SPECIFICITY OF MYCORRHIZAL SYMBIONTS IN FOUR SYMPATRIC *LEPANTHES* SPECIES (ORCHIDACEAE) AND THE POSSIBLE ROLE OF SYMBIONTS IN DRIVING ORCHID DIVERSIFICATION

by

PIOTR TYMON TUCZAPSKI

(Under the Direction of Dorset W. Trapnell)

ABSTRACT

Despite comprising over half of all orchid species globally, mycorrhizal associations in tropical epiphytic orchids remain relatively understudied compared to their terrestrial counterparts. The greatest diversity of these orchids is found in tropical montane cloud forests, where they constitute more than 50% of all epiphytic plants. While the drivers of such high diversity remain uncertain, it has been suggested that mycobiont associations may play a role in this phenomenon. Previous studies on orchid mycorrhizal interactions have generally found little evidence of specialization, with limited research on tropical orchids also indicating generalist associations. However, epiphytic orchids often exhibit narrow distributions, high endemism, and dependency on both phorophyte and fungal partners, making them particularly vulnerable to environmental changes. This highlights the need to examine the effects of phorophyte identity and habitat disturbance on orchid mycobiont composition. In this study, we analyzed root-associated fungal communities in rare, closely related, and narrowly endemic epiphytic orchids from the rapidly diversifying

genus *Lepanthes* within one of the world's most biodiverse hotspots. Our objectives were to: (1) characterize the composition and diversity of mycorrhizal communities associated with four sympatric orchid species, (2) assess the degree of exclusiveness in these associations and the influence of keystone fungi on the structure of orchid-fungal symbioses, (3) determine whether orchids associate with closely related fungal partners by examining the degree of phylogenetic signal at different interaction frequency resolutions, and (4) investigate the impact of phorophyte identity and habitat disturbance on mycobiont composition in 22 orchid populations of a single species. Our findings revealed distinct mycorrhizal communities among the focal orchid species, with variation not uniformly distributed across species. Contrary to previous reports of generalism in tropical orchids, we detected a strong signal of specialization in species interaction networks. Opportunistic interactions influenced the phylogenetic signal, which was stronger in keystone fungal taxa. Additionally, mycobiont communities varied significantly among both phorophyte species and habitat types. To our knowledge, this is the first study demonstrating differing fungal communities in closely related orchid species, supporting phorophyte bias and highlighting the impact of environmental degradation on orchid fungal associates.

INDEX WORDS: mycorrhizae; specialization; orchids; interaction networks; environmental disturbance

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by

PIOTR TYMON TUCZAPSKI

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M.S., Warsaw University, Poland, 2014

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by

PIOTR TYMON TUCZAPSKI

Major Professor:	Dorset W. Trapnell
Committee:	James H. Leebens-Mack
	Travis C. Glenn
	Melissa McCormick
	Y. Anny Chung

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The majority of land plants engage in a mutualistic relationship with mycorrhizal fungi. For example, between 80-90% of plants from most angiosperm and gymnosperm families, including a majority of trees, herbaceous plants, fern sporophytes, and lycopods, form arbuscular mycorrhizas (Smith and Read 2008). In contrast, only about 3% of common seed plants form ectomycorrhiza (Smith and Read 2008). While most plants rely on these fungal partners, the influence of this relationship on plant community structure, niche differentiation, and plant diversification is still not well understood (Sutherland et al. 2013).

Typically, mycorrhizal fungi are capable of colonizing the roots of multiple plant species, and plants often associate with a variety of distantly related fungal species. Therefore, these interactions are usually considered to have low specificity. On the other hand, the orchid relationship with Orchid Mycorrhizal Fungi (OMF) is much more specific compared to other plant-fungal interactions, since the orchid life cycle is heavily dependent on OMF (Leake 1994, Rasmussen 2002, Otero et al. 2011, McCormick et al. 2012, Selosse and Martos 2014, Stöckel et al. 2014). Orchid seeds are dust-like and contain tiny, undifferentiated embryos with no endosperm, meaning they lack the reserves needed to support seedling development (Dearnaley et al., 2016). As a result, orchids must form a mycorrhizal association with fungi capable of accessing complex carbon sources that are otherwise unavailable to the developing protocorm

(Smith and Read 2008, Whigham et al. 2008). The fungus supplies carbon to the plant until it reaches adulthood, when the relationship generally is believed to become more balanced with mutual nutrient exchange (Cameron et al., 2007; Liebel et al., 2015; Schiebold et al., 2018, Read et al. 2024). In contrast, OMF do not depend on orchids, are often free-living decomposers, and are usually widespread (Dearnaley et al. 2012, Cruz et al. 2014). Additionally, orchids can form relationships with a variety of fungal groups, including ectomycorrhizal and non-mycorrhizal fungi (McCormick et al. 2018, Li et al. 2021), offering a broader understanding of fungal diversity and its role in plant ecology. Together with the obligatory nature of the mutualism, this diversity in fungal associations makes orchids an excellent model for studying mycorrhizal interactions, offering valuable insights into plant evolution, speciation, and niche differentiation, particularly in tropical ecosystems where orchid diversity is high (Nadkarni and Wheelwright 2000, Küper et al. 2004). Moreover, understanding these relationships have implications for conservation, as many orchid species are rare or endangered.

Associations with distinct mycorrhizal fungi may facilitate coexistence of orchid species in species-rich environments, potentially leading to resource partitioning and reduced competition (Waterman et al. 2011, Jacquemyn et al. 2014). However, most research has focused on a limited number of terrestrial, often distantly related, temperate orchid species from Europe, North America and Australia that inhabit relatively large geographic areas, ranging from hundreds to thousands of square meters and sometimes spanning thousands of kilometers (e.g., Jacquemyn et al. 2014, Jacquemyn et al. 2012a, Jacquemyn et al. 2012b, Waterman et al. 2011, Waud et al. 2016). Furthermore, prior studies have often overlooked the phylogenetic signal in OMF assemblages and/or lacked a fine-scale taxonomic resolution

of mycobionts, raising questions about the accuracy of reported fungal diversity (Waterman et al. 2011, Herrera et al. 2017). Previously observed differences in mycorrhizal communities among co-occurring orchid species further complicate the study of mycobiont influence on plant speciation, particularly in understanding how plant adaptation to new fungal taxa may drive diversification (Thompson 1987; Cowling et al. 1990; Otero and Flanagan 2006). A potential approach to addressing this challenge is to focus on closely related plant taxa, as the presence of distinct mycorrhizal partners in recently diverged species would support the idea that mutualism plays a key role in speciation (Waterman et al. 2011).

Another area of study that is of great interest to researchers is the degree of mycorrhizal specificity, as it plays a crucial role in conservation initiatives and species reintroduction efforts (Martos et al. 2012, Pandey et al. 2013, Suárez and Kottke 2016). Additionally, understanding specificity can shed light on evolutionary dynamics between interacting species and the stability of ecological networks (Blüthgen et al. 2008). However, studies on orchid mycorrhizal specialization have revealed a complex spectrum of specificity, ranging from highly specific to more generalized associations (McCormick et al. 2006, Shefferson et al. 2006, Shefferson et al. 2008, Pandey et al. 2013, Jacquemyn et al. 2014, Herrera et al. 2017). This variability may be influenced by differences in research methodologies, sampling strategies, sequencing techniques, fungal identification methods, and the phylogenetic resolution used to assess specificity. For example, Herrera et al. (2017) investigated only orchid-Tulasnellaceae interactions within a community of terrestrial and epiphytic orchid species, most of which were sampled only once, and concluded that orchids exhibit generalist associations based on a nested interaction network. In contrast, Waud et al.

(2016) analyzed mycorrhizal communities at the OTU (Operational Taxonomic Unit) level in three coexisting Belgian orchid species and found minimal overlap in OTUs, suggesting a high degree of specificity. In some studies, the number of detected OTUs has been used as an indicator of specialization, with a high number interpreted as evidence of generalism and a low number as specificity (e.g. Jacquemyn et al. 2014). These factors can obscure the true patterns of mycorrhizal relationships, complicating efforts to draw broad conclusions. Thus, there is a need for a more controlled study of mycorrhizal associations with orchids.

Furthermore, while it is broadly accepted that orchids are more specialized on fungi than the fungi are on orchids, that view has been established based on studies of temperate terrestrial species. In epiphytic orchids, fungal symbionts may benefit beyond nutrient exchange, including protection through the water-retention properties of velamen root tissue (Suárez and Kottke 2016). Moreover, since mycobionts may have secondarily adapted to colonizing epiphytic niches, they might be less suited to surviving in harsher soil conditions, making them more reliant on the support provided by their plant partners. Finally, epiphytic orchids, with their greater exposure to light compared to their terrestrial counterparts in shaded forest understories, may exhibit enhanced photosynthetic activity, allowing them to supply more carbon to their fungal partners in return for nutrients (Martos et al. 2012). Together, this implies a potentially tighter partnership between epiphytic orchids and associated mycobionts in comparison to terrestrial counterparts.

Several authors have noted that environmental degradation can significantly alter soil fungal composition, which is crucial for habitat succession and ecological restoration (Harris,

2009; Frouz et al., 2016). Among the organisms affected by habitat degradation are root-associated fungi, which play a key role in shaping plant communities. While endophytic and saprophytic fungal communities tend to develop randomly (Zhang et al. 2017), disturbances to the environment can lead to a significant decline in mycorrhizal species (Dickie et al., 2013). Mycorrhizal fungi play a key role in nutrient absorption, particularly in nutrient-deficient environments (Yang et al. 2016). Understanding how ecosystem disturbances impact species interactions is essential for ecologists, particularly in light of the rapid loss of biodiversity and the anticipated effects of climate change, especially in tropical regions (Barlow et al., 2016; Nadkarni et al., 2023). However, the impact of habitat disturbance on the mycobiont composition of epiphytes remains largely unknown. In tropical montane cloud forests, habitat fragmentation and the removal of large trees lead to a decline in arboreal soil accumulation, which are essential for epiphytes as they enhance resource retention, moisture availability, and nutrient absorption from both atmospheric sources and host trees (Gotsch et al. 2016). Given that fungal community composition is shaped by substrate conditions—including nitrogen and phosphorus availability, acidity, and organic matter content—changes in these factors due to habitat disturbance can profoundly impact fungal diversity and the identity of the available mycobionts (Lauber et al. 2008, Yan et al. 2018).

Other factors can certainly affect the composition of orchid root-associated fungi. For instance, the epiphytic orchid habitat is highly dynamic and experiences fluctuations in light exposure, humidity, and even the physical characteristics of the bark as the phorophyte host ages (Rasmussen and Rasmussen 2018). Moreover, some reports suggest orchid phorophyte bias, where orchids show preference for or against certain tree species (Tremblay et al. 1998**,**

Adhikari et al. 2012). This bias is thought to be linked to variations in mycobiont availability and performance among different tree species, potentially influenced by physico-chemical factors associated with the bark and the microclimate of the tree canopy (Gowland et al. 2013). Moreover, the number of fungal species decreases and their geographic range increases with latitude (Tedersoo et al. 2014), and the diversity of saprotrophic, arbuscular and ectomycorrhizal fungi varies with temperature and other environmental factors (Allen et al. 1995, McGuire et al. 2012, Geml 2017). Thus, the geographic and associated environmental conditions are undoubtedly important factors to consider. However, the search for their impact on orchid mycobiont composition has been, so far, successful only on a global scale as opposed to a regional scale (Kartzinel et al. 2013, Oja et al. 2017, Shefferson et al. 2019).

Compared to terrestrial orchids, little is known about the mycobiont diversity, specificity and factors affecting OMF community distribution in tropical epiphytic orchids (Li et al. 2021, McCormick et al. 2018). Considering the following, the investigation into mycobiont community diversity patterns, the degree of specialization, and impact of phorophyte species and environmental disturbance on root-associated fungi in epiphytes appears warranted as it provides the opportunity to gain novel insights into the ecological and evolutionary significance of these symbiotic relationships. 1) The majority of orchids are found in the tropical forests, with over 80% of these species growing as epiphytes (Givnish et al. 2015). Epiphytic orchids constitute a critical component of the native flora. In the neotropical montane cloud forest, epiphytic orchid diversity reaches its highest levels, with epiphytic orchids comprising over half of all epiphytic vascular plants and more than one-

third of the total plant species (Dodson 1999, Küper et al. 2004, Nadkarni and Wheelwright 2000). The drivers of such high biodiversity in the tropics are poorly understood. 2) Epiphytic orchids may be especially vulnerable to environmental disturbances due to their rarity, restricted distributions, reliance on specific mycobiont partners, and dependence on host trees for survival. 3) Epiphytic orchids may experience stronger selective pressures to form associations with suitable fungal partners compared to their terrestrial counterparts, as they grow in environments with greater water and nutrient limitations. 4) Understanding the identity of fungal mycobionts, and mechanisms governing mycobiont composition is vital to conservation efforts in tropical regions which experience high rates of deforestation (Zahawi et al. 2015).

The goals of this dissertation were to 1) estimate the patterns of mycobiont associations and assess their potential role in speciation of four sympatric orchids through alpha- and beta- diversity analyses, 2) determine the specialization of orchid-fungal interactions of four sympatric orchids through analysis of the phylogenetic signal in mycobiont communities as well as specialization network analysis, 3) investigate the phorophyte and habitat disturbance effects on the composition of root-associated fungi of an epiphytic orchid species. To address these questions, the mycobiont communities of four rare, closely related, and narrowly endemic epiphytic orchid species from the rapidly diversifying (Pérez-Escobar et al. 2017) genus *Lepanthes* were characterized within one of the world's most biodiverse hotspots. Fungal taxonomic units were identified by sequencing the full Internal Transcribed Spacer (ITS) region with two primer sets fused with Pacific Biosciences (PacBio) barcodes.

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CHAPTER 2

CONGENERIC ORCHID SPECIES GROWING IN SYNTOPY ASSOCIATE WITH DISTINCT MYCORRHIZAL ASSEMBLAGES[1]

[1] Tuczapski, P. T. and Trapnell, D. To be submitted to *Molecular Ecology*.

2.1 ABSTRACT

A key mutualistic relationship found in over 80% of land plants is their interaction with mycorrhizal fungi. While most plants rely on mycorrhizal partners, the effects of this mutualism on plant community composition and diversification remain poorly understood. Interactions between orchids and their mycobionts have often been studied to examine the impact of the mycobiome on plants because the orchid life cycle obligatorily depends on mycorrhizae. Historically, studies have emphasized the role of niche partitioning and competition avoidance resulting in distinct mycobiome compositions among coexisting orchids. Since closely related orchid species have been found to associate with similar groups of fungi, it has been speculated that different fungal species are needed for coexistence but not for speciation. However, fungi have often been examined at lower resolution levels (i.e., class, order, family, or genus). Here, we show that the employment of third-generation high-throughput sequencing technology, specifically Pacific Biosciences (PacBio), enables characterization of fungal communities at fine scale with high accuracy. We have evaluated alpha- and beta-diversity of fungal communities in four closely related, narrowly endemic epiphytic orchid species from the rapidly diversifying genus *Lepanthes* in one of the world's richest biodiversity hotspots. We found that focal species have different mycobiont compositions and those differences are not uniformly distributed across orchid species. As far as we know, our analysis provides the first evidence of differing fungal communities in sister orchid species. We suggest that the potential role of fungi in driving speciation in orchids should not be dismissed and deserves reconsideration. We speculate that our results reflect the relatively recent taxonomic divergence of some *Lepanthes* species compared to others.

2.2 INTRODUCTION

A conundrum that has long intrigued the scientific community is what factors have driven the exceptionally high levels of biodiversity in the tropics. Scientists have similarly tried to understand what factors have contributed to the rapid diversification of orchids, the second largest flowering plant family, the majority of which occur in the tropics. One of the most important mutualisms in over 80% of land plants is their interaction with mycorrhizal fungi (Smith & Read 2008). Within the field of plant ecology, there is a growing recognition that this mutualism influences plant community composition, niche partitioning, and plant diversification, warranting further study (Sutherland et al. 2013).

Mycorrhizal symbionts play a vital role in facilitating nutrient uptake and are particularly beneficial in nutrient-poor conditions (Yang et al. 2016). Dust-like orchid seeds lack resources (i.e. endosperm) and thus cannot germinate without nutrients transferred by orchid mycorrhizal fungi (OMF) (Smith & Read 2008). Consequently, orchids have an obligatory relationship with OMF, but little is known about its possible role in structuring orchid communities and in driving orchid diversification. It has been previously argued that presence of distinct mycorrhizal partners in recently diverged orchid species would support the idea that mutualism plays a key role in speciation (Waterman et al. 2011).

Some have speculated that coexistence of orchid species in sympatry might be facilitated by their association with different mycorrhizal fungi, which may result in resource partitioning and reduced competition (Waterman et al. 2011, Jacquemyn et al. 2014). However, most studies of orchid-mycorrhizal relationships target terrestrial orchids from temperate regions, despite the

fact that most orchids are tropical species and 80% of those are epiphytes (Givnish et al. 2015). Additionally, studies examining the role of mycorrhizal symbionts in governing coexistence of orchid species to date have focused on distantly related plant taxa that co-occur over broad geographic areas on the scale of hundreds of square meters to hundreds of square kilometers (Otero et al. 2011, Waterman et al. 2011, Jacquemyn et al. 2012a, Jacquemyn et al. 2014, Waud et al. 2016). In contrast, New World epiphytic orchids often occur in highly localized multi-species communities, sometimes occupying the same tree branches. Thus, the co-occurrence of high orchid diversity at small spatial scales in the New World tropics is a fertile testing ground of the mycorrhizal-mediated niche partitioning hypothesis.

The highest diversity of epiphytic orchids in the Neotropics is found in montane cloud forest habitat at elevations of 300-3000 m above sea level (asl) (Dodson and Escobar 1993). Epiphytic orchids are a critical component of the native flora (Dodson 1999). In Ecuador, at the elevational zone (1000–1500 m asl) that hosts the largest number of epiphytes, an estimated 53% of all epiphytic species are orchids (Küper, Kreft, Nieder, Köster, & Barthlott 2004). In the Monteverde region of Costa Rica, orchids constitute a large portion of the local flora: of the approximately 1700 plant species recorded, about 600 species are orchids (Nadkarni and Wheelwright 2000). The drivers responsible for such high orchid diversity in the region are poorly understood. Pérez-Escobar et al. (2017) have examined whether uplift of the Andes may have been a factor in Neotropical orchid diversification, an idea originally proposed by Gentry & Dodson (1987). In one of the two largest Neotropical orchid groups, subtribe Pleurothallidinae (44 genera and 5100 species, Karremans 2016), they found no correlation between paleo-elevation and diversification, probably because the most recent common ancestor of the subtribe had already

been adapted to montane cloud forest habitats (*c*. 1200 m asl). In addition, the Andes Mountain Range seems to have acted as a source of new lineages for the rest of the continent, with speciation taking place in situ. Finally, uplift of the Andes probably did not act as a barrier to orchid dispersal (Pérez-Escobar et al. 2017). Taken together, these results suggest that factors other than physical barriers must have contributed to the diversification of the Pleurothallidinae, such as shifts in pollinator partners and/or fungal symbionts across an elevational gradient and heterogenous habitat (Lugo et al. 2008).

Here, we characterize the composition and diversity of mycorrhizal communities associated with four closely related, epiphytic orchid species that grow in sympatry in the New World tropics. If shifts in mycobionts play a role in speciation, differences in fungal partners should be detectable in recently diverged orchid species. Our study species belong to the evolutionarily young genus *Lepanthes* Swartz (Orchidaceae) which has the highest speciation rate in the Pleurothallidinae subtribe (Pérez-Escobar et al., 2017). The four focal species (*L. cribbii*, *L. falx-bellica, L. mentosa,* and *L. monteverdensis*) are endemic to the Monteverde region of Costa Rica and often grow sympatrically (i.e. on the same tree). This serves as a powerful system for addressing questions regarding mycorrhizae-mediated niche partitioning and whether shifts in symbionts may contribute to orchid diversification. The aims of the study are to (i) genetically identify fungal root-associates of the four orchid species; (ii) estimate variability of root-associated fungal communities (alpha diversity) within each orchid species; (iii) estimate variability in mycobiont community composition (beta diversity) among orchid taxa; and (iv) compare alpha- and beta-diversity among orchid individuals within species and among species. This research will advance our understanding of the poorly understood relationship between epiphytic orchids and

their symbionts, and address a question that has long mystified biologists, namely what factors contribute to exceptionally high species diversity.

2.3 METHODS

Study Species

Lepanthes Swartz (Orchidaceae) is a Neotropical genus comprised of over 1400 species (Bogarin, Karremans, and Fernandez 2018; POWO 2024), making it one of the largest genera within the family. Members of the genus are distributed from Mexico to Bolivia and northern Brazil with most species restricted to high elevation cloud forests and paramos of the Andes Mountain Range (Pérez-Escobar, Kolanowska and Parra-Sanchez 2013). The genus *Lepanthes* is estimated to have evolved over the last 2.5 million years and has the highest speciation rate in the Pleurothallidinae subtribe (Pérez-Escobar et al. 2017). Scarce reports on the pollination ecology indicate that male fungus gnats are attracted to flowers via sexual deception (Blanco & Barboza 2005, Karremans et al. 2019, Peakall 2023). Many members of the genus are narrow endemics (Pridgeon 2005) and co-occur, making the genus particularly well-suited for studies addressing the question of the role of mycorrhizal specificity and niche partitioning in the diversification of sympatric species (Bayman et al. 1997). Although not documented for the focal species, some *Lepanthes* spp. flower and set fruit continuously throughout the year (Tremblay & Ackerman 2001). The four study species produce one, or occasionally two, inflorescences on the apex of the stem (called a ramicaul) (P. Tuczapski, pers. obs.). Each inflorescence can bear multiple (15 or more) flowers, typically with only one open at a time (P. Tuczapski, pers. obs.). As with most orchids, the tiny seeds are well adapted for wind dispersal (Acevedo et al. 2015, Karremans et al. 2023). While it is unknown how many seeds are produced per capsule; it is not uncommon for

the fruits of other members of the family to produce thousands to millions of dust-like seeds (Ardittii & Ghani 2000). Because the seeds lack endosperm, they cannot germinate without the carbon resources supplied by their mycorrhizal symbionts. *Lepanthes monteverdensis* has been observed to occasionally produce secondary leaf-bearing stems with roots on the apex of ramicauls (P. Tuczapski pers. obs.), although most *Lepanthes* species are thought to not reproduce asexually (Karremans & Vieira-Uribe, 2020). A study of four *Lepanthes* species from Puerto Rico has estimated a highly variable lifespan with an average of 3.4 years (Tremblay 2000). The four focal species (*L. cribbii, L. falx-bellica, L. mentosa, L. monteverdensis*) are epiphytes with pendent habit, endemic to the Monteverde region of Costa Rica, which is characterized as a tropical montane cloud forest. The study species are approx. 6-10 cm in height and flowers are approx. 5 mm long. The four focal species often grow in syntopy (*i.e.*, on the same tree).

Sampling

Collections were made at the beginning of the rainy season in July 2019 and July 2021 (**Fig. 2.1**). Root samples were collected from 10 adult orchids per population, where possible, from eight sites within or near the Monteverde Cloud Forest Biological Reserve. A population is defined as all the conspecific orchids growing within a tree. Study sites are situated at 1470-1756 m asl and are separated from one another by at least 1 km and a maximum of ~6 km. Each site encompasses one to eleven trees (i.e. phorophytes) supporting one to four species of *Lepanthes*. Samples were collected from 50 populations occupying 35 phorophytes belonging to 23 species (**Table 2.1**). Orchid root samples were collected from 102 individuals of *L. monteverdensis* from 11 populations (mean 9.3 individuals/population), 183 individuals of *L. falx-bellica* from 22 populations (mean

of 8.3 individuals/population), 74 individuals of *L. cribii* from 10 populations (mean of 7.4 individuals/population) and 50 individuals of *L. mentosa* from 7 populations (mean of 7.1 individuals/population) (**Table 2.2**). Roots were immediately cleaned, surface sterilized in 10% bleach solution for 5 minutes, stored in 2% CTAB buffer and snap-frozen in liquid nitrogen for transport to the University of Georgia for analysis.

Samples were also collected from tree bark that was in direct contact with sampled orchid roots (**Supp. Table 2.1**). Bark samples were immediately snap-frozen in liquid nitrogen for transport to the University of Georgia for analysis.

Sequencing Library Preparation

Genomic DNA (fungal and orchid) was extracted from macerated root tissue using a modified CTAB protocol (Doyle 1991). Genomic DNA (fungal and tree) was also extracted from bark samples (see **Supp. Table 2.1** for details).

DNA quality and quantity were evaluated using an ND-1000 Nanodrop® spectrophotometer. In order to capture the broadest spectrum of mycorrhizal taxonomic units possible, the entire fungal internal transcribed spacer (fITS) ITS1-5.8S-ITS2 region was PCR amplified with two primer sets in two independent reactions. The first was a universal fungal primer set, ITS1-F_KYO2 (Toju et al. 2012) and ITS4 (White et al. 1990), which allows non-discriminating amplification of the fITS. This primer pair offers improved non-biased coverage across diverse taxonomic groups of fungi while being relatively selective for fungal ITS sequences rather than plant ITS sequences (Toju et al. 2012). The second primer set used is taxon-specific ITS4-Tul which targets orchid-associated *Tulasnella* (Taylor & McCormick 2008) paired with ITS1-F_KYO2. We designed forward and reversed primers from both primer sets to

be fused with PacBio barcodes and applied combinatorial dual-indexed barcoding system in the library preparation, following the approach employed previously in TruSeq and NextEra Illumina library preparation methods (Glenn et al. 2019). Thus, each sample was amplified twice - once with each primer set - and assigned a unique barcode combination per primer set, effectively creating two separate libraries. PCR reactions were carried out in 25 μL reactions containing 12.25 μL molecular grade ddH2O, 5 μL 5x Kapa HiFi buffer, 0.75 μL 10 μM Kapa dNTP's mix, 0.5 μL Kapa HiFi DNA polymerase (Roche, Basel, Switzerland), 2 μL of each forward and reverse 5 μM primers, and 2.5 μL of each sample genomic DNA (10, 5, 2.5. or 1.25 ng/μL dilutions). Most samples required 30 cycles to observe amplicon DNA bands on agarose gels, with some samples needing 35 or rarely 40 cycles. PCR conditions were as follows: initial denaturation (5 min at 95°C); 15 cycles of touchdown (30 s at 94°C, 30 s at 60°C, -1°C/cycle, 30 s at 72°C); followed by 10-25 cycles (30 s at 94°C, 30 s at 50°C, 30 s at 72°C); final extension (5 min at 72°C); hold (12°C). A total of 818 uniquely barcoded root samples were mixed and submitted to Georgia Genomics and Bioinformatics Core for sequencing on PacBio Sequel II platform. Note that we have also submitted together with these samples 239 tree bark samples (**Supp. Table 2.1**), which are included in the report of sequencing data as well as initial denoising and dereplication of reads in the Results section (see below), but otherwise we have not included them in the downstream analyses.

Sequence Quality Control and Amplicon Processing

All reads were re-oriented into 5' to 3' direction by searching for sequences matching the forward primer, with an allowance for ≤ 5 mismatches, using *DADA2* (Callahan et al. 2016) package in R version 4.3.2 (R Development Core Team 2023). Next, primers were trimmed using BBDuk with

allowance for ≤ 2 mismatches. Reads from both primer sets (universal and taxon-specific) were merged for each sample. The remaining Quality Control steps were performed using DADA2. We inspected read quality profiles and applied read dereplication using an error rate learning algorithm, before implementing a core sample inference algorithm that returned amplicon sequence variants (ASVs), i.e. fungal taxonomic units, for each sample. Lastly, we removed chimeric sequences before constructing the ASV count table. Fungal taxonomy was assigned to ASVs using the General Fasta release file from the UNITE 9.0 ITS reference dataset (Abarenkov et al. 2022). The result was a dataset consisting of all fungal ASVs recovered from root extracts. Data generated by this workflow will be referred to as the general fungal (GF) dataset.

We assigned ecological guilds to all recovered fungal taxa from the GF dataset using FUNGuild in R version 4.3.2 (R Development Core Team 2023, Nguyen et al. 2016). From these, we retrieved only those ASVs belonging to guilds that have been classified as mycorrhizal. Data generated by this workflow will be referred to as the classified mycorrhizal (CMF) dataset. All ASVs found with fewer than six reads in both datasets were removed from subsequent analyses due to recovery of artifact ASVs in the mock community (see Results section below) .

Sequencing/PCR Bias Control

Some commonly employed measures of alpha- and beta- diversity in ecological communities rely on the assumption that read abundance recovered from sequencing platforms corresponds to the abundance of given taxon in the sample. In an effort to test this assumption and control for potential PCR and/or sequencing biases, a mock fungal community (SynMock; Palmer et al. 2017) was included in the sequencing run. To create the SynMock community we used 12 *Escherichia coli* strains containing pUC57 ampicillin-resistant plasmids with cloned synthetic

ITS-like sequences (598 – 668 bp). First, cultures were grown in LB medium with ampicillin at 37°C overnight on a shaker table. Next, plasmid DNA was extracted from bacterial colonies using an alkaline lysis method (Cold Spring Harb Protoc; doi:10.1101/pdb.prot093344). Concentrations of DNA were measured with an ND-1000 Nanodrop® spectrophotometer and extracts were subsequently equimolarly mixed to a final concentration of 20 ng/μL for each member in the SynMock community. This was then diluted to 10 ng/μL and PCR-amplified according to the protocol described previously.

Alpha Diversity Analyses

Alpha diversity captures the variability in a single community (i.e. fungal ASVs) and can include richness (number of ASVs) and evenness (relative abundances of ASVs) measures. We estimated the α-diversity of fungal communities present in the roots of individual orchids using QIIME2 (Boylen et al. 2019). We repeated these calculations separately for the GF and CMF datasets.

We used a combination of quantitative and qualitative measures; Faith's phylogenetic diversity (PD) (Faith 1992) to estimate ASV richness, Shannon-Wiener diversity index (*H*) (Shannon 1948) to estimate ASV diversity (combined evenness and richness), and Pielou's evenness (*E)* (Pielou 1966) measure to estimate evenness of ASV distribution. We built a phylogenetic tree from ASV sequences using *phylogeny* plugin's *align-to-tree-mafft-fasttree* pipeline which performs a multiple sequence alignment using MAFFT (Katoh & Standley 2013) in QIIME2. For the purpose of testing for differences in PD we treated roots of an individual plant as a sample unit and orchid species as a group. Since we found that the abundance of reads returned after sequencing is not representative of the abundance of community member sequences (and presumably taxa) present in the sample before the PCR (see Results section below on SynMock)

we used a different approach for calculating quantitative α-diversity measures. Thus, for Shannon-Wiener diversity and Pielou's evenness indices we used presence/absence of ASVs in individual plants. We treated roots of a population as a sample unit and the orchid species as a group. Pairs of orchid species were tested for significant differences in PD, *H* and *E* with the Kruskal-Wallis test (Kruskal & Wallis 1952). We chose non-parametric test (instead of e.g. ANOVA) since group sizes differed and group variances may be heterogenous. To assess whether sampling effort was sufficient to fully capture α-diversity, we plotted rarefaction curves.

Beta Diversity Analyses

Beta diversity captures the variability in community composition (*i.e*., identity of ASVs) among samples, *i.e.* how similar or different two communities are in terms of shared taxa. In this study, we compared fungal diversity among conspecific orchid species. We used ASVs found in individual plants for qualitative (Jaccard and UniFrac) analyses, where individual plant was treated as a sample unit and orchid species as a group. For quantitative (Bray-Curtis) analysis we took the same approach as for Shannon-Wiener diversity and Pielou's evenness indices, *i.e.* we summed up ASV presence/absence data within individual plants per populations. Next, we treated populations as a sample unit and orchid sp. as a group.

The extent of overlap in fungal ASVs associating with different orchid species was visualized with principal coordinate analysis (PCoA) (Gower 1966) and non-metric multidimensional scaling plots using *phyloseq* (McMurdie and Holmes 2013) and *vegan* (Oksanen et al. 2022) packages in R version 4.3.2 (R Development Core Team 2023). The degree of similarity/dissimilarity of ASVs in the four orchid taxa was estimated both quantitatively and qualitatively using Jaccard (S_j) (Jaccard 1912), Bray-Curtis (S_{BC}) (Bran & Curtis 1957), and

UniFrac (U_{AB}) (Lozupone & Knight 2005) coefficients. First, we tested for differences in the composition of fungal communities among orchid species. Jaccard Similarity Index S_j (converted to a distance) was calculated between each pair of orchid individuals (within- and among-*Lepanthes* species). Next, we tested whether there was a difference between fungal communities of orchid species when fungal phylogenetic relationships were considered. We calculated Unweighted UniFrac Distance among each pair of plants. Finally, Bray-Curtis Dissimilarity Index was calculated between pairs of populations (within and among *Lepanthes* species). The significance of the differences between orchid species was tested with permutational multivariate analysis of variance (PERMANOVA) (Anderson 2014) using 999 permutations. All analyses were performed separately for the GF and CMF datasets in QIIME2 as well as packages *phyloseq*, *Biostrings*, and *vegan* in R version 4.3.2 (R Development Core Team 2023).

