UNEARTHING THE ROLE OF ECTOMYCORRHIZAL FUNGI IN PINE CO-INVASIONS ON MAUI

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Abstract

Pines are one of the most invasive trees in the world, invading with the aid of belowground ectomycorrhizal fungal mutualists. *Pinus radiata* is currently invading multiple parts of the Hawaiian Islands, including near the Haleakalā National Park on the island of Maui. While there are no pines or their associated ectomycorrhizal (EM) fungi that are native to Hawai‘i, previous studies have shown EM fungal species, especially suilloid species, associating with *P. radiata* up to 1000 m away from the original plantations. In order to predict areas on Maui that are susceptible to future pine invasions, we must understand how the distribution of EM fungi, specifically *Suillus* spp., varies across the landscape and how these invasive fungi affect pine seedling success. To do so, a bioassay experiment was performed in which *P. radiata* seeds were grown from soil collected at varying distances from the existing plantation at the Kula Forest Reserve. Pine seedling roots were visually analyzed for percent colonization of EM fungi, weighed, and sequenced for EM fungal community composition using Illumina amplicon sequencing. The community of EM fungi found 2000 m away from the plantation was significantly different than the community within and around the plantation, and largely comprised of *Suillus* spp. The percent colonization of bioassay roots by EM fungi increased with distance from the plantation and increased colonization was positively correlated with increased seedling biomass. With the aid of *Suillus* spp., *P. radiata* appears to have the symbiotic partners needed to aid in the dispersal and survivorship of seedling out into this landscape.

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Introduction

The increasing worldwide prevalence of invasive and introduced animals, plants, and microbial species has brought about numerous ecological concerns. These concerns range from the rapid loss of biodiversity due to competition with invasive species, further degradation of already polluted and climate impacted ecosystems, and negative impacts to ecosystem services, all of which negatively impact local flora and fauna, including humans (Pyšek & Richardson, 2010; Vitousek et al., 1996). Not only are invasive species a source of major ecological harm, but they also generate serious economic costs. In 2011, it was estimated that invasive species cost the United States $219 billion, the top costs being managing crop pests and weeds as well as treating human diseases (Pimentel, 2011). By understanding how and why invasive species spread, as well as how to control and eradicate them, billions of dollars could be saved.

Although much of the attention surrounding invasive species is focused on their environmental impacts and costs such as loss of revenue, invasive species can also impact human health and culture. The impacts of invasive species on humans are numerous, including decreased access to drinking water due to the increased water demands of invasive plants, decreased food yields due to pests and pathogens, and decreases in recreational activities like hiking because of the presence of irritating trail plants (Pejchar & Mooney, 2009). All the many negative impacts of invasive species increase motivations to slow their spread.

Invasive plant species around the world have been introduced both by accident, such as on the shoes of hikers along trails, and on purpose, for use in agriculture or as ornamentals (Foxcroft et al., 2019). The introductions of these non-native plant species have led to significant impacts on plant communities, soil, and fire regimes worldwide (Pyšek et al., 2012). Invasive species are particularly problematic on islands, like Hawai‘i, due to their large number of endemic species. There are roughly 1,039 native plant species in Hawai‘i, with 940 of those found nowhere else in the world (Price & Wagner, 2018). Yet, there are now an estimated 1,652 naturalized plant species in Hawai‘i, meaning over half of the plant species found in the Hawaiian Islands are non-native (Imada, 2019).

When plant species are introduced to a new habitat, there are several important hurdles that must be passed before those species become invasive. These steps include: colonization within their new environment by surviving abiotic conditions, establishment of the species in the

environment by overcoming biotic interactions, and finally, landscape spread by dispersal out into the new environment (Blackburn et al., 2011; Theoharides & Dukes, 2007). Once a species has successfully made it over this final hurdle, control and eradication is much more difficult. Further research that aids in the production of active management strategies is essential for stopping introduced species from becoming invasive and keeping those that already are considered invasive in check for the protection of native ecosystems (Larson et al., 2011).

Pine Invasions and Ectomycorrhizal Fungi

Pine trees (family Pinaceae) are just one of the many invasive plants causing major ecological and economic harm across the world, especially in the sub-tropics and Southern Hemisphere (Nuñez et al., 2017; Richardson et al., 1994). Pines are largely native to the Northern Hemisphere in temperate climates, ranging from North America to Western Asia (Keeley, 2012). Pine plantations are used to produce timber and pulp for construction, furniture, and paper making. To meet demands for timber and tree products, pines were intentionally planted into plantations to increase wood yields. As lumber needs continued to grow in the 19th century, pine plantations began being planted in the Southern Hemisphere to create more local sources of timber (Richardson et al., 1994). After initial planting and establishment, many of these new plantations thrived. As an unintended consequence, many pines have dispersed beyond their initial plantation boundaries and are now invading into native ecosystems throughout the Southern Hemisphere and in parts of the tropics. In fact, Pinaceae is considered the most invasive conifer family on earth (Richardson & Rejmánek, 2004). Large scale pine plantations in places like South Africa, New Zealand, and South America are the sources for pine invasions in these regions. There is often a lag between planting and invasion, but once pines begin entering new habitats, they are difficult to control and eradicate (Simberloff et al., 2010). Invasive pines have led to a variety of negative ecological impacts including decreased overall native plant richness, native plant density, and potentially decreases native arbuscular mycorrhizal fungal richness where they invade (Brewer et al., 2018; Franzese et al., 2017; Gazol et al., 2016).

Although pines are very successful invaders, they require the aid of belowground mutualists, known as ectomycorrhizal (EM) fungi (Nuñez et al., 2009). These fungal symbionts largely belong to the Basidiomycota and Ascomycota and grow within and around the roots of their plant host, promoting the exchange of resources between the two partners. The host plant

provides carbon via photosynthesis in exchange for access to growth limiting soil nutrients, like nitrogen and phosphorus, which the EM fungus has greater physical and chemical access to in the soil than the plant's roots alone (Smith & Read, 2008). In areas like Hawai‘i and South Africa, where pines are currently invading, there are no known native pine associated EM fungi (Hayward & Hynson, 2014; Wood, 2017). In fact, several pine plantations in Africa were not able to establish initially due to the lack of appropriate EM fungi (Richardson et al., 1994). Once soil from areas containing pine associated ectomycorrhizal symbionts was introduced, the plantations were able to establish and pines escape (Richardson et al., 1994).

Plant-EM fungal symbioses are horizontally transferred; meaning, the host and symbionts disperse independently and must come into contact in the environment to form the symbiosis. When the root of a plant host encounters an EM fungal spore or hyphae in the soil, the mycorrhizal symbiosis can form (Smith & Read, 2008). Only 2% of all flowering plants species are considered EM, but most of those species are obligately mycorrhizal, meaning that in order for the plant host or fungus to survive, they must establish this symbiosis (Brundrett & Tedersoo, 2018). Pinaceae is one of these obligately ectomycorrhizal families and for any invasion to occur, this symbiosis must establish (Nuñez et al., 2009).

Pine Co-Invasions

For plants that form obligate symbioses with belowground microorganisms, like pines and their EM fungal symbionts, the ways in which either partner can invade into new environments are intimately tied to the success and dispersal of their symbionts. There are key factors about the host plants, mycorrhizal fungi, and the environment that dictate whether an invasion will occur and be successful, including the range of mycorrhizal partners that a host plant can have (Pringle et al., 2009). Although pines require EM fungi in order to survive and thrive, pines can associate with more than one species of EM fungi, especially within their native ranges (Barroetaveña et al., 2007). Several studies have shown that the EM fungal species associating with pines within and around a forest or plantation differ from those associating with pines away from high density pine environments (Hynson et al., 2013; Urcelay et al., 2017). However, only a single species of EM fungus is needed to enable a pine invasion into a new environment (Hayward, Horton, Pauchard, et al., 2015; Santolamazza-Carbone et al., 2019). Therefore, if there is at least one suitable EM fungal partner, pines have the potential to invade.

Another factor that influences the success of plant-fungal invasions is the quality of the relationship between partners. The amount of resource exchange between partners can vary depending on the species involved and the environmental conditions (Bogar & Peay, 2017). EM fungi that aid in the uptake of nutrients and water at the lowest carbon cost to the plant benefit plant host the most and are considered higher quality partners, and may be preferably selected by the plant host (Dickie, 2007; Rasmussen et al., 2017). However, the strongest competitor between EM fungal species may not always be the highest quality fungal partner for a plant host. For example, in order to test for a potential tradeoff between interspecies competition and nutritional acquisition function, one study measured the amount of extracellular enzymes and defense chemicals produced by three different EM fungal species when inoculated into *P. muricata* alone and together. They found that the weakest ectomycorrhizal competitor, *Suillus pungens*, had the highest enzymatic levels in relation to the other species in the study suggesting that there may be a tradeoff between symbiotic competition and symbiotic function (Moeller & Peay, 2016).

The dispersal capabilities of fungal symbionts are another feature that can greatly determine the invasion potential and invasion rate of the plant host. For EM fungi, dispersal distance can range from centimeters to kilometers away (Horton, 2017). Despite this range of distances, one study found that 95% of basidiospores from six EM fungal species fell within one meter of the basidiocarps, indicating that long distance dispersal events are relatively rare (Galante et al., 2011). For pines to invade a habitat, they need to encounter either EM fungal mycelia or spores, most of which should be found very close to their original EM fungal source. While many spores are wind dispersed, some are dispersed via mycophagy by mammals. In fact, non-native mammals feces in New Zealand have been shown to contain the spores of non-native EM fungi species, likely aiding in the invasion of conifers such as *P. contorta* and *Pseudotsuga menzeiesii* (Wood et al., 2015)

Finally, the type of association between plant host and ectomycorrhizal fungal symbiont can influence the success of the plant invasion. For example, an invasive plant could encounter a potential symbiont that is considered a “familiar association”. This occurs when a symbiont found in the plant’s native range is also found in the new environment and can help facilitate the plant’s survival (Dickie et al., 2017). If there are symbionts native to an environment that can associate with the introduced plant, but not found to associate with the host in its native range

this is known as a novel association, and if both species are introduced from different regions but can associate, this is known as a co-xenic novel association. The final way for this symbiosis to occur is if both symbionts are introduced together from the same environment, also known as a co-introduction or co-invasion (Dickie et al., 2017).

