NORTHWESTERN UNIVERSITY

Investigating Specificity and Diversity of Orchid Mycorrhizal Fungi of *Vanilla Planifolia* and *Dendrophylax lindenii*.

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Plant Biology and Conservation

By

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EVANSTON, ILLINOIS

June 2019

ABSTRACT

Conservation efforts start with understanding the life cycle and interactions of species. For example, orchid conservation requires understanding pollinators as well as mycorrhizal fungi because these are mutualisms orchids require. For instance, all orchids require assistance from orchid mycorrhizal fungi (OMF) to germinate and some orchid species continue to be dependent on OMF as adults acquiring nutrients from their fungal associations. The primary goal of my dissertation was to investigate the diversity of OMF in epiphytic orchids (the most common ecology) and the orchid's dependency on this association. This association has been studied less frequently and rigorously in epiphytic orchids compared to terrestrial orchids. Amplicon sequencing was used in Chapter 1 to study the fungal community of epiphytic and terrestrial roots of the hemiepiphytic orchid, *Vanilla planifolia*. The analyses revealed diverse fungal symbionts colonizing both epiphytic and terrestrial roots of *V. planifolia* including OMF and ectomycorrhizal (ECM) fungi. In Chapter 2, I used amplicon sequencing to investigate mycorrhizal helper bacteria associated with the OMF of *V. planifolia*. Mycorrhizal helper bacteria were ubiquitous in epiphytic and terrestrial roots but co-occurred less frequently with OMF compared to ECM fungi. Amplicon sequencing was again used in Chapter 3 to examine the diversity and specificity of OMF as well as the potential role of OMF as drivers of host tree preference in a leafless epiphytic orchid, *Dendrophylax lindenii*. *Dendrophylax lindenii* was found to associate with a single OMF (*Ceratobasidium* sp.) but the data were inconclusive regarding the role fungi play as drivers in influencing host preference. The dependence of *D. lindenii* on fungal symbionts was studied in Chapter 4 using stable isotope data. Stable isotopes ^{2}H and ^{13}C were shown to be enriched for *D. lindenii* compared to its surrounding vegetation which suggests that *D. lindenii* is dependent on fungal derived carbon in its natural habitat.

ACKNOWLEDGEMENTS

I want to thank my advisor Dr. Greg Mueller for setting me on this path and encouraging me to pursue the research I wanted to do. Thank you for your mentorship and guidance.

Thanks to my committee, Dr. Louise Egerton-Warburton, Dr. Andrea Porras-Alfaro, Dr. Norm Wickett, and Dr. Lawrence Zettler, who challenged me along the way, and were helpful with their knowledge, advice and time. Special thanks are needed to Dr. Porras-Alfaro and Dr. Zettler who provided me with access to Mexican, Florida and Cuban collaborations to further develop the dissertation.

Thank you to the Soil Fungal Lab, especially Andy Wilson, Chen Ning, Rui Zhang, and Ben Morgan for their valuable knowledge that helped me to learn skills to complete the dissertation. Additionally, I am grateful for the students who I mentored, Simone Baskerville, Josh Dansie, Theresa Nguyen, Jack Norris and Renata Murg. They were wonderful lab assistants. A special thanks to my "last minute" lab assistant Luesoni Johnson.

I am truly grateful for the time and assistance my collaborators provided me in the field or through long distance phone calls or emails. First, thanks to my Mexican collaborators: Ma. del Carmen A. Gónzalez-Chávez and Rogelio Carrillo-González, and their students, Crystella and Gilberto. Ma. del Carmen provided me with travel funds from the award from the SAGARPA-CONACYT-SNITT, Mexico to support this research as part of Subproject 03, project 2012-04-190442). A special thanks is also needed for the vanilla growers for access to their farms. The Mexican vanilla farmers were also hospitable and I'm grateful for their kindness. Second, thanks to my Florida collaborators: Mark Danaher, Larry Richardson, and Dr. Michael Kane. I am just as equally grateful for the help of Kathy Cahill from the Garfield Park Conservatory for always

being there to help me with vanilla roots and seed pods. A special thank you to Kevin Feldheim at the Pritzker Lab (Field Museum) for allowing me access to conduct my research. Although I did not obtain data from Cuban Ghost orchids, a special thanks is also needed for my Cuban collaborators who were willing to do research with me and housing me while I learned about the Cuban *Dendrophylax lindenii* in Cuba. Thank you Dr. Ernesto Mújica, Dr. Elaine González, Director Jose Bocourt - Soroa Orchid Garden, Alejandro Camejo – Guanahacabibes NP

Lastly, I would like to thank the funding sources for supporting this research: the Fred Case Grant from The Native Orchid Conference, Botany in Action - Phipps Conservatory Fellowship, Fungal Forest Ecology Award provided by the Mycological Society of America, Catherine Beattie Fellowship provided by the Garden Club of America, and the Plant Biology and Conservation Travel and Research Awards. Also, I would like to thank the Illinois Mycological Association, North American Mycological Association and Mycological Association of Washington for providing me with opportunities to share my research.

Thanks also to countless friends and family that supported me throughout my doctoral studies. Mama Lisa for providing me with care packages definitely helped my learning.

I would like to thank my wife, Eva for her endless support along the way.

Finally, I am grateful to my loving father who told me at a young age to be a doctor and always provided support for my education.

DEDICATION

For my dad, Claurence Johnson, who will always be known

to me as “the baddest man on the planet.”

You saw the beginning of the dissertation but were unable to see the end. You never wanted to outlive any of your children, but you were gone way too soon. Know that your impact on my life will forever shape my outlook on life to persevere through the difficult times.

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INTRODUCTION

Mutualisms between land plants, bacteria and fungi play key ecological roles since the Devonian and possibly assisted early plants to colonize land (Brundrett, 2002; Simon et al., 1993). Mycorrhizas are symbioses that plant roots form with fungi, and this mutualism is present among 92% of extant plant families (Wang and Qiu, 2006). Despite the documented importance of mycorrhizal fungi (MF) as drivers of plant community structure, plant productivity, and distribution, mycorrhizal relationships with Orchidaceae are poorly understood and enigmatic (Smith and Read, 2010). While orchid seed germination and juvenile development are dependent on the association with appropriate orchid mycorrhizal fungi (OMF) (Arditti and Ghani, 2000) the role of OMF in adult orchids is less well understood. Mycorrhizal helper bacteria have been shown to improve the mycorrhizal colonization rates in roots of trees and a number of herbaceous plants but have little been investigated for orchid systems (Garbaye, 1994; Frey-Klett et al., 2007; Bonfante and Anca, 2009). Thus, orchid conservation requires understanding pollinators as well as mycorrhizal fungi and their associated bacteria.

Several differences between the morphology and ecology of epiphytic vs. terrestrial orchids are likely to influence fungal symbionts of epiphytes. Epiphytic orchids are adapted to microhabitats that are water stressed, nutrient poor, and have a high irradiance (Benzing, 2008). Because of their habitat, epiphytic orchids have been assumed to have low levels of colonization by mycorrhizal fungi with low species richness, and a lack of specificity (Dearnaley et al., 2012). However, several recent studies of epiphytic orchids report relatively high fungal specificity (Dearnaley et al., 2012; Otero et al., 2002), and fungal colonization (Suárez et al., 2006). While tree host (phorophyte) specificity (i.e., an epiphytic orchids occurrence on a particular tree species) is at least partially due to abiotic factors such as microclimate, substrate stability,

mineral nutrition and toxicity (Wagner et al., 2015), much less research has focused on mycorrhizal fungi as potential drivers of fine-scale distributions of epiphytic orchids.

The four primary objectives of the dissertation were to 1) characterize the orchid mycorrhizal fungal (OMF) communities of epiphytic roots; 2) characterize the bacteria community, especially mycorrhizal helper bacteria that associate with OMF; 3) examine OMF as drivers of host tree specificity for epiphytic orchids; and 4) investigate the dependence of epiphytic orchids on OMF as a carbon source. Diversity and specificity were the focus of Chapter 1 and 3, examining two very different orchid systems, a hemiepiphyte (*Vanilla planifolia*) and a leafless epiphytic orchid (*Dendrophylax lindenii*). Bacterial relationships were examined in Chapter 2 with *V. planifolia*, while OMF dependency was covered in Chapter 4 with *D. lindenii* stable isotope data.

Overall fungal communities of *D. lindenii* and *V. planifolia* provided insights that the morphology of epiphytic roots may not limit their colonization by fungal symbionts compared to terrestrial orchids. While *D. lindenii* showed a high specificity for a single OMF (*Ceratobasidium* sp.) *V. planifolia* associated with diverse OMF and suggests that it can be characterized as a generalist for OMF. Thus, it is too early to form broad generalities regarding fungal diversity, community composition, and OMF specificity for epiphytic orchids.

A unique observation that was observed for *V. planifolia* terrestrial and epiphytic roots were its associations with diverse ECM fungi contrary to *D. lindenii* which was dominated by OMF. The habitat of *V. planifolia* likely has ECM host trees to establish these associations unlike the habitat of *D. lindenii* where the ECM host trees were distant. Alternatively, this may be indicative of the orchid's ecology. ECM fungi have been commonly reported for terrestrial

orchids and the terrestrial roots of the hemiepiphytic *V. planifolia* may have facilitated the colonization of ECM fungi.

Detecting putative MHB co-occurring with *Ceratobasidium* and *Sebacina* supports the few prior reports of bacterial and OMF interactions (McCormick et al., 2014; Novotná and Suárez, 2018). Functional studies will be needed to determine that these interactions are beneficial to both partners. Detecting MHB associating with ECM fungi in orchid roots was not surprising because of their prior detection in other plant hosts. Higher diversity and frequency of interactions of putative MHB with ECM fungi over OMF in both terrestrial and epiphytic roots of *V. planifolia* needs additional investigations to determine if differences in these interactions can be extrapolated to other OMF species and to other orchids species.

The dissertation also reveals the usefulness of methods as well as pitfalls to avoid for future orchid mycorrhizal studies. High-throughput sequencing such as amplicon sequencing are efficient for characterizing fungal and bacterial communities although future work to determine the function of symbionts will be required to determine true relationships. The failure to detect *Tulasnella*, a common OMF is potentially due to a primer bias, so further work using additional primers are needed to detect *Tulasnella* within the habitats of *V. planifolia* and *D. lindenii*. ^{2}H is a more informative for distinguishing nutritional modes of orchids, because of potential confounding CAM photosynthesis signatures for $\delta^{13}C$ values can be misleading when predicting partial mycoheterotrophy of orchids.

This work was part of a larger international collaboration to improve the production of Mexican vanilla and provided insights to improve its cultivation. We determined that farming practices of *V. planifolia* influences the species richness of its fungal symbionts. The presence of higher diversity of fungal symbionts (OMF and ECM fungi) at wild natural farms compared to

highly managed farms is an indication that farming practices should be considered for improving the yields and maintaining healthy *V. planifolia* plants. Results from my study have been provided to our Mexican collaborators for inclusion in their recommendations.

Orchid conservation requires understanding their associations with fungi and bacteria. *D. lindenii* interactions with a single *Ceratobasidium* from its seedling stage to its adult stage indicated that this fungal symbiont is likely essential for its survival in its natural habitat at the FPNWR. Investigations of bark fungal communities for trees with and without *D. lindenii* did not reveal differences in the presence of the specific *Ceratobasidium* which suggests that the fungus is not driving the fine scale distribution pattern of the orchid. Low colonization of the specific *Ceratobasidium* was detected from some of the micropropagated transplants. Further research is needed to determine how this is impacting their fitness.

Chapter 1 - Differences between the mycorrhizal fungal communities in epiphytic and terrestrial roots of a hemiepiphytic orchid, *Vanilla planifolia*

Abstract

Most orchids, 69%, are epiphytic, but little is known about the fungal symbiosis they have compared to terrestrial orchids. To study differences between the fungal symbionts (specifically mycorrhizal fungi) of epiphytic and terrestrial roots we characterized the fungal communities of a hemiepiphytic orchid, *Vanilla planifolia,* from four Mexican farms with different management systems. Fungal communities of epiphytic roots were distinct from those of the terrestrial roots. Mycorrhizal fungal communities, species traditionally reported as orchid mycorrhizal fungi plus ECM fungi, however, did not differ and the mycorrhizal fungal communities of epiphytic roots were a subset of the mycorrhizal fungi found in terrestrial roots. We identified 40 OTUs as putative mycorrhizal fungi, including traditional orchid mycorrhizal fungi (Cantharellales such as *Ceratobasidium*, *Sebacina*, and *Thanatephorus* species), and species of several genera of ectomycorrhizal (ECM) fungi. This is the first report of a diverse community of ECM fungi in association with epiphytic orchid roots. Epiphytic roots had lower abundances of mycorrhizal fungi than terrestrial roots. Our study suggests that ECM fungal associations for *V. planifolia* are common, and further research is needed to understand their importance in the ecology of epiphytic orchids.

Introduction

Orchids are the largest flowering plant family in the world with an estimated 27,800 species (Chase et al., 2015; Christenhusz and Byng, 2016). The family occurs worldwide, and

the majority (69%) are adapted to grow as epiphytes (Zotz, 2013). Although pollinators are essential drivers of orchid diversity (Cozzolino and Widmer, 2005), mycologists have argued that fungi are also involved in fostering orchid diversity and their distribution patterns (Otero and Flanagan, 2006). All orchids require orchid mycorrhizal fungi (OMF) to germinate and in some cases, continue to use fungi as adults (Yoder et al., 2000; Rasmussen et al., 2015; Gebauer et al., 2016). Understanding the relationship between orchid diversity and OMF has received some attention (Taylor et al., 2004; Shefferson et al., 2007; Roche et al., 2010; Waterman et al., 2011). More information is needed to understand fundamental aspects of OMF diversity, including potential differences in OMF composition and abundance between epiphytic and terrestrial orchids.

Orchid mycorrhizal fungi are facultative biotrophs. They are a polyphyletic group of mainly Basidiomycota belonging to the form genus *Rhizoctonia*, a group consisting of Ceratobasidiaceae, Sebacinales (Serendipitaceae), and Tulasnellaceae species (Dearnaley et al., 2012). In general, mature roots of both epiphytic and terrestrial orchids have been reported to associate with OMF with varying degrees of specificity from non-specific to highly specific, e.g., associating with a single phylogenetic clade of *Rhizoctonia*. Additionally, some non-photosynthetic terrestrial orchids frequently associate with ectomycorrhizal (ECM) fungi (Taylor and Bruns, 1997; McKendrick et al., 2000). These orchids are considered mycoheterotrophic where the orchid is achlorophyllous and use the carbon derived from ECM associations with other photosynthetic hosts. These ECM fungi include Ascomycota [e.g., *Tuber*, (Selosse et al., 2004)] and Basidiomycota fungi [e.g., *Inocybe*, *Russula*, and *Scleroderma* (Taylor and Bruns, 1997; Bidartondo et al., 2004; Selosse et al., 2004; Roy et al., 2009; González-Chávez et al., 2018)]. Epiphytic orchid associations with ECM fungi have rarely been reported (Martos et al.,

2012; Kartzinel et al., 2013), although terrestrial orchids that are photosynthetic have been observed in shaded forests to associate with ECM fungi (Julou et al., 2005). Martos *et al.* (2012) hypothesized that epiphytic orchids likely co-evolved with fungal symbionts and carbon is available to fungal symbionts because of the epiphyte's higher photosynthetic capacity in sunlight compared to terrestrial orchids.

Several differences between the morphology and ecology of epiphytic vs. terrestrial orchids are likely to influence fungal symbionts of epiphytes. Epiphytic orchids are adapted to microhabitats that are water stressed, nutrient poor, and have a high irradiance (Benzing, 2008). Microscopy studies of epiphytic roots have shown frequent OMF colonization where the root adheres to the surface of the host tree bark (Smith and Read, 2010). Epiphytic roots have a velamen, an external and hygroscopic tissue layer around the cortex, while terrestrial roots also have a velamen that may be spongy compared to the harder epiphytic roots (Stern and Judd, 1999)

The traditional reliance on culture-based methods has limited the characterization of the total fungal community of orchid roots because many fungi are either unculturable or very slow-growing (Allen et al., 2003; Arnold et al., 2007). This includes the vast majority of ECM fungi. The use of environmental sequencing (i.e., DNA isolated and sequenced directly from an environmental sample) is an improved alternative to culture-based methods for determining mycorrhizal fungi (Manter et al., 2010; Lundberg et al., 2012; Edwards et al., 2015). Recent studies of epiphytic and terrestrial orchids in temperate and tropical habitats have revealed the usefulness of amplicon sequencing of environmental samples to characterize fungal symbionts in orchid roots (Oja et al., 2015; Cevallos et al., 2017; Herrera et al., 2019).

To assess differences in fungal symbionts between epiphytic and terrestrial roots, we compared amplicon sequences of the epiphytic and terrestrial roots from *Vanilla planifolia* Jacks. ex Andrews, a hemiepiphytic orchid. Morphologically the epiphytic roots of *V. planifolia* are chlorophyllous (Díez et al., 2017), while its terrestrial roots are achlorophyllous (Stern and Judd, 1999). In previous studies, *Rhizoctonia*-like fungi such as *Ceratobasidium*, *Thanatephorus*, and *Tulasnella* were observed in both root types of *V. planifolia* (Porras-Alfaro and Bayman, 2003, 2007; Bayman et al., 2010) and a recent study by González-Chávez *et al.* (2018) found *Scleroderma*, an ECM fungus, forming pelotons in the terrestrial roots of *V. planifolia*.

The present study was part of a broader interdisciplinary effort to understand the biotic and abiotic factors that influence the production of *V. planifolia*, an economically valuable crop in Mexico sold worldwide for its aroma and flavor (Herrera-Cabrera, 2016; Havkin-Frenkel and Belanger, 2018). Typically, *V. planifolia* crops are a monoculture with terrestrial roots grown in soil while epiphytic roots are aboveground rooted on "tutors" (supports) of live or dead host trees or inorganic materials (concrete). Therefore, we examined the diversity of fungal symbionts associating with *V. planifolia* epiphytic and terrestrial roots under different farming systems in Mexico. We hypothesized that terrestrial roots of *V. planifolia* have a distinct and more diverse fungal symbiont community compared to its epiphytic roots. We also hypothesized that *V. planifolia* of highly managed farms have lower species richness of fungal symbionts than wild natural farms.

Materials and Methods

Sampling sites and sample collections

Epiphytic and terrestrial roots were randomly sampled from four *V. planifolia* farms in Mexico during March and April 2014 (during anthesis) (Table S1). Epiphytic roots that adhered to substrates were collected. Three of the farms were in the state of Veracruz, and the fourth farm was in the state of Puebla. Farms were in 1 de Mayo, Papantla de Olarte Ocampo, Veracruz (20° 17' 719" N,97° 15' 909" W); 20 Soles, Papantla de Olarte, Veracruz (20° 25' 1.57"N, 97° 18' 8.04" W); two farms at Puntilla Aldama, San Rafael, Veracruz (20° 10' 45.58" N, 96° 54' 13.69" W), and at Pantepec, Puebla (20° 30' 18" N, 97° 53' 22" W). Each vanilla farm was characterized into one of three general categories based on their farming practices: 1) wild natural, 2) traditional, and 3) highly managed (see Fig. S1 and Table S1). For instance, the wild natural farm of 1de Mayo grew *V. planifolia* within a secondary forest. The traditional farms of Puntilla Aldama and 20 Soles used living trees and dead trees as tutors (support) for vanilla. The highly managed farm at Pantepec used cement tutors. All farms grew *V. planifolia* as a monoculture with no other crops interspersed (González-Chávez et al., 2018).

Epiphytic and terrestrial roots of five healthy *V. planifolia* plants were sampled from each of the four farms for a total of 40 root samples (20 epiphytic and 20 terrestrial). The length of each root sample was approximately 5 mm. Each root sample was immediately stored in cetyltrimethylammonium bromide (CTAB) buffer and then placed on ice. Long term storage of root samples was at 4 ℃. Mycorrhizal colonization was confirmed by observing the roots under a microscope. Root samples were surface sterilized with 70 % ethanol and 50 % Clorox® (=2.6 % sodium hypochlorite) following the methods of Bayman *et al.*, (1997) before DNA extraction.

Amplicon library preparations

Genomic DNA was extracted with a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following instructions from the manufacturer. The presence of genomic DNA was visualized using electrophoresis gels.

Amplicon libraries for the Internal Transcribed Spacer (ITS) region 2 were generated using a three-step PCR approach for paired-end sequencing on an Illumina miSeq (Table S2). First, 1 µL of primers ITS86f (5′- GTGAATCATCGAATCTTTGAA-3′; Turenne et al., 1999) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′; White et al., 1990) were used in 25 µL reactions that contained 1 µL of genomic DNA, (5 mM of final concentration), 9.5 µL of PCR-grade water, and 12.5 µL of 2X My Taq Master Mix (Bioline). The thermal cycler conditions were: 2 min at 94 °C, followed by 32 cycles at 94 °C for 45 s, then annealing at 59 °C for 45 s, an extension for 1 min at 72 °C, and a final extension at 72 °C for 5 min. These PCR products were then purified with a concentration of 0.8x AMPure XP beads (Beckman Coulter, Brea CA).

Next, for the second round of PCRs, primers ITS86F-adpt and ITS4-adpt (see Table S2) were used in 25 µL reactions to amplify the PCR productions generated from the first-round of PCRs. This second PCR step used the same reagents and quantities as the first PCRs, however, this PCR step was reduced to 25 cycles. Gel electrophoresis was used to visualize successful PCRs. These PCRs were cleaned with a concentration of 0.8x AMPure XP beads.

For the third-round of PCRs, index adapters (Nextera XT Index Kit v2 Set B, 96 indices, 384 samples, Illumina, San Diego, California) were ligated onto the amplicons generated from the second-round of PCRs. The third round used the same reagents and quantities as the first and second rounds for 8 cycles. PCR products were then purified with a concentration of 1.0x AMPure XP beads then quantified using a Qubit 2.0 Fluorometer (Life Technologies, Burlington, ON, Canada) with the Qubit dsDNA HS kit (Invitrogen). The PCR products were

pooled into equimolar concentrations for a final amplicon library. The approximate bp lengths of the final amplicon library were determined using a Bioanalyzer - Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) with a Bioanalyzer DNA 1000 chip. Sequencing of the final amplicon library was completed on an Illumina miSeq (2 x 250 bp kit) at the Field Museum, Chicago, IL. For the miSeq run, 40 % PhiX (Control v3, Illumina, Inc., San Diego, CA, USA) was spiked at the same equimolar concentration as the final amplicon library. The sequences generated from this study were submitted to NCBI's Sequence Read Archive and associated with BioProject PRJNA540935.

Data processing and statistical analyses

Paired-end reads were processed using the Pipits pipeline (version 2.2, (Gweon et al., 2015). First, forward and reverse sequences were joined (VSEARCH, (Rognes et al., 2016), and quality filtered with FASTQ_QUALITY_FILTER (FASTX-toolkit, (Hannon, http://hannonlab.cshl.edu) using the default settings of Pipits. During this quality filtering step global singletons were removed. Fungal ITS region 2 reads were then detected and retained, (ITSx, (Bengtsson-Palme *et al.*, (2013); HMMER3 (Mistry et al., 2013) while chimeras were removed (VSEARCH, (Rognes et al., 2016). Lastly, reads were clustered at 95% sequence similarity into Operational Taxonomic Units (OTUs) and, taxonomy was assigned with the RDP classifier (Wang et al., 2007) that relied on the UNITE fungal database (Abarenkov et al., 2010; Nilsson et al., 2019). The default setting for a confidence threshold of 85 % for RDP classifier was used in Pipits to assign taxonomy. Finally, to assess functions of OTUs, OTUs were assigned to guilds using the program FUNGuild v1.0 (Nguyen et al., 2016). FUNGuild

assignments did not contain OMF assignments, so OMF assignments were determined based on the OMF taxa reported in Dearnaley *et al.*, (2012).

Rarefaction curves were determined using iNext, (Hsieh et al., 2016) an R package (R Development Core Team, 2012) that interpolated and extrapolated the data. After determining rarefaction curves, OTUs that had <1000 sequences and not present in two or less samples were discarded from the overall analyses. Samples were then normalized with Cumulative Sum Scaling (metagenomeSeq, (Paulson et al., 2013). Principal coordinate analysis (PCoA) with Bray Curtis distances were used to investigate the differences between the fungal communities of each sample as well as differences with a subset of the data that included only putative fungal symbiont sequences. The significance of β diversity metrics was analyzed by permuting the raw data (10000 permutations) using the function adonis in the R package vegan (adonis function is analogous to Anderson's (2001) "permutational manova."

Results

Our analyses of *V. planifolia* roots (i.e., 18 epiphytic and 19 terrestrial root samples passing quality filtering) from four Mexican farms yielded 656,918 quality reads (i.e., epiphytic = 355,299; terrestrial = 301,619). We characterized 834 OTUs after clustering reads at 95% sequence similarity. OTUs were mostly Ascomycota (55%) and Basidiomycota (22%), with the remaining 23% of total reads classified as "unknown" at the phylum level. No OTUs were found at more than 15% of read abundance. The most dominant order for Ascomycota was Sordariomycetes with 27% of the total reads followed by Dothidiomycetes with 12% of the total reads. A dominant *Fusarium* OTU accounted for 8% of the total terrestrial reads and 2% of the

epiphytic reads. For Basidiomycota, Agaricomycetes accounted for 16% of the total reads while the other classes totaled 6%.

Most OTUs (60%) could not be assigned to trophic modes with the program FUNGuild (Table S3). Of the remaining 40% assigned, saprotrophs comprised 12%, symbiotrophs 5%, pathotrophs 6.5%, and multiple guild associations 16.5% (e.g., pathotroph-symbiotroph) (Table S3). FUNGuild is not effective at assigning trophic modes of genera that have multiple trophic modes (e.g., *Ceratobasidium* sp. are assigned as pathogens in FUNGuild but are well documented symbionts in orchids). Dearnaley *et al*. (2012) was used to further resolve trophic modes resulting in 9.8% of total reads being assigned as OMF and ECM fungi (i.e., 40 OTUs as fungal symbionts (Table S4). Mycorrhizal fungi included species of *Ceratobasidium*, *Sebacina*, and *Thanatephorus*, as well as ECM fungi such as *Inocybe*, *Russula, Scleroderma, Tomentella,* and *Tuber*. We also detected saprobic fungi such as *Marasmius*, *Mycena*, and *Gymnopus* that we characterized as putative fungal symbionts for this study based on Dearnaley *et al*. (2012). In prior microscopy of root samples, we detected fungal pelotons in all the epiphytic and terrestrial root samples of *V. planifolia* (González-Chávez *et al*., 2018).

The PCoA ordination of total fungal communities revealed distinct clusters from epiphytic and terrestrial roots in ordination space (Fig. 1). These differences were further highlighted with a PERMANOVA revealing that differences were significant between epiphytic and terrestrial fungal communities ($F_{1, 35} = 3.65$, $r^2 = 0.09$, $p < .05$). Furthermore, rarefaction curves revealed that epiphytic roots had a greater richness in comparison to terrestrial roots (Fig. S2), but OTU richness for each sample was similar with a range of 28 to 227 observed OTUs (Fig. S3).