2.4 RESULTS

Sequencing, Amplicon Processing and Mock Community

The average quality score of raw pre-processed reads returned from sequencing was 98.9%. Screening of 3,141,700 reads yielded 2,826,941 demultiplexed circular consensus sequencing (CCS) reads (i.e. 90% success rate) for an average of 2,584 reads per sample. After trimming primer sequences, 98.7% of demultiplexed reads were retained.

Post implementation of the DADA2 dereplication and denoising algorithm returned 2,678,332 reads. From these, DADA2 inferred 17,116 ASVs, of which 1,778 were chimeras. Thus, 10% of all ASVs were chimeras, however these chimeric ASVs translated to only 3.4% (90,732 reads) of the total number of reads. Removal of chimeras yielded 15,338 ASVs, of which 8,058 originated from root samples (GF). After assigning these ASVs to guilds using FUNGuild, 1,714 ASVs were

classified as mycorrhizal. Of these, 942 ASVs originated from root samples and constitute our CMF dataset.

We recovered all 12 SynMock ASVs with 100% read accuracy for a total of 3,588 sequences. Subsequent to the denoising step in DADA2 and chimera removal, 3,423 sequences remained. After chimera removal there were an unequal number of reads per SynMock ASV (114 to 505 reads per ASV) despite DNA extracts having been mixed equimolarly before PCR reactions (**Table 2.3**, **Fig. 2.2**). For this reason, we did not use a quantitative approach that relies on read counts for calculating α- (*i.e.,* Shannon-Wiener Diversity Index or Pielou's evenness index) and β - (*i.e.,* Bray-Curtis coefficient) diversity. In addition to the 12 true ASVs, we recovered three artifact ASVs at frequencies of 2 to 5 reads each. Thus, we removed from real samples all ASVs with fewer than 6 reads per sample as potentially erroneous, yielding 6,543 features in the GF dataset and 870 features in the CMF.

L. monteverdensis was assigned 3,539 GF ASVs (vs. 274 CMF ASVs), *L. falx-bellica* 2,402 GF ASVs (vs. 356 CMF ASVs), *L. cribbii* 1,083 GF ASVs (vs. 176 CMF ASVs), and *L. mentosa* 837 GF ASVs (vs. 187 CMF ASVs).

We examined rarefaction curves generated by QIIME2 and the *phyloseq* package in R to choose appropriate sampling depth for calculating α- and β-diversity metrics. In both datasets, ASV and Faith's PD accumulation curves reached asymptotes. For the GF dataset we used a sampling depth of 679 which retains 69% (279) of 404 filtered samples (**Fig. 2.3** and **2.4**). For the CMF dataset we used a sampling depth of 445, which retains 62% (236) of 382 filtered samples (**Fig. 2.5** and **2.6**). When populations were used as sample units and ASV occurrences were

summed, we applied a sampling depth of 100 for the CMF dataset which retains 76% (38) of 50 filtered samples (populations). For the CMF dataset we used a sampling depth of 23 which retains 74% (37) of the filtered 50 samples.

Alpha Diversity Analyses

Orchid species had statistically significant different fungal communities in terms of their richness as shown by a Kruskal-Wallis H test performed on the GF dataset (H-value: 18.16, p-value: 0.0004). The root-associated fungal community was the richest in *L. monteverdensis*, measured as phylogenetic diversity in GF communities. Fungal richness differed most significantly between *L. monteverdensis* and *L. falx-bellica* ($\overline{PD}$ = 10.1 and $\overline{PD}$ = 6.4 respectively, Kruskal-Wallis test for Faith's PD p-value = 0.000085). *Lepanthes monteverdensis* associated with richer GF community in comparison also to other orchid species. Specifically, significant difference in richness was found between *L. monteverdensis* and *L. cribbii* ($\overline{PD}$ = 7.5) as well as between *L. monteverdensis* and *L. mentosa* ($\overline{PD}$ = 6.7) (p-value = 0.03 and 0.004, respectively). The other three orchid species had similarly rich mycobiont communities (**Table 2.4**).

A Kruskal-Wallis H test performed on the CMF dataset also showed that orchid fungal communities varied in richness (H-value: 18.58, p-value: 0.0003). More specifically, the analysis supports results of the analysis performed with GF dataset in that *L. monteverdensis* mycobionts make the richest community ($\overline{PD}$ = 2.1). As in analysis with GF dataset, communities varied in richness between *L. monteverdensis* and *L. falx-bellica* ($\overline{PD}$ = 1.7, Kruskal-Wallis test p-value = 0.00003), as well as between *L. monteverdensis* and *L. mentosa* ($\overline{PD}$ = 1.7) (p-value = 0.01). In contrast to alpha diversity analysis with GF dataset, we found no difference between *L.*

monteverdensis and *L. cribbii* ($\overline{PD}$ = 1.9) (p-value = 0.17). Additionally, we found that fungal community associated with *L. falx-bellica* was on average less rich than *L. cribbii* ($\overline{PD}$ = 1.9) (p-value = 0.04) (**Table 2.5**).

Calculating the Shannon-Wiener Diversity Index and Pielou's Evenness Index per population, followed by application of the Kruskal-Wallis test of the ASV count/population showed no significant differences for the GF or CMF datasets.

Beta Diversity Analyses

We found strong evidence for differences in fungal composition (Jaccard Similarity Index S_j) among *Lepanthes* species in the GF dataset (pseudo-F value: 1.92, p-value: 0.001, **Table 2.6**), and the CMF dataset for every PERMANOVA test we performed (pseudo-F value: 3.29, p-value: 0.001, **Table 2.7**, **Fig. 2.8** and **2.10**). PCoA and NMDS clustering generated from Jaccard indices using both GF and CMF ASVs showed the most apparent distinct clustering for mycobionts of *L. monteverdensis* (**Fig. 2.8**-**2.10**, **Supp. Fig. 2.1** & **2.3**, **Supp. Table 2.2** & **2.4**).

When fungal phylogenetic relationships are considered, we again found strong GF differences among *Lepanthes* species (pseudo-F value: 2.13, p-value: 0.001, **Table 2.8; Fig. 2.11 & 2.13**) (Unweighted Unifrac Distance U_{AB}). Specifically, mycobionts of *L. monteverdensis* were phylogenetically different from *L. falx-bellica* (p-value = 0.001), *L. cribbii* (p-value = 0.019) and *L. mentosa* (p-value = 0.001). There was a notable but non-significant difference between *L. falx-bellica* and *L. mentosa* (p-value of 0.079). Test results using the smaller CMF dataset (pseudo-F value: 3.84, p-value: 0.001) were consistent with results obtained from GF dataset. Specifically, *L. monteverdensis* mycobionts had different phylogenetic composition than fungi associated with

L. falx-bellica (p-value = 0.001), *L. cribbii* (p-value = 0.049) and *L. mentosa* (p-value = 0.017) (Table 1.9). Additionally, the CMF community differed significantly between *L. falx-bellica* and *L. cribbii* (p-value = 0.039). PCoA and NMDS clustering using UniFrac distances generally followed the same pattern as that produced by Jaccard indices, except for no apparent clustering in PCoA produced with CMF dataset (**Fig.2.11-2.14, Supp. Fig. 2.2 & 2.4, Supp. Table 2.3 & 1.5**).

Furthermore, we found that GF communities differed when number of ASV interactions were quantified (Bray-Curtis Dissimilarity Index S_{BC}) between *L. monteverdensis* and *L. falx-bellica* (p-value = 0.001) as well as between *L. monteverdensis* and *L. cribbii* (p-value = 0.001). Using the smaller CMF dataset, we found no support for difference in Bray-Curtis Diversity among any of the orchid species.

2.5 DISCUSSION

Full-length ITS sequencing might more accurately characterize a fungal community

In microbial and plant ecology, researchers of fungal communities have traditionally utilized short DNA sequence fragments, typically ranging from 100 to 500 base pairs from either the ITS1 or ITS2 subregions. Due to the reduced number of base pairs in such fragments — compared to the full-length ITS region — it is thought that these minibarcodes are less informative (Kõljalg et al. 2013; Schlaeppi et al. 2016). The ITS2 subregion for example has been found to overestimate the number of fungal taxonomic units, as well as richness and/or diversity measures which could be an artifact of more random errors and hypervariability of the region (Tedersoo et al. 2017).

Third-generation high throughput sequencing technologies, such as the Pacific Biosciences (PacBio) sequencing platform, have the potential to sequence molecules up to 60 kbp and as such have opened up an exciting opportunity to utilize the whole ITS region with low error rate. Tedersoo et al. (2017) found that the full ITS dataset matched reference database records less often than the more limited ITS2 dataset, but when it did, it was more likely to be identified at a species level. This suggests that the majority of fungal DNA information comes from sequencing the ITS2 subregion and secondly that the full ITS dataset offers better taxonomic resolution. Consequently, our GF dataset (prior to mycorrhizal fungal guild assignment) likely represents true fungal taxa and as such is more informative about the fungal community than the CMF dataset which is only 13.3% of the size of GF dataset. These longer sequences have the advantage of covering both hypervariable ITS subregions as well as the more conservative flanking 5.8S, LSU and SSU subregions (Bleidorn 2016). Consequently, their use allows better estimates of fungal diversity as well as more accurate phylogenetic placement. Use of the full ITS barcode improves taxonomic identification at all taxonomic ranks. Tedersoo et al. (2017) found 9% higher resolution at the class level and 33% higher resolution at the genus level. Moreover, there were 2.2 - 3.2 times fewer unidentified Basidiomycota than reported with datasets of shorter ITS1 and ITS2 sequences. This is particularly relevant to our study since all major groups of orchid mycorrhizal fungi (OMF) belong to Basidiomycota.

Interestingly, the only study we are aware of where both CMF and GF datasets were assessed have reported a similar ratio of fungi recovered by both datasets as estimated by one of the ITS2-targeting primers employed (4.4-7.6 fold difference depending on orchid species, 4.5-

9.6 fold in our data) while the other ITS2-targeting primer set potentially performed worse in overall and mycorrhizal fungal recovery (25.6-30.7 fold difference) (Waud et al. 2016). This study recovered about 6x fewer fungal taxonomic units in the whole fungal dataset and about 10x fewer fungal units in a confirmed mycorrhizal dataset, a finding that could be an indication of lower fungal diversity in terrestrial temperate orchids comparing to tropical epiphyte orchids.

ASVs versus OTUs for characterization of fungal communities

A common approach in fungal research is to apply a 97% sequence similarity threshold to circumscribe Operational Taxonomic Units (OTUs) (Hughes, Petersen, & Lickey 2009), a method also used to delimit mycorrhizal species in basidiomycetes (Jacquemyn et al. 2010). It has been argued that Tulasnellaceae OTUs should be delimited using a less conservative 95% threshold due to high sequence diversity in *Tulasnella* spp. (Novotná et al. 2018). We chose instead to use the output generated by *DADA2* denoising and dereplication methods, to identify ASVs for downstream analyses for several reasons. First, the average quality score of raw sequences was reasonably high (98.9%) and all mock community ASVs were 100% accurate. Second, based on our SynMock results we reasoned that any variable length sequences introduced during PCR or sequencing should be in low frequency and/or occurrence, and even if they survived filtering, they would have little impact on α- and β-diversity patterns. Third, previous research has found that the risk of overestimating the number of fungal community members is more likely to happen with the use of ITS2 as opposed to the full ITS region (Tedersoo et al. 2017). Moreover, applying ITS sequence similarity cutoffs is likely to underestimate fungal diversity (Waterman et al. 2011). Lastly, the read coverage we obtained per sample is sufficient to recover all community members as evidenced by rarefaction curves. The

GF curves reach saturation at about 15-35 (5-7 in CMF dataset) ASVs/orchid species which roughly corresponds to the approximate orchid mycorrhizae OTU number reports available in scarce studies of tropical orchids (Novotná et al. 2018; Cevallos et al. 2017). However, contrary to prior studies we recovered many more total fungal taxonomic units, a finding potentially due to more intensive sampling as well as higher sequencing coverage. Differences in methodology could also have contributed to this discrepancy, e.g. Novotná et al. (2018) sequenced only fungi that were previously successfully cultured, potentially omitting non-culturable fungi, while Cevallos et al. (2017) employed a shorter ITS2 minibarcode. Regardless, this would not affect the overall diversity estimates given that the rarefaction curve of Faith's PD reached asymptote. Thus, in addition to the low risk of rare ASVs confounding the results, the ASVs found in the present study are likely to be biologically meaningful. That said, we recognize that some properties inherent to fungal biology might inflate ASV numbers (Palmer et al. 2017).

Mycorrhizal community diversity

To generate a comprehensive understanding of diversity present in the fungal communities among orchid species we selected several metrics of α- and β- diversity. We attempted to include quantitative information by summing up ASV occurrences across orchid populations (Shannon, Bray-Curtis indices). Transforming the data for quantitative diversity estimates was necessary since read abundance does not correspond to fungal abundance as demonstrated by Palmer et al. 2017 and was further corroborated by our SynMock analysis. However, doing so greatly diminished the sample size (from 404 to 88 in the GF dataset and from 382 to 89 in the CMF dataset) as well as sampling depth (from 679 to 100 in the GF dataset and from 445 to 23 in the CMF dataset) potentially decreasing the power to detect any differences; for this reason we

remain skeptical about the results of this set of analyses and thus omitted them from the summary figure (**Fig. 2.15**).

The four sympatric *Lepanthes* species associate with 6,543 GF ASVs and 870 CMF ASVs. The GF ASVs may include fungi that are orchid-specific endophytes, pathogens, or yet-to-be described mycorrhizal symbionts. We suspect however that a number of GF ASVs are truly mycorrhizal but are not included in the CMF dataset for two reasons. First, taxonomical assignment inevitably depends on contributions of previous researchers to databases like UNITE and FUNGuild. Second, tropical OMF are underrepresented due to a limited number of fungal studies in equatorial regions. Finally, it is noteworthy that some recent reports have found fungi outside of widely accepted OMF groups to associate frequently with orchids, that in other plants act as endophytes or even pathogens (Li et al. 2021). These fungi may be beneficial to orchids through supporting growth, development, stress resistance, and some may possibly act as OMF. Interestingly, though, the overall picture of the fungal diversity patterns that emerges from both GF and CMF datasets remains the same. Based on the analyses, *L. monteverdensis* associates with the most distinct and diverse mycorrhizal assemblage relative to its three sympatric congeners. This is evidenced by the number of fungal ASVs as well as different community composition, phylogenetic richness, and phylogenetic distance from mycobionts associated with the other *Lepanthes* spp. While the difference in CMF richness between *L. monteverdensis* and *L. cribbii* (Faith's PD) was not significant, there was a significant difference among these species in community qualitative composition and phylogenetic distance (Jaccard and UniFrac scores, respectively). By comparison to the mycobiont community assemblage of *L. monteverdensis*, *L. falx-bellica* shares some of its mycobiont composition with other *Lepanthes*. While all measures

support its fungal diversity as separate from that of *L. monteverdensis*, this species shares some diversity with that of *L. cribbii* and *L. mentosa*. In the GF dataset, the Kruskal-Wallis test p-values for Faith's PD comparisons among fungi in *L. falx-bellica* - *L. cribbii* individuals were low but not significant. Since this metric measures branch length in a phylogenetic tree, it simply implies that the mycorrhizal communities that these orchid species partner with have fairly similar amounts of evolutionary changes. UniFrac measures for these species were also not significantly different, indicating high amount of shared phylogeny in their fungal assemblages. However, the ASVs that make up those communities are actually different, as evidenced by the qualitative Jaccard measure. By comparison, there was limited evidence emerging from CMF dataset to support that *L. falx-bellica* mycobionts were different from those of *L. cribbii.* The PERMANOVA test detected a significant difference in the amount of unique evolutionary history of those ASVs that belong to each orchid species communities in the CMF dataset (UniFrac index), and *L. cribbii* had higher richness (Faith's $\overline{PD}$). In contrast, all measures in both CMF and GF datasets detected no difference between *L. mentosa* and either *L. falx-bellica* or *L. cribbii*, except for Jaccard. Thus, these results suggest that congeneric orchids investigated 1) differed in their mycobiont composition, and 2) those differences were not uniformly distributed among species.

All four narrowly endemic *Lepanthes* spp. have been found growing on the same phorophyte in different configurations. Out of the sampled populations, *L. falx-bellica* has been found to share the same phorophyte most frequently with *L. cribbii* (17.1% of sampled trees), followed by with *L. monteverdensis* (8.6% of phorophytes). Note that our sampling data (**Table 2.2**) indicates that *L. falx-bellica* was growing by itself on 31.4% of phorophytes, however this

value is overestimated since some of these populations did co-occur on the same tree with *L. cribbii* as well, however it was not recorded (P.Tuczapski, pers. obs.). *L. mentosa* co-occurred with *L. cribbii* on 2.9% of phorophytes. Three species, namely *L. cribbii*, *L. falx-bellica* and *L. monteverdensis* were found on the same phorophyte 2.9% of the time, as were all four species. Among the instances where *Lepanthes* sp. were found growing alone, *L. monteverdensis* occurred on 17.1% of its phorophytes, *L. mentosa* on 14.3%, and *L. cribbii* on 2.9% of its phorophytes. We speculate that niche partitioning might be a mechanism driving segregation of mycobiont partners among orchid species. However, orchids vary in the degree to which their mycobiont composition differs. The frequency of co-occurrence of given orchid species with its congeners does not seem to explain fully this distribution of mycorrhizal differences. Specifically, we might expect that *L. monteverdensis*, which associates with the largest number of ASVs as well as most distinct fungal community, would have the highest co-occurrence rate in the phorophytes sampled. However, that was not the case. *L. falx-bellica* was the species that co-occurred with its congeners the most often (>31.4% vs. 14.3% in the case of *L. monteverdensis* out of 35 sampled trees), yet it shared some of its mycobiont composition with other sister taxa.

We hypothesize that our results reflect the relative recency of taxonomic divergence of some *Lepanthes* spp. versus others. It is likely that *L. monteverdensis* is the oldest of these taxa. Given reliance of orchid life cycle on their OMF, we speculate that apparent range of the degree of mycorrhizal diversity might be explained if these four sister orchid species have not been separated as taxonomic units for long enough for them to "switch over" to different mycobionts, or for mycorrhizal fungi to accumulate enough changes for tests of diversity metrics to detect.

Notably, mycorrhizal partners of *L. mentosa* were different from those of *L. falx-bellica* and *L. cribbii* only as detected by Jaccard indices. This result indicates that while the compositions of the investigated fungal communities were different, phylogenetically they were similar. This could indicate that *L. mentosa* diverged more recently and is at an earlier stage of partitioning the CMF niche space, or its OMF had least time to diverge from *L. falx-bellica*'s and *L. cribbii*'s OMF. Alternatively, perhaps the pressure posed on *L. mentosa* to escape competition for resources was the lowest since it did not occupy the same niche as other orchids. While *L. mentosa*'s range overlaps with other species, it was found to be growing mainly at the lower altitude (1482-1617 m asl) sites. This may explain little difference in the mycorrhizal diversity with *L. cribbii*, which range has little overlap with that of *L. mentosa* and its upper limit (1584-1750 m asl) extends over *L. mentosa*'s range, thus possibly those two species do not commonly co-occur. However, it doesn't explain the same result when compared to mycobionts of *L. falx-bellica*, which was found often on similar altitudes (1502-1712 m asl) and theoretically should be able to compete with *L. mentosa* more often. Finally, since *L. mentosa* was the least sampled species, we advise caution when interpreting data generated from that species. In conclusion, evidence suggests that congeneric sympatric species of orchids associate with different mycorrhizal assemblages, and those differences are more pronounced in some orchid species.

Role of mycorrhizae on New World orchids

This study provides the first fine-scale insight utilizing high-throughput third generation sequencing technology into potential importance of mycorrhizal symbionts for niche partitioning and/or speciation in the understudied system, namely rapidly speciating epiphytic orchids of the New World tropics.

Previous studies revealed segregation of mycorrhizal communities between co-occurring orchid species and have pointed at its possible role in niche partitioning and coexistence. However, this has been investigated mostly in a small number of plants of terrestrial, often distantly related, temperate species that occupy relatively extensive geographical regions (i.e. hundreds to thousands of square meters, sometimes spanning over thousands of kilometers; see e.g. Waterman et al. 2011, Jacquemyn et al. 2012a, Jacquemyn et al. 2012b, Jacquemyn et al. 2014, Waud et al. 2016). Moreover, prior studies have failed to account for the phylogenetic signal in the OMF assemblages and/or fine-scale taxonomic resolution of mycobionts, and their reported number is often dubious. This study provides an extreme case scenario of syntopy in which representatives of sister species often co-occur on the same phorophyte (i.e. host tree) or even branch. Epiphytic orchids are adapted to much harsher abiotic environments (*e.g.*, nutrient and water limitations) relative to that experienced by terrestrial counterparts (Martos et al. 2012). Thus, epiphytic orchids have been hypothesized to experience much stronger selective pressures not only to partner with mutualistic fungi with specific functions, but also that allow to partition limited nutrient resources present in a confined space. In this context, finding an appropriate partner may be crucial for survival.

It has been speculated that plants might speciate by adapting to new fungal taxa (Thompson 1987; Cowling et al. 1990; Otero and Flanagan 2006) that would allow for access to novel sources of nutrients and ecological niches. Furthermore, it has been argued (Waterman et al. 2011) that if a given mutualism acts as an important driver of speciation, one should expect to identify different partners particularly in recently diverged species. To our knowledge, the results

of our analyses provide the first report of different fungal communities in sister orchid species. It indicates that, at least in the hyper-diverse, rapidly diversifying genus *Lepanthes,* sister species indeed do tap into different mycobionts. Our finding provides an interesting contrast to previous study (Waterman et al. 2011) where authors argue that shifts in pollination traits drive plant speciation and are important for coexistence while mycorrhiza allows coexistence but not speciation. While pollinator specialization of *Lepanthes* is out of scope of this study, we acknowledge that it is likely a strong force contributing to diversification rate, given that scarce reports point at sexual deception as a mechanism employed by species of the genus (Blanco & Barboza 2005). Previous studies of sexual deception in orchids have revealed that pollination in this mating system is highly specific, with each orchid species employing males of single pollinator species (Peakall and Whitehead 2013). In contrast, ancestral character trait models performed on the second largest Neotropical orchid group, Cymbidiae, revealed no correlation between shifts in pollinator syndromes and diversification rates, which suggests that pollinator specificity might not be important for orchid fitness, and consequently, speciation (Pérez-Escobar et al. 2017). In that context, the fact that incipient sister orchid species utilize different fungal taxa might be further accelerating the process of producing new plant species. Thus, we suggest that the potential role of fungi in driving speciation in orchids should not be dismissed and deserves reconsideration. We recognize that this study is by no means a comprehensive estimation of mycobiont potential role in speciation process. For instance, it remains to be tested whether neotropical orchids associate with different fungi at different life stages, what is the extent of specialization (reciprocal selectiveness of both partners), whether there are keystone fungal nutrient providers vs. facultative partners, and whether there is a temporal effect on mycorrhizal community turnover e.g. associated with the seasons. Nevertheless, this study offers

insight into the possible role of mycorrhizae in co-existence and diversification of epiphytic orchids.

Conclusions

This study represents the first use of third-generation high-throughput sequencing technology, the Pacific Biosciences (PacBio) sequencing platform, to characterize orchid mycobiont communities. The whole ITS region sequenced allowed for fine-scale taxonomic resolution and high identification accuracy. The number of fungal taxonomic units recovered was several orders of magnitude higher than previously reported. Four narrowly endemic, syntopic epiphytes from a rapidly evolving genus exhibited distinct mycobiont interactions, with these differences unevenly distributed among species. These findings suggest that niche partitioning alone may not fully explain mycobiont selection, highlighting the need to reconsider the role of fungi in orchid speciation.

2.6 FIGURES

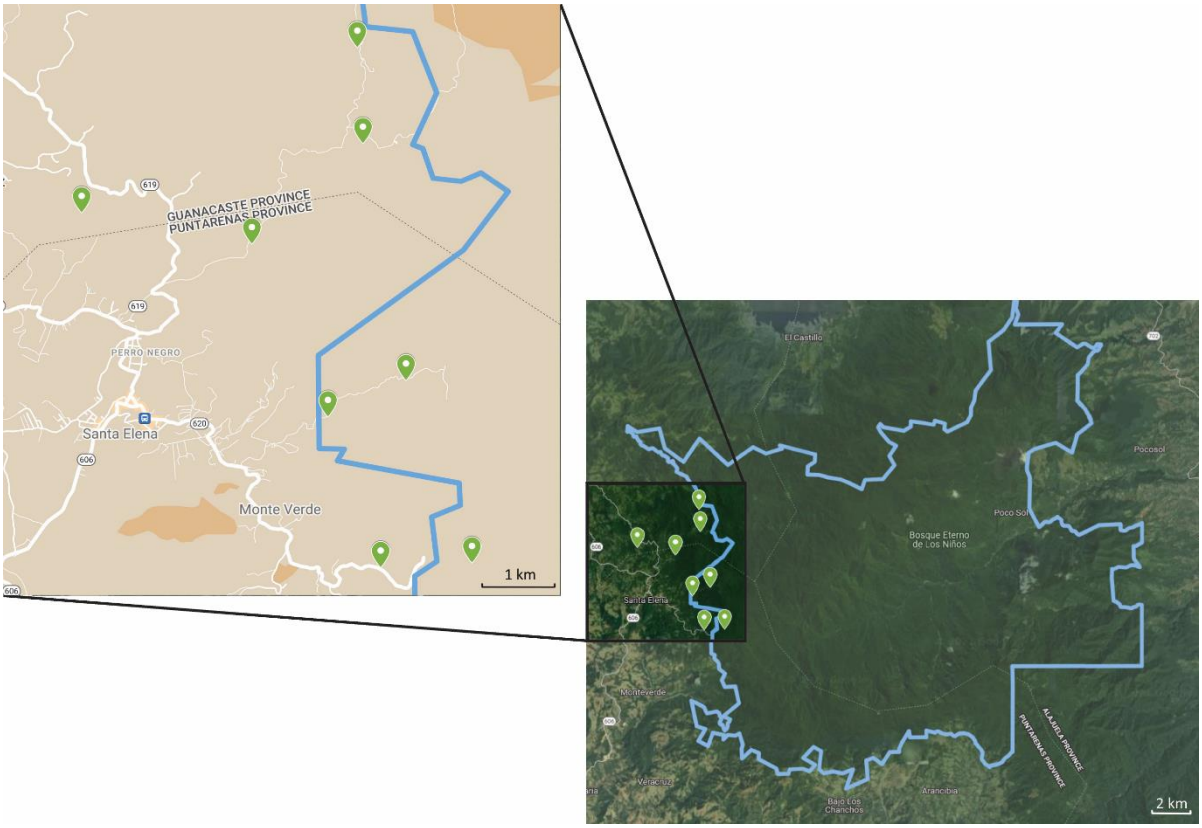


Figure 2.1. **Map of study sites in the Monteverde Cloud Forest Biological Reserve** (outlined in blue). Green pins indicate sampled locations, which span ~6 km. Additional areas to the north and south of the study sites were searched for the four study species without success.

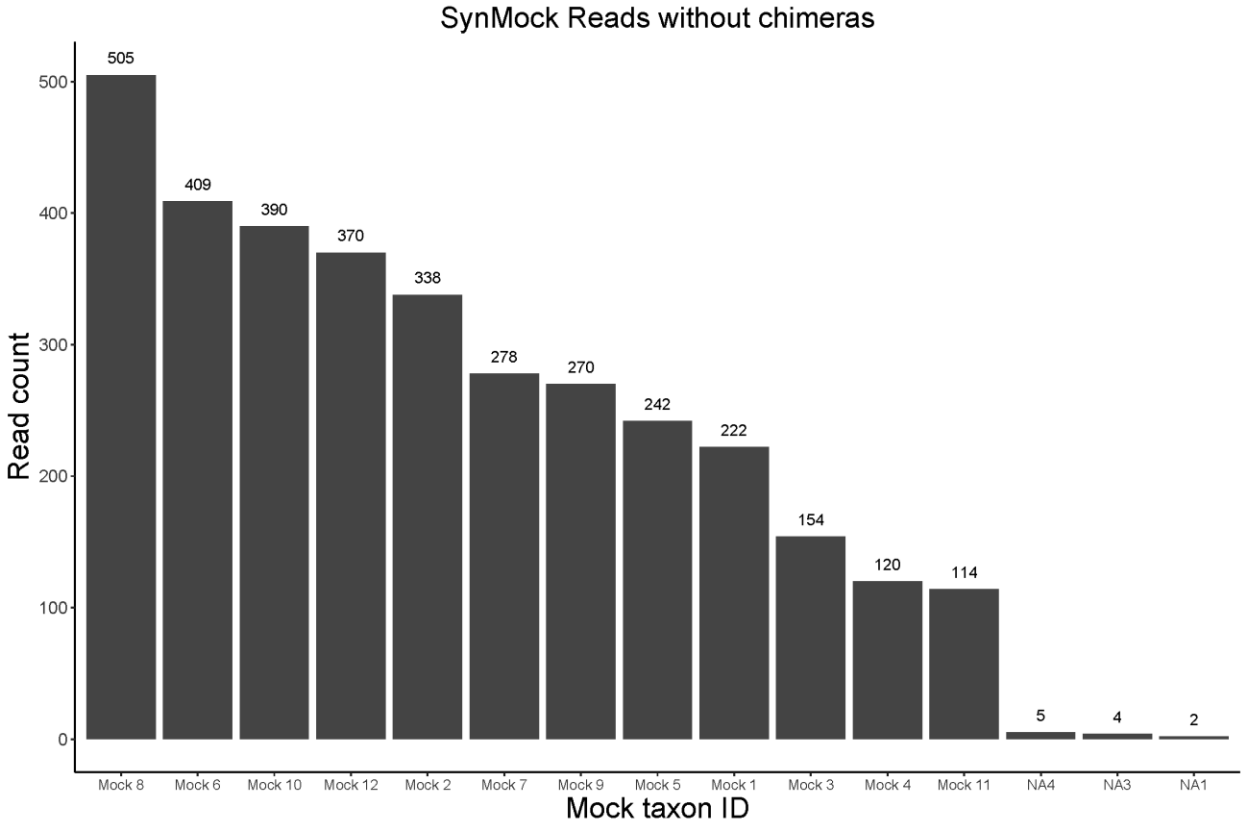


Figure 2.2. Recovered read count of synthetic non-biological fungal community (SynMock) sample members. *NA* = unidentified sequences not used in the community library pool.

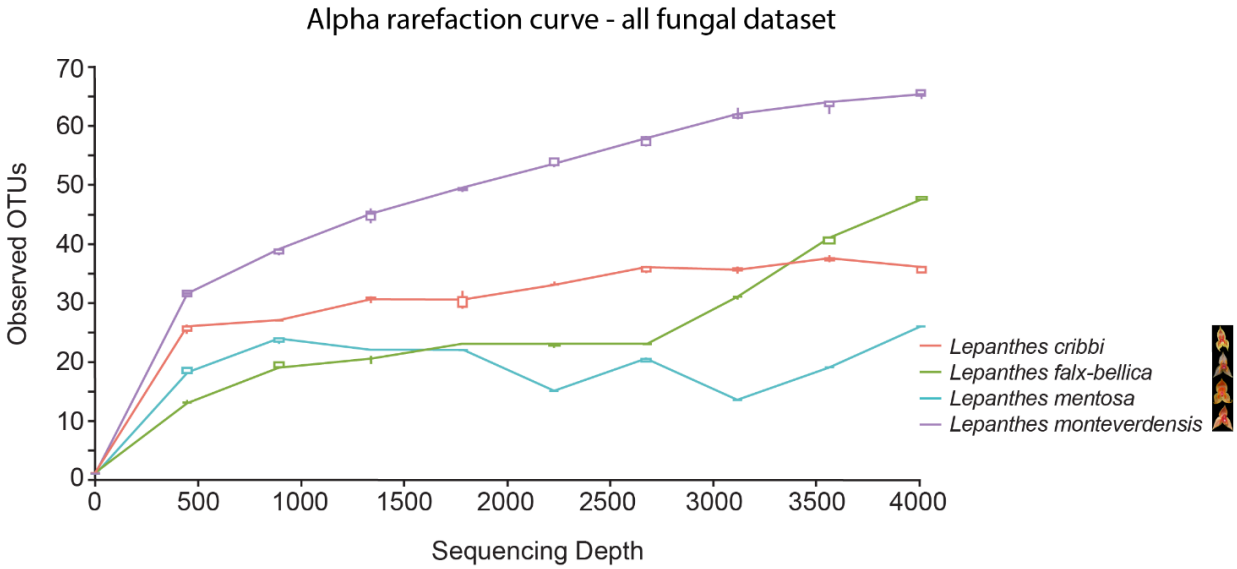


Figure 2.3. Alpha rarefaction curves showing accumulation of fungal OTUs (*i.e.*, ASVs) over sequencing depth. Curves have been constructed based on most comprehensive, all-fungal (GF) dataset by averaging rarefaction curves of 404 samples over a grouping variable of an orchid species.