Co-invasions occur when a plant host establishes in an environment due to their symbionts either arriving with them or they are already present due to prior introduction. EM fungal spores can remain viable in the soil spore bank for at least six years, and potentially longer (Nguyen et al., 2012). This suggests that if EM fungal spores are introduced into an environment, such as soil transferred from another location, they could potentially await a pine host. Pines are often intentionally and unintentionally introduced with EM inoculum. This could be by way of an intact root system that already has EM associations, or with soil that contains EM spores or mycelium (Nuñez et al., 2009; Richardson et al., 1994). Having symbionts already present enables pines to invade out into the environment much more quickly.

When studying woody plant invasions, most invasive woody plants are mycorrhizal and co-invasions with EM fungi were found to be relatively common (Nuñez & Dickie, 2014). Specifically, the invasion of *Pinus* species appears to be dominated by co-invasions (Dickie et al., 2010). Many successful invasions by pines are thought to be due to a co-introduction of pines with their associated EM fungi and have been documented in New Zealand, Argentina, Hawai‘i, and Chile, among other locales (Dickie et al., 2010; Hayward, Horton, Pauchard, et al., 2015; Hynson et al., 2013).

In their native ranges, pines typically associate with a wide range of different EM fungal species but when invading a new environment, one particular group of EM fungi is always present: suilloid fungi (Policelli et al., 2019; D. Lee Taylor et al., 2014). In fact, a review of global pine invasions found that suilloid fungi are likely the main EM fungi driving pine invasions (Policelli et al., 2019). The suilloid group is a monophyletic lineage containing *Suillus*, *Rhizopogon*, *Gomphidius*, *Chroogomphus*, among others (Bruns et al., 1998). Most suilloid species associate specifically with members of the Pinaceae (Bruns et al., 2002), and suilloid species are consistently associated with pines individuals that are the furthest from the initial pine source, whether it is a native forest or plantation (Policelli et al., 2019). Often, these are areas without established EM fungal mycelium, meaning for pine seedlings to become inoculated with EM fungi, they must be colonized by spores (Nguyen et al., 2012; Policelli et al., 2019).

Suilloid fungi have several traits that are believed to explain their frequent association with invasive pines. This group of fungi invests more energy into producing spores and basidiocarps than many other groups, and those spores are often wind dispersed, allowing them to increase their chances of dispersing further from the basidiocarp (Peay et al., 2012). Some species are also dispersed by mammals, which eat the basidiocarps, and deposit viable spores in their feces into the soil (Ashkannejhad & Horton, 2006; Policelli et al., 2019; Wood et al., 2015). Boars that have been shown carrying suilloid spores in their feces can travel up to 12 km in a day, which could lead to spore being disperse very far from their original source (Nuñez et al., 2013). Suilloid spores can also survive for a minimum of 6 years when deposited in the soil, with recent unpublished work showing spore survival up to 15 years (Bruns et al., 2009; Nguyen et al., 2012; Nguyen, 2021). Once pine germlings find the spores in the soil, the spores are quick to germinate and colonize the young plant roots (Dahlberg & Finlay, 1999). It has also been shown that suilloid species produce more of the enzymes necessary to breakdown soil for nutrient acquisition, while also exploring the soil further than other EM fungal species to find these soil pockets of nutrients (Moeller & Peay, 2016; Peay et al., 2011). These resources can then be exchanged with their pine hosts, making them higher quality partners. All these traits of suilloid fungi dispersal, longevity and symbiont efficiency are believed to be aiding in the ability of pines to invade across the world (Policelli et al., 2019).

Pinus radiata

One such tree that is co-invading into new habitats with the help of EM fungi is *Pinus radiata* (Walbert et al., 2010). *Pinus radiata*, or Monterey pine, is a fast-growing species of pine that is widely grown for lumber. The native range of *P. radiata* is limited to three locations: Santa Cruz, the Monterey Peninsula, and San Luis Obispo Counties (Millar, 1999). Monterey pine grows up to 30 m tall in the wild and can live up to 90 years (Cope, 1993), it is considered a closed-cone pine and with serotinous cones (Linhart, 1978).

Interestingly, *P. radiata* is considered endangered in its native range (IUCN, 2021). Monterey pine's decline is largely due to various pathogens. Outside of *P. radiata*'s native range, it has been introduced in places like Australia, South Africa, and New Zealand for timber (Richardson & Rejmánek, 2004). While a successful source of lumber in many of these

locations, *P. radiata* has also escaped out into these new environments and is often considered invasive (Richardson, 1998).

Pinus radiata was introduced to the Hawaiian Islands as a potential local source of timber for the islands (Little & Skolmen, 1989). It was grown in plantations on every island except for Oʻahu (Little & Skolmen, 1989). One of these plantations is in the present-day Kula Forest Reserve located on the leeward side of Haleakalā on Maui. It was originally a native forest and shrub-land, which included plants like koa (*Acacia koa*), ʻōhiʻa (*Metrosideros polymorpha*), and pūkiawe (*Leptecophylla tameiameiae*). In the late 1880s, this area was converted into pastureland for grazing until 1912, when it was converted into a forest reserve. During the 1930s – 1950s, instead of attempting to convert the land back into a native forest, the land was used to grow various economically valuable trees, including *P. radiata*, in hopes that it could be used for timber (State of Hawaiʻi Department of Land and Natural Resources, 2017).

After the plantations were abandoned and no longer maintained, the pines began dispersing out into remaining intact native bunchgrass vegetation and shrublands. *Pinus radiata* individuals are now spreading up towards the summit of Haleakalā, which is known for its endangered endemic species, like the Hawaiian silversword (*Argyroxiphium sandwicense* subsp. *macrocephalum*), and if left unchecked will likely convert the native shrubland into a coniferous forest (Loope et al., 1992). Between 2008 and 2018, land managers spent $1.5 million on pine control on Maui (Mallinson, 2018). In order to protect Haleakalā's unique ecosystem and reduce costs, land managers are eager to better understand how these pines are spreading and what could be used to stop them.

Like other *Pinus* species, *P. radiata* requires EM fungal symbionts to survive and thrive. There are no known pine-associated EM fungi species native to Hawaiʻi (Hayward & Hynson, 2014). This suggests that the EM fungi associated with *P. radiata* were concurrently introduced (Hynson et al., 2013). This "co-invasion" scenario is similar to other regions where non-native pines have only successfully established when their requisite EM fungi were co-introduced (Dickie et al., 2010). Previous research by Hynson et al. (2013), determined the diversity and distribution of *P. radiata*'s co-invading EM fungi in Hawaiʻi and found that there is a viable resident EM fungal spore bank stretching at least 1000 m away from pine plantations. While *P. radiata* can associate with many EM fungal species in its native range, similar to other regions where pines have invaded, this isolated spore bank is primarily made up of two pine-specific

Suillus species (Dickie et al., 2010; Hynson et al., 2013). This suggests that *Suillus* and other long-distance dispersal EM fungal species can disperse out beyond the visible pine invasions and lie in wait for the arrival of new *P. radiata* to colonize.

Premise of the current study

While it is known that the composition of the EM fungal community associating with *P. radiata* changes with distance from the plantation, it is unclear how change in EM fungal community composition impacts the success of *P. radiata*. This is important in order to understand the ways in which EM fungal species may be aiding pine seedlings establishment and ability to thrive. If we can understand how specific EM fungal species are affecting pine success and the patterns in which they are dispersing across the landscape, we may be able to develop better management strategies for slowing this pine's spread.

To observe the fungal community associating with pine seedlings at the Kula Forest Reserve and measure any differences in *P. radiata* seedlings based on the where in this landscape the seedling grew, soil collected from the Kula Forest Reserve pine invasion was used to grow *P. radiata* seedling bioassays. In this study, I tested how increasing distance from the plantation influences (1) the community composition of EM fungal partners of *P. radiata*, (2) the percent colonization of EM fungi on *P. radiata* roots, and (3) the relationship between colonization and total seedling biomass. To do so, *P. radiata* bioassays were grown in a greenhouse with soil collected from the plantation and at various distances up to 2000 m and their roots were analyzed for percent colonization and EM fungal composition. This allowed me to identify which species of EM fungi were present within and around the plantation and compare the EM fungal community to those that occurred at the farthest distances away, while also being able to measure whether there were any significant changes in percent colonization or biomass of the seedlings.

As the distance from the plantation increases, I hypothesized that there would be a significant decrease in EM species richness. I expected the community composition of EM fungi to be significantly different, with the EM fungal community within and near the plantation to be dominated by species that colonize via hyphae and are better competitors and the further distances being largely composed of EM fungi that colonize via spores and are not as

competitive, such as suilloid species, like *Suillus* spp. I also predicted that there would be decreased inoculum potential with increasing distance from the plantation, which would, in turn, lead to a decrease in percent colonization and biomass of *P. radiata* bioassays with distance from the plantation.

Materials and Methods

Field Site

The Kula Forest Reserve is located on the island of Maui on the leeward side of Haleakalā. The reserve ranges from 1,158 to 2,895 meters in elevation. Frequent fog across the reserve contributes greatly to the 83.82 to 101.6 centimeters of annual precipitation. Temperatures range from 5.56 °C – 18.89 °C throughout the year, with an average temperature of 12.22 °C (Lawrimore et al., 2016). There are nine different soil types across the reserve, but the most abundant type is Andisols (State of Hawai‘i Department of Land and Natural Resources, 2017). Historically, this site contained native subalpine shrublands and native bunchgrass vegetation. Starting in 1924, stands of tropical ash (*Fraxinus uhdei*), redwood (*Sequoia sempervirens*), maritime pine (*P. pinaster*), Monterey pine (*P. radiata*), and others were planted in the reserve (State of Hawai‘i Department of Land and Natural Resources, 2017). Between the edge of the *P. radiata* plantation and 500 m from the plantation, there is a mixture of escaped *P. radiata* individuals, non-native vegetation, and native vegetation. At around 500 m from the margins of the plantation the vegetation shifts into dominantly native vegetation such as *Sophora chrysophylla*, *Metrosideros polymorpha*, *Dodonaea viscosa*, and *Leptecophylla tameiameiae* (State of Hawai‘i Department of Land and Natural Resources, 2017).

This site was chosen because there is an active *P. radiata* invasion occurring and to further prior investigations of EM fungi associating with *P. radiata* (Hynson et al., 2013).

