ABSTRACT

Title of Dissertation: **"INVESTIGATING THE HYPERDIVERSITY OF FUNGAL ENDOPHYTES IN WILD RUBIACEAE TROPICAL PLANTS AND COFFEE PLANTATIONS".**

Humberto Castillo-González, Doctor of Philosophy, 2022

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Fungal endophytes are an essential component of a plant's microbiome, their effect spreads to fitness, disease dynamics, stress tolerance, water acquisition and nutrient uptake. Plant ecosystems, from natural forest to plantations bear the indelible signature of its presence. The current investigation was designed to understand the diversity of endophytes in the *Rubiaceae* family, in plants associated to natural and managed ecosystems. The effect of location, leaf developmental stage, tissue type, host genotype, and anthropogenic interference was evaluated through amplicon sequencing. Costa Rica served as base for the sample collection. Leaves and sapwood from a variety of tropical plant species were collected in old-growth natural forests and foliar tissue from domesticated coffee plants were sampled in two plantations under different management. Fungal diversity was assessed by metabarcoding using the ITS2 nrDNA region fITS7 – ITS4, and library sequencing was completed by Ion Torrent. We identified a hyperdiversity of endophytes inhabiting these plants and were able to isolate a total of 659 fungi from coffee leaves. This investigation

provides relevant information about overall community composition, the ecological drivers of community assemblage and the characteristics of the fungal endophytic communities, including potential interactions among the identified taxa. Endophytes may harness the potential to transform agriculture and conservation science, however we currently lack the knowledge to engineer microbial communities through breeding or management. It is imperative to continue the efforts on understanding community functions and dynamics, and how host, endophyte interactions, and other ecological and human-related mechanisms influence their diversity in both forest species and agronomically important crops.

INVESTIGATING THE HYPERDIVERSITY OF FUNGAL ENDOPHYTES IN WILD RUBIACEAE TROPICAL PLANTS AND COFFEE PLANTATIONS

by

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Dedication

A mi mamá, que siempre me alentó a que alcanzara mis sueños. Mi historia comienza en la tuya.

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The culmination of this dissertation wouldn’t have been possible without the support from a group of people who provided company and valuable advice when I needed it. It has been a long journey but I’m grateful I made it to this point.

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Chapter 1: On the significance of plant microbiomes in agricultural and forest ecosystems.

All higher organisms are inhabited by hundreds of thousands, if not, millions of microbes including fungi, bacteria, archaea and viruses that form complex ecological communities that influence their hosts in many ways due to the collective metabolic activities and interactions that they present (Berg et al., 2016; Braga et al., 2016). As our knowledge on microbiomes increase, the potential they harness for transforming modern agriculture continues to be expanded, opening new avenues for a plausible next green revolution, in which agricultural practices will be adapted and tailored not only to the plants' genetic makeup but their associated microbiota as well (Oburger et al., 2022; Strobel, 2018). In recent years, a plethora of studies have demonstrated the crucial role that plant microbiomes play in their hosts, providing a variety of functions and molecular signals needed to maintain proper health and fitness (Foo et al., 2017; Sudheer et al., 2020; Trivedi et al., 2020). This highlights the potential they have for the development of new preventive and management strategies in the plant conservation and agricultural sectors. Yet, microbiome functions and interactions are complex and remain poorly understood.

The diverse and multilevel relationships that exist between plants and the microbes present in their environment can range from beneficial to detrimental (Occhipinti, 2013) and are largely determined by complex physical and chemical interactions. Plants have depended on the assistance of microorganisms since they colonized dry land, about 450 million years ago (Card et al., 2016). Currently, plant tissues constitute one the largest habitat surfaces for microorganisms on Earth (Koskella, 2020), and, to this date, every plant and every tissue and organ of a plant has been found to host microbial communities (Harrison & Griffin, 2020);

to study them, however, researchers have focus on three larger areas: the rhizosphere, the phyllosphere, and more recently, the endosphere (Foo et al., 2017). The microbial diversity coupled to these ecosystems is believed to contribute a number of genes that exceeds those of the host plant, providing a large reservoir of additional genetic information and physiological diversity to the plant, reason thereof to consider it a "second genome" (Braga et al., 2016; Gopal & Gupta, 2016).

Evidence shows that plant associated microorganisms often play roles in plant competition, pathogen defense and stress reduction (Hardoim et al., 2015), they can also help their hosts by suppressing diseases, stimulating growth, and mobilizing nutrients (Mengistu, 2020). A prominent group of microorganisms found to affect plants in these ways are the endophytes. Fungi are the most frequently encountered type and embody a prominent, hyperdiverse and polyphyletic group that can inhabit and thrive asymptomatically in the tissues of the plants, at least for a part of their life cycle, in a complex, transient and symptomless association in which none of the interacting parts is negatively affected (Kusari et al., 2012). However, the origins and reasons that explain this interaction and how equilibrium is attained in this relationship are not yet completely unraveled.

The high diversity of endophytes, from different individual, taxonomic, and functional groups, often found within a small area of plant tissue could indicate that the inside spaces of a plant represent a complex mosaic environment entailing a rich continuum of metabolic profiles and antagonistic and synergistic interactions. To colonize the plant, endophytes must work out strategies to protect themselves from plant defense mechanisms (Khare, Mishra, & Arora, 2018), including the presence of toxic metabolites such as phenols, phenolic glycosides and phytoanticipins, among others. Successful symbionts counter these compounds by mechanisms that include evasion, tolerance, detoxification, neutralization, degradation and may even use them as a carbon/energy source or as cues to propagule attachment and germination (van Bael et al., 2017; Zambell & White, 2017).

While the mechanisms by which endophytes persist within and colonize plant tissues remain poorly understood, ecological adaptations and differences between fungi may influence the community structure and affect overall assemblage over time by enrichment or exclusion of certain species. In this sense, endophytes encompass a huge diversity of microbial adaptations that have been developed in special and sequestered environments (Owen & Hundley, 2004). Their diversity would expand by niche partitioning and plant compartments would host differently adapted populations of fungal taxa. Thus, the host plants may play critical a role in selecting the lineages and functions of the community. To this point, it's been shown that, in the case of horizontally transmitted endophytes, mostly environmental and geographic factors influence their functional and taxonomical diversity (Chagnon et al., 2016a; DeMers & May, 2021; Jones et al., 2019).

A better understanding of these groups and the dynamics of community assembly could transform the way we think about and manage plant stress resistance facing the constant challenges and imminent threats in conservation and agriculture. Plant microbiome characteristics and functions could provide a unique insight into understanding how plants adapt to certain environmental conditions and overcome biotic stress via their microbial communities (Oyserman et al., 2018; Turner et al., 2013). Learning to shape a plant's microbiota to alter its performance could be beneficial in sustainable agriculture to arm our crops with the tools they need to fight off pathogens and grow in stressful environments (Razzaq et al., 2021). This could potentially reduce the number of chemical inputs used in agriculture and the emissions of greenhouse gases, a desirable goal parallel to nourishing the world's growing population.

As a result, there's an increasing interest in host mediated and multi-generational microbiome selection in which beneficial microbes are used to inoculate seeds, seedlings, soil and/or the rhizosphere in order to develop breeding strategies to select for plant varieties that have enhanced symbiosis with these microorganisms and display desirable characteristics (Orozco-Mosqueda et al., 2018).

Beyond enhancing crops performance, plant-associated microbiomes could also contribute to other areas such as human health. As customers patterns reveal a growing interest in consuming fresh raw produce, we must consider that those microbes are part of our diet and they can either improve our health, cause outbreaks of diseases by transferring possible pathogens or on the opposite, mitigate pathogen survival (Akhtyamova, 2013; Jackson et al., 2015). Another field where a plant's microbiota might have an impact is phytoremediation, contaminants that are taken-up by the plant can be detoxified by plant secondary metabolism, that could be modulated by its microbiota, or via stimulation of plant-associated microorganisms (Thijs et al., 2016; Weyens et al., 2015).

In plant disease management, examples of successful cases have been found via transference of microbiome in the rhizosphere. Rather than identifying, testing and applying potential biocontrol agents, research in suppressive soils has attempted to understand how indigenous microbiomes can reduce disease, by mixing disease suppressive soils with disease conducive soils (Schlatter et al., 2017). In a similar fashion, a recent study incorporated foliar microbiomes into plant conservation. Evidence suggests that naturally occurring fungal foliar endophytes partially determine disease severity in plants. If endophytes are modifiers of plant disease, transferring foliar microbiome to endangered plants could be an effective approach to prevent their extinction and support conservation efforts (Christian, Herre, et al., 2017; Zahn & Amend, 2017).

Given that feeding the growing world population and climate change are big challenges to overcome in the near future, the integration of beneficial plant microbiomes seems like a feasible option for optimizing the reliability, resource use, and environmental impacts of food production (Sudheer et al., 2020). Nevertheless, the study of plant microbiomes could be more difficult and complex than expected. Currently, our lack of knowledge on the factors and rules driving community assemblage, the diverse origin of the microorganisms and its interactions, represent a barrier to fully understand the ecology of plant-associated microbiomes and their impact in traits underlying plant homeostasis (de Souza et al., 2016 & Busby et al., 2017).

The recruiting process, maintenance of populations and relationship with the plant and other symbionts, as well as how to prevent lifestyle switches that could lead to pathogenesis are some other gray areas that need further comprehension (Kusari et al., 2012) Considering the above aspects, it remains clear that although microbiomes represent a useful tool for improving agriculture, designing an effective breeding program that includes them it's probably much harder than anticipated. Before applying microbiome transplants to restore a plant's balanced microbial composition we first need to define whether the microorganisms we aim to put accurately represent the populations that are associated with healthy plants in nature. Then, we must link these data to genomic approaches, aiming to contest the host and possible microbial genotypes to optimize their association, for instance, to find out if genetic traits from the host plants respond to specific endophytes or vice versa (Turner et al., 2013).

Fungal endophytes with desired traits and functions could be selected and combined to form “synthetic microbiomes”. One advantage from this approach is that the composition is controllable. However, the development of synthetic microbiomes is limited, first and foremost by our understanding of the core microbiome and second because only a small portion of the plant associated microorganisms is culturable, so synthetic inoculants would be missing important contributors. It might seem like a titanic task, but the next-generation agriculture should aim to customize the practices and tools to the crop individual conditions. The microbiome definitely has the potential to transform agriculture and lead it the way forward to a new age. Optimal therapies should be chosen based on molecular diagnostics precedent, considering the genetic context of the local microbiome and the host with the purpose to improve and stabilize the effects of microbial inoculants (Schlaeppi & Bulgarelli, 2015).

This study aims to assess the diversity of fungal endophyte communities of an important plant family, *Rubiaceae*. With more than 13000 species distributed in 637 different genera, this family is one of the largest of the angiosperms (Kiehn & Berger, 2020). *Rubiaceae* are mainly tropical woody plants (Bremer & Eriksson, 2009) and present a wide diversity of phytochemical substances such as iridoids, alkaloids, anthraquinones, terpenoids, flavonoids and other phenolic derivatives and it has been thoroughly studied from this perspective (Martins et al., 2015; Mongrand et al., 2005). It also contains cultural and economically important genera including *Cinchona*, *Genipa*, *Uncaria*, *Psychotria*, *Rubia* and *Coffea*.

Coffea consists of 125 species of flowering plants, two of which are of major importance as the source of coffee: one of the most consumed beverages in the world. More than 50 countries around the world grow this crop, being Brazil, Vietnam and Colombia the top producers (International Coffee Organization, 2021) and approximately, 25 million small producers rely on this crop for a living. Unfortunately, its low genetic diversity and the peculiar requirements this plant has to grow: the right combination of temperature, soil and rainfall, make it a crop extremely vulnerable to climate change and pathogens, especially coffee rust, a fungal disease, that can easily reach epidemic levels causing millions of losses every year (Merle et al., 2020), threatening the livelihoods of thousands of farmers, especially in underdeveloped countries. Traditional methods have failed to successfully fight and prevent this disease and other threats making it imperative to research different approaches that might lead to more effective ways to deal with these issues.

The present research is driven by some basic ecological questions such as what fungal endophyte species are present in the coffee family, how diverse they are taxonomically, which features are ubiquitous in the communities, and which are unique to certain plants or habitats, and whether fungal interactions can be predicted from the species present. Unfortunately, the mechanisms that drive fungal endophyte communities' assemblage and structure in the tissues of coffee and other plants within *Rubiaceae* are not well understood. This research intends to fill substantial gaps in biodiversity knowledge such as how ecological drivers affect the diversity of foliar microbiomes. Through high throughput sampling of fungal endophyte diversity, we intend to identify niche partitions associated with the host ecosystems, in wild and native habitats, such as natural old-growth forest, and anthropogenic interfered systems such as plantations, as well. Thus, the information here generated could be used to produce or improve strategies of disease management in coffee plantations or support the efforts of conservation in tropical forests. The extent to which the plant host or the environment shape communities of foliar endophytic fungi is an important question. How domestication has affected the diversity of plant associated microbes or the plant's ability to select, recruit and maintain metabolically diverse endophytes is crucial as well. As abiotic factors induce changes in the plant's biochemistry and physiology, fungal communities are expected to adapt accordingly, causing community disbalances. The implications of this context reach and impact agricultural management, and climate change science.

The present dissertation is structured in the following way: Chapter 2 explores the effect of region, leaf developmental stage, and tissue type on community composition and diversity of endophytes spanning a range of tropical plant species within *Rubiaceae*. The fungal diversity of leaves and sapwood tissues collected at several sites in old-growth natural forests, in Golfito and Guanacaste, Costa Rica was assessed via next generation sequencing and metabarcoding approaches. Plant tissues exposed to different environments in time and space could potentially be influenced by structural and chemical properties that change through ontogeny. In order to predict the potential impact of many of these fungi in agricultural and forest ecosystems, it is crucial to understand the degree to which apparent patterns of host colonization are dictated by the host, the endophyte, or other ecological mechanisms.

The interactions between these microorganisms and their host can have important implications for agronomically important plants such as *Coffea arabica*. Chapter 3 presents a comprehensive investigation on how location, coffee varietal, and agricultural management practices can affect the fungal assemblage pertaining to the foliar tissues of this this crop species. To provide data that could be useful in future work for shaping coffee microbial communities, a combination of culture dependent and independent methods was used to ascertain an accurate picture of the coffee-associated endophytes and the potential implications of agricultural management strategies on community assembly.

Plants in farms may have impaired associations with microbes as a result of domestication. Crops might lack the required genetic and physiological features needed to select for beneficial endophytic populations. In agricultural ecosystems, it is expected that the number and type of endophytic interactions will be reduced in comparison to their wild counterparts. In Chapter 4, the associated fungal endophytic communities of wild *Rubiaceae* and domesticated coffee are compared to understand the extent to which the transition from natural to agricultural systems affects the diversity of the foliar endophytic microbiome and whether the fungal endophytic taxa present in these communities display habitat preference. Investigating the degree of ecological coherence across plant species, growth ecosystems and geographical locations would indicate the level of association of endophytic taxa with this family of plants. Endophytes exhibiting recurrence or specificity could potentially play a significant ecological role in forests and plantations.

This chapter also aims to gain a deeper insight into the potential interactions among the identified fungal taxa in native and domesticated habitats. Co-occurrence data does not reproduce the complexity of ecological network interactions happening in the plants but provide interpretable information about community assembly that can be used to manage microbial communities of crops in plant health. Chapter 5 presents the future directions of research in this area. Overall, the purpose of with this work is to determine the fungal groups dominating the endophytic communities of healthy *Rubiaceae* plants, including coffee, the taxa driving the potential differences observed in community assemblages and the putative life strategies associated with each scenario.

As the level of influence and the widespread effects that endophytes have on the physiology of the hosts become more apparent, the contribution of this investigation to the state of the art is not minor. There are still cryptic and undiscovered taxa, whose distribution, presence and ecological roles are unknown. The processes that govern the endophytic assembly dynamics, the myriad of interactions that happen within the plant tissues and the potential ecological services these microorganisms could offer to their host, are vital to understand if we pretend to modify plant associated microbial communities to improve their performance in a given ecosystem, especially under the imminent threat of climate change and the constant challenges in agriculture.

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Chapter 2: Fungal endophytic communities in tropical forests are shaped by geographic location and plant tissue type.

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Keywords: fungal endophytes, ecology, Rubiaceae, tropical forest, diversity, sapwood.

ABSTRACT

Fungal endophytes are major drivers of tropical forest dynamics; they can influence plant fitness by stimulating growth, suppressing diseases, promoting stress tolerance/resistance, and mobilizing nutrients. The current experiment was designed to understand the effect of region, leaf developmental stage, and tissue type on community composition and diversity of endophytes spanning a range of tropical plant species. We hypothesized that endophyte communities would be affected by all these factors and those changes would be detectable using amplicon sequencing. Leaves and sapwood belonging to plants in the Rubiaceae family were collected in old-growth natural forests, in Golfito and Guanacaste, Costa Rica. Fungal diversity was assessed by metabarcoding using the ITS2 nrDNA region fITS7 – ITS4, and library sequencing was completed by Ion Torrent. Most identified

ASVs belonged to the Ascomycota phylum. The orders Botryosphaeriales and Glomerellales contributed the most species to the endophytic assemblages. Although no specific fungal communities were detected at plant level, the total colonization frequency and species richness of endophytic fungi were statistically higher in Golfito. No statistically significant differences were observed between mature and juvenile leaf tissue and, in general, leaves presented richer and more diverse assemblages than sapwood. Since plant tissues were exposed to different environments in time and space our results could be influenced by structural and chemical properties that changed through ontogeny. In order to predict the potential impact of many of these fungi in agricultural and forest ecosystems, it is crucial to continue the efforts on understanding the degree to which apparent patterns of host colonization are dictated by the host, the endophyte, or other ecological mechanisms.

INTRODUCTION

Tropical forests, especially in Latin America, support the highest diversity of terrestrial plants on Earth (Raven et al., 2020). In these biomes, plant tissues, as a source of labile nutrients and niche space (O'banion et al., 2020), can maintain an extraordinary mosaic of microbial assemblages (Arnold & Lutzoni, 2007; Oita et al., 2021; Suryanarayanan et al., 2011), whose distributions influence hosts and drive forest dynamics at diverse levels ranging from mutualism, through commensalism, to pathogenicity (Del Olmo-Ruiz & Arnold, 2017; Donald et al., 2020). Plant fungal endophytes that colonize leaves and wood are abundant and diverse (Oita et al., 2021), although traditionally understudied compared to mycorrhizal and free-living forms.

Although the mechanisms that fungi use to colonize plant tissues remain poorly understood, endophytes are typically transferred horizontally (Chagnon et al., 2016b). Environmental and geographic factors are often described as drivers of fungal diversity, selecting the lineages and functions of the community (Solis et al., 2016). Understanding the factors that determine the distributions and maintenance of species, and the underlying environmental constraints that shape the structure and composition of endophytic assemblages is crucial. Endophytes can positively influence plant fitness and health, protecting them against abiotic stress, herbivores and pathogens (Higgins et al., 2014). Furthermore, they may be new sources of bioactive compounds with novel chemical features (Terhonen et al., 2019).