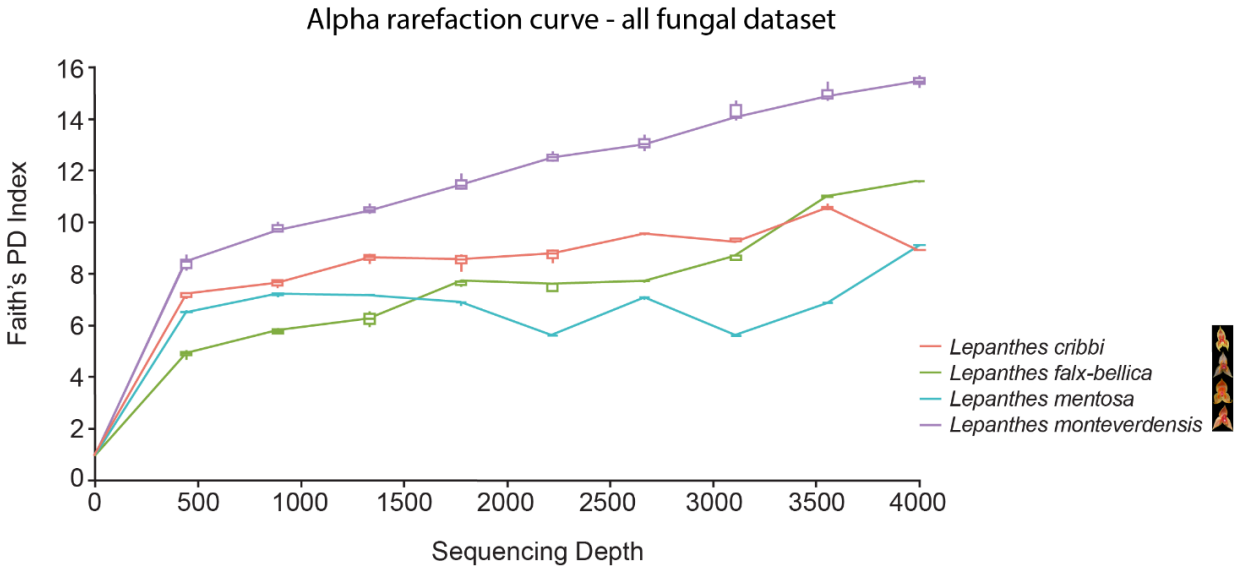


Figure 2.4. Alpha rarefaction curves showing accumulation of Faith's PD Index over sequencing depth. Curves have been constructed based on most comprehensive, all-fungal (GF) dataset by averaging rarefaction curves of 404 samples over a grouping variable of an orchid species.

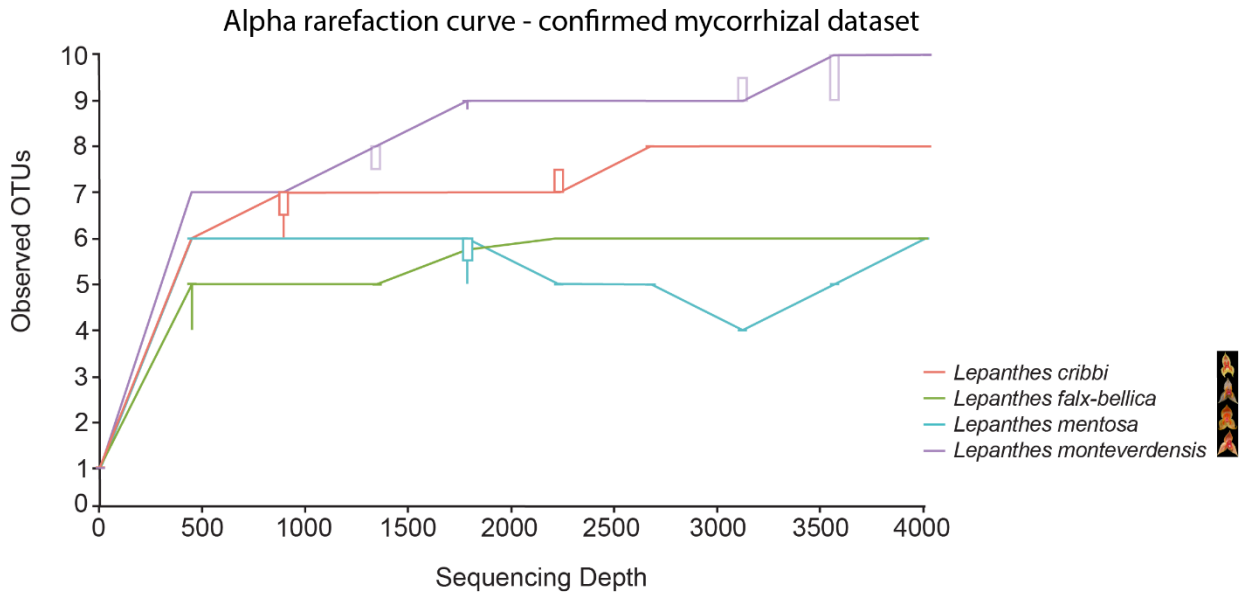


Figure 2.5. Alpha rarefaction curves showing accumulation of fungal OTUs (*i.e.*, ASVs) over sequencing depth. Curves have been constructed based on confirmed mycorrhizal (CMF) dataset by averaging rarefaction curves of 382 samples over a grouping variable of an orchid species.

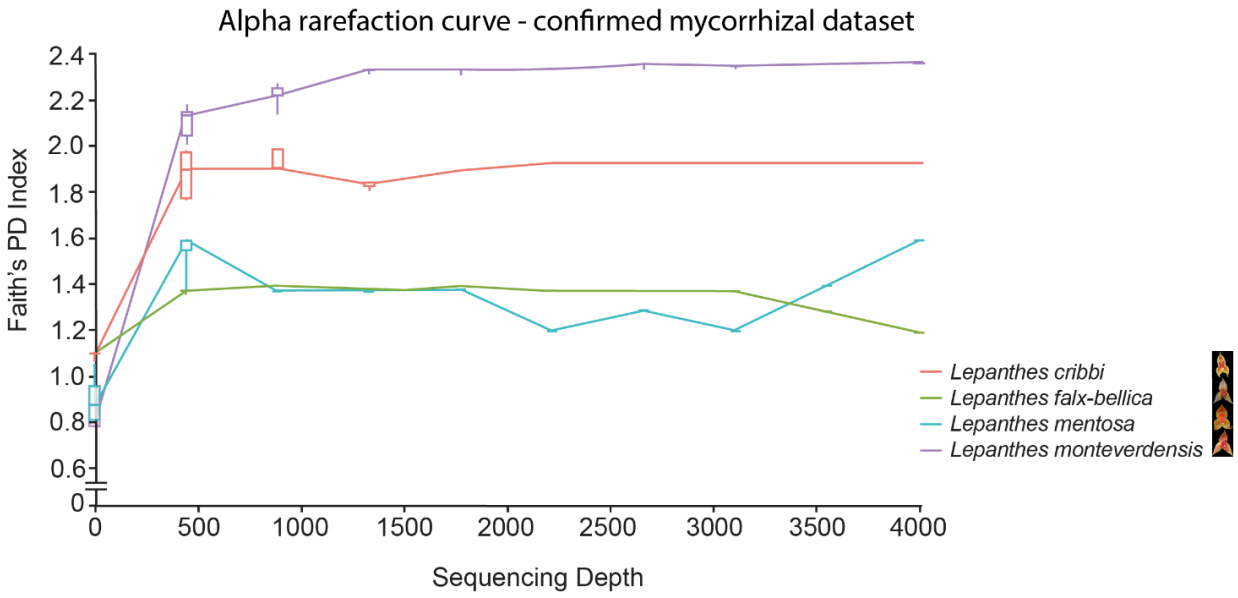


Figure 2.6. Alpha rarefaction curves showing accumulation of Faith's PD Index over sequencing depth. Curves have been constructed based on confirmed mycorrhizal (CMF) dataset by averaging rarefaction curves of 382 samples over a grouping variable of an orchid species.

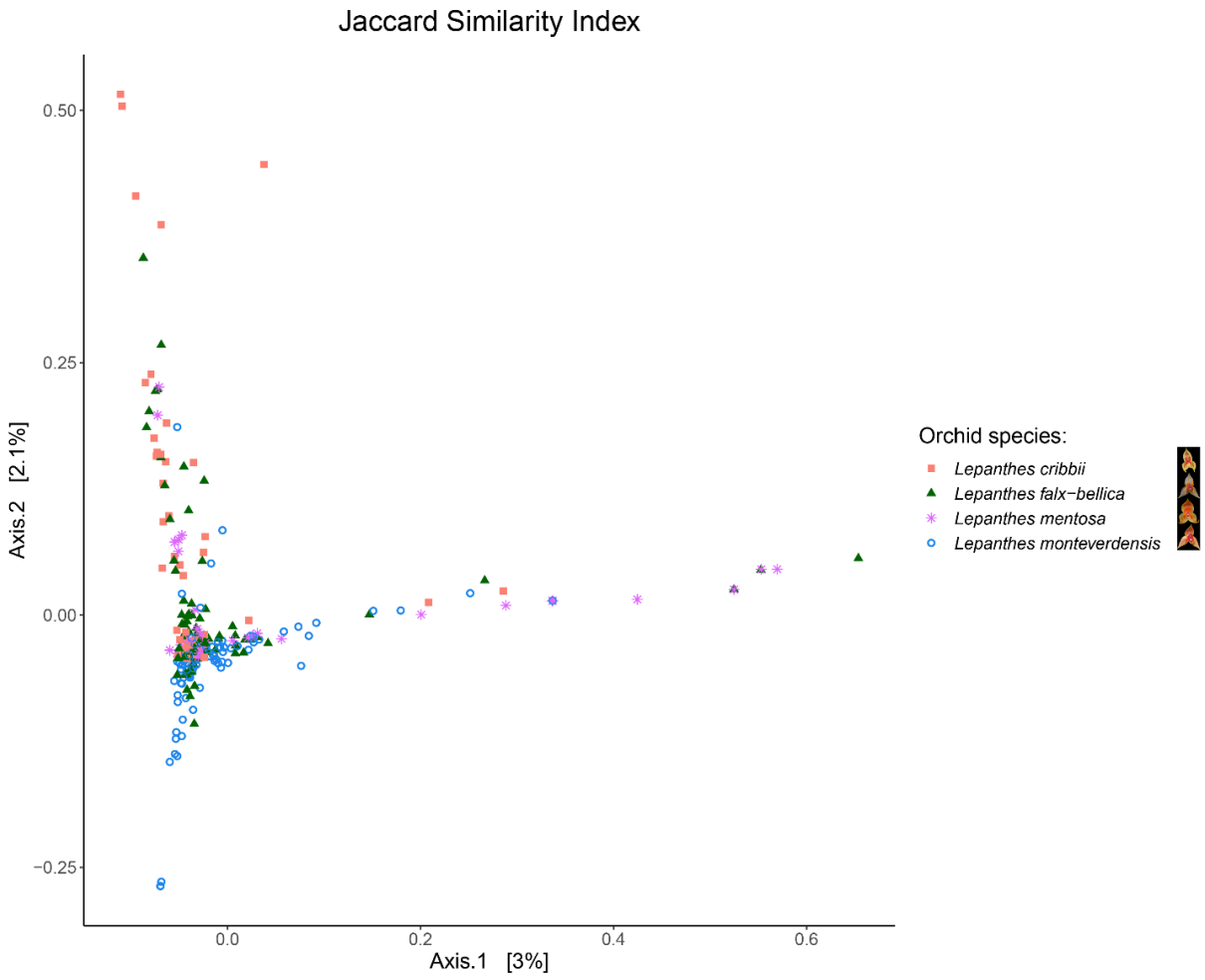


Figure 2.7. Ordination diagram of Principal Coordinate Analysis (PCoA) calculated on Jaccard distances (converted from Similarity Indices S_j) between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. Jaccard distance scores calculated on most comprehensive GF dataset (404 samples) were added into the diagram.

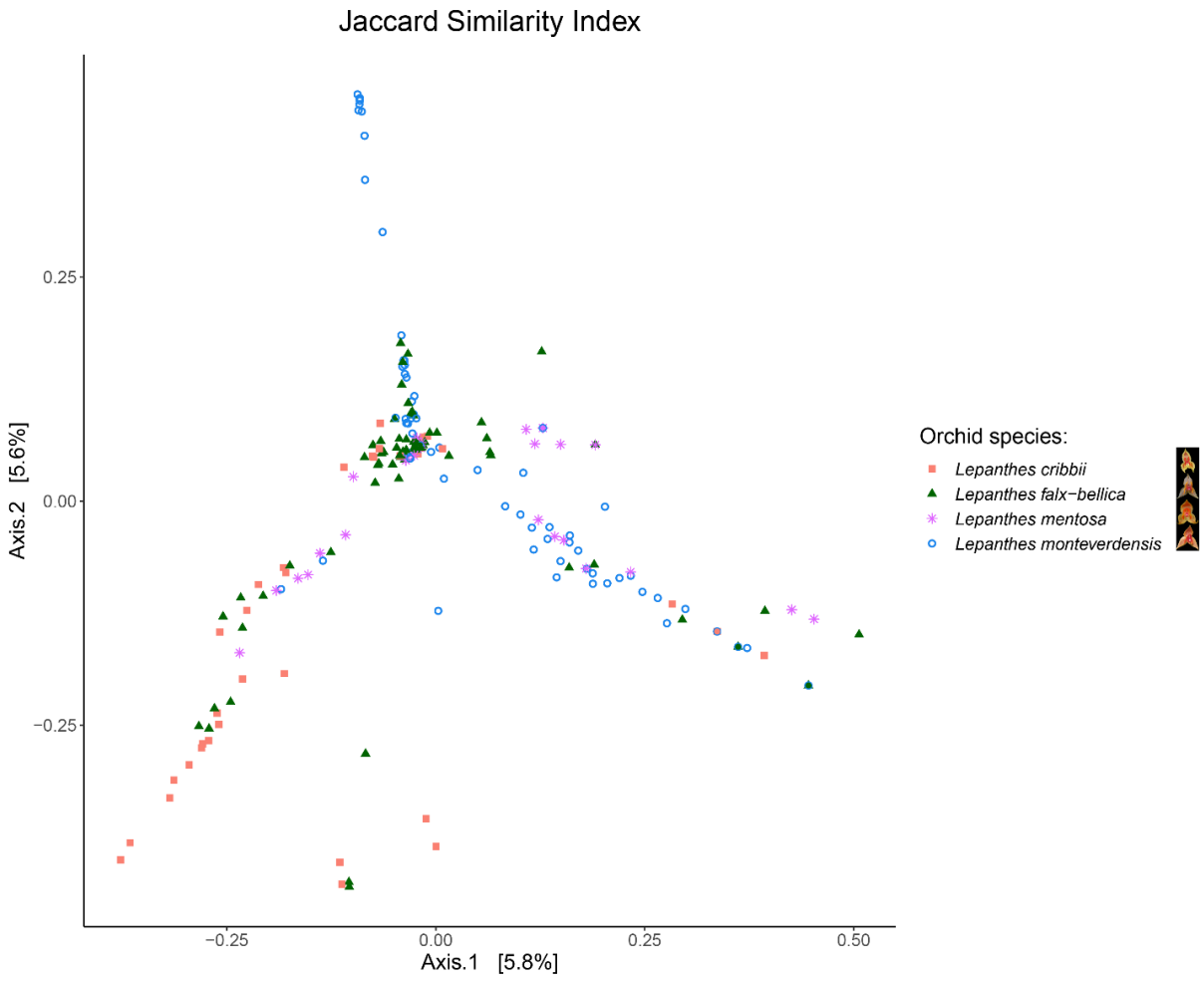


Figure 2.8. Ordination diagram of Principal Coordinate Analysis (PCoA) calculated on Jaccard distances (converted from Similarity Indices S_j) between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. Jaccard distance scores calculated on CMF dataset (382 samples) were added into the diagram.

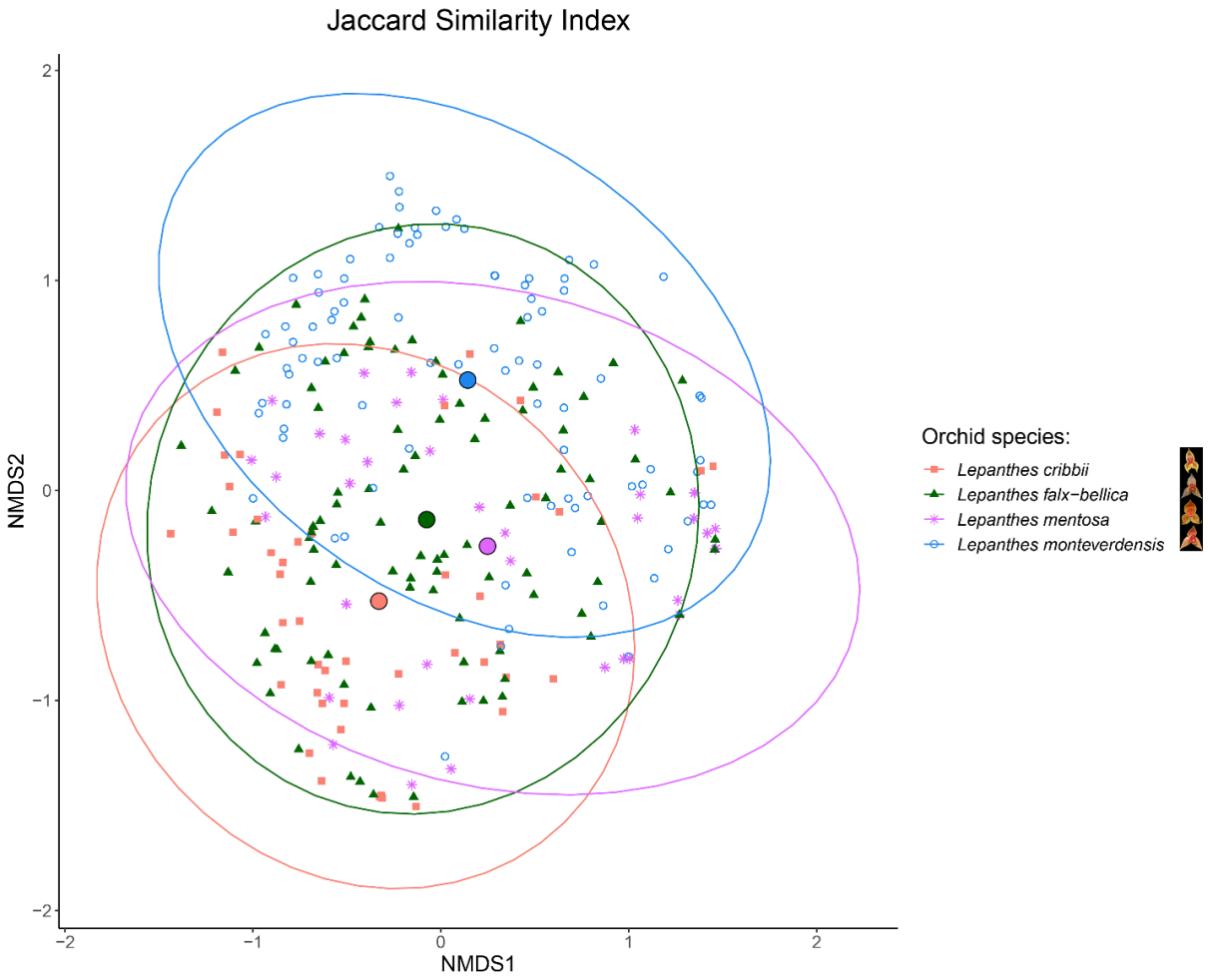


Figure 2.9. Ordination Diagram of Non-metric Multidimensional Scaling (NMDS) calculated on Jaccard distances (converted from Similarity Indices S_j) between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. S_j scores calculated on GF dataset (404 samples) were added into the diagram. Number of

dimensions of k = 5 has been selected for constructing a plot (see **Supp. Fig. 2.1** and **2.3**). Centroids and 95% confidence ellipses have been indicated. Stress value: 0.188.

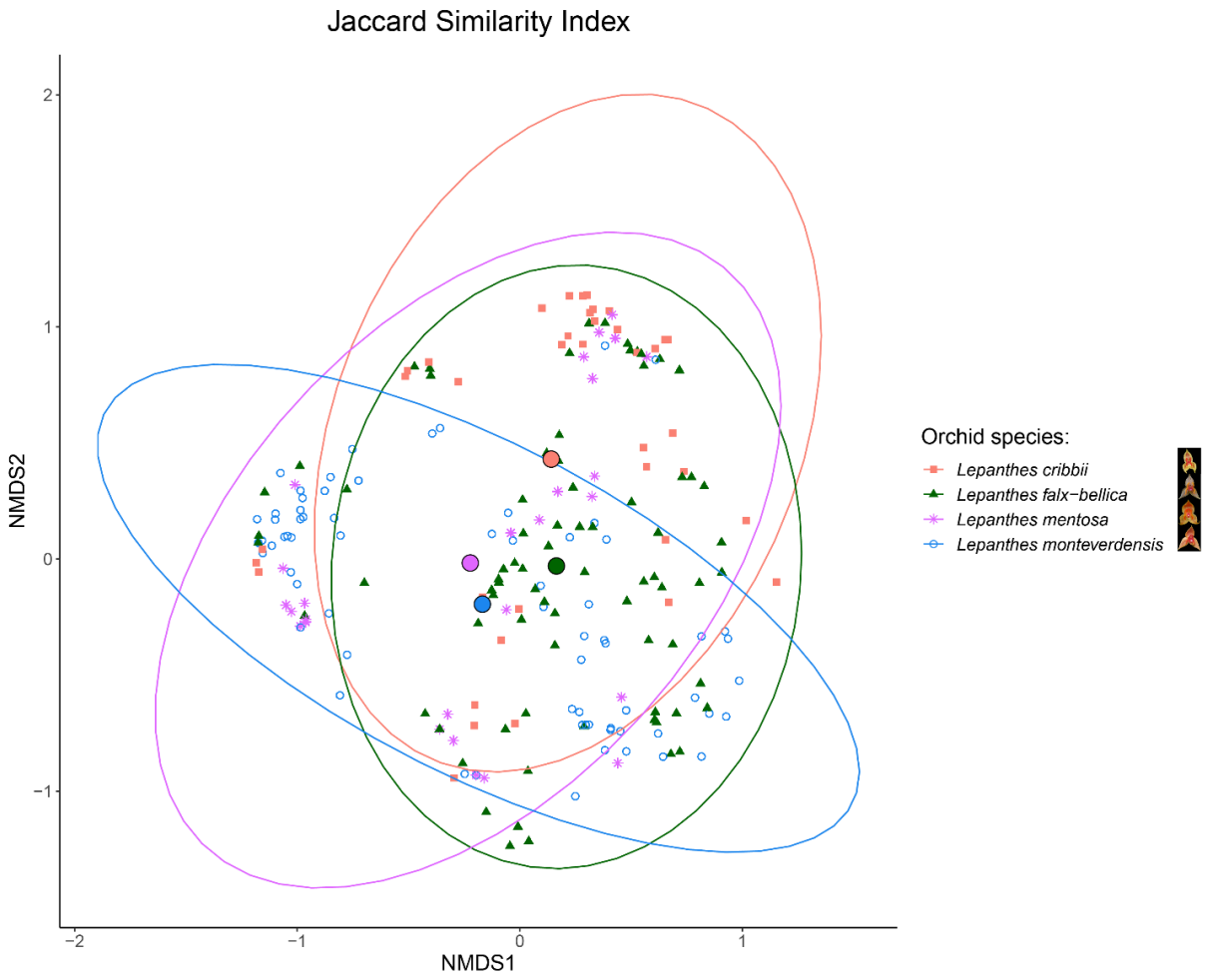


Figure 2.10. Ordination Diagram of Non-metric Multidimensional Scaling (NMDS) calculated on Jaccard distances (converted from Similarity Indices S_j) between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. Jaccard distance scores calculated on CMF dataset (382 samples) were added into the diagram. Number of dimensions of k = 5 has been selected for constructing a plot (see **Supp. Fig. 2.1** and **2.3**). Centroids and 95% confidence ellipses have been indicated. Stress value: 0.188.

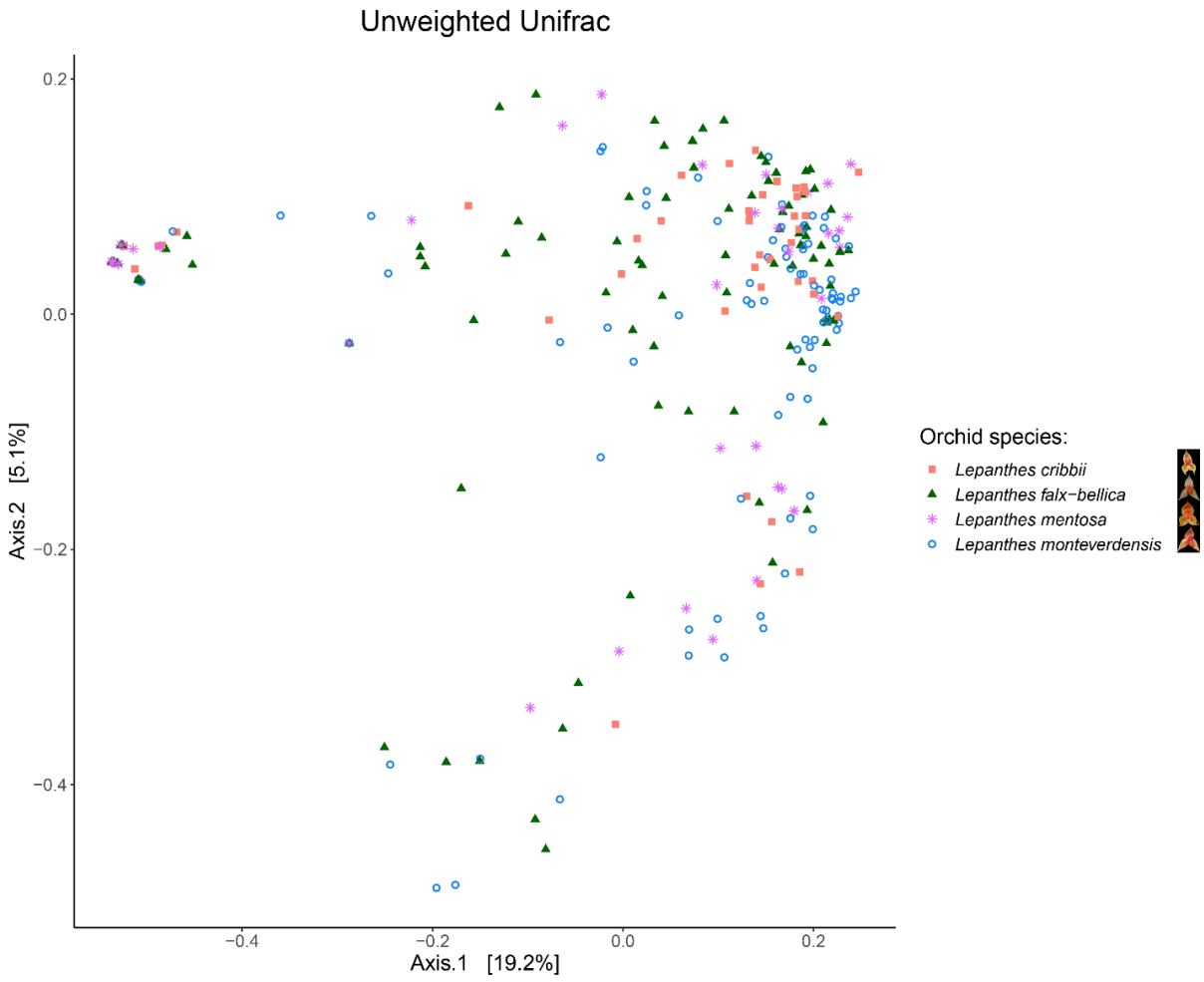


Figure 2.11. Ordination diagram of Principal Coordinate Analysis (PCoA) calculated on Unweighted UniFrac Distances U_{AB} between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. U_{AB} scores calculated on most comprehensive GF dataset (404 samples) were added into the diagram.

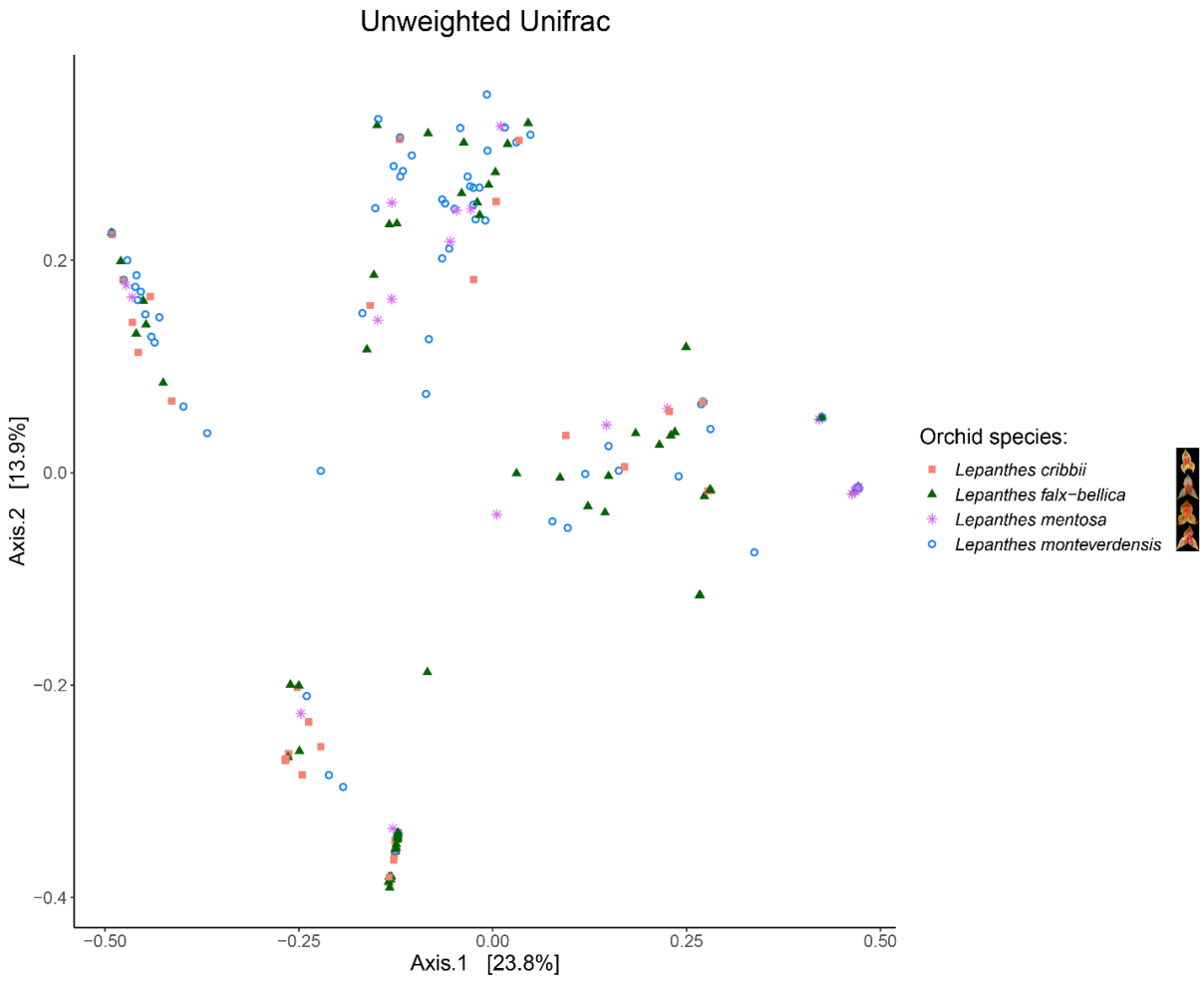


Figure 2.12. Ordination diagram of Principal Coordinate Analysis (PCoA) calculated on Unweighted UniFrac Distances U_{AB} between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. Jaccard distance scores calculated on CMF dataset (382 samples) were added into the diagram.