Conversely, putative fungal symbionts were more diverse in terrestrial roots compared to epiphytic roots (Fig. 2). Epiphytic roots associated with a subset of terrestrial root taxa, sharing 86% of their fungal symbionts (Fig. S4 B). Moreover, we detected lower read abundances for fungal symbionts in most epiphytic root samples (Fig. 2). Fungal symbionts of the total epiphytic fungal community comprised 2% of the total reads whereas the terrestrial community was 19% of the total reads.

Ceratobasidiaceae were the dominant fungal symbionts in *V. planifolia* roots (both epiphytic and terrestrial) (Fig. 2). We further refined the taxonomy of Ceratobasidiaceae OTUs using the species hypothesis on UNITE (Nilsson et al., 2019) and identified several of the OTUs as *Thanatephorus* species. Other fungal symbionts such as *Inocybe* (OTU 131) and *Tuber* (OTU 181) were frequent among terrestrial samples occurring at all farms. Other fungal symbionts such as *Gymnopus* (Omphalotaceae), *Marasmius* (Marasmiaceae), *Mycena* (Trichlomataceae), *Russula* (Russulaceae), *Scleroderma* (Sclerodermataceae), and *Thelephora* (Thelephoraceae) were rare (Fig. 2).

Terrestrial roots revealed higher read abundances of fungal symbionts for the wild natural farm compared to the highly managed farm (Fig. 2). Terrestrial roots of the wild natural farm (1 de Mayo) had increased read abundances for both *Ceratobasidium* (OTU 66) and *Inocybe* (OTU 131) (Fig. S5). *Inocybe* OTU 131 was present at both farms, but the read abundance at the wild natural farm was greater. Also, rare taxa such as *Mycena* and *Marasmius* (non-traditional OMF) were absent at the highly managed farm. OTUs of *Russula* and *Inocybe* were present among both highly managed and wild natural farms. We detected *Gymnopus* at the highly managed farm that was absent from the wild natural farm (Fig. S6). Finally, we observed differences between the

two other farms (traditional farms) in terms of the presence and absence of rare taxa such as *Mycena*, *Marasmius*, and *Scleroderma* OTUs (Fig. S6).

At all farms, fungal symbionts sequenced from terrestrial roots had 10 times more reads than epiphytic roots (Fig. S5). In epiphytic root samples, two *Ceratobasidium* OTUs (OTU 93 and OTU 95, Fig. S6) had abundant reads. Rare fungal symbionts such as *Sebacina* (OTU 225), *Scleroderma* (OTU 53), and *Tuber* (OTU 181) were present within epiphytic roots at multiple farms. Lastly, although epiphytic roots lacked some rare taxa, for example, *Russula* at the traditional farm (Puntilla) and *Sebacina* at the highly managed farm (Pantepec), these rare taxa were present in their corresponding terrestrial roots.

Discussion

Using amplicon sequencing, we made in-depth comparisons between the fungal communities of morphologically distinct epiphytic and terrestrial roots of the hemiepiphytic orchid, *V. planifolia*. Our results document differences in the overall fungal communities of epiphytic versus terrestrial roots. Epiphytic roots had greater OTU richness overall with 692 OTUs compared to 342 OTUs in terrestrial roots (Fig. S4 A, C). However, we did not detect differences in fungal symbiont communities (OMF and ECM fungi) as terrestrial and epiphytic roots shared 86% of their OTUs (Fig. S4 B). We also observed lower species richness of fungal symbionts at the highly managed farm (growing on concrete tutors) than at the wild natural farm (growing on living trees). Lastly, we documented several taxonomically diverse ECM fungi colonizing both epiphytic and terrestrial roots. To our knowledge, this is the first report of ECM fungi forming a significant portion of the fungal community of epiphytic roots.

We detected most of the major traditional OMF genera, i.e., *Ceratobasidium*, *Thanatephorus*, and *Sebacina*. However, we did not detect *Tulasnella,* which was reported from *V. planifolia* by (Porras-Alfaro and Bayman, 2007) and many other orchids (Currah et al., 1997; Suárez et al., 2006; Zettler et al., 2017), our failure to detect *Tulasnella* in our study is likely due to primer bias rather than an absence in the roots. Although the primers we employed were optimized to detect OMF including *Tulasnella* OTUs (Waud et al., 2014), Tedersoo *et al.*, (2015) noted *Tulasnella* sequences having mismatches with these primers, and this could potentially limit the efficiency of these primers to amplify *Tulasnella* species.

A diversity of fungal guilds in addition to potentially symbiotic fungi were detected in the roots we examined. For instance, we repeatedly observed a *Fusarium* OTU within roots of healthy *V. planifolia* plants. Similar to our study, researchers have routinely reported *Fusarium* species as endophytes in *V. planifolia*, although it is also considered a common fungal pathogen of *V. planifolia* in Mexico (Havkin-Frenkel and Belanger, 2018). In contrast, the other principal fungal pathogen of *V. planifolia*, *Colletotrichum*, (Havkin-Frenkel and Belanger, 2018) was rarely detected in our study with <1% of the total reads.

Similar fungal symbiont communities in terrestrial and epiphytic roots suggest that root morphology and physiology does not constrain the fungal symbiotic diversity for either root type. For example, the observation of similarly high fungal symbiont richness in the epiphytic roots of *V. planifolia* that are photosynthetic supports the findings of McCormick *et al.*, (2004) that photosynthetic capacity might not influence mycorrhizal fungi diversity. McCormick *et al.*, observed that terrestrial orchids had lower specificity for OMF than non-photosynthetic orchids. While fungal symbionts in epiphytic roots attached to organic tutors possibly use the bark or wood of these tutors as sources of nutrients, such nutrient sources are lacking at the highly

managed farm (Pantepec) where the epiphyte roots were attached to concrete tutors. This suggests that fungal symbionts of epiphytic roots may use different venues to obtaining nutrients including potential terrestrial sources and may even grow systemically throughout the plant, however, this hypothesis must be tested.

While we did not detect a difference in richness, we did detect a difference in read abundance of fungal symbionts between root types (Fig. S6). This low read abundance is consistent with the findings of (Porras-Alfaro and Bayman, 2007) that observed fewer pelotons in epiphytic roots than terrestrial roots of *V. planifolia*. Other microscopy studies also reported epiphytic roots being colonized less than terrestrial roots and that colonization was restricted to the root surface adjacent to substrates (i.e., host tree bark) (Hadley and Williamson, 1972; Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Porras-Alfaro and Bayman, 2003). This lower read abundance may be due to passage cells in epiphytic roots limiting mycorrhizal fungal colonization as proposed by Chomicki *et al.*, (2014).

Although more research is required, we hypothesize that orchids may be predisposed to forming associations with ECM fungi and therefore ECM fungal colonization of epiphytic orchids may be widespread. Some terrestrial orchids associate with Ceratobasidiaceae and *Tulasnella* species that are connected to photosynthetic trees as ECM fungi (Warcup, 1985; Yagame et al., 2008, 2012; Bougoure et al., 2009). Recent research by González-Chávez *et al.*, (2018) documented pelotons formed by the ECM fungus *Scleroderma* in terrestrial roots of *V. planifolia*. As pelotons are the sites of nutrient exchange in orchid mycorrhizae (Kuga et al., 2014), it is hypothesized that this *Scleroderma* was functioning as an orchid mycorrhiza.

Our results are consistent with Gebauer *et al.*, (2016) who proposed that most orchids, including putative fully autotrophic orchids, are likely partial mycoheterotrophs. Our use of

amplicon sequencing provided a comprehensive characterization of the fungal community of epiphytic and terrestrial roots of *V. planifolia*. The majority of ECM fungi cannot be cultured and were therefore not detected in previous culture-based studies. Studies that employed Cantharellales specific primers would also have missed these taxa (Tedersoo et al., 2015). Additional research is needed to understand the function of ECM fungi in epiphytic orchid roots. Further studies should not overlook root endophytes, pathogens, and fungal symbionts to understand fungal communities of the epiphytic niche. These data are important for improving the management of *V. planifolia* farms, and more broadly, for enhancing *ex situ* orchid conservation.

Chapter 2 - Interactions between putative mycorrhizal helper bacteria and fungal symbionts of a hemiepiphytic orchid, *Vanilla planifolia*.

Abstract

Mycorrhizal fungi are essential symbionts of plants that improve plant growth and fitness. Studies of mycorrhizae have shown that specific helper bacteria associate with mycorrhizal fungi may enhance the rate of mycorrhization. Although mycorrhizal helper bacteria (MHB) are mainly described for ectomycorrhizal (ECM) fungi, little is known about the MHB of orchid mycorrhizal fungi (OMF). Previous investigations from Chapter 1 showed that roots of *Vanilla planifolia,* a hemiepiphytic orchid, were colonized by fungal symbiont taxa that are traditionally classified as OMF (e.g., Ceratobasidiaceae) and ECM fungi (e.g., *Scleroderma* and *Tuber*), therefore we investigated *V. planifolia* roots for the presence of putative MHB. We used 16S amplicon sequencing to characterize the microbiota from DNA extractions from 20 epiphytic and 20 terrestrial root samples from Chapter 1. We used co-occurrence network analyses and a bipartite network to visualize MHB and fungal interactions and to detect modularity. We detected 498 bacterial OTUs (97% sequence similarity) and classified 42 OTUs as putative MHB based on reports from the literature of these bacterial taxa as MHB. All putative MHB were ubiquitous among epiphytic and terrestrial roots and showed no differences between *V. planifolia* root types. The co-occurrence network produced 233 nodes and 734 edges, of which four OTUs were OMF, three were ECM fungi, and 15 OTUs were classified as putative MHB. Three Ceratobasidiaceae OTUs co-occurred frequently with putative MHB, but one Ceratobasidiaceae OTU formed a module with *Pseudomonas* OTUs and the other Ceratobasidiaceae formed a module with a ubiquitous *Rhizobium* OTU. Sebacina OTU co-occurred frequently with putative MHB similar to ECM fungi. Our results suggest that Ceratobasidiaceae have fewer interactions

with MHB taxa compared to ECM fungi. Nonetheless, the presence of putative MHB in *V. planifolia* roots shows that further investigations are needed to reveal whether these putative MHB enhance the function of orchid mycorrhiza or enhance the fitness of *V. planifolia*.

Introduction

About 80% of plants form beneficial symbioses with mycorrhizal fungi that improve their growth and fitness (Smith and Read, 2010). Studies of the mycorrhizal symbiosis have established that plant growth and fitness can also be enhanced by a third interaction with helper bacteria (Garbaye, 1994; Frey-Klett et al., 2007). Mycorrhizal helper bacteria (MHB) interact with the mycorrhizal symbiosis by improving mycorrhizal fungus colonization rates in roots (Frey-Klett et al., 2007). Mycorrhizal helper bacteria are often detected close to mycorrhizal fungi within plant roots and belong to diverse phyla such as Actinobacteria, Firmicutes, and Proteobacteria (Bonfante & Anca, 2009). Roots that form associations with arbuscular mycorrhizal fungi (AMF), and ectomycorrhizal (ECM) fungi show numerous associations with MHB taxa that colonize and assist these mycorrhizal fungi (Frey-Klett et al., 2007). In comparison, orchid mycorrhizal fungi (OMF) are understudied and core MHB taxa that potentially associate with this mycorrhizal symbiosis are largely unknown.

Orchid research has broadly investigated the associations of bacteria with orchid roots (Bayman and Otero, 2006), and bacteria that have been documented in epiphytic orchids include diazotrophs (nitrogen-fixing bacteria) that colonize the rhizoplane (Tsavkelova et al., 2001). These diazotrophic bacteria were hypothesized to acquire nitrogen in nutrient poor microhabitats. These bacteria have not been classified as MHB. Other endophytic bacteria of orchid roots were reported to enhance orchid seed germination (Wilkinson et al., 1989), as well as improve root

growth through the production of indole acetic acid (IAA) (Tsavkelova et al., 2003, 2007). Some recent evidence by Novótona *et al.*, (2018) has documented the OMF, *Serendipita* sp., isolated from an orchid, associates with Firmicutes and Proteobacteria (Novótona et al., 2019). Although the study of (Novotná and Suárez, 2018) did not identify these bacteria as MHB, their evidence suggests the potential of OMF to associate with taxa that have been previously classified as MHB. Moreover, it is still unclear what core MHB potentially interacts with OMF within the orchid root *in situ*.

To determine putative MHB that associate with OMF we employed co-occurrence network analysis to data from amplicon sequencing of fungal and bacterial communities found in *Vanilla planifolia* roots. *Vanilla planifolia* Jacks. ex Andrews is an ideal orchid to investigate the associations of OMF with MHB because our previous investigation (see Chapter 1) of this hemiepiphytic orchid revealed that traditionally classified OMF (*Ceratobasidium*, *Sebacina*, and *Thanatephorus*) and ECM fungi (e.g., *Inocybe*, *Scleroderma* and *Tuber*) colonized both its epiphytic and terrestrial roots. The presence of these mycorrhizal fungi allows for a comparison of significant interactions between MHB of ECM fungi and traditionally classified OMF. The use of co-occurrence networks can provide insights into interactions of microbial communities with statistical support from bipartite networks that supplements our overall study (Faust and Raes, 2012; Dormann and Strauss, 2013). For instance, co-occurrence network analyses have previously hypothesized a variety of interactions of co-occurrences within microbial communities within plants, the human gut, oceans, and soil (Fuhrman and Steele, 2008; Qin et al., 2010; Bakker et al., 2014; Nielsen et al., 2014).

Our primary objective was to characterize the MHB community that potentially interacts with *V. planifolia* fungal symbionts (OMF and ECM fungi). As demonstrated in Chapter 1,

amplicon sequencing is an effective method compared to culture-based methods to characterize root microbiota, especially when <1% of bacteria are culturable (Torsvik et al., 1990). We hypothesized that the same community of MHB associates with OMF and ECM fungi.

Materials and Methods

Vanilla planifolia root samples (i.e., epiphytic and terrestrial roots) were collected in 2014 at four Mexican farms from Pantepec, Puebla, San Rafael, Veracruz, and Papantla de Olarte Ocampo, Veracruz, (see Fig. S1 and Table S1 in Chapter 1). We randomly sampled a total of 20 *V. planifolia* individuals, five plants per farm, and stored root samples at 4°C in microcentrifuge tubes of cetyltrimethylammonium bromide (CTAB) buffer. Epiphytic roots that adhered to substrates were collected. Root samples were surface sterilized following the methods of Bayman *et al.*, (1997) which used 70% ethanol and 50% Clorox® (=2.6% sodium hypochlorite). Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol.

ITS amplicon sequencing

Amplicon libraries for fungal communities were generated from ITS amplification of genomic DNA extracted from the same samples as those used to amplify bacterial communities. We also used s three-step PCR to amplify ITS region 2 with the following primers ITS86f (5′-GTGAATCATCGAATCTTTGAA-3′; Turenne et al., 1999) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990) (Chapter 1).

16S rRNA gene amplicon sequencing and analyses

Amplicon libraries were generated from 16S rRNA genes using a three-step PCR following the methods of Chapter 1. Then the amplicon libraries were sequenced on an Illumina miSeq. Briefly, the first PCR was completed with the primers, 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) (Table S1). Second and third PCR steps used modified primers (515f-adpt / 806r-adpt) and primers from Nextera XT Index Kit v2 Set B to amplify PCR products from the previous PCR step. Thermocycler conditions for each PCR step followed the first PCR step of 3 min at 95°C, followed by 25 cycles at 95°C for 30 s, then annealing at 55°C for 30 s, an extension for 30 s at 72°C, and a final extension at 72°C for 5 min. PCR cycles for the third PCR step were 8 cycles. All successful amplicon libraries for each sample were pooled into equimolar concentrations based on concentrations analyzed from a Qubit 2.0 Fluorometer. The bp lengths of the final amplicon library were assessed using a Bioanalyzer - Agilent 2100. The final amplicon library was sequenced with 40% PhiX in an equimolar concentration, then sequenced on an Illumina miSeq (2 x 250 bp kit) at the Field Museum, Chicago, IL. The sequences generated from this study were submitted to NCBI's Sequence Read Archive and associated with BioProject PRJNA540935.

Paired-end reads were processed with the pipeline USEARCH 9.2.64 (Edgar, 2010). USEARCH quality filtered sequences, detected chimeras, and clustered Operational Taxonomic Units (OTUs) at 97% sequence similarity (Edgar, 2013). Taxonomy was assigned to reads with the UTAX algorithm (Edgar, RC (unpublished) against the RDP training set 15 (Cole et al., 2014) with a confidence of 80%. Operational taxonomic units assigned to Chloroplast, Streptophyta, and "Unassignable" at the phylum and kingdom levels were removed from

downstream analyses. OTU abundances for each sample was normalized with Cumulative Sum Scaling in the R package metagenomeSeq (Paulson et al., 2013)

To detect differences between samples we used weighted UniFrac analysis (e.g., root type) (McMurdie and Holmes, 2013). The significance of the UniFrac analysis relied on 1000 permutations of the raw data with the function adonis that is part of the R package vegan (adonis function is analogous to Anderson's (2001) “permutational manova”). Principal coordinate analysis (PCoA) in the R package phyloseq (McMurdie and Holmes, 2013) was used to visualize the UniFrac analysis in multivariate space.

Co-occurrence networks were generated using the app CoNet (version 1.1 beta (Faust and Raes, 2016) in the Cytoscape program (version 3.7.1, Smoot *et al.*, 2011). We used OTU abundance data of 16S and ITS reads (Chapter 1, see Table S4). All steps of filtering and normalization of the data was performed in CoNet. First, we removed rare taxa, i.e., OTUs present in a single sample and then abundance data were normalized to account for differences in sequencing depth. Co-occurrence statistics to generate significant edges and nodes for the final network was performed in two steps as outlined in Faust and Raes (2016). The network used two correlations, Spearman and Kendall correlation and the final network that was produced retained edges that were significant if one or both of the correlations were retained. Edges for the final network were selected from 25% quantile of scored edges, and the significance of the edges was calculated with 100 permutations. In contrast to recommendations by Faust and Raes (2016), we skipped the last bootstrap step because the stringent settings removed the majority of nodes to make sufficient comparisons for the fungal symbionts. The final network was visualized using Cytoscape. Within the network we classified bacterial OTUs as putative MHB based on the literature reviews of Frey-Klett *et al.*, (2007) Bonfante & Anca, (2009), and Tsavkelova (2011).

A bipartite network was produced to better visualize the interactions between the OMF and ECM fungi nodes, and putative MHB following instructions from the Cytoscape manual (version 3.7.1, Smoot *et al.*, 2011). Additionally, we analyzed a quantitative bipartite network for modules (communities of MHB and OMF and ECM fungi) using the default settings of the function computeModules from the R package bipartite (version, bipartite v2.11, Dormann & Strauss, 2013; Beckett, 2016). The read abundance for each MHB OTU co-occurring with the OMF and ECM fungi was used as the quantitative data matrix input for computeModules.

Results

In total, we obtained 1,009,302 quality filtered reads from 16S amplicon sequencing on an Illumina miSeq. Most, 76% of the total reads, were assigned to Streptophyta (i.e., Chloroplast OTUs that likely represent plant DNA), so we removed these non-bacterial reads from further downstream analyses. We also removed reads that were "unassignable" at the phylum and kingdom levels resulting in a final reduced dataset of 242,417 reads. This reduced dataset clustered into 498 OTUs at 97% sequence similarity.

Proteobacteria was the most abundant phylum which accounted for 73%of the reads (Fig. 1 and 2). Specifically, Enterobacteriaceae OTU-2 was the dominant Proteobacteria OTU in most samples, followed by an unclassified Bacteria OTU (OTU-3) (Fig. 2 and Table S2). Other Bacteria OTUs accounted for less than 5% of reads. PCoA results confirmed a lack of differences between the bacterial communities of epiphytic and terrestrial root samples (Fig. 3)

PCoA results confirmed a lack of differences between the bacterial communities of epiphytic and terrestrial root samples (Fig. 3).

Forty-two OTUs classified as putative MHB were common in epiphytic and terrestrial roots of *V. planifolia* samples and were present in over 50% of all samples. These putative MHB belonged to four phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria), six classes (e.g., Bacilli and Gammaproteobacteria), seven orders (e.g., Burkholderiales and Enterobacteriales), 11 families (e.g., Enterobacteriaceae, Pseudomonadaceae and Streptomycetaceae), and 12 genera (e.g., *Burkholderia* and *Pseudomonas*) (Table S3). Bacteria that were present in more than half of the samples and not classified as putative MHB included an unclassified Bacteria, Rhizobiales species, *Staphylococcus*, and *Sphingomonas*.

A co-occurrence network was generated from 498 bacterial and 40 fungal OTUs (Table 2 and 3). This resulted in a co-occurrence network consisting of 233 nodes (i.e., OTUs) and 724 edges (i.e., co-occurrences) (Fig. 4). The 233 nodes consisted of 225 bacterial OTUs (15 OTUs classified as putative MHB, Fig. 5), and 9 fungal OTUs, *Ceratobasidium* (3 OTUs), *Inocybe* (4 OTUs), *Scleroderma* (1 OTU), and *Tuber* (1 OTU). The majority of putative MHBs (27 OTUs) and fungal OTUs (31 OTUs, OMF and ECM fungi) did not display significant edges and were not retained in the final co-occurrence network because of low read abundances and sample counts (Table 4 and 5).

Co-occurrence network analysis revealed ECM fungi such as *Inocybe*, *Scleroderma*, and *Tuber* OTUs were highly connected to putative MHB that included *Arthrobacter*, *Bacillus*, *Burkholderia, Pseudomonas*, *Rhizobium*, and *Streptomyces* (Fig. 5). In contrast, traditionally classified OMF such as Ceratobasidiaceae OTUs generally co-occurred with *Pseudomonas* OTUs. Another OMF, a *Sebacina* OTU, co-occurred with a single putative MHB, *Paenibacillus* within a subnetwork that was not connected to other bacterial nodes that were connected to the other *Ceratobasidium* and ECM fungi.

The structure of the bipartite network showed differences between the read abundances of the putative MHB that co-occurred with either the OMF or ECM fungi (Fig. 6). Differences were apparent for Ceratobasidiaceae OTUs, which had interactions with putative MHB such as *Pseudomonas* plus *Rhizobium*. These bacteria were recovered at low read abundances when they co-occurred with Ceratobasidiaceae OTUs. In contrast, interactions of putative MHB with ECM fungi included additional putative MHB taxa and these co-occurring putative MHB had higher read abundances (Figs. 6 and 7). Surprisingly, *Sebacina* displayed similar interactions with MHB as ECM fungi, such as more interactions with putative MHB that had high read abundances. The quantitative bipartite network showed four modules. Ceratobasidiaceae OTUs in these modules had the fewest effective partners among the fungi ranging from 1.6 – 3.9, whereas ECM fungi and *Sebacina* had 7.2 – 8.4 effective partners (Table 5). The *Inocybe* OTU interacted strongly with *Bacillus* and *Pseudomonas*, and both *Scleroderma* and *Sebacina* OTUs were generalists with seven MHB taxa (eight OTUs) (Fig. 8).

Discussion

This study investigates interactions (i.e., co-occurrences) between reported MHB and OMF and ECM fungi of *V. planifolia*. The use of co-occurrence networks suggested that some OMF such as *Ceratobasidium* interact less frequently with MHB than with ECM fungi. Our study provides one of the first published reports for interactions between putative MHB and orchid root fungal symbionts (OMF, i.e., *Ceratobasidium* and *Sebacina*, and ECM fungi).

Our findings of *Sebacina* co-occurring with Proteobacteria (*Pseudomonas*) and Acidobacteria were consistent with the report of (Novotná and Suárez, 2018), who reported *Sebacina* (*Serendipita*) co-occurring in cultures with bacteria such as Firmicutes, Proteobacteria

and Acidobacteria. McCormick *et al.*, reported that *Ceratobasidium* isolated from orchid roots associate with endobacteria, however, the bacterial strains were not described (published abstract, (McCormick et al., 2014).

Pseudomonas species have been reported to co-occur with ECM fungi such as *Tuber* species. Mamoun & Olivier (1992) documented that *Pseudomonas* can function as an MHB. Our finding that Ceratobasidiaceae OTU-97 and *Tuber* OTU-181 had similar co-occurrences frequently with *Pseudomonas* OTU-15 suggests that *Pseudomonas* may be functioning as a MHB in *V. planifolia*. Furthermore, although fungal symbionts of orchids co-occur in roots, we did not detect Ceratobasidiaceae OUT-97 and *Tuber* OUT-181 reads in all the same samples. This suggests that *Pseudomonas* co-occurs independently with these fungal symbionts. Tsavkelova *et al.,* (2007) observed *Bacillus, Burkholderia*, and *Pseudomonas* colonizing roots of epiphytic and terrestrial orchids grown in a greenhouse. Tsavkelova *et al.,* (2007) reported that these bacteria produced indole-3-acetic acid (IAA, auxin) in orchid roots. The interaction with *Pseudomonas* and other MHB may provide a source of nutrients for orchids, especially epiphytic orchids that are growing in nutrient poor habitats. For example, putative MHB were detected in epiphytic roots of *V. planifolia* growing attached to concrete supports (Chapter 1).

Co-occurrence networks are a useful method for investigating potential interactions among microbes. But our a-priori designation of putative MHB taxa based on literature reports limits our inferences. We used this a-priori approach because we do not have functional data of any of the bacteria in our study. The majority (211) of the bacteria (non-designated MHB taxa) co-occurring with the fungal symbionts of *V. planifolia* are assumed to be endophytes, commensalists or mycophagic bacteria. However, the function of all bacteria in our system is unknown. For example, *Burkholderia*, was designated as a putative MHB in this study based on

a report by Poole *et al.*, (2001), but it has been reported to have antagonistic relationships with mycorrhizal fungi by Levy *et al.*, (2003). Another limitation of our study is that the low frequency of reads for many of the OMF and ECM fungi reported in Chapter 1 potentially prevented significant interactions from being identified in the co-occurrence analyses.

Chapter 3 - Diversity, distribution, and specificity of orchid mycorrhizal fungi in a leafless epiphytic orchid, *Dendrophylax lindenii*.

Abstract

Orchids grow in diverse habitats worldwide and most of the family, 69%, grow as epiphytes. Some researchers have identified orchid mycorrhizal fungi (OMF) as drivers for terrestrial orchid distribution, but little is known about the role of OMF in influencing fine scale distribution of epiphytic orchids. To investigate this question, we used amplicon sequencing to examine the composition of the OMF community associated with a leafless epiphytic orchid, *Dendrophylax lindenii* and its phorophytes (host trees). We compared the fungal community of *D. lindenii* in its natural habitat in southwestern Florida to the fungal root community of co-occurring leafless epiphytic orchids: *Campylocentrum pachyrrhizum*, *Dendrophylax porrectus*, and foliar epiphytic orchids, *Epidendrum amphistomum*, *Epidendrum nocturnum,* and *Prosthechea cochleata*. We also investigated the fungal community of bark from trees at the sites with and without *D. lindenii.* In total, we recovered 526 OTUs (at 95% sequence similarity) from root samples and 1,077 OTUs from bark samples. Our results suggest that *D. lindenii* has a high specificity for a single OMF (*Ceratobasidium* sp.) not recovered at a high read abundance in co-occurring epiphytic orchids. Phylogenetic analyses showed that this species is conspecific with a reported undescribed species of *Ceratobasidium* that is used to germinate *D. lindenii ex situ*. However, transplanted orchids originating in the laboratory lacked or had lower read abundances compared to naturally occurring plants. While specific to *D. lindenii*, this *Ceratobasidium* was recovered from the bark of trees with and without the orchid at low read abundances. Thus, the fungus may not be driving the fine scale distribution of the orchid.