Endophytes exhibiting recurrence or specificity play a significant ecological role in forests (Guo et al., 2008; Molina et al., 2020). Under the premise that high diversity maintains the overall integrity of an ecosystem (i.e. biological insurance theory), the hidden diversity of endophytes can enhance the fitness of individual trees and by extension shape forest composition and demography (Terhonen et al., 2019). Efforts to understand endophytic diversity are necessary for predicting endophyte function in association with hosts, specific environmental conditions, or entire ecosystems. In this sense, tropical forests, the most diverse and productive biomes on Earth, are an ideal setting for studying patterns and interactions of endophytes with host plants.

Rubiaceae is one of the largest and most complex families of angiosperms with over 13,000 species. Plants in this group range from weedy herbs to large trees and occupy many habitats and biogeographical regions in all continents (Bremer & Eriksson, 2009). This family includes several cultural and economically important genera (e.g., *Cinchona*, *Coffea*, *Genipa* and *Psychotria*). Costa Rica harbors a rich *Rubiaceae* flora that includes the majority of its genera and presents most biogeographic elements from the neotropics (Kiehn & Berger, 2020). Although it extends to only 51,100 km^2 (0.03% of the global land surface) Costa Rica is a kaleidoscope, comprising all main terrestrial ecosystems found in the Central American region. It contains 12 life zones distributed altitudinally and 6 climatic transitions occur, including: tropical rainforest, seasonally dry tropical forest, tropical cloud forest, temperate (oak) forest, and high elevation ecosystems such as *páramo*. Although biodiversity estimates at global scale are imprecise, Costa Rica is home to about 5% of all known species, including at least 2300 fungi and 11500 plants (Kappelle et al., 2016).

Characterizing the endophyte communities within *Rubiaceae* and the processes shaping their assemblages could provide a unique insight into understanding endophyte niche preferences in tropical forests. In this study, we investigated endophytic communities at different locations, at different leaf developmental stages, and between tissue types. We hypothesized that endophytic communities would differ between regions, both in diversity and in composition, as a response to their variable environmental conditions. Secondly, we hypothesized that communities would differ between plant species; and within a tree, endophytes would also differ between young and mature leaves and between leaves and sapwood. In addition, to testing these hypothesizes, we aimed to understand which fungal groups drove such differences.

METHODS

Site description.

Old-growth forests from two locations were sampled. Golfito, in the South Pacific region, was visited in April 2017. All sampling sites there belong to the typical tropical rainforest: no defined dry season, intense rainfall, 2-3 layers, evergreen, and dense. In Guanacaste sampling took place in May 2017. We selected two different regions (North and North Pacific) that included three sub climates: seasonal dry forest, semi-deciduous forest influenced by monsoons and the forested slopes of a volcano, influenced by a mountain system (Solano & Villalobos, 2001) (Figure 1). Climate data, including mean annual temperature, mean annual precipitation, and elevation were obtained from the WorldClim database (Table 1) (Fick & Hijmans, 2017).

Sampling.

Young and mature healthy leaves from 50 Rubiaceae plants (distributed in 47 species from 27 genera, 10 tribes and 3 subfamilies) were collected (Supplemental Information 1). Leaves from three different branches per plant were sampled. In order to identify clearly different developmental stages, one newly emerged and one mature leaf, separated by three nodes were selected.

For 23 of the plants, a sterilized knife was used to cut a sliver of outer bark at shoulder height to expose the alburnum and one piece of living sapwood tissue, sized ca. 30 x 5 mm, was excised from the exposed area. All collected samples were put in a clean plastic bag and placed on ice for transport to the field workstation.

The samples were processed within six hours of collection. A section of each leaf of ca. 20 x 4 mm was cut and surface sterilized using sequential washes of 2% commercial bleach (1 min), 70% EtOH (30 sec) and sterile distilled water (30 sec). Finally, leaves were dried with autoclaved tissue paper, and stored in microtubes containing silica gel before being transferred on ice to the laboratory for DNA extraction. Sapwood samples were only singed and then deposited in the microtubes.

Fungal community profiling.

Samples were transferred to a pre-filled 500-micron Garnet and a 6-mm Zirconium Grinding Satellite bead tube (OPS Diagnostics LLC, New Jersey, USA) and ground using a FastPrep® bead mill (MP Biomedicals, Santa Ana, California, USA.). Each tube was treated to a grinding cycle of speed: 6.0 m/s for 45 seconds, or until no visually recognizable fragments remained. Total DNA was extracted using the QiagenDNeasy Plant Mini Kit® according to the manufacturer's instructions (Qiagen, Hilden, Germany).

DNA library preparation, PCR and next generation sequencing of the ITS2 nrDNA region fITS7 and ITS4 (Ihrmark et al., 2012) were contracted to an external party (Naturalis Biodiversity Centre, The Netherlands) using Ion PGM TorrentTM technology (Thermo Fisher Scientific, Waltham, MA, USA).

ASV classification and taxa assignment.

Primers and barcodes were trimmed from each read using the command line tool cutadapt (Martin, 2011) and subsequentially processed using R v. 4.0.3 in RStudio, version 2022.2.0.443 (RStudioTeam, 2022). Raw reads were subjected to a data curation process including quality filtering, trimming and chimeras checking. Any spurious contigs were removed using the package DADA2 v1.18.0 (Callahan et al., 2016), which was also used to classify the DNA reads into Amplicon Single Variants (ASV). Taxonomic assignment of ASVs was conducted using the UNITE database v8.2 (Abarenkov et al., 2020) training set from the DECIPHER package, v 2.18.1 (Wright, 2016).

Diversity and community structure analyses.

Statistical analyses and plotting were conducted using the phyloseq v1.38.0 (McMurdie & Holmes, 2013), vegan v2.5.7 (Oksanen et al., 2020), microbiome v1.12.0 (Lahti et al., 2017) and ggplot2 v3.3.5 (Wickham, 2016) packages. Endophyte alpha diversity for each of the datasets was characterized by calculating the Shannon, Simpson, and Chao1 indexes for each variable followed by ANOVA.

Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of ASV abundances were used to visualize community patterns (beta-diversity) using the function 'metaMDS' from vegan (100 random starts) and statistical differences in endophyte assemblages between different variables (i.e.: location, tissue, developmental stage) were tested with a PERMANOVA.

Relative abundance of the top families associated with every variable was calculated. The overall prevalence (number of occurrences among samples) of these different taxonomic groups was computed, and to estimate how strongly they deviate from a random preference among the plant partners available at the study sites we calculated the d' index of the specialization using the bipartite package, v 2.17 (Dormann et al., 2008), function 'dfun'. ASVs were statistically classified into habitat generalists or specialists using a multinomial species classification method (CLAMtest) in vegan; and indicator taxa (ASVs with p-values < 0.05) in the different variable categories were identified using the 'multipatt' function in the indicspecies package, v1.7.12 (De Cáceres & Legendre, 2009). Finally, to determine if the specific functional groups of fungi differed between the different habitats, we classified ASVs from the top 17 families into primary and secondary lifestyles using the fungal functional database FungalTraits (Põlme et al., 2020).

RESULTS

A total of 6,474,859 reads passed bioinformatics quality control filters. The mean number of sequences per sample was 20,581 for leaves and 26,490 for sapwood. We identified 3,129 putative taxa (ASVs) belonging to at least 241 different genera. ASV identification rate was 79%, 63.5% and 46% at the order, family and genus levels respectively. The species richness or number of unique ASV recovered per sample (triplicates in the case of leaf tissue) ranged from 2 to 327, with a mean of 66.

All datasets had a high number of single occurrences, and no asymptote was reached in the species accumulation curves (Supplemental Information 2) indicating that the datasets are incomplete, and the number of samples was not sufficient in capturing the expected endophyte diversity (Hill's $q = 0$). However, when focusing on dominant species only (Hill's $q = 2$), all curves, except for sapwood, reached a plateau.

Fungal endophyte assemblages differed in location and tissue type (Table 2). The alpha diversity indices (Shannon, Simpson and Chao1) were higher in the samples from Golfito in comparison to Guanacaste ($p \leq 0.05$). Samples from leaf and sapwood tissues also harbor different fungal communities ($p \leq 0.001$), with leaves having richer assemblages. The endophyte communities in mature leaves were slightly more diverse but still considered similar to those in new leaves. Chao1 was statistically significant between developmental stage, but no differences were detected by Shannon or Simpson (Table 2).

Using non-metric multidimensional scaling (NMDS) we observed differences between location and dispersion, with Guanacaste points appearing more scattered (figure 2A), three outliers were removed for visualization purposes. Community dissimilarities between these variables were corroborated via PERMANOVA, which indicated significant separation in fungal assemblages when comparing between the different locations ($p < 0.001$). In the case of tissue type, the PERMANOVA was also significant ($p < 0.001$), and no clear location effect was observed in the plot, however, the dispersion of the samples varied: leaves converged on the interior of the cluster and sapwood, being more variable, was found mostly around it; two samples were removed for better visualization (figure 2B). Looking at just the leaves at the two different development stages, no effect was detected, there was a high overlap between the fungal communities (figure 2C), and the PERMANOVA indicated no significant differences ($p = 0.8671$). Fungal endophyte community composition did not cluster based on individual plants or at any higher taxonomic level (i.e., genera, tribe, subfamily), the pattern for tribe is shown in Figure 2.

The Ascomycota phylum was most abundant, representing 89% of all fungal ASVs. Within this group, the most abundant and prevalent classes were Sordariomycetes followed by Dothidiomycetes. The classes Tremellomycetes and Agaricomycetes were the most abundant and prevalent Basidiomycota, making up 9.5% of the taxa. We characterized endophytic assemblages dominated by a few abundant dominant taxa and many rare taxonomic groups, e.g., we identified 150 different families (Supplemental Information 3) from which Glomerellaceae, Phyllostictaceae and Sporocadaceae composed the bulk of biodiversity (31.42%) present within the endophytic assemblages (Figure 3).

The most prevalent fungal families were not the most abundant in all cases, but they tended to correspond in most major families (Figure 3). The top 17 families, whose presence made up at least 1% considering all habitats, made up to 69% of the total relative abundance. With the d′ index, we determined that the most prevalent and abundant families had values closer to 0 (no specialization). We also found indication that members of these fungal families interact with a broad host range, although some of the less prevalent taxa had values closer to 1 (perfect specialist). The major fungal families were similar between Golfito and Guanacaste (Figure 3), and most prevalent and abundant families had d' index values close to 0. This indicates that members of these fungal families interact with a broad host range. Some of the less prevalent taxa present a value closer to 1, including Irpicaceae and Rhynchogastremataceae. Some of the groups that presented very low abundance in Golfito, such as Hypocreaceae, Irpicaceae and Rhynchogastremataceae, were more abundant in Guanacaste and such preference is highlighted by the CLAM tests (Figure 3).

We didn't find indicator species among the top 17 families for sapwood, but some of them were identified for leaves. Phyllostictaceae, being the second most abundant and prevalent family overall, was not present in the sapwood tissues sampled. Sporocadaceae made up almost 25% of the relative abundance in some sapwood samples, and most families with less than 5% relative abundance in leaves present clearly higher values in sapwood. The CLAM test classified most families as specialists in one or the other habitat, except for Cladosporiaceae and Trichosphaeriaceae, which happen to be the only two groups that didn't show habitat preference in any of the evaluated instances (Figure 3). Intriguingly, some of the families preferred sapwood over leaves but displayed preference for a developmental stage when found in foliar tissues: Irpicaceae, Schizoporaceae and Rhynchogastremataceae.

Less than half of the families showed habitat preference or specialization and only two were recognized as indicators for mature leaves and none for new. Interestingly, these two families (i.e., Mycosphaerellaceae and Xylariaceae), exhibited habitat preference in all the tested cases: Golfito, leaf tissue and mature stages. And more than half of the families, 9 in total, did not show habitat specialization at all.

Based on genus, 38% of the ASVs within the top 17 families matched a record on the FungalTraits database. Most were identified as potential plant pathogens and litter saprotrophs (figure 4). Less than one-third were recognized as foliar endophytes, and this group included two of the families identified as indicators for leaves: Botryosphaeriaceae and Xylariaceae (Figure 3). There were low numbers of parasites (of other fungi or animals), other saprotrophs (of wood and soil) and decomposers (of animal and other fungi) (Figure 4).

DISCUSSION

A fungus' niche preference is largely determined by the interaction between genotype and environment (Toju et al., 2013). Therefore, it can vary among forests that differ in plant community composition and biogeographic and environmental conditions. Communities of endophytes with a broad host range may be completely shaped by the environment, while for those with host restrictions the plant might exert a stronger influence (David et al., 2016). In high-diversity natural systems, such as tropical forests, the endophyte communities are typically composed of numerous taxa of low relative abundances (Harrison et al., 2021), and tend to be dominated by few fungal taxa with a broad host range. The latter are predicted to have a widespread availability of suitable partners, displaying loose host affiliation, and occupying geographically and taxonomically disparate plant hosts (Oita et al., 2021; Suryanarayanan et al., 2011; Zimmerman & Vitousek, 2012). These patterns are supported by our current study.

Host jumps or shifts by microbial symbionts usually occur among closely related host taxa and are driven by the exploitation of new adaptive zones

(Chaverri & Samuels, 2013). Endophytes not only interact with, but overlap in function with other groups that colonize plant tissues, such as pathogens and saprotrophs (Porras-Alfaro & Bayman, 2011). Cosmopolitan endophytes could represent core members that drive community functioning and ecosystem dynamics (Christian, Whitaker, et al., 2017), and may display host preferences at the plant family (Zuo et al., 2022) or order (Kembel & Mueller, 2014) level. In our study, since all the samples belonged to *Rubiaceae*, factors other than plant taxonomy shaped the community.

Endophyte communities are affected by location.

Consistent with the distribution of higher organisms, samples from Golfito, immersed solely in the tropical rain forest, had higher alpha diversity in comparison to Guanacaste. These differences were observed not only in richness (Table 2) but also in community similarity. Guanacaste had a more scattered pattern, implying that the communities in those samples are more variable (Figure 2A), and this observation could be linked to the differences and variability in the environmental conditions (Table 1) across the region (Figure 1).

If niche theory predicts that environmental conditions are spatially structured, and this is reflected in the distribution of species in different habitats (Y. Chen et al., 2018), community similarities should decrease along ecological gradients, regardless of geographic proximity. Therefore, the environment can serve as a filter and regulate what particular taxa are capable of colonizing and persisting within the hosts (David et al., 2016; Vaz et al., 2014). For example, Rojas-Jiménez et al. (Rojas-Jimenez et al., 2016) found that in wet forests of Costa Rica the composition and diversity of culturable endophytes are specific to different stratums and decrease as elevation increased. In our case, elevation showed a significant value but explained a very low proportion of the total variance found in ASVs distributions according to a Canonical – Correlation Analysis (Supplemental Information 4). In general, we did not find strong associations between environmental variables (i.e., elevation, temperature, precipitation) and the variation in fungal communities (Supplemental Information 5), leading us to assume that neutral processes (e.g., dispersal limitation) are also important in distribution pattern of endophytes. Since endophyte transmission occurs mostly horizontally, host colonization and taxa distribution depend on dispersal and successful establishment in the plant (U'Ren et al., 2019). This process restricts the pool of species available to colonize a given host, hence, similarity of species composition between ecological communities typically decreases as distance increases, a phenomenon known as distance decay (Graco-Roza et al., 2022).

Distance-decay relationships reveal the incredible heterogeneity of endophyte-host associations (Higgins et al., 2014), and although they are frequently interpreted as evidence for neutral community assembly processes, they can also arise from environmental gradients (Clark et al., 2021). Dispersal limitation is not considered strong for endophytes, but distance decay has been previously reported both absent (Vincent et al., 2016) and documented at different magnitudes (DeMers & May, 2021; Oono et al., 2017). Our study suggests that there was a marginally significant interaction between geographic location and community structuring (Supplemental Information 6). Because the endophytic community is more strongly correlated with the geographic distance than the environmental parameters (Supplemental Information 5), we consider that in this system, although dispersal limitation is modest, its effect is comparatively higher than the environment's. In reality, niche and neutral processes are not mutually exclusive, and the inversely proportional relationship between spatial scale and community similarity is somewhat implicit in the species turnover phenomena.

Our results agree that spatial variation plays an important role in selecting lineages of fungi, but it's important to consider that seasonal (temporal) distribution of endophytes has been observed in temperate forests (Quilliam & Jones, 2010) and more recently in the tropics (Oita et al., 2021). This is relevant in light that some of the samples from this study were collected in the tropical dry forest, which due to seasonality must endure several months of drought, presenting semi-deciduous trees. For this reason, there might be taxa that remain dormant during specific periods and were not captured at the time of sampling, reducing the observed local pool of endophyte species in Guanacaste.

However, both locations were dominated by host generalists, characterized by high prevalence and abundance, contrasting what appears to be local restricted specialists, such as Hypocreaceae in Guanacaste (Figure 3A). The availability of suitable hosts might be the limiting factor in the geographical distribution of the examined taxa. However, niche processes filter communities producing variations in taxonomic groups and functional traits, and it wouldn't be unlikely for environmental heterogeneity to be more closely related to trait values than to taxonomy at this broad spatial scale (Crowther et al., 2014; Rueda-Almazán et al., 2021).

Endophyte communities are structured by tissue.

The phyllosphere is a dynamic and heterogeneous environment, whose surfaces are often considered relatively oligotrophic (Liu et al., 2020). The ability to colonize plant tissues is largely conditioned by complex physical-chemical interactions with their hosts. For example, fungi must pass through several lines of defense (Khare et al., 2018) and resist the host production of antimicrobial compounds (Ludwig-Mü, 2015; Van Bael et al., 2017). We observed large differences in endophytes between tissue types (Figure 2). Leaves harbored more robust and rich assemblages than sapwood (Table 1), although the latter is considered rich in organic nutrients and is a target of many different organisms, including fungi (Franceschi et al., 2005). We observed little overlap between foliar and alburnum endophytes, suggesting spatial heterogeneity across plant compartments.

While most of the top families of fungi were present in both tissues, they differed in relative abundance and were not dominated by the same taxa (Figure 3B). The most abundant family in the alburnum tissue was Sporocadeaceae, and interestingly, out of the four families belonging to the Basidiomycota phylum in the top 17 families, three of them preferred sapwood according to the CLAM test. Remarkably, in our study, Phyllostictaceae, the second most abundant family in our dataset was not detected in sapwood, showing that endophytes might not be as ubiquitous. Meanwhile, other families almost imperceptible in leaves, presented higher abundance in this tissue: Rhynchogastremataceae and Apiosporaceae, suggesting that tissue type is correlated with a selective enrichment of specific endophytes.

Bark has the most complex anatomical structure and ontogenetic origin of all plant structures, (Rosell, 2019). Specificity of fungal endophytes in this tissue has been reported before (Wang & Guo, 2007), holding great but underappreciated ecological significance, for these fungi inhabit large surfaces often targeted by insects and pathogens (Menkis et al., 2020; Takemoto et al., 2014) and represent a passive reservoir of spores and latent life that will eventually play an important role in wood decay dynamics (Pellitier et al., 2019). Several studies on endophytic community profiles suggest broad correlations between diversity and select defense mechanisms in bark (Bailey et al., 2011), such as reaction zones with the ultimate goal of protecting the phloem (rich in nutrients), the vascular cambium and the transpiration stream in the sapwood (Morris et al., 2020). Therefore, bark acts as an environmental filter structuring inner bark fungal communities (Pellitier et al., 2019) which might explain the differences in community richness, diversity and composition observed in this study.