Sampling Scheme

Soil sampling occurred October 15 – 18, 2018. Four transects perpendicular from the plantation edge were established at three distances from each transect: 10 m between the first and second transect, 100 m between the second and third transect, and 250 m and between the third and fourth transect. Soil samples were taken at regular intervals: within the plantation, at the

invasion front (on average 304 m from the plantation sampling point), and then an additional 100 m, 500 m, 1000 m, and 2000 m from the invasion front, along each transect. Past ~500 m from the invasion front individual invading pines are rare and absent by 1000 m. Locations were recorded using handheld GPSs (Garmin GPSmap 64s and eTrex 10). Roughly one liter of soil was collected from each interval using a trowel that had been sterilized with 70% EtOH before each use and put into a Ziploc bag, in total there were ~24 liters of soil sampled from the four transects.

Soil was also collected from outside of the plantation (~2000 m away from the nearest soil collection point), but from the same soil type, to be used as a negative control for our bioassay experiment (see below). Collected soil was transported on ice and kept in a cooler until being transferred to an 8°C cold room at the University of Hawai‘i at Mānoa where we set up the greenhouse bioassay experiment. Mature *P. radiata* cones were collected from the plantation to use as a seed source for the bioassays.

Bioassays

Pinecones were heated overnight at ~40 °C to open the cones and release the seeds. The seeds were then removed from the cones, dewinged using sterile forceps, and surface sterilized in 500 ml of 30% hydrogen peroxide and three drops of Tween 20 and stirred for 20 minutes. The sterilized seeds were then rinsed 3x with 400 ml of sterilized water (Hynson et al., 2013). Using sterile technique, the seeds were placed onto moist filter paper into a petri dish and put into a growth chamber on a 12h h : 12 h light : dark cycle at 23.5 °C and allowed to germinate for at least 2 weeks before planting.

To test the EM fungi inoculum potential of this landscape without planting invasive *P. radiata* directly into the soil, greenhouse bioassays of field collected soil and pine seedlings were used to represent our site. Bioassays were set up using 115 ml volume Cone-tainers (Stewe and Son Tangent, OR, USA). Poly-fill was used to fill the drainage holes to prevent soil loss followed by a layer of autoclaved sterilized sand. Each tube was marked with a printed label indicating transect and distance. There were eight replicate bioassays for each sample point along the transects for a total of 192 bioassays. We also set up negative controls for the transects using twice autoclaved field soil that was collected ~2,000 m from the nearest sample point, for a grand total of 224 bioassays.

Each tube was filled with ~75 ml of field soil in preparation for planting. Soils from different distance classes were separated by at least one row in Cone-tainer racks to prevent any cross-contamination. One to three germinated seeds were then planted into each tube using sterilized forceps and then topped with between 1 to 2.5 cm of sterilized sand as an additional measure to prevent cross-contamination. Bioassays were grown in the greenhouse under ambient light and temperatures and watered twice daily using an automated sprinkler system and trays were rotated weekly. After 18 weeks from initial planting tubes were thinned to one seedling. Those that had no surviving seedlings were replanted with three germinated seeds up to three times. All bioassays were planted within 33 days of soil collection. Bioassays were grown for at least six months within the greenhouse before harvesting.

Bioassays were analyzed in the order of when the last seeds were planted, i.e. the oldest seedlings were harvested first. In the lab, the top layer of sand was removed, the roots rinsed with tap water to remove Poly-fill and soil. Once clean, the bioassay roots were kept in a beaker of water until they were analyzed. Bioassay shoots were cut just above the first set of roots and placed into a labeled envelope and dried in a drying oven until there was no detectable additional water loss (72 h) and then weighed to the nearest 1 mg. The roots were then analyzed using the Bubriski "Scoring Percent Colonization" protocol (Bubriski, 2012). To estimate the number of root tips that I needed to count to get a representative sample of the entire root system of each bioassay, the following steps were taken for one bioassay from each distance class from a single transect. A clear small plastic tray was divided into six sections numbered one through six and filled halfway with water. The roots of a bioassay were then cut into six sections and placed randomly into one of the sections of the tray. Then, a random section of the root system was placed under a dissecting scope in a large petri dish of water. Every colonized and uncolonized root tip was counted using a tally counter. This was repeated for each of the six sections. The percent colonization of the entire root system was calculated by dividing the number of root tips colonized by the total number of root tips multiplied by 100, this was repeated for each of the bioassays from each distance class. The average percent colonization was calculated as 65% and from this number I calculated how many root tips needed to be counted to reach this average, which was 185 root tips. The rest of the bioassays were then analyzed by dividing the root system into the plastic tray and using a random number generator to look at random sections. The number of colonized and number of uncolonized root tips were counted and recorded until

185 total root tips were observed. The ratio of colonized root tips to total root tips counted per bioassay was recorded as "percent colonization". After, roots were placed into a labeled and tared 50 ml tube, frozen at -20 °C for at least 24 h, followed by -80 °C for at least two hours, lyophilized, weighed to the nearest mg, and stored for future molecular analyses. Root and shoot biomass measurements were then added together to calculate the total bioassay biomass.

DNA Extraction

To extract DNA from the bioassay roots, I used the Macherey-Nagel Genomic DNA from plant kit (MACHEREY-NAGEL Inc., PA, USA) and followed their protocol with the following modifications. Lyophilized roots were first homogenized using ~3.5 g of Fisherbrand 2.8 mm sterilized ceramic beads, then 10 mg ± 1 mg of the homogenized roots were added into 2 ml extraction tubes. Ninety-one extraction tubes filled with homogenized roots were haphazardly arranged into 96 well plates along with three randomly placed empty extraction tubes (extraction negatives), along with a positive control (previously extracted DNA of *Penicillium* sp.) and one remaining space for a PCR negative control at the end of the plate. Then, 10 µL of RNase A was added to each tube along with the 500 µL of MC 1 Lysis buffer. The next modification occurred during step 2 where we transferred 400 µL of lysate to a sterile Kingfisher Deep Well Plate. The plate was then centrifuged for 6 minutes at 14,000 rpm to help remove any unwanted particulates. The lysate was then transferred to the Bind Plate (Charm Biotech, MO, USA). I used 100 µL of the MC 6 Elution Buffer in the Elution plates. The KingFisher automated pipetting machine (Thermo Fisher Scientific Inc., MA, USA) was used to complete the DNA extraction process. I then transferred the 50 µL of the eluted DNA product into two 96-well PCR microplates which were stored at -20°C.

Library Preparation

PCR was performed according to the following procedure: a master mix of 12.5 µL of 2X KAPA 3G buffer, 1.5 µL of $MgCl_2$, 0.075 µL both of forward and reverse primers, 0.20 µL of KAPA 3G polymerase, and 8.05 µL of deionized water was made. 25 µL of master mix was then added to each of the PCR plate wells. 2 µL of the DNA template was added to each reaction along with the barcoded primers. The plates were then sealed with foil and placed in the

thermocycler at the following cycling conditions: 95°C for 3 minutes; 35 cycles alternating between 95°C for 20 seconds, 53°C for 15 seconds, and 72°C for 30 seconds; then held at 72°C for 3 minutes until finally being held between 4 – 10°C. An agarose gel was then run to determine the size and quantity of PCR product produced.

The Advanced Studies in Genomics, Proteomics and Bioinformatics Laboratory at the University of Hawai‘i at Mānoa sequenced the library on a single run using the Illumina sequencing platform (Miseq V3 600 cycle). A dual index approach was used with 8 bp barcodes on both primers (Illumina Inc., CA, USA).

Bioinformatics

The sequencing run resulted in 19,483,767 reads that were analyzed through the MetaFlowmics for demultiplexing and ITS pipeline (Arisdakessian et al., 2020). This pipeline first extracts the ITS region using ITSxpress (Rivers et al., 2018). Contigs are filtered by removing those smaller than 20 base pairs as wells as any containing ‘N’ nucleotides and then filtered for quality through the Fastx-toolkit (Hannon, 2010). Chimeras are removed using VSEARCH (Rognes et al., 2016). DADA2 was used for error models and denoising (Callahan et al., 2016). Operational taxonomic units (OTUs) were clustered at 97% similarity using VSEARCH. Finally, co-occurrence pattern correction was done using LULU (Frøslev et al., 2017). After these various quality control steps, the amplicon library was left with 9,861,224 reads. OTUs with fewer than three reads were manually removed, leaving 1,100 OTUs (Nguyen et al., 2015).

To assign taxonomy to the OTUs, the seed sequences were run through both the UNITE and the NCBI databases using the Basic Local Alignment Search Tool (Nilsson et al., 2019). Because it is a curated database, taxonomic assignments from UNITE were given preference over NCBI. Our cut off for bit score was 250 and E values less than 1.00E-50. Following Tedersoo et al. 2014, taxonomic assignments were made using the following criterion: ≥95% sequence identity for species, ≥90% sequence identity for genus, and ≥85% sequence identity for family. OTUs that could not be assigned beyond family were removed, with family being the minimum taxonomic distinction needed to determine EM status, leaving 600 OTUs.

To filter only EM fungi species, I compared the taxonomies to lists of known EM fungi species and ran the taxonomies through FUNGuild (Nguyen et al., 2016; Tedersoo et al., 2010;

Tedersoo & Smith, 2013). Any OTUs not matching these lists or not marked as "ectomycorrhizal" were removed. The remaining EM fungal OTUs were then run through MUSCLE sequencing alignment (Edgar, 2004) for each genus with more than one OTU, due to multiple OTUs being labeled the same species. This resulted in two *Tylospora* spp. OTUs being merged due to their close similarity (Supplemental Table 4). *Suillus* spp. were run against a global *Suillus* phylogeny including fully sequenced genomes, leading to two OTUs being merged for *S. brevipes* and three OTUs merged for *S. pungens* (Vilgalys, 2020). This left 20 EM fungal OTUs remaining. There were eight EM fungi OTUs detected within our negative control extraction samples. In order to control for potential contamination, the highest read count per OTU from all the extraction negative controls was subtracted from all of the samples (Nguyen et al., 2015) (Supplemental Table 2). There were four EM fungi OTUs detected within our negative bioassay controls. The read count of the top contaminant, *Trichophaea* sp. 1, made up only 0.4% of the total average reads for this taxon per non-control bioassays, and therefore were still included (Supplemental Table 3).