Introduction

Since the writings of Schimper (1888) over a century ago, the causes of fine scale distribution (e.g., phorophyte specificity) of epiphytes have been a mystery. Specifically, drivers of epiphyte distribution have been debated within the literature (see review by Wagner *et al.*, (2015) and have focused on many abiotic factors (e.g., microclimate, host bark characteristics) and biotic factors (e.g., symbiotic fungi, co-occurrence with moss) without conclusive data to test these hypotheses. Mycorrhizal fungi are well known mutualists that are essential for their plant partners' abundance and distribution (Smith and Read, 2010). However, the potential role that specificity and distribution of orchid mycorrhizal fungi (OMF) of epiphytic orchids plays in their geographical and local distribution is poorly understood (McCormick and Jacquemyn, 2014; Rasmussen et al., 2015). While 69% of orchid species are epiphytic (Zotz, 2013), little is known about the OMF they associate with compared to their terrestrial counterparts. In fact, the importance of OMF and whether epiphytic (adult) orchids require OMF for their survival in their natural habitats is still debated (Dearnaley et al., 2012).

McCormick & Jacquemyn, (2014) showed that most studies of orchid distribution was conducted on terrestrial orchids and concluded that at geographic scales orchids are not constrained by OMF but they may be limited at local scales. In contrast to terrestrial orchids, little is known about drivers of large and fine scale distributions of epiphytic orchids (McCormick et al., 2018).

Gowland *et al.*, (2013) investigated the phorophyte specificity of three different epiphytic orchid species and their OMF and relationships. Another study by Kartzinel *et al.*, (2013) investigated OMF and seed germination of epiphytic orchids but did not investigate the fungal

community of bark from phorophytes or non-phorophytes to examine OMF as the primary cause of their phorophyte specificity (McCormick et al., 2018).

The previous investigations of phorophyte specificity for epiphytic orchids and their OMF can be improved by newer methods of environmental sequencing (McCormick et al., 2018). Researchers have recommended improvements to experimental designs to investigate the abundance and distribution of OMF associating with orchids (McCormick and Jacquemyn, 2014; McCormick et al., 2018). Chapter 1 showed that most non-traditional OMF such as ECM fungi are undetected by early studies that used either culture-based methods or Sanger sequencing. For example, it has been shown that culture-based methods do not detect ECM fungi. Molecular methods such as amplicon sequencing can enhance the study of epiphytic orchids compared to Sanger sequencing (Waud *et al.*, 2016; Jacquemyn *et al.*, 2017; Novotná *et al.*, 2018, Chapter 1 this dissertation).

To examine the role of OMF as drivers of phorophyte specificity for epiphytic orchids, we studied a leafless epiphytic orchid, *Dendrophylax lindenii* (Lindl.) Bentham. ex Rolfe, its co-occurring epiphytic orchids, and bark of trees with and without the orchid (Fig. 1).

Dendrophylax lindenii is restricted to southwestern Florida and the western tip of Cuba (Brown, 2006). The Florida habitats of *D. lindenii* are cypress domes and strand swamps in the Big Cypress Basin (Fig. 1). In Florida, it primarily grows rooted to tree trunks and branches of its phorophytes (*Annona glabra* L. and *Fraxinus caroliniana* Mill.) that are part of a lower canopy under *Taxodium distichum* (L.) Rich. individuals (Brown, 2006; Stewart and Richardson, 2008). Although *D. lindenii* grows in this swamp habitat, it experiences dry periods during the region's dry season which lacks any standing water (Mújica et al., 2018).

Like all orchids, *D. lindenii* requires OMF for germination (Hoang et al., 2016). Early seedling stages of *D. lindenii* have a rudimentary ephemeral leaf. As an adult, the orchid has photosynthetic roots and is leafless and shootless (Benzing and Ott, 1981; Benzing et al., 1983; Hoang et al., 2016). Researchers have identified that the chlorophyllous roots of *D. lindenii* use CAM photosynthesis (Benzing and Ott, 1981). *D. lindenii* associates with a species of *Ceratobasidium* to germinate and mature roots are also colonized by *Ceratobasidium* species (Hoang et al., 2016; Mújica et al., 2018).

Our primary aim for this study was to identify the OMF in *D. lindenii* and to investigate the role of OMF in influencing its fine scale distribution. We investigated two hypotheses: 1) *D. lindenii* has a specific OTU community of OMF compared to co-occurring epiphytic orchids; and 2) the OMF colonizing *D. lindenii* are found in the bark of *D. lindenii* host trees in higher abundances than in the bark of trees without *D. lindenii*. In addition, given that *D. lindenii* is an endangered North America orchid (state listed in Florida), we only sampled root tips to minimize damage to the plant. To investigate if additional OMF were missed with this sampling method we investigated the fungal community of six whole roots.

Materials and Methods

Site Description:

During 2016 and 2018 we collected several root and bark samples from five sites at the Florida Panther National Wildlife Refuge (FPNWR). Four of the sites were natural habitats for *D. lindenii* and the fifth site had *D. lindenii* reintroduced individuals, i.e., explants that were micropropagated under axenic conditions in the lab. Sites were either sloughs and strand swamps and were separated about 1 km from each other. When we collected samples in 2016 (March),

FPNWR sites all had standing water in sloughs and swamps, but when we collected samples in 2018 (April) all sites were dry. In our study we have not disclosed the locations of the sites at the FPNWR because *D. lindenii* and several co-occurring orchids are state listed as endangered in Florida and are prone to being poached. For each site, collecting permits were obtained and permission to access and sample *D. lindenii* populations was granted by the state and federal offices managing the sites at FPNWR.

Root and Bark Sampling (2016 and 2018)

In March 2016, we collected several root samples from four sites at the FPNWR. Root samples collected from epiphytic orchid species included *D. lindenii* (n = 9) and several co-occurring epiphytic orchids: *Campylocentrum pachyrrhizum* (Rchb.f.) Rolfe (n = 3), *Dendrophylax porrectus* (Rchb.f.) Carlsward & Whitten (n = 6), *Epidendrum amphistomum* A. Rich. (n = 4), *Epidendrum nocturnum* Jacq. (n = 1) and *Prosthechea cochleata* (L.) W. E. Higgins (n = 3). Then in April 2018 at the same sites plus one additional site, we collected root samples from only *D. lindenii* (n = 27).

During April 2018 from five sites at the FPNWR, we collected bark samples from random host trees of *D. lindenii* and non-host trees of *D. lindenii*. At each site, we sampled five host tree individuals and five non-host tree individuals. The sampling scheme involved collecting bark samples in relation to the position of *D. lindenii* on the tree. In the case of trees without *D. lindenii* these positions were the same positions compared to the trees with *D. lindenii* (Fig. 2). Positions on the trees included: 1) the base of the tree trunk, 2) above *D. lindenii*, 3) the side of roots of *D. lindenii* root; and 4) the opposite side of the tree trunk, where *D. lindenii* grew. Most

trees sampled were *F. caroliniana*, and then other trees such as *A. glabra*, *Salix* sp. and *Taxodium distichum*.

Root Sections (Pilot study to investigate potential sampling bias)

To determine if we had a sampling bias, we collected root samples from *D. lindenii* and *Prosthechea cochleata* (control species) in February 2018 at the FPNWR. Roots ca. 50 mm long were collected from four *D. lindenii* individuals, one root sample from a juvenile *D. lindenii*, plus a root sample from a mature *P. cochleata*, a foliar orchid. We also collected a root sample from a cultivated orchid as a control. For all root samples, starting from the tip, 5 mm of root tissue was cut and labelled alphabetically (i.e., A, B, C, etc.). For example, the root tip would be "A", while the preceding root section close to the root tip would be labelled "B" (see Fig. 3).

Sample Preparation, DNA Extraction, PCR, and Sequencing

Approximately 5 mm of root and bark tissue was collected and stored into cetyltrimethylammonium bromide (CTAB) buffer. For all samples (including the root sections) we surface sterilized root and bark samples with 70% ethanol, and 50% Clorox® (2.6% sodium hypochlorite) using the method outlined in Bayman *et al.*, (1997). Next, we extracted genomic DNA from root samples using the Qiagen DNeasy extraction kits (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. We extracted genomic DNA from bark samples with the modified CTAB method of Murray & Thompson (1980), and for difficult to extract samples we used the MOBIO Power Soil DNA Extraction kit (MOBIO Laboratories, Carlsbad, CA, USA) following the manufacture's instruction.

For the 2016 root samples the extracted genomic DNA was amplified for the fungal DNA from the fungal barcode using the primers: ITS86f (5′- GTGAATCATCGAATCTTTGAA-3′; Turenne *et al.*, 1999) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′; White et al., 1990). These fungal primers (ITS86F / ITS4) amplify the internal transcribed region ITS for the ITS subregion 2 which is shown to be effective for delimiting OMF such as those in the Cantharellales.

The samples for 2016 were first amplified and generated amplicons that were sequenced on an Illumina miSeq amplicon libraries with a three step PCR sequencing (see Chapter 1 materials and methods). For the 2016 library preparation this included PCR steps that used modified primers with indices from the Nextera XT kit for 96 indices to sequence 2 x 250bp. The final libraries that were generated for root samples were quantified using a Qubit dsDNA HS kit (Invitrogen) and a Bioanalyzer-Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA). Final libraries for root samples were pooled to equimolar concentrations then sequenced on the Illumina miSeq.

Separately, root sections (for the pilot study) and the bark samples for 2018 were PCR amplified using modified primers of the fungal primers ITS86F / ITS4 that contained Novogene supplied barcodes. Then generated amplicon libraries were shipped to Novogene for sequencing on an Illumina hiSeq.

Data Processing and Statistical Analyses

Sequences were quality filtered and OTUs clustered with the default settings of Pipits pipeline (version 1.4.0) (Gweon et al., 2015). Briefly, Pipits joined reads and quality filtered short reads (<50 bp), extracted non ITS fungal reads with the script ITSx, then clustered OTUs at

95% sequence similarity (see supplemental info 2 for more details). Additional Pipits scripts assigned taxonomy to OTUs with the Naïve Bayesian Classifier and the UNITE database. Sequences for the hiSeq dataset was analyzed separately from the 2016 miSeq dataset. The single difference between analysis of the miSeq data analyses and hiSeq data analyses was omitting the ITSx step for the hiSeq data because computations were time consuming.

All statistical analyses were conducted within R (R Development Core Team, 2012). Interpolation and extrapolation curves were produced with the R package iNext, (Hsieh et al., 2016). For rarefaction and iNext sampling curves (interpolation and extrapolation), singletons were retained when curves were generated. After rarefaction and extrapolation (iNext) sampling curves, further data analyses excluded rare OTUs that were less than 1000 sequences.

To investigate differences between fungal communities, the raw read abundances were first normalized with Cumulative Sum Scaling in the R package metagenomeSeq (Paulson et al., 2013). Principal coordinate analysis (PCoA) were generated using Jaccard distances. Significance between fungal communities of *D. lindenii* and epiphytic orchid roots, host tree and non-host tree bark, and sites were determined with "permutational manova" (Anderson, 2001) in R package vegan (adonis function) by first permuting the raw data (10000 permutations).

Phylogenetic trees were generated with sequences from both bark and root fungal communities. In addition, sequences for *Ceratobasidium* and an outgroup sequence of *Tulasnella* were obtained from the UNITE fungal database. We used Muscle for multiple sequence alignments and generated a Maximum Likelihood tree using the default settings of the program FastTree (version 2.1.10).

Results

Sequences were generated from field collected roots of *D. lindenii* and other co-occurring epiphytic orchids in 2016 and 2018, resulting in 537,371 and 1,691,086 reads, respectively. We obtained no amplicon libraries for Site 2 and did not include it in any of the analyses. Clustering at 95% sequence similarity identified 526 OTUs (2016) and 1,077 OTUs (2018). The higher read and OTU number in 2018 is a result of using the hiSeq platform rather than the miSeq platform. *Dendrophylax lindenii* had higher read abundances of root associated fungi per sample compared to other co-occurring epiphytic orchids, but the observed OTU richness was similar (Fig. 4). Sequences generated from sections of whole roots yielded 3,205,959 reads (1,372 OTUs). PCoA showed no distinctions between fungal communities of root sections originating from the same root (5 mm sections starting from root tip). The OMF and ECM fungal community recovered from whole roots matched those recovered from root tips (Fig. 5).

There were three dominant Ceratobasidiaceae OTUs in the fungal communities (Figs. 4 & 6). A Sebacina OTU was also recovered with high read abundance from two root samples of *D. lindenii* in 2018 (Fig. 4). The OTUs that were less abundant consisted of other OMF and ECM fungi, and in most samples were <10% of the relative abundance. OMF detected at low abundances in both 2016 and 2018 were additional Ceratobasidiaceae and Sebacinales. ECM fungi detected included Inocybaceae, Russulaceae, *Scleroderma*, Thelephoraceae, *Tomentella*, and *Tuber* species (Figs. 4 & 6).

The dominant Ceratobasidiaceae OTU in 2016 (OTU 14) and 2018 (OTU 76) of *D. lindenii* were conspecific based on phylogenetic analyses and have >98% sequence similarity (Clade 2, Fig. 7). Additional members of Clade 2 were *Ceratobasidium* sequences (Dlin-394, Hoang *et al.*, 2016) from the study of mature roots of *D. lindenii* that germinated *D. lindenii*. The

other two dominant Ceratobasidiaceae OTUs (19 and OTU22) were abundant in root samples of *D. porrectus* (samples GO-17-21, Fig. 4), and formed different clades, Clade 1 and 3 (Fig. 7).

Differences were observed between naturally grown *D. lindenii*, the cultivated *D. lindenii* and co-occurring epiphytic orchids (Figs. 4 & 5). Naturally grown *D. lindenii* were dominated with Ceratobasidiaceae Clade 2 (Fig. 5). Juvenile *D. lindenii* samples had fewer Ceratobasidiaceae Clade 2 reads compared to mature roots of *D. lindenii*. However, Clade 2 was recovered at low read numbers (<1000 reads) from root samples of explanted *D. lindenii* Site 4 (Fig. 4). Garden cultivated *D. lindenii* had abundant Ascomycota OTU reads (Fig. 5).

Fifty-seven bark samples were successfully sequenced, 15 from trees with *D. lindenii* and 42 without *D. lindenii*. Sequencing of bark samples yielded 7,245,995 (1,141 OTUs). We detected low read abundances for OMF and ECM fungi within all bark samples (Fig. 8). No differences were observed between trees with and without *D. lindenii* for Ceratobasidiaceae Clade 2 (Fig. 14). The total reads in the entire dataset for Ceratobasidiaceae Clade 2 comprised 4% (Supplementary Fig. S1C). PCoA revealed clustering of bark samples that were collected from the base of the trees (Fig. 9).

Discussion

Our study shows that *D. lindenii* has high specificity for a single Ceratobasidiaceae OTU (Clade 2) in its natural habitat at the FPNWR. It was abundant in *D. lindenii* but rare (<1% of total reads) in other epiphytic orchids at FPNWR. The distribution of the fungus in tree bark throughout the orchid's range could influence its fine scale distribution. However, b sequences from bark samples were rare, our results were inconclusive. Out of 200 samples we only

obtained three samples from tree species other than *F. caroliniana*, the orchid's primary phorophyte.

The fungal community of bark from trees with and without *D. lindenii* had Ceratobasidiaceae Clade 2 present, but it was a rare OTU. Additional studies to investigate host tree specificity are needed because Ceratobasidiaceae Clade 2 was widespread among trees in *D. lindenii* habitats at the FPNWR. In addition, our study establishes the usefulness of amplicon sequencing as a method to examine fungal communities of endangered orchids such as *D. lindenii*. Sampling the actively growing root tips provides a potentially non-destructive sampling method for future studies of this and other threatened and endangered orchids.

We detected low read abundances for ECM fungi in the roots of the epiphytic orchids examined. This is in contrast to epiphytic roots of *V. planifolia* which were heavily colonized by ECM fungi (Chapter 1). *Vanilla planifolia* is a hemiepiphytic orchid and it is possible that the ECM fungi in the epiphytic roots are from systemic colonization emanating from the terrestrial roots. ECM fungi have been commonly reported from terrestrial orchids, but except for Chapter 1 ECM fungi have not been reported for epiphytic orchid roots

Dendrophylax lindenii displays a similar extreme fungal specificity as mycoheterotrophic orchids (McKendrick et al., 2002; Selosse et al., 2002), mycoheterotrophic plants (Leake, 2010), and some epiphytic orchids (Otero et al., 2002, 2004; Graham and Dearnaley, 2012). When we define specificity based on the phylogenetic breadth as stated by Taylor *et al.*, (2003), we concluded that Ceratobasidiaceae OTU 14/76 associated with the same *Ceratobasidium* (Dlin-394) that is reported to germinate *D. lindenii* seeds (Hoang *et al.*, (2016). This resolved clade suggests that Ceratobasidiaceae 14/76 and *Ceratobasidium* Dlin-394 are potentially conspecific because we also obtained sequence alignments for these sequences that were >99% similar.

We also observed evidence of possible specificity in other orchids, but sample size was small. These orchids associated with different *Ceratobasidium* (Clade 1 and 2). For example, *Ceratobasidium* OTU 19 and 22 were detected primarily in *D. porrectus*, another leafless epiphytic orchid. We hypothesize, with a caveat of small sample size, that mature roots of leafless epiphytic orchids are dominated by a single OMF unique to that species.

Chomocki *et al.*, (2014) reported through microscopy that passage cells in *D. lindenii* roots will restrict the OMF that is able to colonize its roots. In our study, foliar orchids displayed lower read abundances compared to *D. lindenii* and other leafless epiphytic orchids. Although additional studies are required to understand the low abundance of OMF in foliar orchids, we hypothesize that greater photosynthetic capacity provided by the leaves of these foliar orchids influences the dependence on OMF for fungal carbon.

Bark is the likely source of OMF in epiphytic orchids because OMF need to be present for establishment as they are necessary for seed germination and seedling growth (Rasmussen et al., 2015). However, Ceratobasidiaceae OTUs in bark were recovered at low read abundances, i.e., <5% relative abundance for Ceratobasidiaceae Clade 2 including bark samples collected adjacent to actively growing root tips. Veldre *et al.*, (2013) revealed, through an extensive phylogenetic analysis of Ceratobasidiaceae sequences, that the family Ceratobasidiaceae contained diverse nutritional modes such as pathogens, orchid mycorrhiza, and saprotrophs. If Ceratobasidiaceae Clade 2 is an OMF, then it is likely an inefficient saprobe and are therefore outcompeted by more efficient saprotrophic fungi in the bark fungal community.

Although Ceratobasidiaceae Clade 2 is found in similar abundances in the bark of sampled trees at site 5 as other sites at the FPNWR, there are no naturally occurring *D. lindenii* at this site. Roots of the explant *D. lindenii* at this site had the lowest colonization (i.e. read

abundances) of Ceratobasidiaceae Clade 2. Explants at an adjacent site (Site 5) had Ceratobasidiaceae Clade 2 read abundances similar to naturally occurring plants growing nearby. It is uncertain why site 5 lacks naturally occurring plants since its Ceratobasidiaceae Clade 2 is present in the bark of its preferred phorophyte at the site. Understanding site differences in terms of presence of Ceratobasidiaceae Clade 2 is crucial to sustaining populations of *D. lindenii* and preventing 'senile' populations', an ageing orchid population that lacks seedling recruitment (Rasmussen et al., 2015). An interesting observation by Mújica *et al.*, (2018) has suggested that overall the population of FPNWR has lower percentage of seedlings compared to *D. lindenii* populations of Cuba that have a higher percentage (30.3%) of seedlings. Although McBride's Pond has reintroduced *D. lindenii* further investigations are needed to understand if it sustains *D. lindenii* populations.

Chapter 4 - Mycorrhizal dependence of a leafless epiphytic orchid, *Dendrophylax lindenii*.

Abstract

Dendrophylax lindenii is a leafless epiphytic orchid that is state listed as endangered in Florida where it is restricted. Previous investigations show that *D. lindenii* associates with a single specific *Ceratobasidium* sp. in its natural habitat from early germination stages and adulthood. The dependency it has for fungal-derived carbon to grow in its natural habitat has not been examined. To determine the mycorrhizal dependency of *D. lindenii*, we investigated the natural abundance of hydrogen, carbon, and nitrogen stable isotopes. In our study we recorded enriched $\delta^{2}H$ (-15.1 to -20.8‰) and $\delta^{13}C$ (-14.4 to -15.5‰) for *D. lindenii* relative to its surrounding vegetation which included other co-occurring orchid species that were foliar orchids. ^{15}N stable isotope compositions showed no variations between guilds. While $\delta\,^{13}C$ values for *D. lindenii* are inconclusive because they overlap with expected values for plants that use CAM photosynthesis, and $\delta\,^{2}H$ values suggest that *D. lindenii* is a mycoheterotrophic orchid. Additional studies are required to clarify the ratio that fungal-derived carbon has in the carbon budget of *D. lindenii.* Our study also revealed that the ^{13}C and ^{2}H for root tissue are enriched relative to the leaf tissue for foliar epiphytic orchids, this difference should be considered for studying orchids.

Introduction

Since colonizing land 400 million years ago, plants have associated with microbes to acquire nutrients (Simon et al., 1993; Brundrett, 2002). Most extant plants associate with microbes such as mycorrhizal fungi to obtain nutrients (Wang and Qiu, 2006). Orchids are an excellent example of the reliance plants have on mycorrhizal fungi. Orchids rely on mycorrhizal fungi at an early stage because their seeds lack sufficient nutrients to germinate and use fungi as

a nutrient source instead (Arditti and Ghani, 2000; Rasmussen et al., 2015). Then as orchids mature (depending on the species of orchid) their dependency on mycorrhizal fungi can vary, ranging from mycoheterotrophy, partial mycoheterotrophy or autotrophy (Dearnaley et al., 2012). Understanding the dependency of orchids on mycorrhizal fungi is essential for conservation efforts of threatened or endangered orchids (Swarts & Dixon, 2009; Coopman & Kane, 2018).

North America has 210 native orchids of which 57% are at risk of extinction (Krupnick et al., 2013). One of these threatened orchids is *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, the Ghost Orchid (Raventós et al., 2015). *Dendrophylax lindenii* is native to southwest Florida and the far western tip of Cuba (Brown, 2006; Stewart and Richardson, 2008). In Florida, the natural habitats of *D. lindenii* are restricted to hardwood hammocks, sloughs, strand swamps, and cypress domes. *Dendrophylax lindenii* is a leafless epiphytic orchid that is typically rooted on tree trunks of *Annona glabra* L. and *Fraxinus caroliniana* Mill. (Carlsward et al., 2003, 2006). During early seedling stages, *D. lindenii* has an ephemeral rudimentary leaf, and as an adult, it has photosynthetic roots (Benzing and Ott, 1981; Benzing et al., 1983; Hoang et al., 2016). *Dendrophylax lindenii* employs Crassulacean Acid Metabolism (CAM) photosynthesis (Benzing & Ott, 1981, reported under the synonym *Polyradicion lindenii*). Recent studies (Mújica et al., 2013; Hoang et al., 2016), as well as Chapter 3 of this dissertation, confirmed that *D. lindenii* has specificity for one orchid mycorrhizal fungus (OMF) *Ceratobasidium* species. The dependency on this *Ceratobasidium* by the orchid is unknown.

Stable isotope analysis is has been used to investigate the dependency of orchids on mycorrhizal fungi (Dearnaley et al., 2012). Typically natural abundances for carbon (^{13}C), and nitrogen (^{15}N) are examined to determine the physiologies of fully mycoheterotrophic, partial

mycoheterotrophic, and autotrophic orchids (Gebauer and Meyer, 2003; Selosse and Martos, 2014; Gebauer et al., 2016). However, enrichment of ^{15}N from orchids associating with common OMF like *Ceratobasidium* spp. has not been observed, reducing the value of using this stable isotope and making it challenging to predict partial mycoheterotrophy (Hynson et al., 2013; Gebauer et al., 2016). Recently, Gebauer *et al.*, (2016) recommended the use of natural abundances of $\delta^{2}H$ to differentiate partial and fully autotrophic orchids.

To better understand the reliance that *D. lindenii* has on fungi as a carbon source we examined the natural abundance of these stable isotopes (^{2}H, ^{13}C, and ^{15}N for *D. lindenii*. For this study we hypothesized that the $\delta^{2}H$ and the $\delta^{13}C$ of *D. lindenii* are enriched compared to co-occurring epiphytic orchids with leaves.

Materials and Methods

Study Site and Sampling scheme and species investigated

Sampling was undertaken at four sites at the Florida Panther National Wildlife Refuge (FPNWR, in Collier Country, FL) during March 2016. We collected leaf and root samples from epiphytic orchids including *D. lindenii* and leaf samples from host plants (*Annona glabra* and *Fraxinus caroliniana*) and co-occurring plants (Table 1). Three of the sites were sloughs that were monodominant with the *D. lindenii* host trees *F. caroliniana* and the fourth site was dominated by *A. glabra*. Permits were obtained from state and federal offices managing the sites to access and collect samples from *D. lindenii* populations.

The sampling scheme consisted of: 1) root samples from *D. lindenii* individuals (n = 10); 2) root and leaf samples from co-occurring epiphytic orchids (n = 20) and; 3) leaf samples from host trees (n = 20) (Table 1).

After collecting, samples were stored in coin envelopes and dried at 105°C for a minimum of 24 hours then ground to a fine powder. Samples from the same site were often pooled due to insufficient tissue needed for the analyses. The carbon stable isotope abundances were analyzed via an elemental analyzer/continuous flow isotope ratio mass spectrometry at the Duke Environmental stable Isotope Lab. Hydrogen stable isotope abundances were analyzed via a temperature conversion elemental analyzer isotope ratio mass spectrometer at Northwestern University.

The measured isotope abundances were denoted as δ-values and are calculated according to the following equation: $\delta\ ^{2}H$, $\delta\ ^{13}C$, or $\delta\ ^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard.

For all the statistical tests, R (R Development Core Team, 2012) was used to analyze the stable isotope compositions. Stable isotope data were tested for statistical differences using the non-parametric test Kruskal–Wallis.

Results

Dendrophylax lindenii samples were enriched for both $\delta\ ^{2}H$ and $\delta\ ^{13}C$ compared to surrounding vegetation (Fig 1 and Table 1). *Dendrophylax lindenii* values ranged from -15.1 to -20.8‰ for $\delta\ ^{2}H$, and -14.4 to -15.5 for $\delta\ ^{13}C$ and did not overlap with stable isotope values from other guilds (Fig 1 and Table 1) (Kruskal-Wallis for $\delta\ ^{2}H$: Chi square = 24.3 , $p < 0.005$, df = 6; and Kruskal-Wallis for $\delta\ ^{13}C$: Chi square = 36 , $p < 0.005$, df = 6). Similar to *D. lindenii*, a single sample for *Dendrophylax porrectus* (Rchb.f.) Carlsward & Whitten (a leafless epiphytic orchid)

was enriched for δ ^{2}H with -25.7‰. We did not obtain a ^{13}C value for *D. porrectus* because there was insufficient tissue.

