ABSTRACT

Title of Document: **EVALUATING THE ENDOPHYTIC FUNGAL COMMUNITY IN PLANTED AND WILD RUBBER TREES (*Hevea brasiliensis*)**

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The main objectives of this dissertation project were to characterize and compare the fungal endophytic communities associated with rubber trees (*Hevea brasiliensis*) distributed in wild habitats and under plantations. This study recovered an extensive number of isolates (more than 2,500) from a large sample size (190 individual trees) distributed in diverse regions (various locations in Peru, Cameroon, and Mexico). Molecular and classic taxonomic tools were used to identify, quantify, describe, and compare the diversity of the different assemblages. Innovative phylogenetic analyses for species delimitation were superimposed with ecological data to recognize operational taxonomic units (OTUs) or "putative species" within commonly found species complexes, helping in the detection of meaningful differences between tree populations. Sapwood and leaf fragments showed high infection frequency, but sapwood was inhabited by a significantly higher number of species. More than 700 OTUs were recovered, supporting the hypothesis that tropical fungal endophytes are

highly diverse. Furthermore, this study shows that not only leaf tissue can harbor a high diversity of endophytes, but also that sapwood can contain an even more diverse assemblage. Wild and managed habitats presented high species richness of comparable complexity (phylogenetic diversity). Nevertheless, main differences were found in the assemblage's taxonomic composition and frequency of specific strains. Trees growing within their native range were dominated by strains belonging to *Trichoderma* and even though they were also present in managed trees, plantations trees were dominated by strains of *Colletotrichum*. Species of *Trichoderma* are known for their biocontrol properties, whereas species of *Colletotrichum* have been always associated with plant disease.

EVALUATING THE ENDOPHYTIC FUNGAL COMMUNITY IN PLANTED AND WILD RUBBER TREES (*Hevea brasiliensis*)

By

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Foreword

This thesis is based on the following articles, which are referred in the text as Chapters:

1. **Gazis R.**, Chaverri, P. 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3: 240-254

2. **Gazis R.**, Rehner S., Chaverri P. 2011. Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. Molecular Ecology 20: 3001-3013.

3. **Gazis R.**, Gruner D.S., Chaverri P. 2012. Sampling effect in estimating tropical fungal endophyte diversity: Are we undersampling?

4. **Gazis R.**, Miadlikowska J., Arnold B., Lutzoni F., Chaverri P. 2012. Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of Pezizomycotina (Xylonomycetes).

5. **Gazis R.**, Chaverri P. 2012. Amazonian forests are a reservoir of natural enemies against plant diseases: A comparison of fungi associated with wild and planted rubber trees (*Hevea* spp.).

Other publications deliverable from this study:

- Chaverri P., **Gazis R**. 2010. *Perisporiopsis lateritia*, a new species on decaying leaves of Hevea spp. from the Amazon basin in Peru. Mycotaxon 113: 163-169.
- Chaverri P., **Gazis R**., Samuels, G.J. 2010. *Trichoderma amazonicum*, a new endophytic species on Hevea brasiliensis and *H. guianensis* from the Amazon basin. Mycologia 103: 139-151.
- Chaverri P., **Gazis R**. 2010. Linking *ex planta* fungi with their endophytic stages: *Perisporiopsis*, a common leaf litter and soil fungus, is a frequent endophyte of *Hevea* spp. and other plants. Fungal Ecology 4: 94-102.

Dedication

To my grandparents Julia and Jose Olivas, whom I miss every day.

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Introduction

Endophytic fungi have been defined as fungi that live inside asymptomatic or apparently healthy plant tissue (Petrini 1991, Wilson 1995, Schulz et al. 2002). Unlike mycorrhizal fungi, fungal endophytes reside entirely within plant tissue and may grow within roots, stems and/or leaves, emerging to sporulate at plant or host-tissue senescence (Stone et al. 2004). Fungal endophytes are considered to be ubiquitous and have been found in every plant species examined to date (Arnold 2007 & Lutzoni 2007, Rodriguez et al. 2009). Traditionally, fungal endophytes have been divided into two major groups: clavicipitaceous endophytes (C-endophytes) and non-clavicipitaceous endophytes (NC-endophytes). C-endophyte infections are limited to some cool-and warm-season grasses and produce a systemic intercellular infection. Their transmission is primarily vertical, passing from maternal plants to offspring through seeds. Usually, colonized plants harbor one to a few fungal isolates/genotypes. Therefore, their community is characterized by having low diversity, in terms of number of species and genetic variability. Relationships of C-endophytes and their hosts have been studied extensively due to its mutualistic symbiosis, which typically increases their host fitness and consequently has applications in crop systems (Seiber 2007, Stone et al. 2004, Rodriguez et al. 2009).

NC-endophytes are highly diverse and belong to many lineages, primarily in the Ascomycota and Basidiomycota (Herre et al. 2007, Thomas et al. 2008; Arnold et al. 2009, Parfitt et al. 2010). NC-endophytes have been recovered from multiple plant lineages and from all terrestrial ecosystems, including both agro-ecosystems and biomes ranging from the tropics to the tundra (Higgins et al. 2007, Vega et al. 2010).

In addition, they have been recovered from algae, mosses, ferns, and lichens (Zuccaro et al. 2008, Pressel et al., 2010, U'Ren et al. 2010). The NC-endophytes group has been divided into three classes depending on their host colonization patterns, mechanisms of transmission between generations, *in planta* biodiversity levels, and ecological functions (see Table 1, Rodriguez et al. 2009).

Tropical trees are colonized primarily by NC-endophytes Class 3 (Arnold 2008). Most of these NC-endophytes are transmitted horizontally, traveling among hosts as spores via the air, rain splash, or insect vectors. Hence, successful colonization of host tissues is affected by factors influencing local abundance of aerial and epiphytic fungal propagules (Bayman et al. 1998; Arnold & Herre 2003, Santamaria & Bayman 2005, Saunders et al. 2010). Fungal endophytes invade their host in the same way fungal pathogens do —through direct penetration, wounds, or natural openings such as stomata and lenticels (Seibert 2007, Slippers & Wingfield 2007). Many studies have characterized leaf endophytes' species diversity and infection mechanisms (Bayman et al. 1998, Arnold et al. 2003, U'ren et al. 2009); however, little is known about how and which endophyte species colonize sapwood tissue (Tejesvi et al. 2005, Samuels et al. 2006, Oses et al. 2008, Errasti et al. 2010). Studies have suggested that leaf samples harbor a greater number of fungal endophytes species because leaves have fewer infection barriers than does sapwood (Arnold & Lutzoni 2007). Leaf endophytes mostly originate from aerial fungal inoculum, whereas sapwood endophytes probably invade tree hosts through their root systems, moving from the soil to their new niche inside the plant. The most common endophyte genera found in stems (*Clonostachys*, *Fusarium*, *Trichoderma*, and

Penicillium) tend to be abundant in soil (Evans et al. 2003, Mejia et al. 2008, Hanada et al. 2008). Another mode in which endophytes could colonize the sapwood is by mobilization from the crown into the stem or trunk through the plant's vascular system (Bailey et al. 2008).

The ecological roles and nature of the host-interaction of Class 3 endophytes are still in debate (Arnold 2007, Hyde & Soytong 2008, Yuan et al. 2010). Several studies indicate that this group of endophytes can be mutualistic, despite the fact that several aspects of their biology (i.e., horizontal transmission and high diversity within host) are more frequently associated with parasitic or pathogenic lifestyles (Rodriguez et al. 2009). Endophyte-infected plants have been reported to increase below- and above-ground biomass (Tan & Zou 2001, Hamayun et al. 2010, Bailey et al. 2011). This effect is in part due to the ability of some endophytes to produce phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances or to the fact that endophytes could facilitate the hosts' uptake of nutritional elements such as nitrogen and phosphorus (Lue et al. 2000, Mucciarelli et al. 2003, Pirttilä et al. 2004, Yuan et al. 2010). Endophytic colonization may also improve the ecological adaptability of the host by enhancing tolerance to abiotic stresses (Rodriguez et al. 2004, Redman et al. 2005). Conversely, Shultz and colleagues (1998, 1999) demonstrated that some species have a negative effect in plant growth, and Arnold & Engelbrecht (2007) and Ren & Clay (2009) showed that some seedlings lost a greater amount of water during drought when plants were infected by Class 3 endophytes.

Based on the diversity of lineages included within Class 3 endophytes, a broader and a more complex scenario should be contemplated when their ecology is examined. This group of endophytes might not be increasing host fitness directly or immediately (i.e., promoting growth); rather, they might be protecting their host against predators (Carroll 1988, 1995, Aneja et al. 2005, Newcombe et al. 2009). Webber (1981) showed that colonization of *Ulmus glabra* (Elm) by the endophyte *Phomopsis oblonga* (an endophyte that was recovered in 75% of the healthy elms) not only reduced the number of galleries produced by elm bark beetles (vector of Dutch elm disease) but also reduced the beetle's larval development. Later, Arnold and colleagues (2003) inoculated *Theobroma cacao* endophyte-free leaves with fungal endophytes isolated from natural infected *T. cacao.* They observed a significant decrease of both, leaf necrosis and mortality, when endophyte-inoculated seedlings were challenged with a pathogenic strain of *Phytophtora*. Hanada and colleagues (2010) also performed trials on the *Theobroma* species, to test the biocontrol potential of fungal endophytes on black pod disease (*Phytophtora palmivora*), finding a significant decrease in disease severity. Additionally, Class 3 fungal endophytes have been reported to induce both systemic and localized resistance to a variety of plant pathogens (Harman et al. 2004). On the other hand, other studies have shown that tree endophytes can decrease resistance to herbivory (Saikkonen et al. 1999). Because herbivores may promote horizontal transmission and infection via damage or gut passage, selection for tolerance or facilitation of herbivory rather than resistance is expected (Faeth & Hammond 1997). Class 3 endophytes have also been considered as ecologically unimportant or neutral inhabitants (Sounders et al. 2010). Nonetheless,

endophytes are obligate heterotrophs and they densely colonize tissues of hosts that might be under carbon-limited circumstances (such as an understory plant in a tropical forest). Another hypothesis on endophyte-host interaction is that they might be mildly parasitic, subtly costing to their host plants fitness in ways not yet understood (Arnold 2005, Slippers & Wingfield 2007). Since this group of endophytes comprises very diverse fungal lineages, which at the same time present very diverse characteristics, no generalization about their ecological role can be stated. In summary, it appears that the Class 3 endophytes have a diversity of roles, ranging from mutualistic to pathogenic. Fungal endophytes of tropical trees may be either neutral in terms of cost, or mildly parasitic, but they might also confer upon their host a benefit that compensates, at least under certain conditions, for costs of endophyte infection (Arnold, 2005, Rodriguez et al. 2009).

Most endophyte studies have been conducted in temperate forests. Studies conducted in tropical regions have revealed an immense diversity of species, and a diversity gradient towards higher values in the tropics has been established (Arnold & Lutzoni 2007). For instance, Arnold and colleagues (2001) recorded 242 fungal endophytes morphotypes for *Heisteria concinna* and 259 for *Ouratea lucens* and Vega and colleagues (2010) recovered 257 unique ITS genotypes for *Coffea arabica*. Moreover, the overall diversity estimation for tropical fungal endophytes has been increased exponentially with the development of non-culture-dependent techniques such as direct PCR from leaves or sapwood (Hanada et al. 2010).

Endophyte research has increased in the last decade due to their vast array of potential uses, especially with the promise of discovering new bioactive compounds

(Strobel, 2003, Hyde & Soytong 2008, Guo et al. 2008, Suryanarayanan et al. 2009, Tejesvi & Pirttilä 2011). Nevertheless, a small number of studies have been conducted in the host's native habitat (Evans et al. 2003, Samuels et al. 2006, Sánchez et al. 2008). Arnold et al. (2003) evaluated the fungal endophytic community of *T. cacao* in the natural forests of Barro Colorado Island (BCI), Panama. However, all relatives of domesticated *Theobroma* are native to northern Amazonia in South America (Young, 1994; Evans et al. 2003). In Panama, cacao is cultivated intensively occurring at low frequency in primary and secondary forests in BCI (Arnold & Herre 2003) probably as a result of an ancient human introduction (Motamayor et al. 2002). Even though the individuals used in the mentioned study were distributed in natural forests, the species under investigation is not native from the area. Therefore, the community assemblage found in this survey may not share a coevolutionary history with the host, given that the time they have been associated might not been long enough to develop a mutualistic symbiosis (Thompson 2010). Evans et al. (2003) studied *Theobroma* within its native range; however, endophytes were sampled from *T. gileri*; thus, direct comparisons cannot be conducted until more is known about host specificity in this class of fungal endophytes. To date, only one study has compared the fungal endophytic community of a plant species within its native distribution range (or natural habitat) versus outside (either in plantations or forests). This study was conducted by Helander and colleagues (2006) and they compared the diversity and abundance of the horizontally transmitted fungal endophytic community of Silver birch (*Betula pendula*) in east central Finland. They found that the old

natural forest tended to have the most diverse community, but the difference was not statistically significant.

Endophytes as mutualistic facilitators

Through several studies it has been demonstrated that symbionts coevolve with their hosts and their diversity and composition is shaped through their interactions along their life history (Thrall & Burdon 2004, Thrall et al. 2006, Barrett et al. 2007, 2008, Guimaraes et al. 2007, Donoghue, 2008). The "mutualist facilitation" hypothesis argues that the replacement of lost mutualists/commensals from plants' native ranges with new mutualists/commensals in their introduced ranges is important for the establishment and success of introduced plants (Richardson et al. 2000, Mitchell et al. 2006). Changes in the mutualistic species composition may limit success or even prevent establishment and naturalization of a plant in a new area, if the pool of mutualists available to it does not include species with which it coevolved (Schemske & Horvitz 1984, Nadel et al. 1992, Bever, 2002, Klironomos 2003). Since tropical endophytes are transmitted horizontally, when the seeds are removed from their natural habitat into plantations, these potentially mutualistic or coevolved endophytes might be left behind.

Endophytes as potential biological control agents

The ubiquity of endophytic fungi among plants and within plant tissues, and the observation that endophytic fungi have been associated with plants since the first colonization of land, suggest that plants and endophytes likely share a long and intimate history of coevolution (Seiber 2007, Krings et al. 2012). Biological control

has been defined by Cooke and Baker (1983) as follows: “Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man.” Classical biocontrol aims to restore the ecological imbalance by introducing coevolved natural enemies, selected for specificity and biocontrol activity, from the evolutionary center of origin of the invasive plant, alien pest or pathogen (Eilenberg et al. 2001, Thomas et al. 2008). Natural enemies, the agents used in biological control, are the fundamental resource with which biological control success is achieved. Biological control should be an important component of an integrated pest management strategy (Evans 2007, Gentz et al. 2010). The introduction of natural enemies into crops to reduce the pathogen population is one of the most common use alternatives in biological control. The ecological basis relies on the principle that many populations are limited in their native habitat by the action of upper trophic level organisms or, in the case of some plant pathogens, by competitors within the same trophic level. Species in these upper trophic levels are often considered beneficial organisms because of their action in suppressing a pest population (Driesche & Bellows 1996, Hajek 2004). In biological control, the species used to suppress the target plant pathogen is called “antagonists” and are defined as biological agents with the potential to interfere in the life processes of plant pathogens. Antagonists are equivalent to natural enemies. An antagonist may use more than one form of antagonism, and the action of the antagonist may fit under more than one mechanism (Pal & McSpadden 2006, Tjamos et al. 2010). Table 2 illustrates the different types of antagonism.