Data Analysis

For data analysis I used R (R version 4.0.2) statistical software program (R Core Team, 2020). To examine the relationship between distance from the plantation, biomass of the pine seedlings, and the percent colonization of seedling roots by EM fungi, I used a general linear model. A type III ANOVA was used to test for statistical significance at an $\alpha \leq 0.05$. Percent colonization was then regressed against each distance class and seedling biomass for each bioassay. Bioassay age was also positively correlated to percent colonization, as well as distance from the plantation, but when included in the linear model, age was not significant, therefore any effect of age should also be accounted for by distance from the plantation.

To examine the diversity and community composition of EM fungi in my dataset, I pooled sample reads by distance and calculated the average richness at each distance class along the four transects. An ANOVA was run to test for significance between richness and distance and another to test for significance between richness and seedling biomass. Richness was then regressed against each distance class and seedling biomass for the pooled samples. A heat map was generated to observe the change in the relative abundance of each EM fungal species across the distance classes, using the *pheatmap* package (Kolde, 2015).

To test for dissimilarity in community composition across distance from the plantation, Bray-Curtis values were calculated using the *vegan* package (Oksanen et al., 2019). A PERMANOVA was used to test for a significant relationship between distance and community dissimilarity. The *vegan* and *phyloseq* packages were used to generate an NMDS ordination of the community based on the Bray-Curtis values and the different distance classes, (McMurdie & Holmes, 2013). To test for the contribution of dispersion of the Bray-Curtis values within a distance class relative to other distance classes a beta dispersion test was conducted. A regression was done to show the relationship between community dissimilarity and log of the distance between points.

Results

EM Fungi Community Composition

Of the 190 bioassay roots analyzed, there were ~1400 OTUs identified. Once OTUs with fewer than three reads were removed, ~1,100 OTUs remained. According to FUNGuild, 40% of OTUs could not be assigned to a guild. Most OTUs could not be classified to species or genus, which limited FUNGuild's ability classify those OTUs with just one guild, but ~56% of the OTUs were marked as some type of saprotroph, followed by ~25% pathogen,~12% endophyte, ~9.5% parasite, ~5% ectomycorrhizal, and ~4% arbuscular mycorrhizal (Supplemental Table 6). After filtering for only EM fungal species based on the reports by Tedersoo and Smith in addition to FUNGuild and removing bioassay and DNA extraction negative control contaminants, only 20 OTUs were found (Supplemental Table 5).

A total of five suilloid species were found along the transects: *Rhizopogon.* cf. *mohelnensis*, *R.* cf. *salebrosus*, *S. brevipes*, *S. luteus*, and *S. pungens*. *Suillus brevipes* was found in every transect for the 500 m, 1000 m, and 2000 m distance classes, where it peaked in relative abundance, making up 92% of the OTU sequences (Table 1). *Suillus brevipes* was also present at somewhat low levels, 7% relative abundance, at the 100 m distance class within transect 2 and 4, and at the invasion front distance class within transect 3 (Table 1, Supplemental Table 5). *Suillus pungens* was the most widespread suilloid species, occurring at a minimum of one transect for each distance class and peaked in relative abundance with 53% of the OTU sequences at the 500 m distance class (Table 1, Figure 1, Supplemental Table 5). *Suillus pungens* generally increased

with distance from the plantation, until the 500 m distance class, where *S. brevipes* began to dominate, but remained present even at 2000 m from the plantation with 7% relative abundance (Table 1). *Suillus luteus* was only found in transect 2 in the 500 m distance class, where it had a relative abundance of 10% (Supplemental Table 5). *Rhizopogon* cf. *mohelnensis* was found within the plantation in each transect except for transect 1 and within the invasion front at transects 1 and 2. *Rhizopogon* cf. *salebrosus* was only found within transect 1 and 2 in the 100 m distance class. Both species of *Rhizopogon* maintained relative abundances less than or equal to 1% (Supplemental Table 5).

	Plantation	**Invasion Front**	**100 m**	**500 m**	**1000 m**	**2000 m**
Rhizopogon cf. *mohelnensis*	<0.001	0.004836	0	0	0	0
Rhizopogon cf. *salebrosus*	0	0	<0.001	0	0	0
Sistotrema sp.	0	0	0	0	<0.001	0
Suillus brevipes	0	0.07081	0.07341	0.2647	0.6312	0.9207
Suillus luteus	0	0	0	0.02460	0	0
Suillus pungens	0.01139	0.3012	0.3089	0.5278	0.1835	0.07795
Thelephora cf. *terrestris*	0.08561	0.1644	0.01250	0.1474	0.01800	<0.001
Tomentella cf. *lateritia*	0.06760	0	0	0	0	0
Trichophaea sp. 1	0.4238	0.2984	0.5742	0.02875	0.06397	0
Trichophaea sp. 2	0	0.03231	0	0	0	0
Trichophaea sp. 3	<0.001	<0.001	0	0	0	0
Trichophaea sp. 4	<0.001	<0.001	0	0	0	0
Trichophaea sp. 5	0	<0.001	0	0	0	0
Tylospora sp. 1	0.001434	0.04345	<0.001	<0.001	0.01906	<0.001
Tylospora sp. 2	0	0.01237	<0.001	0.005898	0.08321	0.001076
Tylospora sp. 3	0	<0.001	0	<0.001	<0.001	<0.001
Tylospora sp. 4	0	0	0	0	<0.001	0
Tylospora sp. 5	0	<0.001	0	0	0	0
Wilcoxina sp. 1	0.4100	0.0710	0.03090	0	0	0
Wilcoxina sp. 2	<0.001	<0.001	0	0	0	0

Table 1: Average relative abundance of each ectomycorrhizal fungal sequences observed in this study. Distance classes are pooled by transect, with increasing distance from the invasive P. radiata *plantation at the Kula Forest Reserve.*

There were four other Agaricomycetes genera found in this study: *Sistotrema*, *Thelephora*, *Tomentella*, and *Tylospora* (Table 1, Figure 1). One species of *Sistotrema* was found, only occurring at the 1000 m distance class within transect 4 with less than 1% relative abundance (Table 1, Figure 1). *Thelephora* cf. *terrestris* was found in at least two transect at every distance class (Supplemental Table 5). The relative abundance of *T.* c.f. *terrestris* varied

across the distance classes, peaking within the invasion front at 16% and then the 500 m distance class at 15%, before quickly decreasing to less than 1% at the 2000 m distance class (Table 1, Figure 1). *Tomentella* c.f. *laeterita* was only detected within the plantation within transects 2 and 3 with a relative abundance of 7% (Table 1, Supplemental Table 5). Finally, there were five species of *Tylospora* found throughout the study site. *Tylospora* sp. 1 was found in at least one transect of every distance class, but always maintained a relative abundance of less than 2% (Table 1, Supplemental Table 5). *Tylospora* sp. 2 appeared within the invasion front and beyond, peaking at the 1000 m distance class with an 8% relative abundance (Table 1). *Tylospora* sp. 3 was found within the transect 4 invasion front and transect 2 500 m distance classes, as well as two transects for the 1000 m and 2000 m distance classes, but never with a relative abundance greater than 1% (Table 1, Supplemental Table 5). Both *Tylospora* sp. 4 and 5 were each found at single distance classes, 1000 m, and the invasion front, respectively with less than 1% relative abundance (Table 1).

Two genera from the Pezizales were found in this study site: *Trichophaea* and *Wilcoxina*. Five OTUs of *Trichophaea* were detected, with *Trichophaea* sp. 1 being the most prevalent one. *Trichophaea* sp. 1 occurred within every transect in the plantation and invasion front, and 100 m distance classes, except for transect 4 100 m, and then was only found at transect 2 500 m and transect 4 1000 m (Supplemental Table 5). The relative abundance of *Trichophaea* sp. 1 was the greatest with 57% at the 100 m before falling to the single digits for the 500 m and 1000 m distance classes (Table 1). None of the other *Trichophaea* species were found beyond the invasion front distance class and never above 3.2% relative abundance (Table 1, Figure 1). *Wilcoxina* was found within the plantation, invasion front, and 100 m distance classes, but only within one transect each (Table 1, Supplemental Table 5). *Wilcoxina* sp. 1 was the second most abundant species within the plantation with 41% relative abundance, before greatly decreasing in relative abundance beyond (Table 1, Figure 1). *Wilcoxina* sp. 2 made up less than 1% of the OTU reads within the plantation and invasion front distance classes (Table 1).

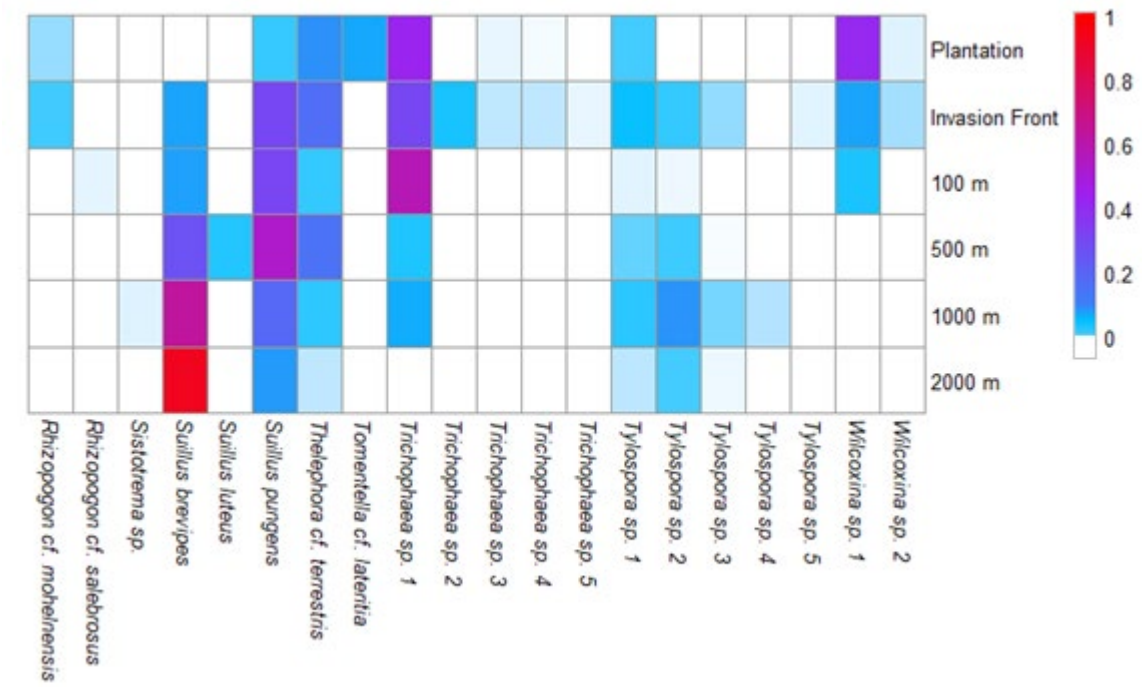


Figure 1: Heatmap of the average relative abundance of each ectomycorrhizal fungal sequences observed in this study. Distance classes are pooled by transect, with increasing distance from the invasive P. radiata plantation at the Kula Forest Reserve. Color represents the differences in average relative abundance. Red hues indicate a relative abundance closer to 1 while light blue and white hues are closer to 0 or no abundance.