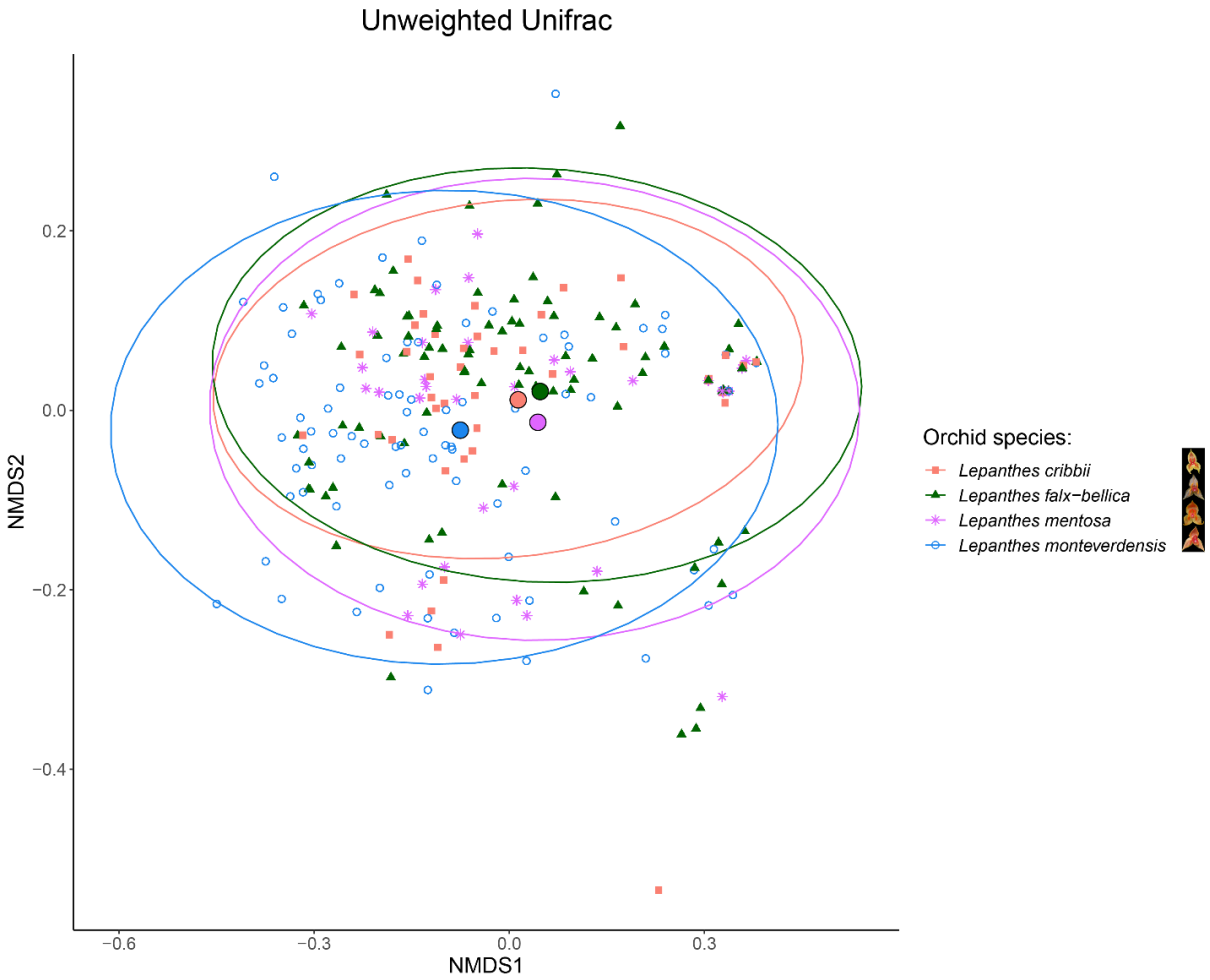


Figure 2.13. Ordination Diagram of Non-metric Multidimensional Scaling (NMDS) calculated on Unweighted UniFrac Distances U_{AB} between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. U_{AB} scores calculated on GF dataset (404 samples) were added into the diagram. Number of dimensions of k = 3 has been selected for constructing a plot (see **Supp. Fig. 2.2** and **2.4**). Centroids and 95% confidence ellipses have been indicated. Stress value: 0.142.

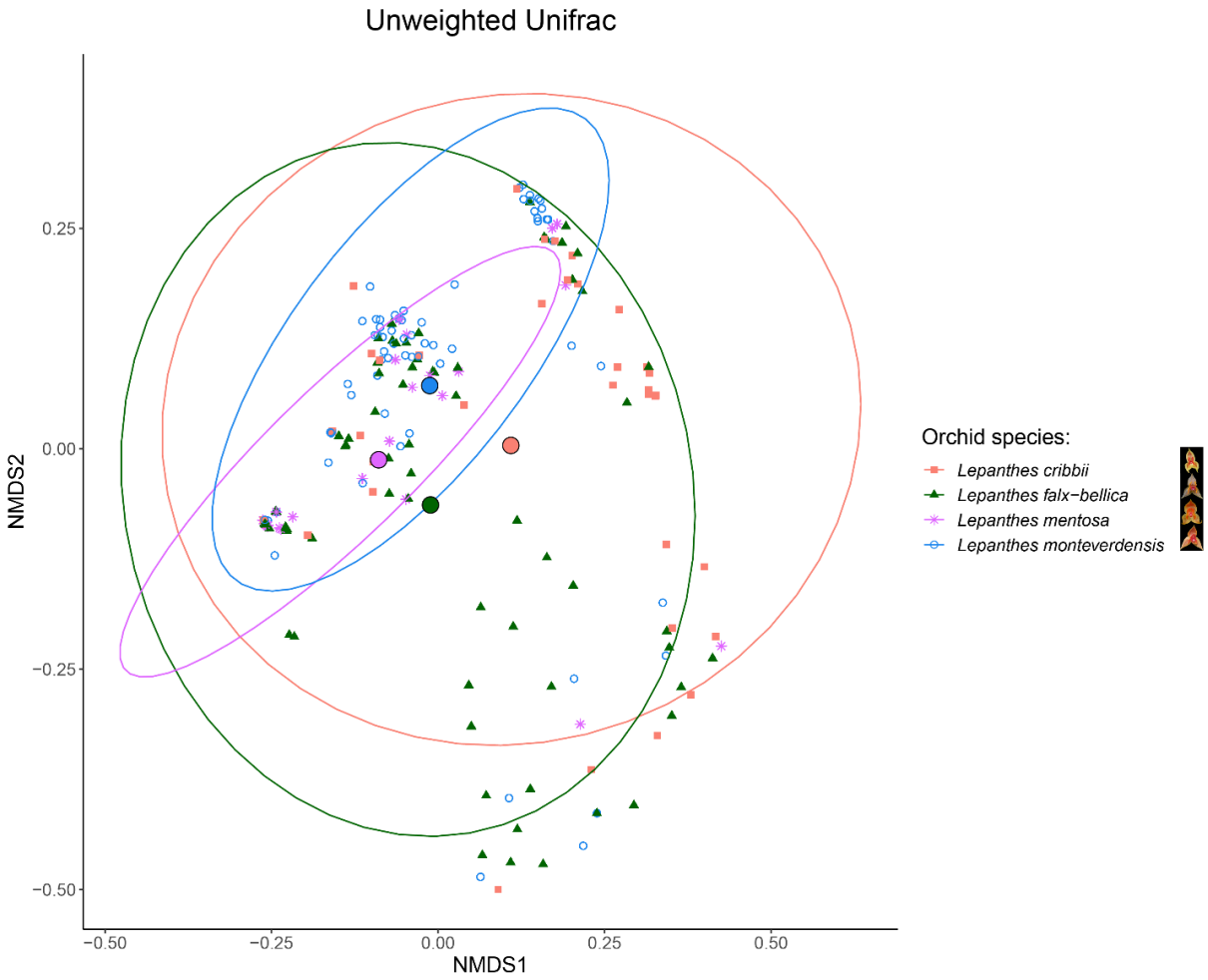


Figure 2.14. Ordination Diagram of Non-metric Multidimensional Scaling (NMDS) calculated on Unweighted UniFrac Distances U_{AB} between individual orchid plant fungal communities. The classification of samples into one of the four orchid sister species is displayed by different color and symbol of individual community scores. U_{AB} scores calculated on CMF dataset (382 samples) were added into the diagram. Number of dimensions of k = 3 has been selected for constructing a plot (see **Supp. Fig. 2.2** and **2.4**). Centroids and 95% confidence ellipses have been indicated. Stress value: 0.167.

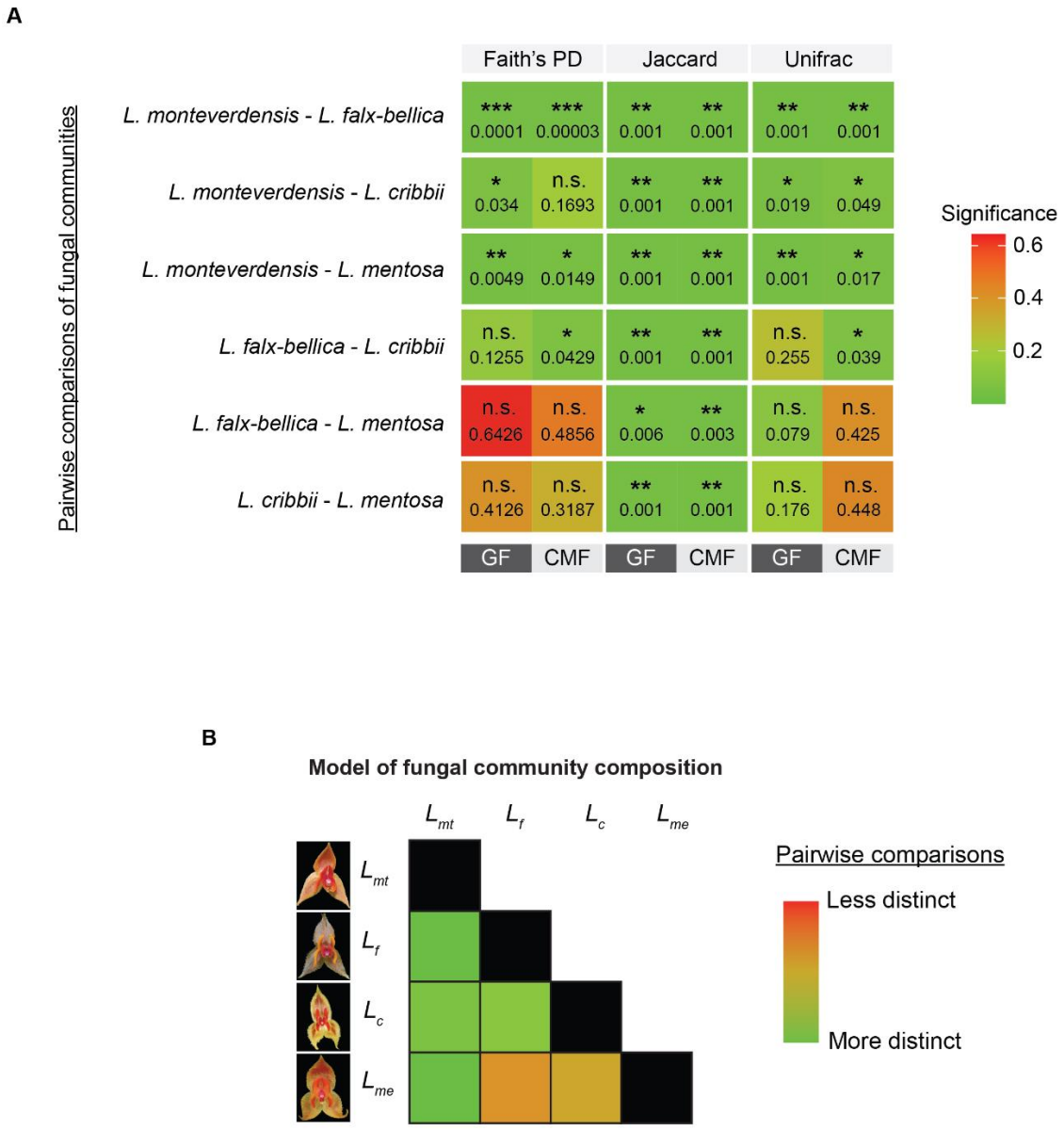


Figure 2.15. Mycorrhizal community diversity patterns associated with four *Lepanthes* sister species. (**A)** Summary of the results for the α- (Faith's PD) and β- (Jaccard, Unweighted UniFrac) diversity analyses. GF = results of analyses performed on general fungal dataset, CMF = results of analyses performed on classified mycorrhizal dataset, Significance = p-value of respective test (Kruskal-Wallis or PERMANOVA). Significance levels: *** ≤ 0.0005, ** ≤ 0.005, * ≤ 0.05, n.s. = nonsignificant. **(B)** Conceptual model of orchid fungal communities in the order of most to least distinct.

2.7 TABLES

Table 2.1. Total number of individuals and populations sampled of four focal orchid species.

Orchid species	No. of samples	No. of populations	No. of sites
L. monteverdensis	102	11	5
L. falx-bellica	183	22	7
L. cribbii	74	10	5
L. mentosa	50	7	2
Total	**409**	**50**	**8**

Table 2.2. Sampling data for four *Lepanthes* species. Site ID = name of sample site, Pop ID = identity of population (i.e. tree) sampled, Tree species = phorophyte species, Lc = *Lepanthes cribbii*, Lf = *Lepanthes falx-bellica*, Lme = *Lepanthes mentosa*, Lmt = *Lepanthes monteverdensis*, N_O = number of orchid individuals sampled. The last row contains column totals.

Site ID	Pop ID	Tree species	*No*			
			Lc	Lf	Lme	Lmt
B	1	*Pouteria exfoliata, cf.* (Sapotaceae)	-	-	-	10
B	2	*Quercus brenesii (= Q. cortesii)* (Fagaceae)	-	10	-	10
C	3	*Miconia oerstediana* (Melastomataceae)	-	5	-	10
D	4	*Eugenia valerii* (Myrtaceae)	-	-	-	11
D	5	*Calyptranthes monteverdensis* (Myrtaceae)	-	7	-	10
H	6	*Calyptranthes monteverdensis* (Myrtaceae)	10	9	-	-
H	7	*Calyptranthes monteverdensis* (Myrtaceae)	9	10	-	-
H	8	*Calyptranthes monteverdensis* (Myrtaceae)	6	10	-	-
H	9	*Rondeletia buddleioides* (Rubiaceae)	10	9	-	9
H	10	*Calyptranthes pittieri, cf.* (Myrtaceae)	5	-	-	-
J	11	*Sapium rigidifolium* (Euphorbiaceae)	-	-	5	-
J	12	*Trichilia havanensis* (Meliaceae)	10	-	6	-
J	35	*Miconia conorufescens* (Melastomataceae)	-	-	10	-
K	13	*Cojoba costaricensis* (Fabaceae)	10	10	-	-
K	14	*Miconia durandii* (Melastomataceae)	-	8	-	-
K	15	*Eugenia austin-smithii* (Myrtaceae)	10	10	-	-
K	16	*Monteverdia recondita* (Celastraceae)	-	7	-	-
K	17	*Guatteria verrucosa* (Annonaceae)	-	8	-	-
K	18	*Sapium rigidifolium* (Euphorbiaceae)	-	7	-	-
K	19	*Sapium rigidifolium* (Euphorbiaceae)	-	9	-	-
N	20	*Guarea kunthiana* (Meliaceae)	-	10	-	-
N	21	*Gonzalagunia rosea* (Rubiaceae)	-	-	8	-
N	22	*Miconia durandii* (Melastomataceae)	-	-	9	-
N	23	*Miconia oerstediana* (Melastomataceae)	-	-	10	-
N	24	*Miconia oerstediana* (Melastomataceae)	-	-	-	10
N	25	*Myrsine coriacea* (Myrsinaceae)	1	5	2	4
N	26	*Pouteria exfoliata* (Sapotaceae)	-	-	-	10
N	27	*Viburnum venustum* (Viburnaceae)	-	-	-	8
N	32	*Salacia petenensis* (Hippocrateaceae)	-	8	-	-
N	33	*Salacia petenensis* (Hippocrateaceae)	-	8	-	-
N	34	*Clusia sp.* (Clusiaceae)	-	-	-	10
U	28	*Pouteria exfoliata* (Sapotaceae)	-	10	-	-
U	29	*Monteverdia recondita* (Celastraceae)	-	10	-	-
U	30	*Guarea rhopalocarpa* (Meliaceae)	3	8	-	-
U	31	*Pleurothyrium palmanum* (Lauraceae)	-	5	-	-
8	**35**	**23**	**74**	**183**	**50**	**102**

Table 2.3. Sequencing data of synthethic non-biological fungal community (SynMock) sample submitted to sequencing with real samples as PCR/sequencing control. ID = recovered community member, N with chimeras = number of sequences recovered prior to chimera removal step, N after chimera removal = number of sequences recovered post chimera removal step as implemented with DADA2, Similarity with original seq = similarity percentage with original published synthetic sequence (Palmer et al. 2017), C = concentration of a mock community member, Length = sequence length

ID	N with chimeras	N after chimera removal	Similarity with original seq (%)	*C* (ng/μL)	Length (bp)
NA1	2	2	-	-	
NA2	3	-	-	-	
NA3	4	4	-	-	
NA4	5	5	-	-	
NA5	10	-	-	-	
NA6	12	-	-	-	
NA7	18	-	-	-	
Mock 11	114	114	100%	20	668
Mock 4	120	120	100%	20	668
Mock 3	154	154	100%	20	668
Mock 1	222	222	100%	20	668
Mock 5	242	242	100%	20	668
Mock 9	270	270	100%	20	598
Mock 7	278	278	100%	20	598
Mock 2	338	338	100%	20	668
Mock 12	370	370	100%	20	668
Mock 10	390	390	100%	20	598
Mock 6	409	409	100%	20	668
Mock 8	505	505	100%	20	598

Table 2.4. Results for Kruskal-Wallis tests applied pairwise to test for difference in fungal community richness as measured by Faith's PD. Orchid species were treated as grouping factor, N = number of samples, and H = Kruskal-Wallis test statistic. Faith's PD has been calculated with the most comprehensive GF dataset.

Orchid sp. 1	Orchid sp. 2	H	p-value
L. monteverdensis (N=92)	*L. falx-bellica* (N=100)	15.450451	0.000085
L. monteverdensis (N=92)	*L. cribbii* (N=48)	4.496050	0.033973
L. monteverdensis (N=92)	*L. mentosa* (N=39)	7.917431	0.004896
L. falx-bellica (N=100)	*L. cribbii* (N=48)	2.346913	0.125531
L. falx-bellica (N=100)	*L. mentosa* (N=39)	0.215409	0.642561
L. cribbii (N=48)	*L. mentosa* (N=39)	0.671335	0.412587

Table 2.5. Results for Kruskal-Wallis tests applied pairwise to test for difference in fungal community richness as measured by Faith's PD. Orchid species were treated as grouping factor, N = number of samples and H = Kruskal-Wallis test statistic. Faith's PD has been calculated with the CMF dataset.

Orchid sp. 1	Orchid sp. 2	H	p-value
L. monteverdensis (N=82)	*L. falx-bellica* (N=83)	17.225904	0.000033
L. monteverdensis (N=82)	*L. cribbii* (N=40)	1.888888	0.169327
L. monteverdensis (N=82)	*L. mentosa* (N=31)	5.932504	0.014864
L. falx-bellica (N=83)	*L. cribbii* (N=40)	4.099110	0.042906
L. falx-bellica (N=83)	*L. mentosa* (N=31)	0.486287	0.485588
L. cribbii (N=40)	*L. mentosa* (N=31)	0.994119	0.318738

Table 2.6. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference in the qualitative composition of orchid fungal communities based on ASV identity. Analysis has been applied to the matrix of Jaccard distances (converted from Similarity Indices S_j) calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic. Jaccard distances have been calculated with the most comprehensive GF dataset.

Orchid sp. 1	Orchid sp. 2	Sample size	Permutations	pseudo-F	p-value
L. monteverdensis (N=92)	*L. falx-bellica* (N=100)	192	999	1.862384	0.001
L. monteverdensis (N=92)	*L. cribbii* (N=48)	140	999	2.360626	0.001
L. monteverdensis (N=92)	*L. mentosa* (N=39)	131	999	2.043075	0.001
L. falx-bellica (N=100)	*L. cribbii* (N=48)	148	999	1.765900	0.001
L. falx-bellica (N=100)	*L. mentosa* (N=39)	139	999	1.470963	0.006
L. cribbii (N=48)	*L. mentosa* (N=39)	87	999	2.106545	0.001

Table 2.7. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference in the qualitative composition of orchid fungal communities based on ASV identity. Analysis has been applied to the matrix of Jaccard distances (converted from Similarity Indices S_j) calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic. Jaccard distances have been calculated with the CMF dataset.

Orchid sp. 1	Orchid sp. 2	Sample size	Permutations	pseudo-F	p-value
L. monteverdensis (N=82)	*L. falx-bellica* (N=83)	165	999	3.043544	0.001
L. monteverdensis (N=82)	*L. cribbii* (N=40)	122	999	5.357175	0.001
L. monteverdensis (N=82)	*L. mentosa* (N=31)	113	999	2.693063	0.001
L. falx-bellica (N=83)	*L. cribbii* (N=40)	123	999	3.279371	0.001
L. falx-bellica (N=83)	*L. mentosa* (N=31)	114	999	1.899696	0.003
L. cribbii (N=40)	*L. mentosa* (N=31)	71	999	3.591191	0.001

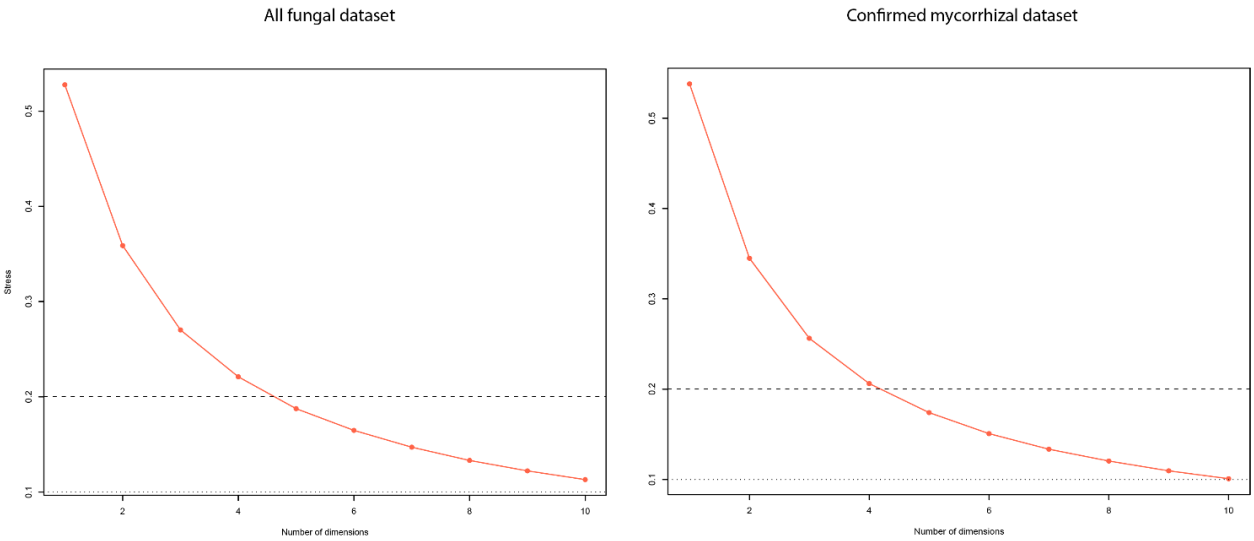
Table 2.8. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference in the qualitative composition of orchid fungal communities using phylogenetic relationships. Analysis has been applied to the matrix of Unweighted UniFrac Distances U_{AB} calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic. Unweighted UniFrac Distances have been calculated with the most comprehensive GF dataset.

Orchid sp. 1	Orchid sp. 2	Sample size	Permutations	pseudo-F	p-value
L. monteverdensis (N=92)	*L. falx-bellica* (N=100)	192	999	3.501245	0.001
L. monteverdensis (N=92)	*L. cribbii* (N=48)	140	999	2.013795	0.019
L. monteverdensis (N=92)	*L. mentosa* (N=39)	131	999	2.621555	0.001
L. falx-bellica (N=100)	*L. cribbii* (N=48)	148	999	1.111374	0.255
L. falx-bellica (N=100)	*L. mentosa* (N=39)	139	999	1.456744	0.079
L. cribbii (N=48)	*L. mentosa* (N=39)	87	999	1.264895	0.176

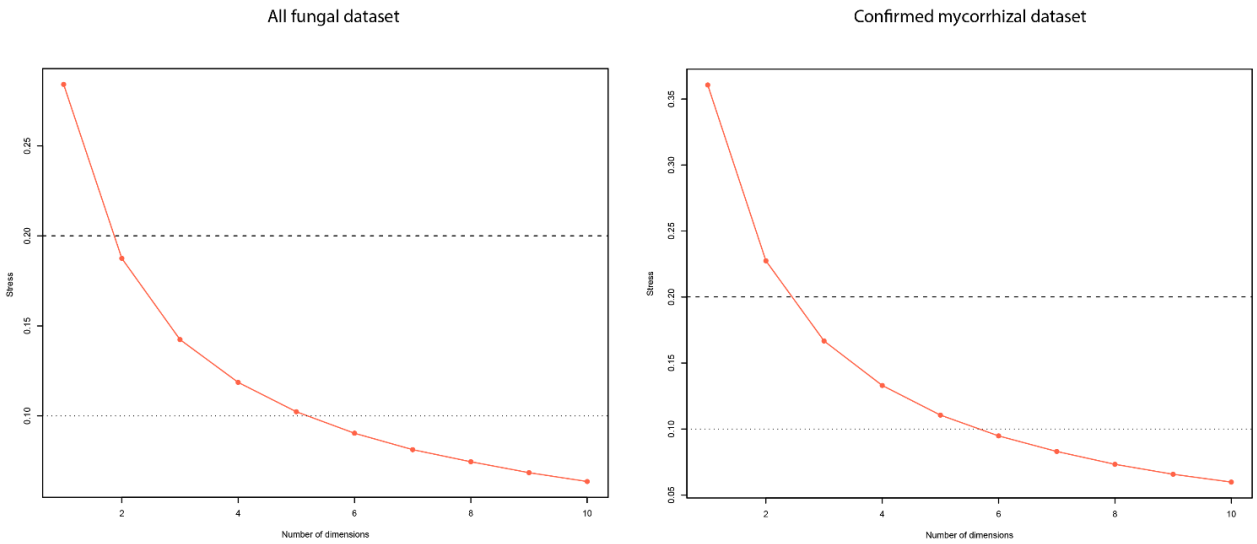
Table 2.9. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference in the qualitative composition of orchid fungal communities using phylogenetic relationships. Analysis has been applied to the matrix of Unweighted UniFrac Distances U_{AB} calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic. Unweighted UniFrac Distances have been calculated with the CMF dataset.

Orchid sp. 1	Orchid sp. 2	Sample size	Permutations	pseudo-F	p-value
L. monteverdensis (N=82)	*L. falx-bellica* (N=83)	165	999	9.533601	0.001
L. monteverdensis (N=82)	*L. cribbii* (N=40)	122	999	2.383633	0.049
L. monteverdensis (N=82)	*L. mentosa* (N=31)	113	999	2.958624	0.017
L. falx-bellica (N=83)	*L. cribbii* (N=40)	123	999	2.758029	0.039
L. falx-bellica (N=83)	*L. mentosa* (N=31)	114	999	0.921529	0.425
L. cribbii (N=40)	*L. mentosa* (N=31)	71	999	0.865445	0.448

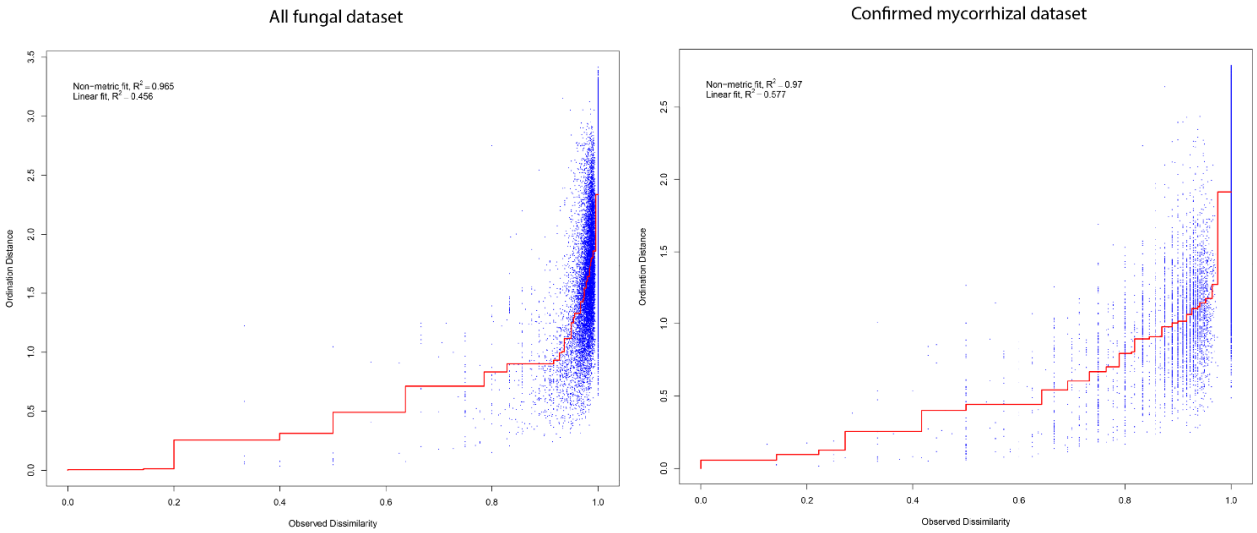
2.8 SUPPLEMENTARY FIGURES



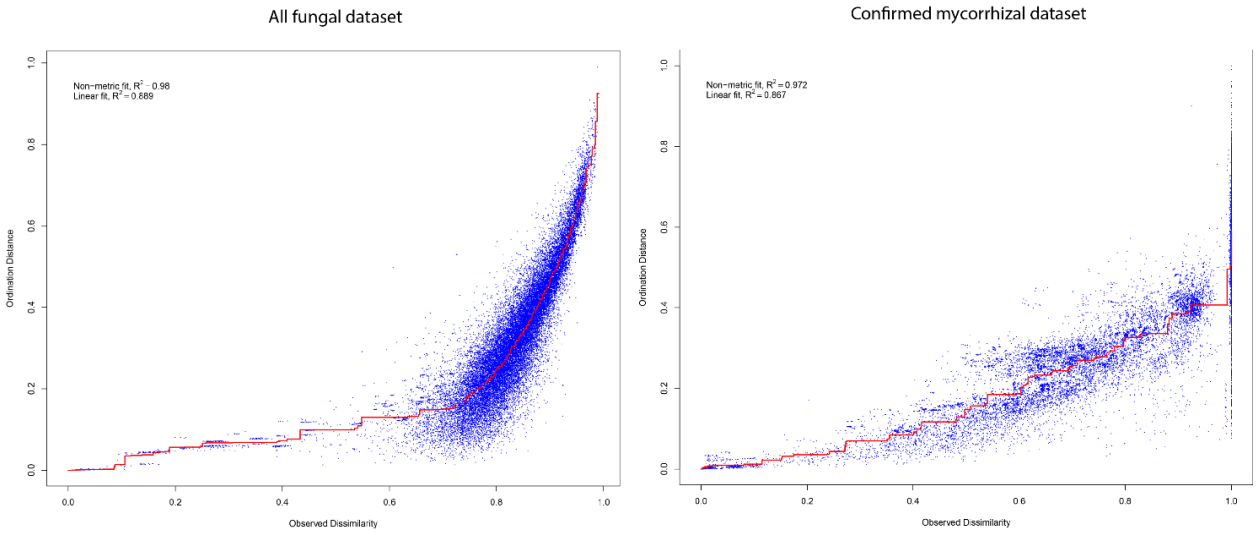
Supplementary Figure 2.1. Plots showing stress as a function of number of selected dimensions k used in a series of NMDS runs. Jaccard S_j score distance matrix calculated on GF dataset (left) and CMF dataset (right) was subject to 10 runs of an NMDS algorithm.



Supplementary Figure 2.2. Plots showing stress as a function of number of selected dimensions k used in a series of NMDS runs. UniFrac U_{AB} score distance matrix calculated on GF dataset (left) and CMF dataset (right) was subject to 10 runs of an NMDS algorithm.



Supplementary Figure 2.3. Shepard diagram of the GF dataset (left, 404 samples) and CMF dataset (right, 382 samples) showing fit among Jaccard distances between pairs of samples (Observed Dissimilarity) and the Euclidean distances between the same pairs in the 3-dimensional ordination space (Ordination Distance). Correlation statistics indicating the fit between ordination distances and observed dissimilarities are displayed.



Supplementary Figure 2.4. Shepard diagram of the GF dataset (left, 404 samples) and CMF dataset (right, 382 samples) showing fit among UniFrac distances between pairs of samples (Observed Dissimilarity) and the Euclidean distances between the same pairs in the 3-dimensional ordination space (Ordination Distance). Correlation statistics indicating the fit between ordination distances and observed dissimilarities are displayed.

