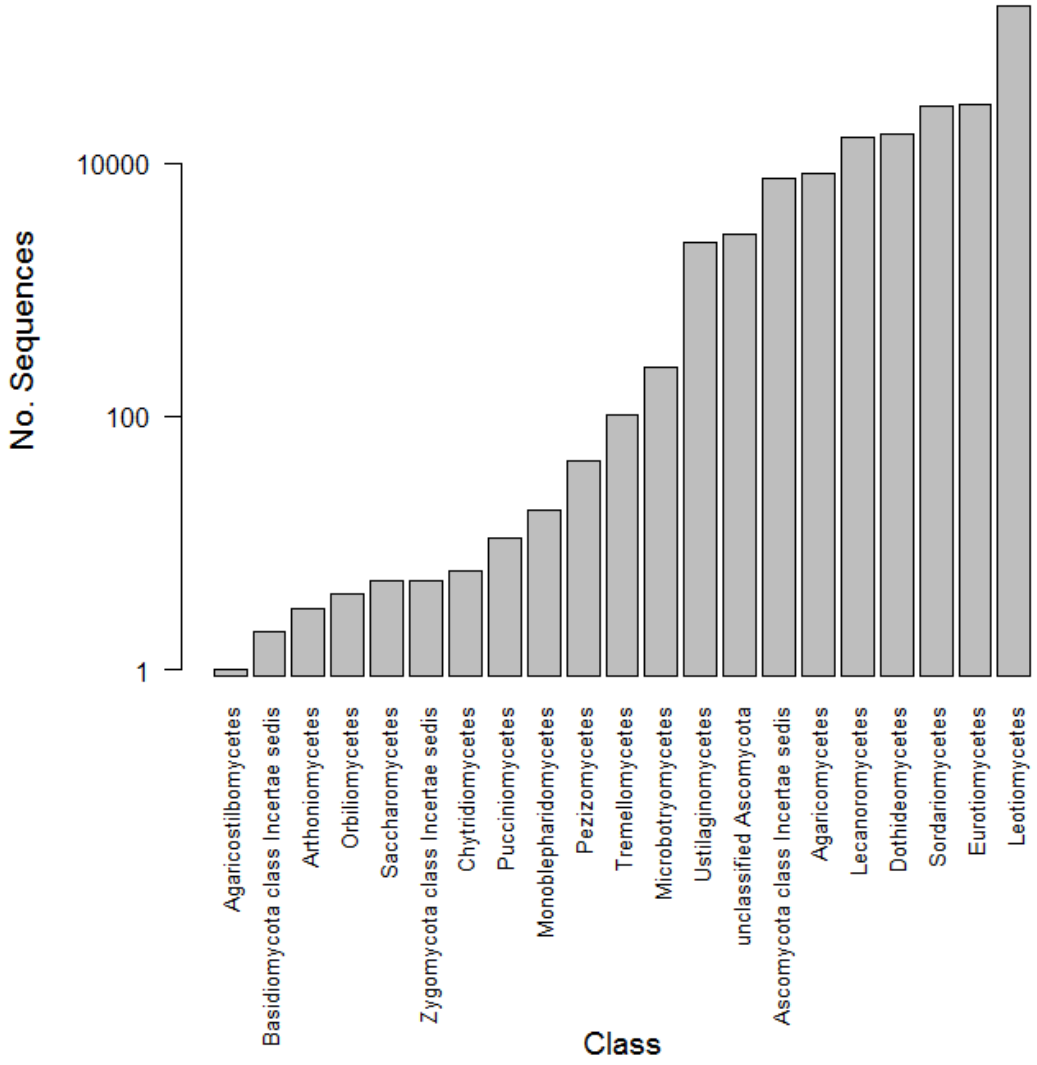

Fig. 1. Log_{10} transformed frequency of sequences aligning to a given fungal taxonomic Class.