The results of the PERMAONVA test were highly significant (p <0.001, Supplemental Table 8), indicating that there is a significant relationship between distance and EM fungi community composition. In the NMDS plot, samples from the 2000 m point form a tight cluster relative to all other distance classes, whereas the invasion front shows the most dispersion, encompassing almost all other points (Figure 2). The 2000 m cluster is nested within the 1000 m cluster which is then largely nested within the 500 m point and invasion front point, indicating that the species found within the 2000 m distance class are also found within the 1000 m distance class, and all those species are largely found within the 500 m distance class. The plantation is nestled within the invasion front and roughly half of the plantation ellipses overlaps with the 100 m distance class. Overall, the similarity of EM fungal community composition decreased with distance from the plantation ($F_{1,149} = 22.9$, p <0.001, Figure 3).

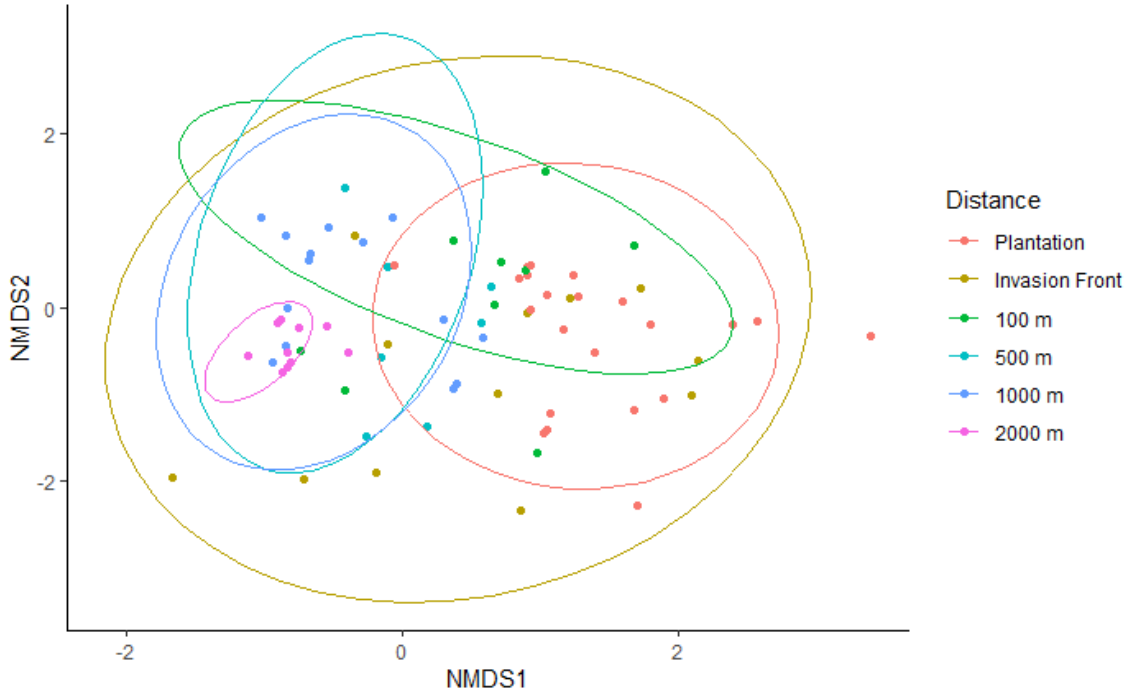


Figure 2: NMDS ordination of ectomycorrhizal fungal community similarity. Each point represents the community found within one P. radiata *bioassay grown in soil taken from increasing distances along transects away from the* P. radiata *plantation at the Kula Forest Reserve. Color represents each distance class. Points more closely together have more similar ectomycorrhizal communities than those further apart.*

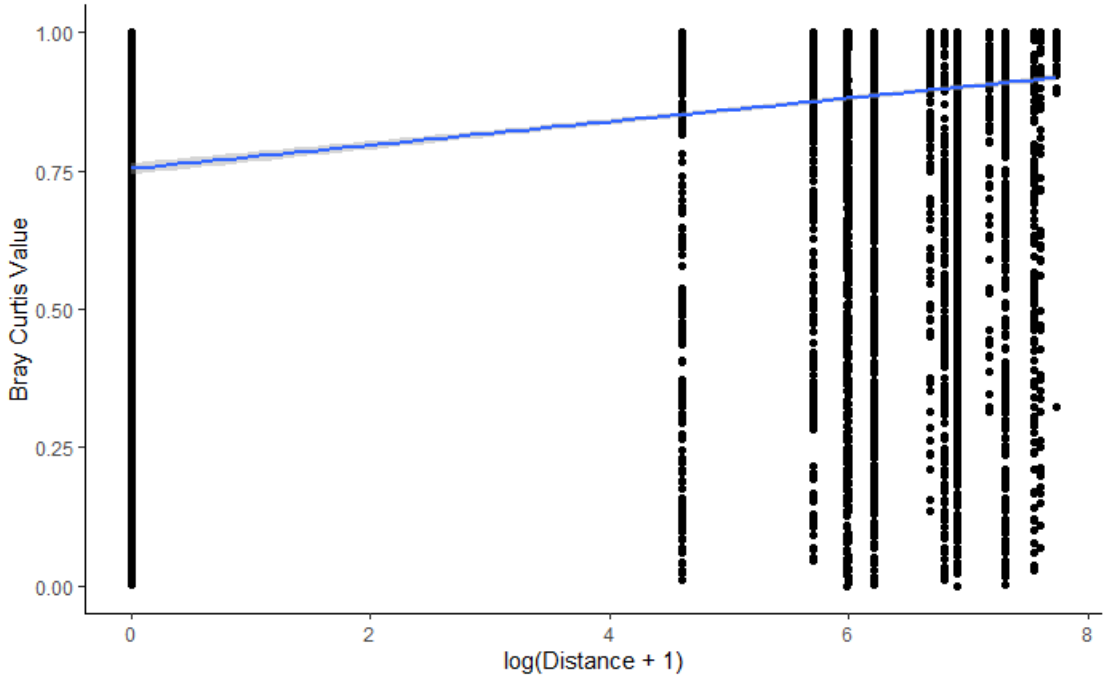


Figure 3: Bray-Curtis ectomycorrhizal fungal community dissimilarity values compared to the log of the distance between sampling points. Trend line shows significant positive correlation ($p < 0.001$).

Percent Colonization, Distance, and Biomass

Out of the 209 seedlings planted, 190 survived, for an overall 91% survival rate. There was no correlation to distance class for bioassay survival, but there was some correlation to transect, with 8 bioassays dying for transects 1 and 2, 7 dying for transect 3, and 6 dying for transect 4 (Supplemental Table 1). Of the surviving non-control seedlings, the average percent of colonization by EM fungi was 45.5±22.2%. Percent colonization by EM fungi was significantly correlated with both the total biomass of the pine seedlings ($F_{1,171} = 25.1$, $p < 0.001$) and distance from the plantation ($F_{1,171} = 63.8$, $p < 0.001$; Fig. 4, 5, Table 2). As distance from the plantation increased, so did percent colonization (Figure 4) and biomass (Figure 5). However, the interaction of distance and biomass was not a significant predictor of percent colonization (Table 2). Combined, distance and biomass explained 33.1% of the percent colonization data (conditional $R^2 = 0.331$, Figure 4).

	SS	***df***	F	*P*
Distance (m)	2.12	1	63.8	<0.001***
Biomass (g)	0.832	1	25.1	<0.001***
Residuals	5.671	171		

Table 2: Summary results of ANOVA type III test between percent colonization of bioassays, total bioassay biomass, and distance from the plantation. Stars represent significance.

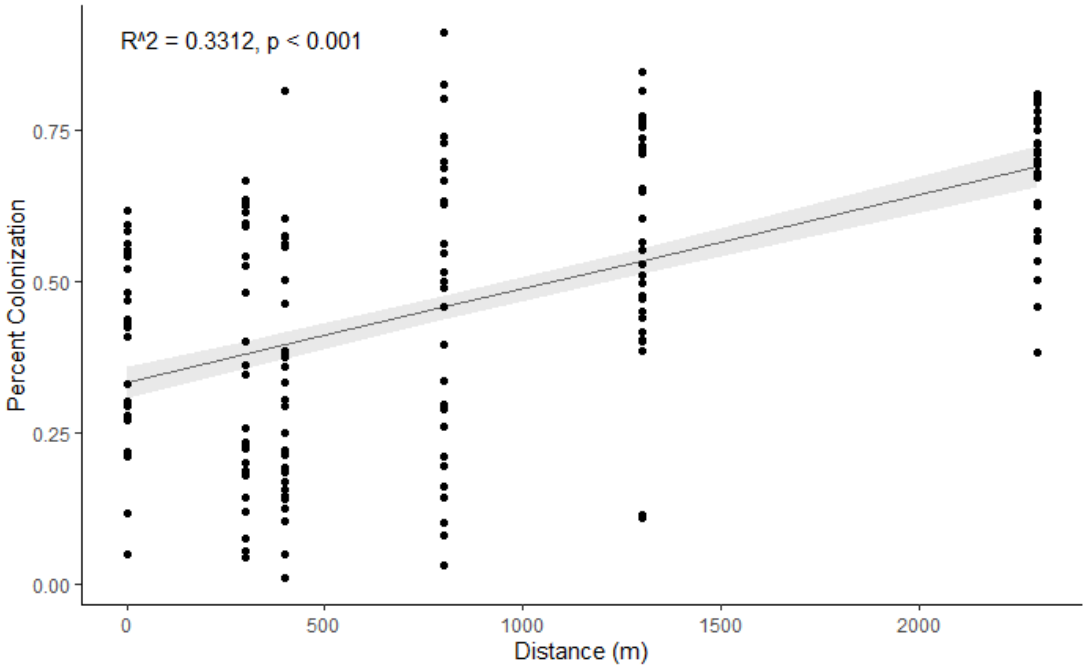


Figure 4: Percent colonization by ectomycorrhizal fungi in pine seedling bioassays roots grown soil collected from increasing distance from a pine plantation regressed against distance from that pine plantation. Each point represents an individual bioassay. Trend line shows a significant positive relationship with the shadowed area representing standard deviation.