2.9 SUPPLEMENTARY TABLES

Supplementary Table 2.1. Sampling data for bark samples. Site ID = name of sample site, Pop ID = identity of population (i.e. tree) sampled, Tree species = phorophyte species, Lc = *Lepanthes cribbii*, Lf = *Lepanthes falx-bellica*, Lme = *Lepanthes mentosa*, Lmt = *Lepanthes monteverdensis*. N_B = number of phorophyte bark samples collected in proximity of the orchid sampled. The last row contains column totals. Tree bark samples were collected from the immediate vicinity of 58 individuals of *L. monteverdensis* from 6 populations (mean 9.7 bark samples/population), 95 individuals of *L. falx-bellica* from 11 populations (mean 8.6 bark samples/population), 70 individuals of *L. cribbii* from 8 populations (8.8 bark samples/population), and 11 individuals of *L. mentosa* from 2 populations (5.5 bark samples/population). Bark samples were processed for sequencing as follows. One-tenth of a gram of each bark sample was mixed with 0.8 mL of DNA extraction buffer (0.1 M Na_2HPO_4 (pH 5.6)) for 30s at 30 Hz. Supernatant was discarded after centrifugation (1 min, 18,000 rpm), and samples were subjected to bead milling (45s, 30 Hz) with 0.5 mL of phosphate buffer (0.1 M Na_2HPO_4 (pH 5.6) and 0.1 mL of 10% SDS (w/v). The supernatant was collected after centrifugation at 18,000 rpm for 3 min and mixed with 0.25 mL KCl (2.5 M) and incubated at 4°C for 15 min. The supernatant was collected after centrifugation at 18,000 rpm for 5 min, and 0.6 mL of chloroform : isoamyl alcohol (24 : 1) was added, mixed, and centrifuged at 18,000 rpm for 5 min twice. The supernatant obtained was precipitated by addition of 0.8 volume of 100% isopropanol. Finally, the pellet was recovered by centrifugation at 18,000 rpm for 30 min, washed in cold 70% ethanol and dissolved in 30 µL TLE buffer (Tris-HCl 10 mM; EDTA 0.1 mM).

Site ID	Pop ID	Tree species	N_B			
			Lc	Lf	Lme	Lmt
B	1	*Pouteria exfoliata, cf.* (Sapotaceae)	-	-	-	10
B	2	*Quercus brenesii (= Q. cortesii)* (Fagaceae)	-	10	-	10
C	3	*Miconia oerstediana* (Melastomataceae)	-	5	-	10
D	4	*Eugenia valerii* (Myrtaceae)	-	-	-	9
D	5	*Calyptranthes monteverdensis* (Myrtaceae)	-	4	-	10
H	6	*Calyptranthes monteverdensis* (Myrtaceae)	10	9	-	-
H	7	*Calyptranthes monteverdensis* (Myrtaceae)	9	10	-	-
H	8	*Calyptranthes monteverdensis* (Myrtaceae)	6	10	-	-
H	9	*Rondeletia buddleioides* (Rubiaceae)	10	9	-	9
H	10	*Calyptranthes pittieri, cf.* (Myrtaceae)	5	-	-	-
J	11	*Sapium rigidifolium* (Euphorbiaceae)	-	-	5	-
J	12	*Trichilia havanensis* (Meliaceae)	10	-	6	-
J	35	*Miconia conorufescens* (Melastomataceae)	-	-	-	-
K	13	*Cojoba costaricensis* (Fabaceae)	10	10	-	-
K	14	*Miconia durandii* (Melastomataceae)	-	8	-	-
K	15	*Eugenia austin-smithii* (Myrtaceae)	10	10	-	-
U	29	*Monteverdia recondita* (Celastraceae)	-	10	-	-
U	30	*Guarea rhopalocarpa* (Meliaceae)	-	-	-	-
U	31	*Pleurothyrium palmanum* (Lauraceae)	-	-	-	-
7	**19**	**16**	**70**	**95**	**11**	**58**

Supplementary Table 2.2. Tukey test results conducted after a test for multivariate homogeneity of group dispersions (p-value: 2.58e-07) implemented by *betadisper* function in *vegan* package in R. Orchid species were treated as groups and Jaccard distances were calculated between pairs of samples (individual orchids) of the GF dataset. Diff = difference among observed mean distance-to-centroid of the levels of the grouping factor, lwr = lower bound of the 95% confidence interval, upr = upper bound of the 95% confidence interval, p adj = p-value after adjustment for the multiple comparisons.

Groups compared	diff	lwr	upr	p adj
Lepanthes falx-bellica-Lepanthes cribbii	0.013739	0.006343	0.021135	1.55e-05*
Lepanthes mentosa-Lepanthes cribbii	-0.00032	-0.00933	0.008688	9.997e-01
Lepanthes monteverdensis-Lepanthes cribbii	0.009907	0.002457	0.017356	3.776e-03*
Lepanthes mentosa-Lepanthes falx-bellica	-0.01406	-0.02196	-0.00616	3.855e-05*
Lepanthes monteverdensis-Lepanthes falx-bellica	-0.00383	-0.00989	0.002229	3.61e-01
Lepanthes monteverdensis-Lepanthes mentosa	0.010229	0.002277	0.01818	5.527e-03*

Supplementary Table 2.3. Tukey test results conducted after a test for multivariate homogeneity of group dispersions (p-value: 1.823e-3) implemented by *betadisper* function in *vegan* package in R. Orchid species were treated as groups and UniFrac distances were calculated between pairs of samples (individual orchids) of the GF dataset. Diff = difference among observed mean distance-to-centroid of the levels of the grouping factor, lwr = lower bound of the 95% confidence interval, upr = upper bound of the 95% confidence interval, p adj = p-value after adjustment for the multiple comparisons.

Groups compared	diff	lwr	upr	p adj
Lepanthes falx-bellica-Lepanthes cribbii	0.007771	-0.01352	0.029061	0.781252
Lepanthes mentosa-Lepanthes cribbii	-0.01435	-0.04028	0.01159	0.481644
Lepanthes monteverdensis-Lepanthes cribbii	0.018841	-0.0026	0.040285	0.107378
Lepanthes mentosa-Lepanthes falx-bellica	-0.02212	-0.04486	0.000626	0.06001
Lepanthes monteverdensis-Lepanthes falx-bellica	0.011069	-0.00638	0.028518	0.357935
Lepanthes monteverdensis-Lepanthes mentosa	0.033188	0.010298	0.056078	0.001244*

Supplementary Table 2.4. Tukey test results conducted after a test for multivariate homogeneity of group dispersions (p-value: 5.959e-05) implemented by *betadisper* function in *vegan* package in R. Orchid species were treated as groups and Jaccard distances were calculated between pairs of samples (individual orchids) of the CMF dataset. Diff = difference among observed mean distance-to-centroid of the levels of the grouping factor, lwr = lower bound of the 95% confidence interval, upr = upper bound of the 95% confidence interval, p adj = p-value after adjustment for the multiple comparisons.

Groups compared	diff	lwr	upr	p adj
Lepanthes falx-bellica-Lepanthes cribbii	0.043293	0.019197	0.067389	3.399e-05*
Lepanthes mentosa-Lepanthes cribbii	0.016872	-0.01356	0.047306	0.478603
Lepanthes monteverdensis-Lepanthes cribbii	0.029903	0.005475	0.054331	0.009412*
Lepanthes mentosa-Lepanthes falx-bellica	-0.02642	-0.05349	0.000647	0.058576
Lepanthes monteverdensis-Lepanthes falx-bellica	-0.01339	-0.03347	0.006689	0.312355
Lepanthes monteverdensis-Lepanthes mentosa	0.01303	-0.01433	0.040395	0.606556

Supplementary Table 2.5. Tukey test results conducted after a test for multivariate homogeneity of group dispersions (p-value: 4.043e-4) implemented by *betadisper* function in *vegan* package in R. Orchid species were treated as groups and UniFrac distances were calculated between pairs of samples (individual orchids) of the CMF dataset. Diff = difference among observed mean distance-to-centroid of the levels of the grouping factor, lwr = lower bound of the 95% confidence interval, upr = upper bound of the 95% confidence interval, p adj = p-value after adjustment for the multiple comparisons.

Groups compared	diff	lwr	upr	p adj
Lepanthes falx-bellica-Lepanthes cribbii	-0.02144	-0.11072	0.067845	0.925019
Lepanthes mentosa-Lepanthes cribbii	-0.1509	-0.26366	-0.03813	0.003562*
Lepanthes monteverdensis-Lepanthes cribbii	-0.09681	-0.18732	-0.00629	0.030867*
Lepanthes mentosa-Lepanthes falx-bellica	-0.12946	-0.22975	-0.02916	0.005386*
Lepanthes monteverdensis-Lepanthes falx-bellica	-0.07537	-0.14977	-0.00096	0.045818*
Lepanthes monteverdensis-Lepanthes mentosa	0.05409	-0.04731	0.155485	0.512424

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CHAPTER 3

HIGH SPECIFICITY CHARACTERIZES MYCOBIONT INTERACTIONS WITH RARE ENDEMIC EPIPHYTIC ORCHID SPECIES[2]

[2] Tuczapski, P. T. and Trapnell, D. To be submitted to *New Phytologist*.

3.1 ABSTRACT

Given anthropogenic pressure imposed on tropical ecosystems, it is of high interest to understand mechanisms contributing to the exceptionally high diversity in tropical hotspots. Likewise, researchers have been investigating the causes of the rapid diversification of orchids, the second-largest family of flowering plants, predominantly found in tropical regions. Since many tropical orchids are narrow-ranged endemics that constitute a large fraction of local flora, it is of interest to understand factors affecting their life history and distribution, with implications for restoration and conservation efforts. Symbiosis of orchids with mycorrhizal partners is particularly interesting since plant life cycle depends on forming association with mycobionts. This study examined specialization of those interactions in 50 populations of four co-occurring, endemic orchid species of a hyper-diverse, evolutionarily young genus with a rapid speciation rate. Unlike some previous evidence of generalism in orchids and tropics, we identified significant specialization signal in the network including 6543 fungal taxonomic units. This signal increased with only keystone fungi investigated. Moreover, we found opportunistic interactions affecting phylogenetic signal in fungal communities, with higher signal in keystone taxa. Examined orchids ranged from specialist, through moderate specialists (apparent generalists), to generalist. It has been previously hypothesized that closely related orchids might share interaction traits determining them to associate with certain mycobionts (phylogenetic constraint). We suggest that a host-jumping mechanism, potentially accelerated by niche partitioning, is responsible for an escape from phylogenetic constraint. Epiphytism may be an important contributor to tight specialization, mycobiont ecological assemblages, and ultimately distribution of tropical orchids.

3.2 INTRODUCTION

It is estimated that most terrestrial plants associate with mycorrhizal fungi. For instance, 80-90% of angiosperms and gymnosperms, most tree species, most herbaceous species, as well as fern sporophytes and lycopods form arbuscular mycorrhizal associations, while only about 3% of widespread seed plants form ectomycorrhiza (Smith and Read, 2008). As a rule, fungal species are able to colonize roots of multiple plant species and plant species associate with a large number of distantly related fungi. Thus, these interactions are generally believed to be of low specificity. In contrast, orchids form an association with orchid mycorrhizal fungi (OMF) that is believed to be more specific due to dependence of orchid life cycle on OMF. Orchid dust seeds contain minute, undifferentiated embryos. Because of the lack of endosperm to support seedling development, orchids must associate with mycorrhizal fungi to access nutrients required for germination. Upon fungal colonization of the orchid, the OMF transfers carbon to the plant until the orchid matures, at which stage there is more reciprocal nutrient exchange where plant supplies carbon to fungus in exchange for nitrogen and phosphorus (Cameron et al., 2007; Liebel et al., 2015; Schiebold et al., 2018, Read et al. 2024). Germination trials show that germination is most effective when seeds are inoculated with fungal strains similar to, or the same as, isolates found in mature plants (McCormick et al. 2004, Otero et al. 2011). Furthermore, seed germination and seedling establishment are major determinants of the spatial distribution of adult plants, as seed dispersal can result in germination only if appropriate OMF are present (Jacquemyn et al. 2012a, Jacquemyn et al. 2012b).

OMF are mostly free-living saprophytes that are not dependent on their orchid partners (Rasmussen, 1995; Roberts 1999). Orchid mycobionts belong mainly to three groups: Ceratobasidiaceae, Tulasnellaceae and Sebacinales. These have historically been assigned to the

anamorphic genus Rhizoctonia, however other groups have been reported since, such as Thelephoraceae, Ascomycetes and Atractiellomycetes (McCormick and Jacquemyn 2014, Suárez and Kottke 2016), various root endophytes and pathogens of other plant families (Martos et al. 2012, Waud et al. 2016, Shefferson et al. 2019, Li et al. 2021). *Tulasnella* fungi are reportedly the most common and widespread associates of green orchids (Suárez and Kottke 2016). Phylogenetic analysis has shown that OMF from the Tulasnellaceae form narrow clades, are taxonomically complex, and are characterized by high genetic diversity (Shefferson et al. 2005, Cruz et al. 2016).

The specificity of orchid mycorrhizal interactions results from three adaptive phenomena: it improves germination rates, may promote efficient nutrient exchange among orchids and OMF, and affects orchid distribution patterns and by extension gene flow (Tremblay et al. 2015, Li et al. 2021). However, OMF research has mostly focused on terrestrial temperate orchids, thus little is known about the OMF in tropical epiphytes, even though over 80% of tropical orchids are epiphytes (Givnish et al. 2015, Li et al. 2021). Some have proposed that since the number of fungal species decreases and their range sizes increase with increasing latitude (Tedersoo et al., 2014), specialization should be more common with increasing latitude (Shefferson et al. 2019). Moreover, it has been argued that survival in nutrient-poor niches with little water and high irradiation might require interaction with multiple, narrowly distributed OMF (Jacquemyn et al. 2010). It follows that epiphytic orchids should be under selective pressures favoring generalized interactions under resource-deprived conditions.

Knowledge of the degree of mycorrhizal specificity is of great interest to researchers due to its importance for conservation and reintroduction efforts (Martos et al. 2012, Pandey et al. 2013, Suárez and Kottke 2016). Additionally, it may enhance understanding of evolutionary

dynamics between interacting partners and the stability of ecological networks (Blüthgen et al. 2008). To date, studies of orchid mycorrhizal specialization have revealed complex patterns that range from low to high specificity. The emerging picture might be obscured by differences in sampling scheme, sampling effort, fungal identification (i.e. molecular vs. morphological identification of cultured isolates), molecular markers used, and sequencing depth. Variable resolution of fungal phylogeny might also affect specificity inference. For instance, Blüthgen et al. (2006) pointed out that a plant species pollinated by multiple moth species may be inappropriately described as a generalist, while a plant interacting with few insect species from different orders may be labelled a specialist. Similarly, Herrera et al. (2017) focused on orchid-Tulasnellaceae interactions among community of terrestrial and epiphytic orchid species and concluded that they had a generalist partnership, due to a nested network structure. On the other hand, Pandey et al. (2013) concluded generalism in a rare terrestrial orchid, since its 26 fungal OTUs belonged to the three major OMF groups (Ceratobasidiaceae, Sebacinaceae and Tulasnellaceae). Waterman et al. (2011) found a strong preference for different clades of fungi by six Australian orchid lineages. Since sister orchid species were associated mostly with the same clade of fungi, they inferred that OMF were unlikely to drive speciation of these orchids, although the phylogenetic signal at OTU-level resolution within those clades was not estimated. In contrast, Waud et al. (2016) compared mycorrhizal communities at an OTU-level in three co-occurring Belgian orchid species and found few shared OTUs, concluding high specificity. In some cases, a high number of detected OTUs have been interpreted as a sign of generalism and a low number as specificity. For instance, Jacquemyn et al. (2014) found that nine coexisting terrestrial orchid species each associated with several to > 15 OTUs, inferring that multiple associations and low specificity are common.

Vogt-Schilb et al. (2020) analyzed the role of mycorrhizal specificity in seven orchid species in restored and semi-natural grasslands using the Shannon-Wiener diversity index and the Bray-Curtis dissimilarity index. They interpreted high index values as an indication of generalism, and low index values, together with low number of associated OTUs, as specificity; however, these metrics do not account for phylogenetic relatedness of OMF or orchids.

More recently, Shefferson et al. (2019) offered a novel conceptual framework for estimating specificity in ecological interactions using orchid-mycorrhizal interactions as an example. The authors pointed out that most species interact with multiple partners and even the most specialized species have broad interactions, which give the impression of lack of specialization. Furthermore, the degree of specialization of a focal species likely depends on whether an interacting partner has the ability to fulfil a niche requirement, the number of potential partners with that ability, and the range of partners. Thus, in the case of orchid-OMF interactions, fungal host switches, if present, should involve new mycobionts that are closely related to the original host because they are likely to provide similar services (Tedersoo et al. 2013). Consequently, such interactions will be under selective pressure and should be detectable by testing the evolutionary history of the mycobionts. Thus, specificity should be testable by analyzing the phylogenetic breadth of partners with which a focal species interacts. In assemblage specialization, it should be expected that orchids interact with a community of closely related mycobionts that cluster phylogenetically. In the case of true generalism, the OMF of an orchid species should lack a phylogenetic signal. Finally, in apparent generalism, stabilizing selection should result in one or a few keystone OMF species that provide unique resources, and other

functionally redundant symbionts. This would yield phylogenetic signal only for keystone symbionts, but no signal for the full assemblage.

This study aimed to investigate the degree of specialization, as well as the presence and impact of keystone OMF on specificity in four congeneric epiphytic orchid species in the biodiversity hotspot of Monteverde, Costa Rica. We constructed a large binary matrix of orchid-symbiont interactions between 6543 fungal taxonomic units and 404 orchid individuals from four narrowly endemic, co-occurring miniature orchids from evolutionarily young and extremely speciose genus *Lepanthes* (Karremans 2016, Pérez-Escobar et al. 2017). We also examined network-wide specialization at the resolution of orchid-keystone root-associated fungi. Furthermore, we tested whether these orchids tended to associate with closely related partners by investigating the degree of phylogenetic signal at different resolutions: (i) all fungal associates; (ii) keystone fungi as determined by frequency of interactions at different constraint levels. Finally, we tested the degree of exclusiveness at two levels of partners: (i) fungi interacting with orchids, and (ii) orchids interacting with fungi.

3.3 METHODS

Our dataset is comprised of root-derived fungi (mycorrhizal and other endophytes) isolated from 404 individuals (mean = 8 individuals/population) from 50 populations of *Lepanthes cribbii*. *Lepanthes falx-bellica*, *Lepanthes mentosa*, and *Lepanthes monteverdensis*. Amplicon Sequence Variants (ASVs) were assigned taxonomic identities where possible. We have tracked which fungi have been found in which plant individuals. See Chapter 1 for further details on the Study Species, Sampling, as well as laboratory and analytical methods pertaining sequence processing (Library Preparation, Quality Control, Amplicon Processing, PCR Bias Control). ASVs have been derived

as a result of processing sequence data in *DADA2* (Callahan et al. 2016) package in R version 4.3.2 (R Development Core Team 2023).

Mean Pairwise Distance Analysis

The degree of specialization in orchid – fungal interactions was estimated by calculating mean pairwise distances (MPDs) in fungal communities, and more precisely, standardized effect size of mean phylogenetic pairwise distance:

$$SES.MPD = (MPDobs - \text{mean } MPDnull)/sdMPDnull ,$$

where: SES.MPD = Standardized Effect Size of MPD, MPDobs = observed value of MPD in the empirical data, MPDnull = MPD values computed from a null model, and sdMPDnull = standard deviation of MPDs obtained from a null model. SES.MPD < 0 and a low quantiles (p-value < 0.05) indicate phylogenetic clustering, i.e. taxa in the community are more related than expected by chance; SES.MPD > 0 and high quantiles (p-value > 0.95) indicate phylogenetic evenness, i.e. taxa in the community are less related than expected by chance; whereas SES.MPD = $(-\infty, \infty)$ and a p-value = (0.05, 0.95) indicate that the pattern of phylogenetic relatedness of community members is indistinguishable from that generated by chance alone (see Kembel 2010). The MPD-related calculations were performed in *picante* package (Kembel et al. 2010) in R version 4.3.2 (R Development Core Team 2023). To generate the null phylogenetic distance distribution (MPDnull), we randomly shuffled the tip labels of a phylogenetic tree of fungi associated with all 404 plants (*L. cribbii* = 73 plants, *L. falx-bellica* = 180 plants, *L. mentosa* = 49 plants, *L. monteverdensis* = 102 plants) 100 times, and recalculated phylogenetic distances after every reiteration. If orchid species are specialized in their mycorrhizal associations, they should show preference for a narrow taxonomic group of fungi (which may, but not necessarily, possess

ecological traits well suited to fulfilling the orchid's metabolic requirements). On the other hand, mycobionts of orchids that are generalists will span a wider taxonomic range and yield a pattern of phylogenetic evenness, i.e. overdispersion, which may indicate ecological dissimilarity of partnering fungi. Fungal phylogenetic trees were constructed from ASV sequences in QIIME2 (Boylen et al. 2019) using *phylogeny* plugin's *align-to-tree-mafft-fasttree* pipeline which creates a masked multiple sequence alignment with MAFFT (Katoh & Standley 2013), prior to tree reconstruction. We did not focus on any specific group of fungi and have included all root-associated fungal ASVs. We calculated a phylogenetic distance matrix using the "cophenetic" function and the phylogenetic tree of fungi imported from QIIME2. We summed up all ASV occurrences (as opposed to read number) per orchid species and used this fungal matrix together with phylogenetic distances to calculate standardized effect size of MPD using "ses.mpd" function.

Some mycobionts in interaction networks may be more essential due to the ecological service they provide the orchid, while other mycobionts may fill a more supplemental role (Shefferson et al. 2019). We would expect to find the more obligatory associates in the orchid species of interest more often. In the context of orchid mycorrhizae, these would be keystone fungi. We evaluated the phylogenetic relatedness of mycobionts and the presence of keystone fungi using a multi-staged approach.

For each orchid species we retained only ASVs found ≥ 2 individuals (following methodology of Shefferson et al. 2019) using *filter-feature* pipeline from *feature-table* plugin in QIIME2 (Boylen et al. 2019). A single feature table of all ASV occurrences per orchid species was generated to estimate relatedness of each orchid mycobiont in the context of mycobionts retrieved

from all four orchid species. Finally, we computed SES.MPD only for these pre-defined keystone fungi using phylogenetic distances calculated from a phylogenetic tree of fungi found associated with all 404 orchid individuals. We repeated these steps, selecting for keystone fungi associating with at least 3 and 4 plant individuals per orchid species.

Network Analysis of Specialization

To measure specialization in an entire plant-fungal interaction network we calculated a standardized interaction diversity metric (H_2') (Dormann et al. 2009), which is derived from Shannon diversity of links (i.e. interactions) in the network (H_2). We chose this metric since, unlike other weighted and unweighted metrics (e.g. connectance, nestedness, degree distribution, interaction strength), it has been reported (Blüthgen et al. 2008) to be robust against the impact of sampling intensity and the underlying species abundance distribution, as well as network size and shape, on strength of the specialization signal. The analysis is standardized against a null model, using studied network as a reference. Specialization is quantified by estimating how much realized interactions deviate from the expectation. To achieve this, the total number of observed interactions for each partner, as well as a grand total of observed interactions, are fixed. The frequency distribution is then compared between null model and the empirical network. We used Patefield's (1981) null model algorithm where interaction marginal totals (per species of each interacting partner in the contingency table) are fixed and interactions are randomly distributed among available partners. This strategy allows for heterogenous distribution of the number of links where species observation records define encounter probability. Therefore, specialization is decoupled from an underlying asymmetry in abundance of interacting partners and rare species do not inflate the specialization metric. We generated 1000 permutations for each analyzed network

using function "r2dtable" in *bipartite* package (Dormann et al. 2008) in R version 4.3.2 (R Development Core Team 2023) and calculated the H_2' index for each of the randomized networks using "H2fun" function. The degree of specialization present network-wide ranges from $H_2' = 0$ for extreme generalization and $H_2' = 1.0$ for maximum specialization (i.e., exclusiveness of interacting partners).

To evaluate the role of keystone fungi on the degree of specialization, we measured mutual specialization among orchids and mycorrhizal fungi in four networks: a network with all ASVs detected, with ASVs found in at least two plants per orchid species, three plants per orchid species, and four plants per orchid species. Additionally, we analyzed specialization in a network containing only fungi belonging to the Tulasnellaceae. *Tulasnella* species are believed to be a major clade of orchid-associated fungi (Taylor et al. 2002, McCormick and Jacquemyn 2014), as well as the most common and widespread mycobionts of green (autotrophic) orchids (Kottke et al. 2016, Herrera et al. 2017). Furthermore, a number of *Tulasnella* species promote orchid growth (McCormick et al. 2004) and some species may have a role in determining orchid specificity (Smith and Read 2008). Taxonomic identification was performed by blasting across the UNITE 9.0 ITS reference dataset (Abarenkov et al. 2022) in *DADA2* (Callahan et al. 2016) package in R version 4.3.2 (R Development Core Team 2023). In each network, an observation of a given ASV in an individual plant was counted as one interaction record. To calculate the statistical significance of observed network structure we calculated the z-score as follows:

$$\text{z-score} = (H_2\text{'obs} - \text{mean } H_2\text{'null})/\text{sd}H_2\text{'null},$$

where: H_2'obs = observed value of H_2' in the empirical network, H_2'null = H_2' values (n = 1000) computed from a null model, sdH_2'null = standard deviation of H_2' values obtained from a null

model (see Dormann 2020). Z-score expresses the difference between observed index and the mean obtained from null model in terms of standard deviations. Finally, we calculated two-sided p-value for significance of the H_2' specialization index by calculating its percentile in the null distribution with *2*pnorm(-abs(z-score)* function in R version 4.3.2 (R Development Core Team 2023).

To measure specialization of the elements of the network, we calculated standardized Kullback-Leibler distance d_i' for interacting partners at each level (orchids and fungi) (Dormann 2011). This species-level specialization index compares the distribution of the interactions of each partner at the respective level (i.e. proportion of its pairwise interactions) to the partner availability (measured as proportion of all its interactions to all interactions present in the network). Next, the index is normalized by accounting for a theoretical minimum value. Thus, d_i' measures how strongly observed interaction records deviate from a scenario where all partners are used, proportional to their availability (see Blüthgen et al. 2006). A fungus associated only with one abundant orchid with many other partners would receive low index value, whereas a fungus associated with rarer orchid would receive higher value. Similarly, an orchid partnering with a small number of abundant fungi that also associate with other orchids, would receive a low index value, but a high value when partnering with rare fungi. Standardized specialization index d_i' ranges from 0 (most generalized) to 1.0 (most specialized).

We measured species-level specialization for both fungi and orchids for three networks: a network with all fungal ASVs, a network with ASVs present in ≥ 4 orchid individuals, and a network with

Tulasnella species only. All analyses were performed using "dfun" function in *bipartite* package (Dormann et al. 2008) in R version 4.3.2 (R Development Core Team 2023).

Indicator Species Analysis

We performed Indicator Species Analysis (ISA) (Dufrêne & Legendre 1997) to cross-validate the idea that keystone fungi, defined as fungi frequently associated with orchid species, are likely ecologically important from the orchid perspective and thus potentially contribute to orchid specificity. Thus, instead of applying a fixed pre-defined cutoff to the ASV feature table, defined as a minimum number of plants per orchid species in which ASVs are required to be found, we searched for keystone fungi by identifying species (i.e. ASVs) that are strongly associated with a particular orchid species and then used those fungi for Network Analysis of Specialization. We employed simplified ISA for this search, where we first transformed the ASV community data matrix to binary, presence/absence data, and then performed the analysis with the "multipatt" function in *indicspecies* package in R version 4.3.2 (R Development Core Team 2023). For this analysis, individual orchid plants were treated as sites, and orchid species as site groups. To identify indicator species, we used Pearson's phi coefficient of association (Chytrý et al. 2002), corrected for unequal number of plants between *Lepanthes* species by employing the "r.g" function. The statistical significance of the list of indicator species found for each orchid species was tested using 9999 random permutations.

Finally, we used a list of fungal ASVs detected by ISA to create a network consisting only of those indicator ASVs and used it to calculate network-wide reciprocal specialization (H_2'), following the same procedure as described above for the Network Analysis of Specialization.

3.4 RESULTS

Mean Pairwise Distance Analysis

The strength of the phylogenetic signals in the mycobiont communities of the four *Lepanthes* species was variable. The strongest phylogenetic signal was found in *L. monteverdensis* mycobionts, as indicated by the significantly negative MPD value which reflects a shared evolutionary history (**Table 3.1**). This signal remained consistent as more stringent thresholds for the keystone fungi were applied (**Table 3.2-3.4**). These results suggest strong specificity of mycobiont-orchid interactions in *L. monteverdensis*. Conversely, the phylogenetic signal of *L. cribbii* mycobionts was significantly positive when all ASVs were considered, suggesting generalized interactions (**Table 3.1**). However, when singleton mycobionts were omitted, the MPD value was significantly negative (**Table 3.2**), and remained negative with the application of more stringent thresholds (**Table 3.3-3.4**). This suggests that *L. cribbii* may be an apparent generalist, with some keystone, as well as an array of exchangeable fungi. A similar trend was found for *L. falx-bellica*. When all ASVs were considered, there was no phylogenetic signal, suggesting generalized mycobiont interactions (**Table 3.1**). Although not significant, the signal trended negative at the two last most stringent thresholds (**Table 3.3-3.4**). Most notably the phylogenetic signal almost reached significant level at the third threshold (**Table 3.3**, p-value = 0.07). Thus, *L. falx-bellica* revealed more generalized interactions, as well as associations with a few keystone mycobionts. In *L. mentosa* there was a complete absence of a phylogenetic signal at any level, indicating true generalism (**Table 3.1-3.4**).

Network Analysis of Specialization

Network analysis of specialization showed different levels of reciprocal specialization, as well as species-level specialization. Each orchid-fungal interaction network analysis indicated a significant degree of network-wide specialization. All-fungal network differed significantly from the null model of random interactions (average $H_2'_{ran}$ = 0.26 ± 0.006 (mean ± SD of 1000 simulations, p-value <0.001). Thus, the associations within empirical network were significantly more specialized than if the interactions were opportunistic. When we evaluated the effect of keystone fungi on the specialization signal, the network-level specialization index value shifted most dramatically (from H_2' = 0.41 to 0.52) when the singleton mycobionts were omitted (**Fig. 3.1A** and **B**) and remained almost unchanged (H_2' = 0.51) despite applying more conservative thresholds to keystone fungi (**Fig. 3.1C** and **D**). Interestingly, average specialization in null models (completely opportunistic interactions) decreased with each consecutive threshold (from $H_2'_{ran}$ = 0.26 to $H_2'_{ran}$ = 0.1 on average). All realized associations in keystone fungal networks differed significantly from the null expectation (average $H_2'_{ran}$ = 0.2 ± 0.006 [mean ± SD of 1000 simulations], 0.14 ± 0.006, and 0.1 ± 0.007, respectively), again suggesting specialized interactions. Thus, focal orchid species are likely to engage in many opportunistic associations, while maintaining partnerships with some keystone fungal taxa.

Examination of the species-level specialization index corroborated these results and revealed structural properties of the networks. Standardized Kullback-Leibler distance d_i' yields 0 for most generalized cases and approaches 1.0 for species that interact exclusively with their partners, thus do not associate with their competitors (reciprocal specialization). When all fungi were subject to evaluation, orchid-mycorrhizal network was dominated by moderately specialized fungi ($<d'_{fungi}>$ = 0.45), which constituted 36.5% (2365 of 6483 ASVs) (**Fig 3.2A**). This category

was followed by many opportunistic singleton mycobionts. These highly generalized mycobionts made up 25.7% of all ASVs (<d'$_{fungi}$> = 0.0, 1669 ASVs). The third and fourth largest groups of partners were specialists (<d'$_{fungi}$> = 0.7 & 1.0; 638 & 523 ASVs, respectively). In contrast, when only keystone fungi were considered (i.e. fungi found in ≥ 4 plants per orchid species), the interactions were mostly with specialists (<d'$_{fungi}$> = 0.65, 151 of 260 ASVs, 58%) (**Fig. 3.3B**). The weighted mean degree of specialization was higher in the network with keystone fungi (<d'$_{fungi}$> = 0.52) than in all-fungal web (<d'$_{fungi}$> = 0.38).

From the plant perspective, the degree of specialization of orchid species was also higher in the network with keystone fungi (**Fig. 3.2C** and **D**). The respective weighted means were <d'$_{orchids}$> = 0.48 and <d'$_{orchids}$> = 0.53. Thus, plants on average appeared more specialized than fungi when all ASVs were considered, but when only keystone fungi were accounted for, the specialization of plants and fungi was similar. Notably, *L. monteverdensis* was the most specialized of the four orchids.