The root samples of foliar epiphytic orchids were enriched for both δ ^{2}H and δ ^{13}C compared to their leaf samples, but not as enriched as the leafless species (Fig 2). For example, the ^{13}C values of root samples for *E. amphistomum* ranged from -16.2 to -20.6‰ and the ^{13}C values for its leaf samples ranged from -20.7 to -23.6‰. Delta ^{2}H values for both leaf and root samples for *E. amphistomum* also showed the same correlation. For example, root δ ^{2}H values were -27.9 to -42.2‰ while leaves were depleted with values of -60.4 to -86.2‰.

Delta ^{2}H and δ ^{13}C values for all leaf samples for *Fraxinus caroliniana* and ferns (reference plants) were depleted compared to other guilds and had an average of -29.5‰ (standard deviations were ±1.5) for ^{13}C and -73.3‰ (standard deviations were ±11.8) for δ ^{2}H. Bromeliads were enriched for δ ^{13}C (mean = -17.5, standard deviations were ± 0.7) but not for δ ^{2}H (mean = -68.9, standard deviations were ± 6.3) (Table 1).

In contrast to δ ^{2}H and δ ^{13}C, the δ ^{15}N values for most guilds overlapped within a range of 4 to -4‰ (Fig 2). The δ ^{15}N values for host trees and orchids (leafless and foliar) had the most overlap among all guilds. Bromeliads were the most depleted in δ ^{15}N compared to other plant stable isotope values.

Discussion

Our results from stable isotope analyses suggests that the leafless epiphytic orchid, *D. lindenii*, is dependent on fungal-derived carbon for its growth. Specifically, the natural abundances of δ ^{2}H and δ ^{13}C of *D. lindenii* were more enriched than in the leaves of its

phorophytes, and surrounding vegetation, including leaf and root samples of co-occurring epiphytic orchids (Fig 2).

^{13}C enrichment is indicative of fungal-derived carbon but also of the CAM photosynthetic pathway. *Dendrophylax lindenii* has previously been documented to use CAM photosynthesis (Benzing *et al.* 1981). The natural abundance of ^{13}C values reported in this study ranged from -15.1 to -20.8‰ which is consistent with previously reported ^{13}C values for CAM photosynthetic plants (Smith and Epstein, 1971; Bender et al., 1973; O'Leary, 1981; Cernusak et al., 2013). Silvera *et al.*, (2005) reported $\delta^{13}C$ values that ranged from -11.8 to -32.3‰ for 87 foliar orchid species that used CAM photosynthesis. More specifically, (Winter et al., 1983) reported two Australian leafless epiphytic orchids, *Chiloschista phyllorhiza* (F.Muell.) Schltr. and *Taeniophyllum malianum* Schltr. had $\delta^{13}C$ values of -14.5‰ and -15.8‰ respectively. Additionally, the bromeliads in our study showed the same level of enrichment for ^{13}C as *D. lindenii*. Many bromeliads use the CAM photosynthetic pathway and we assume that this is the reason we recorded the $\delta\,^{13}C$ values reported here. Thus, the enriched ^{13}C values for *D. lindenii* are inconclusive regarding potential mycoheterotrophy. Similar to $\delta\,^{13}C$ values, the $\delta\,^{15}N$ values for *D. lindenii* did not distinguish *D. lindenii* from other co-occurring foliar orchids and reference plants. Others report similar findings with $\delta\,^{13}C$ values, the $\delta\,^{15}N$ values (Hynson et al., 2013; Gebauer et al., 2016).

$\delta\,^{2}H$ is a more reliable indicator of a plants carbon source (Gebauer et al., 2016). An elevated ^{2}H is suggestive of mycoheterotrophy in orchids (Gebauer et al., 2016; Schiebold et al., 2017). Recorded values of -15.1 to -20.8‰ for *D. lindenii* are consistent with values of a fully mycoheterotrophic orchid (Gebauer et al., 2016), however, *D. lindenii* has photosynthetic roots (Benzing and Ott, 1981). In addition, Coopman and Kane (2019) reported that cultivated *D.*

lindenii grown via asymbiotic micropropagation in the greenhouse developed and survived without associating with OMF. This suggests that *D. lindenii* has a photosynthetic capacity to sustain its growth without OMF, at least under optimum conditions. Thus, the carbon budget of the plant is likely met by some combination of fungal-derived carbon and photosynthetic carbon. The ratio of these two carbon sources is currently unknown.

A limitation of our study was the relatively small number of root samples we were able to collect because *D. lindenii* is a state endangered plant. However, our results are consistent with those of an earlier study by D.L. Taylor (unpublished data) that recorded enriched $\delta^{13}C$ values of *D. lindenii* compared to its surrounding vegetation.

An interesting finding of our study is the consistent enrichment of natural abundances of ^{2}H and ^{13}C for epiphytic roots of foliar orchids compared to their photosynthetic leaves. Foliar orchid leaf ^{2}H values that were -95.8 to -40.8‰ whereas, root samples were -76.3 to -27.9‰ (Fig 1). Previous research has shown that natural abundances of stable isotopes between different plant tissue occurs due to fractionation (Badeck et al., 2005). Future studies of the natural abundances of stable isotope in orchids should account for potential differences of plant tissues.

Understanding the ecological role that OMF have in influencing the distribution and establishment of orchids has important conservation implications both for managing existing populations and increasing the success of reintroductions (Swarts and Dixon, 2009; McCormick and Jacquemyn, 2014; Heilmann-Clausen et al., 2015). Given that the majority (69%) of orchids are epiphytic and are in habitats that create nutrient and water stress, partial mycoheterotrophy may be essential for establishment and their persistence.

Figures and Tables for Chapter 1

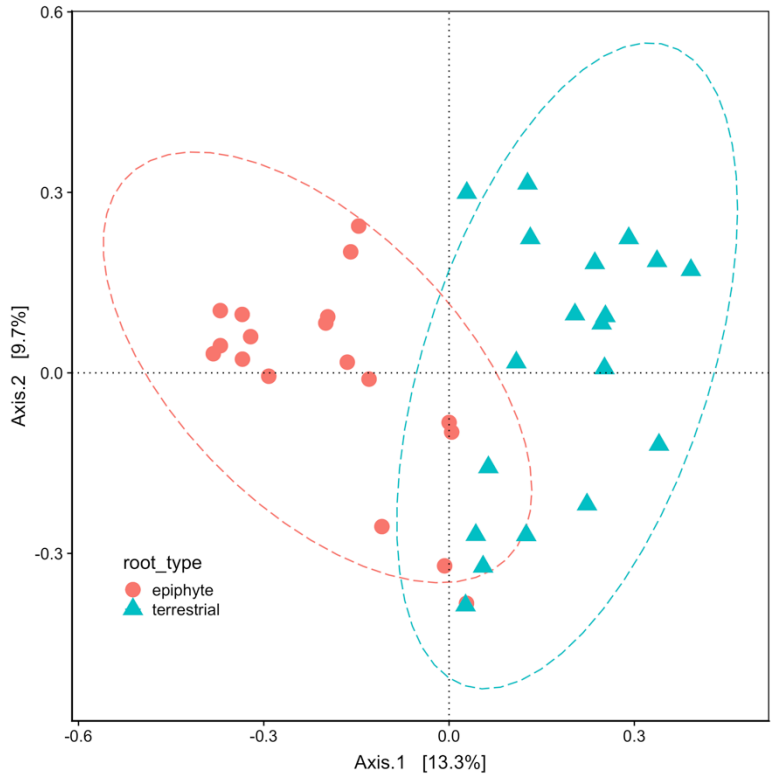

Figure 1.1. Principal coordinate analysis of fungal community composition in all root samples (epiphytic = ○, and terrestrial = Δ) using Bray-Curtis dissimilarity on abundance data (i.e. CSS normalized abundance data). 95% confidence ellipses show 1 standard deviation around the epiphytic and terrestrial centroids.

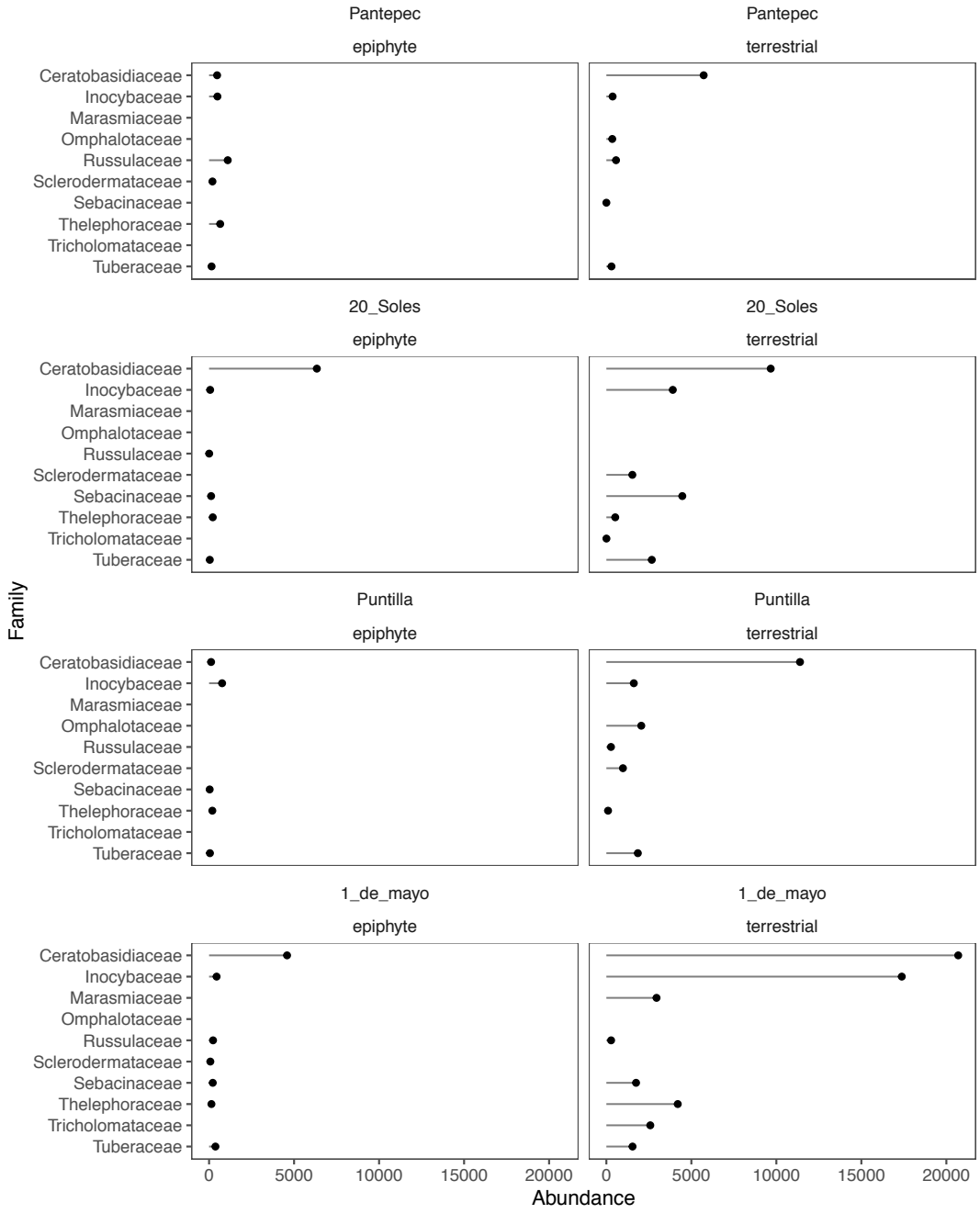

Figure 1.2. OMF read abundances of *V. planifolia* across 4 different farms: Pantepec (highly managed farm), 20 Soles (traditional farm), Puntilla (traditional farm), 1 de Mayo (Wild natural farm).

Figures and Tables for Chapter 2

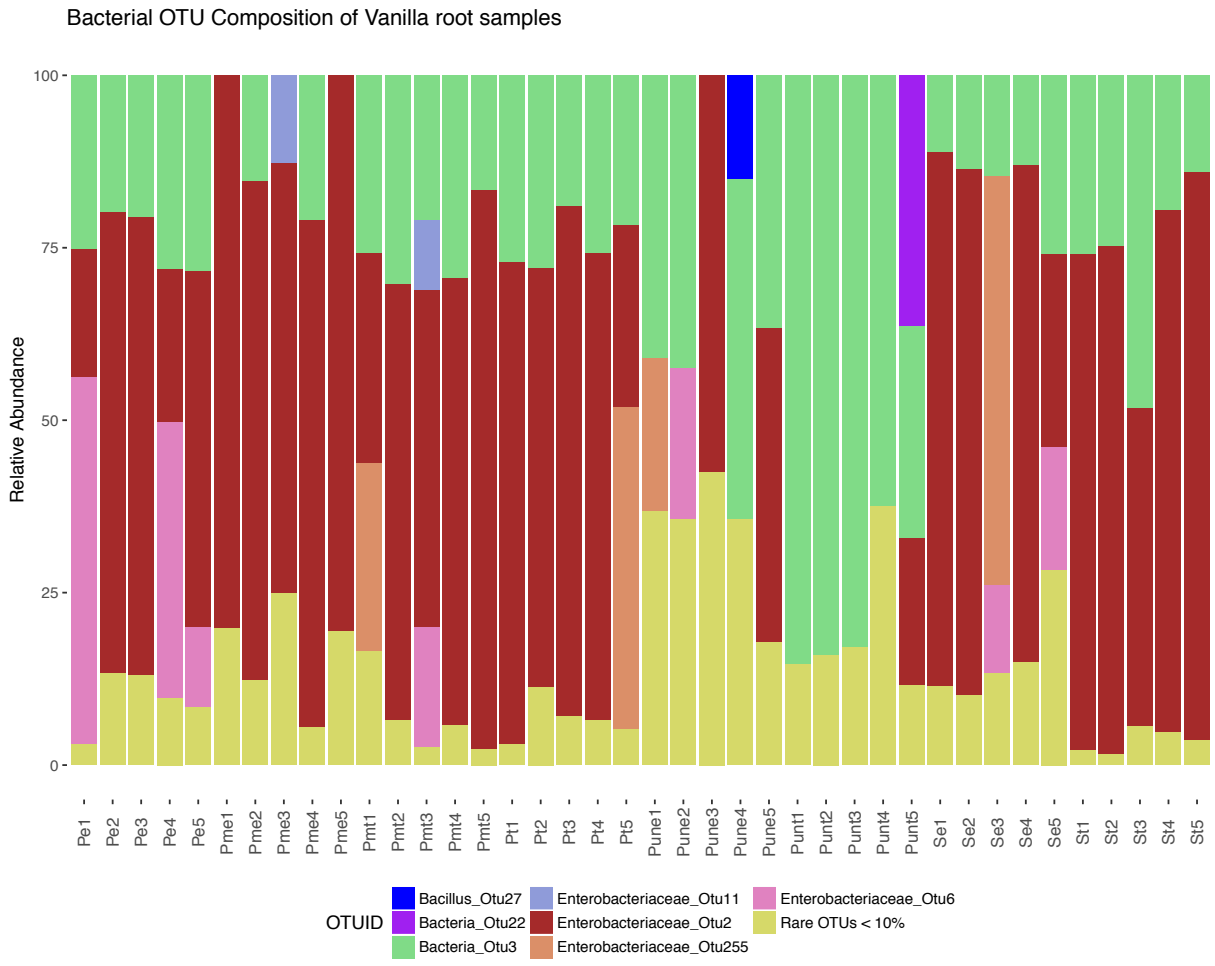

Figure 2.1. The relative abundance of bacterial OTUs from root samples collected from four Mexican *V. planifolia* farms.

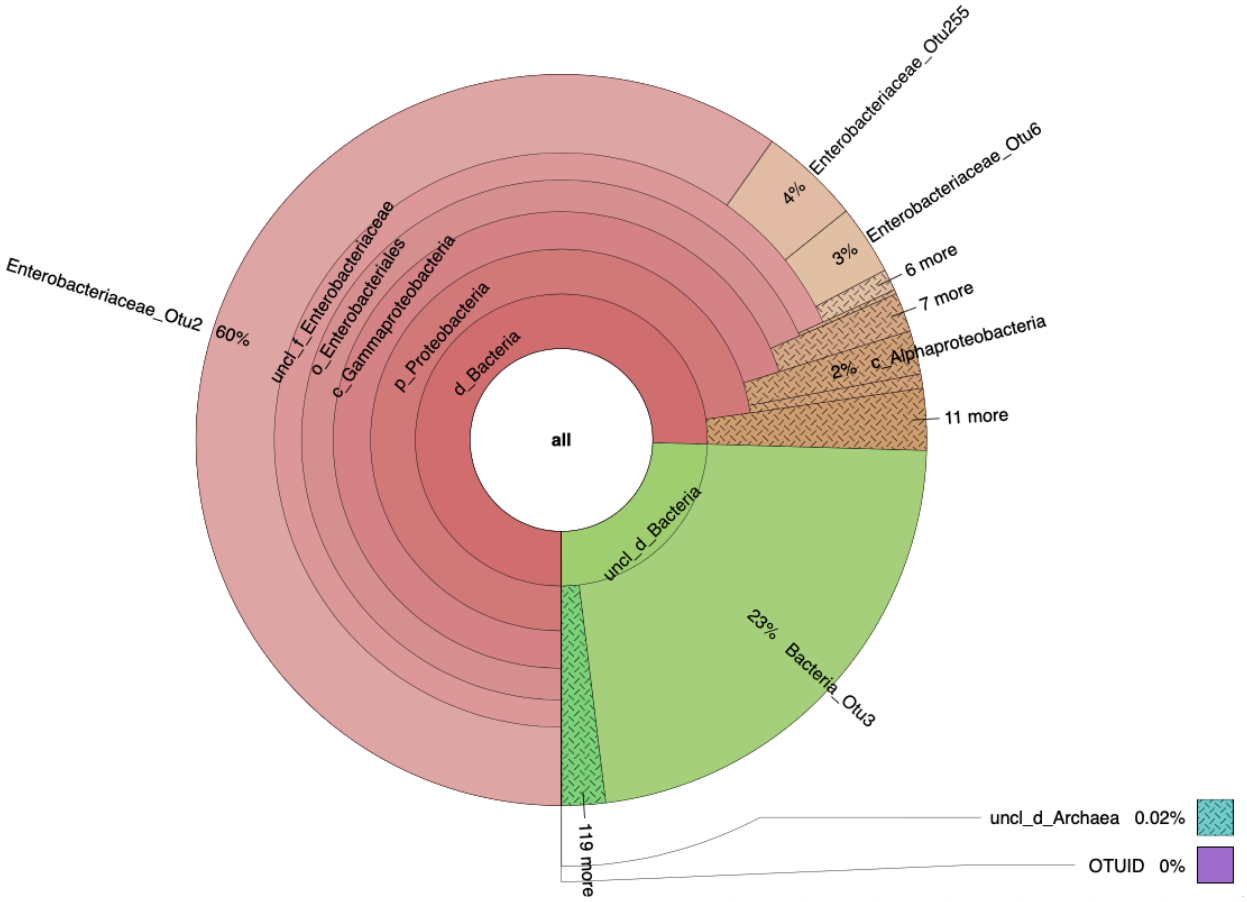

Figure 2.2. Krona chart representing 16S sequences from *Vanilla planifolia* roots.

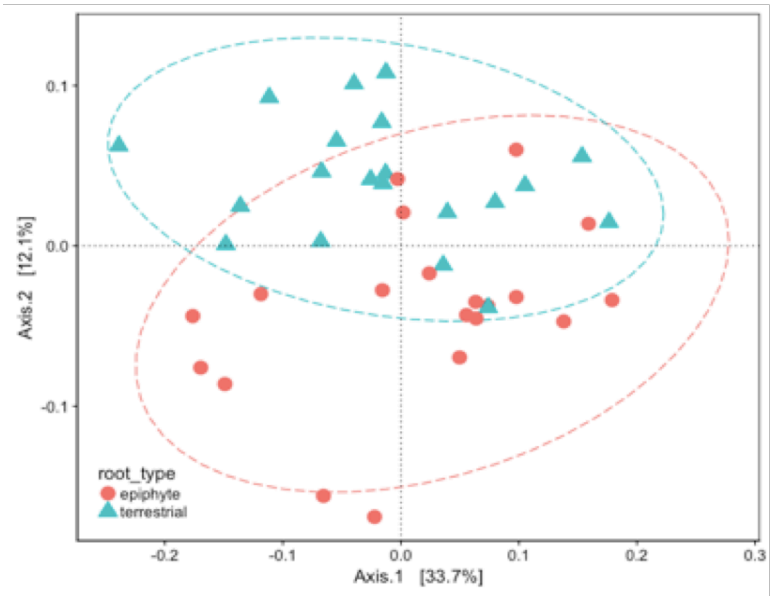

Figure 2.3. Principal coordinate analysis of bacterial community in *V. planifolia* roots using UNIFRAC (weighted) to examine differences between epiphytic (pink) and terrestrial (blue) root samples. Ellipses represent 1 SD around the epiphytic and terrestrial centroids.

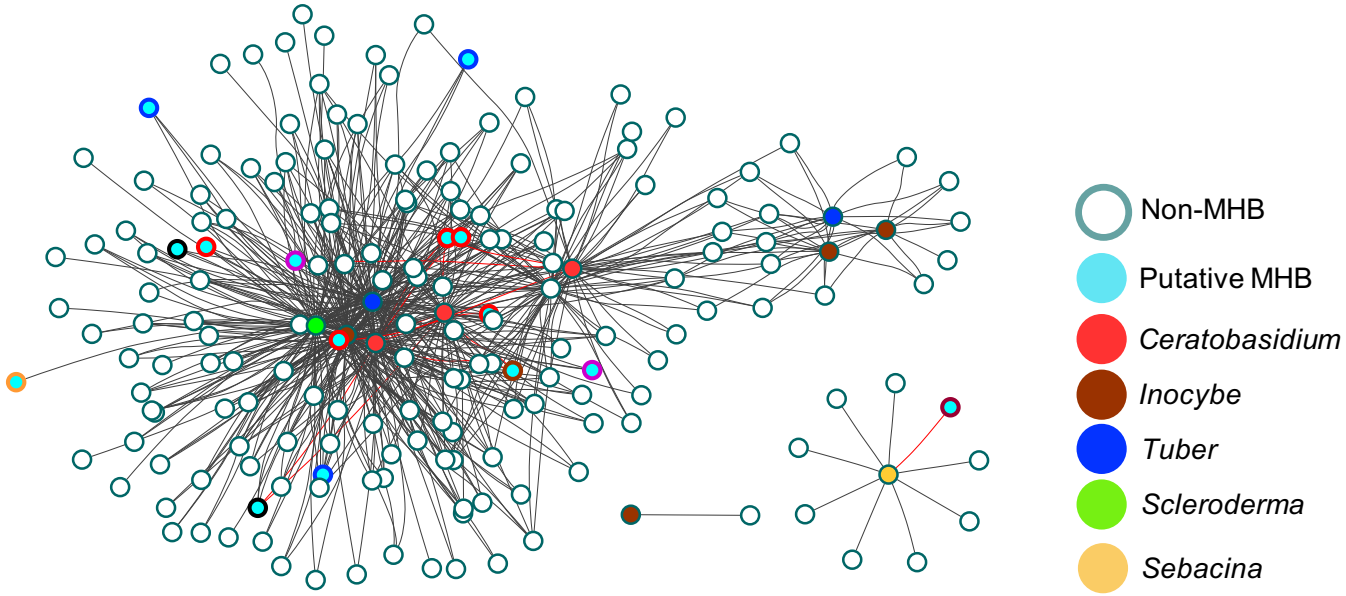

Figure 2.4. Co-occurrence network of putative MHB OTUs and fungal symbionts. Network analysis was generated using an ensemble approach of two correlation methods. Edges represent significant correlations ($p \leq 0.05$). Nodes represented are either bacterial or fungal OTUs. Open circles ○ represent bacteria that are not putative MHB. Filled circles ● represent bacteria that are putative MHB. Fungal symbionts are the following filled circles: *Ceratobasidium*: ●; *Inocybe* ●; *Scleroderma* ●; *Sebacina* ●; *Tuber*: ●

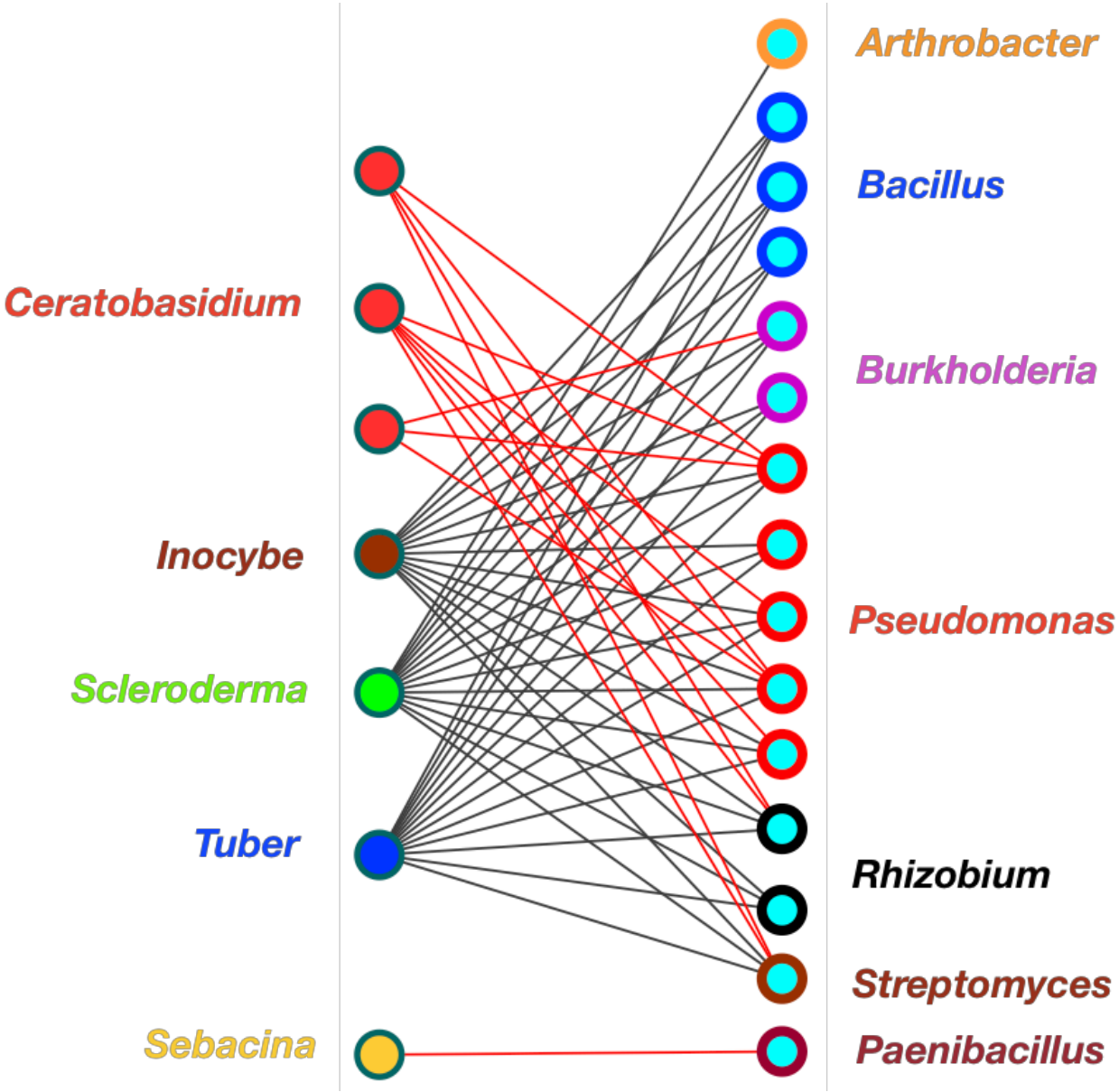

Figure 2.5. Bipartite network produced from main subnetwork of co-occurrence network of OMF and putative MHB OTUs. Red lines represent co-occurrences between traditional OMF, *Ceratobasidium* and putative OMF.