Several studies have shown the ability of fungal endophytes to be antagonists on plant pathogens through many of the mechanisms shown in Table 2. Extensive research has been conducted in biological control of cacao diseases using fungal endophytes (Arnold et al. 2003, Samuels et al. 2006, Mejia et al. 2008, among others). In a preliminary assessment of endophytes from *Theobroma gileri*, a relative of *T. cacao*, in its center of genetic diversity, Evans and colleagues (2003) found that several fungal endophytes isolates from trees growing in natural forests were parasitic on *Moniliophthora roreri* (frosty pod rot of cacao), giving protection to the tree against this disease. Rubini et al. (2005) isolated fungal endophytes from cacao trees from orchards located in Brazil and screened *in vitro* and *in planta* for antagonism against *Crinipellis perniciosa* (the causal agent of witches' broom disease). He found that the fungal endophyte *Gliocladium catenulatum* reduced the symptoms *in planta* under greenhouse conditions. Previous research has shown that genera such as *Fusarium*, *Acremonium*, *Trichoderma*, and *Clonostachys*, have better potential for biological control than other fungi (Papavizas 1985, Driesche & Bellows 1996, Burgess & Keane 1997, Xue 2003, Harman 2004, 2006, Odintsova et al. 2009), all of which are known to be endophytes of tropical trees and are hypothesized to inhabit species of *Hevea*. The best examples are in the genus *Trichoderma*. For instance, the cacao endophytes *Trichoderma ovalisporum* and *T. koningiopsis* both collected in Ecuador and Brazil showed to be relatively effective against *M. roreri* (Holmes et al. 2004). Samuels et al. (2006) isolated *T. theobromicola* and *T. paucisporum* from wild cacao (*T. cacao*) and tested their biological control potential. Both species were found to produce a volatile/diffusible antibiotic that inhibits development of *M. roreri in*

vitro and on pod trials. *Trichoderma asperellum* has shown to be moderately effective against *Phytophthora megakarya* (cacao black pod disease) in Africa (Tondje et al. 2007). Bailey and colleagues (2008) not only tested the antagonistic effect (antibiosis and mycoparasitism) of several isolates of *Trichoderma* against cacao diseases, but they also tested *Trichoderma* colonization and establishment success within the host. They found that most of the *Trichoderma* isolates were able to become endophytic in cacao seedlings and that many of them produced inhibitory metabolites or were able to parasitize cultures of *M. roreri*. Antifungal compounds have been also been found in species of endophytic fungi harbored in other plant species besides cacao species (Verma et al. 2009, Gond et al. 2010, Yu et al. 2010).

Hevea brasiliensis: the study system

Natural rubber (NR) is synthesized by numerous plant species (over 2,000) belonging to 300 different genera. However, "Para rubber" (*Hevea brasiliensis*) produces the best quality latex contributing to 90% of the world's NR (Mooibroek & Cornish 2000, Priyadarshan et al. 2003). *Hevea* spp. (Euphorbiaceae) exhibits much morphological variability and inhabits a wide range of ecological sites. Its members range from emergent trees to shrubby, sometimes even prostrate plants. They can be found in deeply flooded alluvial land, in acidic boggy sites, on high well-drained uplands and on the tops of xerophytic quartzitic mountains. *Hevea*'s natural variability has led to a disagreement on the number of species contained in the genus. The number or recognized species varies from 8 to 11, depending if hybrids are included (Seibert 1947, Shultes 1970, Goncalves et al. 1983, Pires et al. 2002). *Hevea brasiliensis* is a fast growing medium-sized to large tree, which frequently reaches 45

meters in height, being an essential part of the Amazonian emergent canopy. *H. brasiliensis* presents the largest diameter in the genus sometimes reaching 1–1.5 meters. Its habitat varies depending on the region. Through much of its range, *H. brasiliensis* is associated with periodically inundated conditions, but in its southwestern-most limits of distribution, it is found on the well-drained plateau areas above the rivers (Seibert 1947).

Hevea brasiliensis is primarily used for its latex, which is usually tapped for a period of 10 to 20 years, but the tree can be productive for as long as 40 to 45 years, depending on the intensity of tapping and the amount of carbon uptake (Lieberei 2007, Goncalves et al. 2009, Chantuma et al. 2011, Teoh et al. 2011). This species is cultivated on a large commercial scale in several countries within the tropics, amounting to 9.5 million hectares worldwide (Evans 2004, FAO 2010). The production of NR is a vital agricultural commodity used in the manufacture of more than 50,000 different products: adhesives, high-quality tires, surgical gloves, health equipment and accessories, condoms, among others (Rippel & Galembeck 2009). In addition, its wood is used for small boards, matches, packing boxes, compressed wood textiles, round arches (Silpi et al. 2006, Hameed & Daud 2008, Teoh et al. 2011), and its seeds are consumed by Amazonian people and used as an alternative to petroleum fuels (Seibert 1947, Shultes 1956, Zhu et al. 2011). Even though synthetic rubber has replaced NR in many artifact manufacturing, NR still accounts for 40% of the rubber's world's consumption (Priyadarshan et al. 2009). NR production plays a major role in the socioeconomic fabric of many developing countries. Over 20 million families in Africa and Asia depend on rubber cultivation as their source

income (FAO 2007; Umar et al. 2011). Worldwide rubber demand is constantly growing and NR will always be a product on demand because its typical physicochemical properties have not been achieved yet by synthetic products (Cornish 2001, IRSG 2009).

In contrast to the geographical distribution of natural rubber production (i.e., Asia and Africa), the recognized biological center of origin or native habitat of the rubber tree is within the Amazon basin (Lieberei 2007). The annual production of NR in Brazil represents only 1% of the world's production and is far below the national needs. Brazil occupies the sixth position in the ranking of rubber consumption, representing 3.5% of the world's rubber consumption (ISRG 2009). The reason for this inconsistency is the fact that natural rubber in Asia, India, and Africa can be grown in plantations on a large scale, while in Brazil and in other South American countries, NR is almost completely extracted from wild-growing rubber trees (Verheye 2010). Plantations in South America have never reached full production because they are destroyed by a devastating fungal disease (*Microcyclus ulei*) before the trees reach the physiological maturity necessary for harvesting (Mariau 2001, Garcia et al. 2006, Lieberei 2007). The disease caused by *M. ulei* is generally called South America Leaf Blight (SALB). Repetitive infection of this fungus induces successive defoliation, leading to tree mortality. Currently, this disease extends from Southern Mexico (180 latitude North) to Sao Paulo State in Brazil (240 latitude South), covering Brazil, Bolivia, Colombia, Peru, Venezuela, Guiana, Trinidad, Tobago, Haiti, Panama, Costa Rica, Nicaragua, Salvador, Honduras, Guatemala and Mexico (Weber 1973, Holiday 1980, Mariau 2001, Liberei 2007).

The native range of *Hevea* (especially *H. brasiliensis*) species is clearly defined in the Upper Amazon basin (Schultes 1956, Wycherley 1976, Besse et al. 1994) (see Figure 1). Molecular analyses of genetic diversity revealed a high diversity and clear structure of Amazonian populations in accordance with geographic origin, further supporting the center of genetic diversity in the Upper Amazon (Luo et al. 1995, Bicalho et al. 2008). These studies also show that the genetic diversity (i.e., polymorphic loci, heterozygosity, rare alleles) is significantly higher in wild rubber populations compared to plantations. The proliferation of rubber beyond its native range has been extensive. Rubber germplasm from Brazil was introduced to Southeast Asia by the British in 1876 (Onokpise 2004, Lieberei 2007). The rubber genetic stock used to establish plantations in Asia came from a few seeds. Consequently, plantations were mostly clonal, which eventually led to a gradual erosion of genetic variability (Priyadarshan et al. 2003, Onokpise 2004, Priyadarshan et al. 2009).

Objectives

- Characterize and compare the fungal endophytic community of *Hevea*, from populations distributed within its native range (Amazon Basin) and under management settings (plantations).
- Evaluate the collection, processing, and analytical techniques used in the study of fungal endophytes (with emphasis in tropical habitats) with the aim of proposing standardized protocols that can contribute to the communication between scientists and to increase the comparative utility between studies.

- Based on phylogenetic inference and their restrictive presence in wild trees, propose fungal endophytic strains with potential use in biological control of *Hevea brasiliensis* diseases.

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Summary of chapters

Even though *Hevea brasiliensis* represents one of the most economically important crops and has been qualified as a high pest risk, its associated fungal community has not been studied in detail. This is especially true for its endophytic community, which could harbor beneficial and mutualistic species with biological control potential and applications. Since there was no previous knowledge of any aspect of the fungal endophytic community for any species of *Hevea* neither in the wild nor in plantations when this project started (2007), Chapter I presents a preliminary assessment of the fungal endophytes inhabiting leaf and sapwood tissue of a wild rubber tree population distributed within the Peruvian Amazon. Sampling techniques were tested, especially in regard to sapwood endophyte isolation. Collection sites involving wild rubber populations were all located in remote areas, where facilities for media and culture preservation represented a constant challenge. Chapter I describes the techniques used in endophyte isolation and isolate identification, and gives a preliminary overview of the diversity and abundance of the fungal endophytic species associated to wild *H. brasiliensis*. It also illustrates the compositional difference between the fungal endophytic community of leaf and sapwood tissues.

From Chapter I, an appreciation of the high diversity of fungal endophytes and the difficulty in their species delimitation process came to light. Many groups of fungi known to contain species complexes were found to be common components of the endophytic community of *H. brasiliensis*. Therefore, in Chapter II, species delimitation concepts and techniques commonly used in the study of fungal diversity were explored and tested. The main objective of Chapter II was to demonstrate how

the choice of a species delimitation concept influences not only the estimation of diversity but also the biogeographic and ecological inferences drawn from those values. Based on several approaches and using three unlinked genetic markers, we were able to conclude that three of the most common groups of endophytes (i.e., *Colletotrichum*, *Pestalotiopsis*, and *Trichoderma*) are really species complexes, harboring more than one cryptic species. Based on the results from this chapter, a higher ITS (Internal Transcribed Spacer genetic marker) sequence similarity threshold was recommended (up to 99% in sequence similarity).

Sampling intensity can also affect the estimation of diversity and, like the choice of species delimitation criteria, it can influence further inferences and hypothesis on ecology and biogeography. Chapter III explores the incongruence of sampling designs among fungal endophyte studies and its effects on diversity estimation. Based on two *H. brasiliensis* populations (for a total of 100 individuals), the sampling effort needed to reach an asymptotic species accumulation curve was estimated. These populations of *H. brasiliensis* were also used to compare the sampling effort needed in natural (wild) versus artificial ecosystems (plantations). Furthermore, in this chapter, the performance of the most commonly used diversity indices were evaluated, within the hyperdiversity context imposed by tropical fungal endophytes.

This dissertation represents one of the few studies involving a fungal endophyte survey from a Neotropical tree species distributed within its native range. Moreover, is one of the few that has explored the fungal endophytic community of sapwood. The fungal Kingdom is believed to hold a great percent of unknown

species; therefore, it was not surprising that through this research, new lineages were discovered. Chapter IV describes a new fungal lineage within the Pezizomycotina (Ascomycota), corresponding to a class rank. The new lineage, named Xylonomycetes showed distinctive morphological, ecological, and molecular characteristics that set it apart from all the other recognized ascomycetous classes. Chapter IV highlights the incompleteness of our current knowledge of the "Fungal Tree of Life" and stresses the need to conduct more explorations in remote tropical areas and poorly explored niches (e.g. sapwood).

One of the main objectives of this dissertation was to explore for potential biocontrol agents that can be use in the fight against economically important rubber diseases. For this purpose, we sampled fungal endophytes extensively (endophytes were isolated from 190 individual *Hevea* trees). Chapter V describes and compares the fungal endophytic community of wild and planted *Hevea* trees and proposes potential strains for future *in vitro* and *in planta* assays. This last Chapter emphasizes the vast and distinctive diversity of fungal endophytes harbored only in wild *Hevea* trees, while it raises the awareness of the potential loss of these symbionts due to land use change, viz. deforestation.

Importance of the study

- Previous to this work, there was no information available on the biodiversity of rubber fungal endophytes neither in their host's native habitat nor in introduced areas (plantations). Since the project started there has been only one study involving fungal endophytes of *Hevea* (Rocha et al. 2011) conducted in rubber plantations.

Tropical fungi and especially fungal endophytes are poorly known, particularly in remote areas such as the ones selected as field sites for this study. Therefore, findings reported here will improve the taxonomy of many understudied fungal groups. This is especially true for sapwood endophytes, which have been surveyed far less than leaf endophytes.

- One of the outcomes of this study was the addition of a new lineage to the Fungal Tree of Life, represented by the description of a new class within the Pezizomycotina. This will improve our knowledge not only in fungal diversity but also in fungal evolution.

- This study proposed few endophytic species as promising candidates for future biocontrol *in vitro* and *in planta* studies, against rubber diseases. Biocontrol assays and screening for antifungal compounds may uncover potential endophytes for biocontrol strategies, improving our understanding of the role of endophytes play within their host. In addition, the finding of useful biocontrol agents can add a component to the integrated pest management of this crop. Alternative means of control of rubber diseases are in great need, especially in developing countries where natural rubber production is the main source of income.

- This project has resulted in a collection of more than two thousand fungal cultures. Selected cultures have been deposited in various public culture collections (i.e., CBS, BPI) that can be accessed by scientists, academics, or commercial groups interested in studies related to biocontrol of rubber and fungal biodiversity.

- The DNA sequences of the identified endophytes are available in GenBank, where tropical fungi are still underrepresented. In addition, multi-locus alignments

produced by this project are accessible through public online databases (i.e., TREBASE) and morphological and ecological descriptions are available on the Internet (i.e., UMD website, Mycobank). This is expected to facilitate the identification of fungi by other people studying microbial diversity and will aid in the dissemination of information about fungal diversity, ecology and systematics, to scientists and other interested individuals working in various fields.

- The unique taxonomic composition of the fungal endophytic assemblage within wild rubber trees in addition to the presence of a greater number of fungal strains with potential uses for biological control, demonstrates the importance of identifying and conserving endophytic fungal diversity in the host-plant's native habitat. Results from this study reinforce and add "reservoir of potential mutualistic symbionts" to the list of ecological services that natural forests provide to humans. The latter has implications in conservation efforts.

Chapter 1: Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru

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Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru

Abstract

Endophytic fungi isolates from foliage and sapwood of *Hevea brasiliensis* were studied to determine the total diversity of endophytes inhabiting leaves and sapwood, and differences, between respective endophyte communities found in leaves and sapwood. Endophytes were recovered from 72% (161) of the 225 samples, with a total of 175 isolates. Sequence data from ITS and LSU nrDNA revealed 58 distinct OTUs. Ascomycota was dominant, representing almost 97% of the isolates. In contrast, Basidiomycota and "Zygomycota" were represented by 1% and 2%, respectively. Among the genera isolated *Penicillium, Pestalotiopsis* and *Trichoderma* were the most frequently isolated. A greater diversity of endophytes was found in sapwood as compared to leaves. However, endophytic colonization frequency was greater in leaves than in sapwood. Comparisons between leaves and sapwood demonstrated a spatial heterogeneity in endophyte assemblages among plant parts and sites.