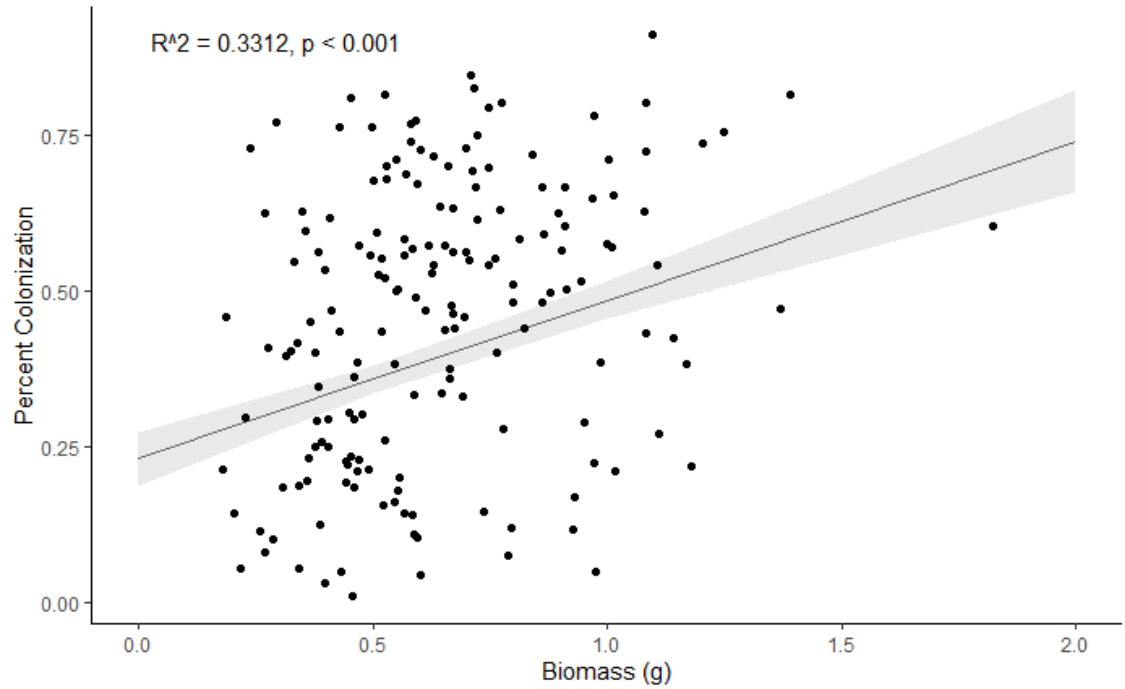


Figure 5: Percent colonization by ectomycorrhizal fungi in pine seedling bioassays roots grown soil collected from increasing distance from a pine plantation regressed against total bioassay biomass. Each point represents an individual bioassay. Trend line shows a significant positive relationship with the shadowed area representing standard deviation.

When testing for a relationship between observed species richness and distance from the plantation, I found no significant relationship between the two (Supplemental Figure 2). The relationship between total seedling biomass and observed species richness was also not significant (Supplemental Figure 1). Despite the non-significant relationship between richness and distance, there appears to be a general negative trend in species number as distance from the plantation increases (Supplemental Figure 2).

Discussion

My results show that there is increased dissimilarity in EM fungal communities as distance between transect sampling points increases (Figure 5). This result is similar to other studies where community dissimilarity increases with geographic distance (Bahram et al., 2013; Goldmann et al., 2016; Hynson et al., 2013). When looking at the NMDS ordination (Figure 2) we can see that the bioassay communities that are present 2000 m away from the plantation are more similar to each other than those within the plantation and invasion front, suggesting that the invasion front is the most diverse and the 2000 m distance class is the least diverse.

The overall community composition in our site is unique, due to fact that the EM fungi species that are present are descendants of those who were able to survive being transported to Hawai‘i. I was unable to find information about source of the initial pine seedlings for this plantation, but it possible that they came either from the US mainland, where *P. radiata* is native, or from stock of another foreign plantation, like in New Zealand where *P. radiata* is cultivated for timer, meaning that the source of EM fungi associated with these pines could be from various places, leading to a composition of EM fungi found nowhere else. Our reads of *Suillus* were placed within a super tree for the genus and found that our species are closely related to those in the mainland US and Asia, suggesting that these communities may be different than any found elsewhere.

As expected, suilloid species of EM fungi were the dominant species present at the furthest distance classes (500 - 2000 m). Specifically, *S. brevipes* with 63% and 92% of all the OTU sequences at the 1000m and 2000 m distance class respectively, and *S. pungens* with 53% of the OTU sequences at the 500 m distance class (Table 1). While these species were present around the plantation, they only occurred at very low abundances (Figure 1). Many studies have shown that suilloid fungi are found associating with invasive pines, especially when they are

young (Hynson et al., 2013; Policelli et al., 2019; Urcelay et al., 2017). In fact, it has been shown that just one species of *Suillus*, *S. luteus*, was enough to facilitate an invasion of pines in Chile (Hayward, Horton, Pauchard, et al., 2015). Although we found two *Rhizopogon* species in this study site, they were not detected passed the 100 m distance class and maintained less than 1% relative abundance (Table 1). *Rhizopogon* species are dispersed via mammals, and the potential dispersers at the Kula Forest Reserve are the non-native axis deer, goats, and pigs (Ashkannejhad & Horton, 2006; Bruns et al., 2009; State of Hawai‘i Department of Land and Natural Resources, 2017). Mammal dispersal may have led to patchiness in the dispersal of *Rhizopogon* spores outside of the plantation, which may have not been sampled by our study.

There are several traits of suilloid species that are thought to promote and support invasion with pines. These traits include long-distance dispersal via wind and mammals, rapid colonization, resistant spore banks, and long distance exploration type capable of exploiting soil nutrient patches meters from the host roots (Policelli et al., 2019). Suilloid species, specifically *Suillus*, are also known for their ability to produce mass quantities of spores, which increase the likelihood that some spores will be carried further away and are able to establish a robust spore bank in the soil (Peay et al., 2012). Previously, it has been shown that in a native *Pinus muricata* forest in California, *S. brevipes*, while being a colonizer of young forests, can also persist within more mature forests and *S. pungens* was found associating with the younger forests (Nguyen et al., 2012). Within our study, this was reverse, with *S. pungens* being found within the plantation and *S. brevipes* dominating at the further distance classes. Preliminary evidence suggests that *S. brevipes* spores may survive longer in the spore bank, which may be playing a role in the lower abundance of *S. pungens* further out in the landscape (Nguyen et al., 2012).

While the relative abundance of *Suillus* increased with distance, I found that percent colonization of individual *P. radiata* bioassay roots and total bioassay biomass increased as well (Figure 4, Figure 5). Increasing percent colonization was contrary to my original hypothesis, which stated that there would likely be a decrease in colonization of bioassay roots with increasing distance from the plantation, due to low inoculum potential (Nuñez et al., 2009; Peay et al., 2012). Percent colonization of the bioassays was likely driven by community composition at the different distance classes. In this study *Suillus* species are present at the distance classes closer to the plantation but did not dominate until at least 500 m from the invasion front and became the most prevalent genus by a vast margin at the 1000 m and 2000 m distance classes.

The bioassays that were grown in soil from the 2000 m distance class, where *S. brevipes* and *S. pungens* were more abundant, may have been colonized quicker and more robustly than the bioassays that were grown in soil from the distance classes closer to the planation, due to the combination of less competition between EM fungi for root tips and the ability of *Suillus* to colonize quickly. Competition between EM fungi species may have prevented more colonization within the plantation and invasion front, despite the plentiful number of species present. The bioassays with more biomass from these further distance classes are likely to be colonized, at least in part, by *Suillus* species, which has been shown to be a high-quality partner and rapid colonizer (Moeller & Peay, 2016; Policelli et al., 2019). *Suillus*'s ability to disperse outwards and its propensity for rapid colonization has led to robust colonization in pine seedlings and increased biomass, which is furthering enabling *P. radiata*'s escape outward into the landscape.

Another group of fungi found in this study were all our crust forming fungi. It is not surprising that *Thelephora.* c.f, *terrestris* was found in at the Kula Forest Reserve, as it is common in forest nursey soils and has been found associating with invasive pines in Argentina, New Zealand, as well as Hawai‘i (Hayward, Horton, & Nuñez, 2015; Hynson et al., 2013; Smith & Read, 2008; Urcelay et al., 2017). Studies have found that *T. terrestris* is a relatively strong competitor among EM fungal species, particularly *S. pungens*, but *T. terrestris* does not produce as many spores nor does disperse as far as *S. pungens*, possibly explaining its prevalence within the middle distance classes (Table 1) (Moeller & Peay, 2016; Peay et al., 2012). Another Agaricomycete in this study was *Tomentella* c.f. *lateritia*, which was only found within the plantation. There isn't very much functionally known about *Tomentella* species but they may be dispersed by arthropods, which could explain the limited spread in our study (Lilleskov & Bruns, 2005; Smith & Read, 2008). *Tomtenella* species are also widespread and seems to be significantly associated with forests, so further research is needed to understand how they may be aiding pines and their invasion (Binder et al., 2013; Jakucs & Erős-Honti, 2008). The other prevalent Agaricomycete genus in this study is *Tylospora*, which despite maintaining a relatively low abundance of less than 9% at any distance class, had species ranging from the plantation out to the 2000 m distance class (Table 1). This genus has been found associating with alien Pinaceae within native Pinaceae ranges and may be able to access otherwise inaccessible organically bound nutrients and further research may be needed to see if *Tylospora* species are more key players in pine invasions (Erland & Taylor, 1999; Vlk et al., 2020).