The Tulasnellaceae-only interaction network revealed significant specialization (H'_2 = 0.34 (**Supp. Fig. 3.2**), with average $H_2'_{ran}$ = 0.13 ± 0.009 (mean ± SD of 1000 simulations, p-value<0.001). The weighted mean degree of specialization of the lower level of partners (*Tulasnella* species) was <d'$_{Tul}$> = 0.4, with many *Tulasnella* ASVs assigned the lowest specialization value, indicating generalized interactions (<d'$_{Tul}$> = 0.0, 133 out of 484 ASVs constituting 27.5% of all ASVs). However, the next two biggest categories of *Tulasnella* were moderate and high specialists (<d'$_{Tul}$> = 0.45 and <d'$_{Tul}$> = 1.0, 87 and 84 respectively out of 484 ASVs constituting together 35% of all ASVs; **Supp. Fig. 3.1A**). Thus, although many interactions in the Tullasnellaceae network were opportunistic, the next two species-rich groups consisted of

moderately and highly specialized *Tulasnella* fungi. From the plant point of view, the weighted degree of specialization of the higher level of partners (orchid species) was $<d'_{orchids}>$ = 0.38 (**Supp. Fig. 3.1B**).

Indicator Species Analysis

From the pool of 6543 candidates, ISA returned a list of fungal ASVs with statistically significant indicator values for each orchid species. ISA identified 42 ASVs (12 of which were Tulasnellaceae) associated with *L. cribbii*, 8 ASVs (2 Tulasnellaceae) associated with *L. falx-bellica*, 40 ASVs (8 Tulasnellaceae) associated with *L. mentosa*, and 176 ASVs (16 Tulasnellaceae) associated with *L. monteverdensis*. Many of these indicator fungi could not be taxonomically classified, and for three of the orchid species (excepting *L. cribbii*) these unidentified ASVs often had the most significant indicator values (p-value<0.001). Out of 266 taxa identified as indicator species, 54 were assigned to mycorrhizal guild by FUNGuild (Nguyen et al. 2016), 33 to endophytes, 52 to saprotrophs, 17 to pathogens, 8 were lichenized and 102 were unassigned; however, many taxa were assigned multiple guilds. Of mycorrhizal fungi, 38 belonged to Tulasnellaceae, 3 to Sebacinales, and 2 to Ceratobasidiaceae. The degree of specialization calculated for the network of orchid-keystone fungi as identified by ISA was significant with H_2' = 0.45 (average for $H_2'_{ran}$ = 0.14 ± 0.008 [mean ± SD of 1000 simulations, p-value<0.001]) (**Supp. Fig. 3.3**).

3.5 DISCUSSION

***Lepanthes* specialization on Tulasnellaceae**

We found speciose categories of moderately and highly specialized *Tulasnella* fungi, although we also detected a high number of opportunistic interactions. The weighted mean degrees of specialization of both levels of partners were lower than when all groups of fungi were considered. It should be noted however that the specialization index should be interpreted within the context of a network. We caution against interpreting this result as evidence of higher generalism within the Tulasnellaceae than all-mycobiont network. This pre-selected clade has narrow phylogenetic breadth and is one of the most important groups forming orchid mycorrhizas, thus by definition consists presumably of specialists. Our analyses suggest that even within specialized OMF exists a range of specialization signals, with some *Tulasnella* species interacting more frequently than others, creating modular network architecture (Blüthgen et al. 2006). This finding is the opposite of a network analysis of interactions with Tulasnellaceae in the tropics that detected nested pattern (Herrera et al. 2017). The discrepancy in findings potentially may be explained by smaller sampling effort or a nestedness index used (NODF), since heterogeneous abundance distribution alone creates a significantly nested pattern, as do networks created with null models based on unweighted links (Fischer and Lindenmayer 2002, Lewinsohn et al. 2006, Blüthgen 2008).

Specialization was present in all *Lepanthes*-fungal interaction networks

Network analysis of specialization performed on the keystone fungi identified by Indicator Species Analysis corroborated specialization level obtained from analyses of networks with all and keystone mycobionts. Notably, many recovered fungal taxa are not classified as conventional OMF groups and may represent fungi specific to orchids that act as endophytes and pathogens of other plants or fungi, as well as saprophytes, a finding reported previously (Waud et al. 2016, Shefferson et al. 2019, Li et al. 2021). Noteworthy, the biggest recovered category was unclassified

fungi, and some unidentified species were classified as mycorrhizal based on their assigned genus or family. Thus, further study of these fungal taxa in the tropics is clearly needed.

Mycobiont specialization as a possible mechanism for orchid diversification

We hypothesize that *Lepanthes* orchids are phylogenetically constrained, meaning that closely related orchid species likely share interaction traits due to shared ancestry, leading them to interact with closely related fungal taxa. A similar pattern has been previously reported in terrestrial orchids (Jacquemyn et al. 2014, Waterman et al. 2011). Given the more recent diversification of orchids compared to fungi (Otero et al. 2011), the ancestor of *Lepanthes* may have already developed mycorrhizal associations retaining the affinity to certain fungal groups as *Lepanthes* diversified. Some authors have argued that host-jumping rather than strict codivergence might be a mechanism responsible for, at least to some extent, orchid diversification (Thompson 1987; Cowling et al. 1990; Otero and Flanagan 2006, Otero et al. 2011). If such mechanism takes place, it follows that one should be able to detect different preferences for mycorrhizal fungi in recently diversified sister orchid species (Waterman et al. 2011). Here, we show that this condition was met by studying specialization signals in four endemic orchid species of a hyper-diverse, evolutionarily young genus, with the highest speciation rate in the Pleurothallidinae subtribe (Pérez-Escobar et al. 2017).

Epiphytic orchids may be more specialized on OMF than terrestrial counterparts

It has been previously proposed that orchid mycorrhizal specialization increases with latitude and decreases in nutrient-deprived conditions and thus may be stronger in temperate terrestrial orchids. Our findings, however, show moderately high OMF specificity in the tropics. Indeed, we speculate that epiphytic orchids might be subject to stronger selective pressures for host-switching than their

terrestrial counterparts for several reasons. First, epiphytic orchids grow under challenging conditions of water and nutrient limitations, thus finding appropriate mycobionts is critical to survival (Suárez and Kottke 2016). This is true particularly for miniature orchids like *Lepanthes* that presumably experience high dehydration pressure. In addition to finding a partner able to fulfil its needs, expansion in the number of fungal partners might facilitate orchid persistence, particularly if fungal habitat range is small or a given mycobiont is rare (Shefferson et al. 2019). Finally, epiphytic orchids often grow sympatrically, i.e. share the same niche with other epiphytes. In our study, all four *Lepanthes* species were often found growing on the same tree or even branch. Thus, the ability to interact with a larger number of mycobionts may reduce competition. If sympatric orchids can employ a mycobiont jumping mechanism to better partition resources, this might ultimately open access to new niches for emerging species of orchids. Together, we propose that our finding of variation in the specificity of mycobiont associations may allow the orchids to escape evolutionary constraint.

Escape from ecological constraint is an alternative, but unlikely mechanism to explain the pattern found in our study, since 1) the studied orchids are co-occurring narrow endemics and experience the same environmental variables, and 2) in the broader context, tropical orchids should engage in generalist interactions if specificity is explained solely by environment. Interestingly, a similar specialization pattern was found in lichens in the genus *Peltigera* between fungi and photosynthetic cyanobacteria (Chagnon et al. 2019). The authors found a phylogenetic signal in microbial partners of fungi, and that fungal speciation resulted in the development of new combinations of fungus and cyanobiont. In the case of sympatric orchids, niche partitioning might be accelerating the process leading to such new interactions.

Co-evolutionary potential of orchid-mycobiont associations

Our network analyses suggest potential for co-evolutionary processes in *Lepanthes*-mycobiont interactions. While Otero et al. (2011) did not find strict co-cladogenesis between the terrestrial Australian Pterostylidinae orchids and their mycobionts, Martos et al. (2012) found a stronger phylogenetic signal in epiphytic than terrestrial orchids and concluded that epiphytes may co-evolve more. As pointed out by Blüthgen et al (2008), high specialization implies lower ecological redundancy and suggests that network architecture is shaped by co-evolutionary processes. From a fungal perspective, partnering with orchids may provide a benefit of protection by the water-retention capacity of velamen root tissue (Suárez and Kottke 2016). It has been argued that orchid mycobionts secondarily evolved the ability to colonize epiphytic niches and thus are not as well adapted to living in these harsher conditions and must rely on protection from plant partners. It has been also argued that epiphytic orchids have higher photosynthetic activity due to better access to light than terrestrial orchids, thus are able to provide more carbon to mycobionts (Martos et al. 2012). Thus, orchid-fungal associations could be evolutionarily advantageous to both partners.

Significance

Understanding the identity of mycobionts and the degree of specificity in their symbiotic relationships is critical for understanding the evolution of orchids. This knowledge is also a prerequisite for conservation efforts, especially in biodiverse tropical regions such as the montane cloud forest of Monteverde, Costa Rica, where the approximately 600 species of orchids account for about 35% of all plant species (Nadkarni and Wheelwright 2000).

Mycorrhizal specificity has been a central focus in orchid research, as this obligate relationship is essential for seed germination and plant development, and hence understanding it is vital for conservation initiatives (Cribb et al., 2003; Dearnaley et al., 2012).

Identifying the fungal species that support the survival and growth of orchids, particularly those that are threatened or endangered, enables conservation and restoration efforts to target specific fungi. Instances of high reciprocal specialization may warrant focused attention in conservation (Blüthgen et al. 2008). A higher specialization in terms of interaction exclusiveness suggests lower redundancy of species and a higher possibility of negative effects on consumer species (e.g., orchids) if mycobiont species decline. Additionally, taxon-specific research within the Orchidaceae family is crucial, particularly to inform conservation strategies for rare species such as those found in *Lepanthes* with its many narrow endemics.

Conclusion

We have found that mycorrhizal specificity varies at a much finer scale than previously thought and found sister orchid species differ in their mycobionts at the Amplicon Sequence Variant resolution. Given the young evolutionary history of the *Lepanthes* genus and its rapid diversification, it is likely that mycobiont-switching is facilitating speciation by allowing emerging orchid species escape evolutionary constraints.

3.6 FIGURES

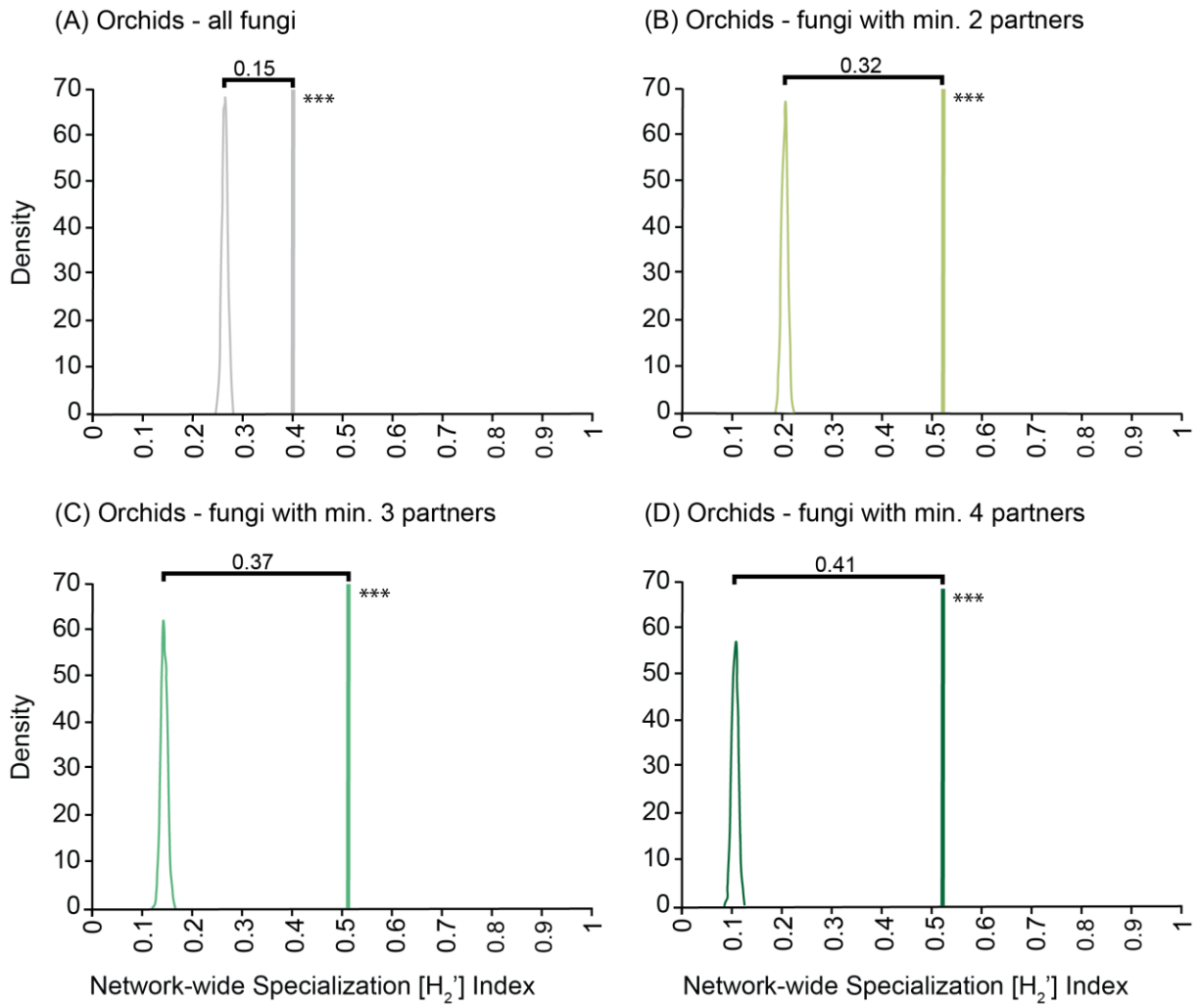


Figure 3.1. Network-wide specialization within orchid-fungal networks. In each plot, straight line represents observed H_2' value calculated from experimental network, and bell-shaped curve represents frequency distribution of $H_2'_{ran}$ values generated from 1000 randomized networks. (A) interaction network between all orchids and all sampled fungi; (B-D) interaction networks between all orchids and keystone fungi defined as ASVs with min. two (B), three (C), and four (D) partners within each orchid sp. $H_2' = 0$ indicates extreme generalization and $H_2' = 1.0$ indicates maximum specialization. *** indicates p-value ≤ 0.001.

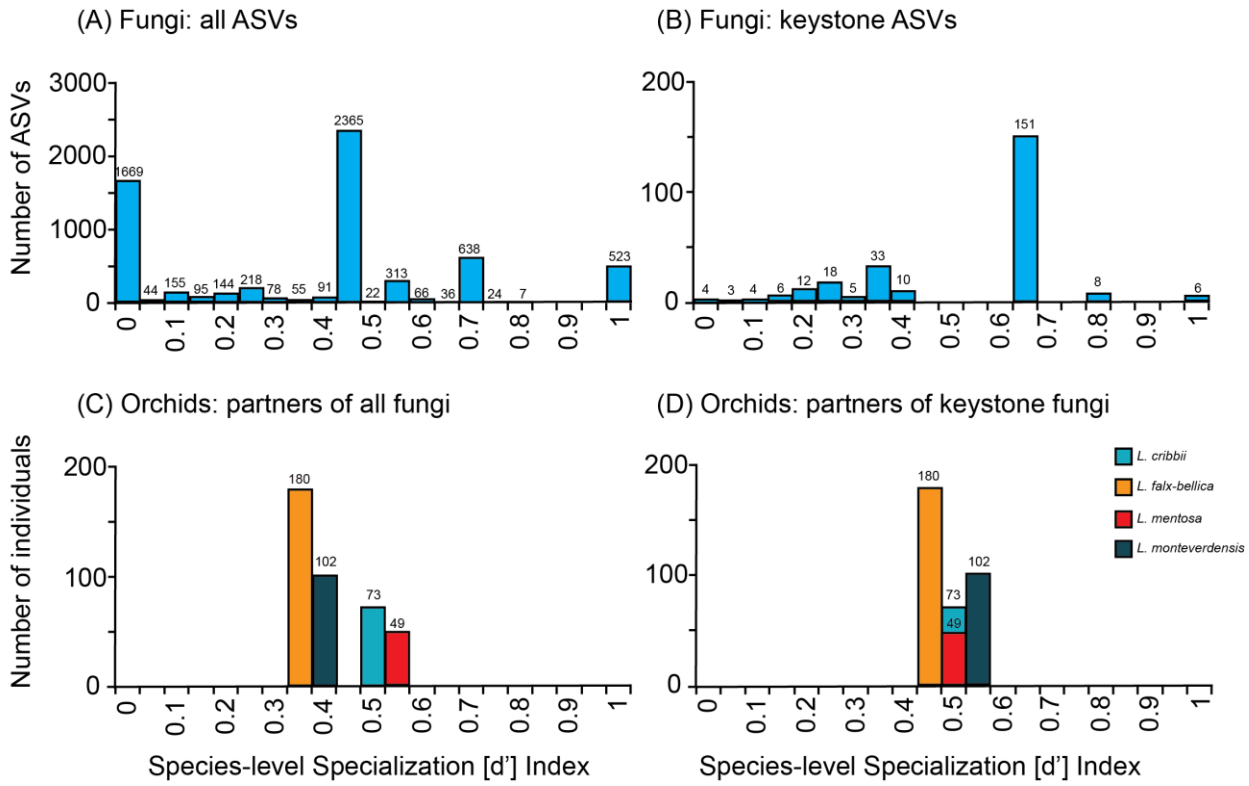


Figure 3.2. Patterns of species-level specialization within orchid-fungal networks. Frequency distribution of the species-level specialization index (d') for all fungi (**A**), keystone fungi interacting min. with four plant individuals within each orchid sp. (**B**), orchid sp. interacting with all fungi (**C**), and orchid sp. interacting with keystone fungi only (**D**). Bars show the number of fungal taxonomic units (**A** and **B**) or plant individuals (**C** and **D**) in each category with total number in each category indicated on top (label '0' defines $0.00 \leq d' < 0.05$, etc.). Standardized specialization index $d_i' = 0$ indicates highest generalization and $d_i' = 1.0$ indicates most specialized taxa.

3.7 TABLES

Table 3.1. Results for Mean Pairwise Distance (MPD) analyses test for presence of a phylogenetic signal in orchid fungal communities consisting of all sampled fungi. Orchid sp. = focal orchid species interacting with fungal community subject to the analysis, nASVs = number of fungal taxonomic units in a community, mpd.obs = observed MPD value in community, mpd.rand.mean = mean MPD value in null communities, mpd.rand.sd = standard deviation of MPD in null communities, mpd.obs.z = standardized effect size of MPD, mpd.obs.p = p-value of observed MPD, runs = number of randomizations. mpd.obs.z < 0 and p-value < 0.05 indicate phylogenetic clustering, i.e. taxa in the community are more related than expected by chance; mpd.obs.z > 0 and p-value > 0.95 indicate phylogenetic evenness, i.e. taxa in the community are less related than expected by chance; whereas mpd.obs.z = $(-\infty, \infty)$ and p-value = (0.05, 0.95) indicate that the pattern of phylogenetic relatedness of community members is indistinguishable from random. * indicates significant result.

Orchid sp.	nASVs	mpd.obs	mpd.rand.mean	mpd.rand.sd	mpd.obs.z	mpd.obs.p	runs
L. cribbii (N=73)	1083	1.1044	1.0501	0.0188	2.8799	1*	100
L. falx-bellica (N=180)	2402	1.0587	1.0540	0.0110	0.4224	0.6436	100
L. mentosa (N=49)	837	1.0676	1.0512	0.0182	0.9011	0.8119	100
L. monteverdensis (N=102)	3539	0.9949	1.0531	0.0080	-7.2991	0.0099*	100

Table 3.2. Results for Mean Pairwise Distance (MPD) analyses test for presence of a phylogenetic signal in orchid fungal communities consisting of fungi interacting with at least two plant individuals within orchid species. Abbreviations and interpretation as in **Table 3.1**.

Orchid sp.	nASVs	mpd.obs	mpd.rand.mean	mpd.rand.sd	mpd.obs.z	mpd.obs.p	runs
L. cribbii (N=73)	154	0.9645	1.0570	0.0540	-1.7120	0.0297*	100
L. falx-bellica (N=180)	464	1.0546	1.0516	0.0284	0.1055	0.5446	100
L. mentosa (N=49)	106	1.1098	1.0548	0.0560	0.9830	0.8218	100
L. monteverdensis (N=102)	785	0.9720	1.0572	0.0209	-4.0833	0.0099*	100

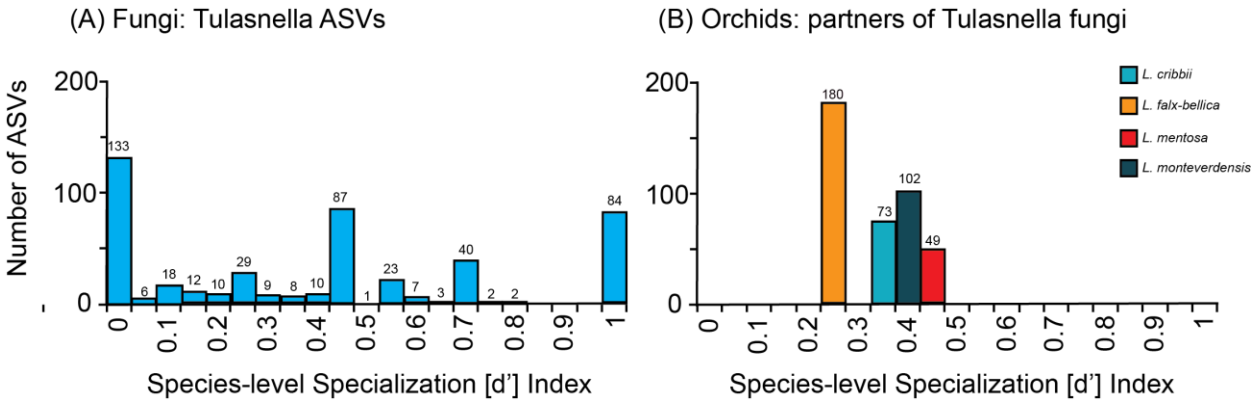
Table 3.3. Results for Mean Pairwise Distance (MPD) analyses test for presence of a phylogenetic signal in orchid fungal communities consisting of fungi interacting with at least three plant individuals within orchid species. Abbreviations and interpretation as in **Table 3.1**.

Orchid sp.	nASVs	mpd.obs	mpd.rand.mean	mpd.rand.sd	mpd.obs.z	mpd.obs.p	runs
L. cribbii (N=73)	60	0.9879	1.0621	0.0910	-0.8159	0.2079	100
L. falx-bellica (N=180)	174	0.9788	1.0507	0.0528	-1.3643	0.0693	100
L. mentosa (N=49)	40	1.1544	1.0568	0.1057	0.9234	0.8317	100
L. monteverdensis (N=102)	329	0.9353	1.0484	0.0384	-2.9472	0.0099*	100

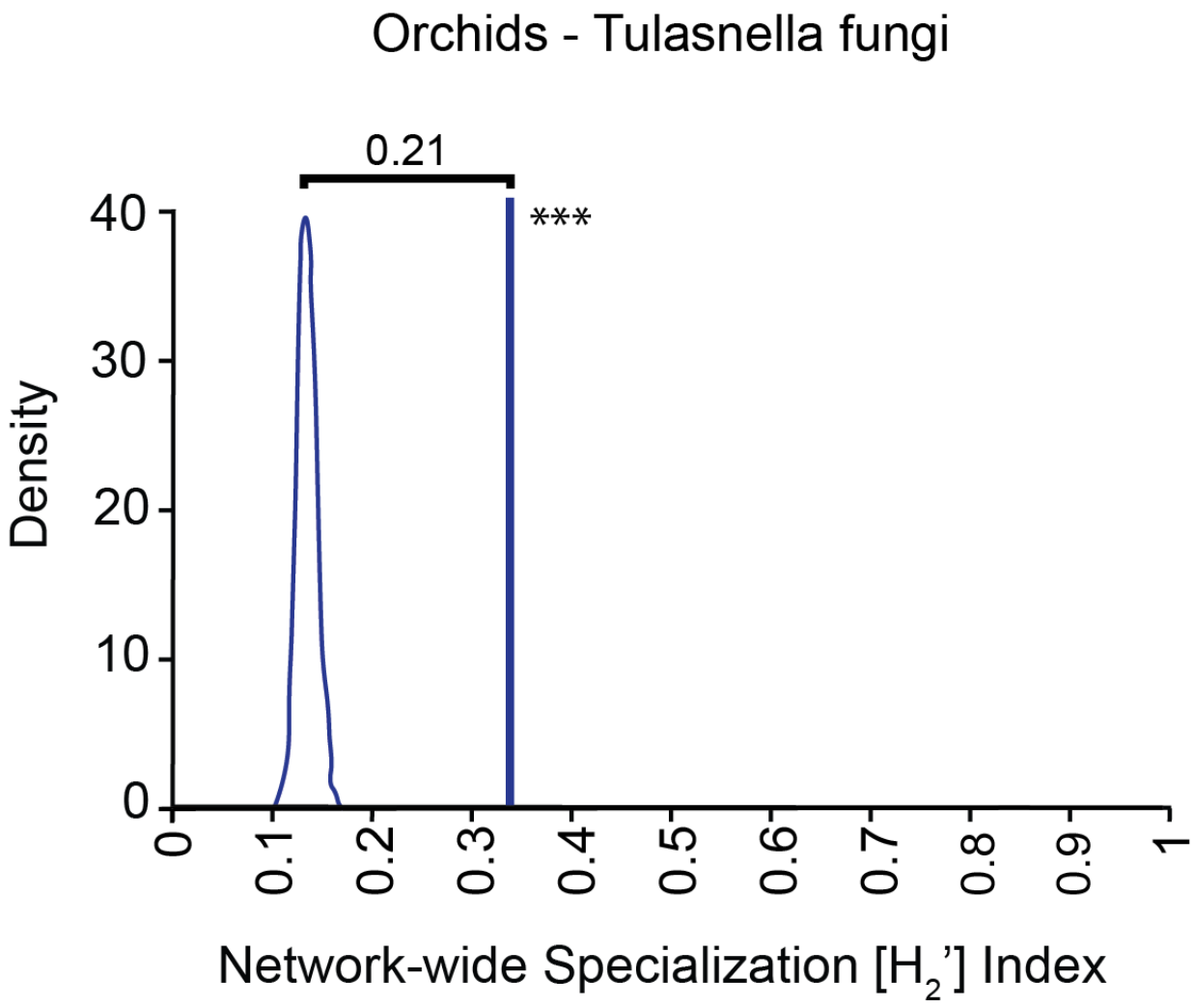
Table 3.4. Results for Mean Pairwise Distance (MPD) analyses test for presence of a phylogenetic signal in orchid fungal communities consisting of fungi interacting with at least four plant individuals within orchid species. Abbreviations and interpretation as in **Table 3.1**.

Orchid sp.	nASVs	mpd.obs	mpd.rand.mean	mpd.rand.sd	mpd.obs.z	mpd.obs.p	runs
L. cribbii (N=73)	37	0.9991	1.0640	0.1090	-0.5955	0.3069	100
L. falx-bellica (N=180)	94	1.0004	1.0610	0.0736	-0.8236	0.1980	100
L. mentosa (N=49)	21	1.1553	1.0747	0.1462	0.5514	0.7921	100
L. monteverdensis (N=102)	189	0.9198	1.0599	0.0407	-3.4446	0.0099*	100

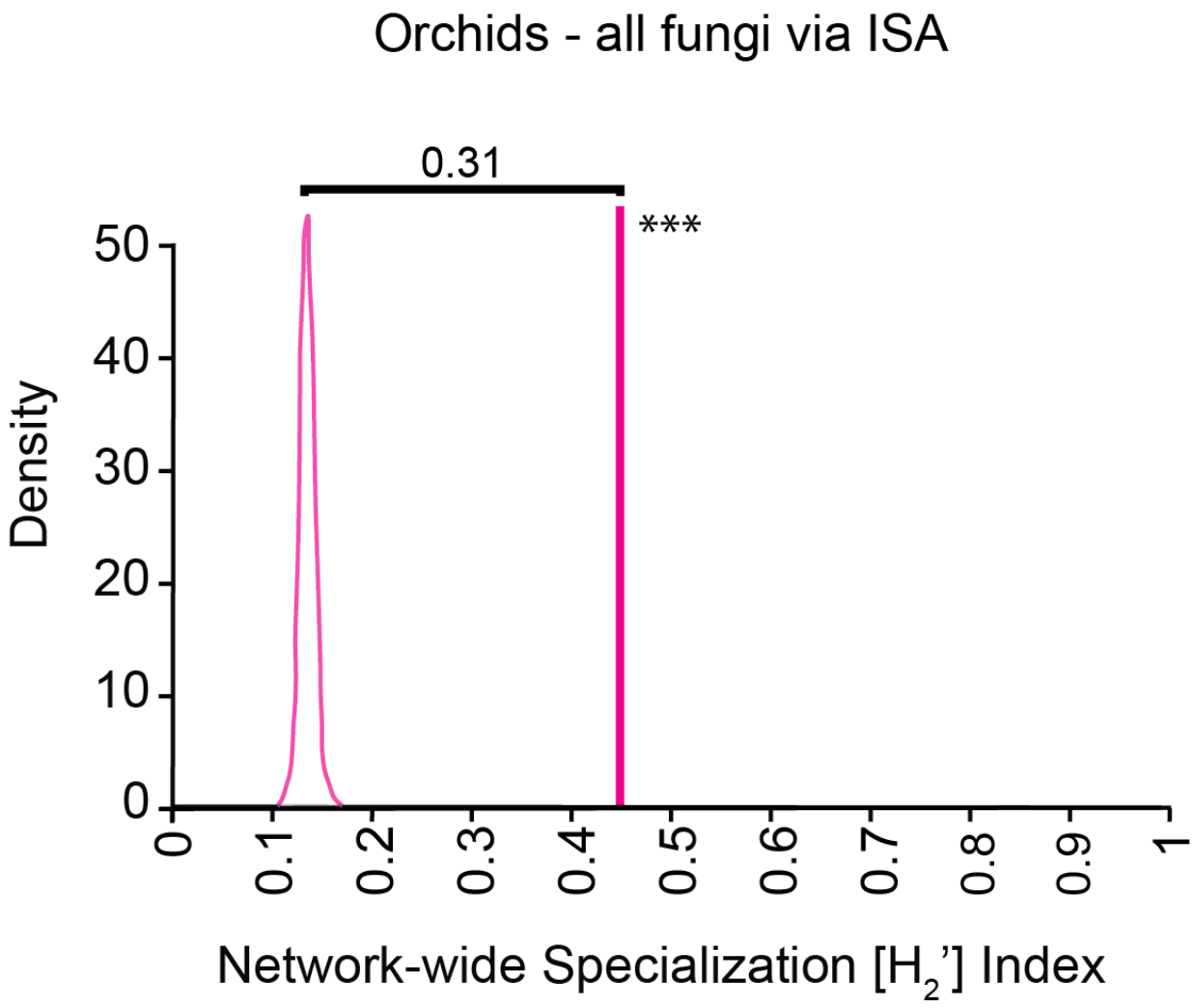
3.8 SUPPLEMENTARY FIGURES



Supplementary Figure 3.1. Patterns of species-level specialization within orchid-*Tulasnella* network. Frequency distribution of the species-level specialization index (d') for *Tulasnella* fungi (**A**), and plants (**B**). Bars show the number of fungal taxonomic units (**A**) or plant individuals (**B**) in each category with total number in each category indicated on top (label '0' defines $0.00 \leq d' < 0.05$, etc.). Standardized specialization index $d_i' = 0$ indicates highest generalization and $d_i' = 1.0$ indicates most specialized taxa.



Supplementary Figure 3.2. Network-wide specialization within orchid-*Tulasnella* network. Straight line represents observed H_2' value calculated from experimental network, and bell-shaped curve represents frequency distribution of $H_2'_{ran}$ values generated from 1000 randomized networks. $H_2' = 0$ indicates extreme generalization and $H_2' = 1.0$ indicates maximum specialization. *** indicates p-value ≤ 0.001.



Supplementary Figure 3.3. Network-wide specialization within the network of orchids and keystone fungi as identified by Indicator Species Analysis (ISA). Straight line represents observed H_2' value calculated from experimental network, and bell-shaped curve represents frequency distribution of $H_2'_{ran}$ values generated from 1000 randomized networks. $H_2' = 0$ indicates extreme generalization and $H_2' = 1.0$ indicates maximum specialization. *** indicates p-value ≤ 0.001

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CHAPTER 4

SITE DISTURBANCE AND PHOROPHYTE IDENTITY IMPACT THE MYCORHIZAL COMMUNITY IN ORCHID SPECIES[3]

[3] Tuczapski, P. T. and Trapnell, D. To be submitted to *American Journal of Botany*.