Keywords: Ascomycota, biocontrol, conservation, fungal ecology, Hypocreales, systematics.

Introduction

Understanding the role of fungal species in ecosystems has been hampered by little sampling and lack of characterization of fungal diversity (Hyde *et al*., 2007). Endophytes are among the poorly understood groups of fungi even though they are

thought to be important in plant populations and communities (Arnold *et al*. 2003; Arnold & Engelbrecht 2003; Arnold *et al*. 2007; Saikkonen 2007; Rodriguez *et al*. 2009). Endophytes reside asymptomatically within most living plant tissues examined to date (Schulz *et al*. 2002; Li *et al*.2007; Tao *et al*. 2008) and are found in diverse habitats, ranging from coastal mangroves (Kumaresan & Suryanarayanan, 2001) to temperate and alpine areas (Espinosa-Garcia & Langenheim 1990). Endophyte research expanded in recent years from cataloguing species to examining the nature of the endophyte/plant interaction (Hyde & Soytong 2008; Mejia *et al.* 2008; Tao *et al*. 2008; Rodriguez *et al.* 2009;) with particular emphasis on studying endophytes of medicinal plants in order to discover novel compounds (Huang *et al*., 2008, 2009; Mitchell *et al*., 2008). Nevertheless, few studies have been conducted in the host's natural habitat (e.g. Cannon & Simmons 2002; Samuels *et al.* 2006) or from a biological control perspective (e.g., Hanada *et al*. 2008). It has been hypothesized that as plants are moved from their native range they lose endophytes and, moreover, coevolved endophyte confer on plants beneficial effects such as resistance to equally coevolved pathogens (Evans 2002). Sampling and characterizing fungal endophyte diversity is an emerging challenge and promises to lead to the discovery of new species, novel compounds and a better understanding of their role in ecosystems (Arnold & Lutzoni 2007; Saikkonen 2007; Sieber 2007; Rodriguez *et al*. 2009). This study focused on characterizing endophytic fungal diversity in wild *Hevea brasiliensis* (Willd.) Muell. Arg. (Euphorbiaceae) in the Amazon basin. The latex produced by this tree is converted into rubber. Even though *H. brasiliensis* is of great economical importance, few have studied its associated fungi (Schwob *et al.* 1999;

Araujo *et al*. 2004; de Mello *et al*. 2006; de Melo *et al*. 2008; Evueh & Ogbebor 2008). Natural rubber has been an important commodity for the past 100 years. It is synthesized by several plant species (over 2000) belonging to 300 different genera. However, Pará rubber (*H. brasiliensis*) produces the best quality and contributes to 90% of the world's natural rubber and 40% of the rubber world's consumption (60% is delivered by synthetic rubber).

The proliferation of *H. brasiliensis* beyond its wild distribution is extensive due to cultivation (Seibert 1947; Wycherley 1976; Wright 1998; Onokpise 2004). Today, rubber is primarily produced in large-scale plantations in Southeast Asia and Africa (FAO 2005). In the Americas, the production of rubber in plantations has failed largely because of one disease the South American Leaf Blight (SALB) caused by the fungus *Microcyclus ulei* (Henn.) Arx (Lieberei 2007; Holliday 1980; Mariau 1999). Because SALB is only found in the Americas, there are concerns about possibility of global proliferation of this disease to plantations in Asia and Africa, which would certainly result in significant economic losses. No cure exists for SALB and chemical fungicides have largely failed (Lieberei *et al*. 1988; Priyadarshan & Goncalves 2003; Le Guen *et al*. 2006; Guyot *et al.* 2008). Even though potential impacts of SALB are large, few alternative methods of control exist (Junqueira *et al*. 1992; Garcia *et al*. 1999; de Mello *et al*. 2006; Guyot *et al.* 2008; de Mello *et al.* 2008). Endophytic fungi from leaves (Herre *et al.* 2007; Mejia *et al.* 2008) and sapwood (Holmes *et al.* 2004; Samuels *et al.* 2006; Hanada *et al.* 2008) have shown promise in the control of disease in another tropical crop, cacao (*Theobroma cacao* L.), thus leading to the possibility of a biological control against SALB disease of rubber as well.

The objective of the current study was to characterize endophytic fungi in leaves and sapwood of wild *H. brasiliensis*. We used a traditional technique to isolate the endophytes but molecular sequence data to provide a more accurate idea of the taxa (Wang *et al*. 2005; Thomas *et al*. 2008). The study will create a reservoir of strains that may be assayed for biological control potential while at the same time beginning to catalogue the fungi that are asymptomatically associated with above ground parts of healthy rubber trees.

Materials and Methods

Study Site and Sampling Strategy

The present study was conducted in June 2007 at "Picaflor Research Centre", a station located on the banks of the Tambopata River in the Department of Madre de Dios, southeast Peru (13°08'S - 69°36'W). The area was selected because it is located within the native range of *H. brasiliensis* (Seibert 1947) and various authors have reported that areas near Tambopata River are among the few known to have disease resistant rubber trees (Schultes 1956; Evans 2002). Moreover, the sampling area is inside the buffer zone of the Tambopata National Reserve and Bahuaja-Sonene National Park, two of the most biodiverse and undisturbed areas in the world (Conservation International 1994; Wilson & Sandoval 1996; Naughton-Treves *et al*. 2003).

A nested design was used to survey fungal endophytic diversity from *H. brasiliensis*. Three sites were selected within the sampling area, which were 1–5 km apart from each other. From each site, five trees (15–70 cm diam.; 10–40 m in height) were

randomly chosen for endophyte collection. To eliminate as much as possible the effects of plant genetic variation on endophyte species composition, trees within *Hevea* patches were sampled. Localized wild populations of *H. brasiliensis* trees have been found to be largely genetically homogeneous probably due to the limited autochorous dispersal (*ca*. 30 m radius) by their explosive fruits (Wycherley 1976; Besse *et al.* 1994; Swarbrick 1997*)*. Thus, *H. brasiliensis* trees were found in patches, with a large "mother" tree surrounded by many younger and smaller trees. Fifteen rubber trees were sampled, with a total sample number of 225 (n=225).

Isolation of Endophytic Fungi

Fungal endophytes were obtained from living leaves and sapwood tissue (= vascular cambium and phloem). Three apparently healthy leaflets were collected randomly from the crown of each tree. Once in the station's laboratory and within six hours of the collection, three segments from each leaflet (lamina tip, midrib, and lamina base) were surface-sterilized through sequential immersion in 2% (v/v) sodium hypochlorite (bleach) solution, 70% (v/v) ethanol, and sterilized water (Arnold *et al*. 2001; 2003).

Endophytes from the living sapwood were collected according to methods used in Evans *et al*. (2003). A sterilized knife was used to cut three slivers of ca. 3 × 6 cm of dead bark from each tree at shoulder height and from different parts of the circumference of the tree, to expose the living sapwood. Three pieces of ca. 5 mm of living sapwood tissue were excised from the exposed areas and transferred immediately to Petri plates containing CMD (BBL ™ corn-meal-agar + 2% dextrose).

To eliminate bacterial endophytes, an antibiotic solution was added to the CMD (1% solution of Neomycin–Penicillin–Streptomycin, Sigma-Aldrich, St. Louis, MO, U.S.A). The surface sterilization of the leaves as well as the location of the sapwood segments was done to eliminate unwanted epiphytes and other superficial contaminants. Although the leaf-print control was not used to test for growth of epiphytes (Shultz *et al.* 1998), the sterilization procedure followed in this work has been widely used in endophyte research (Lodge *et al.* 1996; Arnold *et al*. 2001; 2003; Cannon & Simmons 2002, Santamaria & Bayman 2005).

Petri plates were kept at low temperature (ca. 4–8°C) until they were processed in the laboratory located at the Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, USA. The plates were incubated for several days (up to 2 months) and the emerging colonies were subcultured to obtain pure isolates.

Morphological Characterization of Endophytes

Isolates were first identified to morphospecies. Cultures were grown in different media depending on their ability to sporulate. At first the isolates were grown on Difco™ potato-dextrose-agar (PDA) and CMD in vented plastic Petri plates and incubated at 25°C with 12 h fluorescent light and 12 h darkness. In cases where the endophyte isolate did not sporulate on PDA or CMD, they were grown in Difco™ oat-meal-agar (OA) and MP Biomedicals™ malt-extract-agar (MEA) to promote sporulation. If the isolates did not sporulate on PDA, OA or MEA, the following techniques were used to induce sporulation: adding *Hevea* leaf extract to the media, placing a sterile *Hevea* leaf or filter paper in the media, and changing drastically the

intensity of light exposure. For the latter, the cultures were kept in an incubator with no light for 2 weeks and later moved to a 12h fluorescent light/darkness incubator. The following characters were used for the characterization and identification of morphospecies: colony appearance, mycelium color and structure, type of anamorph, conidiomata, conidia and conidiophore morphology (size, color, shape, ornamentation, etc.), and conidiogenous cells.

Phylogenetic Analyses

For the molecular identification, isolates from pure cultures were grown on Difco™ potato-dextrose-broth under 25 °C for 4 d to 2 wks depending on their growth rate. Genomic DNA was extracted from the mycelial mat using Power Plant™ DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, U.S.A). *Internal Transcribed Spacers* (ITS) and *Large Subunit* (LSU) of the nuclear ribosomal DNA were amplified in a single reaction using ITS5 and LR5 primers (White *et al*. 1990). For the isolates that were identified as *Trichoderma* spp. based on their ITS sequence, an additional gene was amplified: *translation elongation factor* 1α (*tef1*) using the primers EF-728 (Carbone & Kohn 1999) and EF2 (Jacobs *et al*. 2004). The PCR reaction conditions were conducted following Chaverri & Samuels protocol (2003). *Tef1* was selected because this gene region which contains introns gives greater resolution at species level than does ITS (Chaverri & Samuels 2003). PCR reactions were set-up using the following ingredients for each 50 µl reaction: 25 µl of GoTaq®Green Master Mix 2× (Promega corporation, WI, U.S.A), 2.5 µl of 10 µM reverse primer, 2.5 µl of 10 µM of forward primer, 1 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, U.S.A), 5 µl of 100× Bovine Serum

Albumin (BSA, Sigma-Aldrich, St. Louis, MO, U.S.A), a maximum of 25 ng/µl of genomic DNA, and double distilled water to complete the total volume. The reaction conditions were as follows: 94 °C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min and primer extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min. PCR products were purified using ExoSAP-IT® (USB corporation, Cleveland, OH, U.S.A.) following the manufacturer's instructions. Clean PCR products were sequenced at University of Maryland Sequencing Facility using ITS5, ITS4, LR5, and LROR primers (White *et al*. 1990).

Sequences were edited and assembled using Sequencher™ 4.8 (Gene Codes Corporation, MI, U.S.A). Consensus regions (ca. 400–500 bp for ITS and ca. 800 bp for LSU) were compared against GenBank's database using their Mega BLAST program. The closest hit sequences were then downloaded in FASTA format and aligned with the sequences produced for this study using MAFFT 6.0 (Katoh & Hiroyuki 2008) and later refined by hand using Mesquite 2.6 (Maddison & Maddison 2009). Sequences that presented ≥98% similarity in ITS sequences were considered to belong to the same "operational taxonomic unit" (OTU), based on ITS region, and were placed in the same branch in the phylogenetic tree. Sequences with <98 % similarity were considered to belong to a different OTU, and depending on the percentage, they were assigned to the taxon with the closest BLAST match. These percentages were selected based on studies that reported an ITS intraspecific variation of 1.96 % for Ascomycota (Nilsson *et al*. 2008). Other studies have suggested 95% in ITS similarity to delimit species or OTUs boundaries (Arnold & Lutzoni 2007; U'Ren

et al. 2009). However, since in many fungal lineages ITS sequences present a high interspecific similarity (Lacap *et al*. 2003; Chaverri & Samuels 2003; Seifert *et al.* 2007), OTUs boundaries were set to 98% similarity. Sequences were also compared between each other to detect species frequency. Sources of the fungal specimens used for the molecular analyses and the database accession numbers for their DNA sequences are provided in the publication. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers FJ884070–FJ884196 for ITS and FJ890363–FJ890428 for LSU.

Maximum Likelihood (Felsenstein &Churchill 1996) phylogenetic trees with bootstrap analysis were constructed with RAxML version 7.0.4 (Randomized Axelerated Maximum Likelihood; Stamatakis *et al.* 2008) using the general time-reversible (GTR) evolutionary model and the Gamma model of rate heterogeneity settings. Unordered characters, random taxon addition sequences, gaps treated as missing data and the tree bisection-reconnection (TBR) branch swapping were used in the analyses. Two species belonging to the Basidiomycota also collected in this survey were used as outgroup for the phylogenetic analysis: *Trametes gibbosa* (Pers.) Fr. and *Coprinus* aff. *radians* (Desm.) Fr. Topological incongruence was examined with a reciprocal 70% bootstrap threshold (Mason-Gamer & Kellogg 1996, Reeb *et al*. 2004). After ITS and LSU phylogenies were analyzed for congruence, a combined tree was produced using RAxML under maximum likelihood approach. Trees were visualized in TreeView (Page 1996) and exported to graphics programs.

Endophyte Diversity Analyses

Species richness and evenness were calculated for the endophyte diversity analyses. Diversity of the total area (e.g. Tambopata) was calculated using Shannon-Wiener, Simpson's, Chao-1, and Chao-2 indices. Richness of OTUs between sites and plant parts were estimated using the following similarity indices: Classical Jaccard, Sorensen, and Morisita-Horn. However, only Bray-Curtis and Morisita-Horn estimates will be considered for results interpretation. The latter were chosen because Jaccard's and Sorensen's classic similarity estimates perform poorly when there are many rare species (i.e. singletons) within the sample (Chao *et al.* 2005). Data used for comparison purposes were at first combined (leaf and sapwood). However, because of their low similarity, the data had to be partitioned into two groups: data from isolates collected from leaves and data from isolates collected from sapwood. All the diversity analyses were done using EstimateS Win800, version 8.0 (Colwell 2005). Singletons were considered for all the analyses because they might be keystone organisms for the endophyte and plant community and therefore, important for the purposes of this study. Nevertheless, some of the ecological tests performed by the software automatically eliminate singletons in their analysis. To compare species richness among samples of unequal size, the data was rarefied to the smallest sample size. A species accumulation curve was also developed.

Results

Diversity of Endophytic Fungi

A total of 225 samples from *H. brasiliensis* leaves and sapwood were collected from 15 trees. Seventy two percent (161) of the samples had endophyte growth, with a total of 175 isolates (given that some of the samples presented more than one endophyte). A total of 58 OTUs were identified. The distribution of isolates among the 58 OTUs approximated a log-normal pattern, with a few common taxa and many rare taxa (Fig 1.1).

A greater number of isolates were recovered from leaf samples showing 90% occurrence. On the contrary, it was found that many sapwood samples did not present fungal growth; endophyte growth was observed on 60% of the sapwood samples. Filamentous Ascomycota (Pezizomycotina) dominated the fungal endophytic community in *H. brasiliensis*, representing 96.6% of the isolates. Few isolates belonging to the Basidiomycota and "Zygomycota" were collected, representing only 1.1% and 2.3%, respectively.