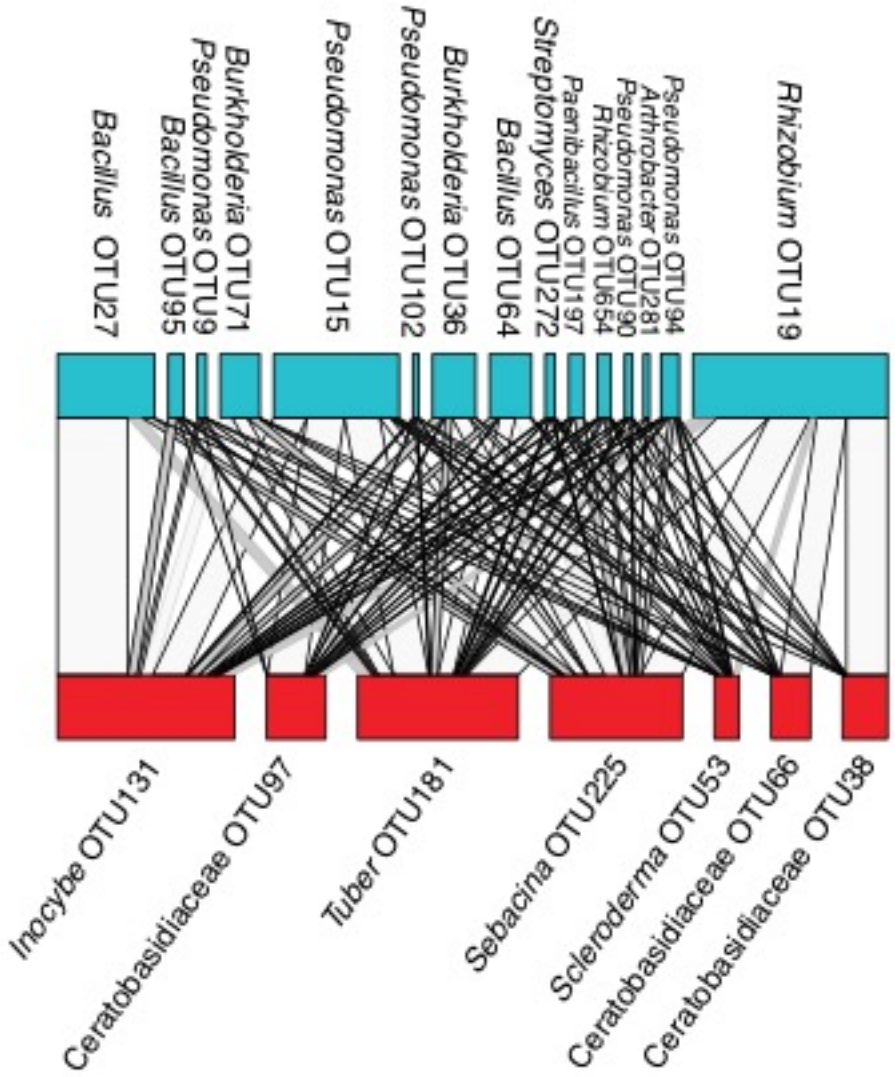

Figure 2.6. Quantitative bipartite network produced from co-occurrences between OTUs of putative MHB (top) and fungal symbionts (bottom, OMF and ECM fungi). The width of links (edges) represents the read abundances for the putative MHB co-occurring with the fungal symbiont.

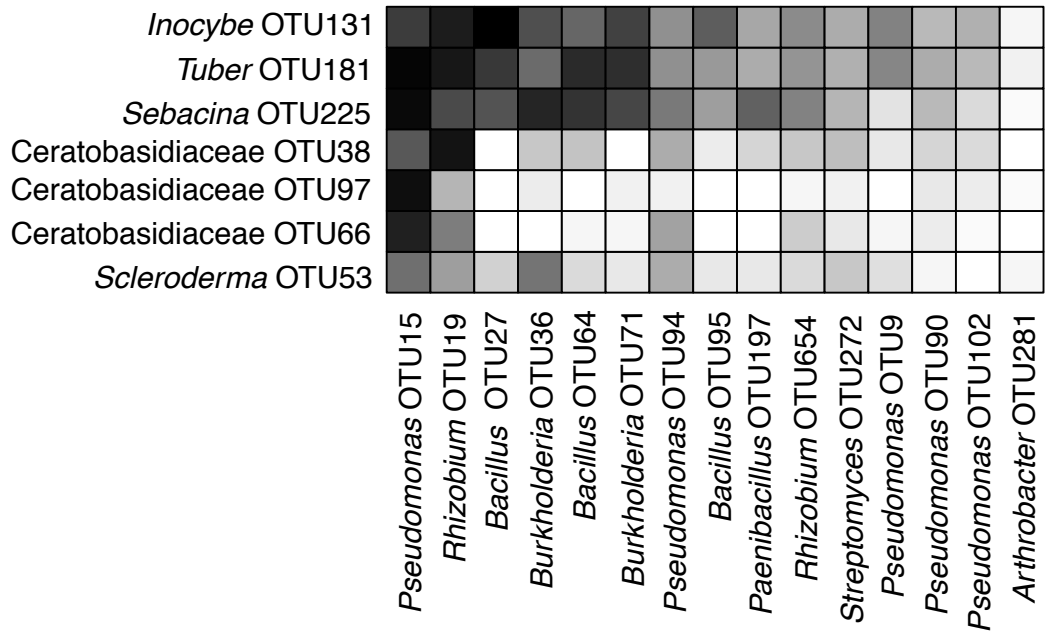

Figure 2.7. Interaction matrix of the co-occurrences between putative MHB and fungal symbionts (OMF and ECM fungi). This incidence network represents the same information visualized in Figure 6.

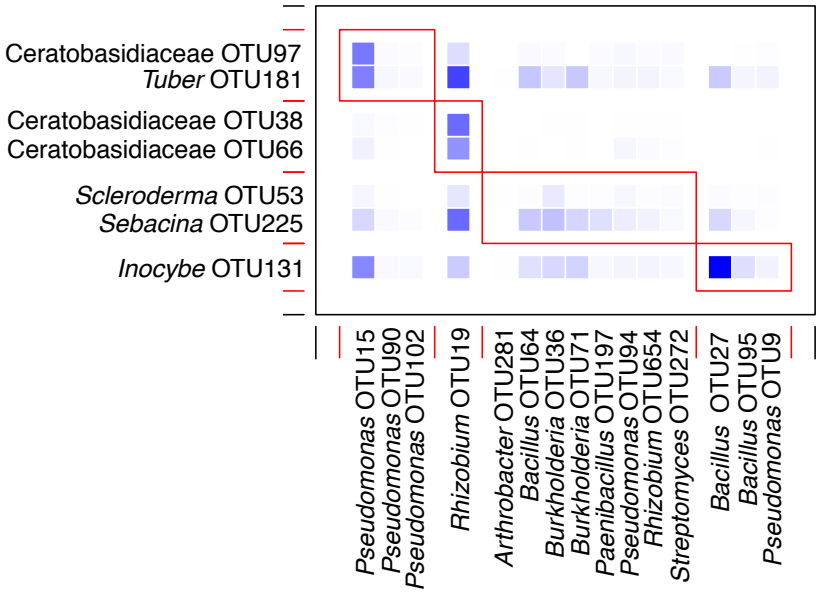

Figure 2.8. A visualization of co-occurrences between OTUs of putative MHB and fungal symbionts (OMF and ECM fungi) from bipartite analyses. Modules are identified in red.

Table 2.1. All primers used to generate amplicon libraries for Illumina miSeq. Rows show the completed primer construct i.e. the Nextera XT kit binding region and the linker. Annealing temperatures are for the entire primer construct.

Primers	Primers (standard desalt)	Nextera XT kit binding region	linker and locus specific primer 5'---3'	Annealing Temperature °C
515f_adpt	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCCAGCMGCCGCGGTAA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GTGCCAGCMGCCGCGGTAA	63.8-66.5
806r_adpt	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGGGCACTGAGGGACTACHVGGGTWTCTAAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	GTGGGCACTGAGGGACTACHVGGGTWTCTAAT	62.6-66.6
515f	GTGCCAGCMGCCGCGGTAA			
806r	GGACTACHVGGGTWTCTAAT			

Table 2.2. List of putative MHB detected in *V. planifolia* roots (terrestrial and epiphytic).

OTU ID	Epiphytic (read number)	Terrestrial (read number)	Phylum	Class	Order	Family	Genus
OTU2	77436	113831	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU6	7714	2178	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU255	7147	7062	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU11	1037	1045	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU36	555	11	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*
OTU579	215	136	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU71	174	43	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*
OTU95	142	104	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
OTU197	93	0	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
OTU474	83	407	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU183	64	0	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
OTU654	49	34	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
OTU203	24	37	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU9	17	67	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU73	16	23	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	uncl_Enterobacteriaceae
OTU94	9	112	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU192	5	4	Proteobacteria	Betaproteobacteria	Burkholderiales	uncl_Burkholderiales	uncl_Burkholderiales
OTU272	4	37	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*
OTU97	3	6	Proteobacteria	Betaproteobacteria	Burkholderiales	uncl_Burkholderiales	uncl_Burkholderiales
OTU622	2	6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	uncl_Enterobacteriales	uncl_Enterobacteriales
OTU422	2	1	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*
OTU382	0	16	Proteobacteria	Betaproteobacteria	Burkholderiales	uncl_Burkholderiales	uncl_Burkholderiales
OTU216	0	2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*

Table 2.3. List of OMF and ECM fungi identified from Chapter 1 as traditionally classified OMF and ECM fungi that are used in the co-occurrence network analysis.

OTU ID	Phylum	Class	Order	Family	Genus
OTU131	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU93	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU108	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	*Tomentella*
OTU88	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU132	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	*Mycena*
OTU38	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU97	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU181	Ascomycota	Pezizomycetes	Pezizales	Tuberaceae	*Tuber*
OTU95	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU66	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU225	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	*Sebacina*
OTU98	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	*Ceratobasidium*
OTU117	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	
OTU53	Basidiomycota	Agaricomycetes	Boletales	Sclerodermataceae	*Scleroderma*
OTU14	Basidiomycota	Agaricomycetes	Agaricales	Omphalotaceae	*Gymnopus*
OTU33	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	*Russula*
OTU169	Ascomycota	Pezizomycetes	Pezizales	Tuberaceae	*Tuber*
OTU157	Basidiomycota	Agaricomycetes	Sebacinales		
OTU553	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU403	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU84	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	*Sebacina*
OTU274	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU10	Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	*Marasmius*
OTU109	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	*Tomentella*
OTU170	Ascomycota	Pezizomycetes	Pezizales	Tuberaceae	*Tuber*
OTU223	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU205	Basidiomycota	Agaricomycetes	Sebacinales		
OTU94	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	*Russula*
OTU57	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU91	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	*Thanatephorus*
OTU105	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	*Tomentella*
OTU213	Basidiomycota	Agaricomycetes	Sebacinales		
OTU86	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU99	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU116	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	
OTU296	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU89	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU61	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	
OTU133	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*
OTU439	Basidiomycota	Agaricomycetes	Agaricales	Inocybaceae	*Inocybe*

Table 2.4. List of fungal symbionts (OMF and ECM) fungi in co-occurrence network. Red text represents OTUs that were retained after the network was generated. Sample count is the presence in each of the 37 root samples the OTU was present; Read abundance is the number of reads for the OTU after quality filtering; and the Degree is the number of bacterial nodes that is connected to the fungal OTUs.

OTU ID	Family	Sample count	Read Abundance	Degree
OTU133	Inocybaceae	1	2	
OTU439	Inocybaceae	1	2	
OTU403	Inocybaceae	2	518	
OTU274	**Inocybaceae**	**4**	**316**	**1**
OTU296	**Inocybaceae**	**5**	**13**	**21**
OTU553	**Inocybaceae**	**5**	**560**	**27**
OTU131	**Inocybaceae**	**19**	**4725**	**144**
OTU10	Marasmiaceae	2	117	67
OTU14	Omphalotaceae	2	659	
OTU132	Tricholomataceae	2	3135	
OTU53	**Sclerodermataceae**	**9**	**744**	**92**
OTU57	Ceratobasidiaceae	1	187	
OTU61	Ceratobasidiaceae	1	3	
OTU86	Ceratobasidiaceae	1	42	
OTU91	Ceratobasidiaceae	1	81	
OTU98	Ceratobasidiaceae	1	90	
OTU89	Ceratobasidiaceae	2	9	
OTU223	Ceratobasidiaceae	3	95	
OTU95	Ceratobasidiaceae	3	1518	
OTU88	Ceratobasidiaceae	4	4119	
OTU99	Ceratobasidiaceae	4	34	
OTU93	Ceratobasidiaceae	5	5934	
OTU66	**Ceratobasidiaceae**	**2**	**1400**	**60**
OTU38	**Ceratobasidiaceae**	**4**	**2681**	**68**
OTU97	**Ceratobasidiaceae**	**9**	**1671**	**79**
OTU94	Russulaceae	4	229	
OTU33	Russulaceae	5	641	

Table 2.5. Bipartite network statistics produced from computeModules analyses (function in R package bipartite). See the following page for the definitions of the column headings.

OTU ID	Degree	Species strength	Species Specificity index	Fisher alpha	Partner diversity	Effective partners	d
CeratobasidiaceaeOTU38	10	0.54	0.90	1.79	0.48	1.62	0.33
CeratobasidiaceaeOTU66	10	0.56	0.75	1.85	0.92	2.51	0.23
CeratobasidiaceaeOTU97	12	1.25	0.63	2.11	1.36	3.91	0.26
Inocybe OTU131	15	4.37	0.40	2.23	1.98	7.21	0.21
Scleroderma OTU53	14	0.99	0.32	3.17	2.13	8.40	0.11
Sebacina OTU225	15	3.49	0.31	2.36	2.16	8.68	0.09
Tuber OTU181	15	3.81	0.35	2.27	2.04	7.69	0.03
Arthrobacter Otu281	5	0.01	0.33	4.63	1.52	4.59	0.05
Bacillus Otu27	4	0.62	0.50	0.53	0.82	2.27	0.28
Bacillus Otu64	6	0.33	0.17	0.98	1.29	3.64	0.08
Bacillus Otu95	5	0.11	0.33	0.98	1.12	3.07	0.08
Burkholderia Otu36	6	0.52	0.17	0.98	1.44	4.24	0.11
Burkholderia Otu71	6	0.28	0.17	0.99	1.20	3.33	0.09
Paenibacillus Otu197	5	0.13	0.33	0.99	1.18	3.27	0.11
Pseudomonas Otu9	6	0.09	0.17	1.43	1.37	3.94	0.06
Pseudomonas Otu15	7	1.37	0.00	0.97	1.53	4.64	0.15
Pseudomonas Otu90	7	0.08	0.00	1.84	1.71	5.52	0.00
Pseudomonas Otu94	7	0.27	0.00	1.43	1.80	6.04	0.05
Pseudomonas Otu102	6	0.06	0.17	1.62	1.54	4.67	0.02
Rhizobium Otu19	7	2.82	0.00	0.91	1.75	5.77	0.22
Rhizobium Otu654	7	0.17	0.00	1.54	1.73	5.65	0.02
Streptomyces Otu272	7	0.14	0.00	1.70	1.80	6.04	0.03

Definitions for Table 5 column headings:

Degree:	Number of links (edges) from the OTU to other OTUs.
Species Specificity index:	The number of dependencies for each OTU. The purpose is to quantify an OTUs importance across all its partners.
Fisher alpha:	Fisher's alpha diversity for each OTU.
Partner diversity:	Calculated Shannon diversity.
Effective partners	The logbase to the power of “partner diversity”. This value determines if partners are unique.
d	A calculated number of the specialization of the OTU.

Figures and tables for Chapter 3

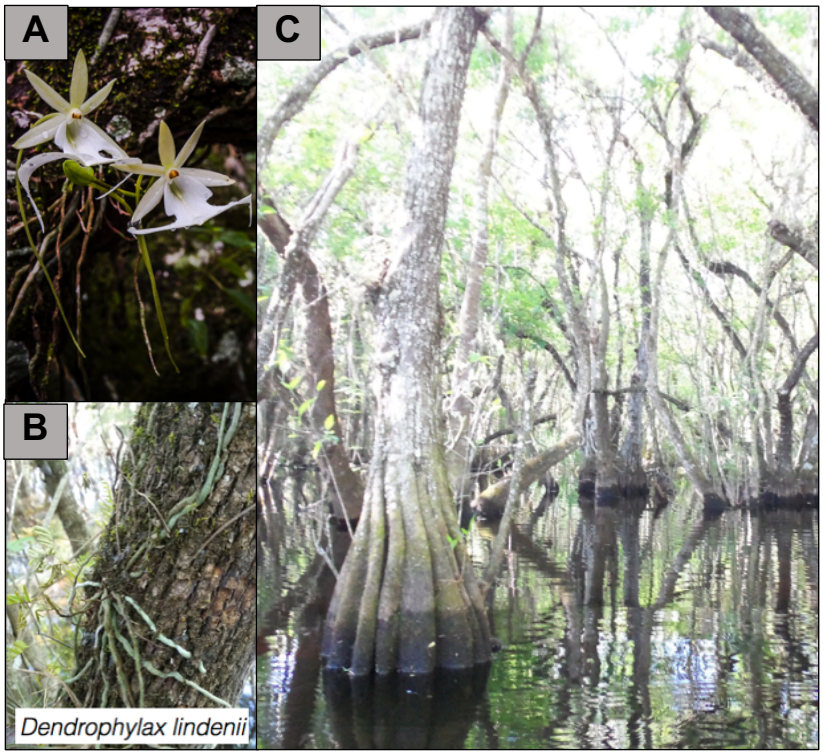

Figure 3.1. *Dendrophylax lindenii* and its habitat. A) Flowers of *D. lindenii*. B) *Dendrophylax lindenii* growing on its host tree. C) Habitat of *D. lindenii* in the Florida Panther National Wildlife Refuge.

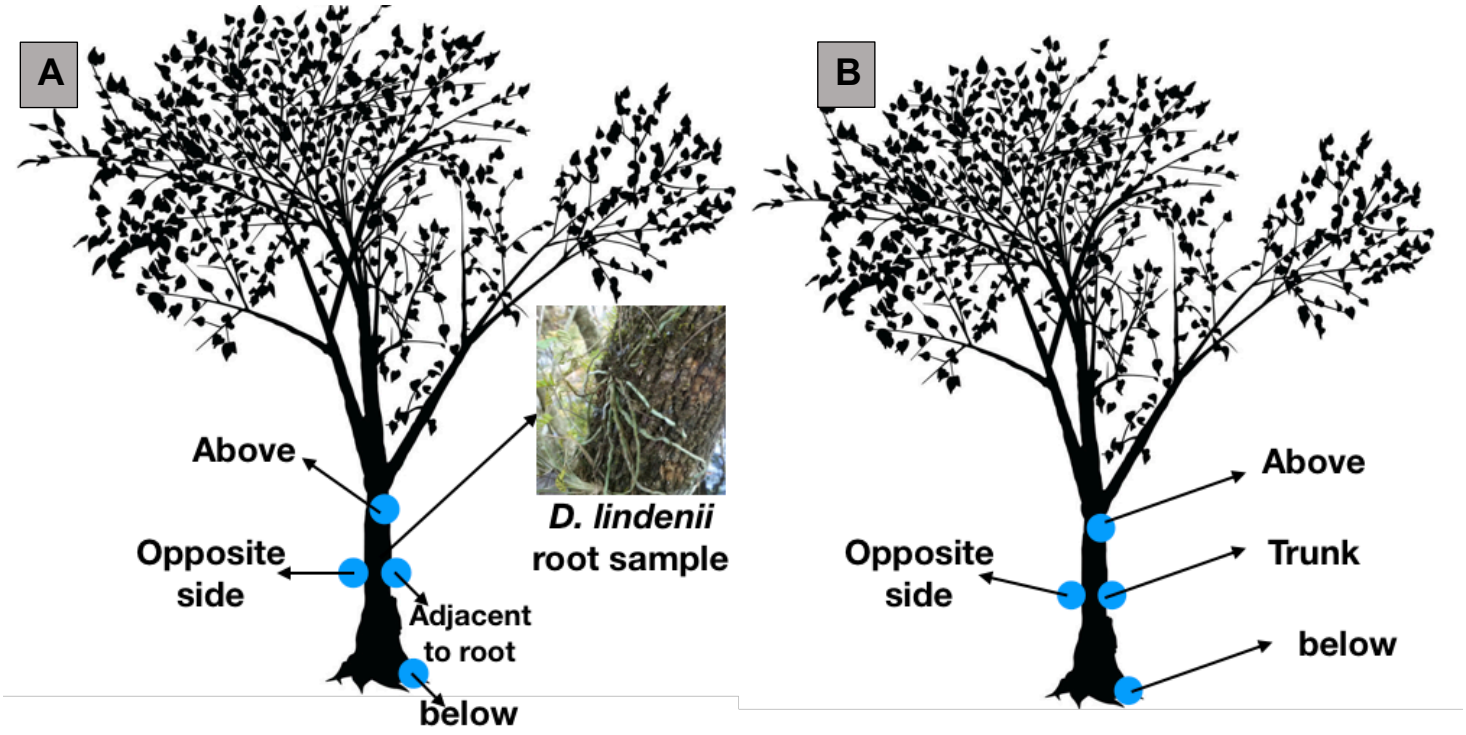

Figure 3.2. A) Sampling scheme to collect bark from trees with *D. lindenii*; and B) collecting bark samples from trees without *D. lindenii*.

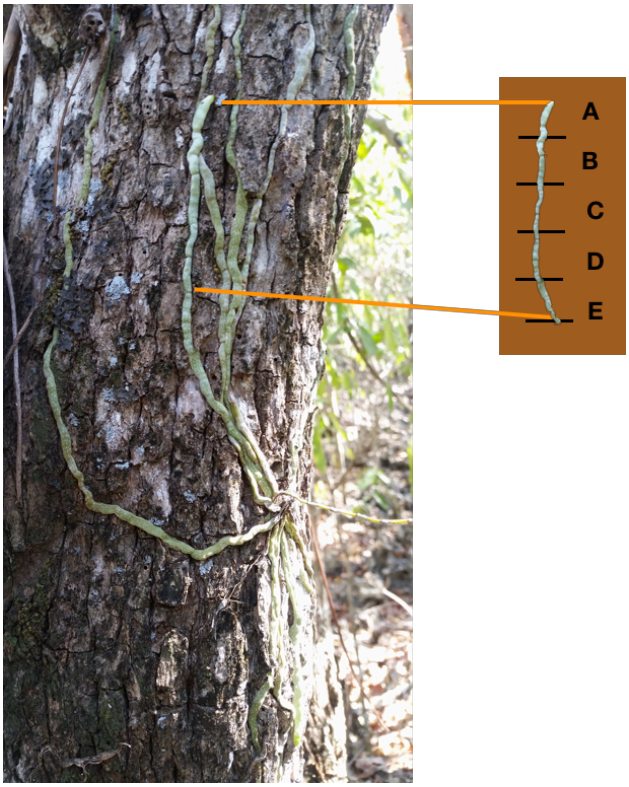

Figure 3.3. Representation of sampling root sections from *D. lindenii* individuals.

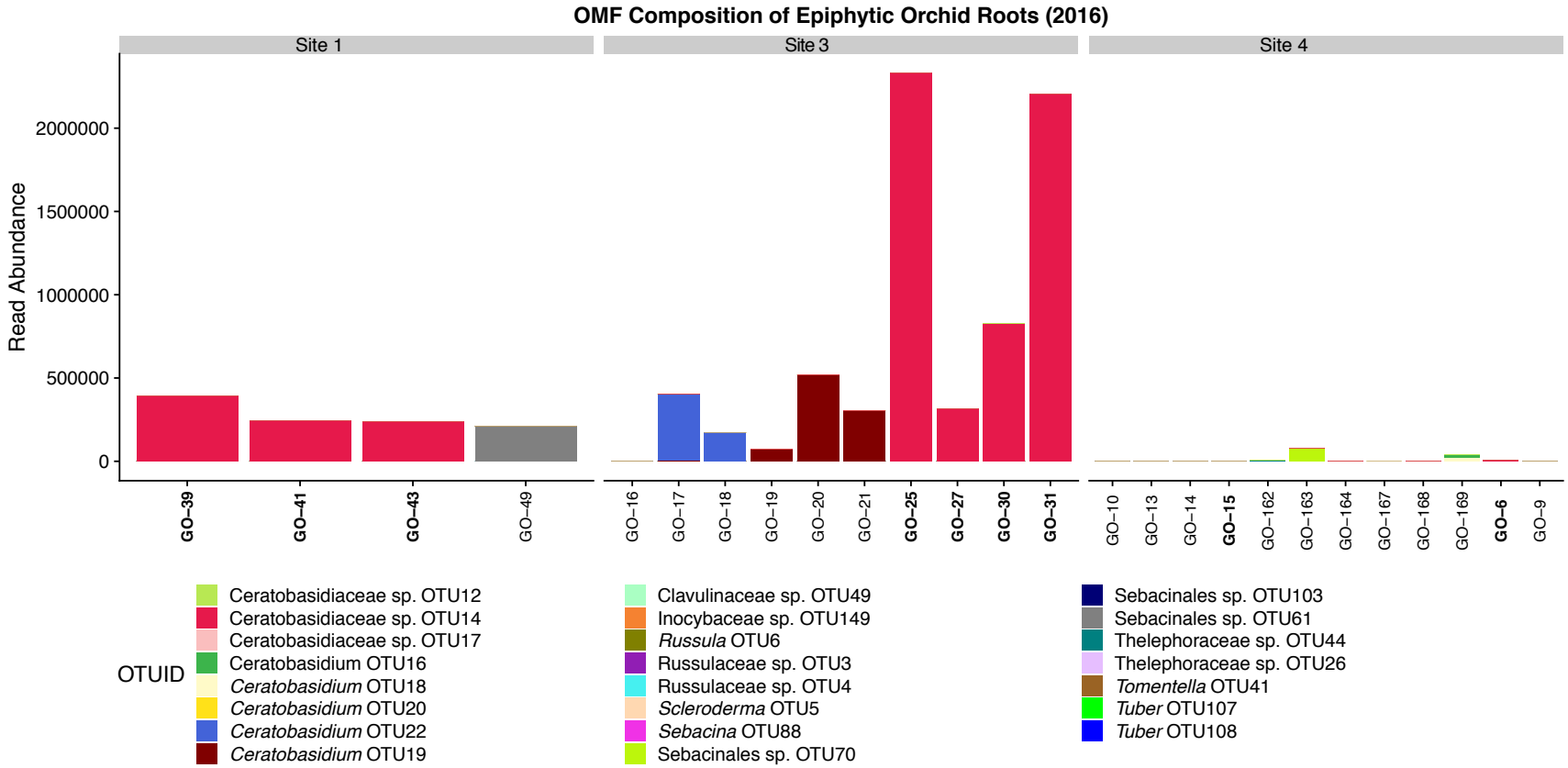

Figure 3.4. Read abundance of OMF and ECM fungal OTUs obtained from root samples of *D. lindenii* and co-occurring epiphytic orchids at the FPNWR in 2016. *Dendrophylax lindenii* samples are in bold.