The two genera belonging to the Pezizales, *Trichophaea* and *Wilcoxina*, were found within and near the plantation and invasion front distance classes. Within the plantation, *Trichophaea* sp. 1 and *Wilcoxina* sp. 1 dominated, making up 42% and 41% of the OTU reads, respectively (Table 1). Not much is known about the ecology of *Trichophaea*, although at least one species may be heat resistant, which could mean that burning the landscape in order to eliminate *P. radiata* and any EM fungal inoculum might not be effective (Šimonovičová et al., 2014). However, *Trichophaea*'s relative, *Wilcoxina*, is more well known. *Wilcoxina* is widespread colonizer of pine nursery seedlings with the ability to survive in spore banks for at least six years and has even been shown to colonize seedlings after a stand replacing wildfire (Baar et al., 1999; Barroetaveña et al., 2007; Leski et al., 2010; Nguyen et al., 2012). These characteristics lead to *Wilcoxina* being found in relatively young stands of pines and often considered an early successional colonizer (D. L. Taylor & Bruns, 1999). Although this genus is a quick and robust EM fungal colonizer of pines, it often colonizes vegetatively producing chlamydospores belowground, limiting dispersal distance (Glassman et al., 2015; Yu et al., 2001). This may be why *Wilcoxina* spp. are only seen up to the 100 m distance class and not beyond. Lastly, these species were previously only known to be present and colonizing pines in the Holarctic realm, but this study is evidence that they have reached beyond and into the soils of Maui (Tedersoo et al., 2010).

I expected the plantation to have the most species and for species number to decrease with distance, as the plantation is reasonably the source for the EM species in this system as there are no known native pine-associating EM fungi in Hawai'i (Hayward & Hynson, 2014). Although there was no statistical significance between observed richness and distance, the richness of species peaked at the invasion front (15 species, Supplemental Table 7) and was lowest 2000 m away (6 species, Supplemental Table 7). This may in part due to the change in root density between the plantation and invasion front distance classes. Previous work researching EM fungi communities and root density found that areas of higher root density had a higher prevalence of short-distance exploration type EM fungal species, whereas on the edges of forests where root density was lower, longer-distance exploration type EM fungi were more prevalent (Peay et al., 2011). The two most abundant species within the plantation, *Trichophaea* and *Wilcoxina*, are known to be strong hyphal colonizers, which may have given them an edge in the plantation due to the closer proximity of roots to outcompete some of the other species who

rely more on spore colonization and longer distance exploration types (Yu et al., 2001). In our study, the invasion front distance class pines were much further apart than within the plantation, which may have allowed for more opportunities for longer distance exploration type or weaker competitor EM fungal species, such as *Suillus pungens*, which increased from 1% relative abundance within the plantation distance class to 30% relative abundance in the invasion front distance class (Table 1) . The species present in the invasion front classes are also likely present within the plantation distance class, but at such low abundances that they were not detected by this study's sampling methods. For example, *Sistotrema* sp. 1 likely occurred elsewhere within the Kula Forest Reserve but was only detected at a very low abundance at one sampling point (Supplemental Table 5).

The percent colonization trend line showed no signs of reaching a plateau at the 2000 m distance class, which was opposite of what I expected (Figure 4). This could indicate that there is still high colonization potential beyond 2000 m from the plantation, and higher up Haleakalā, despite few to no currently established pine trees. Saplings of three pine species, including *P. radiata,* have been found near the crater of Haleakalā, and National Park Service is already working to remove them (Strohecker, 2016). As more and more pines encroach towards the crater of Haleakalā, the endemic species found there, and in the Kula Forest Reserve, continue to be threatened. Our study helps to show that there is inoculum potential, particularly by *Suillus*, at least 2000 m from the *P. radiata* plantation at the Kula Forest reserve, and likely beyond. If a seed lands in the soil at these distances and other abiotic and biotic conditions are favorable for germination, it is likely to be colonized. Currently, there are no known feasible ways to decrease or remove EM fungal spores or mycelium from the soil, limiting the management options available to control the spread of *P. radiata* via management of the EM fungal symbiont.

The possibility that the invading pines may not encounter their required EM fungal symbionts as they get further away from the plantation appears to not be the case, which ultimately impacts management decisions. The limiting factor of this invasion is likely *P. radiata* seed dispersal and not symbiont availability, meaning that more active management of the pines is necessary. Further study into pine dispersal on this landscape is underway, but unless managers can prevent seeds from reaching further and further away from the plantation, this invasion will likely progress upwards towards Haleakalā and encroach on the unique ecosystem found there.

Overall, our study found that the community of EM fungi occurring at increasing distances from the Kula Forest Reserve plantation are very different than within and around the plantation, and the most abundant species found at those far distances were *S. brevipes* and *S. pungens*. *Suillus*, and other suilloid species, have been shown to enable pine invasions globally and are likely the main drivers of the *P. radiata* invasion at the Kula Forest Reserve (Policelli et al., 2019). The long-distance exploration type, plentiful spore production, increased spore longevity and dispersal distance, and increased nutrient acquisition ability of *Suillus* species are likely why we detect them associating with the seedlings growing the furthest away, and why these seedlings are able to survive and thrive (Agerer, 2001; Moeller & Peay, 2016; Nguyen et al., 2012; Policelli et al., 2019). This study also saw an increase in percent colonization and biomass of *P. radiata* bioassays with increasing distance from the plantation, suggesting that these pines can continue invading out into the environment and up towards the Haleakalā crater. If managers aim to limit the spread of *P. radiata*, active management strategies, such as removal of pine individuals who have already invaded beyond the plantation and the removal of the pine plantation in order to reestablish native forest plants, need to be taken against the pines, due to the availability of ideal symbionts in this landscape.

Appendices

Transect	Distance Class	Number Survived	Number Died
1	Plantation	8	1
1	Invasion Front	7	1
1	100 m	7	1
1	500 m	6	2
1	1000 m	7	1
1	2000 m	6	2
2	Plantation	7	1
2	Invasion Front	8	0
2	100 m	8	0
2	500 m	7	1
2	1000 m	8	0
2	2000 m	8	0
3	Plantation	7	1
3	Invasion Front	7	1
3	100 m	8	0
3	500 m	8	0
3	1000 m	7	1
3	2000 m	7	1
4	Plantation	7	1
4	Invasion Front	8	0
4	100 m	8	0
4	500 m	8	0
4	1000 m	7	1
4	2000 m	8	0
	Control	13	3

Supplemental Table 1: The total number of P. radiata *bioassays that survived and died per transect and distance class over the course of this study.*

EM Fungal Species	Extraction Negative Highest Value
Suillus brevipes	28
Suillus pungens	136
Trichophaea sp. 1	61
Wilcoxina sp. 3	89
Thelephora cf. *terrestris*	2
Tylospora sp. 1	14
Tylospora sp. 2	4
Suillus pseudobrevipes	139

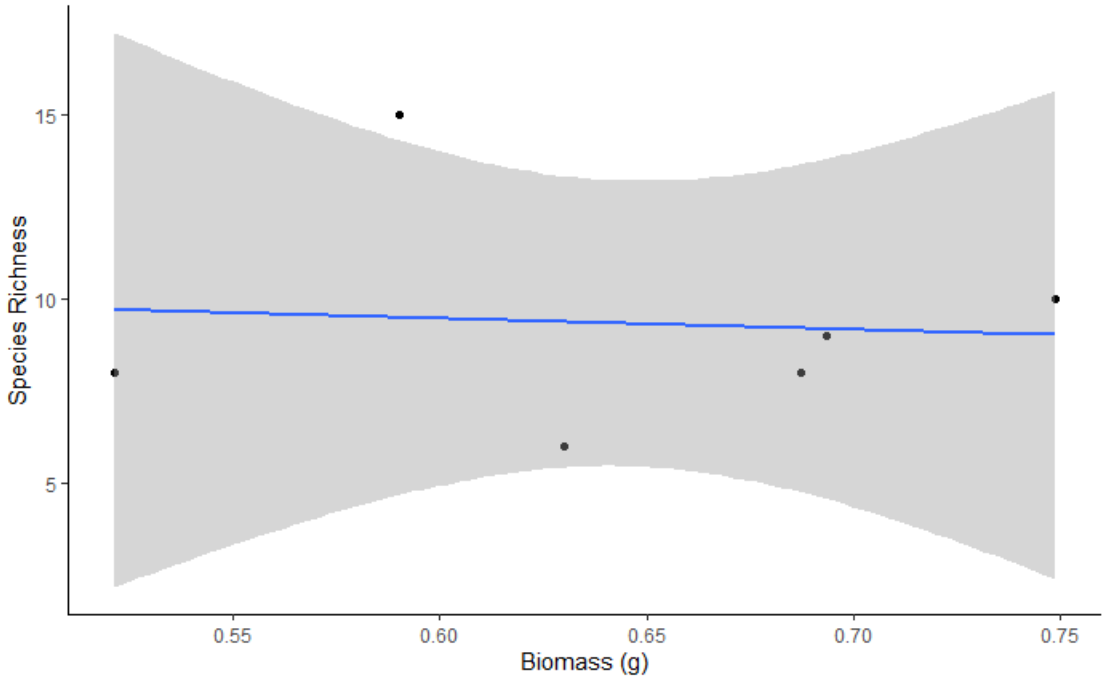
Supplemental Table 2: The highest number of OTU reads for each EM fungal OTU found in the extraction negative controls. These amounts were then subtracted from each sample for each EM fungal OUT to control for contamination.