Within the Ascomycota, three genera were the most common occurring in all three sites: *Pestalotiopsis* (23% of all isolates), *Trichoderma* (22% of all isolates), and *Penicillium* (18% of all isolates). Other identified genera were rarely isolated, each between 1% and 3% of frequency. These genera were *Alternaria, Annulohypoxylon*, *Cladosporium, Cochliobolus, Colletotrichum*, *Endomelanconiopsis, Entonaema*, *Epicoccum, Fusarium*, *Guignardia, Leptosphaerulina*, *Khuskia,* and *Umbelopsis*. *Pestalotiopsis* aff. *palmarum* (Cooke) Steyaert and *Trichoderma harzianum* Rifai were the most common species found as endophytes. Based on ITS and LSU, all the

Pestalotiopsis isolates represented a single OTU and its closest BLAST match was *P. microspora* (Speg.) G.C. Zhao & N. Li. However, based on morphology, the isolates were more similar to *P. palmarum*. Maximum Likelihood phylogenetic tree of the combined regions (ITS and LSU) is shown in Figure 1.2. Because of the low sporulation rate, in most of the cases, isolates were identified using molecular techniques. The identified OTUs and their abundance are listed in Table 1.1.

The total fungal species richness associated with *H. brasiliensis* in Tambopata was 58 with an evenness of 0.78 (Table 1.2). All the indices suggest a relatively high diversity of the fungal endophytic community, as evidenced by the OTU accumulation curve that did not reach an asymptote (Fig 1.3). The Shannon-Wiener (H') estimates a diversity of 3.16 for the study area (the H' index is usually between 1.5 and 3.5, 1.5 representing the lowest diversity and 3.5 the highest). Simpson's diversity index (λ) resulted in a diversity of 0.1 (the λ index ranges between 0 and 1, being 1 a population with 1 species). The Chao-1 formula estimates the number of missing species based on the number of singletons and doubletons in a sample. This estimator suggests that the endophyte community has been undersampled, giving an estimated number of missing species of 120 with a standard deviation of 22.

Characterization and Comparison of Endophytic Fungi from *H. brasiliensis*

Between sapwood and leaves

In total, 90 samples from leaves and 135 from sapwood were collected. After rarefying the data, it was found that endophyte species richness (S) coming from sapwood was greater than that from leaves. However, this difference was not

statistically significant (sapwood = 36 vs. leaves = 35). Species evenness measure resulted in 0.85 and 0.75 for the sapwood's and leaf's community, respectively. Fisher's alpha diversity, Shannon's and Simpson's indices suggested that sapwood harbors a greater diversity than leaves. The number of missing species calculated using Chao-1 and Chao-2 estimates showed that the fungal endophyte community from sapwood was undersampled by 64 and 305 species and the leaf community by 85 and 100 species, respectively (Table 1.2). Neither the leaf's nor the sapwood's endophyte community reached an asymptote (Fig 3). Although the highest species richness was found in sapwood, the curve representing the accumulation of species in leaves showed a steeper slope. Thirteen species (24%) were found to inhabit both sapwood and leaves (Table 1.3). On the other hand, 21 species (36 %) were only isolated from leaves and 23 species (39.7%) only from sapwood.

Abundance revealed a different pattern. Samples isolated from leaves showed a greater occurrence of fungal endophytes. Almost 41% of the samples obtained from sapwood did not show any colony growth; compared to the 10% found in the leaf's samples.

Between sites

The greatest number of species was found in site 3 (30 species), with slightly lower numbers in the other two sites: 27 species in site 1 and 23 species in site 2 (Table 1.2). Seven species were found in the three sites: *Cladosporium cladosporoides*, *Perisporiopsis* aff. *melioloides*, *Pestalotiopsis* aff. *palmarum*, *Penicillium brevicompactum* Dierckx, *Penicillium paxilli* Bainier, *Penicillium sclerotiorum* J.F.H. Beyma, and *Trichoderma harzianum*. Some of the species were unique to a particular

site: 16 species were only collected from site 1, 15 species from site 3, and 12 from site 2. Combined data were used to calculate diversity indexes for each site (the partition of the data agreed with the combined data). Based on Shannon-Wiener, Simpson's, evenness, and Chao-1, site 3 presented the highest diversity. However, Chao-2 calculated a higher value for site 1.

The combined data showed that the highest similarity was between site 1 and site 2 and the lowest similarity index was between site 1 and site 3. Different results were obtained when data was divided into leaf and sapwood isolates. Under this scenario, sites 2 and 3 showed the highest similarity and site 1 and 2 the lowest. All similarity estimates are shown in Table 1.3.

Discussion

Diversity of Fungal Endophytes in *Hevea brasiliensis*

Accumulation curves were not asymptotic, demonstrating that endophyte species diversity in wild *Hevea brasiliensis* were not exhaustively sampled. Therefore, more sampling would be needed to obtain an accurate notion of the culturable endophytic fungal community diversity. Moreover, to assess the entire fungal endophytic community, environmental PCR techniques (e.g. DNA cloning: Guo *et al*. 2001; Arnold *et al*. 2007; Seena *et al*. 2008; DGGE or T-RFLP: Nikolcheva & Bärlocher 2005; Duong *et a*l. 2006; Curlevski *et al*. 2009; or PCR product pyrosequencing: Nilsson *et al*. 2009) should be applied. Environmental PCR and sequencing of endophytes would potentially detect species that are not able to grow in artificial media or that their growth is so slow that are overgrown by other endophytes (Arnold & Lutzoni 2007; Hyde & Soytong 2008). In addition, the use of additional nuclear

single-copy genes with a faster evolution rate and hence more variable (e.g. *tef1* introns), will detect more species within species complexes. The ITS region has shown high interspecific similarity in many groups of Ascomycetes such as Hypocreales (Chaverri & Samuels 2003), *Colletotrichum* (Crouch *et al*. 2009) and Botryosphaeriales (Saldanha *et al*. 2007; Lazziera *et al.* 2008) among others (Lacap *et al.* 2003). Therefore, the number of OTUs found in this study is most likely underestimated.

The indices of extrapolated species richness, Chao -1 and Chao-2, are based in the concept that rare species carry the most information about the number of missing ones (Colwell & Coddington 1994). Chao-1 uses the number of singletons and doubletons in a sample and Chao-2 considers unique and duplicate species. The main difference in these two estimates is that Chao-1 relies on species abundance data and Chao-2 on presence and absence data (Magurran 2008). The objective of calculating Chao-1 and Chao-2 is to estimate the number of missing species and use it to calculate the diversity of the entire sample. Chao-1 and Chao-2 estimated the number of missing species to be 120 ± 20 and 117, respectively. In both cases the number of missing species doubled the number of species collected in the present survey. Same estimates were calculated when considering separate subsamples, such as sites or plant parts. In all the cases, the expected number of species was not close to the actual number of species collected and the species accumulation curves did not achieve a plateau in any of the cases. Based on the above estimators and the total number of fungal endophytes found (58 OTUs overall), a total species richness of 150 ±30 would be expected.

The species evenness for the studied area was 0.78 (in a scale 0–1, being 1 the most evenness and hence more diverse), showing that the fungal endophyte community is distributed in a relative equitable fashion. Other studies have shown similar diversity estimates in tropical areas. For example, based on morphological data, Arnold *et al.* (2001) recorded 242 fungal endophyte morphospecies for *Heisteria concinna* Standl. and 259 for *Ouratea lucens* (Kunth) Engl. Gamboa *et al.* (2002) recovered 11.7 ± 3.4 morphospecies of endophytes per leaf in a study involving five tropical plant species. Lodge *et al.* (1996) recovered 17 species from a single leaf blade of *Manilkara bidentata* (A. DC.) A. Chev. in Puerto Rico. *Theobroma* spp. is one of the few Amazonian tropical trees that its fungal endobiota has been evaluated in their natural range as well as in plantations. Evans *et al.* (2003) reported 80 species from healthy stems and pods of *Theobroma gileri* from 40 trees located in the wild. Rubini *et al.* (2005) isolated from 30 morphospecies from 30 trees in plantation. Arnold *et al*. (2003) found 47.5 ± 4.9 morphospecies in only nine leaves from individual trees.

Community Composition and Assemblage

The spatial heterogeneity of tropical endophytes is still in debate. A constant challenge is the prevalence of singletons species, even in large-scale surveys (Arnold *et al*. 2001; Gilbert *et al.* 2002; Arnold & Lutzoni 2007). The present study faced the same situation, because 64% of the identified species were singletons. Consequently, most of the taxa found in this study were considered to be rare (only isolated once or twice) and few species dominated the community (Fig 1.1). Ascomycota certainly was predominant, comprising 96.6% of the isolates and represented by 54 species.

Basidiomycota and "Zygomycota" were also isolated from leaf and sapwood but in very low occurrence. The predominance of ascomycetes appears characteristic of endophytic mycota (Stone *et al*. 2004; Rubini *et al*. 2005; Neubert *et al*. 2006; Higgins *et al*. 2007; Hoffman 2008). However, basidiomycetes also seem to be normal components of the endophytic mycota of diverse plant species, albeit in lower numbers (Crozier *et al*. 2006; Rungjindaimai *et al*. 2008; Thomas *et al*. 2008).

Different parts of the tree were dominated by different endophytic species. *Pestalotiopsis* aff. *palmarum* constituted the greatest percent of isolates that came from leaf samples, and it was also present in sapwood isolates but in lower numbers (32 vs. 9). *Trichoderma harzianum* species complex was the dominant species within the sapwood isolates but was also an important component of the leaf isolates (19 vs. 13). As mentioned before, since singletons comprise a great percent of the isolates, it is difficult to make assumptions not only about the distribution of fungal endophytes in a geographic area or among sites but also challenges the assessment of the their distribution within the plant itself. More sampling in different geographic localities is needed to make further assumptions about the allocation of fungal endophytes within the tree.

Before combining the data (sapwood and leaves), they were partitioned into two groups per site: one for sapwood isolates and one for leaf isolates. After all the analyses, it is concluded that the data can be combined to estimate specific diversity indexes. Therefore, data coming from sapwood and leaves were combined to estimate overall diversity, such as diversity of a site. All the estimates indicated that site 3 held the most diverse community. Nevertheless, contrasting results were encountered

when Chao-1 and Chao-2 were compared. Chao-1 calculated highest projected species richness for site 3 but Chao-2 calculated a highest value for site 1. As mentioned previously, Chao-1 relies on species abundance data to make the estimate whereas Chao-2 uses presence and absence data. Since tropical communities always present a log-normal pattern (with few common taxa and many rare taxa), Chao-2 was considered to be the most suitable estimate. Consequently, for this study site 1 was considered to harbor the highest diversity.

In terms of abundance, leaf samples showed greater occurrence of endophytes. Almost 41% of the samples originating from sapwood showed no fungal growth compared to the 10% with no growth found in leaf samples. Low frequency of endophytes in inner sapwood has been reported in other studies (Tejesvi *et al*. 2005; Verma *et al*. 2007; Oses *et al*. 2008). This phenomenon is probably due to their dispersal mechanism. Tropical fungal endophytes of dicotyledonous plants, in contrast to monocotyledonous plants, are mainly transmitted horizontally. Above-ground fungal endophytes of woody plants travel among hosts as spores through the air, rain splash, or insect vectors. Hence, successful colonization of host tissues is affected by factors influencing local abundance of aerial and epiphytic fungal propagules (Bayman *et al.* 1998; Arnold & Herre 2003; Santamaria & Bayman 2005). Fungal endophytes invade their host in the same way fungal pathogens do: through direct penetration, wounds, or natural openings (stomata or lenticels). *Hevea brasiliensis* above- and below-ground parts posses several protective mechanisms that may act against the penetration of, and later colonization by, fungi. These can be structural, chemical, or biotic. Structural barriers include lignin layers, cuticular

waxes, and outer bark. Chemical barriers include induced lignin, cyanide, hevein (protein present in the latex which has antifungal properties) and scopoletin (phytoalexin produced in the leaves) and niche competition with mutualistic endobionts (i.e. arbuscular endomycorrizas can prevent the invasion of other fungi) is a biotic barriers (Martin 1964; Lieberei *et al*. 1988; Parijs *et al.* 1990; Garcia *et al.* 1995; Silva *et al*. 2001; Kliromonos 2003). Little is known about how sapwood endophytes colonize the plant. Sapwood endophytes probably invade tree hosts through their root system, moving from the soil to their new niche inside the plant since the most common species in the stems (*Trichoderma* and *Penicillium*) are also abundant in soil (Chaverri *et al.* unpubl.).

Studies have suggested that leaf samples harbor the greater number of species because leaves appear to have less infection barriers when compared to sapwood (Arnold & Lutzoni 2007). However, all the diversity indices and species richness estimates from the present study suggest that the diversity of sapwood is not only comparable to the diversity of leaves, but can even be higher. Several species present a niche overlap and were isolated from sapwood and from leaves.

Leaf samples were dominated by the presence of *Pestalotiopsis* aff. *palmarum,* the taxon being present even in the companion of a second endophyte species. Moreover, when the distribution of species within subsamples was analyzed —meaning the species distribution in each individual fragment (one fragment was taken from each the lamina tip, middle rib and lamina base of each leaflet) — it was found that in several cases *P*. aff. *palmarum* was present in the three fragments. Since only the ITS and LSU regions were sequenced, it cannot be firmly concluded that all the isolates

belong to a clonal unit, instead of being different strains or even different species (if ITS does not encode enough variation for that particular taxon). However, it is possible that many fungal endophytes can colonize a relatively large area creating a barrier against other endophyte species by competing (Bandara *et al.* 2006) or by producing antagonistic substances (Strobel 2002; Tejesvi *et al.* 2005; 2007; Gang *et al.* 2008; Li *et al.* 2008).

In terms of genera, many of those found in this study coincide with genera reported in other studies (Lodge *et al*. 1996; Cannon *et al*. 2002; Gamboa 2002; Arnold *et al.* 2003; Santamaria & Bayman 2005), e.g. *Alternaria*, *Botryosphaeria, Colletotrichum*, *Fusarium, Pestalotiopsis/Pestalotia,* and *Xylaria,* among others. However, this study found other genera that are not commonly isolated such as *Corallomycetella, Fimetariella*, *Perisporiopsis,* and *Rubrinectria*. *Trichoderma* species have been reported from *Theobroma cacao* and *T. gileri* Cuatrec. as well, but only from sapwood isolates. In contrast, in our study, *Trichoderma harzianum* was isolated many times from leaves and sapwood. In addition, at least seven species of *Penicillium*, a genus that is noted for their chemical diversity and antibiotic activity, were identified.

Most published surveys have focused on leaf endophytes and only few have compared leaf and sapwood endophytes (Fisher *et al.* 1993, 1994; Gond e*t al.* 2007; Verma *et al.* 2007). The present study is one of the first to characterize and compare leaf and sapwood endophytes obtained from a plant species in its native range and using DNA and morphological methods combined. When the abundance of commonly isolated genera is compared, the results vary greatly to those from other

studies. For instance, Arnold & Lutzoni (2007) and Gamboa *et al.* (2002) reported *Botryosphaeria*, *Colletotrichum*, *Phomopsis,* and *Xylaria* as being the most abundant isolates in leaves. However, in the present study, *Pestalotiopsis* and *Trichoderma* were the most abundant genera found in leaves. The diversity, composition and abundance found in sapwood agreed with those obtained by Evans *et al*. (2003) and Tejesvi *et al.* (2005) in which a high abundance of *Trichoderma* isolates was reported for *Theobroma gileri* and *Terminalia arjuna* W. & A., respectively. In the present study, in addition to *Trichoderma* spp., *Penicillium* spp. were the most common in sapwood.