4.1 INTRODUCTION

Understanding the consequences of ecosystem disturbances on species interactions is crucial for ecologists, given the rapid rate of biodiversity loss and the expected effects of climate change, especially in the tropics (Barlow et al., 2016; Nadkarni et al., 2023). Environmental degradation can affect soil fungal composition, which plays a critical role in habitat succession and ecological restoration. One group of organisms that is particularly affected by habitat degradation is root-associated fungi, which, in turn, shape plant composition. For example, research on nutrient-poor soils, such as lava deposits and soils exposed by retreating glaciers, has shown that early colonization begins with the presence of a few generalist ectomycorrhizal or endophytic species, as well as little to no vegetation, and becomes more complex over time (Nara et al., 2003; Cázares et al., 2005; Blaalid et al., 2012). Within the environmental disturbance framework, non-disturbed sites are colonized by ectomycorrhizal trees or ericoid mycorrhizal shrubs, which thrive in soils rich in organic matter with less mobile nutrients. In disturbed areas, arbuscular mycorrhizal grasses exploit inorganic nitrogen sources. At the most severe disturbance levels, soil is colonized by non-mycorrhizal plants characterized by high fecundity and short generation times, allowing them to rapidly utilize available nutrients (Smith & Read, 2008). Additionally, the presence of suilloid ectomycorrhizal fungi has been found to facilitate pine invasion, suggesting that the identity of the mycobiont partner plays a key role in the successful establishment of plants (Policelli et al., 2019).

However, little is known about the effects of habitat disturbance on the mycobiont composition of epiphytes, despite the fact that, in some ecosystems (e.g., tropical montane cloud forests), they constitute up to one-third of vascular plant species (Gentry & Dodson, 1987). Epiphytes and their associated biota are often characterized by small population sizes, rarity, low

germination rates, and reliance on other organisms, such as fungal symbionts and pollinators (Bartels & Chen, 2012). Due to these characteristics, they are typically sensitive to environmental stress and are therefore used as bioindicators of ecosystem health (Jovan & McCune, 2006). Thus, a better understanding of the interactions between epiphytes and other community members is needed to predict the consequences of disturbance and to guide conservation efforts.

In terms of community stability, a focal species' ability to interact with multiple partners from different guilds suggests higher functional redundancy of species and, consequently, a lower potential for negative effects in the event of a population decline caused by the unavailability of partners (Estrada, 2007). Conversely, high dependence on specific partners can put associated species at risk of extinction, posing conservation challenges for those that rely on specialized interactions (Blüthgen et al., 2008). This risk is exacerbated if associated partners have a narrow geographic range, are available only during specific time windows (Poulin et al., 2011; Slatyer et al., 2013), or if a keystone partner is lost. For instance, the loss of managed honeybee has been found to have a highly negative effect on plant survival, even though its contribution to overall pollinator visitation was low (Traveset et al., 2017).

Orchids, with an estimated 30,000–35,000 species, constitute approximately 10% of all angiosperms and represent the second-largest flowering plant family after Asteraceae (Li et al., 2021). More than half of these species are concentrated in tropical regions. Epiphytic orchids contribute significantly to tropical plant diversity, with an epiphytic-to-terrestrial ratio as high as 5:1 (Givnish et al., 2015; Zhang et al., 2015). Fungal symbionts are critical to the orchid life cycle, as they provide carbon and nutrients to developing dust-like seeds, which lack reserves (Dearnaley

et al., 2016). As a result, orchid germination is obligatorily reliant on mycobionts for establishment, and adult plants continue to maintain these associations (Dearnaley et al., 2012).

From the mycobiont's perspective, fungal partners are likely less dependent on the interaction, though dependence may be higher in epiphytic life forms. First, there is limited evidence of photosynthetically derived carbon transfer from orchids to fungi (Cameron et al., 2007; Liebel et al., 2015; Schiebold et al., 2018, Read et al. 2024). Additionally, in epiphytes velamen tissue may protect symbiotic fungi from environmental stress (Suárez & Kottke, 2016), and epiphytes may translocate more carbon to mycobionts due to their higher photosynthetic activity (Martos et al., 2012).

Thus, epiphytic orchids may be particularly susceptible to environmental disturbances, as they tend to be rare, have narrow distributions, require specific mycobiont partners, and depend on host trees for persistence. Moreover, a phorophyte bias has been observed in epiphytic orchids (Rasmussen & Rasmussen, 2018). For example, around 40% of 105 orchid species were found to inhabit fewer than five types of phorophytes (Silva et al., 2010), and even orchids without an apparent strong preference seem to avoid certain local tree species (Migenis & Ackerman, 1993).

We investigated the mycobiont associations of the rare, narrowly distributed, epiphytic orchid *Lepanthes falx-bellica*. We consider this species rare because it is endemic to the montane cloud forest in a small region west of the Monteverde Cloud Forest Biological Reserve (MCFBR) in Monteverde, Costa Rica (Diego Bogarín, pers. comm.). However, this species is locally abundant when found on host tree species (P. Tuczapski, pers. obs.) and is capable of colonizing

primary forest sites as well as roadside and pasture habitats. Using DNA-based methods, we identified fungal taxonomic units and investigated the mycobiont composition of 22 orchid populations from both disturbed and undisturbed areas. Specifically, we asked (i) Do fungal associates differ among orchids growing on different tree species?; (ii) Do fungal communities differ among populations sampled in different habitat types? And (iii) Is the effect of the habitat type retained when phorophyte effect is accounted for?

4.2 METHODS

For the analyses included here, we used a dataset comprising mycorrhizal and other root-endophytic fungi derived from 22 populations and 183 individuals (mean = 8 individuals/population) of *Lepanthes falx-bellica* orchid species endemic to the Monteverde area. Orchid individuals were found growing epiphytically on 16 tree species (**Table 4.1**). The study was carried out at the western border of the MCFBR and its immediate vicinity in the Cordillera de Tilarán mountain range of Andes, Costa Rica. Sampled plants were found at the altitude of 1502-1712 m asl that encompasses two overlapping life zones: a lower montane wet forest and a lower montane rain forest (Haber 2000). Generally, lower parts of the protected primary evergreen forest fall within the former zone (1450-1600 m asl), with the natural vegetation characterized by dense tall canopy 30-40 m high. The latter zone occurs above the lower montane wet forest (1550-1850 m asl) with a canopy slightly shorter and less dense at 15-30 m high. Frequent wind-blown mists and clouds that migrate from the Atlantic side of the Continental Divide are characteristic of both zones. Annual precipitation is about 3000 mm, and the mean temperature is 12-17°C. A total of 3021 vascular plant species has been recorded for the Monteverde area above 700 m asl, and 442 tree species above 1200 m asl (Haber 1991, Appendix 1). We sampled orchid populations at

seven sites. The farthest sites were ~6 km apart and the closest 1 km apart (~3 km on average). A population is defined as all the orchid individuals growing on a tree. Root samples were assigned to three categories of habitat depending on disturbance level: undisturbed primary forest within the MCFBR (33 plants), secondary forest on the sideroad but within MCFBR boundaries (45 plants), and a pasture/partially cleared land (105 plants). We determined Amplicon Sequence Variants (ASVs) and assigned them fungal taxonomical identities where possible. We ascribed fungal taxonomic units to communities found in individual plants. See Chapter 1 for further details on Study Species, Sampling, and laboratory and analytical methods pertaining to sequence processing (Library Preparation, Quality Control, Amplicon Processing, PCR Bias Control). Amplicon Sequence Variants (ASVs) were derived as a result of processing sequence data in the DADA2 (Callahan et al. 2016) package in R version 4.3.2 (R Development Core Team 2023). Fungal taxonomic units were identified based on the UNITE 9.0 ITS reference dataset (Abarenkov et al. 2022) and fungal guilds were assigned according to FUNGuild in R version 4.3.2 (R Development Core Team 2023, Nguyen et al. 2016).

Assessment of Fungal Diversity

We generated species rarefaction curves to evaluate whether the sampling effort was sufficient to capture the total fungal community richness using the *rarecurve* function in the *vegan* package (Oksanen et al. 2022) in R version 4.3.2 (R Development Core Team 2023). Before proceeding with the downstream analyses, we rarefied the dataset to an even sampling depth of 679 reads, which is the lowest value at which rarefaction curves for most samples appear to level off (**Fig. 4.1**) indicating that in most samples increasing sequence sampling would not notably affect the number of fungal taxonomic units recovered.

We evaluated whether *L. falx-bellica* individuals from habitats of varying disturbance levels differ in their mycobiont composition through beta diversity analyses. First, we assessed if fungal host communities were qualitatively different (i.e., based on the presence-absence of fungal taxonomic units). We calculated Jaccard distances (S_j) (Jaccard 1912) treating the fungal community of each individual orchid plant as a sampling unit and habitat type as a group. If the disturbance level of a habitat affects its fungal composition, orchid's interaction traits (i.e., traits which may control range of suitable partners, Martos et al. 2012) must enable it to associate with fungi that are different from those in undisturbed sites for successful colonization. A dominant pattern in ecological research is the phylogenetic signal, where closely related species tend to exhibit more similar trait values (Shefferson 2019, Shefferson et al. 2019). Consequently, the available fungal associates in disturbed sites will likely be closely related to the orchid's preferred partners, thereby maintaining the ecological traits essential for its survival. Under that scenario Jaccard indices would indicate different mycobiont assemblages of orchid populations inhabiting different habitat types, however, they could be similar in terms of the amount of shared phylogeny. To examine this possibility, we calculated the UniFrac distance **(**U_{AB}) (Lozupone & Knight 2005) for each pair of individual plant fungal root communities, treating habitat type as a group. Unweighted UniFrac coefficients were generated based on a phylogenetic tree built from ASV sequences using *the phylogeny* plugin's *align-to-tree-mafft-fasttree* pipeline in QIIME2 (Boylen et al. 2019) which first performs a multiple sequence alignment with MAFFT (Katoh & Standley 2013). The difference between habitats in mycobiont composition as measured by Jaccard and UniFrac measures was tested for significance with permutational multivariate analysis of variance (PERMANOVA) (Anderson 2014) using 999 permutations in QIIME2.

Non-metric multidimensional scaling (NMDS) plots were generated with dissimilarity metric measures, Jaccard and UniFrac, to visualize the differences among habitats in fungal composition using *vegan* package (Oksanen et al. 2022) in R version 4.3.2 (R Development Core Team 2023). To test for the homogeneity of variance between groups (here, habitat types) we performed an analysis of multivariate homogeneity of group beta diversity dispersions, followed by ANOVA and Tukey's Honest Significant Difference (HSD) test implemented in the *vegan* package to examine which group variances differ. Additionally, since sideroad and pasture mycobiont communities were found to overlap more than either one with primary forest fungi in NMDS plot (see Results section), principal coordinate analysis plot clustering UniFrac diversity measures was generated to cross-validate that result using the *vegan* package.

Finally, we calculated the relative abundance of fungal families found in orchid roots at each habitat type and visualized its distribution in the *phyloseq* package in R version 4.3.2 (McMurdie and Holmes 2013, R Development Core Team 2023).

The Effects of Phorophyte and Habitat Disturbance on Mycobionts

Previous research has found that mycobiont assemblages may be affected by phorophytes in epiphytic orchid life form (Martos et al. 2012, Wang et al. 2017, Xing et al. 2019). To test whether the effect of habitat type on mycobiont community is retained when phorophyte species are accounted for we employed multivariate PERMANOVA (i.e., non-parametric permutational MANOVA) using *adonis2* function in *vegan* package. We assessed the main effects of two explanatory variables simultaneously – tree species and habitat type – on partitioning distance matrices among sources of variation. We repeated the analysis using the model separately with

two distance matrices, one composed of Jaccard and the other of UniFrac coefficients. To test for significance, 9999 permutations were generated in each analysis. Significant effect of a factor was determined when p-value ≤ 0.05.

4.3 RESULTS

Fungal Diversity in Disturbed vs. Undisturbed Habitats

As a result of dataset rarefaction, 83 samples were excluded from further analysis, leaving 23 samples from the primary forest habitat (hereafter referred to as 'primary forest'), 20 samples from the secondary forest (hereafter referred to as 'sideroad'), and 57 samples from pasture. We have recovered the following number of fungal ASVs from the roots of *L. falx-bellica* orchids in the respective habitat types: 231 ASVs from primary forest, 598 from sideroad, and 1383 from pasture.

PERMANOVA test results strongly suggested that mycobiont communities of orchid populations differed in their composition among habitat types, when identities of fungi were considered (pseudo-F value: 1.83, p-value: 0.001, **Table 4.2**). When the influence of habitats on fungal community composition was visualized with NMDS ordination plots, communities from primary forest appeared the most distinct from either pasture or sideroad (**Fig. 4.2A**). Mycobiont communities from both primary forest (pseudo-F value = 2.27, p-value = 0.001) and sideroad (pseudo-F value = 1.43, p-value = 0.001) overlapped with a subset of pasture communities, and to lesser extent with each other, indicating that the subset of fungal taxa found in the pasture were similar to the other two sites. At the same time, composition of mycobionts was less similar between primary forest and sideroad when fungal identities were considered (pseudo-F value = 1.74, p-value = 0.005). The homogeneity of variance test indicated homogeneous dispersion of

Jaccard beta diversity for all habitat types except among primary forest and pasture (**Fig. 4.2B**, **Supp. Table 4.1**). Interestingly, primary forest had much wider dispersion of Jaccard diversity values than either sideroad or pasture, indicating that mycobiont composition was more uniformly distributed among individual plants in the disturbed sites.

The PERMANOVA test based on the UniFrac measures found mycobiont communities to be phylogenetically dissimilar among disturbance levels (pseudo-F value: 5.08, p-value: 0.001, **Table 4.3**). NMDS ordination plot based on UniFrac values showed approximately similar pairwise overlap between communities, indicating similar shared amount of phylogenetic diversity between fungal associates found in all habitat types. However, the centroid of UniFrac scores calculated from mycobionts found in primary forest was slightly farther from other two habitats (for primary forest-sideroad comparison pseudo-F value = 5.78, p-value = 0.001; for primary forest-pasture pseudo F-value = 8.21, p-value = 0.001) than either sideroad or pasture centroids were to each other (pseudo-F value = 1.83, p-value = 0.006), indicating that the community from the primary forest was phylogenetically more distinct (**Fig. 4.3A**). This is further supported with principal coordinate analysis (PCoA) plot, which shows small overlap of primary forest community UniFrac scores with any other habitat type, while pasture and sideroad communities show bigger overlap (**Supp. Fig. 3.1**). The homogeneity of variance test indicated a similar dispersion of phylogenetic diversity in all habitat types (**Fig. 4.3B**),), however the difference between sideroad and pasture was low but not significant (p-value: 0.06, **Supp. Table 4.2**). Thus, the difference between habitat types found in the PERMANOVA test was supported. Notably, the first two principal coordinate axes of the PCoA explained a fairly small amount of variation (6.1%

and 4.3%), suggesting that potentially other factors might have an impact on the fungal composition.

The relative abundance analysis identified that ASVs classified to Tulasnellaceae make up the largest category within each habitat type (**Fig. 4.4**). *Tulasnella* fungi contribute the biggest fraction of the total mycobiont composition in the primary forest (81%), followed by secondary forest, i.e. sideroad (69%), and pasture (45%). Fungi unclassified to any family made the second largest group in each habitat (9% in primary forest, 15% in sideroad, and 22% in pasture). The remaining ASVs were classified into 39 families in primary forest (10%), 98 families in secondary forest (16%), and 121 families in pasture (33%).

The Effects of Phorophyte and Habitat Disturbance on Mycobionts

Both phorophyte species and habitat type were significantly associated with variation in the mycobiont composition of the studied orchid populations. A non-parametric permutational MANOVA (Adonis) revealed that, among the two explanatory factors tested, phorophyte species accounted for a greater proportion of the variation in fungal diversity, as measured by Jaccard distances (17.4%, p-value: 0.0001; **Table 4.4**). However, the effect of habitat was retained after adjusting for tree species, explaining a smaller but significant fraction of variation in the data (1.38%, p-value: 0.0014). When the test was conducted to evaluate the main effect of habitat type on the phylogenetic diversity of mycobionts while controlling for phorophyte species, we found that habitat effect was significant (1.17% of variation explained, p-value: 0.0185) and tree species explained 16.7% of total variation (p-value: 0.0001; **Table 4.5**).

4.4 DISCUSSION

Our results indicate that the disturbance level of the habitat may influence the mycobiont community in *L. falx-bellica* orchids. Similar findings have been documented in terrestrial orchids whose mycobiont composition differed across semi-natural and restored grasslands (Vogt-Schilb et al. 2020), as well as in epiphytic orchids from sideroad and pasture sites (Kartzinel et al. 2013). Traditionally, peloton-forming orchid mycorrhizal fungi (OMF) (classified within the saprotrophic form-genus *Rhizoctonia*) belong to three groups, namely Ceratobasidiaceae, Serendipitaceae (previously known as Sebacinales group B), and Tulasnellaceae, although other mycorrhizal groups have also been reported, encompassing 17 families of Basidiomycetes and five family/genera of Ascomycetes (Li et al. 2021). We found Tulasnellaceae to be the main fungal group across the examined habitats, corroborating with the fact that Tulasnelloid fungi are the main widespread mycobiont partners of orchids (Suárez and Kottke 2016). The relative abundance of *Tulasnella* spp. was the highest in the mycobiont community composition of the orchid individuals growing in the primary forest of the MCFBR. The relative abundance of Tulasnellaceae was lower in the roots of plants sampled from the sideroad within the borders of the MCFBR and lowest in the pasture habitat (**Fig. 4.5**), suggesting that disturbance affects prevalence of Tulasnellaceae. Corroborating with our finding, the number of Tulasnellaceae taxa was significantly higher in relatively undisturbed semi-natural grasslands in comparison to post-arable sites in the Carpathian Mountains of Europe (Vogt-Schilb et al. 2020).

Emerging evidence suggests orchid associations with ectomycorrhizal and non-mycorrhizal fungal (ONF) groups as well (Li et al., 2021). We also detected a group of fungal taxa that were not classified as mycorrhizal, thus potentially acting as endophytic ONF. In orchids,

ONF have been reported to have much higher diversity than OMF and cover over 110 genera (Sudheep and Sridhar, 2012; Ma et al., 2015). Notably, non-rhizoctonia OMF together with ONF made up the highest proportion of overall relative abundance in the fungal community of orchids sampled in the pasture habitat, followed by orchids found in the sideroad secondary forest, and were least abundant in the primary forest populations. Together, our results correspond to general patterns of fungal colonization in response to environmental disturbance, with symbiotrophs in least disturbed sites being replaced by saprophytes in most disturbed sites, suggesting that mycobiont composition may mirror the availability of the hosts in the environment (Yan et al. 2017, Zhang et al. 2017).

The ability of Tulasnellaceae species to access some nutrient sources, such as nitrate, is limited in comparison to other OMF (Nurfadilah et al. 2013). For instance, Ceratobasidiaceae species have been hypothesized to facilitate orchid colonization due to their fast growth and display of multiple trophic modes, including the ability to utilize inorganic nitrogen (Warcup and Talbot 1971, Veldre et al. 2013, Vogt-Schilb et al. 2020). Moreover, *Tulasnella* fungi were found to lack genes allowing access to nitrate and nitrite, in contrast to *Ceratobasidium* (Kohler et al. 2015). In our study, Ceratobasidiaceae were absent from mycobionts of primary forest orchid populations, and were the most abundant in pasture populations, although their overall fraction remained low. Thus, different mycobiont patterns detected among habitat types might be reflective of different abiotic conditions that impact the composition of available fungi, depending on their abilities to tap into given nutrient sources (Nurfadilah et al., 2013). Previous study has shown that the availability of fungal partners in disturbed habitats may be a greater limitation to orchid establishment than edaphic conditions (Klimešová 2019).

While endophytic and saprophytic communities develop randomly (Zhang et al. 2017), habitat disturbance may lead to a reduction of mycorrhizal species (Dickie et al. 2013). In the context of the tropical montane cloud forest, habitat fragmentation and removal of large trees result in reduced amount of arboreal soils, as less canopy cover leads to less plant litter captured within the tree canopy by inner branches and epiphytes. In the Monteverde cloud forest, canopy soils accounted for 63% of the total epiphytic biomass in the primary forest, while in the secondary forest, they made up only 3% (Nadkarni 1984, Hofstede et al. 1993, Nadkarni et al. 2004). Arboreal soils are a vital source of nutrients for epiphytes, as they increase resource retention and interception (Gotsch et al. 2016). In terms of chemical properties, canopy soils are dominated by organic material (histosol-type soil) originating from decomposing epiphytes, plant litter, and canopy biota (Coxson & Nadkarni 1995). Nutrients captured from intercepted plant material and the atmosphere are stored in an organic, less mobile form (Vance and Nadkarni 1990). In Monteverde montane forest canopy, nitrate retention rate is 80%, with bryophytes and nitrogen-fixing organisms likely having a large role in the process (Forman 1975, Clark et al. 1998, Clark et al. 2005). Thus, the nutrient capital is much less in the areas devoid of arboreal soils such as the examined roadside and pasture sites. In Monteverde, the nutrient capital of epiphytic material (including canopy soil) was 440x higher relative to non-epiphytic material in primary forest than in the secondary forest (Nadkarni et al. 2004). We speculate that pasture, sideroad, and primary forest habitats investigated here likely differ in terms of nutrient pools and water retention capacities, similar to the patterns outlined above, resulting in greater water runoff and nutrient leaching at disturbed areas and possibly leading to the differences in OMF composition (Gotsch et al. 2016). Similarly, soil nutrients have influenced mycobiont taxa and diversity in two *Bipinnula*

species from Chile (Mujica et al. 2016). While the chemical properties of the substrate in the investigated sites were not the focus of this study, fungal community composition is known to be influenced by substrate conditions, such as available nitrogen, phosphorus, acidity, and organic matter content (Lauber et al. 2008, Yan et al. 2018).

It is worth considering that the disturbed areas we investigated possibly consisted of younger trees, and the observed difference in mycobiont composition in fact reflects the effect of different microclimates of younger trees. Previous studies have pointed out that the epiphytic orchid habitat is an extremely dynamic environment, and that an orchid experiences during its lifetime changes in irradiation, humidity, and even bark features it is attached to due to the growth of a phorophyte host (Rasmussen and Rasmussen 2018). As the tree ages, the microclimate in a given position generally becomes more shaded, and water-repellent properties of bark along with its acidity increase (Rambo 2010). Additionally, with proximity to the foliage relative humidity increases (Cardelús & Chazdon, 2005). These changes may be more dynamic in younger trees whose relative growth rate is higher than old trees, creating a stressful environment for epiphytic biota (Rasmussen and Rasmussen 2018). We speculate that these fluctuations in microclimate could influence the composition of fungi capable of colonizing the bark surface.

Our results indicate that the identity of phorophytes has a stronger effect on the orchid mycobiont composition than the habitat type. Orchid phorophyte bias for and against certain tree species is a well-documented phenomenon (see Rasmussen and Rasmussen 2018 for a compilation of cases, Tremblay et al. 1998, Adhikari et al. 2012). Here, we found 22 populations of *L. falx-bellica* to associate with 16 tree species, hence while strict preference is not obvious, we cannot

exclude the existence of unsuitable hosts, given that the Monteverde area has a documented 442 tree species above 1200 m asl. It has been previously suggested that phorophyte bias might be related to different mycobiont availability and/or performance among phorophytes (Gowland et al. 2013) due to physico-chemical factors related to bark and crown microclimate. Aqueous bark extracts from host tree species were previously found to enhance orchid seed germination and mycobiont growth, while non-phorophyte bark extracts were inhibitory (Harshani et al. 2014); additionally, mycobiont composition differed among phorophytes of Chinese *Dendrobium sinense* (Wang et al. 2017). Notably, many tree species in our study were sampled only once, thus clearly more targeted sampling is needed. *In situ* seed baiting, transplantation of seedlings, and identification of OMF in phorophyte bark might help to assess the effects of tree hosts and mycobionts on orchid germination and growth.

It is important to consider the effect of the environment on the differential mycobiont assemblages across the geographical continuum (Tedersoo et al. 2014). One of the reasons we selected *L. falx-bellica* as a study species is for its narrow distribution both in altitude and area of occurrence. This species is endemic to the Monteverde region and is locally abundant when found on host trees (P. Tuczapski, pers. obs.). Since sampled populations were found within ~6 km and efforts to find populations outside this range were unsuccessful, we speculate that the environmental conditions in the area do not vary significantly. Similarly, Kartzinel et al. (2013) found that mycorrhizal communities were unrelated to environmental and geographic heterogeneity in epiphytic Costa Rican orchids, while mycobionts were different in disturbed pasture and roadside habitats.

Our data suggests the presence of a core community of preferred Tulasnellaceae mycobionts. A few previous studies have found orchid interaction networks consisted of facultative keystone as well as opportunistic fungal partners (Cevallos et al. 2018, Shefferson et al. 2019). The ability of focal species to interact with multiple partners may enhance community stability, especially if the strength of interactions is asymmetric, with some hosts being more frequent partners (Dunne et al. 2002, Estrada 2007). In our study, *L. falx-bellica* populations interacted with more total fungal taxonomic units in disturbed habitats, and a bigger fraction of all interactions were not with typical OMF (**Fig. 4.5A**, third bar) in comparison to populations from MCFBR (**Fig. 4.5A**, first bar). A similar pattern was found in arbuscular and ectomycorrhizal fungi, where disturbance may result in the prevalence of a few opportunistic species (Jansa et al., 2002; Dickie et al., 2013). A relatively wide breadth of interactions indicates the functional redundancy of some partners in *L. falx-bellica*. Moreover, individual plants differed in their associated mycobiont community more within the undisturbed forest, even though their partners were mainly Tulasnellaceae (**Fig. 4.2B** and **4.3B**, first bar). Thus, by colonizing habitats that undergo abiotic environment fluctuations, an orchid may encounter a subset of its preferred mycorrhizal partners, possibly compensating the lack of preferred partners by associating with more redundant mycobionts. It remains to be tested whether these shifts in mycobiont composition have consequences for the orchid fitness.

Habitat degradation can have a severe impact, especially on epiphytic orchids due to their likely higher dependence on mycobionts in comparison to terrestrial orchids (Martos et al. 2012). Although flexibility in partner choice offers some protection from extinction (Memmott et al. 2004, Sodhi et al. 2008), environmental degradation poses a serious threat depending on host

sensitivity to abiotic changes, and from an orchid perspective, availability of appropriate phorophyte and fungal partner to sustain a plant from germination to adulthood. Costa Rica is a leader in sustainable ecotourism and has successfully implemented many conservation plans. However, deforestation still outpaces reforestation, even though the province of Puntarenas, which includes Monteverde, has recorded a net increase in forest cover over the last two decades (Zahawi et al. 2015). Since disturbance affects the orchid distribution and germination rates (Beltrán-Nambo et al. 2012, Zhang et al. 2017, Klimešová 2019, Vogt-Schilb et al. 2020), and its effects can persist for decades (Tapia-Armijos et al. 2015, Gotsch et al. 2016), a better understanding of consequences of degradation is needed to assist ecologists and land managers in protecting an exceptional orchid diversity in the tropics.

Conclusion

We have found that the mycobiont composition of endemic, narrow-ranged *Lepanthes falx-bellica* varies with habitat disturbance and phorophyte identity. Tulasnellaceae fungi were the main group of fungi across all habitats, and their relative abundance was lower in disturbed sites. The contribution of endophytic non-mycorrhizal fungi and non-rhizoctonia mycorrhizal fungi was the highest in pasture habitat. Our results correspond to general patterns of fungal colonization in response to habitat disturbance. Shift in mycobiont composition may represent orchid ability to adapt to habitats which lack preferred partners by associating with more functionally redundant partners.

4.5 FIGURES

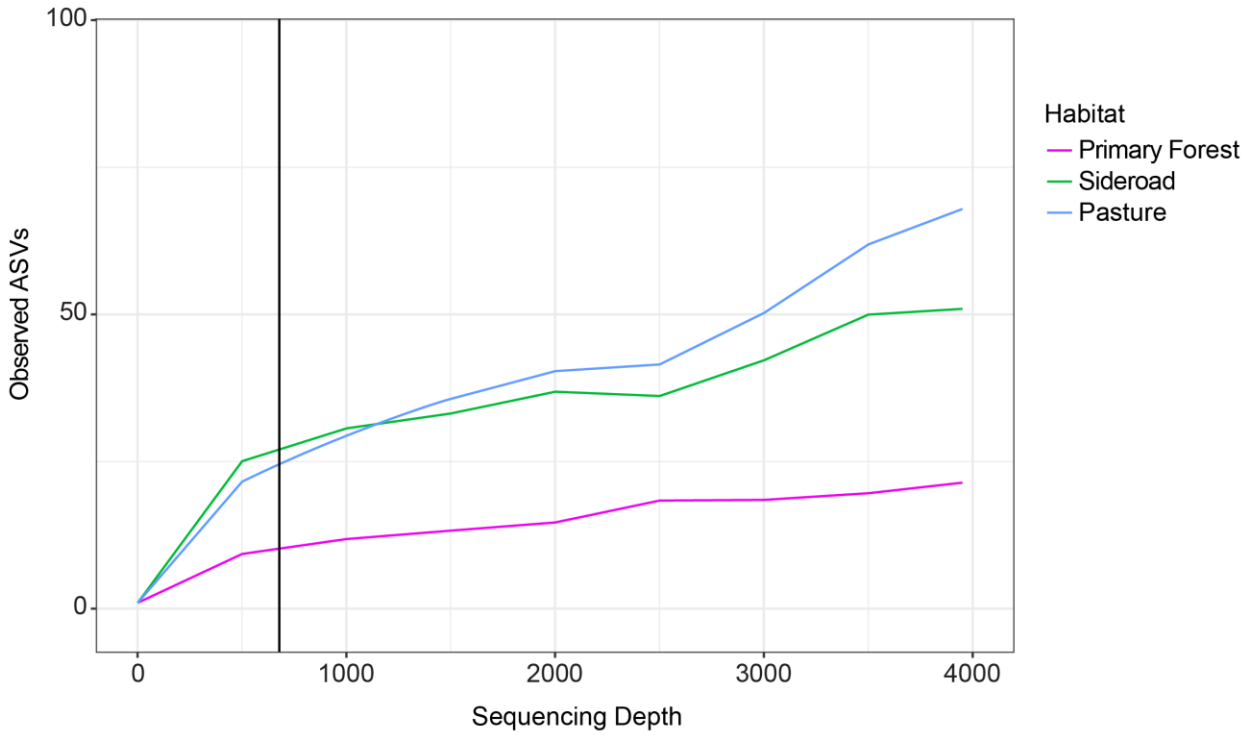
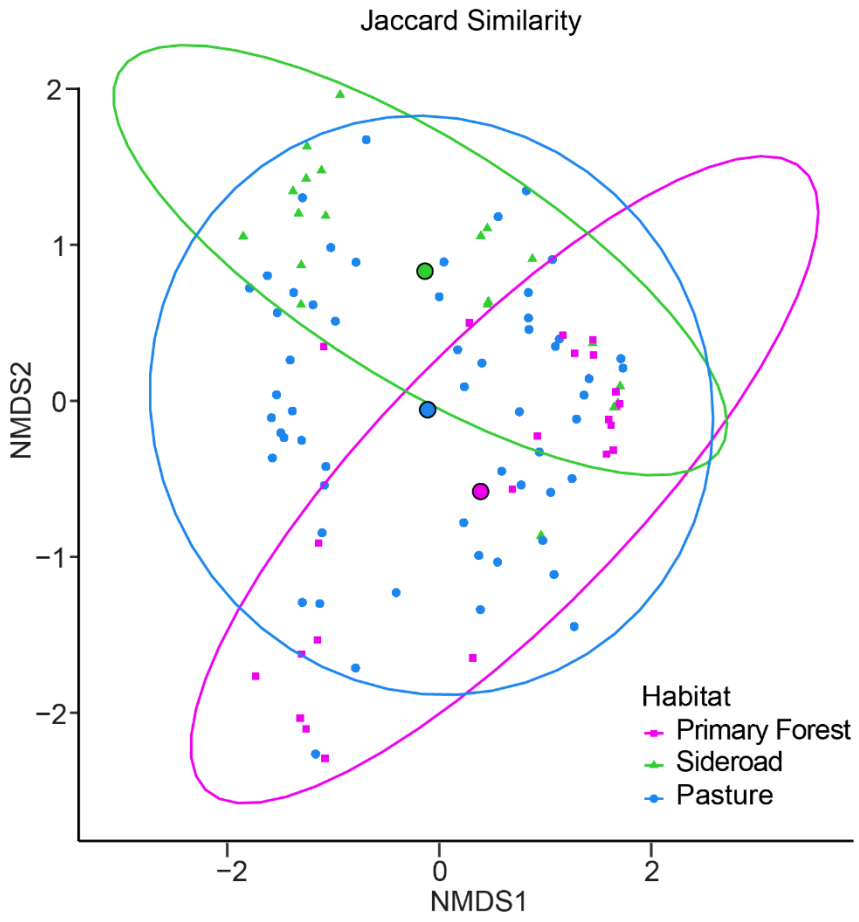


Figure 4.1. Rarefaction analysis performed on the root-associated-OTUs. Alpha rarefaction curves represent richness obtained from sequencing depth of *Lepanthes falx-bellica* orchid individuals at primary forest, sideroad, and pasture habitats. Black vertical line indicates a sampling depth of choice (679 reads) to which the data was rarefied.