Endophyte communities in leaves are not affected by developmental stage.

Ontogeny-related shifts in endophyte diversity are associated with changes in genetic traits that influence tissue chemistry and topology (H. Liu et al., 2020), plant hormonal and/or physiological properties and physical characteristics such as tissue structure and geometry (Gupta et al., 2022). We did not observe consistent differences in relative richness and diversity between developmental stages (table 2), although, new leaves were predicted to present lower abundance of endophytes in response to an expected higher concentration of chemical defenses (Koricheva & Barton, 2012; Zhang et al., 2009).

Although some studies have found large qualitative and quantitative differences in metabolomic composition between expanding and mature leaves (Wiggins et al., 2016) (i.e., Optimal Defense Theory), this is not always the case (Sedio et al., 2019). Research in tropical trees has found low variation in defense traits and weak shifts in secondary chemistry during leaf maturation (Barton et al., 2019). It's been documented that the turnover times of leaf pools across these biomes exhibit an inverse relationship to growth ("grow fast, die fast" hypothesis). Some theories suggest that this trade-off happens in agreement with the plant investment strategies, adjusting carbon allocation to balance early growth against defenses (Bhadra & Cai, 2019; H. Yang et al., 2021).

If that's the case, plants in the tropics not necessarily experience strong selective pressure to evolve higher levels of chemical defense against biotic threats (S. C. Chen & Moles, 2018). We assessed taxa relative abundance and notice no significant higher rates of foliar endophytic fungi increasing with developmental stage. Both habitats were dominated by the same families, which can be considered prominent components of foliar endophyte communities in *Rubiaceae* tropical plants and likely play a significant role in ecosystem functioning. The CLAM test detected *Xylariaceae* and *Mycosphaerellaceae* as the two families presenting habitat preference, in the mature leaves (Figure 3); besides foliar endophytes, these family have members known as pathogens, and litter and wood saprotrophs (Figure 4).

CONCLUSIONS

The diversity of fungi found in the phyllosphere of tropical plants belonging to the *Rubiaceae* family did not have specific host affiliation. High abundance community members seemed to be consistently present within the microbiome across different habitats and diverse host species. Fungal communities in foliar tissues appear to be stable in early tissue development and persist on mature stages. As expected, tissues contained distinct habitats for symbionts. The mechanisms of community assemblage at regional scales are not clear, however, overall, we found weak evidence for dispersal limitation and environmental partitioning, thus the observed patterns may be a result of unknown environmental variables that are correlated with different geographic locations.

With the imminent threat of climate change and the constant challenges in conservation and agriculture, endophytes have the potential to transform the way we think about and manage plant stress resistance. Understanding their distributions and functioning in association with different host plants and ecosystems, and their response to environmental disturbances, is key to predicting the potential impact of many of these fungi in agricultural ecosystems and climate change science. How to translate this acquired knowledge for forest conservation and agricultural management remains a challenge. There are still cryptic and undiscovered taxa, whose presence has gone unnoticed and whose ecological roles are unknown. It is imperative to continue the efforts to understand the degree to which apparent patterns of host colonization and selection for metabolically diverse endophytes are dictated by the host, the endophyte or other ecological mechanisms.

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FIGURES AND TABLES

Figure 1. Map of Costa Rica showing climatic subregions and sample locations. Made per request by the National Meteorological Institute in Costa Rica. (Legend: RN1 – Eastern slopes of the Guanacaste and Tilarán mountain range (very humid subtropical forest), PN2 – North Pacific Central subregion (tropical dry forest), PN3 – Slopes and foothills of the Guanacaste and Tilarán mountain range subregion (monsoon-influenced rainforest), PS5 – Pacific slopes of the Talamanca mountain range (low montane rainforest).

Table 1. Characterization of collecting sites, including mean environmental variables in locations (± SE).

Location	Sites	Plants (n)	Annual Mean Temperature (ºC)	Annual Precipitation (mm)	Elevation (m)
Guanacaste	15	23	22.94 ± 0.12	2718.32 ± 45.03	749.56 ± 41.72
Golfito	8	27	24.65 ± 0.19	3522.09 ± 21.17	124.18 ± 12.23

Table 2. Summary of values of alpha diversity indices for the evaluated factors (± SE) based on Amplicon Single Variants (ASV). Anova p-values reported.

Variable	Category	Sample size	Obs. Species richness	Shared number of ASVs	Shannon	Simpson	Chao1
Location	Golfito	66	2068	317	2.93 ± 0.11	0.87 ± 0.02	77.61 ± 7.60
	Guanacaste	57	1378		2.52 ± 0.14	0.81 ± 0.03	52.61 ± 6.63
	p-value *				**0.022**	**0.021**	**0.016**
Tissue type	Leaf	46	1477	102	2.68 ± 0.14	0.84 ± 0.02	61.76 ± 6.80
	Sapwood	23	237		1.57 ± 0.17	0.66 ± 0.05	11.78 ± 1.64
	p-value ***				**7.25E-06**	**1.85E-04**	**2.60E-06**
Development stage	Mature	50	2170	901	3.08 ± 0.11	0.89 ± 0.01	90.48 ± 8.24
	New	50	1746		2.95 ± 0.12	0.88 ± 0.02	66.52 ± 7.61
	p-value				0.429	0.582	**0.033**

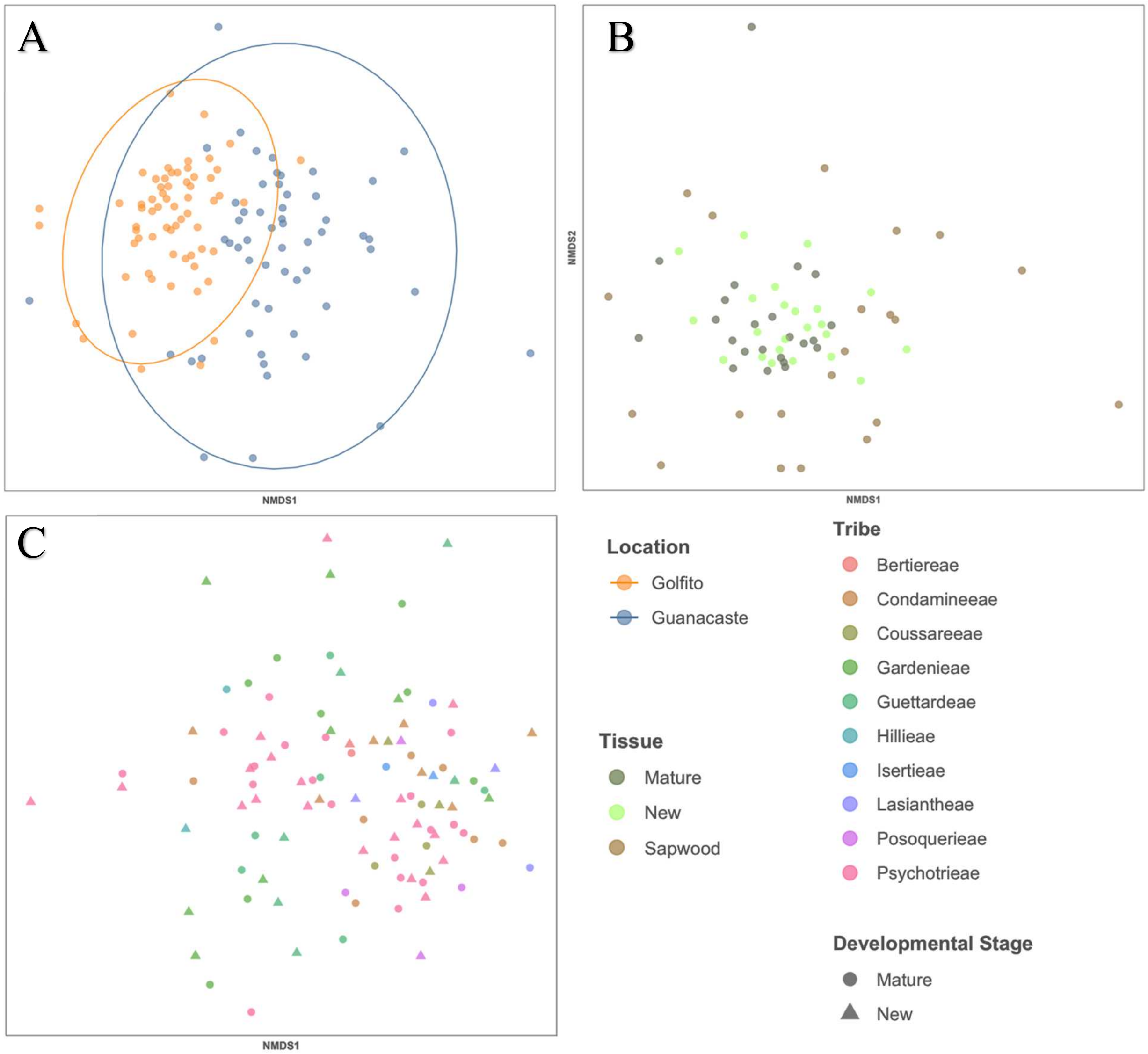


Figure 2. NMDS of Bray Curtis distances showing dissimilarities between the structure of endophyte community assemblages from (A) different locations, (B) different tissues and (C) different plant tribes, developmental stage represented here by shape.

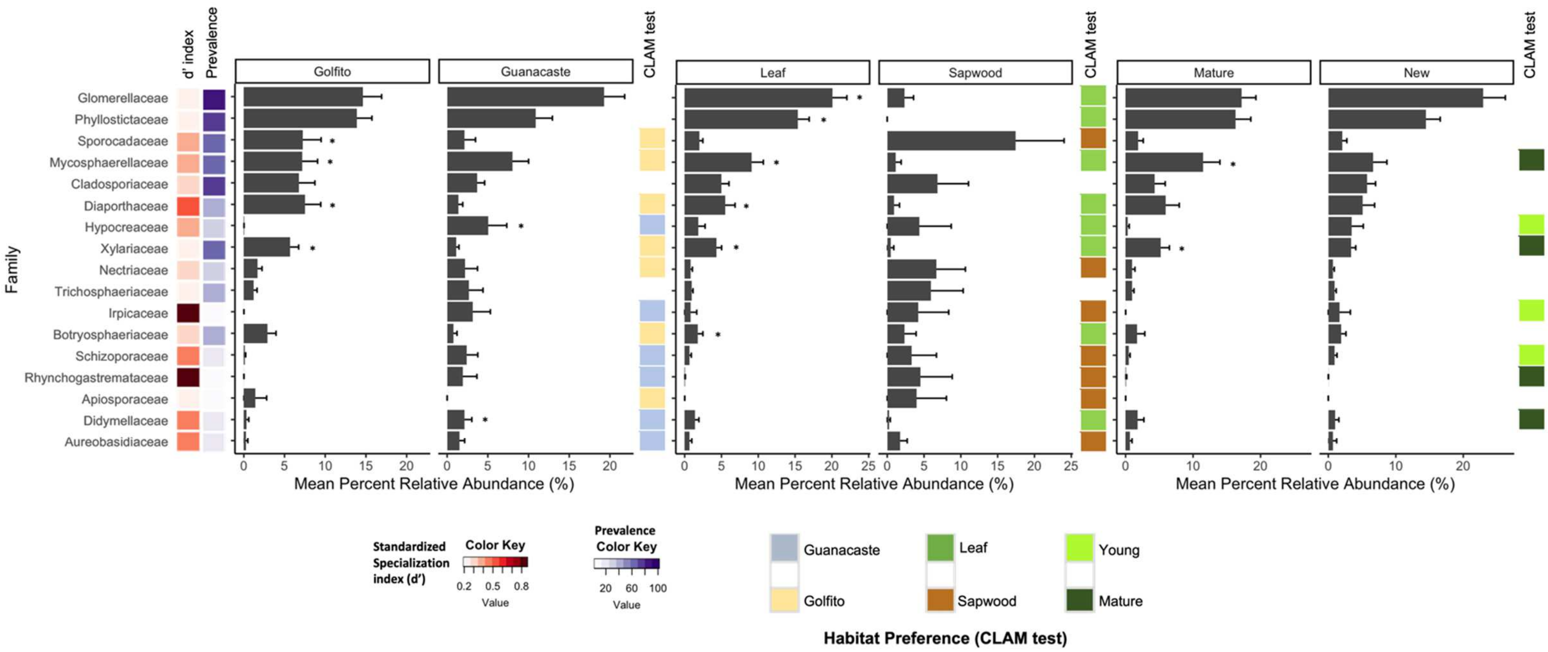


Figure 3. Prevalence and mean percent relative abundance of the 17 most abundant families weighting all habitats within location, tissue and foliar developmental stage. Mean abundance is plotted with error bars representing the standard error. *d'*index calculates how specialized the identified taxa are in relation to the 47 plant putative species. Denoted with a star sign (*) are the families with indicator species within the different habitats; and habitat specialization or generalization is indicated by the CLAM test.

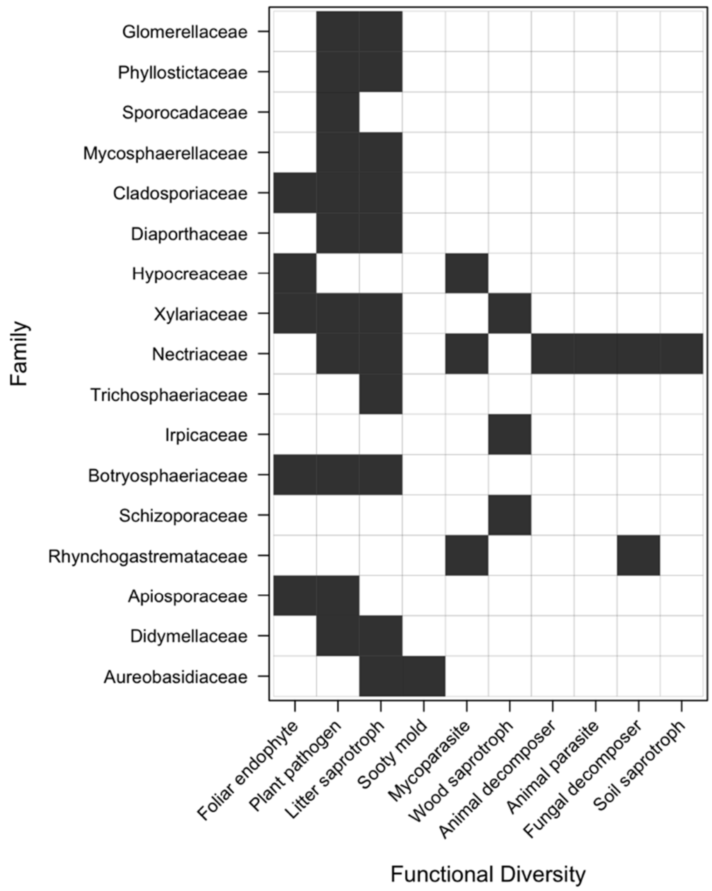
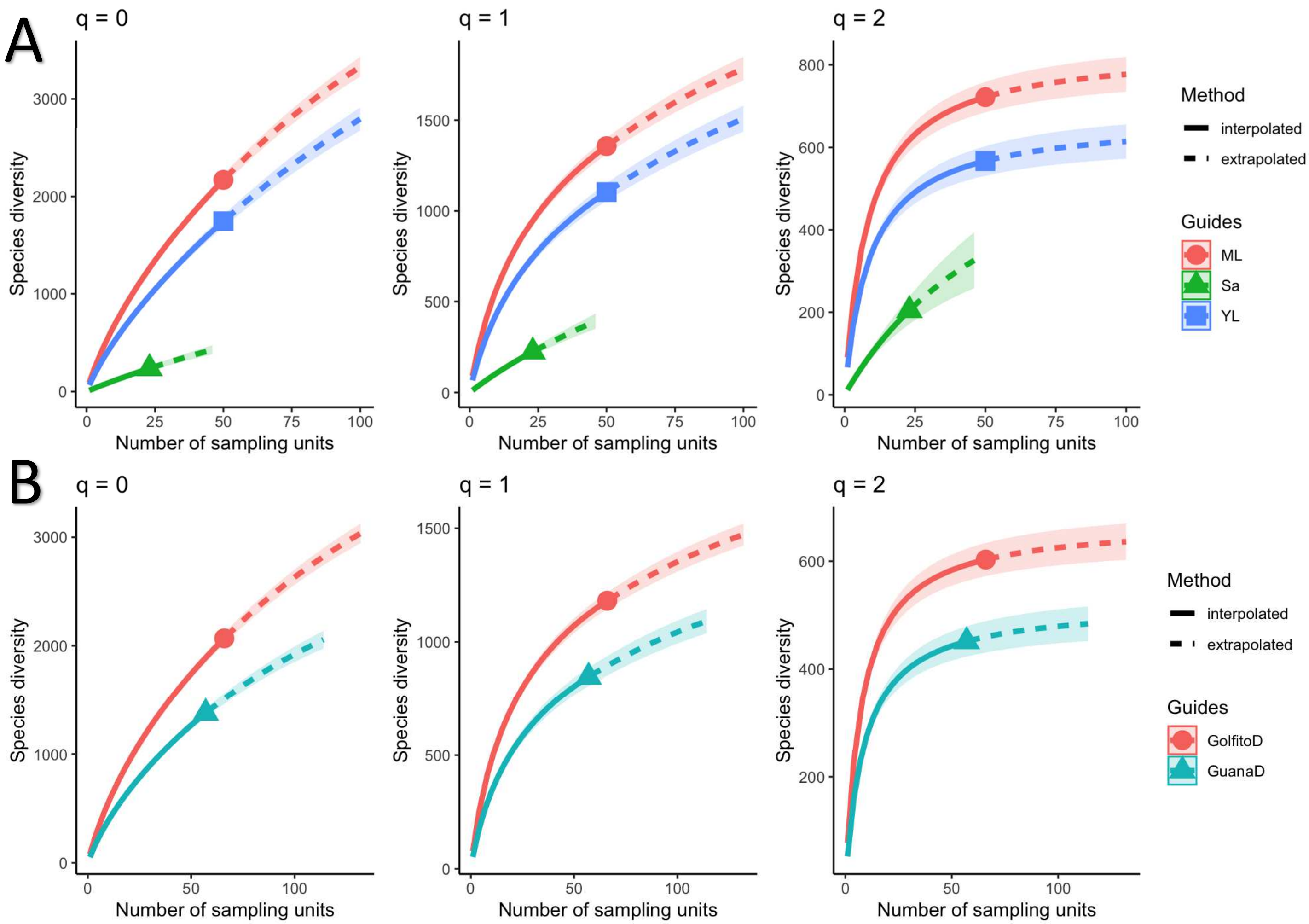


Figure 4. Main lifestyles (primary and secondary) of the genera identified among the top 17 families.

Supplemental Information

Supplemental Information 1. List of putative plant species collected, based on morphology. Bolden are the species collected twice.