Sites were compared to estimate their similarity. Data were partitioned into sapwood and leaf since the results were being influenced by the most diverse group (Table 1.3). For instance, if combined data were used, Morisita-Horn estimate gave the greatest similarity to site 1 and site 2. However, when data was partitioned, site 2 and site 3 showed a greater similarity for the sapwood community and site 1 and site 2 for the leaf community. Even though the number of *H. brasiliensis* individuals limits the power of the analysis, it is interesting to mention that site 2 and site 3 were closer to each other than site 1. More sampling will elucidate the factors that might be influencing the assemblage of the community. Some of these factors might be geographic location (such as within the host species native range or outside), tree diameter, tree height, forest structure, disease occurrence, among others. This study attempted to evaluate the correlation of those factors with the distribution and abundance of fungal endophytes but found no correlation, probably due to the low number of samples used in the analyses.

Endophytic Fungi as Potential Biocontrol Agents against *H. brasiliensis* diseases

The use of fungal endophytes as agents for biological control has received increased attention (see Bailey *et al*. 2008; Mejia *et al*. 2008; Hanada *et al*. 2008; Paparu *et al*. 2009). *Hevea brasiliensis* harbors a high morphological and phylogenetic diversity of fungal endophytes. However, only further sampling will allow precise estimates as well as a better understanding of the community assemblage, especially niche partitioning within the plant. This study also suggests a complex interaction between the tree and its fungal inhabitants. Species and genera isolated in this study are known as plant pathogens (usually weak and opportunistic), as saprobes, and as potential mutualists.

Several of the fungal endophytic species found may potentially be tested as biocontrol agents against *H. brasiliensis* diseases. Few studies have investigated the use of biological control against *Hevea* diseases (Sudirman *et al.* 1992; de Mello *et al.* 2006; Evueh *et al.* 2008). However, none of the mentioned studies have used fungal endophytes as antagonistic agents. The following three genera could be considered for future antagonistic assays: *Colletotrichum, Pestalotiopsis,* and *Trichoderma*. Some strains of *Trichoderma* such as *T. harzianum, T. stromaticum* Samuels & Pardo-Schulth., and *T. asperellum* Samuels *et al*., have been proven to have antagonistic effects against some diseases caused by fungi (De Meyer *et al.* 1998; Watanabe *et al.* 2007; DeSouza *et al.* 2008; Hanada *et al*. 2008). There are several proposed modes by which *Trichoderma* can protect their host: direct parasitism, antibiosis, nutrient competition, enhanced plant growth, or induced resistance (Howel

2003; Harman *et al.* 2004; Holmes *et. al.* 2004; Khan *et al*. 2004; Bailey *et al.* 2008). All these characteristics, in addition to its strong competitiveness, recommend *Trichoderma* for evaluation of biocontrol potential.

Pestalotiopsis and *Colletotrichum* species are well known as weak or opportunistic pathogens forming leaf spots. Recently, some *Pestalotiopsis* and *Colletotrichum* species were reported as prolific producers of bioactive substances, many of them showing antifungal activity against plant pathogens (Inacio *et al.* 2006; Ding *et al.* 2008; Tejesvi *et al.* 2007; Li *et al.* 2008). Because of their antibiotic properties and the ubiquity shown by *Pestalotiopsis* in this study, this species may play an important role in their host's development and should not be considered as a neutral symbiont.

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Chapter 2: Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences

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"Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences"

Abstract

The estimation of species diversity in fungal endophyte communities is based either on species counts or the assignment of operational taxonomic units (OTUs). Consequently, the application of different species recognition criteria affects not only diversity estimates but also the ecological hypotheses that arise from those observations. The main objective of the study was to examine how the choice and number of genetic markers and species delimitation criteria influences biodiversity estimates. Here, we compare approaches to defining species boundaries in three dominant species complexes of tropical endophytes, specially *Colletotrichum gloeosporioides* agg., *Pestalotiopsis microspora* agg., and *Trichoderma harzianum* agg., from two Amazonian trees: *Hevea brasiliensis* and *H. guianensis*. Molecular tools were used to describe and compare the diversity of the different assemblages. Multi-locus phylogenetic analyses (*gpd*, ITS, and *tef1*) and modern techniques for phylogenetic species delimitation were overlaid with ecological data to recognize putative species or OTUs. The results demonstrate that ITS alone generally underestimates the number of species predicted by other nuclear loci. These results question the use of ITS and arbitrary divergence thresholds for species delimitation.

Keywords: barcode of fungi, fungal biodiversity, genealogical sorting index, genetic markers, species complex, species identification

Introduction

Endophytes are microorganisms that live for all, or part of their life cycle, within aboveground plant tissues without causing visible signs of infection. Studies have shown that individual plants may harbor dozens of endophytic fungal species (Arnold & Lutzoni 2007). Fungal endophytes contribute to the hyperdiversity of Fungi (Hawksworth 2001; Arnold 2008) and surveys in tropical moist forests suggest that the majority of the "undiscovered" endophyte diversity occurs in tropical trees (Frolich & Hyde 1999; Arnold *et al.* 2000; Arnold & Lutzoni 2007; Arnold 2008).

Comprehensive surveys of fungal endophyte diversity are challenging because taxonomic literature for many fungal genera does not exist, particularly for tropical taxa. This lack of taxonomic resources reflects in part a prior lack of taxonomic investigation and awareness of these fungi. At a more fundamental level, taxonomic progress in these groups has been hampered by difficulties in formulating effective species recognition criteria (Arnold 2008). Morphological species recognition (MSR) (Burnett 2003; Lacap *et al.* 2003) criteria have been difficult to develop for many endophyte taxa because they are phenotypically simple and often do not develop taxonomically informative vegetative, sexual (Wang & Guo 2007; Thomas *et al.* 2008) or asexual (Reynolds 1993; Taylor *et al.* 1999) reproductive structures *in vitro*. The lack of morphological synapomorphies may be a consequence of genetic isolation preceding the appearance of phenotypically diagnostic character states (Taylor *et al.* 2000). Biological species recognition (BSR) (Mayr 1942, 1963) criteria are also difficult to frame because mating systems are often unknown and most species cannot be induced to produce their sexual states in culture. In addition,

closely related species can retain interspecific interbreeding as ancestral character (Zervakis *et al.* 2004; Dettman *et al.* 2008).

In contrast to both BSR and MSR, the "phylogenetic species recognition" (PSR) criterion uses nucleic acid variation to circumscribe species of phenotypically uniform, apparently asexual, and even unculturable fungal lineages (O'Donnell *et al.* 1998; Arnold & Lutzoni 2007). According to this concept, species are considered to be an 'irreducible cluster of organisms diagnosably different from other such clusters and within which there is a parental pattern of ancestry and descent' (Cracraft 1983, 1989). However, in single-gene genealogies, deciding where to place the species boundaries is subjective, creating uncertainty on species' limits (Taylor *et al.* 2000). In spite of clear evidence that a single gene genealogy does not necessarily reflect the organism's phylogeny (Rosenberg 2002), most published endophyte diversity studies base their species delimitation on the PSR inferred from a single locus (Guo *et al.* 2003; Murali *et al.* 2006; Promputtha *et al.* 2007).

The Internal Transcribed Spacers (ITS1 and ITS2) and 5.8S region of the nuclear ribosomal repeat unit (ITS) is the most widely used molecular marker in endophyte diversity studies (Guo *et al.* 2003; Murali *et al.* 2006; Promputtha *et al.* 2007; U'ren 2009). The use of ITS has many indisputable advantages, the main advantage being the ease by which it is amplified among all lineages of fungi using universal primers (Nilsson *et al.* 2008) and the large size of the available database (Vilgalys 2003; Lutzoni *et al.* 2004). The use of ITS as a species delimiter has several disadvantages, one being the range of intraspecific variation reported in the literature (Lieckfeldt & Seifert 2000; Lacap *et al.* 2003; Nilsson *et al.* 2008). In an effort to

standardize the delimitation of species, investigators have proposed the use of a sequence similarity percentage as species proxy (Arnold & Lutzoni 2007; Higgins *et al.* 2007; Hoffman & Arnold 2008). However, many studies have demonstrated that ITS is not sufficient for species delimitation, especially in rapidly evolving or highly diverse genera or species complexes (Lacap *et al.* 2003; Hoffman & Arnold 2008).

To avoid subjectivity in delimiting species boundaries, Avise and Ball (1990) and later Baum and Shaw (1995) proposed using more than one gene genealogy and to rely on their concordance to recognize reproductive isolated units (Genealogical Species Concept, GSC). The GSC defines a species as a "basal group of organisms all of whose genes coalesce more recently with each other than with those of any organism outside the group." Species genealogies should be concordant due to the effects of genetic isolation and drift with associated lineage sorting and coalescence (Fisher *et al.* 2002). Recombination between individuals of the same species can create conflict among gene trees, and the transition between concordance to conflict determines the limits of species (Taylor *et al.* 2000). The use of GSC to determine species limits is now commonly used in fungal systematics (Miller & Huhndorf 2004; Schoch *et al.* 2006); however, it is not widespread in endophyte research.

Diversity estimates are currently based on species counts or on the assignment of Operational Taxonomic Units (OTUs). Consequently, any variation in this number may not only affect the diversity estimates, but also the ecological hypotheses that may arise from those observations (Gotelli & Colwell 2001; Agapow *et al.* 2004; Magurran 2008). For instance, if two communities (e.g. different hosts or same host distributed in different areas) are compared, the similarity index will be affected by

how accurately it can be determined whether two individuals belong to the same species unit or if they represent unique entities.

The main objective of the present study was to determine how reliance on a single genetic marker (i.e. ITS) can influence biodiversity estimates and the understanding of a community's ecology (species diversity, abundance, composition, and distinctiveness) and biogeography. To test the hypothesis that the most common groups of endophytes contain more than one cryptic species, phylogenetic species recognition criteria were applied by exploring the congruence of gene genealogies for three independent nuclear loci: ITS nrDNA, translation elongation factor 1α (*tef1*), and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*). The second objective was to determine if there is concordance between ecological, biogeographic, and phylogenetic data. To accomplish this objective, other lines of evidence such as geographic location or management type were overlaid on the multi-locus phylogeny to determine if there is a geographic structure within the studied groups. We hypothesized that delimitation of cryptic species would correlate with host, distribution, and land management type. To achieve our objectives we studied three groups of endophytic taxa: *Colletotrichum gloeosporioides* species complex or "aggregate" (hereafter, *C. gloeosporioides* agg.), *Pestalotiopsis microspora* species complex or "aggregate" (hereafter, *P. microspora* agg.), and *Trichoderma harzianum* species complex or "aggregate" (hereafter, *T. harzianum* agg.).

Materials and Methods

Study Taxa

Colletotrichum gloeosporioides (Ascomycota, Sordariomycetes, Glomerellaceae), *Pestalotiopsis microspora* (Ascomycota, Sordariomycetes, Xylariales, Amphisphaeriaceae), and *Trichoderma harzianum* (Ascomycota, Sordariomycetes, Hypocreales, Hypocreaceae) species complexes were chosen as model organisms for this study due to their common occurrence and abundance as endophytes (Evans *et al.* 2003; Jeewon *et al.* 2004; Lu *et al.* 2004; Rubini *et al.* 2005; Rojas *et al.* 2010). Each of these species complexes is depauperate in morphological characters useful in species recognition and little is known about their reproductive biology, thus neither MSR nor BSC are effective for species identification in these taxa. In addition, low innate ITS nucleotide variability within these complexes hampers attempts to delimit cryptic species (Lieckfeldt & Seifert 2000; Jeewon *et al.* 2004; Lu *et al.* 2004; Rojas *et al.* 2010).

Source of Endophytic Isolates

Endophytic isolates were collected from leaves and sapwood of two *Hevea* species (*H. brasiliensis* and *H. guianensis,* Euphorbiaceae). Trees were located in Peru (Iberia, Iquitos, Los Amigos, and Tambopata) and Cameroon (Ekona). *Hevea brasiliensis* trees were distributed under two types of management: wild (Iquitos and Tambopata) and plantations (Iberia and Ekona); whereas the *H. guianensis* population was only sampled from the wild (Los Amigos). At each locality, 15 trees were

sampled for endophytes. Collection techniques and sampling protocols are described fully in a previously published study (Gazis & Chaverri 2010). All strains were initially identified to genus using morphological characters, and by sequencing the ITS locus and referencing it to GenBank database. The abundance of the three taxa varied among sampling localities. Refer to publication for number of isolates, collection locality, host, and GenBank accession numbers.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from the mycelial mat using Power Plant™ DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, U.S.A). ITS region was amplified using ITS5 and ITS4 primers (White *et al.* 1990); *tef1*was amplified using the primers EF-728 (Carbone & Kohn 1999) and EF2 (Jacobs *et al.* 2004); and the *gpd* region was amplified using the primers GPD1 and GPD2 (Berbee *et al.* 1999). The PCR reaction conditions for the ITS, *tef1*, and *gpd* amplification are described in previous publications (Berbee *et al.* 1999; Gazis & Chaverri 2010; Rojas *et al.* 2010). PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, U.S.A.) and sequenced at the University of Maryland Sequencing Facility.

Diversity and Phylogenetic Analyses

Bidirectional sequences were assembled and edited with Sequencher™ 4.9 (Gene Codes Corporation, MI, U.S.A). Sequence for each taxon and locus were aligned with MAFFT version 6 using the E-INS-i strategy (Katoh *et al.* 2005) and refined manually using MESQUITE version 7.2 (Maddison & Maddison 2009). The software DOTUR (Schloss & Handelsman 2005) and MEGA version 4 (Tamura *et al.* 2007)

were used to assess the amount of genetic variation (distance) within and among putative species ("phylogenetic species" or OTUs recognized in this study). MEGA was also used to calculate the number of nucleotide differences and the p-distance between the established OTUs. P-distance is the proportion of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. In MEGA, distances were calculated directly from the nexus file but for DOTUR, a distance matrix generated in Phylip version 3.68 (Felsenstein 1989, 2008) was required. The furthest neighbor algorithm was used for the clustering of OTUs.

Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI) analyses were performed on separate and concatenated data. Maximum Likelihood phylogenetic trees with bootstrap analysis were constructed with RAxML version 7.0.4 (Stamatakis *et al.* 2008) using the general time-reversible (GTR) evolutionary model and the Gamma model of rate heterogeneity settings. Unordered characters, random taxon addition sequences, gaps treated as missing data and the tree bisection-reconnection (TBR) branch swapping were used in the analyses. Maximum Parsimony analysis was conducted by heuristic search in PAUP* 4.0 (Swofford 2002) with the following settings: all characters were equally weighted, gaps were treated as missing characters, starting trees obtained by random addition with 1000 replicates, and TBR branch swapping algorithm. Nodal support for MP and ML was determined by non-parametric bootstrapping, performing 1000 replicates with a heuristic search consisting of 100 stepwise random addition replicates and TBR branch-swapping for each bootstrap replicate. MrBayes version 3.1 (Ronquist & Huelsenbeck 2003) was

used to construct phylogenies under BI. All searches were done using four chains for a total of 10'000000 generations with trees sampled every 100 generations. Convergence of log likelihoods (-Ln) was assessed with TRACER version 1.4 (Rambaud & Drummond). *Trichoderma* spp. sequences were used as outgroup in each dataset.