A


Jaccard Similarity
NMDS2
NMDS1
2
1
0
−1
−2
−2
0
2
Habitat
Primary Forest
Sideroad
Pasture

B

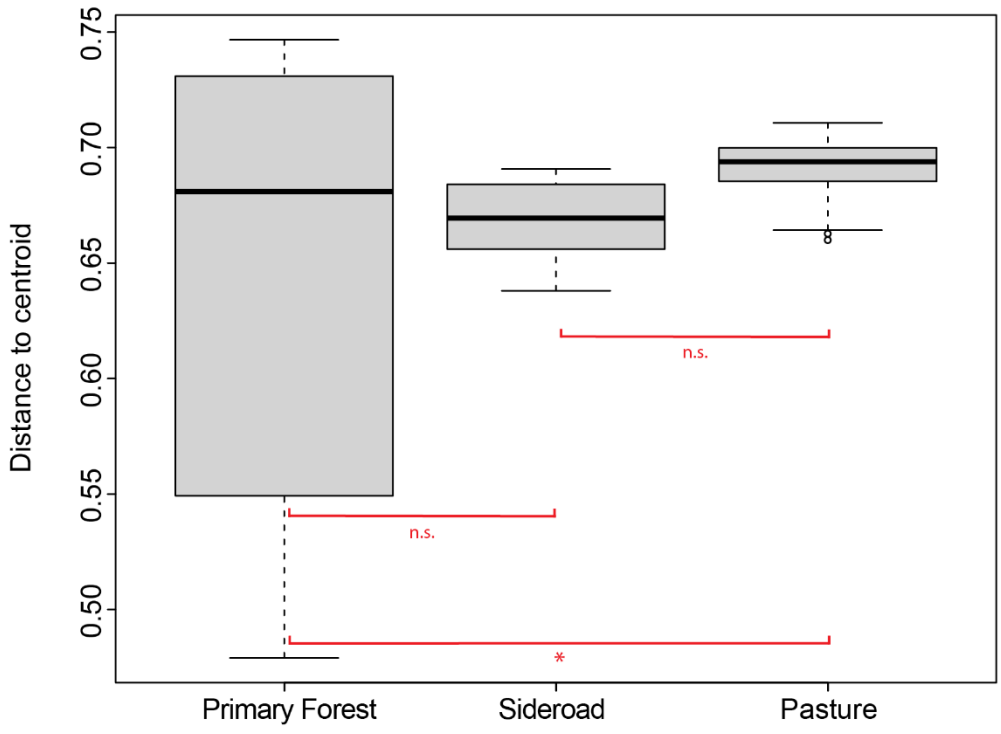
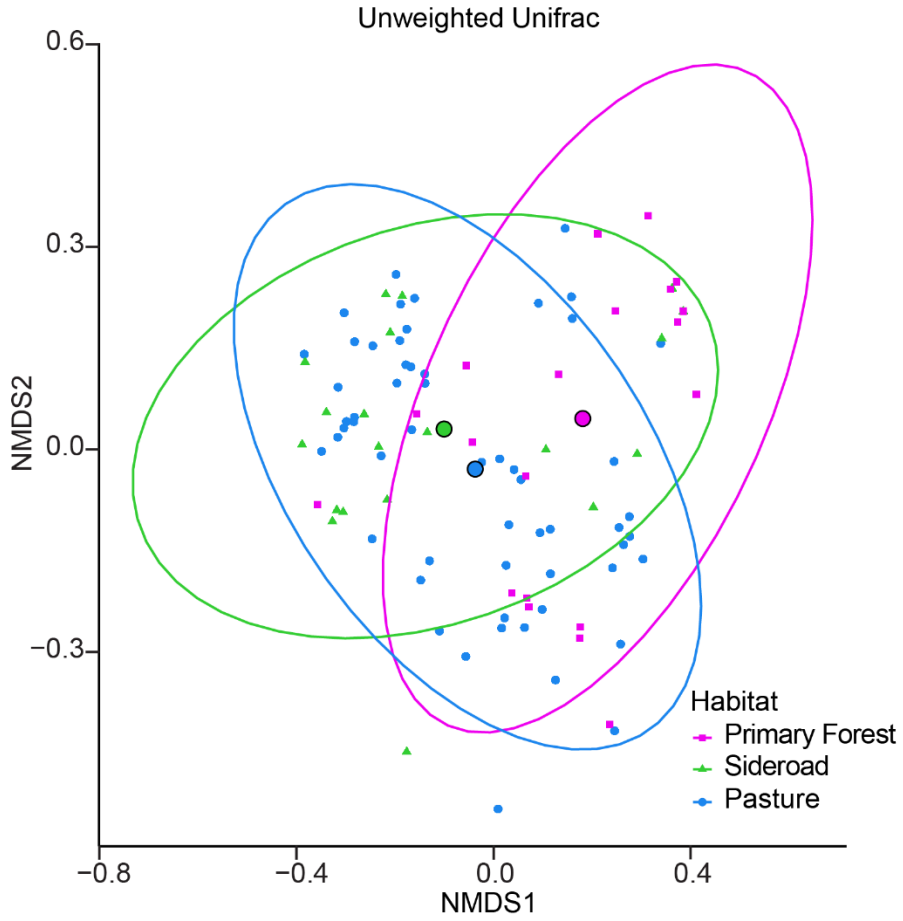

Distance to centroid
0.75
0.70
0.65
0.60
0.55
0.50
n.s.
n.s.
*
Primary Forest
Sideroad
Pasture

Figure 4.2. (**A**) Non-metric multidimensional scaling (NMDS) plot of Jaccard Similarity (converted to distances) matrix with diversity indices calculated between individual orchid plant communities. The classification of samples into one of the three habitat types is displayed by different colors and symbols of individual community scores. The 95% confidence interval ellipses are shown. Stress value: 0.16. (**B**) A boxplot representation of the analysis of multivariate homogeneity of group (i.e. habitat type) dispersions of Jaccard distances. Red bracket with an asterisk indicates a significant result for Tukey's HSD test, non-significant result is indicated with n.s.

A


Unweighted Unifrac
0.6
0.3
0.0
−0.3
NMDS2
−0.8
−0.4
0.0
0.4
NMDS1
Habitat
Primary Forest
Sideroad
Pasture

B

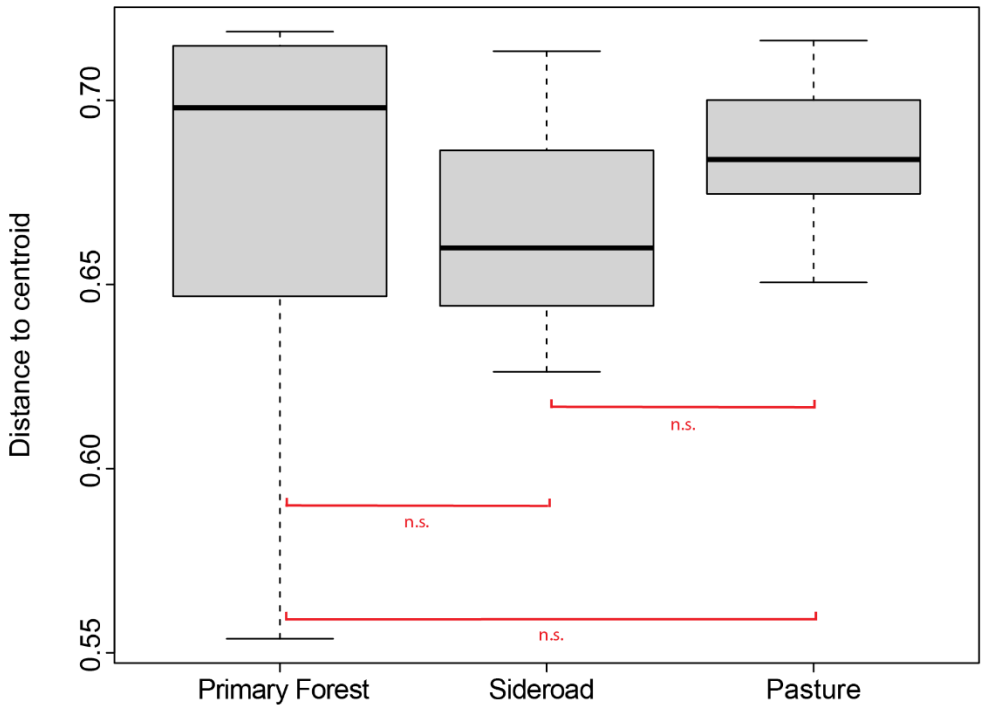
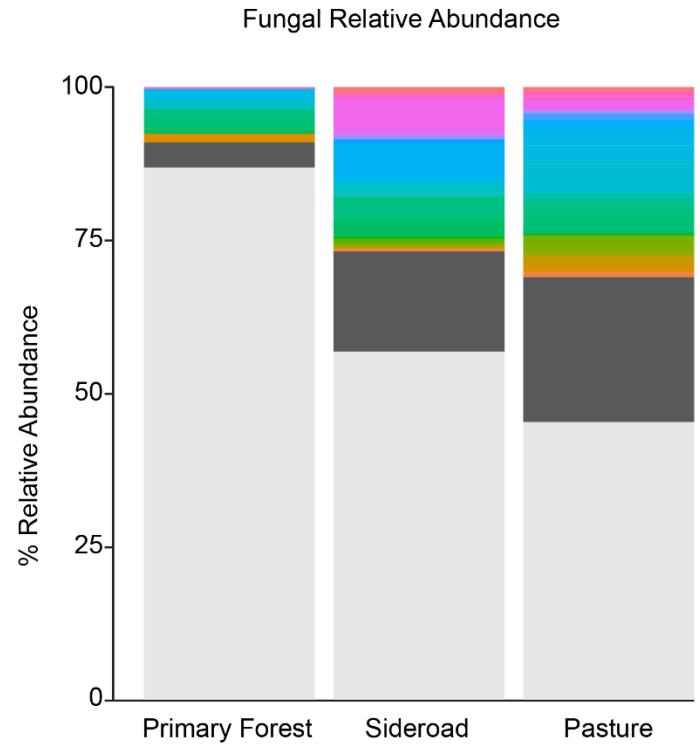

Distance to centroid
0.70
0.65
0.60
0.55
n.s.
n.s.
n.s.
Primary Forest
Sideroad
Pasture

Figure 4.3 (**A**) Non-metric multidimensional scaling (NMDS) plot of Unweighted UniFrac matrix with diversity indices calculated between individual orchid plant communities. The classification of samples into one of the three habitat types is displayed by different colors and symbols of individual community scores. The 95% confidence interval ellipses are shown. Stress value: 0.15. (**B**) A boxplot representation of the analysis of multivariate homogeneity of group (i.e. habitat type) dispersions of UniFrac distances. Red bracket with an asterisk indicates a significant result for Tukey's HSD test, non-significant result is indicated with n.s.

A



B

Family	Primary Forest	Sideroad	Pasture
Tulasnellaceae	81	69	45
Unclassified	9	15	22
Acarosporaceae			0.01
Acrocalymmaceae	0.01		0.01
Agaricaceae		0.01	
Amorosiaceae		0.01	
Amphisphaeriaceae		0.1	0.1
Archaeorhizomycetaceae		0.01	
Aspergillaceae	0.03	0.03	0.2
Beltraniaceae			0.1
Bionectriaceae	0.01	0.03	0.1
Botryosphaeriaceae		0.02	0.1
Bulleraceae		0.01	0.004
Cainiaceae		0.01	
Calloriaceae		0.04	0.03
Capnodiaceae	0.01		0.1
Ceratobasidiaceae		0.04	1
Chaetomellaceae			0.01
Chaetomiaceae			0.01
Chaetosphaeriaceae			0.04
Chrysozymaceae		0.005	0.03
Chytriomycetaceae			0.2
Cladosporiaceae		0.03	1
Clavariaceae		0.05	
Clavicipitaceae		0.01	0.01
Coniophoraceae		0.01	
Conlariaceae	0.01	0.005	
Cordycipitaceae		0.1	0.03
Crepidotaceae			0.01
Cryptococcaceae			0.01
Cucurbitariaceae		0.03	1
Cunninghamellaceae			0.1
Cyphellophoraceae	0.04		0.1
Dacrymycetaceae		0.02	
Debaryomycetaceae		0.1	0.002
Dermateaceae		0.04	0.02
Diaporthaceae		0.03	2
Didymellaceae			2
Didymosphaeriaceae	0.1	0.1	0.1
Discinellaceae			0.01
Distoseptisporaceae		1	0.1
Dothioraceae	0.01		
Entolomataceae		0.1	0.1
Entomophthoraceae			0.01
Eocronartiaceae			0.01
Erythrobasidiaceae			0.003
Exidiaceae		0.2	0.3
Exobasidiaceae		0.01	0.01
Extremaceae		0.02	0.1
Filobasidiaceae		0.02	0.01
Ganodermataceae		0.02	0.01
Glomerellaceae	0.1		0.03
Graphidaceae		0.01	
Helotiaceae	3	1	0.4
Herpotrichiellaceae	0.5	1	0.3
Hyaloriaceae	0.01		0.02
Hyaloscyphaceae	0.4	0.2	2
Hydnaceae		0.005	0.2
Hydnodontaceae	2	2	1
Hypocreaceae	0.1	0.1	0.2
Hypoxylaceae		0.003	0.03
Hysteriaceae			0.04
Incrustoporiaceae			0.3
Irpicaceae		0.04	0.03
Jianyuniaceae			0.005
Lachnocladiaceae		0.01	1
Lentitheciaceae		0.05	0.2
Leotiaceae	0.01		0.03
Leptodontidiaceae		0.01	
Leucosporidiaceae			0.1
Lophiostomataceae			0.004
Lophiotremataceae	1	0.3	0.1
Loramycetaceae	1	0.4	0.1
Lycoperdaceae		0.03	
Malasseziaceae	0.3	0.2	2
Metschnikowiaceae	0.03		0.2
Mollisiaceae			0.2
Mortierellaceae		0.01	0.1
Mucoraceae	0.6	0.2	1
Muyocopronaceae	0.1		0.2
Mycenaceae	0.002		5
Mycosphaerellaceae	0.01	0.01	0.1
Myriangiaceae		0.005	0.001
Myxotrichaceae		0.003	
Nectriaceae	0.2	3	1
Neopyrenochaetaceae	0.02	0.1	0.4
Nigrogranaceae			0.02
Omphalotaceae		1	
Ophiocordycipitaceae		0.004	0.01
Orbiliaceae	0.1	0.1	0.1
Pannariaceae	0.1	0.2	0.1
Paracladophialophoraceae	0.03	0.1	0.2
Peniophoraceae			0.3
Periconiaceae			0.01
Pestalotiopsidaceae	0.1	0.1	0.1
Phaeomoniellaceae		0.04	0.02
Phomatosporaceae		0.002	0.003
Pilocarpaceae	0.03		
Pisorisporialesfam.incertaesedis			0.01
Plectosphaerellaceae		0.02	0.2
Pleosporaceae		0.004	0.1
Pleurotheciaceae	0.3	0.3	0.2
Ploettnerulaceae			0.01
Pluteaceae		0.01	
Podosporaceae	0.01		0.01
Psathyrellaceae		0.02	0.02
Pyrenulaceae		0.04	
Ramalinaceae		0.4	0.03
Rhizopodaceae			0.1
Rhytismataceae			0.05
Rosettozymaceae			0.003
Ruineniaceae			0.01
Schizoporaceae			0.1
Sclerococcaceae		0.1	
Septobasidiaceae		0.02	0.3
Serendipitaceae		2	3
Sporidiobolaceae		1	0.004
Stephanosporaceae		0.04	
Stereaceae			0.1
Stictidaceae	0.1	0.03	0.02
Stropharіaceae	0.1	0.04	0.3
Symbiotaphrinaceae		0.3	0.1
Sympoventuriaceae	0.02	0.01	0.004
Teratosphaeriaceae	0.1	0.2	0.4
Testudinaceae			1
Thelephoraceae			0.003
Thyridariaceae			0.01
Togniniaceae			0.01
Tricholomataceae		0.04	0.2
Trichosphaeriaceae		0.1	0.3
Trimorphomycetaceae		0.01	0.01
Tubulicrinaceae		0.01	
Tympanidaceae			0.1
Umbelopsidaceae		0.1	0.2
Verrucariaceae		1	0.1
Xenasmataceae			0.04
Xenodevriesiaceae	0.04	0.1	0.5
Xylariaceae	0.1	0.4	0.2

% Relative Abundance

Figure 4.4. Relative abundance of fungal families recovered from each habitat type. The specific percentage value of each family is given in (**B**). Fungal families are in the same order within each box of the boxplot (**A**). The order of presented families is the same in (**A**) and (**B**), with the family at the bottom of the boxes presented as first in (**B**). Missing relative abundance value in (**B**) indicates absence of given family in a habitat type.

4.6 TABLES

Table 4.1. Sampling data for *Lepanthes falx-bellica*. Site ID = name of sample site, Pop ID = identity of population (i.e. tree) sampled, Tree species = phorophyte species, N_O = number of orchid individuals sampled, Habitat = name of habitat sampled. The last row contains column totals. Tree species identified by William A. Haber (P. Tuczapski, pers. comm.).

Site ID	Pop ID	Tree species	*No*	Habitat
B	2	*Quercus brenesii (= Q. cortesii)* (Fagaceae)	10	Pasture
C	3	*Miconia oerstediana* (Melastomataceae)	5	Pasture
D	5	*Calyptranthes monteverdensis* (Myrtaceae)	7	Sideroad
H	6	*Calyptranthes monteverdensis* (Myrtaceae)	9	Sideroad
H	7	*Calyptranthes monteverdensis* (Myrtaceae)	10	Sideroad
H	8	*Calyptranthes monteverdensis* (Myrtaceae)	10	Sideroad
H	9	*Rondeletia buddleioides* (Rubiaceae)	9	Sideroad
K	13	*Cojoba costaricensis* (Fabaceae)	10	Pasture
K	14	*Miconia durandii* (Melastomataceae)	8	Pasture
K	15	*Eugenia austin-smithii* (Myrtaceae)	10	Pasture
K	16	*Monteverdia recondita* (Celastraceae)	7	Pasture
K	17	*Guatteria verrucosa* (Annonaceae)	8	Pasture
K	18	*Sapium rigidifolium* (Euphorbiaceae)	7	Pasture
K	19	*Sapium rigidifolium* (Euphorbiaceae)	9	Pasture
N	20	*Guarea kunthiana* (Meliaceae)	10	Pasture
N	25	*Myrsine coriacea* (Myrsinaceae)	5	Pasture
N	32	*Salacia petenensis* (Hippocrateaceae)	8	Pasture
N	33	*Salacia petenensis* (Hippocrateaceae)	8	Pasture
U	28	*Pouteria exfoliata* (Sapotaceae)	10	Forest Reserve
U	29	*Monteverdia recondita* (Celastraceae)	10	Forest Reserve
U	30	*Guarea rhopalocarpa* (Meliaceae)	8	Forest Reserve
U	31	*Pleurothyrium palmanum* (Lauraceae)	5	Forest Reserve
7	**22**	**16**	**183**	**3**

Table 4.2. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference between habitats in the qualitative composition of orchid fungal communities based on ASV identity. Analysis has been applied to the matrix of Jaccard distance Indices Sj calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic.

Habitat. 1	Habitat 2	Sample size	Permutations	pseudo-F	p-value
Primary Forest (N=23)	Sideroad (N=20)	43	999	1.74	0.005
Primary Forest (N=23)	Pasture (N=57)	80	999	2.27	0.001
Sideroad (N=20)	Pasture (N=57)	77	999	1.43	0.001

Table 4.3. Results for pairwise permutational non-parametric multivariate analysis of variance (PERMANOVA) tests to test for difference between habitats in the qualitative composition of orchid fungal communities using phylogenetic relationships. Analysis has been applied to the matrix of Unweighted UniFrac Distances U_{AB} calculated between orchid individual plant fungal communities. Permutations = number of permutations applied, pseudo-F = PERMANOVA test statistic.

Habitat. 1	Habitat 2	Sample size	Permutations	pseudo-F	p-value
Primary Forest (N=23)	Sideroad (N=20)	43	999	5.78	0.001
Primary Forest (N=23)	Pasture (N=57)	80	999	8.21	0.001
Sideroad (N=20)	Pasture (N=57)	77	999	1.83	0.006

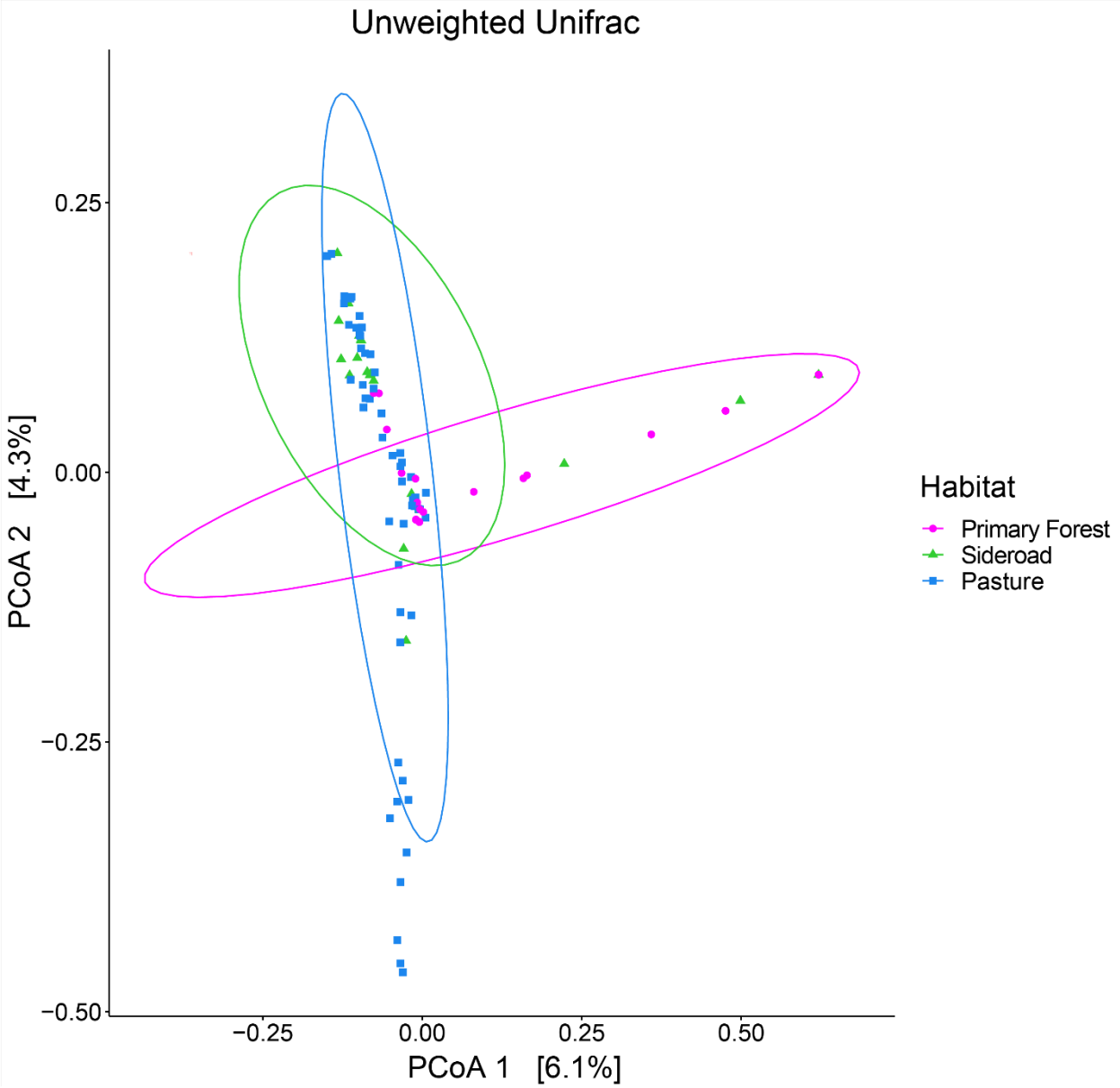
Table 4.4. Results for multivariate non-parametric analysis of variance using distance matrix of Jaccard Similarity Indices calculated between orchid individual plant fungal communities (Adonis test). Factor = factor in a fitted model, Df = degrees of freedom, SumOfSq = sum of squares, R2 = R-square value, i.e. coefficient of determination (the proportion of variance in the response variable that is explained by the independent variable), F = F-value. Number of permutations: 9999.

Factor	Df	SumOfSqs	R2	F	p-value
Phorophyte sp.	14	8.29	0.17401	1.31	0.0001
Habitat type	1	0.66	0.01379	1.45	0.0014
Residual	83	37.62	0.78990		
Total	99	47.62	1		

Table 4.5. Results for multivariate non-parametric analysis of variance using distance matrix of Unweighted UniFrac Distances calculated between orchid individual plant fungal communities (Adonis test). Factor = factor in a fitted model, Df = degrees of freedom, SumOfSq = sum of squares, R2 = R-square value, i.e. coefficient of determination (the proportion of variance in the response variable that is explained by the independent variable), F = F-value. Number of permutations: 9999.

Factor	Df	SumOfSqs	R2	F	p-value
Phorophyte sp.	14	7.99	0.16711	1.24	0.0001
Habitat type	1	0.56	0.01167	1.22	0.0185
Residual	83	38.07	0.79595		
Total	99	47.83	1		

4.7 SUPPLEMENTARY FIGURES



Supplemental Figure 4.1. Principal Coordinate Analysis (PCoA) plot depicting ordinations calculated on Unweighted UniFrac Distances U_{AB} between individual orchid plant fungal communities. The classification of samples into one of the three habitat types is displayed by different color and symbol of individual community scores.

4.8 SUPPLEMENTARY TABLES

Supplemental Table 4.1. Results of Tukey's HSD test performed following the analysis of multivariate homogeneity of group Jaccard distance score dispersions with habitat type treated as a group. Diff = difference between two means, lwr = lower bound of a confidence interval for the difference between means, upr = upper bound of a confidence interval for the difference between means, p adj = adjusted p-value.

Comparison	diff	lwr	upr	p adj
Primary Forest - Sideroad	0.02539167	-0.008839320	0.05962266	0.1866442
Primary Forest - Pasture	0.04767376	0.020016582	0.07533093	0.0002490
Sideroad - Pasture	0.02228209	-0.006815511	0.05137968	0.1675901

Supplemental Table 4.2. Results of Tukey's HSD test performed following the analysis of multivariate homogeneity of group UniFrac Distance score dispersions with habitat type treated as a group. Diff = difference between two means, lwr = lower bound of a confidence interval for the difference between means, upr = upper bound of a confidence interval for the difference between means, p adj = adjusted p-value.

Comparison	diff	lwr	upr	p adj
Primary Forest - Sideroad	-0.004032413	-0.0291402531	0.02107543	0.9226782
Primary Forest - Pasture	0.016894676	-0.0033913835	0.03718073	0.1220327
Sideroad - Pasture	0.020927089	-0.0004154942	0.04226967	0.0558767

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Unlike previous evidence suggesting generalism in orchids, we found significant specialization and phylogenetic signal in the mycobiont symbioses of sister orchid species within the evolutionarily young and rapidly diversifying epiphytic genus *Lepanthes*. Genetic data provide support for the hypothesis that a host-jumping mechanism, potentially accelerated by niche partitioning, is responsible for an escape from phylogenetic constraint in some orchids. Moreover, this study provides the first evidence of differing fungal communities in sister *Lepanthes* orchid species. Thus, niche partitioning alone may not fully explain mycobiont interactions, highlighting the need to reconsider the role of fungi in orchid speciation. Lastly, we found that the composition of root-associated fungi of *Lepanthes falx-bellica* varied with habitat disturbance and phorophyte identity. The focal orchid taxa belong to a group that is underrepresented in research, epiphytic neotropical orchids from one of the most biodiverse regions of the world, namely tropical montane cloud forest. Notably, this research represents the first use of third-generation high-throughput sequencing technology, the Pacific Biosciences (PacBio) sequencing platform, to characterize orchid mycobiont communities.

Four studied orchid species, *Lepanthes cribbii, L. falx-bellica, L. mentosa* and *L. monteverdensis* had significantly different communities of root-associated fungi in terms of richness (Faith's PD) (Kruskal-Wallis test, H-value: 18.16, p-value: 0.0004), presence-absence composition of

taxonomic units (PERMANOVA, pseudo-F value: 1.92, p-value: 0.001) (Jaccard Index) and amount of unique evolutionary history (PERMANOVA, pseudo-F value: 2.13, p-value) (Unweighted Unifrac Distance) among 50 populations. When mycobiont communities were reviewed pairwise, that of *L. monteverdensis* was the most distinct and diverse, followed by, in order, *L. falx-bellica* , *L. cribbii*, and *L. mentosa*. Specifically, richness was the highest in *L. monteverdensis* and differed most significantly between *L. monteverdensis* and *L. falx-bellica* ($\overline{PD}$ = 10.1 and $\overline{PD}$ = 6.4 respectively, Kruskal-Wallis test for Faith's PD p-value = 0.000085), next between *L. monteverdensis* and *L. cribbii* ($\overline{PD}$ = 7.5) as well as between *L. monteverdensis* and *L. mentosa* ($\overline{PD}$ = 6.7) (p-value = 0.03 and 0.004, respectively). Differences in richness between pairwise comparisons of the other three orchid species were not significant. When fungal phylogenetic relationships were considered, mycobionts of *L. monteverdensis* differed significantly from *L. falx-bellica* (p-value = 0.001), *L. cribbii* (p-value = 0.019) and *L. mentosa* (p-value = 0.001). There was a notable but non-significant difference between *L. falx-bellica* and *L. mentosa* (p-value of 0.079). We repeated these analyses with a smaller, confirmed mycorrhizal subset of the dataset, which largely corroborated the results and additionally revealed significant differences among mycobionts of *L. cribbii* and *L. falx-bellica*. This finding is consistent with some previous findings that hypothesized the role of niche partitioning on segregation of mycorrhizal communities between co-occurring orchid species (Waterman et al. 2011). However, in contrast to these studies we found different associates in recently diverged orchid species (Pérez-Escobar et al. 2017) and thus posit that the previously speculated (Thompson 1987; Cowling et al. 1990; Otero and Flanagan 2006) potential role of root-associated fungi in driving speciation in orchids should not be dismissed.

We also used phylogenetic signal and specialization network analysis to study a broad spectrum of fungal communities, as well as keystone fungi, the *Tulasnella* fungal group, and fungi identified by Indicator Species Analysis (ISA). Phylogenetic signal in the fungal host communities was the strongest and maintained when keystone fungi were investigated for *L. monteverdensis,* indicating specialization on a narrow group of its mycobionts. The signal was initially nonsignificant for the *L. cribbii* whole mycobiont assemblage, and significant for its keystone fungi, indicating apparent generalism. For *L. falx-bellica*, the signal was high but nonsignificant for keystone fungi (p-value = 0.07). Interactions of *L. mentosa* were not different from randomized, indicating generalism. We also identified significant specialization signal in the network comprising all 6543 fungal taxonomic units. This signal increased when only keystone fungi were considered (from $H_2' = 0.41$ to $H_2' = 0.52$). Both ISA and *Tulasnella* – based network analyses supported these results. Our findings are in line with the proposed conceptual model of specialization in ecological interaction networks (Shefferson et al. 2019), with examined orchids ranging from specialist (*L. monteverdensis*), through moderate specialists (apparent generalists) (*L. cribbii*, *L. falx-bellica*), to generalist (*L. mentosa*). We suggest a host-jumping mechanism potentially accelerated by niche partitioning is responsible for an escape from phylogenetic constraint in the studied orchids. Epiphytism may be an important contributor to tight specialization, mycobiont ecological assemblage, and ultimately distribution of tropical orchids.

Our data further illustrates that mycobiont composition in populations of the rare, endemic epiphytic orchid (*Lepanthes falx-bellica*) differs among habitats with three levels of disturbance, as well as among phorophyte species. Two separate analyses using different diversity metrics showed that both phorophyte identity and habitat disturbance had a significant effect on mycobiont

composition among 22 studied populations. These results are consistent with previous reports of phorophyte bias in epiphytic orchids (Tremblay et al. 1998, Rasmussen and Rasmussen 2018), as well as general patterns of shifts in fungal composition in disturbed areas (Dickie et al. 2013, Zhang et al. 2017). Tulasnellaceae fungi contributed the largest fraction of the total mycobiont composition in all sites. With increasing levels of environmental degradation, Tulasnellaceae fungi count decreased while conversely, the number of non-mycorrhizal root-associated fungi increased. Different mycobiont patterns may reflect the availability of fungal hosts in the environment and might indicate varying abiotic conditions among habitat types. The increase in interactions with non-mycorrhizal endophytes in disturbed habitats may reflect opportunistic interactions and the absence of some key orchid mycorrhizal fungi. In summary, we demonstrated potential role of niche partitioning in segregating fungal communities in orchids, we found evidence in orchid – mycorrhizal networks, as well as impact of habitat degradation in structuring mycobiont communities. Understanding these interactions offers valuable insight into the dynamics of ecological networks and the evolutionary processes shaping orchid diversity. Most importantly, this knowledge is crucial for effective orchid conservation and reintroduction efforts, particularly in biodiverse tropical regions where orchids are a vital component of the local flora.

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