Golfito	Guanacaste
Chimarrhis latifolia	***Alibertia edulis***
Chomelia microloba	*Allenanthus erythrocarpus*
Coussarea loftonii	*Arachnothryx costaricensis*
Coussarea talamancana	*Bertiera bracteosa*
Duroia costaricensis	*Chomelia costaricensis*
Faramea tamberlikiana	*Cosmibuena macrocarpa*
Gonzalagunia osaensis	*Genipa americana*
Isertia haenkeana	*Guettarda macrosperma*
Macrocnemum roseum	*Palicourea adusta*
Notopleura polyphlebia	*Palicourea eurycarpa*
Posoqueria grandiflora	*Pentagonia costaricensis*
Posoqueria latifolia	*Psychotria berteroana*
Psychotria brachiata	*Psychotria callophyla*
Psychotria chagrensis	*Psychotria guianensis*
Psychotria mortoniana	*Psychotria horizontalis*
Psychotria panamensis	*Psychotria marginata*
Psychotria solitodinum	*Psychotria pubescens*
Ronabea emética	*Psychotria subssesilis*
Ronabea latifolia	*Psychotria valerioana*
Rudgea cornifolia	*Randia aculeata*
Rustia costaricensis	*Randia grandifolia*
Simira maxonii	*Randia lasiantha*
Sommera donnell-smithii	
Tocoyena pittieri	
Warszewiczia coccinea	



Supplemental Information 2. Species accumulation and diversity curves for endophytes sampled from **(A)** young leaves (YL), mature leaves (ML) and sapwood (Sa) tissues, and; **(B)** Golfito and Guanacaste forests. Metrics include richness (q = 0), Shannon (q = 1) and Simpson (q = 2).

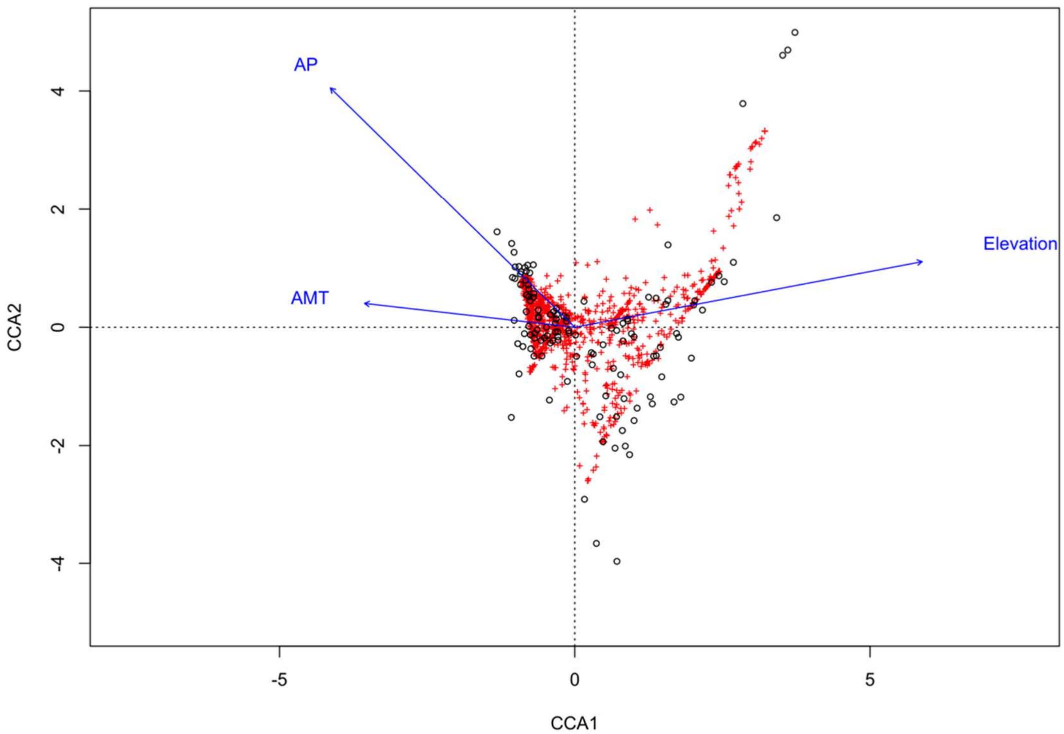
Supplemental Information 3. List of identified fungal families among endophyte communities.

	Family
1	Acrospermales_fam_Incertae_sedis
2	Agaricaceae
3	Agaricostilbaceae
4	Amplistromataceae
5	Amylocorticiaceae
6	Apiosporaceae
7	Aspergillaceae
8	Atheliaceae
9	Aureobasidiaceae
10	Auriculariaceae
11	Australiascaceae
12	Beltraniaceae
13	Bionectriaceae
14	Bolbitiaceae
15	Boliniales_fam_Incertae_sedis
16	Botryobasidiaceae
17	Botryosphaeriaceae
18	Brachybasidiaceae
19	Bulleribasidiaceae
20	Cenangiaceae
21	Ceratobasidiaceae
22	Chaetomiaceae
23	Chaetosphaeriaceae
24	Chaetothyriales_fam_Incertae_sedis
25	Chrysozymaceae
26	Cladosporiaceae
27	Clathraceae

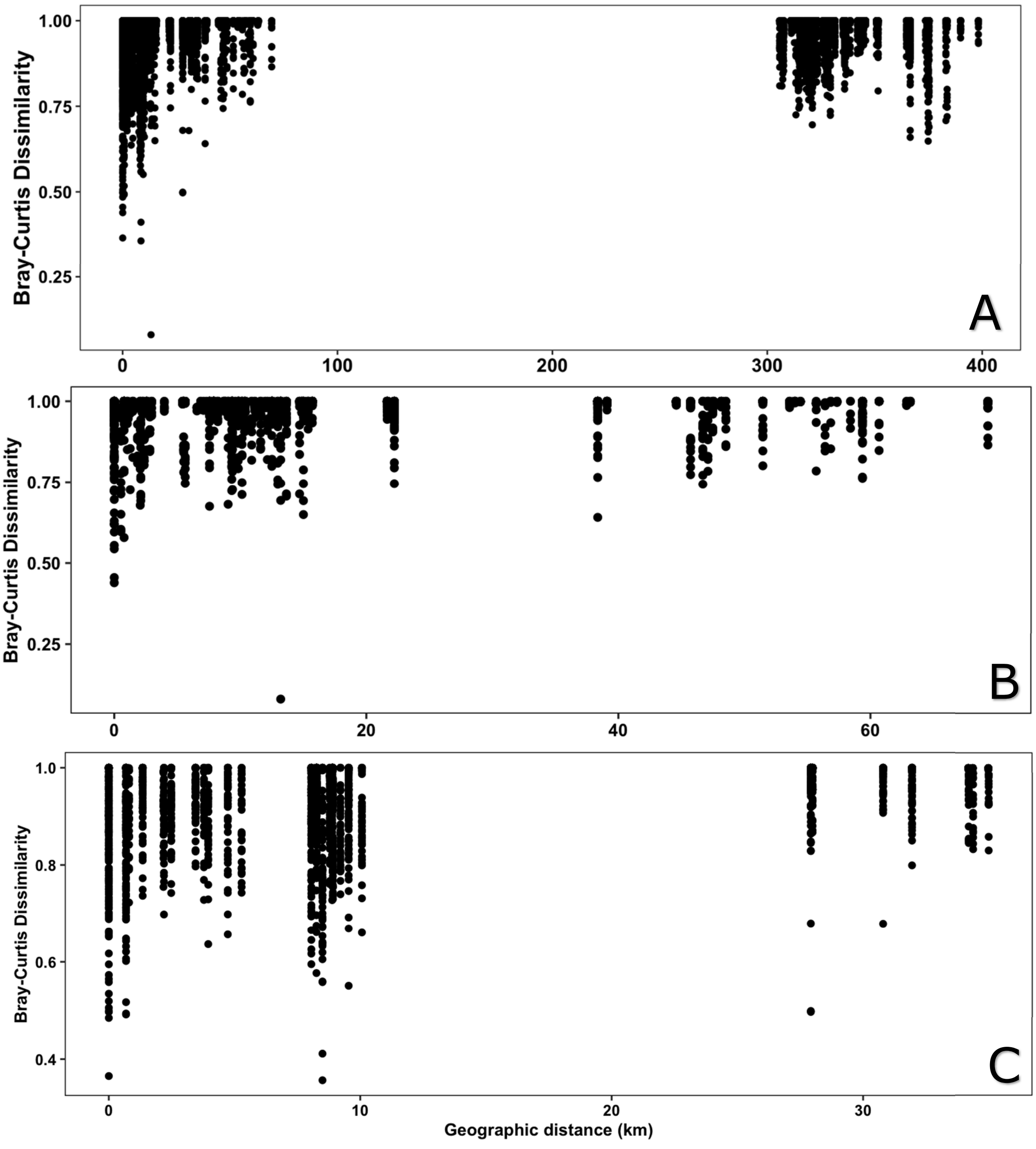
	Family
76	Lentitheciaceae
77	Leucosporidiaceae
78	Lophiostomataceae
79	Lophiotremataceae
80	Lycoperdaceae
81	Magnaporthaceae
82	Malasseziaceae
83	Marthamycetaceae
84	Massarinaceae
85	Meripilaceae
86	Meruliaceae
87	Microbotryomycetes_fam_Incertae_sedis
88	Microdochiaceae
89	Microstromatales_fam_Incertae_sedis
90	Mrakiaceae
91	Mycosphaerellaceae
92	Nectriaceae
93	Ophiocordycipitaceae
94	Ophiostomataceae
95	Orbiliaceae
96	Peniophoraceae
97	Periconiaceae
98	Phaeococcomycetaceae
99	Phaeosphaeriaceae
100	Phanerochaetaceae
101	Phyllostictaceae
102	Physciaceae

28	Clavicipitaceae
29	Coniophoraceae
30	Cordycipitaceae
31	Corticiaceae
32	Corynesporascaceae
33	Cryptococcaceae
34	Cucurbitariaceae
35	Cuniculitremaceae
36	Cyphellophoraceae
37	Cystobasidiaceae
38	Cystofilobasidiaceae
39	Debaryomycetaceae
40	Dermateaceae
41	Diaporthaceae
42	Diatrypaceae
43	Dictyosporiaceae
44	Didymellaceae
45	Didymosphaeriaceae
46	Dissoconiaceae
47	Dothideales_fam_Incertae_sedis
48	Dothioraceae
49	Entolomataceae
50	Erythrobasidiaceae
51	Exidiaceae
52	Filobasidiaceae
53	Fomitopsidaceae
54	Ganodermataceae
55	Geastraceae
56	Glomerellaceae
57	Glomerellales_fam_Incertae_sedis
103	Piskurozymaceae
104	Plectosphaerellaceae
105	Pleomassariaceae
106	Pleosporaceae
107	Pleosporales_fam_Incertae_sedis
108	Pluteaceae
109	Polyporaceae
110	Psathyrellaceae
111	Pyrenulaceae
112	Ramalinaceae
113	Rhynchogastremataceae
114	Rhytismataceae
115	Rickenellaceae
116	Saccharomycetales_fam_Incertae_sedis
117	Sarcoscyphaceae
118	Schizoparmaceae
119	Schizophyllaceae
120	Schizoporaceae
121	Sclerotiniaceae
122	Sordariaceae
123	Sordariales_fam_Incertae_sedis
124	Sordariomycetes_fam_Incertae_sedis
125	Sporidiobolaceae
126	Sporocadaceae
127	Sporormiaceae
128	Stachybotryaceae
129	Steccherinaceae
130	Stereaceae
131	Stictidaceae
132	Strophariaceae

58	Halosphaeriaceae	**133**	Sympoventuriaceae
59	Halotthiaceae	**134**	Teichosporaceae
60	Helminthosphaeriaceae	**135**	Teloschistaceae
61	Helotiales_fam_Incertae_sedis	**136**	Teratosphaeriaceae
62	Herpotrichiellaceae	**137**	Tetraplosphaeriaceae
63	Holtermanniales_fam_Incertae_sedis	**138**	Thyridariaceae
64	Hyaloscyphaceae	**139**	Tilachlidiaceae
65	Hydnodontaceae	**140**	Togniniaceae
66	Hymenochaetaceae	**141**	Trematosphaeriaceae
67	Hymenochaetales_fam_Incertae_sedis	**142**	Tremellaceae
68	Hyphodermataceae	**143**	Trichocomaceae
69	Hypocreaceae	**144**	Tricholomataceae
70	Hypocreales_fam_Incertae_sedis	**145**	Trichomeriaceae
71	Hypoxylaceae	**146**	Trichosphaeriaceae
72	Irpicaceae	**147**	Trimorphomycetaceae
73	Kondoaceae	**148**	Wallemiaceae
74	Lasiosphaeriaceae	**149**	Xylariaceae
75	Lecanoromycetes_fam_Incertae_sedis	**150**	Xylariales_fam_Incertae_sedis



Supplemental Information 4. CCA analysis for correlation between Amplicon Single Variants distribution and environmental variables: Elevation, Annual Mean Temperature and Annual Precipitation). Red crosses represent ASVs and black circles represent samples. p-values: Elevation 0.001, AMT 0.061, AP 0.116; CCA1 0.005, CCA2 0.148.



Supplemental Information 6. Distance decay of taxonomic similarity along spatial distance, every point represents pairwise similarities between samples. Panels A: combined; B: Guanacaste; C: Golfito.

Chapter 3: Do agricultural practices influence the foliar endophytic communities in coffee plants of different varieties?

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Keywords: coffee, Obatá, Catuaí, Caturra, agroforestry, monoculture, organic, agriculture.

ABSTRACT

Fungal endophytes, an essential component of a plant's microbiome, can impact plant health and fitness. The interactions between these microorganisms and their host can have important implications for agronomically important plants such as Coffea arabica, but there are many unknowns. How geography, coffee variety, and farming practices can affect the fungal assemblage in the foliar tissue is of considerable interest. For this investigation, leaves from several coffee plants were collected in plantations from two producing areas in Costa Rica such that they represented different coffee varieties (i.e., Obatá, Catuaí, Caturra), agricultural management practices (organic vs conventional), sun regime (exposed to sunlight/monoculture vs natural shade/agroforestry) and tissue developmental stage (newly emerged and mature asymptomatic leaves separated by three *nodes). Fungal isolates were purified from the leaves and mycelium was harvested from PDA cultures for DNA extraction, PCR amplification and sequencing to assign taxonomy at genus level. Culture independent approach was performed as follows: fungal ITS genes were amplified and sequenced using metabarcoding on an Ion Torrent and taxonomic and functional diversity was assessed using a variety of packages in RStudio (i.e., DADA2, phyloseq, DECIPHER, microbiome, etc.). We ascertained that coffee-associated endophytes are more diverse in mature leaves and in conventionally grown plants and were influenced to a lesser extent by variety. Sun regime had no significant effect on the endophytic diversity. We highlight this finding because of the potential implications in countries like Costa Rica, where coffee is mostly grown in agroforestry. Although the plant microbiome is important, we currently lack the knowledge to construct endophytic communities through breeding or management. This study seeks to understand how the plant and agricultural practices influence their diversity in coffee crops and to provide data that could be useful in future work for shaping coffee microbial communities.*

INTRODUCTION

The sustained and increasing demand for coffee at a global scale makes it one of the most widely consumed tropical beverages (Voora et al., 2019). It is also one of the most traded agricultural commodities in the world, linking producing countries in the southern hemisphere with consuming countries in the northern hemisphere (International Coffee Organization, 2020). Coffee is considered one of the most valuable agronomic exports from the developing nations (FAO, 2021). Brazil, Vietnam and Colombia represent the top producing countries, responsible for more than 60% of the world production (International Coffee Organization, 2021). The coffee sector provides jobs for approximately 125 million people with an estimated annual revenue that exceeds $200 billion (International Trade Center, 2021).

Grown in the world's most biodiverse regions, along the bean belt, coffee is commercially produced in more than 50 countries. *Coffea arabica* and *Coffea canephora* (popularly known as Robusta) are the two main cultivated species, and many countries also grow disease-resistant hybrids. The two major categories of these, Catimor and Sarchimor, are based on crosses between the Timor natural hybrid (*C. arabica x C. canephora*) and the *C. arabica* varieties Caturra and Villa Sarchi, respectively. C. arabica is native to Ethiopia and northern Kenya and due to its autogamy is considered to have low levels of genetic diversity, making it a highly vulnerable crop, often affected by several diseases that include the devastating coffee leaf rust (Talhinhas et al., 2017), and particularly susceptible to climate change (Birkenberg & Birner, 2018). This species, however, accounts for 60% of the world's coffee production, making up for most of the inventory from smaller market participants such as Costa Rica (International Coffee Organization, 2021).

First brought to Costa Rica in the late 17th century, coffee production flourished after independence from Spain (Babin, 2019), becoming the first and main product of exportation until 1890 (Vogt, 2019). Although it is no longer the backbone of the country's economy, only 2.3% of Costa Rica's total export revenue, coffee still has a significant impact on the country and has since been a cultural cornerstone. While Costa Rica's total tons of produced coffee is relatively low, currently contributing to less than 1% of the world's production; coffee is the pound for pound most revenue generating agricultural export (Blackman & Naranjo, 2012; Hospidales, 2021). In 2021, coffee exports were valued at $322 million and the United States was the main destination (Gonzalez, 2022).

Coffee production in Costa Rica is dedicated mostly to two high yield hybrid cultivars: Caturra and Catuaí, which displaced traditional varieties, such as Villalobos, Villa Sarchí, and Typica (Arabigo), in the mid-20th century (Smith, 2018). However, it is not uncommon to find other varieties such as Bourbon, Centroamericano, Milenio (H10), Geisha, and Obatá (Quesada-Román et al., 2022). All these varietals are considered *C. arabica* species, but the latter belongs to the Sarchimor group, thus it incorporates genetic material from Robusta (World Coffee Research, 2019). The country's coffee industry, often recognized for its environmentally friendly practices has been developing the infrastructure needed to obtain high prices for small lots of high-quality coffee. Organic farming has been suggested as one approach. Although the socioeconomic impacts are complex, the environmental positives for organic farming include reduced chemical inputs (i.e., pesticides, herbicides, fungicides, hormones, antibiotics, growth regulators and inorganic fertilizers) and adoption of environmentally friendly management practices, such as, the use of compost, soil management, mechanical weed control and crop diversification (Blackman & Naranjo, 2012; Caldwell et al., 2015; De Resende et al., 2021).

In contrast to monocultures, agroforestry systems have been proposed to improve the cultivation of coffee. This practice involves multi-cropping, in some cases with woody-perennial species, resulting in ecological and economic interactions between the different species. Some of the benefits include improving and conserving soil quality by the continuous deposition of plant biomass and turnover of leaf litter (Araujo et al., 2011). The shade covers for the coffee may attract natural pest control in the plantations (Quesada-Román et al., 2022) and the higher plant biodiversity may also increase crop pollination and result in improved yield rates (Ricketts et al., 2004).