JMODELTEST was used to select the models of nucleotide substitution for the ML and BI analyses (Posada 2008). To determine if the datasets could be combined, highly supported clades were compared among trees (reciprocal bootstrap support) generated from different datasets to detect conflicts (Mason-Gamer & Kellog 1996). High support refers to bootstrap support values ≥ 70%. If no conflict exists between the highly supported clades, this suggests that the genes sequenced share similar phylogenetic history and resolution, and combining the datasets can ultimately increase support.

Species Recognition/Delimitation

Three methods were used for species delimitation: (1) genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al.* 2000), (2) the Genealogical Sorting Index (*gsi*) (Cummings *et al.* 2008), and (3) reticulate networks (Huson & Bryant 2006, 2010). The GCPSR assumes a complete sorting of lineages and thus reciprocal monophyly. However, studies suggest that even if speciation is effective immediately, the time required might not be enough for evolutionary changes to appear allowing two distinct lineages to be recognized (Knowles & Carstens 2007; Cummings *et al.* 2008; O'Meara 2010). The genealogical sorting index (*gsi*) is a statistic that measures the degree of genealogical divergence in a specified group of

taxa. The *gsi* supplements the bootstrap support and the posterior probability of monophyly, calculated from the multilocus analysis, by providing an independent measure of monophyly on a scale between zero and one. Interpretation of monophyly based on the *gsi* statistic follows tests using the posterior probability of monophyly through permutation testing (Cummings *et al.* 2008). Because uneven sample sizes among species can shift P-values downward for smaller groups, significance of the *gsi* was inferred at $P < 0.001$ (Polihronakis 2009). The *gsi* was implemented using the web interface (http://www.genealogicalsorting.org/). Rooted, weighted trees were used in the analysis. *Gsi* was calculated for nodes that contain putative or hypothetical species, even if their bootstrap support was low (e.g. 50–70%). Reticulate networks based on the combined dataset were developed to detect potential recombination events between groups (Holmes *et al.* 1999). This analysis was undertaken using the algorithms implemented in SplitsTree program version 4.11.3 (Huson & Bryant 2010). The GTR character substitution model and the reticulation network split transformation under the recombination 2007 method was used. Bootstrap support for each split was estimated for 1000 replicates.

Terminal clades that were highly supported in the combined analyses (ITS + *tef1* + *gpd*) were considered as species and recognized as unique reproductively isolated units. Individual nodes were considered well supported by the data and analyses when both MP and ML bootstrap presented values ≥70% and when Bayesian posterior probability (BPP) was ≥0.90. Monophyly was supported by the *gsi* when values were ≥0.90 and were significant at $P < 0.001$ (Cummings *et al.* 2008; Polihronakis 2009). Some lineages were not considered in the analyses because of

limited sample size and the inability to determine statistical support of their monophyly (e.g. LA11 and T7 in Figure 2.2c); although it is suspected that these lineages likely represent additional species. In ecological surveys these lineages are regarded as "singletons" and are generally excluded from diversity estimates (Arnold & Lutzoni 2007; Davis & Shaw 2008).

Effects of Species Delimitation on Endophyte Diversity Measures: A Case Study

After defining species limits, the ITS alignment was reviewed for each taxonomic group and the sequence divergence was determined between and among identified OTUs. Furthermore, a selected dataset was used as a case study to investigate the effects of varying the ITS similarity threshold (90–100%). This dataset is composed of 106 ITS sequences belonging to endophytic Ascomycota and 2 Basidiomycota collected from leaves and stems of wild *H. guianensis*.

Results

Phylogenetic Analyses: Single Locus

ITS, *tef1*, and *gpd* each produced discrete amplicons and sequence chromatograms and thus behave as single-copy genes. Overall, this study yielded 484 new sequences; all of them have been deposited in GenBank (see publication for accession numbers). All methods used in phylogenetic inference (MP, ML, BI) identified the same lineages within each group; the MP trees are presented with ML and MP bootstrap values and BI posterior probabilities indicated at each node (Figure 2.1-2.2). Results from the comparison of 70% bootstrap trees from the individual-gene analyses did not

reveal any major conflicts among phylogenies. Phylogenetic analyses based on ITS suggest that the *P. microspora* agg. dataset is composed of three distinct putative species or OTUs, *T. harzianum* agg. by two OTUs, and that all the isolates from *C. gloeosporioides* agg. dataset belong to a single OTU (Figure 2.1, a-c).

In the distance analyses conducted with DOTUR and MEGA, the number of OTUs varied depending on the locus used and on the similarity threshold applied to the dataset. For the *C. gloeosporioides* agg. and *T. harzianum* agg. datasets, the ITS region was the least informative of the three loci. For *C. gloeosporioides* agg., ITS grouped all isolates in one clade, even when the sequence similarity was increased to 99%. The ITS region was also invariable in the *T. harzianum* agg. dataset, grouping all isolates into a single OTU at 98% similarity. The number of OTUs in *T. harzianum* agg. increased to three when the similarity threshold was raised to 99%. By contrast, the ITS was a relatively variable region for the *P. microspora* agg. group for which the use of a 95% threshold revealed three OTUs. Of the three loci examined, the *tef1* region was the most informative and variable. The *gpd* locus was more variable than ITS, but less variable than *tef1*, especially for *C. gloeosporioides* agg.

Phylogenetic Analysis: Multi-Locus Approach and Species Delimitation

No incongruence was observed among gene trees for the three taxa, therefore the three loci were concatenated and multi-locus phylogenies were inferred using ML, MP, and BI. These and the results from the genealogical sorting index (*gsi*) are presented in Figure 2.2. The combination of phylogenetic analyses and the *gsi* values suggest that the *P. microspora* agg. dataset included four putative species, the *C.*

gloeosporioides agg. dataset included ten species, and the *T.* cf. *harzianum* dataset included at least six species (Figure 2.2). The *T. harzianum* agg. dataset presented several terminal clades of unknown taxonomic position or clade affiliation. This pattern of diversification within the *T. harzianum* complex has been reported in another study (Druzhinina *et al.* 2010). One working hypothesis for the occurrence of these isolated groups is that they are "relict lineages" (Druzhinina *et al.* 2010) but other factors such as sampling bias or chance inclusion of a geographic or ecological migrant can also create this phylogenetic pattern (Heath *et al.* 2008). Therefore, for purposes of this study, these terminal unsupported clades are considered as singletons and excluded from diversity analyses.

Results from the reticulate network analysis supported the multi-locus grouping. Reticulation events were observed in clades that showed low bootstrap support for the combined analysis as well as low *gsi* values. Phylogenetic networks for the three datasets are shown in Figure 2.3a-c.

Estimation of Intra- and Interspecific Divergence

After the isolates of each group were assigned to OTUs, sequence divergence for the ITS region was calculated within and between species. Overall, ITS was the least variable of all three genes, with a mean sequence divergence of 0.0042% for sister taxa and 0.0177 % for non-sister taxa. Interspecific divergence was calculated as the average distance between the sister and non- sister clades. *Pestalotiopsis microspora* agg. had the highest interspecific ITS sequence divergence (0.037%), whereas *C.* cf. *gloeosporioides* and *T. harzianum* agg. had the lowest interspecific sequence divergence (0.00191% and 0.0045%). Intraspecific sequence divergence was

significantly lower for all groups with a mean of 0.001%. The locus with highest variability was *tef1*, with the highest level of polymorphism in *C. gloeosporioides* agg. *Gpd* provided better resolution and higher sequence divergence than ITS but much lower than *tef1*.

Effects of Species Delimitation on Ecological and Biogeographic Inferences

Overall, the combined analyses for all three taxa revealed correspondence between resolved clades and plant host association or geographic origin. Host specialization can be clearly observed in the *P. microspora* agg. (Figure 2.2a). In this group, the combined analyses revealed four putative species or OTUs. Two of the OTUs, OTUs 3 and 4, are restricted to *H. guianensis*, whereas, with two exceptions, OTU 1 includes isolates from *H. brasiliensis*. Biogeographic structure among collecting localities was not observed. In contrast, OTUs in *C.* cf. *gloeosporioides* did not display host specificity (Figure 2.2b). Isolates from *H. guianensis* are intermixed with isolates from *H. brasiliensis*, and there are no OTUs that contain only endophytes from *H. guianensis*. Nevertheless, the combined analyses revealed a high geographic structure (Figure 2.2b). For instance, OTUs 8 and 9 contained isolates from Peru and mainly from wild habitats. On the other hand, six lineages contain only Cameroonian isolates (OTUs 1, 2, 4, 5, 7 and 10). Host association was not apparent for any *T. harzianum* agg. OTU, although a correlation was found between geographic origin and management type (Figure 2.2c). For instance, OTU 3 is composed only of Cameroonian isolates and OTUs 4 and 5 by isolates from wild trees distributed within the locality of Tambopata.

Effects of Species Delimitation on Endophyte Diversity Measures

To assess the effect of ITS sequence similarity threshold on OTU diversity estimates, data for 106 endophytic Ascomycota collected from sapwood and leaves of wild *H. guianensis* were used. These data were selected because *Colletotrichum*, *Pestalotiopsis*, and *Trichoderma* were all present in relatively high abundance at this site. Since DOTUR estimates are reported to be sensitive to the alignment (U'ren *et al.* 2009), the MAFFT alignment was not refined manually but subjected to analysis in DOTUR directly. The alignment for the analysis consisted of 730 bp including indels. For the phylogenetic analysis, two isolates belonging to the Basidiomycota, also collected from wild *H. guianensis*, were used as outgroup (LA216 and LA240). The number of OTUs inferred by the analysis depended on the ITS similarity percentage threshold value. The resulting numbers of OTUs were as follow: 90%: 25; 91%: 25; 92%: 26; 93%: 26; 94%: 29; 95%: 31; 96%: 31; 97%: 33; 98%: 35; 99%: 38; and 100%: 50.

The following groups were affected by the increase in the ITS similarity threshold and thus an increase in their OTU numbers: *Beauveria* (95%: 2; 99%: 4), *Bionectria* (95%: 2; 99%: 4), *Fusarium* (95%: 2; 99%: 3), *Phomopsis* (95%: 1; 99%: 2), and *Xylaria* (95%: 1; 99%: 2). The groups in which OTU numbers were most affected are those with known low ITS interspecific variability (O'Donnell 1998; Rehner & Buckley 2005; Tejesvi *et al.* 2009; Menezes *et al.* 2010). However, the majority of clades were unaffected and OTU estimates remained constant over different ITS thresholds.

The clades containing *Colletotrichum* and *Trichoderma* isolates were not affected by the increase in similarity threshold due to their extremely low interspecific ITS variability. The isolates belonging to each of the mentioned clades remained as one OTU even after increasing the cutoff to 99% similarity. However, as shown in Figures 2.2b-c, *Colletotrichum gloeosporioides* agg. and *Trichoderma harzianum* agg. isolates from Los Amigos belong in 2 and 2–4 OTUs, respectively. In the case of *Pestalotiopsis microspora* agg., the use of 95% sequence similarity concurred with the combined analysis revealing two OTUs for the Los Amigos dataset.

Discussion

The ITS region has been adopted by many fungal ecologists as the genetic marker of choice for species delimitation, even though its limitations for this purpose are widely acknowledged. ITS is useful for several reasons, among them the size of the available database in GenBank and the ease in which it is amplified along distant fungal lineages. However, several studies have demonstrated that for some groups (e.g. Ascomycota) the ITS region is insufficiently variable to resolve terminal species-level clades clearly resolved by other commonly used nuclear markers (O'Donnell *et al.* 1998; Inderbitzin *et al.* 2009; Pavlic *et al.* 2009; Druzhinina *et al.* 2010). In addition, field researchers face the challenge of having to examine large numbers of isolates, thus, multi-locus analyses are usually not an affordable or efficient option for conducting pilot surveys of these organisms. Consequently, investigators need to compromise between the numbers of markers used to delimit species and the number of isolates included in the study. The choice of molecular marker and its use (e.g.

distance vs. character based criterion, similarity threshold, or phylogeny) is fundamental for accurately characterizing diversity. Segregation of species into units that reflect their genetic affinities can reveal patterns of biogeography and niche partitioning that may be overlooked otherwise. The failure to notice these patterns is generally a consequence of lumping potential species (or independent units) into large groups and assuming their homogeneity. In this study, all *Colletotrichum* sequences "blasted" with *C. gloeosporioides* with 100% similarity in the GenBank database. Only in the combined gene analyses was it apparent that there are several endophytic species lineages and none are closely related to *C. gloeosporioides sensu stricto* (data not shown, but see Rojas *et al.* 2010 for an example). Assuming that the investigated endophytic strains were indeed *C. gloeosporioides* has further implications, since this species is an important and common pathogen of tropical crops. And, unless a multi-approach analysis was conducted, one could have assumed that this species is also a common endophyte of rubber trees.

Species Delimitation in the Selected Species Complexes

Experimental results reported here confirm that all three fungal genera represent species complexes. The combined analyses show that the isolates belonging to *P. microspora* agg. form four distinct clades (Figure 2.2a). ITS distance analyses diagnosed just three of those putative species (OTU 1, OTU 2/OTU 3, and OTU 4) at a 95% similarity threshold. Only when the threshold was increased to 99% did the number of putative species inferred from ITS sequences equal the number of species revealed by the combined analyses. Phylogenetic analyses based on the ITS locus did reveal a bifurcating pattern, although the reciprocal monophyly of OTU 2 and OTU 3

was only weakly supported (Figure 2.1a). This might be a case of incomplete lineage sorting, in which some ancestral characters are being kept in both populations as a consequence of recent diversification events (Machado & Hey 2003, Koblmüller *et al.* 2010). The reticulate network analysis was mostly concordant with the multi-locus phylogeny, and grouped the isolates into the same OTUs as with the other species delimitation criteria. The only disagreement between these two methods, was in the case of OTU2 and OTU3, were a non-supported reticulation event was detected (Figure 2.3a). This reticulation is reflected in the low MP and BI values estimated in the combined phylogeny. Unfortunately, with the available data it is not possible to determine if the latter represents recombination events between members of OTU2 and OTU3 or if it is the case of incomplete lineage sorting (Morrison 2010). Since the bootstrap value was low (< 70%), the multi-locus and *gsi* analysis was followed, dividing OTU2 and OTU3 into different entities.

The case of *C. gloeosporioides* agg. is more complex. The isolates formed ten well-supported lineages in the combined analysis (Figure 2.2b). The only lineage that lacked significant support was OTU 6 (Figure 2.2b). This OTU was weakly supported by MP, ML, and BI, but the *gsi* analysis revealed the monophyly of the group. For this group, the ITS marker showed extremely low interspecific variability, and all the isolates were included in one OTU, even when using 99% of similarity. When sequences were grouped by unique haplotypes, eight groups were resolved. However, these ITS haplotype groups conflicted with the OTUs inferred by the phylogenetic multi-locus approach (Figure 2.2b). The reticulate network analysis showed similar results to those obtained by the combined phylogeny. The low branch support

between OTU5 and OTU6 was concordant with a reticulation pattern between the mentioned OTUs (Figure 2.3b). As mentioned before, with the available data, it is not possible to determine if this pattern is due to recombination between populations or incomplete lineage sorting. However, in this case incomplete lineage sorting seems to be the most plausible explanation. The latter because OTU5 is only composed by isolates from Cameroon and OTU6 by isolates from Peru, consequently due to the large geographical distance between these two populations, recent recombination events appear unlikely.