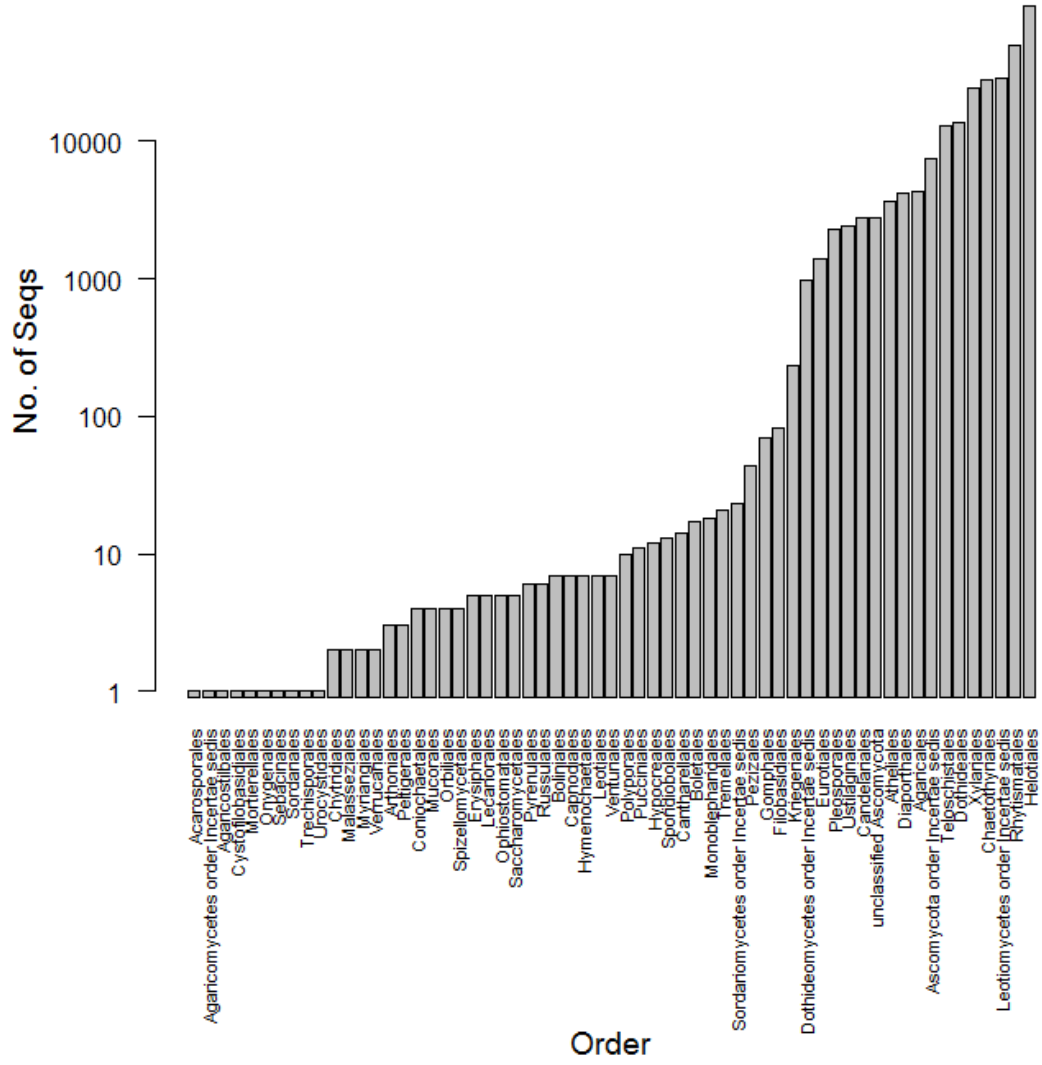

Fig. 2. Log_{10} transformed frequency of sequences aligning to a given fungal taxonomic Order.