EM Fungal Species	Control_104570	Control_104589	Control_104738	Control_104858
Tylospora sp. 2	0	0	0	2
Thelephora cf. *terrestris*	5	9	0	0
Trichophaea sp. 1	48	0	0	0
Rhizopogon cf. *mohelnensis*	0	0	2	2

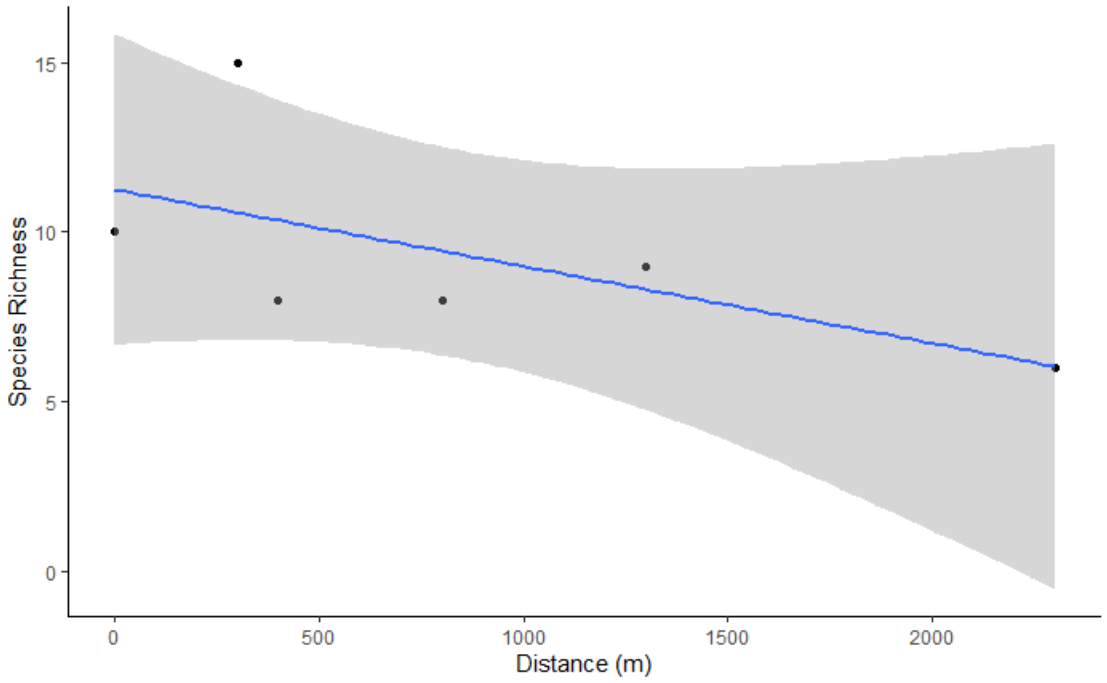
Supplemental Table 3: The total number of EM fungi OTU reads found within the P. radiata *bioassay negative controls.*

OTU Sequencing ID	Species	OTU Sequencing ID				
		OTU97_450	OTU97_574	OTU97_7	OTU97_10	OTU97_82
OTU97_450	Tylospora sp. 5	100	93.87	96.93	94.48	95.09
OTU97_574	Tylospora sp. 6	93.87	100	96.95	95.73	96.39
OTU97_7	Tylospora sp. 1	96.93	96.95	100	97.56	98.17
OTU97_10	Tylospora sp. 2	94.48	95.73	97.56	100	99.4
OTU97_82	Tylospora sp. 2	95.09	96.39	98.17	99.4	100

Supplemental Table 4: The likelihood that any two of these OTUs are the same. OTUs highlighted in yellow were merged due to high likelihood of same identity.



Supplemental Figure 1: Average species richness of ectomycorrhizal fungi in pine seedling bioassays roots grown soil collected from increasing distance from a pine plantation regressed against average biomass. Each point represents averages pooled by distance. Trend line shows no significant relationship with the gray shadow representing standard deviation.



Supplemental Figure 2: Average species richness of ectomycorrhizal fungi in pine seedling bioassays roots grown soil collected from increasing distance from a pine plantation regressed against distance from the plantation. Each point represents averages pooled by distance. Trend line shows no significant relationship with the gray shadow representing standard deviation.

Transect	Distance	*Rhizopogon* cf. *mohelnensis*	*Rhizopogon* cf. *salebrosus*	*Sistotrema* sp.	*Suillus brevipes*	*Suillus luteus*	*Suillus pungens*	*Thelephora* cf. *terrestris*	*Tomentella* cf. *lateritia*	*Trichophaea* sp. 1	*Trichophaea* sp. 2
1	Plantation	0	0	0	0	0	0	0.3599	0	0.6393	0
2	Plantation	0.0002	0	0	0	0	0.0528	0	3.88E-05	0.9470	0
3	Plantation	0.0007	0	0	0	0	0	0	0.4905	0.4996	0
4	Plantation	0.0008	0	0	0	0	0	0.0023	0	0.0004	0
1	Invasion Front	0.0001	0	0	0	0	0	1.12E-05	0	0.7109	0
2	Invasion Front	0.0130	0	0	0	0	0.7199	5.88E-06	0	0.2671	0
3	Invasion Front	0	0	0	0.2112	0	0	0.4533	0	0.0253	0.0964
4	Invasion Front	0	0	0	0	0	0.3474	0.1251	0	0.5275	0
1	100 m	0	8.15E-05	0	0	0	0.2583	8.15E-05	0	0.5532	0
2	100 m	0	7.57E-05	0	0.0010	0	0	0	0	0.9990	0
3	100 m	0	0	0	0	0	0.4641	0	0	0.5359	0
4	100 m	0	0	0	0.3055	0	0.6420	0.0522	0	0	0
1	500 m	0	0	0	0.2822	0	0.6085	0.1093	0	0	0
2	500 m	0	0	0	0.2502	0.0947	0.0571	0.4615	0	0.1107	0
3	500 m	0	0	0	0.1190	0	0.8810	0	0	0	0
4	500 m	0	0	0	0.4048	0	0.5952	0	0	0	0
1	1000 m	0	0	0	0.3777	0	0.6223	0	0	0	0
2	1000 m	0	0	0	0.9999	0	0	1.32E-05	0	0	0
3	1000 m	0	0	0	0.2129	0	0.3635	7.77E-05	0	0	0
4	1000 m	0	0	8.97E-05	0.6803	0	0.0748	0.0317	0	0.1127	0
1	2000 m	0	0	0	0.9817	0	0.0183	0	0	0	0
2	2000 m	0	0	0	0.5812	0	0.4188	3.90E-05	0	0	0
3	2000 m	0	0	0	0.9951	0	0.0012	0	0	0	0
4	2000 m	0	0	0	0.9965	0	0.0021	0.0002	0	0	0

Supplemental Table 5: Average relative abundance of each ectomycorrhizal fungal sequences observed in this study. Averages are pooled by transect and distance class.

Transect	Distance	*Trichophaea* sp. 3	*Trichophaea* sp. 4	*Trichophaea* sp. 5	*Tylospora* sp. 1	*Tylospora* sp. 2	*Tylospora* sp. 3	*Tylospora* sp. 4	*Tylospora* sp. 5	*Wilcoxina* sp. 1	*Wilcoxina* sp. 2
1	Plantation	0	0	0	0.0007	0	0	0	0	0	0
2	Plantation	0	0	0	0	0	0	0	0	0	0
3	Plantation	0	0	0	0.0092	0	0	0	0	0	0
4	Plantation	8.82E-05	4.07E-05	0	0	0	0	0	0	0.9963	0.0001
1	Invasion Front	0	0	0	0.2227	0.0634	0.0026	0	0.0002	0	0
2	Invasion Front	0	0	0	0	0	0	0	0	0	0
3	Invasion Front	0.0003	0.0003	0.0001	0	0	0	0	0	0.2118	0.0012
4	Invasion Front	0	0	0	0	0	0	0	0	0	0
1	100 m	0	0	0	0	4.08E-05	0	0	0	0.1882	0
2	100 m	0	0	0	0	0	0	0	0	0	0
3	100 m	0	0	0	0	0	0	0	0	0	0
4	100 m	0	0	0	0.0002	8.39E-05	0	0	0	0	0
1	500 m	0	0	0	0	0	0	0	0	0	0
2	500 m	0	0	0	0.0031	0.0227	4.42E-05	0	0	0	0
3	500 m	0	0	0	0	0	0	0	0	0	0
4	500 m	0	0	0	0	0	0	0	0	0	0
1	1000 m	0	0	0	0	0	0	0	0	0	0
2	1000 m	0	0	0	0	5.30E-05	0	0	0	0	0
3	1000 m	0	0	0	0.1734	0.2452	0.0040	0.0009	0	0	0
4	1000 m	0	0	0	0.0002	0.0994	0.0004	0.0003	0	0	0
1	2000 m	0	0	0	0	0	0	0	0	0	0
2	2000 m	0	0	0	0	0	0	0	0	0	0
3	2000 m	0	0	0	0.0007	0.003	4.18E-05	0	0	0	0
4	2000 m	0	0	0	8.96E-06	0.0011	4.18E-05	0	0	0	0

Supplemental Table 5 (Continued): Average relative abundance of each ectomycorrhizal fungal sequences observed in this study. Averages are pooled by transect and distance class.

Major Groups	OTUs	Percentage of OTUs
Unknown	428	0.391225
Undefined Saprotroph	321	0.293419
Plant Pathogen	165	0.150823
Endophyte	129	0.117916
Animal Pathogen	114	0.104205
Wood Saprotroph	113	0.103291
Dung Saprotroph	64	0.058501
Soil Saprotroph	59	0.053931
Ectomycorrhizal	57	0.052102
Fungal Parasite	52	0.047532
Lichen Parasite	47	0.042962
Arbuscular Mycorrhizal	46	0.042048
Plant Saprotroph	27	0.02468
Litter Saprotroph	22	0.02011
Orchid Mycorrhizal	18	0.016453
Leaf Saprotroph	12	0.010969
Epiphyte	11	0.010055
Lichenized	11	0.010055
Ericoid Mycorrhizal	4	0.003656
Animal Parasite	3	0.002742
Bryophyte Parasite	2	0.001828
Undefined Biotroph	2	0.001828
Animal Endosymbiont	1	0.000914
Clavicipitaceous Endophyte	1	0.000914
Root Associated Biotroph	1	0.000914

Supplemental Table 6: Number of OTUs in this study categorized by FUNGuild into each guild category. The right column shows what percentage of OTUs fit into that guild.

Distance Class	Richness
Plantation	10
Invasion Front	15
100 m	8
500 m	8
1000 m	9
2000 m	6

Supplemental Table 7: Species richness of each distance class in this study.

	SS	***df***	**F**	***R²***	***P***
Distance (m)	11.93	5	6.980	0.1951	<0.001***
Residuals	49.21	144		0.8049	

Supplemental Table 8: Summary results of PERMANOVA test between community dissimilarity and distance from the plantation. Stars represent significance.

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