Trichoderma harzianum agg. presents an even more complicated scenario, which challenged the delineation of species within the complex. Even when using three independent loci and several approaches, it was not possible to assign some lineages to particular clades (or OTU). For some of these lineages, even though they had more than one representative, their monophyly was not supported by the analyses. Therefore, they were left as singletons until more isolates or additional markers are collected and relationships can be inferred more clearly. Nevertheless, the combined analysis resolved six well-supported clades. The ITS region in the *Trichoderma* dataset displays low polymorphism. Distance methods grouped all the isolates into one clade under 95% and 98% of similarity, irrespectively. Only when the threshold was increased to 99%, the isolates were grouped into three OTUs; however, this grouping did not correspond with the lineages inferred in the combined phylogenetic analysis. OTU 6 encompassed the highest interspecific variability, and was recognized even by the ITS phylogeny (Figure 2.1c). Overall, phylogenetic analyses provided more resolution than distance methods. As in the case of the

Colletotrichum dataset, all the *Trichoderma* sequences blasted to *T. harzianum* using the GenBank database. The latter illustrates the importance of using phylogenetic methods when identifying isolates. Reticulate network analysis was consistent with the multi-locus phylogeny; however, it showed low bootstrap values for the delimitation of OTU3–5 (Figure 2.3c). This might be a reflection of the lack of branch support for the internal nodes encompassing these three groups (Figure 2.2c). All the "singleton lineages" found in the combined analysis were also detected as singletons in the reticulate network but all of them were placed, with support, more closely to OTU3–5. OTU6 was clearly defined by this method, giving additional support to its segregation.

Does Phylogeny and Species Delimitation Reflect the Ecology of Endophytes?

Given the close relationship between ecological divergence and reproductive isolation and consequently the presumed role of ecological adaptation in the speciation process, an evaluation of the studied organism's ecology should play a significant role in species delimitation (Chaverri *et al.* 2003; Nosil & Crespi 2006). The issue is whether genetic divergence in the absence of ecological change or adaptive divergence of some other type is sufficient for species delimitation (Davis *et al.* 2003; Bond & Stockman 2008; Frenkel *et al.* 2010). In the three species groups studied here, some OTUs of each group inferred in the combined analyses are characterized by unique ecological /biogeographic characteristics. For instance, OTU 3 and OTU 4 from the *P. microspora* agg. only include isolates collected from wild *H. guianensis*. This case demonstrates an agreement between genetic and ecological divergence, and the combined analysis revealed a host association between the species of *Hevea* and

the endophytic group. Nevertheless, the majority of the *Pestalotiopsis* isolates were grouped under OTU 1, composed of strains collected from different localities and management types. The combined analysis of the *C. gloeosporioides* agg. isolates also revealed a correlation between their genetic divergence and their ecological niche. For instance, OTUs 1–5, 7 and 10 include only isolates collected from trees in plantations mainly in Cameroon. On the other hand, OTU 6 and 8 contain mainly isolates from wild trees (*H. guianensis* and *H. brasiliensis*). Even though OTU diagnosis in *T. harzianum* agg. was more challenging, the combined analyses also uncovered ecological affinities. This is the case of OTU 6, composed only of isolates collected in Peru and mainly from plantations located in Iberia. All mentioned examples demonstrate how the use of phylogenetic approaches based on multiple loci assist in revealing ecological patterns of diversification. The latter is true especially for groups like *Colletotrichum* and *Trichoderma*, that harbor a great genetic diversity and for which genetic sorting appears to be an active process.

Effects of Species Delimitation on Endophyte Diversity Measures

There is no doubt that the ITS region will continue to be the genetic marker of choice for many future endophyte surveys. Therefore, this study investigated how an increase in the similarity threshold would impact the diversity estimation. Commonly, endophyte surveys use a conservative 95% similarity threshold for species delimitation. The effect of increasing the similarity percentages (90–100%) on the resulting species richness estimation was tested. It was found that most of the groups were not affected by the increase, because the majority of clades were phylogenetically distant, bearing many differences in nucleotides, and therefore an

increase in similarity did not affect their grouping (e.g. LA72a, LA89, LA227, and LA264). The group most affected was the Hypocreales (i.e. *Beauveria, Bionectria*, and *Fusarium*). These results coincide with others that also reported low ITS interspecific variability for the mentioned groups (O'Donnell *et al.* 1998; Davis *et al.* 2003; Rehner & Buckley 2005; Tejesvi *et al.* 2009). It is concluded that an increase of similarity threshold (based on ITS) has an important effect in the community's diversity estimate. For this example dataset, the increase from 95 to 100% showed an overall increase of 19 OTUs, from 31 to 50 OTUs, respectively.

Is Molecular Data Enough for Species Delimitation?

There is an ongoing controversy regarding how much (e.g. how many genes, how many morphological characters) and which type of data (e.g. molecular, morphological, ecological) should be used to circumscribe a species. For some species, morphologically distinctive characters were found only after those lineages were segregated and established by molecular data. A clear example can be drawn from this study. *Trichoderma harzianum* agg. is considered a species complex in which its members cannot be distinguished by using conventional morphological characters (e.g. conidiophore branching, conidia size/shape, phialide size/shape) (Chaverri *et al.* 2003; Druzhinia *et al.* 2010). Nevertheless, after the combined analysis confirmed the distinctiveness of one of the internal clades (OTU 6 in Figure 2.2c), a more intensive examination was performed to search for cryptic morphological differences that could support the molecular data. Few morphological differences between members of the clade OTU 6 and the rest of the members of the *T. harzianum* complex were revealed. For instance, besides its endophytic habitat and

its apparently specific host association (only found in *Hevea* species) members of OTU 6 produce a diffusing brown pigment on artificial media and clustered chlamydospores. Recently, OTU 6 has been described as the new species *Trichoderma amazonicum* (Chaverri *et al.* 2011). Even though for additional clades treated in this study monophyly was highly supported, the proposal of new species is beyond the scope of the present work.

Results from this and other studies advocate the use of more than one gene to delineate species. However, when many isolates are involved, sequencing several genes becomes a challenge. Therefore, for studies that aim to evaluate the diversity of a group of endophytes, the use of the ITS region is recommended for a first screening in which isolates can be sorted into different clades. Since many lineages will be singletons or will form definite clades, a more rigorous examination would only be necessary for speciose groups. On the other hand, if the objective of the study is to answer ecological (e.g. comparison between sites, specificity evaluation, species range estimation) or evolutionary questions, the use of more than one gene is essential and several species limits approaches should be applied to the data. If the sequencing of additional markers is not feasible for a project, the use of a higher ITS similarity threshold (99% to almost 100% or unique haplotypes) is recommended for those speciose clades. The application of a higher ITS similarity threshold will most likely lead to a more accurate estimate of diversity.

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Chapter 3: Sampling effect in estimating tropical fungal endophyte diversity: Are we undersampling?

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Sampling effect in estimating tropical fungal endophyte diversity: Are we undersampling?

Abstract

Fungal endophytes are hyperdiverse and ubiquitous. Ecological studies attempting to estimate their diversity face the challenge of extensive sampling. Low sample size affects the accuracy and precision of diversity estimation and the ability to address ecological questions. Unfortunately, standardized sampling designs are rare, limiting the capability to compare biodiversity estimates from published reports. Furthermore, the fundamental heterogeneity intrinsic to any endophyte community, such as host genetic background and spatial diversity, remain little known. Using the sapwood endophytic fungal community inhabiting *Hevea brasiliensis* as a model, we set the following objectives: (1) evaluate the effects of sample size on commonly used diversity indices, (2) compare sample size effects between managed and wild habitats, (3) estimate the loss of phylogenetic diversity coverage when sample size is low, and (4) calculate the sampling efforts necessary to reach asymptotic species accumulation curves. Using a combination of approaches we determined that all the diversity indices were strongly influenced by sample size and by the number of singleton species. A sample size of fifty only covered a fraction of the overall estimated diversity. This research contributes to the standardization of endophyte surveys and proposes the inclusion of the "estimated diversity coverage" when comparing habitats.

Keywords:

Diversity index, experimental design, fungal endophytes, phylogenetic diversity, sample size.

Introduction

Endophytic fungi are microorganisms that inhabit aboveground plant tissues without inducing symptoms of disease (Petrini, 1991). Leaf endophytes are thought to be especially diverse in tropical forests (Arnold et al., 2000; 2001; Arnold & Lutzoni, 2007; but see Suryanarayanan et al., 2002) and recent surveys have demonstrated that sapwood tissue can also contain a highly diverse fungal community (Evans et al., 2003; Gond et al., 2007; Thomas et al., 2008; Gazis & Chaverri, 2010). Tropical trees harbor fungal endophytes that are mainly transmitted horizontally (Arnold et al., 2003; Santamaria & Bayman, 2005), and although the exact relationship with their hosts has not been elucidated, studies have provided evidence of their central role in shaping the community they inhabit (Redman et al., 2002; Arnold et al., 2003; Rodriguez et al., 2009; Alvarez et al., 2011; Bittleston, 2011). Horizontally transmitted endophytes have been found in all plant species studied to date (Schulz et al., 2002; Arnold et al., 2007; Li et al., 2007; Tao et al., 2008), and in contrast to the vertically transmitted endophytes, a single plant host can harbor numerous species (see Lodge et al., 1996; Gamboa & Bayman, 2001).

The most widely accepted estimate for the global number of fungal species is 1.5 million (Hawksworth, 2001), but more recently extrapolations have raised the number to as many as 5.1 million (O'Brien et al., 2005). Unfortunately, only a small

fraction has been described, resulting in large number of “missing Fungi” (Blackwell, 2011; Jones et al., 2011). Tropical endophytes are thought to represent a significant portion of the yet-undescribed fungi (Hawksworth & Rossman, 1997; Fröhlich & Hyde, 1999; Arnold et al., 2000; Hawksworth, 2001; Smith et al., 2008). Although endophyte research has flourished over the past decade, insufficient knowledge of their ecology precludes predictions about their overall diversity. Studies addressing host specificity and spatial structure are rare and results are frequently contradictory, making the identification of patterns difficult. For instance, some studies have reported evidence of host specificity (Beilharz & Cunnington, 2003; Chaverri et al., 2011; Gazis et al., 2011) while others have supported a host generalist habit (Cannon & Simmons, 2002; Pandey et al., 2003; Arnold & Lutzoni, 2007).

Estimates of species richness are the most straightforward measurements of diversity but are difficult to calculate accurately (Buzas & Hayek, 1996; Hellmann & Fowler, 1999; Lande et al., 2000; Gotelli & Colwell, 2001; Brose et al., 2003; Chao et al., 2009; Beck & Schawanghart, 2010). "An important reason for this difficulty arises from the sampling process itself or the variation in sampling intensity among habitats that vary in size and spatiotemporal complexity" (Lande et al., 2000; Walther & Moore, 2005; Magurran, 2008; Dornelas et al., 2009; Schreiber & Brauns, 2010). Sample size, or sampling effort, can greatly affect the comparison of species’ richness, and small sample sizes are likely to underestimate differences among sites (Buzas & Hayek, 1996; Cao et al., 2002; Connolly, 2005; Walther & Moore, 2005; Mazaris et al., 2008). To estimate the diversity of a habitat with accuracy and precision, sample size should be sufficiently large to span the variation harbored

within it and reflect the taxonomic composition and relative abundance of its members (Cao et al., 2002).

Ecological studies are frequently concerned with the efficacy of a sampling scheme to capture the taxonomic composition and relative abundance of the targeted community (Hughes et al., 2001; Cao et al., 2002). Sample representativeness is negatively correlated with spatial heterogeneity and positively correlated with the evenness of a community's underlying species abundance distribution (Colwell & Coddington, 1994; Hughes & Hellman, 2005). Tropical regions are known for their high spatial heterogeneity, which is reflected in their great diversity of microhabitats (Chave, 2008; Baraloto & Couteron, 2010; Dyer et al., 2010). In addition, tropical plant populations distributed within their native range often present high genetic diversity (Besse et al., 1994; Luo et al., 1995; Amsellem et al., 2000; De Walt & Hamrick, 2004; Bicalho et al., 2008; Le Guen et al., 2009; Souza et al., 2009; Clement et al., 2010; Lambertini et al., 2010). Therefore, endophytic symbionts living within each individual host face distinct environmental conditions that contribute to variation in endophyte abundance and composition.

The diversity of a community can be estimated with confidence when sufficient independent, randomized samples allow a species accumulation curve to reach an asymptote (Walther & Moore, 2005; Magurran, 2008). However, in endophyte research, attempts to standardize the sampling design (e.g., for sampling effort) are rare (Gamboa et al., 2002). This lack of methodological consensus limits the ability to compare published reports (Table 3.1). For instance, Rubini et al. (2005) isolated endophytes from 300 fragments (subsamples) from each of five individuals.

In contrast, Rosa et al. (2010) isolated endophytes from three leaf fragments from 180 individuals. Other procedures used during sampling that can affect the estimation of diversity are: the size of the fragment (Gamboa et al., 2002), the kind of artificial media used to isolate and grow the endophytes (Hyde & Soytong, 2008), and the concept used to delimit ecological units (Gazis et al., 2011). The overall goal of this study is to raise awareness of these key issues in microbial/fungal biodiversity estimation.

Using the endophytic fungal community inhabiting *Hevea brasiliensis* (Euphorbiaceae) as a model, we set the following main objectives: (1) to investigate the effects of sample size on the estimation of diversity comparing diversity indices, (2) to test the effect of sample size on managed (plantation) or wild habitats, (3) to estimate the loss of phylogenetic diversity when sample size is low, and (4) to calculate the sampling effort necessary to reach the asymptote of both habitats' species accumulation curves. We used a combination of approaches, relying on parametric and nonparametric measurements, to estimate the diversity of each habitat at different sample sizes. Finally, with the goal of facilitating the comparison among studies, we stress the importance of specifying the sampling design and recommend the estimation of the sample size coverage.

Materials and Methods

Host

Hevea brasiliensis (Euphorbiaceae) is an emergent tropical tree native to the Amazon basin (Schultes, 1956; Wycherley, 1976; Besse et al., 1994). Commonly known as the

"rubber tree," this species is of great economic importance due to its production of high-quality latex, which makes it nearly the only commercial source of natural rubber (Priyadarshan & Goncalves, 2003; Onokpise, 2004). The cultivation of this crop beyond its native range is extensive, primarily in Asia (> 90%) and to a lesser extent (~ 6%) in Africa (Onokpise, 2004; Lieberei, 2007). Plantations in Central and South America have never reached full production, mainly because of the devastating endemic fungal disease, South American Leaf Blight (SALB) (Garcia et al., 1995, 1999; Le Guen et al., 2006, 2009).

Sampling design and isolation techniques

Hevea brasiliensis trees from two localities were sampled for endophytic fungi. The first locality is situated close to the city of Cardenas in the state of Tabasco, Mexico, (17°58'32.53"N, 93°23'13.65"W) while the second is situated in the province of Loreto, Peru, (3°37'14.90"S, 72°14'48.33"W). The locality of Tabasco represents a managed setting, outside the host's native range, where *H. brasiliensis* stands are cultivated under a monoculture system. In contrast, the second locality represents a site with wild *H. brasiliensis* trees growing under undisturbed primary forest within its native range. In the wild, *H. brasiliensis* is reported to have a scattered distribution (Holliday, 1970; Lieberei, 2007). However, in this and in previous explorations (Gazis & Chaverri, 2010; Gazis et al., 2011), adult rubber trees were found to grow in clusters or stands, forming "subpopulations" and those stands showed a scattered distribution. In plantations, rubber trees are usually grown in monoculture systems (Evans & Turnbull, 2004; Mann 2009).