The phyllosphere is an oligotrophic environment where endophytes are exposed to potential stress conditions due to solar radiation, temperature fluctuations, and water availability (Moulas et al., 2013), but it also contains multifaceted niches, comprising a variety of different habitats for diverse fungal communities that are important for plant health and productivity (de Sousa et al., 2018; Prior et al., 2017). Unfortunately, except for some fungal plant pathogens, information on how agronomic practices affect phyllosphere fungi is limited. Plants in natural communities maintain symbiotic associations with endophytic microbes that aid them in coping with biotic and abiotic stresses. During long-term cultivation these associations might be lost or reduced (White et al., 2019), the causes could be various, including modified mechanisms involved in microbiota assembly and the continuing exposure to agrochemicals which might prevent microbial colonization (Sahu & Mishra, 2021a).

Elucidating how farming practices can be correlated with the assemblage of endophytic fungi in the foliar tissue of coffee plants is therefore of considerable interest. We set out to test four main hypotheses: (i) diversity and community composition of foliar endophytes differ between young and mature tissues, (ii) fungal endophyte communities change between cultivars, (iii) fungal endophyte assembly respond differently towards management practices (organic vs conventional) and (iv) shade vs sun regimens affect the community patterns of fungal endophytes. Additionally, we aimed to determine the fungal groups driving such differences and to understand the putative life strategies associated with each scenario. To the best of our knowledge no other study has investigated the fungal endophyte communities of coffee leaves using culturable-independent strategies.

METHODS

Sampling.

Due to Costa Rica's diversity of climatic profiles, there are several production areas in the country: Coto Brus (Brunca), Los Santos, Perez Zeledón, Turrialba, Central Valley, Tres Rios, Orosi, Western Valley and Northern Zone (Guanacaste). For this study, we chose two areas: Los Santos and Brunca. A total of 24 *Coffea arabica* plants, from 3 different varieties: Caturra, Catuaí and Obatá, were sampled. For the first cultivar, the largest (USDA) certified-organic coffee producer in Costa Rica: Hacienda La Amistad, located in Las Mellizas, Puntarenas was selected, and for the other two varieties, a conventional farm from the large co-op Coope Tarrazú, in San Marcos de Tarrazú, San José, was sampled (Table 1).

A total of 48 healthy leaves were collected for each cultivar. The sampling scheme in both farms included finding plots with two different shading conditions: plants exposed to sunlight and plants grown under shade provided by other plants. For each of the three varietals, 4 plants grown under each condition were chosen at random. We selected three branches per plant and from each branch, one young and one mature asymptomatic leaf were collected. Newly emergent leaves with a fully expanded lamina located on the first node from the apex were defined as 'young', and leaves on the third node down were defined as 'mature'.

All samples were individually placed in a clean plastic bag and transported in narrow time window to the field workstation where they were processed immediately. A section of each foliar tissue of ca. 20 x 4 mm was cut and surface sterilized using sequential washes of 2% commercial bleach (1 min), 70% EtOH (30 sec) and sterile distilled water (30 sec). Afterwards, leaf segments were dried with autoclaved tissue paper, and stored in microtubes containing silica gel before being transferred on ice to the laboratory for DNA extraction to provide a culture-independent approach.

For the culture-dependent approach, five segments from each leaf (including lamina tip and base, midrib, and left and right margins) were cut and surface-sterilized in accordance with the previously described immersions. After being dried out, segments were placed in Petri dishes containing PDA medium, and subcultured to obtain pure isolates.

Fungal sequencing and bioinformatics.

Pre-filled 500-micron Garnet and a 6-mm Zirconium Grinding Satellite bead tubes (OPS Diagnostics LLC, New Jersey, USA) were used to disrupt the foliar tissue using a FastPrep® bead mill (MP Biomedicals, Santa Ana, California, USA). The grinding cycle was set to a speed of 6.0 m/s for 45 seconds. Total DNA was extracted using the Qiagen DNeasy Plant Mini Kit® following the manufacturer's instructions (Qiagen, Hilden, Germany).

DNA library preparation, PCR and next generation sequencing of the ITS2 nrDNA region *f*ITS7 and ITS4 were contracted to external parties: Naturalis Biodiversity Centre, The Netherlands, for the samples from CoopeTarrazú and Penn Genomic Analysis Core, University of Pennsylvania, processed the samples from La Amistad. Both companies used the same protocol and Ion PGM Torrent™ technology (Thermo Fisher Scientific, Waltham, MA, USA).

The command line tool cutadapt was used to trim primers and barcodes from every read (Martin, 2011). Reads were processed in RStudio, version 2022.2.0.443 (RStudioTeam, 2022), R v 4.0.3, following a data curation process that included quality filtering, trimming, and chimeras/spurious contigs removal using the package DADA2 v1.18.0 (Callahan et al., 2016), which was also used to classify the DNA reads into Amplicon Single Variants (ASV). The taxonomic assignment of each ASVs was conducted using the UNITE database v8.2 (Abarenkov et al., 2020) training set from the DECIPHER package, v 2.18.1 (Wright, 2016).

For the fungal cultures, mycelium from all fungi was taken directly from the PDA cultures for DNA extraction using PrepMan® Ultra Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions. PCR amplification of the nrITS regions was performed using the primers ITS5 and ITS4 following these conditions: initial denaturing, 1 cycle of 3 minutes at 95°C; denaturing, 35 cycles of 30 seconds at 95°C, annealing, 35 cycles of 30 seconds at 49°C; elongation, 35 cycles of 1 minute at 73°C; and one final extension cycle during 6 minutes at 73°C before cooling down to 12°C.

PCR products were kept in storage at 4°C and sent out for Sanger sequencing, which was contracted to a third party: Genewiz (Azenta Life Sciences, Frederick, Maryland). The returned sequences in ab1 format were submitted to quality control in the command line using emboss, cutadapt, TrimGalore and FASTQC and the passing reads were compared to the NCBI databases using their online BLAST® services.

Statistical analysis.

Statistical analyses and plotting were performed using the R packages: phyloseq v1.38.0 (McMurdie & Holmes, 2013), vegan v2.5.7 (Oksanen et al., 2020), microbiome v1.12.0 (Lahti et al., 2017) and ggplot2 v3.3.5 (Wickham, 2016). Alpha diversity was assessed using the Chao1 index for each of the evaluated variables (farming system, cultivar, shading condition, foliar age) followed by a Mann-Whitney Wilcoxon test. Community composition was determined, and the top 10 families of each coffee varietal were plotted showcasing differences in relative abundance between foliar ages.

Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of ASV abundances were used to visualize community patterns (beta-diversity) and to test for community differences between shading conditions and leaf developmental stages using the function 'metaMDS' in vegan (100 random starts). Outliers were removed for visual effects but included in a permutational analysis of variance (PERMANOVA). As for the farming system and varieties, a differential abundance analysis using the R package ANCOM-BC was performed to identify differential abundance of ASVs. To determine the relative abundance of the putative functional traits of the fungal communities associated with each habitat we compared the ASVs to the fungal functional database FungalTraits and classify them according to their reported primary lifestyle (Põlme et al., 2020).

RESULTS

After removal of low-quality and low-frequency reads, PCR artifacts and singletons, a total of 529,087 reads were used in the analysis. Samples with zero reads after quality trimming were discarded, leaving a sample size of 19 new and 23 mature leaves (treatments included 19 exposed to sun and 8 shaded, 10 organic and 32 conventional, and 10 Caturra, 16 Catuaí and 16 Obatá leaves from each variety. The number of sequences per sample varied between 7 and 74,326 with an average of 12,597. They were putatively classified into 611 amplicon sequence variants (ASV).

The endophytic communities were dominated by a few abundant dominant taxa and many rare taxonomic groups including at least 2 phyla, 13 classes, 39 orders, 91 families, 124 genera, and 130 species. The identified ASVs belonged primarily to Ascomycota (mean abundance across all samples: 81.65%) and Basidiomycota (14.72%). At family level, Cladosporiaceae dominated the assemblages making up almost half of the ASVs (46.13%), followed by Glomerellaceae (10.49%). At genus level, Cladosporium (45.47%) was predominant, with Colletotrichum encompassing 7.91% of the ASVs, another abundant genus was Tylospora (4.35%) from the Basidiomycota. We identified several ASVs belonging to genera such as: Cercospora, Mycena, Fusarium, Trichoderma and Lecanicillium.

Species accumulation curves (Supplementary Figure S1) showed that not all the fungal diversity was represented in our surveying effort, as no asymptote was reached. This suggests that the library size was not large enough to reflect the actual foliar endophytic fungal diversity in coffee plants. Therefore, we present alpha diversity, with the caveat that more sequencing could change our observations. As per alpha diversity (measured by Chao1 index) statistically significant differences were detected in the fungal assemblages when comparing farming management system (p=0.0027), foliar age (p=0.0071) and the coffee varieties Catuaí and Caturra (p=0.0029). Obatá was not statistically significant different from Catuaí (p=0.0861) or Caturra (p=0.0632) (Figure 1). Conventionally grown, mature leaves, from the Catuaí variety had a richer assemblage than their respective counterparts. The communities from plants under shaded regime tended to appear more diverse but were not statistically different from those exposed to full sun (p=0.57).

Dispersion and location effects were observed in the non-metric multidimensional scaling (NMDS) between the two farming systems and the coffee varieties: the organic farm had more point-to-point variation (figure 1), and the two conventional varieties overlapped. There seems to be a marginal dispersion effect in response to foliar developmental stage in the conventional farming; however, it was not captured by PERMANOVA (p-value=0.4695; individually, Catuaí=0.2927; Obatá=0.2947). Also, a location effect on sun regime for the organic variety was observed and confirmed by statistical analysis (p=0.027). However, the unequal number of samples (3 exposed vs 7 shaded) could have contributed to this result. It is important to note that two samples from the exposed to sun treatment were removed from the plot because they were distant from all other points. The effect of sun regime and foliar developmental stage on community dissimilarity was assessed via PERMANOVA, but no differences were determined, the p-value for the former was 0.14 and for the latter 0.81.

The coffee varieties had different numbers of putatively identified fungal families: 21 for Caturra, 70 for Catuaí and 66 for Obatá. The top 10 most abundant families per variety were plotted to look for differences in dominant taxa (Figure 2). Some of the families were present only in one of the leaf developmental stages. Overall, only three of the dominant families were found in common among all varieties: *Glomerellaceae*, Didymellaceae and *Phaeosphaeriaceae*. The conventional cultivars also both contained *Cladosporiaceae*. To further inspect the differences in community composition we looked for differential abundance of ASVs belonging to these families and established that only *Nectriaceae* had a positive log fold change in the organic farming (Figure 3). As for the conventional cultivars, several families were more differentially abundant in Catuaí, and only *Trichosphaericeae* in Obatá.

We compared the primary lifestyle of the identified ASVs that matched the FungalTraits database to look for differences in the lifestyle strategies in each community. We identified fungi from 14 ecological guilds, including saprotrophs, endophytes, plant pathogens, mycoparasites, etc. (Figure 4). Overall, most fungi were identified as potential litter saprotrophs, followed by potential plant pathogens. The differences between foliar age were not pronounced, however, the new leaves seem to host a more diverse array of lifestyles, including animal parasites and ectomycorrhizal. Leaves from the organic farm harbored more plant pathogens, whereas in the conventional farm litter saprotrophs were more abundant. The organic also had a higher abundance of mycoparasites and wood and nectar saprotrophs. Finally, the communities in the different sun regimes differed in the percentages of plant pathogens, being higher in the exposed plants, as well as soil saprotrophs and animal parasites. While the shaded agroforestry benefited litter saprotrophs, mycoparasites, ectomycorrhizal and sooty molds.

For the endophytes in pure culture, we were able to identify to genus level 659 isolates, belonging to 55 different genera (Table 2), from which 32 were unique to the culture-dependent approach and not found via metabarcoding. Catuaí variety under shade had the largest number of isolates and Caturra leaves exposed to sunlight harbored the least. Each varietal presented from 14 to 26 different genera and only 3 of them: Colletotrichum, Cladosporium and Fusarium were found in every instance. However, other genera including Trichoderma, Xylaria, Pestalotiopsis, Cercophora, Didymella and Nigrospora were common to all three cultivars.

DISCUSSION

A recent review on coffee endophytic microbiota document 4 fungal phyla, 39 orders, 149 genera, and 253 fungal species present in coffee. Most endophytes belonged to the Ascomycota with the *Cladosporium*, *Colletotrichum*, *Aspergillu*s, *Penicillium*, *Fusarium*, and *Trichoderma* being the most cited genera (Duong et al., 2020). Our findings are comparable, except our metabarcoding efforts did not identify the cosmopolitan *Aspergillu*s or *Penicillium* that have been reported before (Vega et al., 2010a). Several *Penicillium* were obtained in our pure cultures (Table 2) however, showcasing the importance of combining both culturable dependent and independent methods to survey fungal diversity in the leaves. Based on DNA results, *Cladosporiaceae* was the dominant family in the conventionally grown coffee and *Nectriaceae* was most abundant the organic system. In both cases *Glomerellaceae* was the second more prevalent with *Cladosporium*, *Cosmospora* and *Colletotrichum* as main representatives. *Colletotrichum* is perhaps the most common endophytic taxon in coffee (James et al., 2016), and our culturing results agreed, suggesting that easily grows on PDA medium and might outcompete other taxa. In the metabarcoding survey, it was not the most abundant, but was still the most common. Several unidentified species in this genus were detected, as was C. *sydowii*, existing in both farms, under both sun regimes, distributed in leaves at different developmental stages, and present in every coffee variety. This suggests that some endophytic fungi are ubiquitous in coffee-growing regions as a consequence of seed/plant exchange or because of the intrinsic distribution of the fungi themselves (Vega et al., 2010b).

Our results in the conventional varieties agreed with previous studies that determined those genera were also the most abundant in leaves (Oliveira, 2014). Many of the taxonomic groups we detected were also isolated as foliar endophytes before, including *Pestalotiopsis*, *Xylaria*, and *Guignardia* (now *Phyllosticta*) (Santamaría & Bayman, 2005). Similar genera have also been found in wild coffee species (Zananirina et al., 2021), and associated with seed and fruits (Vaughan et al., 2015), root (Fulthorpe et al., 2020), soil (Jurburg et al., 2020; Rao et al., 2020) and overall, with coffee plants (Lu et al., 2022), and coffee farms (Pregolini et al., 2021). The diversity of endophytes from this investigation differed somewhat from a study in Veracruz, Mexico, that also found other genera, including *Phomopsis*, *Beauveria*, *Coniosporium* and *Diplodia* (Saucedo-García et al., 2014). Since the assembly of endophytic communities involves filtering at different spatial and biological scales (van Bael et al., 2017), the differences in composition might be due to mechanisms of community assemblage and species coexistence, such as dispersal limitation. As well as specific conditions present in the plant external and internal environment that endophytic fungi must endure to survive in the plantations.

Leaves may be at their most vulnerable during the expansion period (Dalling et al., 2020), and display phytochemical changes through their phenology (i.e., optimal defense theory) (McKey, 1979; Rhoades, 1979) which could represent a bottleneck for fungal colonization. We observed differences in young and mature leaves (Figure 2). The time one internode takes to develop was approximately one month in the plants from this study, therefore, the mature leaves were exposed to the environment between 2.5 and 3 months before the newer leaves. Other studies comparing species richness as leaves developed and senesced found mixed results in the endophyte community diversity (Oono et al., 2015; Yu et al., 2021). Why we observed differences when others observed no changes or saw the opposite trends in species richness is not known. Given the horizontal mode of transmission of endophytes, the process of community assembly in the leaf, might be influenced by priority effects, that ultimately affect the final composition of fungal communities at mature stages (Christian et al., 2015).

No evidence of a conserved endophytic community in the leaves of the three coffee varieties was found and cultivars tended to have distinct assemblies of fungal lineages (Figure 2). Only three taxa at the top 10 family level were found to be shared: *Glomerellaceae*, *Didymellaceae* and *Phaeosphaeriaceae*. The lowest diversity was found in Caturra (Figure 1). Due to low quality sequencing rounds, we discarded several samples however, and therefore it is possible that some of the abundance was not captured, and that diversity was higher than reported. The genetic conformity of the cultivars in this study included direct lineage as Catuaí results from the cross between Caturra x Mundo novo, and a shared base between Catuaí and Obatá, as the latter was developed by crossing Sarchimor x Mundo Novo (World Coffee Research, 2019). Interestingly, Catuaí, followed by Obatá contained the highest diversity; both of these varieties were grown in the same plantation under the same management system (conventional) and had similar alpha and beta diversity levels (Figure 1). This suggests that environment or proximity plays a larger role in determining endophytic communities than plant genetics. A previous study found higher diversity and prevalence of bacterial endophytes in Obatá than in Catuaí and Guariní. In that case the leaf-associated microbiota was correlated with the levels of manganese and calcium on leaves, which were dependent on the plant genotype (de Sousa et al., 2018).

In our case, location and/or farming management system are acting as a predominant source of variation (Figure 1). Although the farms shared similar environmental conditions (Table 1), they were located in different production areas, separated by hundreds of kilometers. As a result, the endophytic populations that inhabit coffee plants might be limited by dispersal, selection, and drift of spores/propagules (Gdanetz et al., 2021). Unfortunately, farming system, production area and cultivar are confounded in the organic production, so our results present limitations. Several investigations have shown that difference in agricultural management (i.e., organic and conventional farming systems) affect endophytic communities in wheat (Karlsson et al., 2014; Wachowska et al., 2018), (R. M. Stuart et al., 2010), sugarcane (R. M. Stuart et al., 2010), soybean (Batzer & Mueller, 2020; A. K. da C. Stuart et al., 2018), common bean (Prior et al., 2017) Karlsson et al., 2014), apple (Walter et al., 2007), maize (Fadiji et al., 2020; Noel et al., 2022), corn, tomato, pepper, and watermelon (Xia et al., 2019), tea

(Cernava et al., 2019), grapevines (Pancher et al., 2012) and grass (Wemheuer et al., 2019). In coffee production the studies are limited. One investigation reported that both fungal and bacterial communities in the soil of coffee farms, clustered significantly according to whether they were organically or conventionally managed, but no difference in richness was found (Jurburg et al., 2020). Another group of researchers found that root fungal communities in conventionally managed coffee had lower richness and a loss of rare taxa when compared to organic fields (Sternhagen et al., 2020). At the same time, it has been suggested that arbuscular mycorrhizal fungal richness and root colonization do not differ between organic and conventionally managed agroecosystems (Aldrich-Wolfe et al., 2020). One study found that organic farming reduced the prokaryotic diversity and richness levels in the coffee rhizosphere microbiome (Caldwell et al., 2015). In the current study abundance and diversity of leaf endophytes in the organic farm was significantly lower than that in the conventional plantation (Figure 1). This is in contrast to a comparison of culturable fungal endophytes that reported little variation between cropping systems (Oliveira, 2014). Clearly more research is needed to gain consistent understanding of fungal endophyte assembly under different management practices.