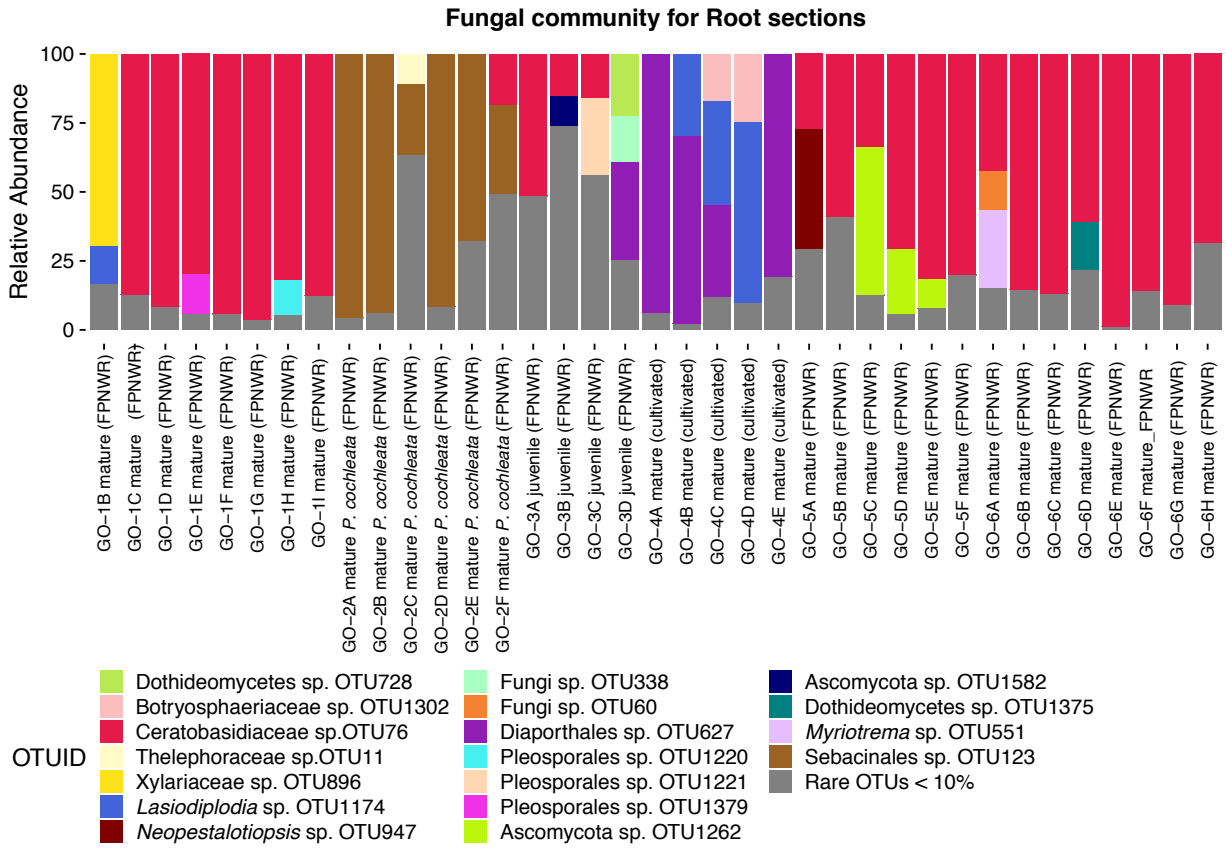

Figure 3.5. Relative abundance of fungal community in root sections of *D. lindenii.* Juvenile *D. lindenii* root samples are GO-3; the cultivated *D. lindenii* root samples are GO-4; mature root samples from naturally growing *D. lindenii* are GO-1, GO-5, GO-6; and *P. cochleata* root samples are GO-2.

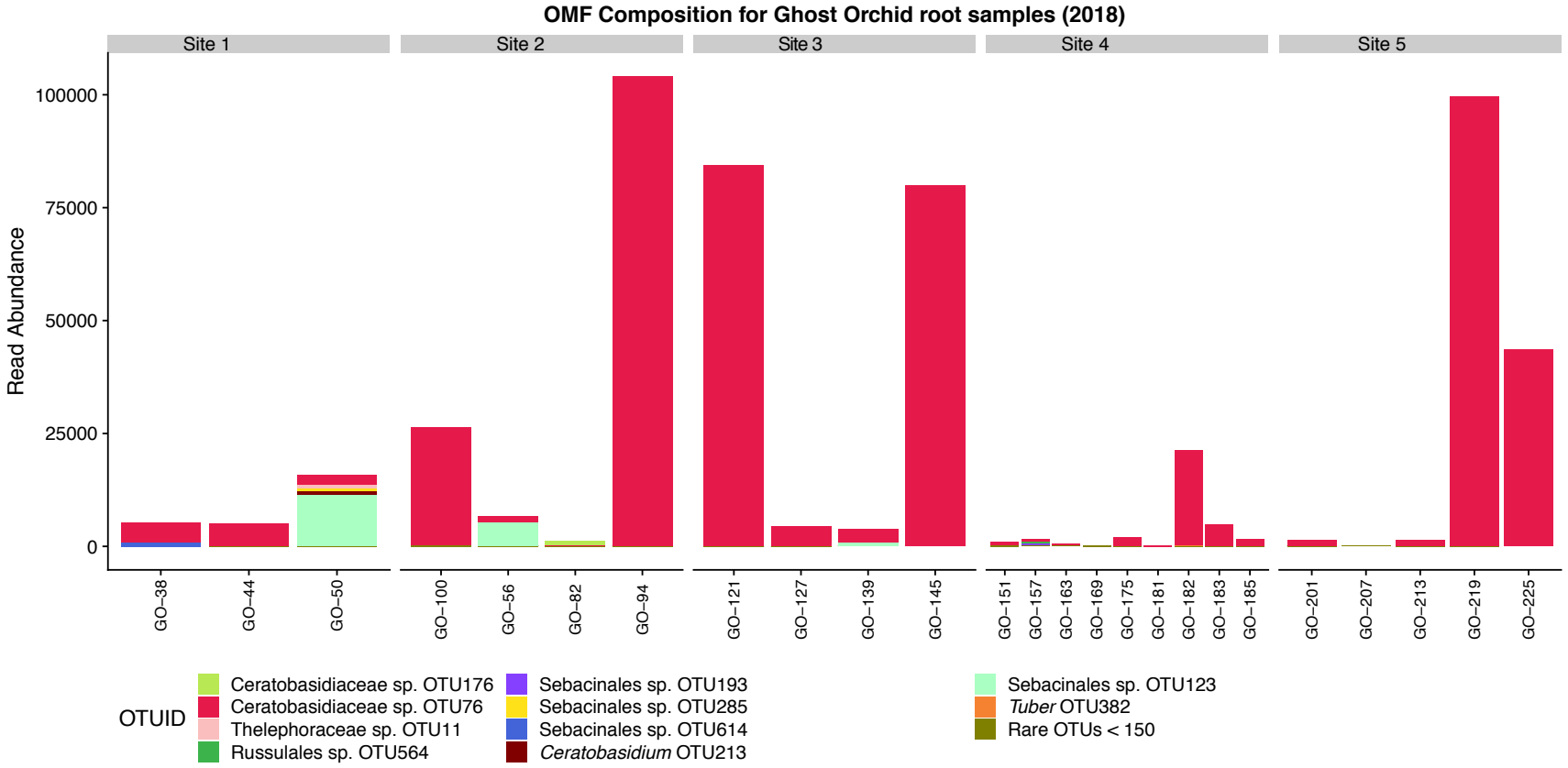

Figure 3.6. Read abundance of OMF and ECM fungal OTUs obtained from root samples of *D. lindenii* collected at the FPNWR in 2018.

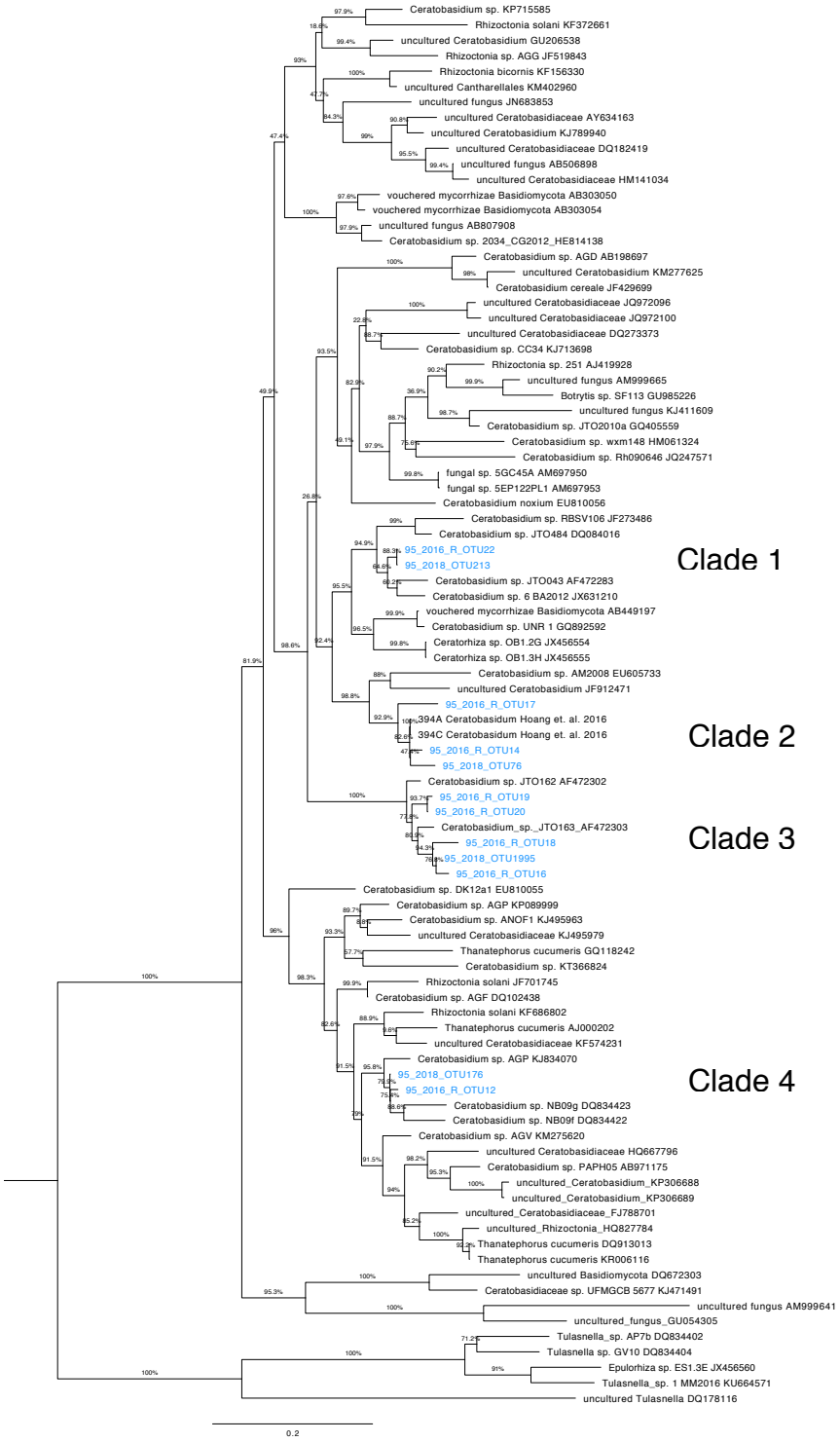

Figure 3.7. Maximum likelihood tree of putative Ceratobasidiaceae species collected from root and bark samples from the FPNWR in 2016 and 2018. Tip labels in blue represent Ceratobasidiaceae reads collected from this study.

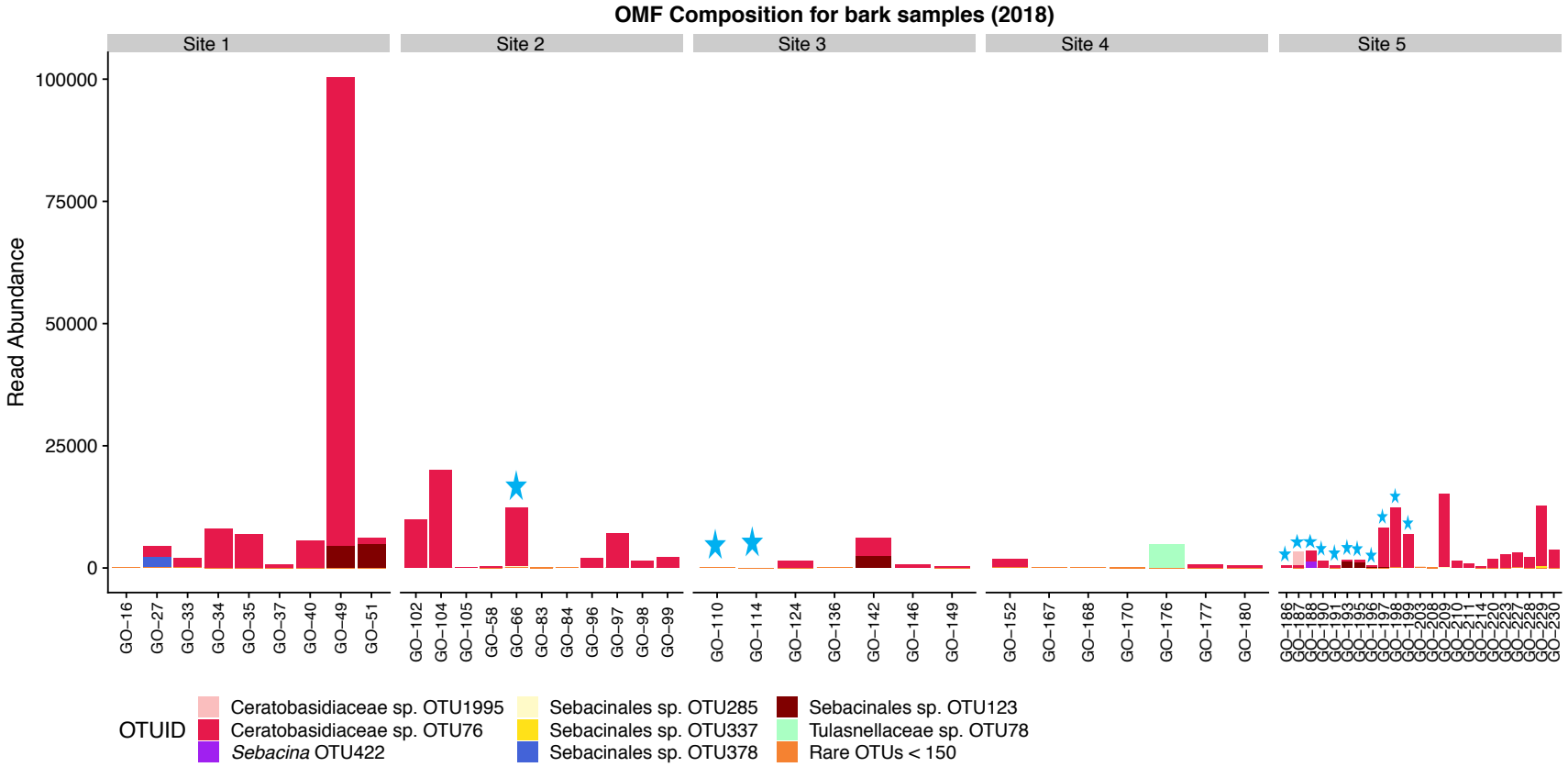

Figure 3.8. Read abundance of OMF and ECM fungal OTUs obtained from bark samples collected from trees with and without *D. lindenii* at FPNWR in 2018. Trees without *D. lindenii* is represented by a blue star: ★

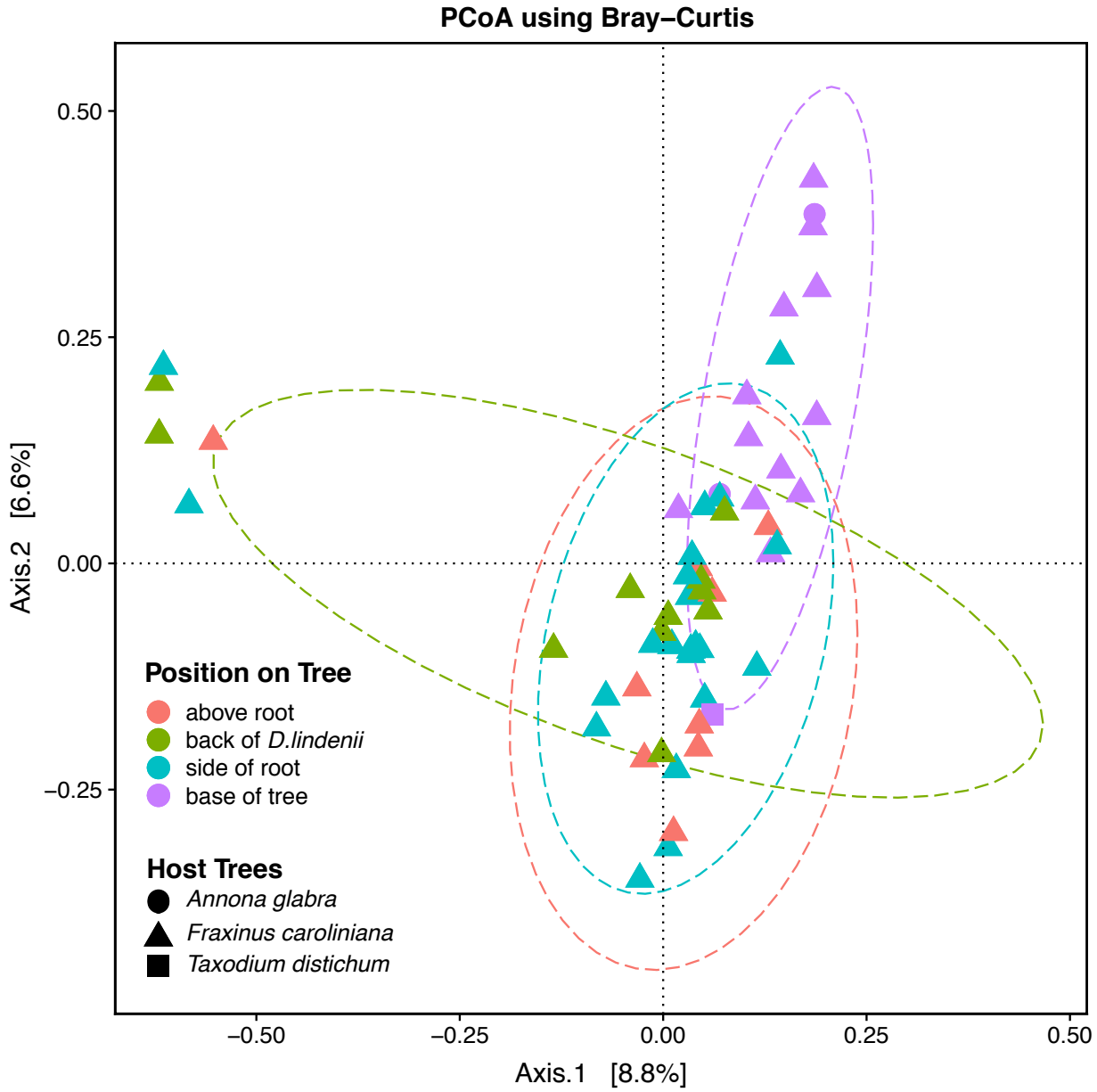

Figure 3.9. PCoA plot of bark samples from trees with and without *D. lindenii* collected at the FPNWR in 2018. Colors represent the positions bark samples were collected from the trees and shapes represent the tree species.

Figures and tables for Chapter 4

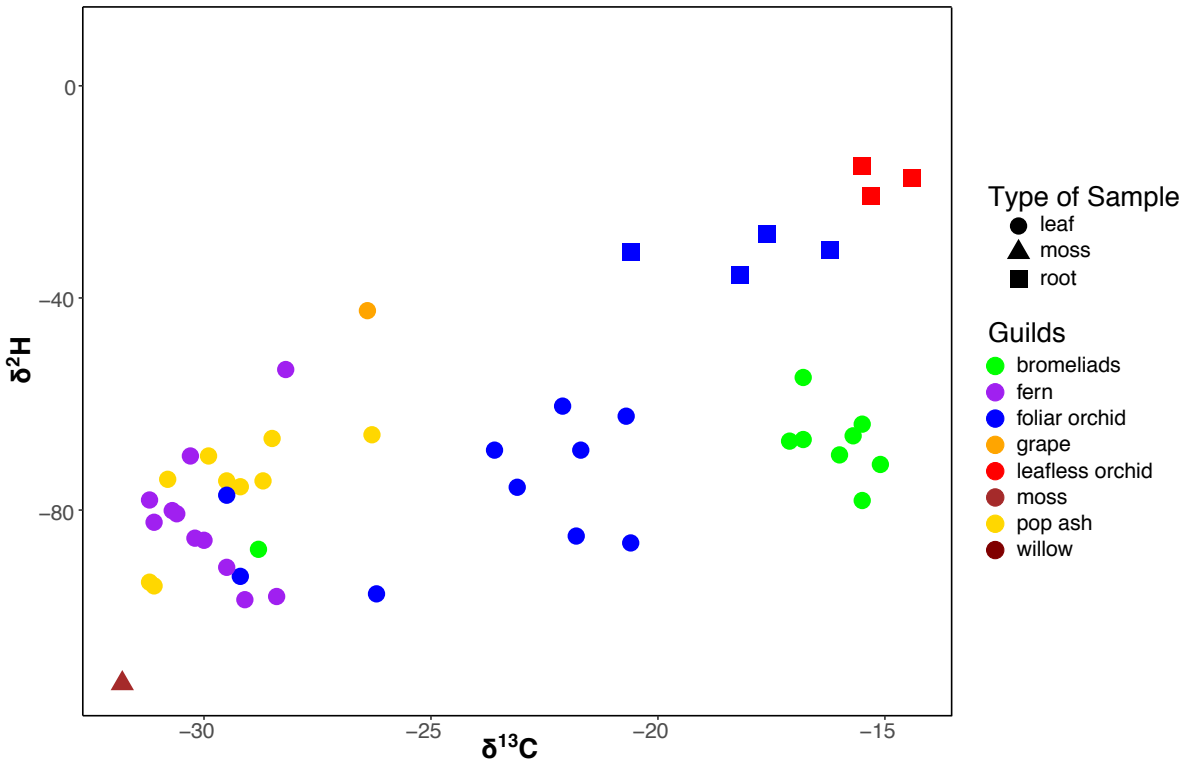

Figure 4.1. The natural abundance of carbon and hydrogen stable isotopes of different guilds sampled at the Florida Panther National Wildlife Refuge in 2016.

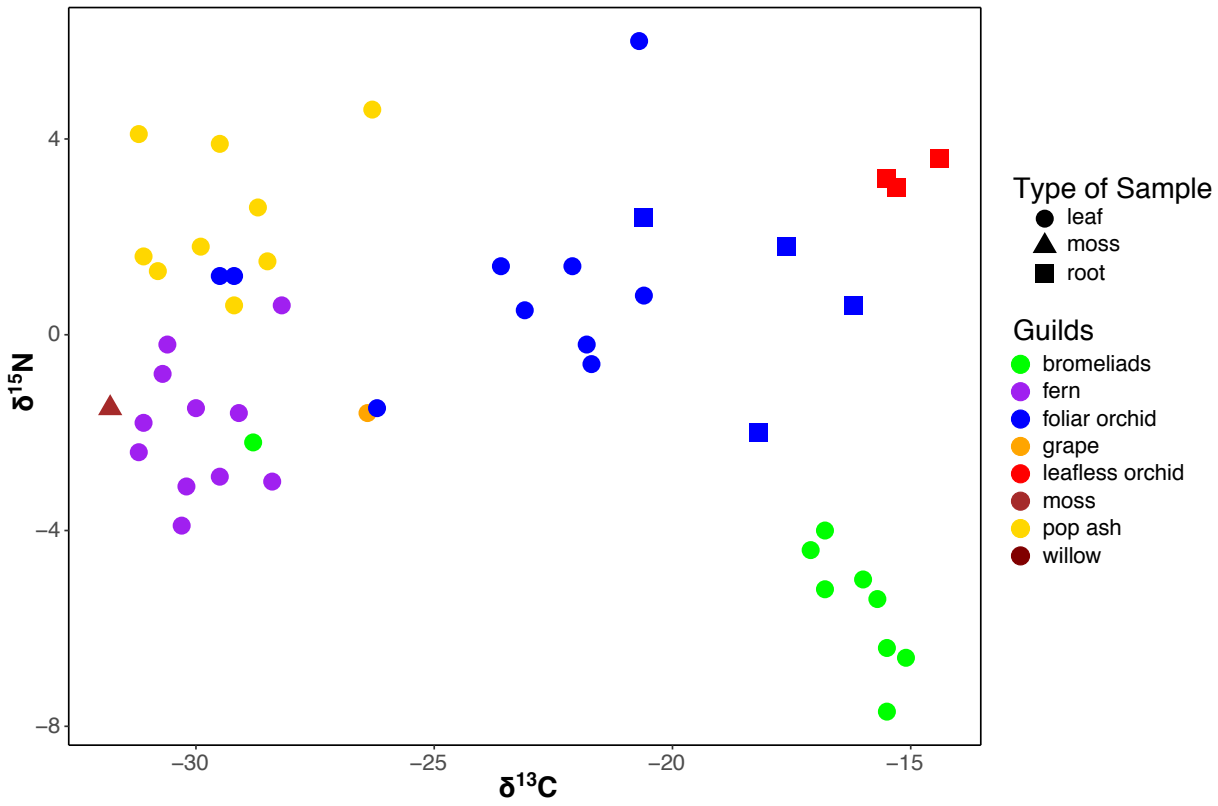

Figure 4.2. The natural abundance of carbon and nitrogen stable isotopes of different guilds sampled at the Florida Panther National Wildlife Refuge in 2016.

Table 4.1. Natural abundances of δ 2H δ13C, and δ15N for *D. lindenii*, co-occurring epiphytic orchids and reference plants collected from the Florida Panther National Wildlife Refuge in 2016. Along with replicates (n), the mean, and standard deviations (s.d.) are calculated for each guild's stable isotope.