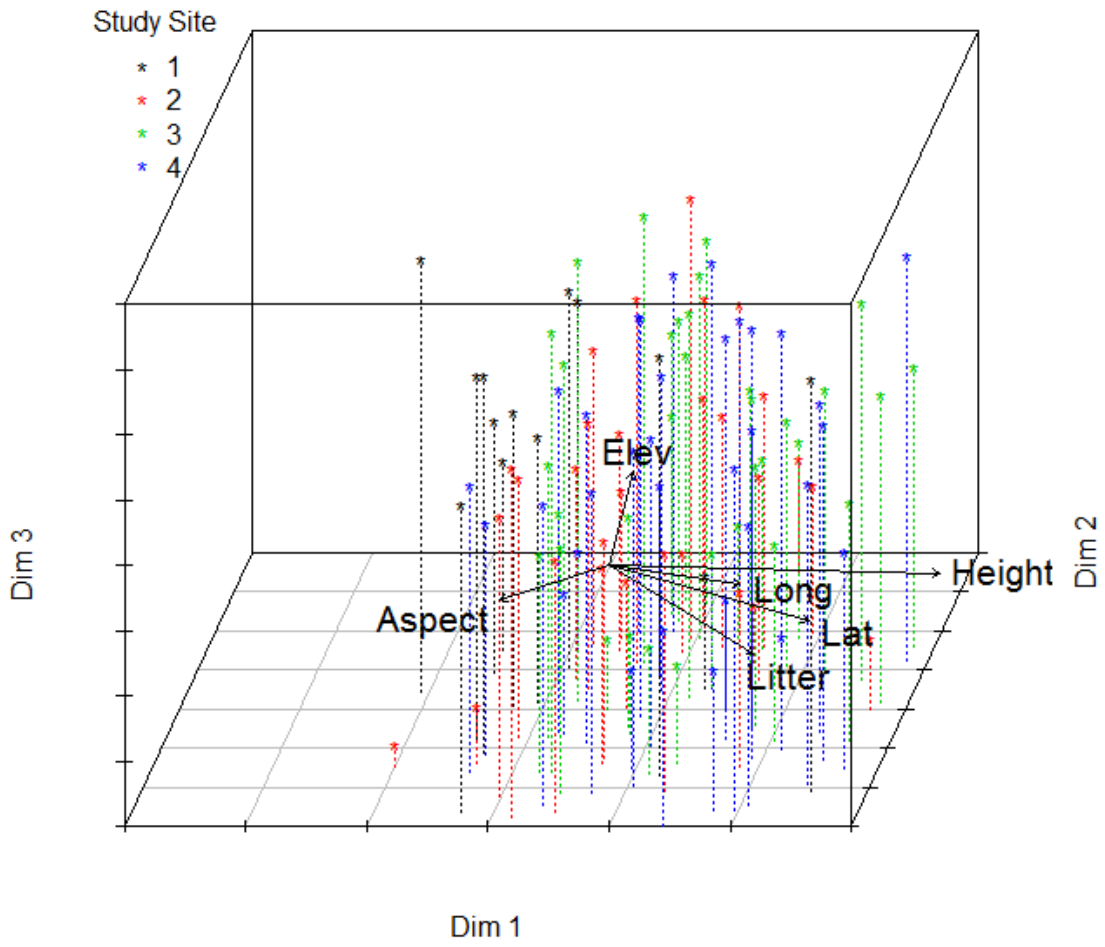

Fig. 3. NMDS ordination of communities in species-space. Endophyte communities are colored according to their study site. Predictive environmental and demographic variables point toward the direction of the greatest gradient change for each given variable.

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Bioinformatics Workflow

- Combine forward and reverse reads one set at a time, remove primer sequences with an 'oligos' file (implemented after each respective contig is made above) via:
    - *trim.seqs* & *make.contigs*
        - this also eliminates any reads that do not contain the forward and reverse primer seqs
- Merge all 96 trimmed contig-libraries into ONE fasta file using *merge.files*
    - operation must be broken into subsets, & subsets merged
- Make group file, which parses each contig-library by sample (tree)
    - operation must be broken into subsets, & subsets merged
- standardize seq lengths using *chop.seqs*
- unique seqs
- screen seqs for minimum length and maximum homopolymers using *screen.seqs*
- populate seqs in name file with seqs in group file to ensure both files contain same seqs using: *list.seqs(name=current)* and *get. seqs(group=225or.good.groups, accnos=current)*
- pre-cluster seqs (works only when seqs are all the same length) using *pre.cluster* with a fasta, group and names file
- chimera identification and removal with: *chimera.uchime* and *remove.seqs*
- subsampling at this point ensures that the same normalized datasets are used for all OTU and taxonomic analyses, and also reduces library size considerably for the clustering step
    - Find largest common no. of reads per sample-library with *count.groups*
    - subsample all libraries to that minimum number of seqs using *sub.sample* with a fasta, name and group file, and persample=true
    - confirm that subsample command operated correctly using *count.groups*
- classify sequences using *classify.seqs*
- Cluster by taxonomic level using *cluster.split* with a fasta, name and taxonomy file, and method=average, taxlevel=4, cutoff=0.3
- make shared file using *make.shared*
- classify OTUs using *classify.otu*