The disparities we observed in the diversity of species present in the different farming systems might be due to endophytes species exploiting dissimilar chemical niches. Defense against pests and pathogens is largely the product of chemical diversity, which is driven by host genetics and inducive environments (Sedio et al., 2019). By restricting or eliminating the use of agrochemicals in the organic system, the threat of pests and diseases can increase (De Resende et al., 2021). This might lead to a stronger selective pressure to evolve higher levels of chemical defense against biotic threats. The lower number of ASVs in the organic plants might indicate a high efficiency against fungal pathogens that extends to prevent colonization from the endophytic equivalents. In fact, there are several reports of reduced fungal disease in organic production, but the mechanisms behind it remain poorly understood (Karlsson et al., 2017). On the other hand, it is important to highlight the remarkable resilience of fungal communities and their ability to respond and recover rapidly after agrochemical applications. In some cases, an increase in population density is triggered by a decrease in the numbers of other organisms, reduced competition, and/or novel chemical niches (Knorr et al., 2019). For example, it has been reported that some widespread endophytic fungi species (e.g., *Colletotrichum* and *Xylaria*) can develop resistance against fungicides, and some potential plant pathogens can survive as endophytes in their hosts before manifesting disease symptoms (Mohandoss & Suryanarayanan, 2009).

Growing coffee in the shade as an understory plant, has been praised because it builds habitat complexity in an otherwise low-diversity environment, promoting an active pollinator community (Ricketts et al., 2004) and the presence of natural enemies for pests and pathogens (James et al., 2016). Full sun exposed plants in monoculture provide a different microclimate due plant density and vegetation abundance, influencing the dynamics of fungal disease development (Karlsson et al., 2017) and possibly the colonization of plants by endophytes. Local habitat differences could drive species sorting in these systems (Rao et al., 2020). However, our results don't show statistical differences in the richness or diversity of endophyte communities as a response to shading or sunlight exposure (Figure 1). Similar results have been found in the root microbiome (Fulthorpe et al., 2020). Nonetheless, the incidence of solar radiation can lead to changes in the plants' internal metabolites (Veloso et al., 2020; L. Yang et al., 2018), disturbing the balance with fungal endophytes (Davitt et al., 2010). As a result, coffee monocultures could present reduced presence of potentially beneficial microbes, such as mycorrhizae (Arias et al., 2012; Sewnet & Tuju, 2013; Zhao et al., 2018).

When investigating the lifestyles diversity in our communities, a higher percentage of potential pathogens was found in exposed plants and more sooty molds and potential ectomycorrhizal fungi were found under shade (Figure 4). Overall, we found a high relative abundance of several types of saprotrophs in the conventional farm (Figure 4), in comparison to an increased percentage of potential pathogens in the organic. Future research should address the role of these conventional species on disease suppression. Indigenous antagonistic microorganisms may contribute to the observed differences, saprotrophs and pathogens dynamically compete for nutrients in a naturally occurring biocontrol process in the phyllosphere (Karlsson et al., 2017). Interestingly, although taxonomic composition changed (Figure 1, Figure 2), the functional diversity in the leaves did not seem to change profoundly with developmental stage. Changes from mutualism and commensalism in younger leaves to saprotrophic and decomposition as the leaf gets older could be expected but that was not the case, suggesting that several taxa could occupy the same functional niche and that ecological equilibrium is attained early on.

CONCLUSION

This study highlights how multiple aspects of crop management alter the endophytic communities in coffee agroecosystems. Some of the observed differences in the abundances of the predominant fungal families seem to result from differences relative to the host plant such as tissue developmental stage. Overall, the dominant fungal community differed according to cropping system or geographical location, and to a lesser extent between coffee variety. We did not find a conserved community but some of the taxa were present in all the evaluated habitats, indicating that some members of the phyllosphere mycobiome of coffee are cosmopolitan over large geographical distances and across different farming practices. We found that conventional as opposed to organic practices could increase the abundance and species diversity of endophytic fungi. The primary lifestyles detected comprised mostly litter saprotrophs and plant pathogens, but several functional guilds differed between the farming management and sun regimes. For instance, agroforestry may result in reduced potential pathogens and increased levels of symbiotic microbes, and organic practices might reshape the community at taxonomic and functional levels.

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FIGURES AND TABLES

Table 1. Environmental characterization of collecting plantations.

Location	Farming system	Coffee varieties	Annual Mean Temperature (°C)	Annual Precipitation (mm)	Elevation (m)
Las Mellizas	Organic	- Caturrá	17.1	2690	1250
San Marcos de Tarrazú	Conventional	- Catuaí - Obatá	21.4	2865	1380

Table 2. Fungal diversity of culturable endophytes at genus level based on their origin varietal. E = Exposed to sunlight, S = Shaded. In parenthesis the number of isolates per sun regime.

Genus	Catuaí		Caturra		Obatá	
	E (n=96)	S (n=135)	E (n=92)	S (n=99)	E (n=119)	S (n=118)
Acremonium					1	
Annulohypoxylon	2			1		
Arthrinium			1			
Bipolaris		3			1	
Biscogniauxia				1	2	
Bjerkandera				2	4	1
Boeremia		4				
Cercophora		6	5	1		1
Chromocleista	1				1	
Cladosporium	19	28	4	1	1	29
Colletotrichum	52	63	55	78	71	55
Coniochaeta					1	1
Coprinelllus	1	1			1	1
Diaporthe			2	2		
Didymella	4		2	1	1	1
Epicoccum						1
Fusarium	1	2	2	2	1	2
Gaeumannomyces				1		
Glomerella						2
Hypomontagnella					3	
Hypoxylon			2			1
Lasiosphaeria			1			
Leptosphaerulina			1	1		
Irpex					1	
Myxospora			1			
Nemania			2			
Neopyrenochaeta						1
Nigroporus						2
Nigrospora	1		5		2	3
Nothophoma				1		

Paecilomyces					1	
Penicillium	3	6			2	3
Perenniporia			1			
Periconia						1
Pestalotiopsis		4	1		2	1
Phanaerochete	1				1	
Phoma			1			
Phlebiopsis					1	
Phyllosticta	2					
Pseudopestalotiopsis			1			
Purpureocillium	1	4			1	
Pyrenochaetopsis		1		1		
Pyricularia		4	1			
Sarocladium						4
Schizophyllum					4	
Setophoma					1	
Shiraia		1				
Sordaria					2	
Stagonosporopsis	1			1		
Trametes					2	
Trichoderma	5	8		1	6	3
Ustilaginoidea					1	
Verticillium						1
Vesiculozygosporium					1	
Xylaria	2		4	4	3	4

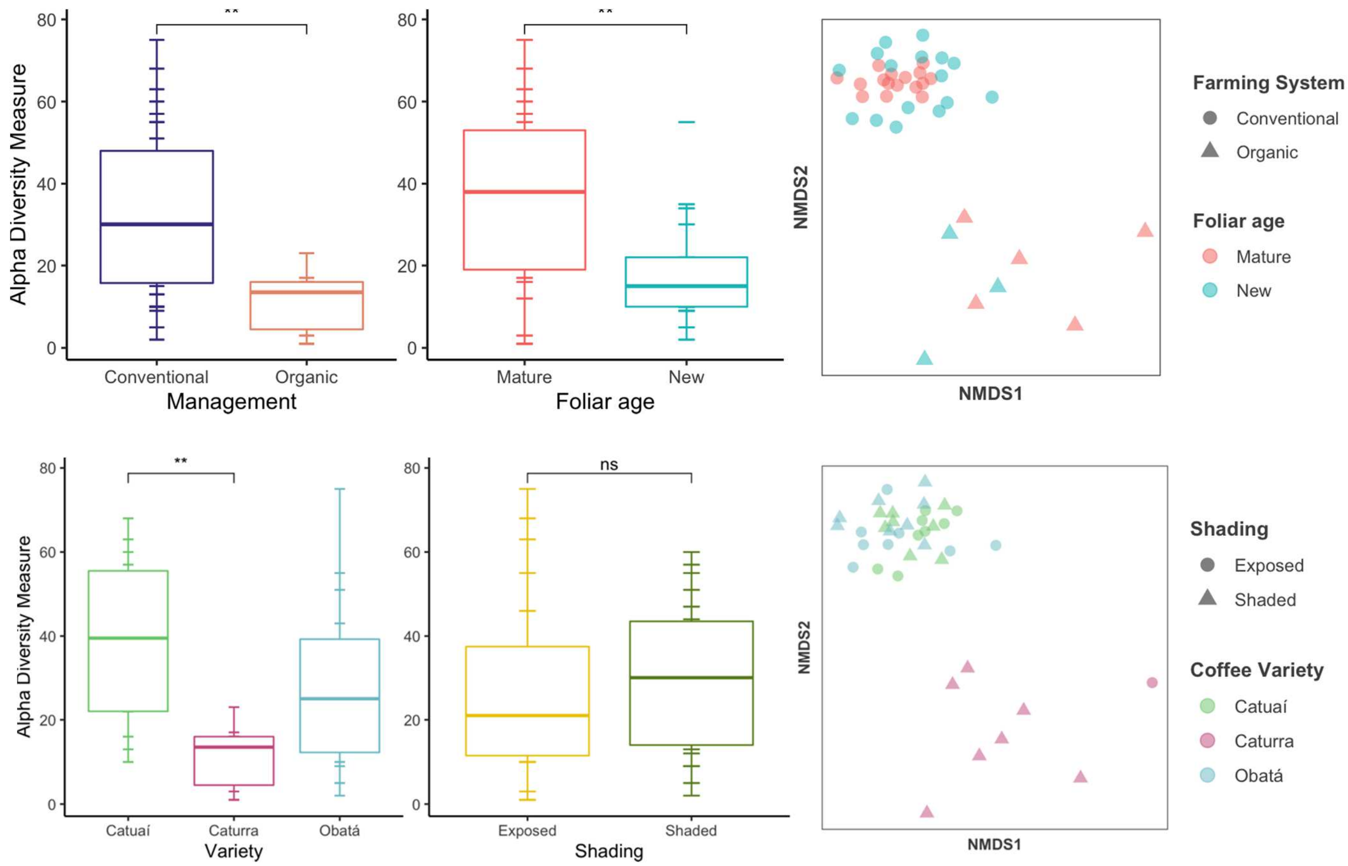

Figure 1. Alpha (Chao 1 index) and beta diversity (Bray-Curtis NMDS) of fungal communities modeled through different habitats and agricultural practices. Statistical significance is shown.

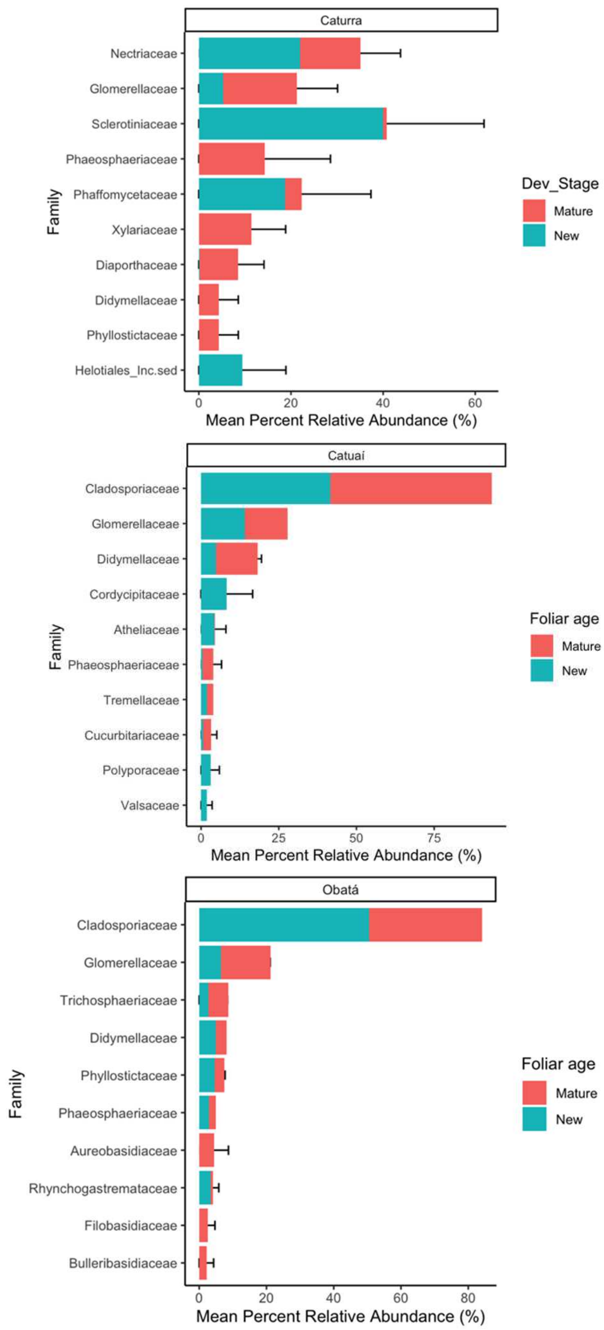

Figure 2. Percentage mean relative abundance of the taxonomic groups comprising the top 10 families in each coffee cultivar. Changes according to tissue developmental stage are displayed.

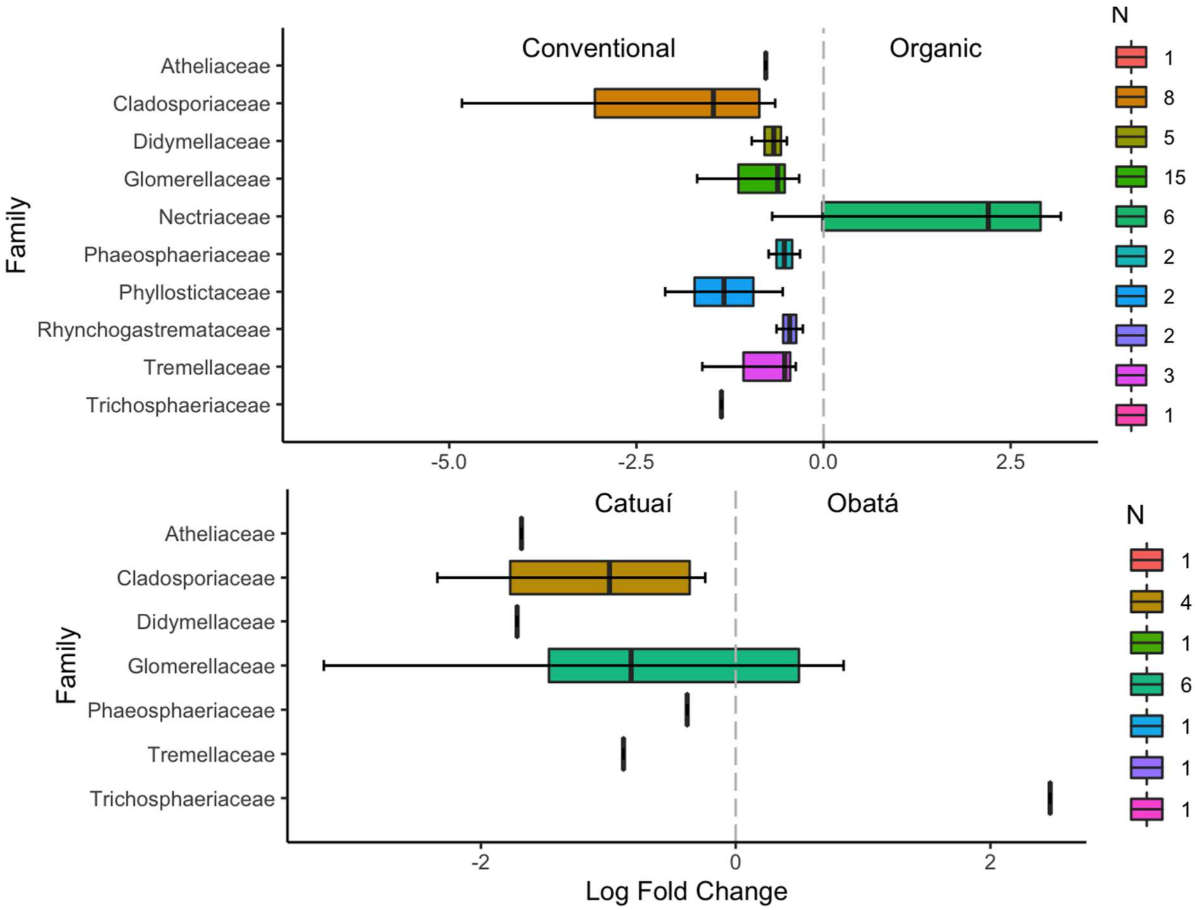

Figure 3. Differential abundance of Amplicon Single Variants (ASV) belonging to the most abundant families in conventional vs organic farming, and the two conventional varieties catuaí and obatá. Number of ASVs responsible for the changes in each family (N) is presented.

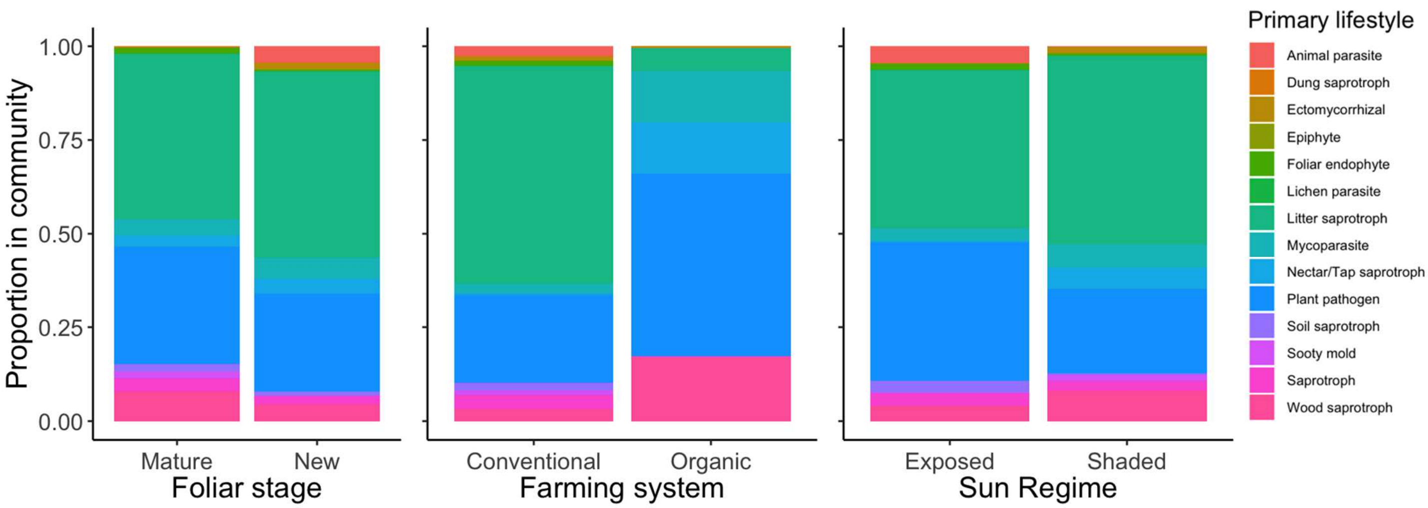

Figure 4. Relative abundance of the different lifestyles displayed by the community members in each environment.

Chapter 4: Insight into the interactions within the endophytic communities of domesticated coffee and its wild relatives from *Rubiaceae*.