Species Name	Guilds		δ ^{13}C [‰]	δ ^{15}N [‰]	δ ^{2}H [‰]	Type of plant tissue
Epidendrum amphistomum A. Rich.	foliar orchid		-23.6	1.4	-68.7	leaf
			-23.1	0.5	-75.7	leaf
			-22.1	1.4	-60.4	leaf
			-21.8	-0.2	-84.9	leaf
			-21.7	-0.6	-68.7	leaf
			-20.7	6.0	-62.3	leaf
			-20.6	0.8	-86.2	leaf
			-20.6	2.4	-31.4	root
			-17.6	1.8	-27.9	root
			-16.2	0.6	-30.9	root
					-78.8	leaf
					-67.8	leaf
					-42.2	root
					-40.7	root
		Mean	-20.8	1.4	-59.0	
		s.d.	2.3	1.8	20.5	
		n	15	15	15	
Epidendrum Nocturnum Jacq.	foliar orchid		-18.2	-2.0	-35.7	root
Encyclia tampensis (Lindl.)	foliar orchid				-59.2	leaf
					-40.8	leaf
		Mean			-50.0	
		s.d.			13.0	

		n				
Prosthechea cochleata (L.) W. E. Higgins	foliar orchid		-29.5	1.2	-77.2	leaf
			-29.2	1.2	-92.5	leaf
					-93.7	leaf
					-76.3	root
					-71.3	root
					-65.3	root
					-62.7	root
		Mean	-29.4	1.2	-77.0	
		s.d.	0.3	0.0	12.2	
		n	8	8	8	
Polystachya concreta (Jacq.) Garay & H.R.Sweet	foliar orchid		-26.2	-1.5	-95.8	leaf
					-94.4	leaf
					-75.1	leaf
					-50.3	root
		Mean	-26.2	-1.5	-78.9	
		s.d.			21.3	
		n	5	5	5	
Dendrophylax Lindenii (Lindl.) Bentham. ex Rolfe	leafless		-15.5	3.2	-15.1	root
			-15.3	3.0	-20.8	root
			-14.4	3.6	-17.3	root
		Mean	-15.0	3.2	-17.7	
		s.d.	0.6	0.3	2.9	
		n	4	4	4	
Dendrophylax Porrectus (Rchb.f.) Carlsward & Whitten	leafless				-25.7	root
Bromeliad	Bromeliaceae		-28.8	-2.2	-87.4	leaf

			-17.1	-4.4	-67.0	leaf
			-16.8	-5.2	-66.7	leaf
			-16.8	-4.0	-55.0	leaf
			-16.0	-5.0	-69.6	leaf
			-15.7	-5.4	-66.0	leaf
			-15.5	-7.7	-63.8	leaf
			-15.5	-6.4	-78.2	leaf
			-15.1	-6.6	-71.4	leaf
					-63.8	leaf
		Mean	-17.5	-5.2	-68.9	
		s.d.	0.7	1.2	6.3	
		n	11	11	11	
Fern	fern		-31.2	-2.4	-78.1	leaf
			-31.1	-1.8	-82.3	leaf
			-30.7	-0.8	-80.1	leaf
			-30.6	-0.2	-80.7	leaf
			-30.3	-3.9	-69.8	leaf
			-30.2	-3.1	-85.3	leaf
			-30.0	-1.5	-85.7	leaf
			-29.5	-2.9	-90.8	leaf
			-29.1	-1.6	-96.9	leaf
			-28.4	-3.0	-96.3	leaf
			-28.2	0.6	-53.5	leaf
					-86.5	leaf
		Mean	-29.9	-1.9	-82.2	
		s.d.	1.0	1.4	11.8	
		n	12	12	12	
Fraxinus caroliniana Mill.	pop ash		-31.2	4.1	-93.6	leaf
			-31.1	1.6	-94.3	leaf
			-30.8	1.3	-74.2	leaf
			-29.9	1.8	-69.8	leaf
			-29.5	3.9	-74.5	leaf
			-29.2	0.6	-75.6	leaf
			-28.7	2.6	-74.5	leaf
			-28.5	1.5	-66.5	leaf
			-26.3	4.6	-65.8	leaf

					-59.0	leaf
					-58.7	leaf
		Mean	-29.5	2.4	-73.3	
		s.d.	1.5	1.4	11.8	
		n	16	16	16	
moss	moss		-31.8	-1.5	-112.7	moss
					-75.8	moss
						moss
		Mean	-31.8	-1.5	-94.2	
		s.d.				
		n	3	3	3	
grape	grape		-26.4	-1.6	-42.4	leaf

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Appendix I: Supplemental Information for Chapter 1

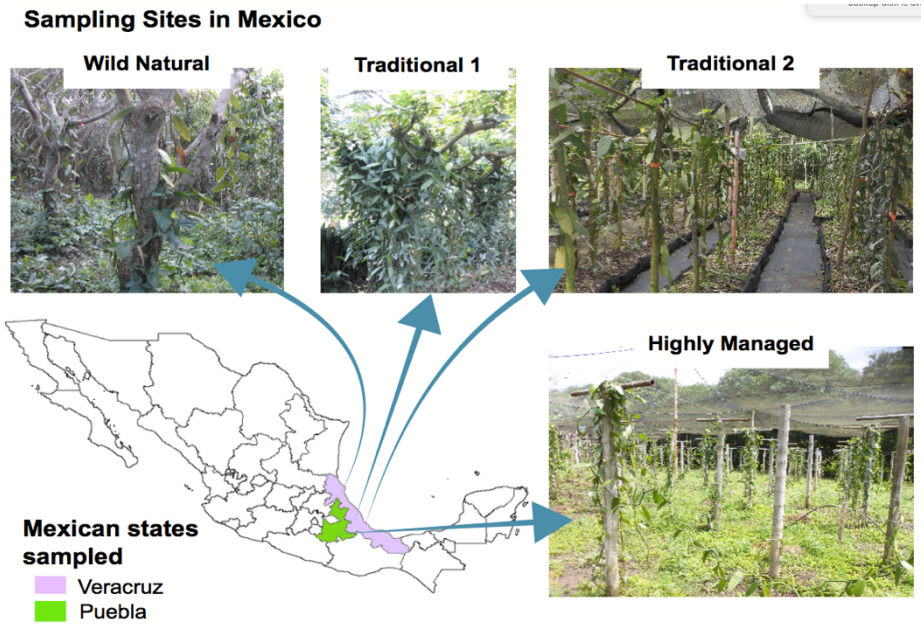


Figure 1.S1. Map of Mexican states where this study collected root samples from *V. planifolia* farms.

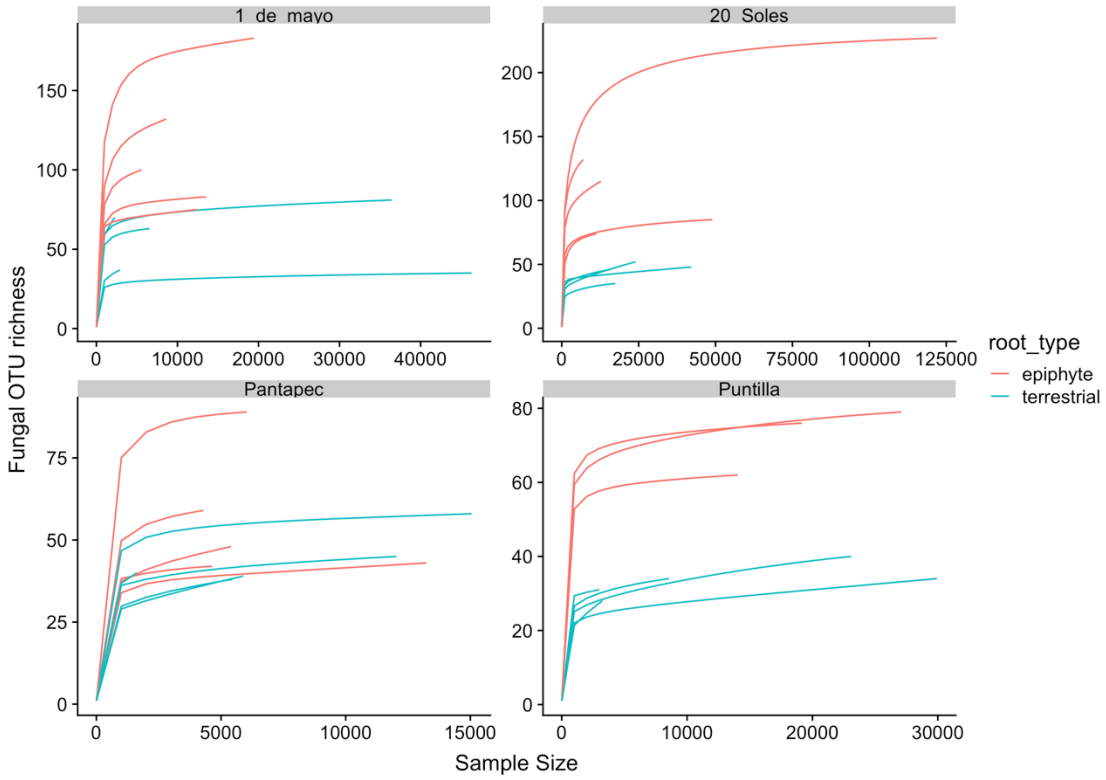


Figure 1.S2. Rarefaction curves of fungal OTUs in *V. planifolia*. Mexican vanilla farms: Pantepec (highly managed farm), 20 Soles (traditional farm), Puntilla (traditional farm), 1 de Mayo (wild natural farm).

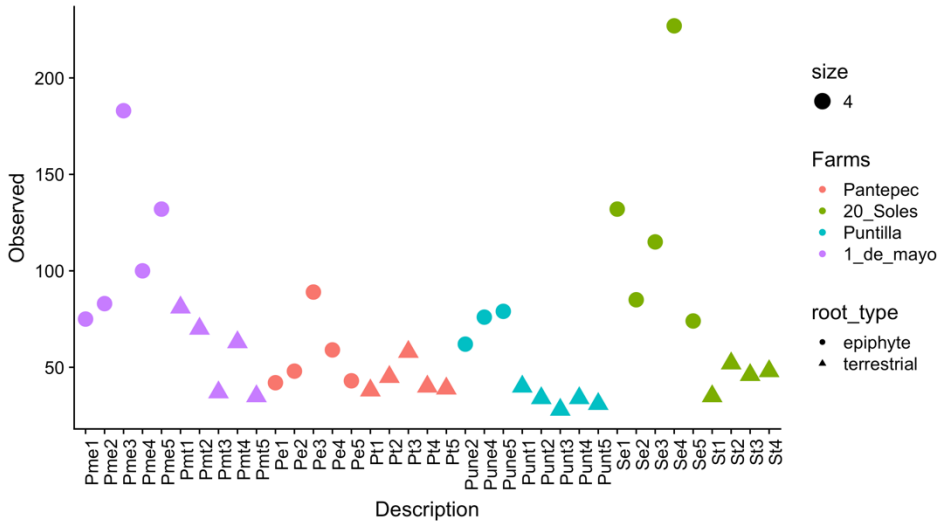


Figure 1.S3. Observed species richness of fungi sequenced from roots of *V. planifolia* from four Mexican farms: Pantepec (highly managed farm), 20 Soles (traditional farm), Puntilla (traditional farm), 1 de Mayo (Wild natural farm).

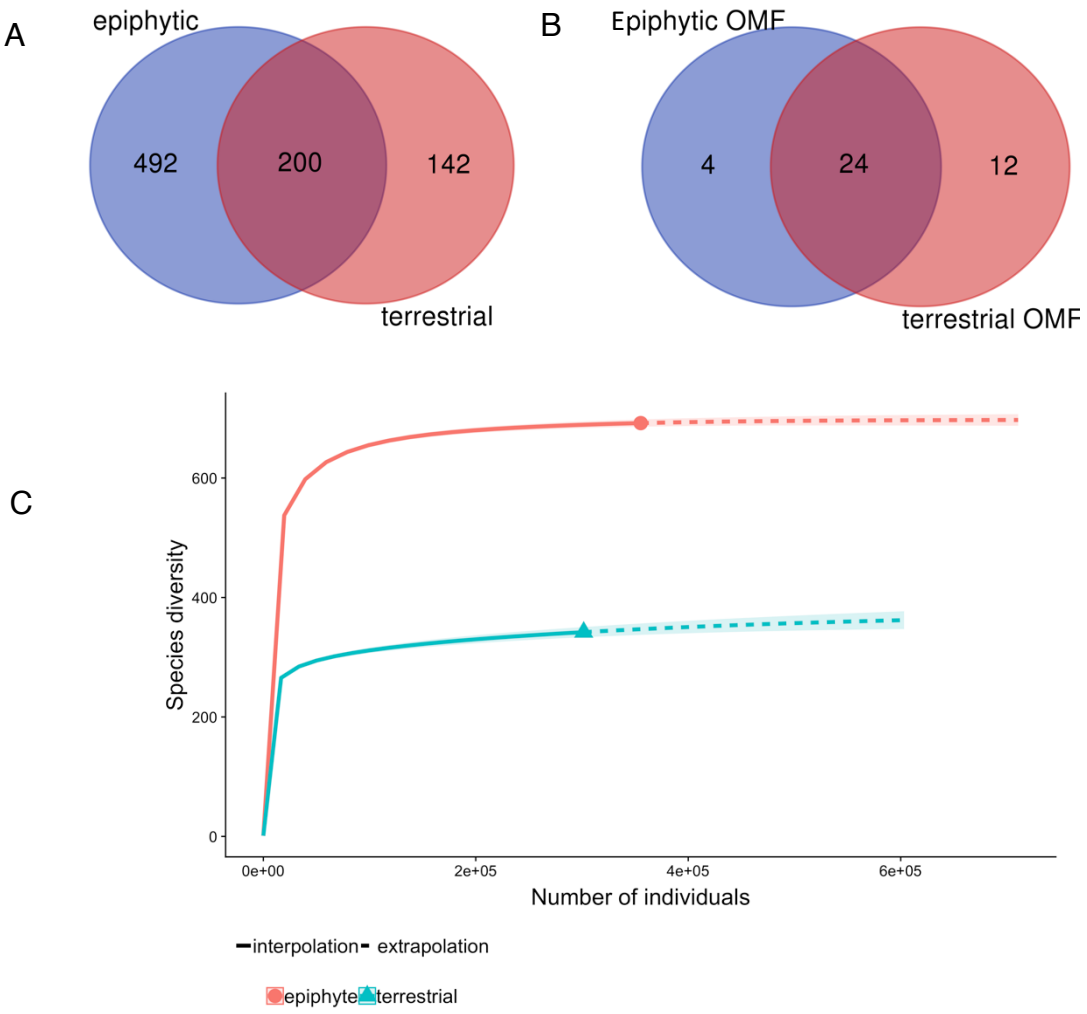


Figure 1.S4. Venn diagrams comparing A) fungal OTUs and B) OMF OTUs in *V. planifolia* roots. C) Species richness of fungal communities of *V. planifolia* roots. Continuous lines represent values interpolated from the data and the dashed lines represents values extrapolated from the data.

Table 1.S1. Geographic locations of Mexican farms and the farming practice used at each *V. planifolia* farm.

Locality	coordinates	n	Farming practice
1 de Mayo, Papantla de Olarte Ocampo, Veracruz	20° 17' 719" N, 97° 15' 909" W	5	Wild-natural, substrate = tree (live) bark
Pantepec, Puebla	20° 30' 18" N, 97° 53' 22" W	5	Highly managed, substrate = cement
20 Soles, Papantla de Olarte, Veracruz	20° 25' 1.57"N, 97° 18' 8.04" W	5	Traditional, substrate = dead wood
Puntilla Aldama, San Rafael, Veracruz	20° 10' 45.58" N, 96° 54' 13.69" W	5	Traditional, tree (live) bark

Table 1.S2. All primers used in this study. Each row shows the completed primer construct i.e. the Nextera XT kit binding region and the linker. The annealing temperature represents the entire primer construct.

Primers	Primers (standard desalt)	Nextera XT kit binding region	linker and locus specific primer 5'---3'	Annealing Temperature C
ITS86F-adpt	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAGCAGGCGGTGTGAATCATCGAATCTTTGAA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GCAGCAGGCGGTGTGAATCATCGAATCTTTGAA	64.8
ITS4-adpt	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGGAGGATCCTCCGCTTATTGATATGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	AGGGAGGATCCTCCGCTTATTGATATGC	61.3
ITS86F	GTGAATCATCGAATCTTTGAA			
ITS4	TCCTCCGCTTATTGATATGC			

Table 1.S3. Fungal guilds detected in roots of *V. planifolia*.

Assigned guilds	Total OTUs
No assigned guild	498
Pathogen-Saprotroph-Symbiotroph	5
Pathotroph	52
Pathotroph-Saprotroph	40
Pathotroph-Saprotroph-Symbiotroph	67
Pathotroph-Symbiotroph	14
Saprotroph	103
Saprotroph-Symbiotroph	11
Symbiotroph	44

Table 1.S4. Fungal symbionts (ECM fungi and OMF) identified in *V. planifolia* roots, Fungal symbionts unique to roots are denoted by the following uppercase letters: Epiphytic roots = E; Terrestrial roots = T.

OTU	Family	UNITE assigned Taxon	UNITE Species Hypothesis ID	Putative Guild*
OTU10[E]	Marasmiaceae	*Marasmius*	SH1543540.08FU	Saprobic fungus
OTU14[E]	Omphalotaceae	*Gymnopus*	SH1542667.08FU	Saprobic fungus
OTU33	Russulaceae	*Russula*	SH1509944.08FU	ECM fungus
OTU38	Ceratobasidiaceae	*Ceratobasidium*	SH1514712.08FU	OMF
OTU53	Sclerodermataceae	*Scleroderma*	SH1526178.08FU	ECM fungus
OTU57[E]	Ceratobasidiaceae	*Thanatephorus*	SH1551727.08FU	OMF
OTU61[E]	Ceratobasidiaceae	*Ceratobasidium*	SH1514712.08FU	OMF
OTU66[E]	Ceratobasidiaceae	*Ceratobasidium*	SH1551745.08FU	OMF
OTU84	Serendipitaceae	*Sebacina*	SH1510988.08FU	OMF
OTU86[E]	Ceratobasidiaceae	*Ceratobasidium*	SH1551745.08FU	OMF
OTU88	Ceratobasidiaceae	*Thanatephorus*	SH1551752.08FU	OMF
OTU89	Ceratobasidiaceae	*Rhizoctonia*	SH1505353.08FU	OMF
OTU91[E]	Ceratobasidiaceae	*Thanatephorus*	SH1551733.08FU	OMF
OTU93	Ceratobasidiaceae	*Ceratobasidium*	SH1551778.08FU	OMF
OTU94	Russulaceae	*Russula*	SH1569727.08FU	ECM fungus
OTU95	Ceratobasidiaceae	*Ceratobasidium*	SH1608943.08FU	OMF
OTU97	Ceratobasidiaceae	*Ceratobasidium*	SH1510176.08FU	OMF
OTU98[E]	Ceratobasidiaceae	*Ceratobasidium*	SH1551738.08FU	OMF
OTU99	Ceratobasidiaceae	*Ceratobasidium*	SH1551778.08FU	OMF
OTU105	Thelephoraceae	*Tomentella*	SH1528520.08FU	ECM fungus
OTU108	Thelephoraceae	*Tomentella*	SH1502723.08FU	ECM fungus
OTU109[T]	Thelephoraceae	*Tomentella*	SH1528461.08FU	ECM fungus
OTU116	Thelephoraceae	Thelephoraceae sp.	SH1502742.08FU	ECM fungus
OTU117	Thelephoraceae	Thelephoraceae sp.	SH1611050.08FU	OMF
OTU131	Inocybaceae	*Inocybe*	SH1557838.08FU	ECM fungus
OTU132[E]	Tricholomataceae	*Mycena*	SH1554462.08FU	Saprobic fungus
OTU133[E]	Inocybaceae	*Inocybe*	SH1557838.08FU	ECM fungus
OTU157	Serendipitaceae	Sebacinales sp.	SH1572434.08FU	OMF
OTU169	Tuberaceae	*Tuber*	SH1645328.08FU	ECM fungus
OTU170	Tuberaceae	*Tuber*	SH1648385.08FU	ECM fungus
OTU181	Tuberaceae	*Tuber*	SH1648386.08FU	ECM fungus
OTU205[T]	Serendipitaceae	*Sebacinales*		OMF
OTU213[E]	Serendipitaceae	*Sebacina*	SH1563273.08FU	OMF

OTU223 [T]	Ceratobasidiaceae	Ceratobasidiaceae	SH1609969.08FU	OMF
OTU225	Serendipitaceae	*Sebacina*	SH1515904.08FU	OMF
OTU274	Inocybaceae	*Inocybe*	SH1524174.08FU	ECM fungus
OTU296	Inocybaceae	*Inocybe*	SH1505632.08FU	ECM fungus
OTU403[T]	Inocybaceae	*Inocybe*	SH1553922.08FU	ECM fungus
OTU439 [E]	Inocybaceae	*Inocybe*	SH1553919.08FU	ECM fungus
OTU553	Inocybaceae	*Inocybe*	SH1553919.08FU	ECM fungus

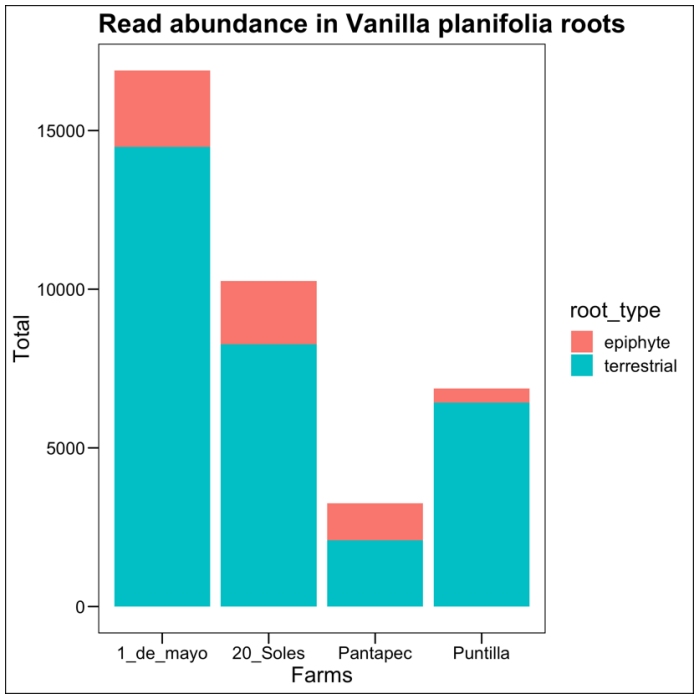


Figure 1.S5. Sequence abundance of only OMF in epiphytic and terrestrial roots of *V. planifolia* from four Mexican farms: Pantepec (highly managed farm), 20 Soles (traditional farm), Puntilla (traditional farm), 1 de Mayo (Wild natural farm).

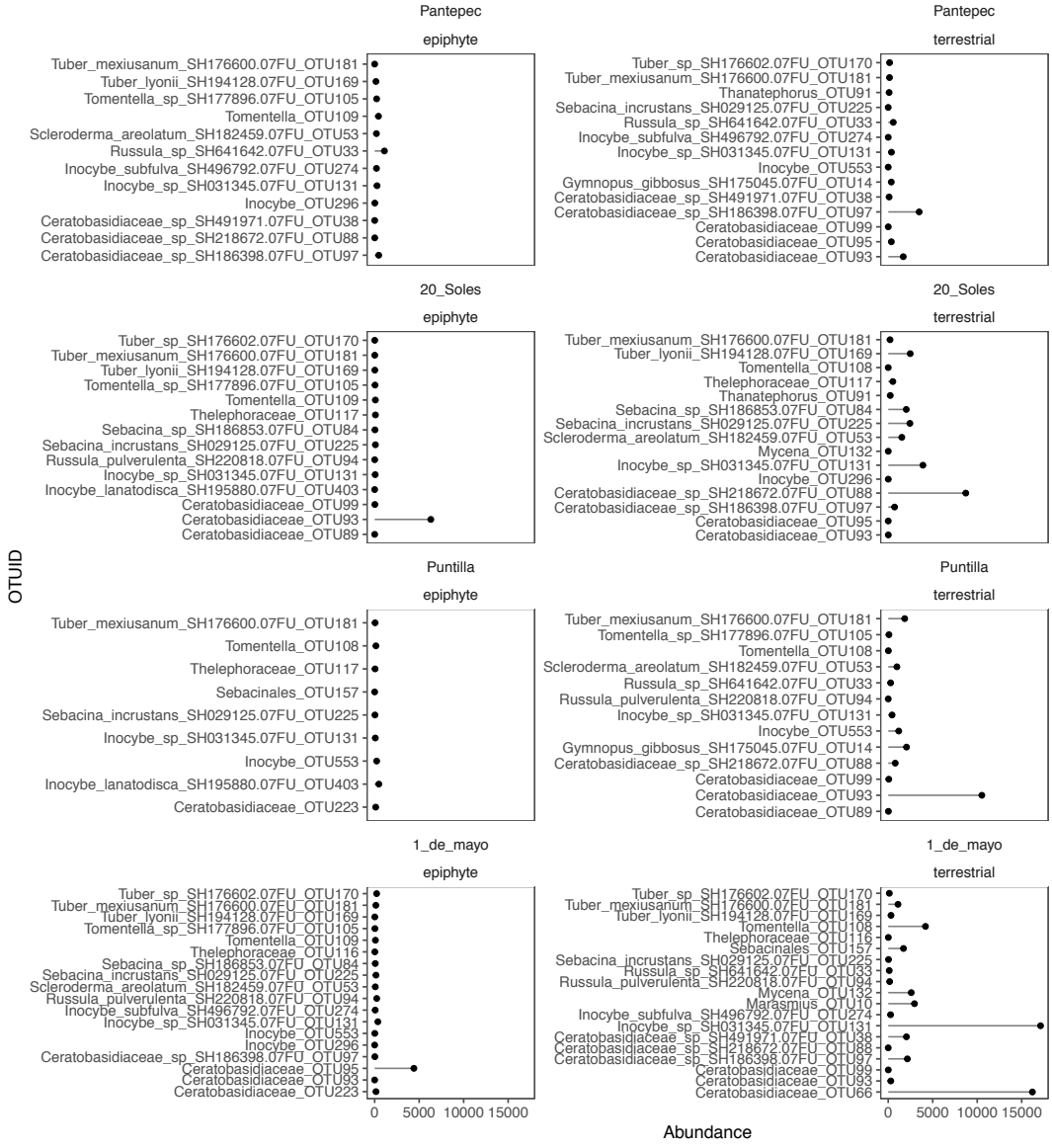
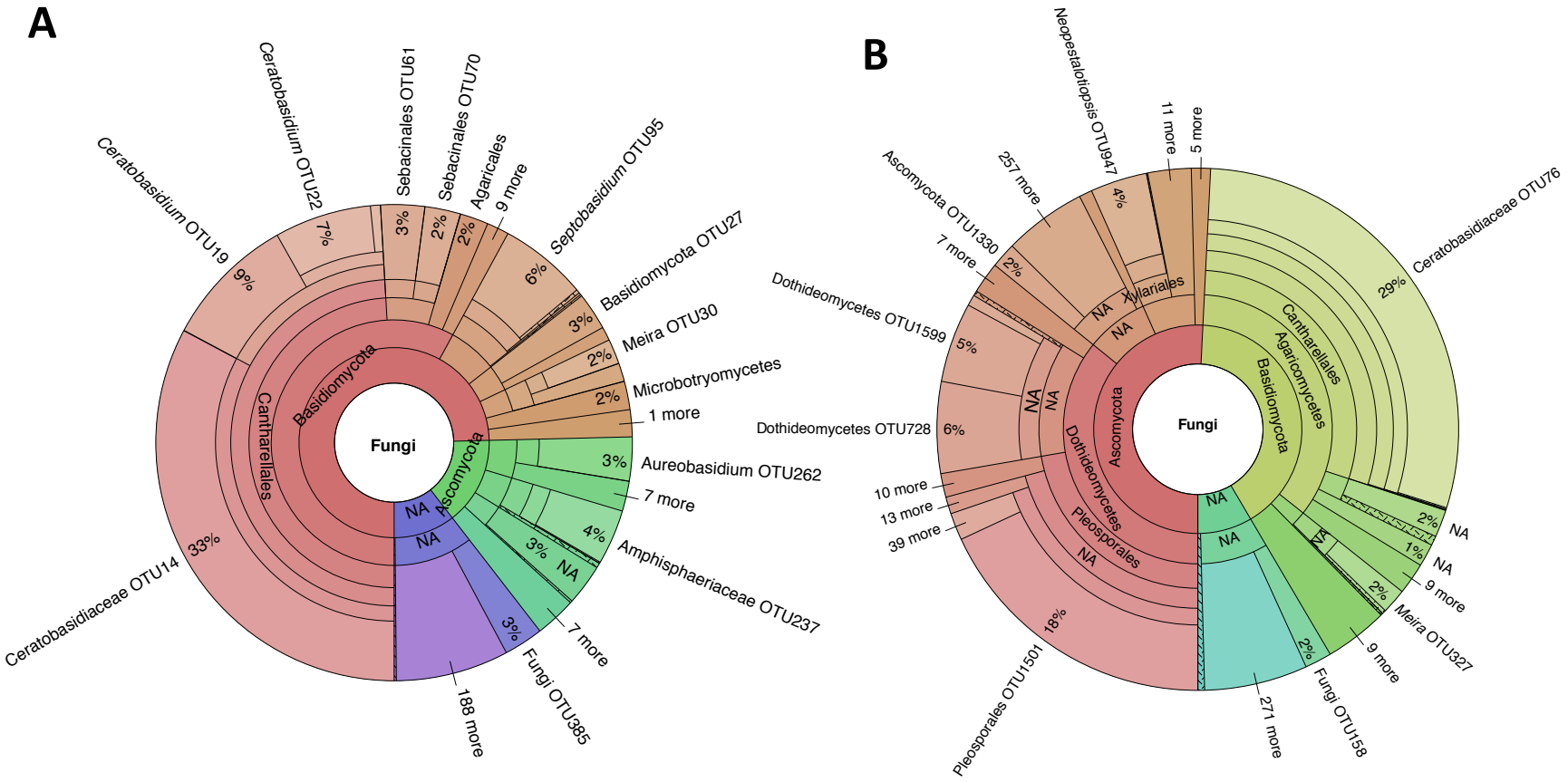


Figure 1.S6. Read abundances for the fungal symbiont OTUs sequenced from *V. planifolia* roots across 4 different farms: Pantepec (highly managed farm), 20 Soles (traditional farm), Puntilla (traditional farm), 1 de Mayo (Wild natural farm).


A
Fungi
Basidiomycota
Ascomycota
Cantharellales
NA
Ceratobasidium OTU19
9%
Ceratobasidium OTU22
7%
Sebacinales OTU61
3%
Sebacinales OTU70
2%
Agaricales
2%
9 more
6%
Septobasidium OTU95
3%
Basidiomycota OTU27
2%
Meira OTU30
2%
Microbotryomycetes
1 more
3%
Aureobasidium OTU262
7 more
4%
Amphisphaeriaceae OTU237
3%
NA
7 more
3%
Fungi OTU385
188 more
3%
NA
Ceratobasidiaceae OTU14
33%
B
Fungi
Basidiomycota
Ascomycota
Agaricomycetes
Cantharellales
Dothideomycetes
Pleosporales
Xylariales
NA
Ceratobasidiaceae OTU76
29%
11 more
5 more
Neopestalotiopsis OTU947
4%
257 more
Ascomycota OTU1330
2%
7 more
Dothideomycetes OTU1599
5%
Dothideomycetes OTU728
6%
10 more
13 more
39 more
Pleosporales OTU1501
18%
271 more
2%
Fungi OTU158
9 more
2%
Meira OTU327
9 more
1%
NA
2%
NA

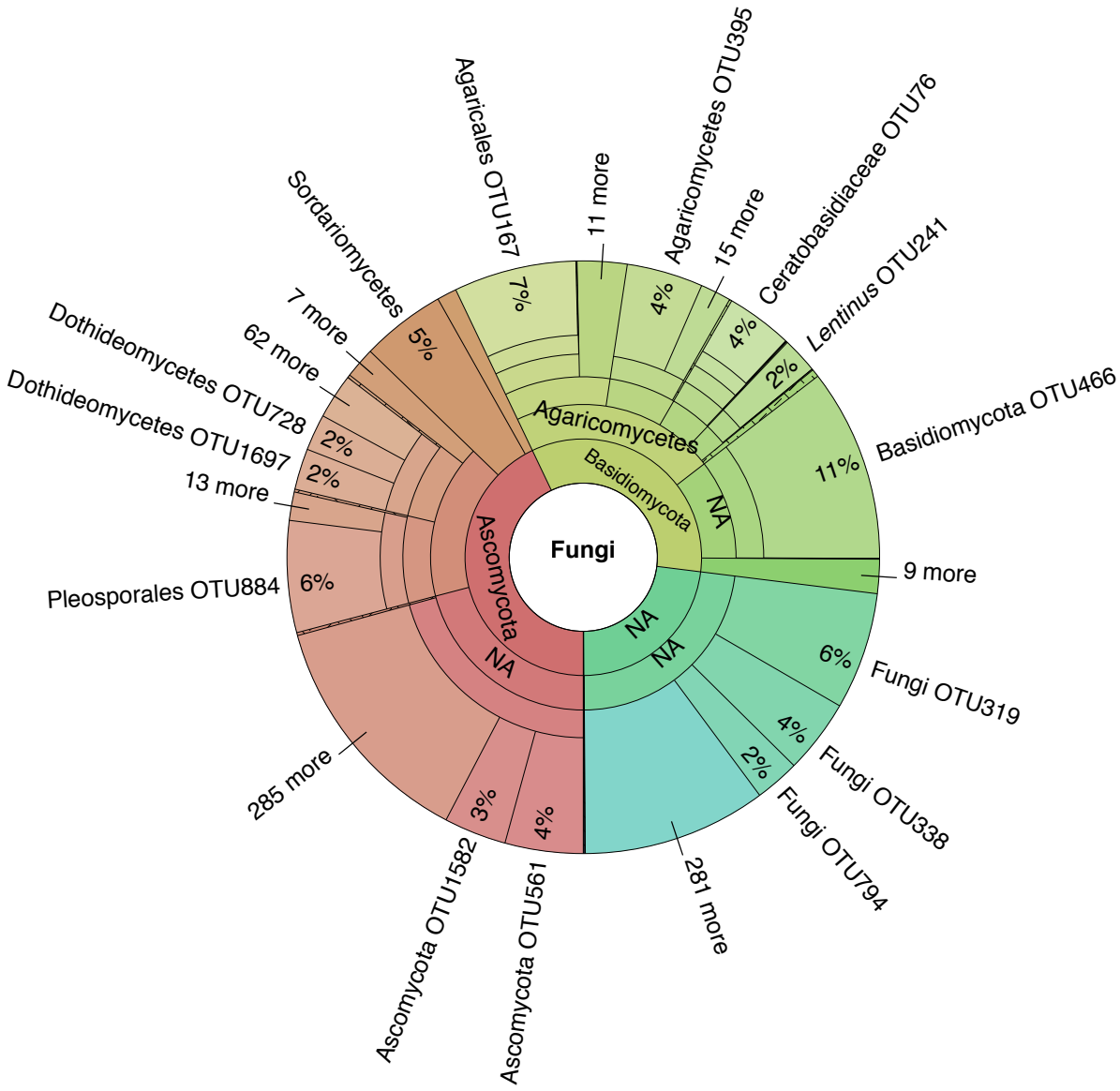


Figure 3.S1. Krona charts of the taxonomic affiliation of samples derived from A) epiphytic orchid roots and *D. lindenii* collected at the Florida Panther National Wildlife Refuge (FPNWR) in 2016. B) *Dendrophylax lindenii* root samples collected at the FPNWR in 2018. C) bark samples collected from trees with and without *D. lindenii* at the FPNWR in 2018.

2016 Root Dataset

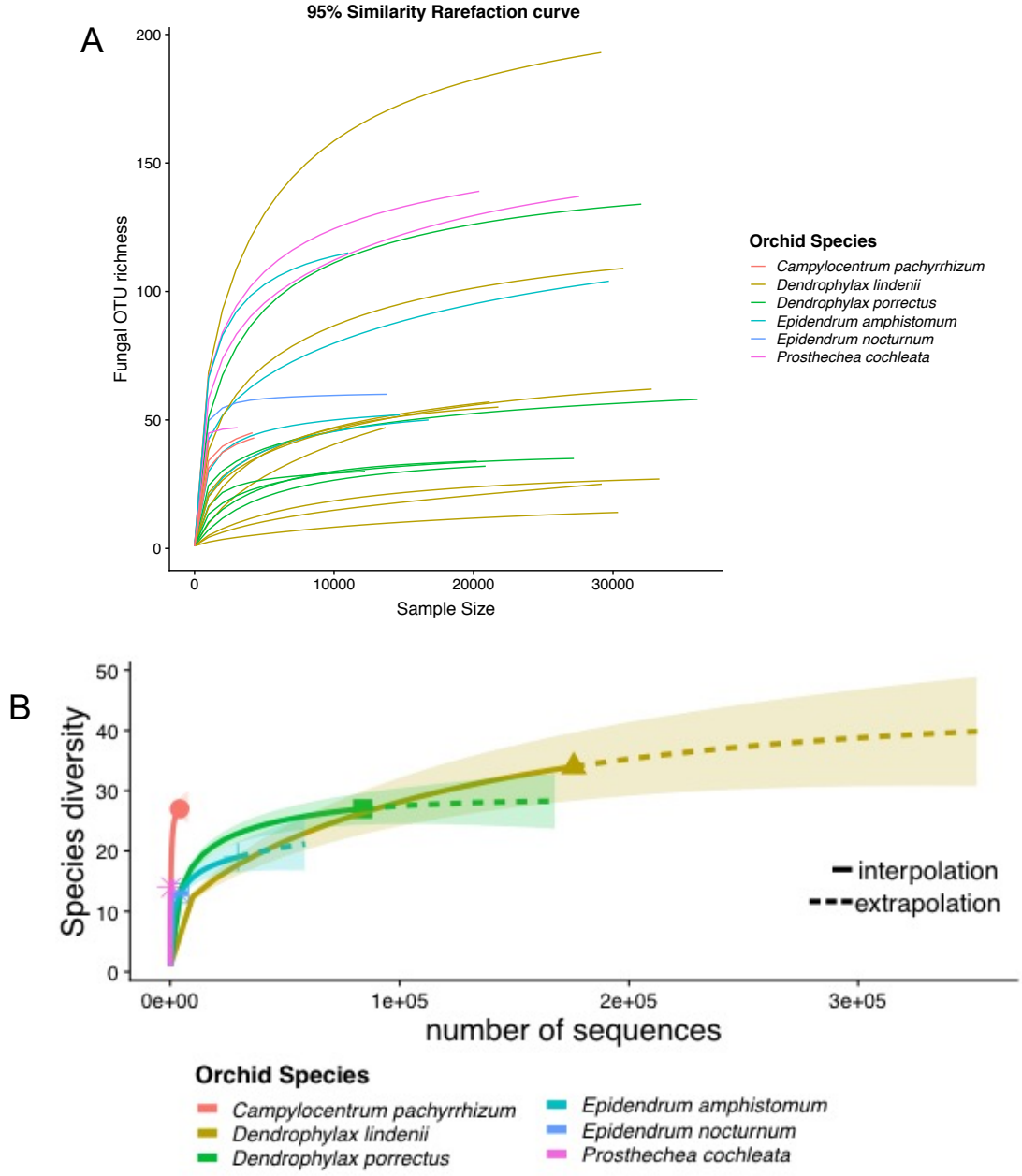


Figure 3.S2. A) Rarefaction curves for root samples of epiphytic orchids including *D. lindenii* collected from FPNWR in 2016 and B) extrapolation and interpolation curves for epiphytic and *D. lindenii* collected at FPNWR in 2016.

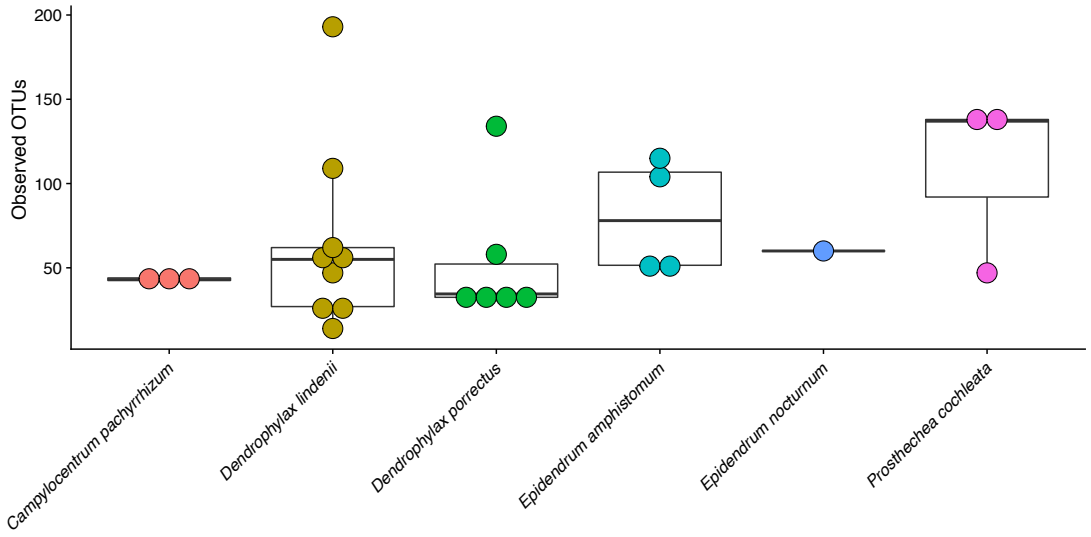


Figure 3.S3. Observed fungal OTU richness for root samples collected from *D. lindenii* and co-occurring epiphytic orchids at the FPNWR in 2016.

2018 Root Dataset

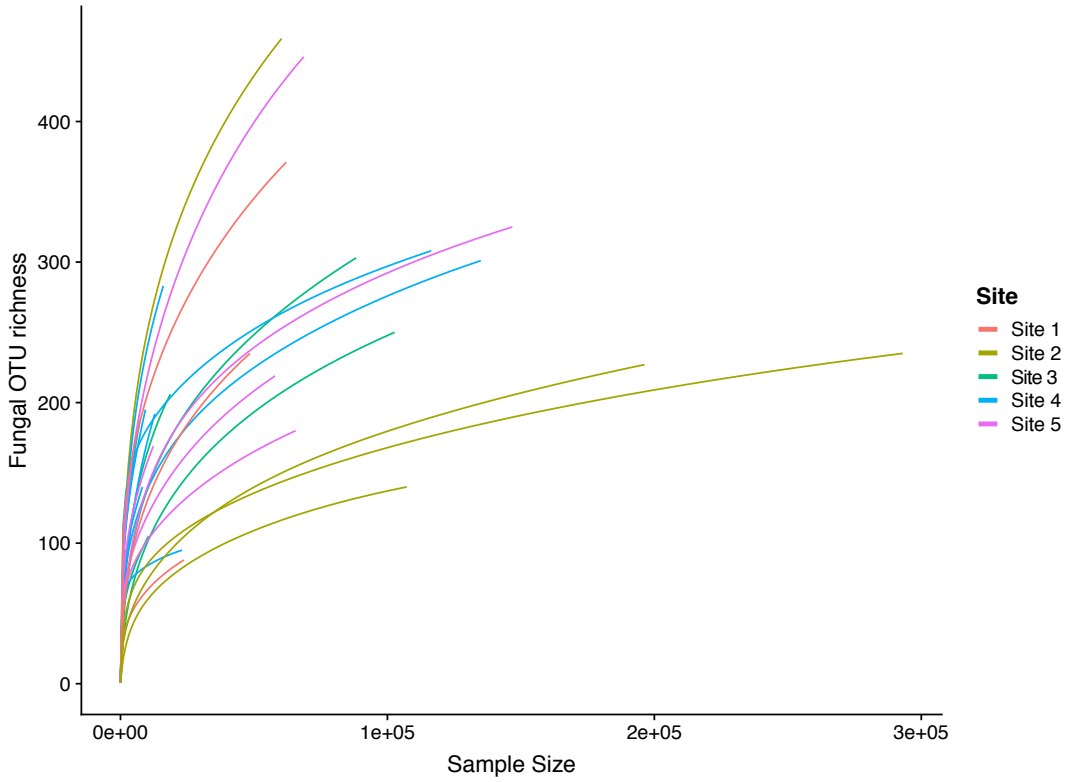


Figure 3.S4. Rarefaction curves of fungal reads obtained from *D. lindenii* root samples collected at several sites at the FPNWR in 2018.

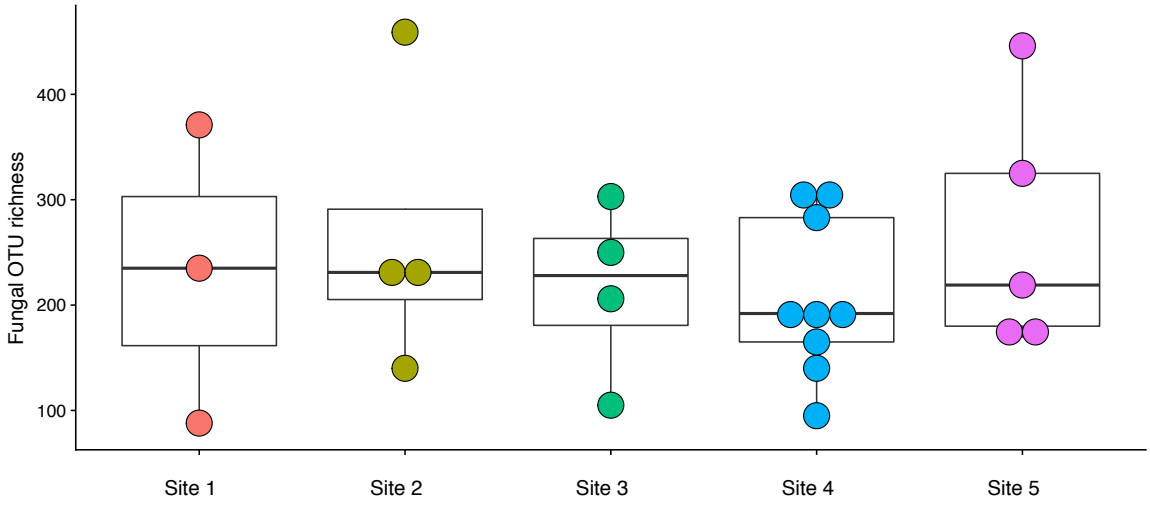


Figure 3.S5. Observed fungal OTU richness for *D. lindenii* root samples collected at the FPNWR in 2018.

2018 Bark Dataset

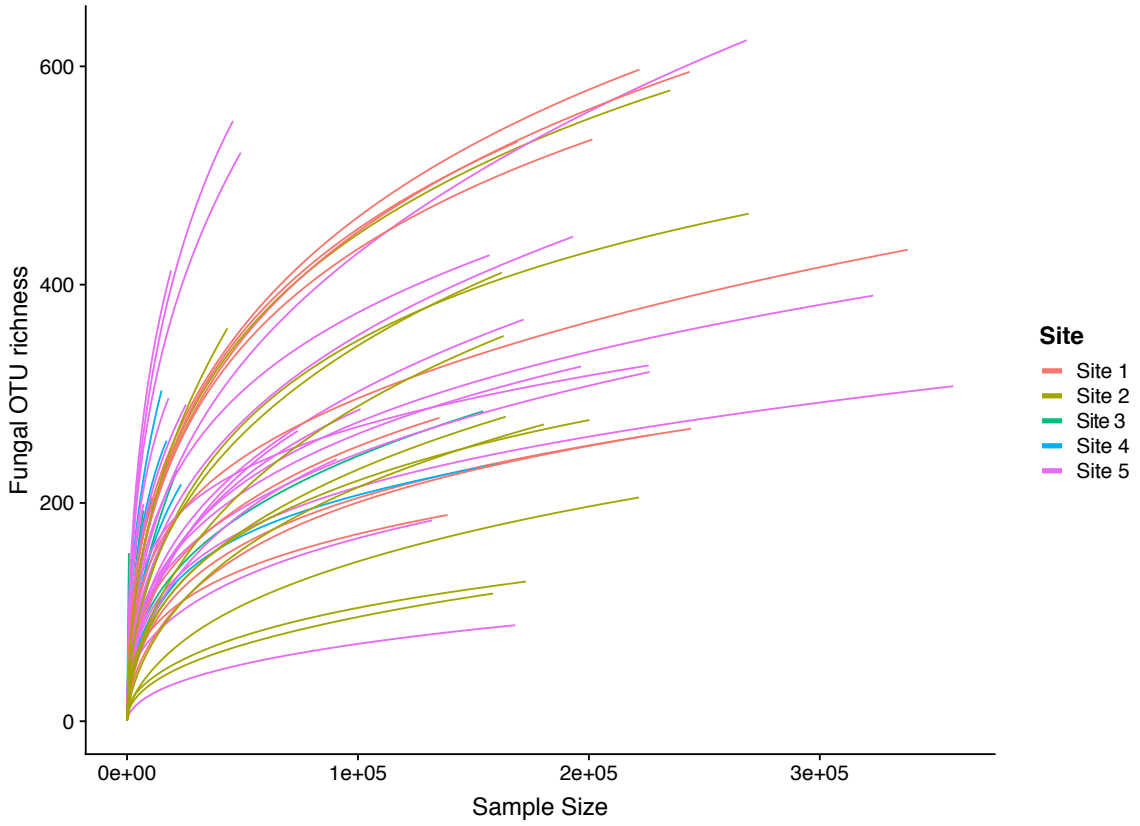


Figure 3.S6. Rarefaction curves of fungal reads obtained from bark samples collected from trees in *D. lindenii* habitats at the FPNWR in 2018.

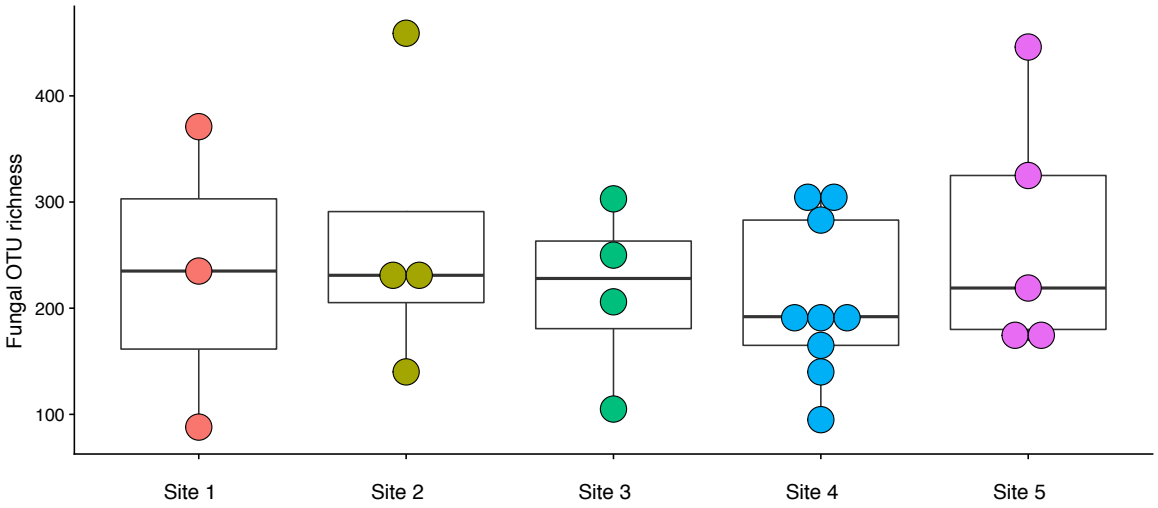


Figure 3.S7. Observed fungal OTU richness for bark samples collected from trees with and without *D. lindenii* at the FPNWR in 2018.

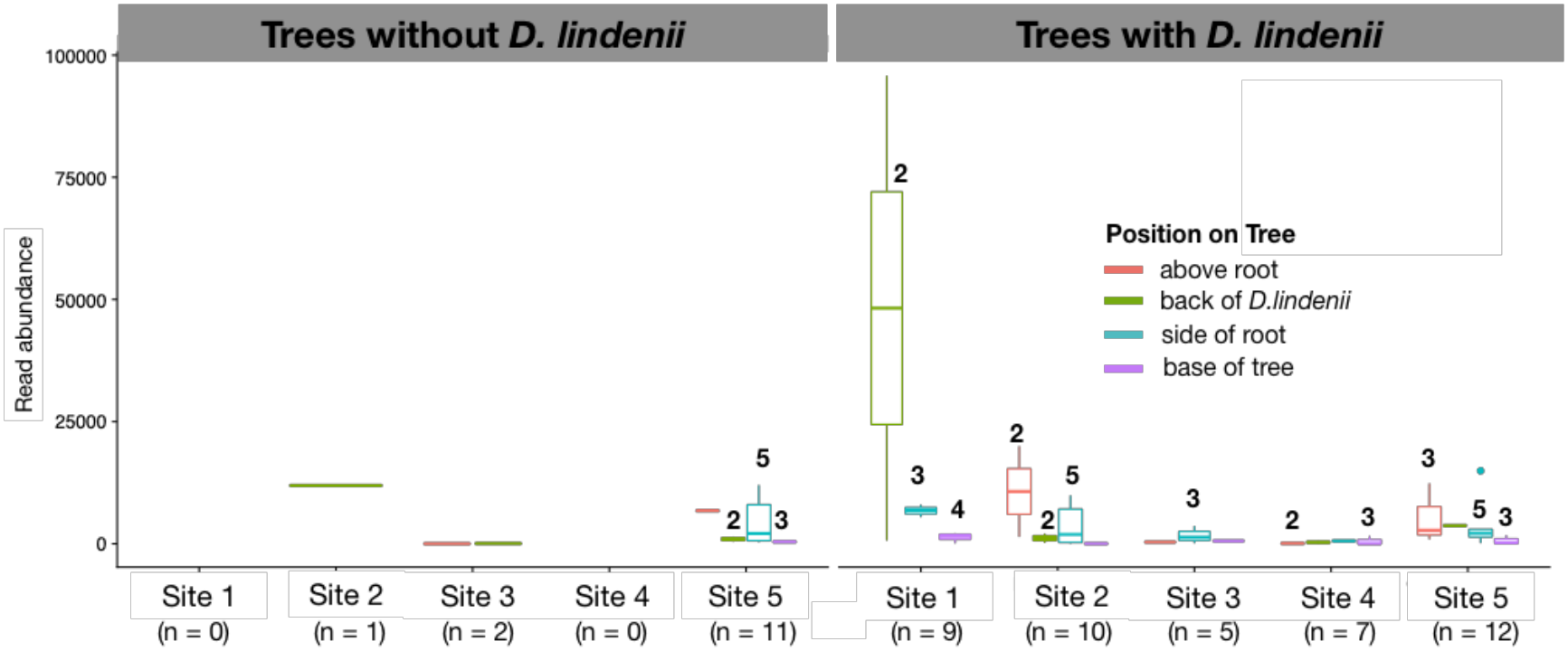


Figure 3.S8. Box and Whisker plots of the read abundance obtained from bark samples of trees with and without *D. lindenii* collected at the FPNWR in 2018.