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ABSTRACT

Plant domestication may have altered the microbiomes and the number of associations plausible in managed ecosystems compared to their wild counterparts. Taking advantage of the current flexible and affordable sequencing technologies, the readily available comprehensive taxonomic databases and the multitude of bioinformatic tools, we characterized the associated fungal endophytic communities of wild Rubiaceae and domesticated coffee to understand the extent to which domestication affected the diversity of the foliar microbiome and tested whether the fungal endophytic taxa in these communities displayed habitat preference. Leaves of coffee had lower species richness but contained similar taxonomic groups to leaves of wild Rubiaceae. Data sets in this paper were previously analyzed in papers that provided information about overall community composition but failed to capture the potential interactions among the identified

fungal groups. In this study, we gain a deeper insight into the interactions among the members of the endophytic population in coffee plantations and forests, finding evidence for both positive and negative co-occurrence relationships that can lead to new opportunities to better manage microbial communities in plant health.

INTRODUCTION

During domestication, the phyllosphere of agronomically important crops is believed to have endured substantial and unforeseen changes (White et al., 2019). For example, the selection of pathogen-resistant plants may have impaired their associations with other microbes. Compared to their wild relatives, modern cultivars present different defense responses and some lack protective microbiota and the required characteristics needed to recruit and sustain beneficial microbial populations (Abdullaeva et al., 2021). The environment and management practices also change plant growth conditions leading to exposure by novel microorganisms that could become plant colonizers (Pérez-Jaramillo et al., 2015). Hybridization and intercropping may favor an interchange of new symbionts (Martínez-Romero et al., 2020), but generally, modern agricultural relies on external inputs to sustain high yields and overcome biotic and abiotic stresses which translates to increased disturbance and reduced number of available ecological niches (Kleijn et al., 2011).

Therefore, the transition from natural to agricultural systems may have hampered the complexity and composition of plant associated microbiota, which depends on other factors including environment, geography, climate, host genotype and developmental stage (Trognitz et al., 2016). Fungal endophytes, a pivotal component of the plant microbiome, are important for plant growth dynamics, plant health, stress tolerance and productivity (Niu et al., 2017). We still know relatively little regarding the properties of the communities they form, however. For example, endophytic communities could represent random assemblages of the species pool available at a specific location or consist of nonrandom species assemblages derived from interspecific interactions (Pan & May, 2009).

Individual plants represent complex systems whose productivity is a function of interactions not only with their abiotic environment but with the diverse organisms associated with them as well. As sequencing technologies continue to evolve, research on plant microbiomes has gained momentum highlighting its crucial role in plants. The holobiont exceeds the genetic and physiological diversity of the host plant and thereof should be considered a second genome (Braga et al., 2016; Gopal & Gupta, 2016). Endophytes have been isolated from all the plants examined to date and are present in all of their tissues, indicating an intimate level of a symbiotic relationship (Sahu & Mishra, 2021b). Records of plant – fungi associations date back more than 400 million years (Card et al., 2016), However, the large number of putative taxa recovered from microbiome studies pose a challenge to understand the nuances of community dynamics, structure, and function (Poudel et al., 2016).

Populations of microorganisms in the environment rarely consist of a single species; microbial interactions are inherent to the establishment of communities. Fungal—fungal interactions are a key to colonization and persistence in a variety of different environments, including plant tissues (Braga et al., 2016). Endophytes create a complex ecological network by interacting with each other to promote or hinder coexistence of multiple species in the same trophic level, such as mutualism, competition, antagonism, predation, and parasitism (Siepielski & McPeek, 2010). Significant spatial co-occurrence is considered evidence of positive interactions, and co-exclusion is evidence of negative interactions. Co-occurrence patterns can also be interpreted in terms of resilience and species response to environmental factors or dispersal limitation, however, revealing not only similar habitat preferences but the strength of the biotic interactions when the environmental processes strongly shape the species distributions (Freilich et al., 2018). In the area of plant microbiome research, relatively few efforts have been made to compare the potential interactions and the resulting ecological function of endophytes in native and domesticated habitats (Sun et al., 2020; Tian et al., 2020).

The microbiome has been proposed as a promising tool for the next green revolution. Efforts to discern the underlying mechanisms that drive the assembly, composition and function of plant microbiomes would open new avenues for sustainable agriculture (Suman et al., 2022). Elucidating the maintenance of endophytes in wild relatives of crop species may lead to the exploitation of beneficial interactions for crop enhancement as these fungi represent a remarkable reservoir of ecological functions. To investigate how the diversity of endophytic fungal communities change from widely consumed, domesticated *Coffea arabica* to its wild relatives in *Rubiaceae*, a comparative analysis of reported data on the composition and diversity of endophytic foliar microbiomes was conducted. This study aims to provide a better understanding of how endophytic communities change after domestication and ultimately contribute to the efforts on restructuring the microbiome of plants in agricultural systems, specifically coffee production. We hypothesize that i) communities of fungal endophytes assemble in response to location and not growing ecosystem (wild vs plantation), ii) wild relatives recruit and sustain a richer and more diverse community of endophytes compared to domesticated coffee, iii) the dominant species differ in coffee because of domestication and iv) the most abundant fungal families exhibit positive and negative co-occurrence patterns rather than random niche overlap.

METHODS

We evaluated two datasets previously described in Castillo-González *et al.* (2022a) and Castillo-González *et al.* (2022b) by combining and reanalyzing the sequence libraries using the R package phyloseq v1.38.0 (McMurdie & Holmes, 2013). In the first study, the authors evaluated the effect of location, leaf developmental stage, and tissue type on the community composition and diversity of endophytes spanning a range of tropical plant species belonging to the *Rubiaceae* family. In the second study, the endophyte communities of foliar samples from different coffee varieties, agricultural management practices, sun regime and tissue developmental stage were examined.

Characteristics of the sampling locations, strategy and processing are therein described. In brief, 6 leaf samples were collected from 50 individual wild plants in Golfito and Guanacaste, and 24 individual coffee plants in San Marcos and Las Mellizas, all of them in Costa Rica. Sections of the tissue were excised and disinfected to reduce external contamination. The total DNA was extracted using the Qiagen DNeasy Plant Mini Kit® (Qiagen, Hilden, Germany) in a FastPrep® tissue homogenizer. The DNA extracts were PCR-amplified and sequenced with ITS2 nrDNA primers: *f*ITS7 and ITS4, on the Ion PGM Torrent™ platform (Thermo Fisher Scientific, Waltham, MA, USA).

These publications described responses in fungal diversity and community composition in the context of ecological drivers, with no analysis of the potential interactions among the observed putative taxa. In the current study, statistical analyses and plotting were conducted in RStudio, version 2022.2.0.443

(RStudioTeam, 2022), R v 4.0.3. To test for uniqueness in the community composition we classified the ASVs according to location using a Venn diagram, with the MicEco package v 0.9.17 (Russel, 2021). We compared the alpha diversity in each of the sampling locations by calculating the Shannon, Simpson, and Chao1 indexes, followed by an ANOVA test. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of ASV abundances were used to visualize community patterns (beta-diversity) according to sampling location and statistical differences in endophyte assemblages were tested with a PERMANOVA.

The identified families were statistically classified into habitat generalists or specialists of the growing ecosystem (forest vs plantation) using a multinomial species classification method (CLAMtest) in vegan v2.5.7 (Oksanen et al., 2020), and the top 10 most abundant families in the dataset were plotted in a Sankey diagram showcasing differences in relative abundance between crop and wild habitats, using the networkD3 v0.4 package (Allaire et al., 2017).

A fungus – fungus incidence matrix was created based on presence/absence of the taxa in the different plants, and the number of coincidences of the top 10 families was graphed in a chord diagram using the package circlize v0.4.15 (Gu et al., 2014). The package cooccur v1.3 (Griffith et al., 2016) was used to classify the co-occurrence between each and all pairs of families as having positive, negative, or random associations. The positive interactions of the five most abundant families were represented in an arc diagram using the package arcdiagram v0.1.12 (Sanchez, 2018). Finally, we applied the Spearman correlations on abundance data of these five families to test for spatial association between taxa using the microeco v0.11.0 (C. Liu et al., 2021). The sign and magnitude of the association is that of the correlation coefficient.

RESULTS

The sequences library constituted 6,389,279 reads that passed bioinformatics quality control filters, the number of samples (i.e., plants) was reduced to 68, and the average number of sequences per sample was 93,960. We encountered 3,209 ASVs belonging to at least 17 classes, 66 orders, 154 families and 250 different genera. In Golfito we identified the highest number of putative taxa, followed by Guanacaste, San Marcos, and Las Mellizas. The latter presented the highest percentage of exclusive ASVs (88%), and San Marcos the highest percentage of shared taxa (55%). The locations in the wild habitats shared the greatest number of ASVs. And remarkably, none of the putative taxa was shared by all locations (Figure 1A). In terms of richness, the communities differed between collecting site. The alpha diversity indices (Shannon, Simpson and Chao1) were higher in the samples from natural environments in Golfito and Guanacaste than in coffee plantations (Figure 1B). The Shannon and Simpson indices were not different between the wild habitats, and the plantations appeared to be statistically similar to each other (Table 1).

Five of the families made up the bulk of the community population, almost 60%. The ten most abundant families in the dataset, in order were *Glomerellaceae*, *Phyllostictaceae*, *Mycosphaerellaceae*, *Cladosporiaceae*, *Xylariaceae*, *Diaporthaceae*, *Sporocadaceae*, *Didymellaceae*, *Botryosphaeriaceae* and *Nectriaceae*; all but the second to last were shared between wild and cultivated plants (Figure 2A). *Cladosporiaceae* was the major group in coffee, while *Glomerellaceae* dominated the wild plants. *Glomerellaceae* contributed only one genus to the overall diversity, i.e., *Colletotrichum*, and *Xylariaceae* encompassed 13 different genera (Table 2).

Most putatively identified ASVs were found to be wild habitat specialists (71.2%) (Figure 2B). Interestingly, from the 10 most abundant families a majority were classified in this category, none were classified as generalists. The crop specialists included ASVs from *Cladosporiaceae*, *Didymellaceae*, and *Nectriaceae*. Rare taxa such as *Amylocorticiaceae* and *Hyphodermataceae* were too low in abundance to classify them as specialist or generalists. *Glomerellaceae* presented the highest number of co-occurrences with the remaining families from the top 10 most abundant, with *Cladosporiaceae* it coincided the most, in 61 instances and with *Botryopshaerieacea* overlapped the least, only in 26 cases. *Didymellaceae* co-occurred in the smaller number of samples with the rest of the families in this group, sharing habitat in 30 occasions with *Glomerellaceae* and 9 times with *Botryopshaerieacea* (Figure 3A).

We investigated the pairwise co-occurrence patterns between all families detected in the dataset and while most of them were categorized as random, the 5 most abundant families (i.e., *Glomerellaceae*, *Phyllostictaceae*, *Mycosphaerellaceae*, *Cladosporiaceae*, *Xylariaceae*) presented positive interactions with other 24 other families (Supplemental Figure 1). Xylariaceae had the highest number of these interactions, with 18 other families and *Glomerellaceae* presented the least, only 4 (Figure 3B). We also observed negative interactions involving these families, for example, *Phyllostictacea* and *Mycosphaerellacea* with *Neodevriesiaceae* and *Xylariaceae* with *Tremellaceae* (Supplemental Figure 1).

In partial agreement with these results, we found that Spearman correlation coefficients between the dominant families were mostly positive (Table 3). Only Xylariaceae was negatively correlated with *Cladosporiaceae* at a very negligible level, however, this interaction was not significant. And while none of the pairwise correlations showed a very strong relationship (≥ 0.70), *Xylariaceae* presented strong relationships with *Phyllostictaceae* and *Glomerellaceae*, which at the same time presented a moderately high correlation between them.

DISCUSSION

Overall, the leaves collected from different sampling sites contained locally endemic fungal endophytes (Figure 1A), suggesting that geographic location is the main factor shaping phyllosphere fungal communities. As expected, the undisturbed communities in forests had higher species richness than those in the agroecosystems (Figure 1B), but no differences were observed in richness between the two natural habitats or between the two plantations (Table 1). Whether *Coffea arabica* has lost the genetic cues governing the assembly processes, as a result of domestication, remains an open question. The higher diversity of endophytes found in native environments could suggest that they play a more critical role in these plants; for example, warding off pests and pathogens (Q. L. Chen et al., 2021). Despite differences at the ASV level, when sequences were classified at the family level the composition of the communities in both ecosystems (wild vs crop) were governed by the same taxonomic groups (i.e., *Glomerellaceae*, *Phyllostictaceae*, *Mycosphaerellaceae*, *Cladosporiaceae* and *Xylariaceae*) (Figure 2A). The presence of a preserved microbiome despite plant domestication shows that some degree of ecological coherence exists across plant species, ecosystems, and geographical locations. Whether the taxa found in crops preserve the same traits as those in wild forms remains unclear.

The reduced species richness found in the domesticated coffee could be due to an alteration of the mechanisms involved in microbiota assembly. Endophytes must be able to resist the internal foliar environment, bypass plant defense mechanisms and endure the external factors that affect their transmission and colonization (Mengistu, 2020). The use of agrochemicals may therefore impact agricultural microbiomes (Prior et al., 2017; A. K. da C. Stuart et al., 2018). Compared to their wild relatives, the disease pressure in coffee plants is higher, and plants are exposed to several agricultural practices that may impact endophyte diversity and function (Sahu & Mishra, 2021b). Several studies have concluded that use of fertilizer, crop rotation, tillage, and other agronomic practices affect the diversity and composition of endophytes (Cernava et al., 2019; Gdanetz & Trail, 2017; Pancher et al., 2012; Wemheuer et al., 2019).

Not many studies have compared the microbiome of crops to their wild counterparts. In the case of coffee, studies have been limited to other species in the *Coffea* genus, for instance, a study comparing the leaf surface mycobiome of *Coffea arabica* with wild species *C. racemosa* and *C. stenophylla* using ITS sequencing found less abundance in the cultivated crop (de Sousa et al., 2021). Another study on rhizosphere microbiomes indicated that *C. arabica* and *C. stenophylla* have different microbiomes, but no differences were detected with three other *Coffea* species (de Sousa et al., 2022). Comparable results were found for wild rice which harbored more root endophytic fungi than the cultivated species (Tian et al., 2021). Similar results were also found in a study analyzing the fungal endophyte communities of wheat and two of its ancestors, revealing that some fungal taxa were common to both the cultivated and wild varieties, but that there was greater species richness in the wild relatives (Sun et al., 2020). Interestingly, another investigation found the opposite: a more diverse microbiota was associated to the seeds of modern cereals in comparison to their wild ancestors, suggesting that breeding led to compositional shifts in the plant-associated microbiome as a result of soil conditions, and/or crop management methods (Abdullaeva et al., 2021).

Investigating management and plant breeding strategies that could improve agricultural resiliency is imperative as anthropogenic pressures increase (Grünig et al., 2020). Fungal taxa able to adapt to wide geographic settings and different habitats may be used for enhancing crops in the future. Although many endophytic taxa have ecological niche preference, generalist species might be suitable prospects as they present larger potential distribution that may result in an increased number of possible associations. The ability to sustain a stable network of interactions in a community is an important buffer to perturbations. An integrated network supports the concept of a holobiont, in which plants act as sources of microorganisms that ensure communication of the plants, with each other, and their environment and contributes to the adaptability and/or fitness of the hosts (Sánchez-Cañizares et al., 2017). Many fungal species play redundant roles by altering or manipulating the distribution of the same resource (Wutkowska et al., 2019) and fungi compete naturally with other groups of fungi to occupy niches (del Carmen H. Rodríguez et al., 2021). Understanding the extent of functional redundancy in endophytic communities should be a priority for future research focused on identifying fungi that could enhance agronomic production.

We initially hypothesized that endophyte community assembly would be dominated by positive and negative species interactions in contrast to assemblages determined by chance. We did observe co-occurrence interactions, but most were weak correlations. In other words, a large number of pairwise species co-occurrences in these communities might be the result of chance (Supplemental Figure 1). Fungal taxa in the dominating families seemed to overlap in a high number of samples (Figure 3A). Co-presence is not always an indicator of strong positive interactions. In our study, however, Spearman coefficients revealed moderate to strong significant positive interactions among these 5 taxonomic groups in more than half of the cases (Table 3). Pairwise comparison showed the presence of even more positive interactions with other families in these communities (Figure 3B). Observations of positive species co-occurrences are usually attributed to interactions such as facilitation. In fungal endophyte communities, it could mean that once taxa from a family colonizes a plant it leads to the host becoming more vulnerable to infection by the other taxa that presented the positive interactions (Freilich et al., 2018). Positive co-occurrences could also be attributed to different species having similar host requirements or a lack of competitive exclusion (Lee et al., 2019). Given the competitive potential and high adaptability harbored in fungal communities, the nature of co-occurrence interactions would be better understood across time, which would also inform about the structure of the communities and the dynamics of fungal succession.

CONCLUSIONS

At finer level, the assembly of fungal endophytes was unique to each collecting site, however at the family level the wild fungal communities were more similar to each other than to those in plantations, regardless of geographic location. Plants in wild environments were able to recruit and sustain a richer and more diverse community of endophytes compared to domesticated coffee. Some fungal taxa displayed habitat preference for the crop environment and similar taxonomic groups were found in the composition of the communities from both habitats, with certain differences in the relative abundance of the dominating groups, *Glomerellaceae* in the forests and *Cladosporiaceae* in the farms, which might be due to domestication. The dominant fungal families exhibit positive and negative co-occurrence patterns but overall, the incidence of endophytes in the plants might be just the product of chance and random niche overlap. Co-occurrence data does not reproduce interactions but provide interesting and interpretable information about community assembly that can be used to manage the microbial communities of crops in sustainable agriculture.

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FIGURES AND TABLES

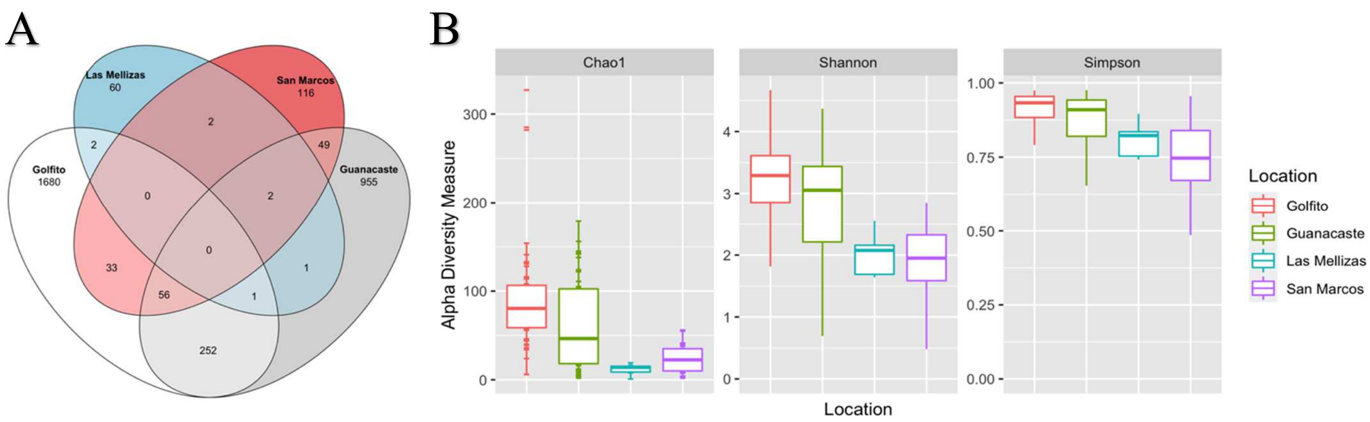


Figure 1. A) Number of unique and shared ASVs in the four locations sampled. B) Alpha diversity (Chao1, Shannon, and Simpson indexes) representing richness in the sampling sites.

Table 1. Reported p-values for pairwise Wilcoxon rank sum tests between alpha diversity values from different indexes for the different sampling locations.

Index		*Golfito*	*Guanacaste*	*Las Mellizas*
Chao1	*Guanacaste*	0.0116	-	-
	Las Mellizas	3.6e-05	0.0038	-
	San Marcos	1.5e-11	0.0024	0.0602
Shannon	*Guanacaste*	0.05613	-	-
	Las Mellizas	0.00014	0.01052	-
	San Marcos	1.3e-09	0.00014	0.93397
Simpson	*Guanacaste*	0.07717	-	-
	Las Mellizas	0.00069	0.02500	-
	San Marcos	1.3e-08	7.7e-05	0.47468

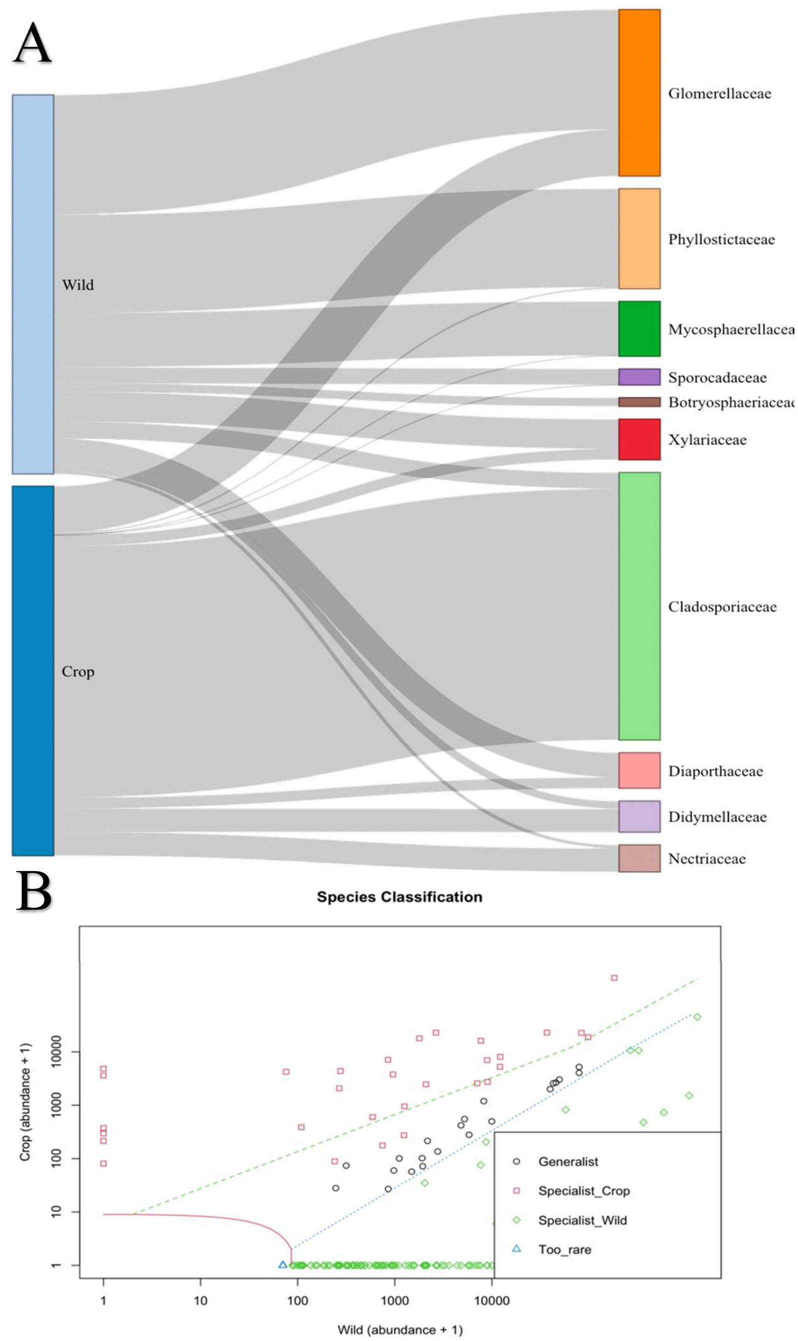


Figure 2. A) Classification of identified fungal families in habitat specialists or generalist as indicated by a CLAM test. B) Distribution of the relative abundance of the top 10 most abundant families according to growing ecosystem (crop vs wild).

Table 2. Identified fungal genera and species in the most abundant families.

Family	Genus	Species
Glomerellaceae	*Colletotrichum*	*C. sydowii, C. aciculare, C. magnisporum, C. gloeosporioides, C. arxii, C. fusiforme*
Phyllostictaceae	*Phyllosticta*	
Mycosphaerellaceae	*Pseudocercospora*	*P. luzardii, P. leandrae-fragilis, P. paranaensis*
	Zasmidium	*Z. dalbergiae, Z. musae*
	Passalora	*P. haldinae*
	Australosphaerella	*A. nootherensis*
	Phaeophleospora	*P. hymenocallidicola*
	Cercospora	
	Neoceratosperma	
Cladosporiaceae	*Cladosporium*	*C. sphaerospermum*
	Toxicocladosporium	
Xylariaceae	*Annulohypoxylon*	*A. stygium*
	Daldinia	*D. starbaeckii*
	Nemania	*N. abortive, N. bipapillata*
	Zygosporium	*Z. pseudogibbum*
	Anthostomella	
	Biscogniauxia	*B. citriformis, B. capnodes*
	Circinotrichum	*C. cycadis*
	Muscodor	*M. suthepensis*
	Xylaria	*X. discolor*
	Hypoxylon	*H. subrutiloides, H. erythrostroma, H. investiens, H. anthochroum, H.*

		notanum, H. hypomiltum
	Stilbohypoxylon	*S. elaeicola, S. quisquiliarum*
	Zygosporium	
	Kretzschmaria	*K. pavimentosa*

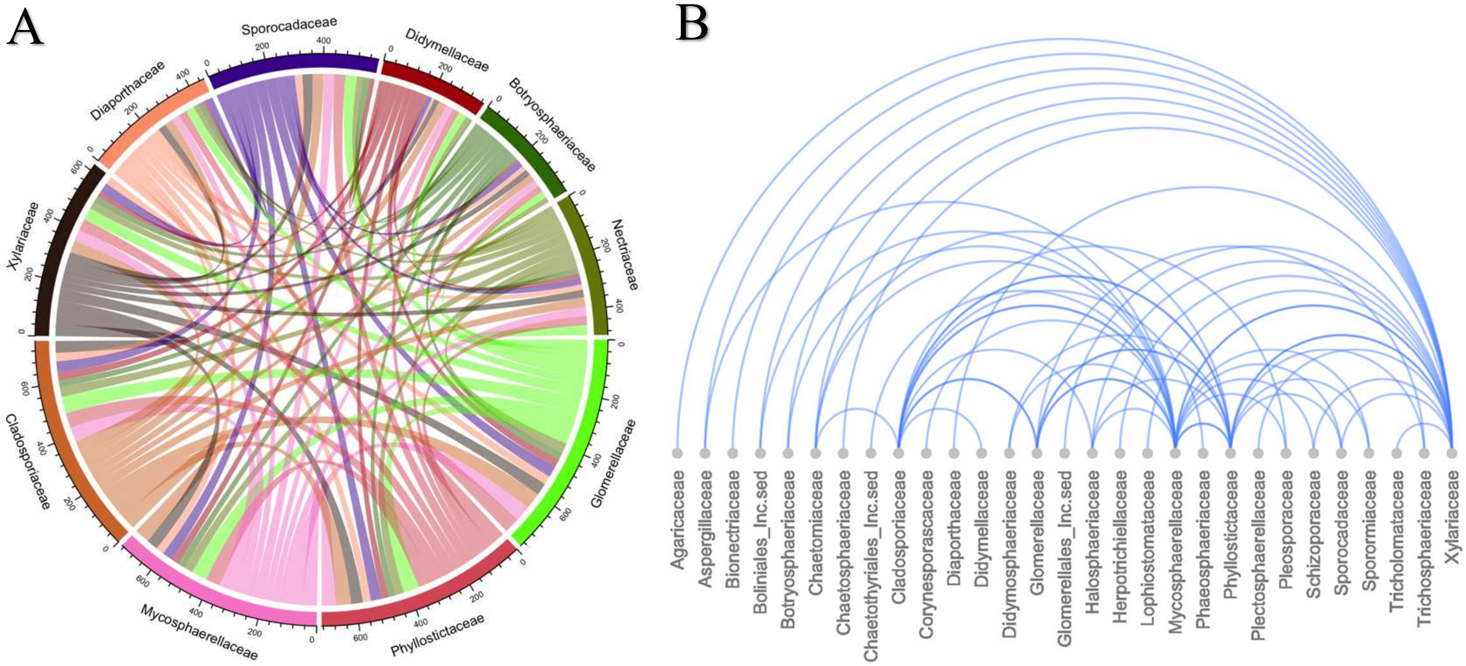
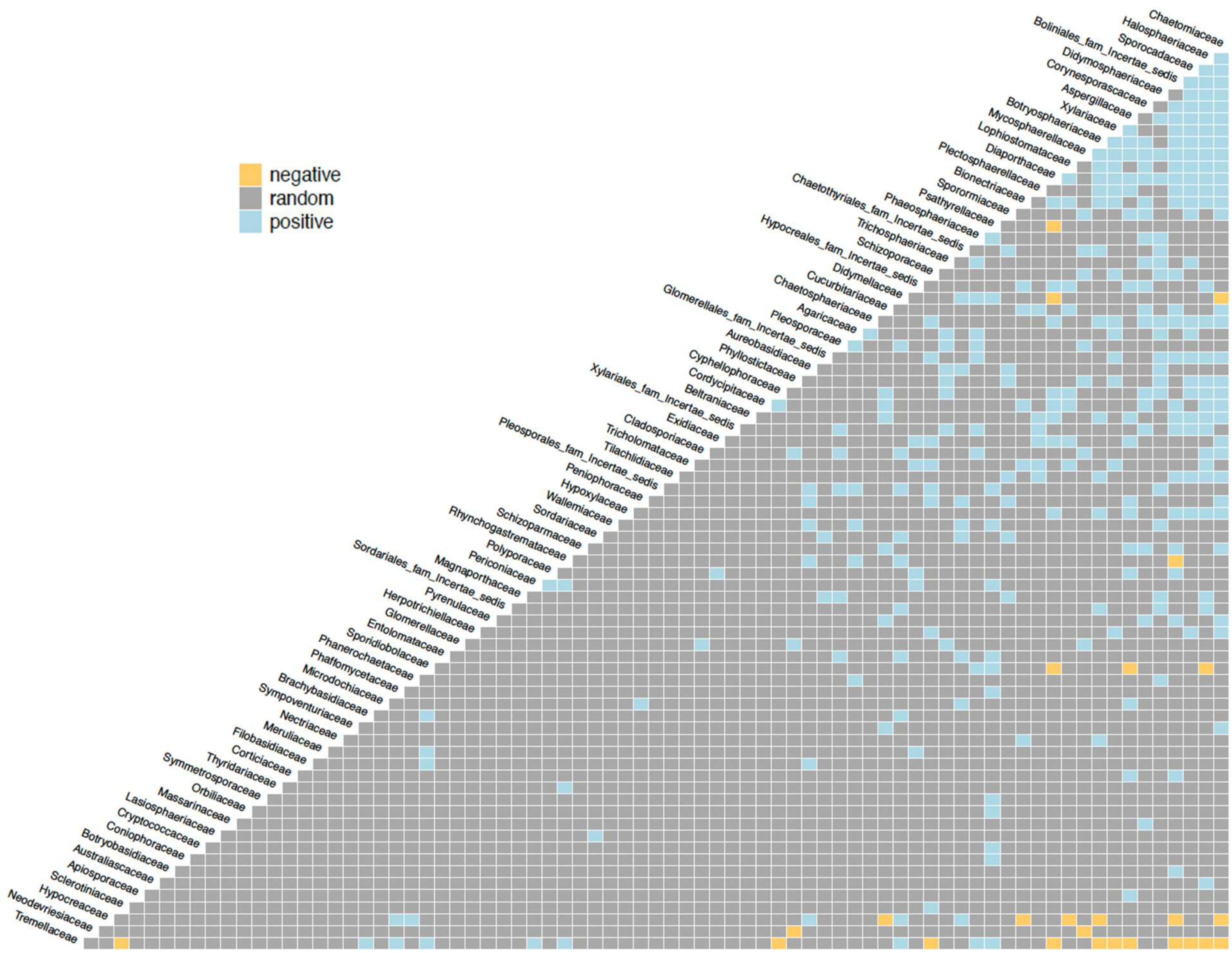


Figure 3. A) Number of co-occurrences within the 10 most abundant families. B) Diagram of the families presenting a positive interaction with at least one of the 5 most abundant families.

Table 3. Spearman correlation coefficient for co-occurrence interactions among the 5 most abundant families. Significant correlations (p<0.05) are in bold and italics.

	Phyllostictaceae	**Glomerellaceae**	**Cladosporiaceae**	**Mycosphaerellaceae**	**Xylariaceae**
Phyllostictaceae	1.0000	***0.6038***	0.0623	***0.4890***	***0.6525***
Glomerellaceae		1.0000	0.0082	***0.4875***	***0.6547***
Cladosporiaceae			1.0000	0.0611	-0.0681
Mycosphaerellaceae				1.0000	***0.4834***
Xylariaceae					1.0000

SUPPLEMENTAL INFORMATION



Supplemental Figure 1. Species co-occurrence matrix, showing pairwise negative, neutral and positive interactions between fungal families.

Chapter 5. Concluding remarks and future directions of research.

An increasing body of research has focused on the associations and relationships between endophytes and host plants. Although some progress has been made in recent years, the gap on knowledge is still substantial and the modification of microbial communities or microbiome engineering in plants is still far from being a normality. While comprehensive, the approach presented in this investigation, of identifying key fungal taxa and their interactions, comparing microbial communities among different host plants in different habitats is not the panacea that will enhance crop productivity. However, it provides the opportunity to assess plant-microbial associations through an interesting optic. How to translate this acquired knowledge for forest conservation and agricultural management remains a challenge.

In Chapter 2 we explored how region, tissue type and leaf developmental stage affect community composition and diversity of endophytes in a broad range of tropical plant species within *Rubiaceae* and found no specific host affiliation. Plant tissues, exposed to different environments in time and space, present different structural and chemical properties that can influence symbionts, however, the most abundant fungal taxa seemed to be consistently present within the microbiome. The endophytic communities found in foliar tissues appear to be reach stasis early on during tissue development and persist to mature stages, but differences were observed between different tissue types, likely because of niche partitioning. At regional scales, the mechanisms of community assemblage are not clear, we found weak evidence for dispersal limitation and environmental partitioning, therefore our results might have resulted due to unknown environmental variables correlated with the different sampling locations.

Understanding the ecology of plant – microbe associations is important considering the potential implications for agronomically important plants such as *Coffea arabica*. In Chapter 3, multiple factors of coffee production agroecosystems, such as location, coffee cultivar, and agricultural management practices were studied to comprehend their effect on fungal assemblage. Some of the observed differences seemed to be nested in the cultivar, for example, as a result of tissue developmental stage. However, cropping system or geographical location were the main drivers of differences in community abundance and composition, and to a lesser extent the coffee variety. No evidence of a conserved community was found but some of the taxa were cosmopolitan over large geographical distances and across different farming practices. Conventional farming as opposed to organic presented a higher abundance and diversity of endophytic fungi at taxonomic level. Nevertheless, several important functional guilds differed between the farming management and sun regimes, underlining how farming practices can shape the structure of endophytic communities,

In farms, plants may have impaired associations with microbes. Domestication could result in the loss of the genetic and physiological features needed to select for beneficial endophytic populations. In Chapter 4, the associated fungal endophytic communities of wild *Rubiaceae* and domesticated coffee were compared to understand the extent to which communities in natural to agricultural ecosystems change. This chapter also explored the potential co-occurrence interactions among the identified taxa. It was observed that at finer level, the composition of fungal endophytes was unique to each collecting site, however at the family level the wild fungal communities were more similar to each other than to those in plantations. Plants in the forests harbored a richer and more diverse community of endophytes compared to those in farms, but some endophytes displayed habitat preference for the crop environment. *Glomerellaceae* was the most abundant family in the forests and *Cladosporiaceae* in the farms. The dominant fungal families exhibited positive and negative co-occurrence patterns but overall, the incidence of endophytes in the plants might be just the product of chance and random niche overlap.

It's important to highlight the effect of several edaphic and environmental factors in modulating the phyllosphere biodiversity and the distribution and performance of endophytes in the field, both in wild habitats and in plantations. Endophytes harness the potential to transform the way we think about and manage plant stress resistance. Understanding their distributions and functioning in association with different host plants and ecosystems, and their response to environmental disturbances, is key to predicting the potential impact of many of these fungi in agricultural ecosystems and climate change science. This work provided new insights into complex microbial interactions and highlighted the importance of integrating fungal endophytes into microbial ecology for sustainable agriculture and conservation science research.

Future research on plant and endophyte associations in connection to crop plants should focus on the following areas:

- Test the prediction that the foliar phytochemical diversity plays a role in selecting the fungal endophytic lineages and phylotypes. Chemical profile in leaf tissues may predict possible niche adaptation; if so, the presence of certain fungi is expected to correlate with the metabolome.
- In that line, understanding the mechanisms underlying the utilization of plant compounds by the microbiota would be important.

- Investigate how endophytes are related to host inherited traits and whether endophytes might have co-evolved with their hosts.
- Compare the fungal phenotypes of similar species found in crops and wild relatives to assess differences between the strains and conserved traits and functions.
- Evaluate the interactions between host plants and endophytic fungi at different stages of the plants and in the presence of biotic stress. The perturbation of the endophytic community with pathogens will help determine how fragile or resilient is the system to external.
- As external inputs are expected to perturbate the plant-endophyte system eliciting different responses it would be interesting to determine how the use of agrochemicals, especially fungicides, affect the community structure.
- Untangle the assembly rules to identify specific mechanisms that influence endophyte community dynamics for example, successional and temporal change.
- Transcriptome approaches can aid elucidate how and which genes in both endophytes and the plant are expressed (up and down regulated) under stress.
- Investigate the multi species interactions and fungal traits that mediate them, such as the production of chemical compounds and their competitive modes (e.g., mycotrophy, competition, antibiosis).
- Network models to determine the presence of keystone taxa or hubs, as well as the overall relationships and significance of each node in the community.

- Cross-feeding interactions could be determined. Detecting metabolic interactions, dependencies and the nature of exchanged metabolites.