In each locality, fifty individuals that satisfied the following criteria were selected: (1) a diameter of at least 10 cm and (2) a distance between two individuals of at least 15 m. In the case of the managed habitat, the sample comprised three small plantations all situated in the same locality.

Sapwood endophytes were collected following the methods described in Evans et al. (2003) and Gazis and Chaverri (2010). Briefly, a sterilized knife was used to cut three slivers of ca. 3 x 6 cm of dead bark from each tree at shoulder height (~1.50 m), exposing the living sapwood. Three pieces of *ca.* 5 mm of living sapwood tissue from three sections, i.e. a total of 9 pieces per tree, were excised from each exposed area and quickly transferred to Petri plates containing CMD (BBL ™ corn-meal-agar + 2% dextrose). To eliminate bacterial endophytes, we added an antibiotic solution to the media (1% solution of neomycin-penicillin-streptomycin, Sigma-Aldrich, St. Louis, MO, USA). Petri plates were kept at low temperature (*ca*. 4–8°C) until they were processed at the Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, USA. Once in the laboratory, plates were incubated for several days to up to 2 months, and the emerging colonies were subcultured in Difco™ potato-dextrose-agar (PDA) to obtain pure isolates.

Replicates refer to individual trees screened in each locality. Subsamples represent sapwood fragments obtained from the exposed living sapwood around the tree trunk. Sample size (also referred as sampling effort) is equal to the number of replicates (N).

DNA extraction, amplification, and sequencing

Once in pure culture, each isolate was grown in Difco™ potato-dextrose-broth (PDB) at 25°C for 4 d to 2 wks. Genomic DNA was extracted from the mycelial mat using a Power Plant™ DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, USA). The procedure was performed according to the manufacturer's instructions with the following modifications: mycelial tissue was stored at -80°C for several days prior to the extraction and in replacement of the vortex, a FastPrep®-24 (MP Biomedicals, Solon, OH, USA) was used for a better tissue lyses but only for 2 min.

The complete Internal Transcribed Spacer (ITS) and a region of *ca*. 900 bp of the Large Subunit (LSU) of the nuclear ribosomal DNA were amplified in one reaction, using ITS5 and LR5 primers (White et al., 1990). All PCR reactions were set up using the following ingredients for each 25 µl reaction: 12.5 µl of GoTaq® Green Master Mix (Promega Corporation, WI, USA), 1.25 µl of 10 µM reverse primer, 1.25 µl of 10 µM of forward primer, 1 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), a maximum of 25 ng/µl of genomic DNA, and double distilled water to complete the total volume. The PCR reaction conditions for the ITS+LSU amplification were as follows: 94°C for 2 min followed by 15 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and primer extension at 74°C for 1 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and primer extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA.) and sequenced at MCLAB laboratories

(www.mclab.com). Due to the large number of isolates, only one direction was sequenced (ITS5 forward) yielding an average of 700–900 bp.

Sequencher™ ver. 4.9 (Gene Codes©, MI, USA) was used to assess the sequences' chromatograms quality and for sequence edition, if needed. Sequences were aligned with MAFFT version 6 using the E-INS-i strategy (Katoh et al., 2009). Alignments were visualized using MESQUITE version 7.2 (Maddison & Maddison, 2009) and the 5' and 3' ends were trimmed to a uniform length. The alignment was performed separately for the Ascomycota and Basidiomycota datasets.

Species recognition/delimitation

Initially, sequences were identified using the BLASTn algorithm implemented in Genbank (www.ncbi.nlm.nih.gov/BLAST). Matches that had 99% or more in similarity (ITS), belonging to ex-type cultures or published studies were considered reliable. FASTA files of the top reliable hits were downloaded and included in the dataset alignments for taxonomic identification through phylogenetic reconstruction. To group isolates into Operational Taxonomic Units (OTUs), we used the furthest neighbor algorithm implemented in MOTHUR (Schloss et al., 2009: www.mothur.org). Ascomycota and Basidiomycota datasets were analyzed separately. OTU assignment and delimitation were based on a sequence similarity threshold of 99% using the ITS region. This cutoff was selected based on previous studies that demonstrated a low ITS sequence variability in many fungal endophyte groups (Gazis et al. 2011). In this study, OTUs inferred by the 99% ITS sequence similarity are treated as species and used as units in the diversity analyses.

Diversity and statistical analyses

Characterization of the datasets

We first evaluated if our sampling was sufficient or if an increased sample size would detect additional unrecorded species. If the localities were sampled exhaustively, their species accumulation curves should plateau at an asymptote (Gotelli & Colwell 2001). Species accumulation curves for each locality were created in EstimateS ver. 7.5 (Colwell, 2005: http://viceroy.eeb.uconn.edu/estimates) using MOTHUR 99% ITS sequence similarity OTU groups. Since the order of the isolates were not obtained in a systematic manner, the species accumulation curves were randomized (using 100 randomizations with replacement) to create "smooth curves." To avoid any influence of density on the comparisons between localities (Colwell et al., 2004), we checked for significant differences on the number of isolates obtained by each sample size. The number of isolates per sample size was also calculated in EstimateS (using 100 randomizations) and later tested for significance and plotted in SPSS® ver. 13.0 for windows.

Overall diversity estimation

Species accumulation curves were extrapolated under the logarithmic model. We used the logarithmic model because it has successfully been applied in previous studies covering hyperdiverse communities (Fisher, 1999, Thompson et al., 2003, Rohr, 2007). This model assumes that species accumulation curves have no asymptote and will increase indefinitely. However, the model allows statements about changes in inventory efficiency (i.e., increase in sampling effort) (Soberón & Llorente, 1993; Longino & Colwell, 1997). The projection of the species

accumulation curves was conducted in BioDiversity Professional ver. 2 (McAleece et al., 1997; www.nhm.ac.uk/zoology/bdpro). As an additional parametric measurement we calculated the Fisher's alpha (α) diversity index. The diversity of each locality was also estimated using several nonparametric indices (no distribution model assumed; Magurran, 2008) such as ACE (Abundance-based Coverage Estimator), Chao 1, and Shannon. The choice of indices was based on their common usage in microbial diversity studies. All diversity indices were calculated using EstimateS. Evenness (E) was calculated based on the following formula $E = e^H / S_{obs}$ (Heip, 1974), where e^H represents the exponential of Shannon index and S_{obs} represents the number of observed species. Analyses were repeated after deleting singletons to assess the effect of single observations (or rare species)

Datasets comparisons

We investigated the similarity between the Tabasco and Loreto datasets (complete and truncated). Sequences belonging to both localities were aligned and grouped into OTUs (see species recognition/delimitation section). Isolates from different localities that grouped together, under a 99% ITS sequence similarity, we treated as the same taxon and consequently classified under "shared species." On the other hand, species isolated only from one locality were classified as "unique species." Nonparametric similarity indices based on presence/absence (Sorensen's indices) and frequency data (Morisita-Horn indices) were calculated using SPADE (Species Prediction And Diversity Estimation, Chao & Shen, 2010).

The Mann-Whitney U-test was used to assess if there was a statistical difference between the means of the different indices among different groups. In

preparation for the analyses, replicates (individual trees) were segregated into four groups according to their origin (group 1 = Loreto complete, group 2 = Tabasco complete, group 3 = Loreto truncated, and group 4 = Tabasco truncated) (complete = with singletons; truncated = excluding singletons). Tests were conducted in SPSS, using EstimateS results from 100 randomizations. Significance was determined based on $P \leq 0.05$.

Effects of sample size on diversity estimation

To examine the effect of sample size (the number of sample units pooled) on diversity estimation, we calculated the species richness, parametric and non-parametric diversity indices, evenness, and the number of singletons for a range of sample sizes (number of trees): 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50. Trees used for the analyses were selected from the pool of samples using a randomization procedure of resampling with replacement. This process was repeated 100 times. For each locality, the complete and truncated datasets were analyzed separately. All calculations were conducted in EstimateS, except for the estimation of evenness, which was calculated following Heip's formula. Results were exported to SPSS for plotting, and curves were investigated for signs of stabilization.

Phylogenetic diversity and sample size

We constructed ITS phylogenies for Tabasco and Loreto datasets using strains drawn from different samples sizes (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50) to investigate how much phylogenetic diversity could go undetected when sampling is insufficient.

Randomly selected samples (using Excel RANDMETWEEN function) were withdrawn from the complete datasets and phylogenetic trees were built based on the individuals left for each sample size. Based on ITS, phylogenetic trees were constructed using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). All searches were done using four chains for a total of 10'000000 generations with trees sampled every 100 generations. MrBayes files were submitted to CIPRES Science Gateway for processing (Miller et al., 2010). Phylogenetic Diversity (PD) as defined by Faith (1992) was estimated for each of the ITS phylogenies based on the strains gathered at the different sample sizes (the number of strains included in the phylogenies depended on which trees were randomly withdrawn). For the latter, we used the Phylogenetic Diversity Analyzer (PDA) version 0.5 (www.cibiv.at/software/pda). To capture the variation in the estimates, we used the last 100 Bayesian trees produced by MrBayes (50 from each run) to calculate PD. No outgroup was used, and PD was calculated for unrooted trees. PD values were then exported to SPPS for parametric statistics analysis. Due to the low abundance of Basidiomycota isolates, all the mentioned analyses were conducted only for the Ascomycota strains.

Sample representativeness and adequate sample size

To determine if the sample size obtained in each locality (N=50) was representative, we followed Chao et al. (2009). We estimated the coverage of the sample and the minimum sample effort required to reach the asymptotic richness estimated by Chao1 (*g*). Formulas were applied to each locality's dataset using several values for *g* (fraction of the estimated species richness that is desired to cover): 0.50, 0.60, 0.70,

0.80, 0.85, 0.90, 0.95, and 0.99. Since the Chao1 diversity estimator uses the number of singletons and doubletons to estimate the diversity of the entire sample, only complete datasets were used.

Results

Diversity of sapwood endophytes in wild and managed populations of *Hevea brasiliensis*

A total of 100 individuals of *Hevea brasiliensis* were sampled for fungal endophytes; 50 of these were distributed in the locality of Loreto and 50 in the locality of Tabasco. A summary of the results can be found in Table 3.2. ITS sequences from isolates representing distinctive OTUs have been deposited in GenBank. A total of 702 culturable endophytic fungi were obtained from both localities: 360 from Loreto and 342 from Tabasco. A greater percentage of the subsamples taken from Loreto showed endophytic growth (71%), in comparison to the subsamples taken from Tabasco (68%). Overall, Loreto presented a higher number of species (169) in comparison to Tabasco (132) for a total of 277 species. In both cases, most species belong in the Ascomycota (133 in Loreto and 102 in Tabasco) although the Basidiomycota were also present (34 in Loreto and 32 in Tabasco). Species from the Phylum Zygomycota were isolated only from the locality of Loreto (2 species).

Significant difference in density (number of isolates in relation to sample number) was detected between Loreto and Tabasco; therefore, the x-axis in the accumulation curves was re-scaled to the number of isolates. For both localities, the sampling was not sufficient. Species accumulation curves did not reach an asymptote for any of the complete datasets especially for the Loreto dataset. On the other hand,

species accumulation curves based on the truncated datasets did show a stabilization trend (Figure 3.1, A-B). Overall, the frequency distribution of species was highly right skewed (*data not shown),* with the great majority of species occurring just once (singletons). In both localities, the percentage of the OTUs classified as singletons ranged from 60 to 70% of the total number of species recorded. Even for truncated datasets, the distribution of species (based on rank/abundance plots) did not follow a log-normal distribution and was also right skewed, showing the largest number of species under the doubletons category (*data not shown*). The frequency of occurrence of the most common species was 90 in Loreto and 39 in Tabasco; both OTUs belong to the genus *Trichoderma*.

Results from all the diversity indices applied in this study agreed in that the community of fungal endophytes inhabiting wild trees distributed in the locality of Loreto was more diverse than the community inhabiting trees growing under plantation in the locality of Tabasco. Table 3.3 shows results from the diversity indices when applied to complete and truncated datasets. We found that the complete dataset from Loreto was significantly more diverse than the complete dataset from Tabasco based on ACE, Chao-1, Fisher's alpha, and species observed (Sobs); however, based on Shannon (H') we found no significant differences ($P \leq 0.05$). On the other hand, when truncated datasets were used as input data, we found that the Tabasco community was significantly more diverse than Loreto based on all indices except Fisher's α. In terms of evenness we found no significant difference between complete datasets, but the truncated version of Tabasco presented a significantly more even community than Loreto.

Datasets comparisons

Overall, we found low similarity between the Tabasco and the Loreto datasets. From the total number of species recovered in both collections (277), only 24 species (8.5%) were found in both localities (Table 3.4). The Ascomycota showed the most number of shared species compared to the Basidiomycota (20 vs. 4). The two species of Zygomycota recovered in this study were only found in Loreto. From the shared genera, *Trichoderma* was found to be the genus with the highest frequency at both localities. When truncated datasets were used as input data for the similarity analyses, we found that only 13 species were shared between localities and that Sorensen's classic overestimated the similarity between Loreto and Tabasco (Table 3.4).

Effects of sample size on diversity estimation

All diversity indices used in this study were affected directly by the increase in sample size. Diversity indices applied to complete datasets were more affected by sample size than their truncated versions, especially the dataset from the locality of Loreto. The diversity measure that was affected the most by sample size was Species Observed (S_{obs}). The latter was especially true for the complete datasets from both localities. Truncated versions showed signs of stabilization (reduction on the slope's angle) when N was close to 50. Sample size did not drastically affect the Fisher's alpha diversity estimation when it was applied to the truncated datasets. Nevertheless, when data from complete datasets was used as input, its estimation significantly increased with sample size showing no signs of stabilization. For all datasets, the Shannon index (H') increased with sample size until N=45, after which it started to show a stabilization trend.

For the complete datasets, Chao1 and ACE were drastically influenced by sample size. For the Loreto complete dataset, and in contrast to the Tabasco complete dataset, we detected no signs of stabilization was found even when N > 45. On the other hand, with truncated datasets these estimators were less affected by sample size, appearing to plateau after N > 20. For all cases evenness decreased with sample size, but the effect was stronger when using complete datasets as input data.

Phylogenetic diversity and sample size

The phylogenetic reconstruction using the ITS marker showed that both localities present a highly diverse sapwood endophytic community. Sample size had a significant effect on the estimated Phylogenetic Diversity (PD). The latter index decreased as samples were randomly removed from the analysis (Table 3.5). PD was found to be significantly higher for the Tabasco dataset when N < 35; but PD for the Loreto dataset was estimated to be significantly greater when the sample size was increased. No signs of stabilization were found for either of the localities, even at N = 50. The Loreto dataset was affected more by sample size than the Tabasco dataset.

Sample representativeness and adequate sample size

Following Chao et al. 2009, we estimated that the sampling size used in this study (N=50) only captured 50% and 40% of the endophytic diversity in the locality of Tabasco and Loreto, respectively (Table 3.6). The number of estimated species (based on Chao 1 diversity index) for the locality of Loreto was higher than for the locality of Tabasco (422 vs. 262); therefore, the estimated number of undetected species was also higher (252 vs. 125). The number of additional samples needed to capture all the

estimated number of species (100% coverage or g =1) was calculated to be higher for Loreto than for Tabasco (894 vs. 614).

Discussion

Although numerous surveys have focused on leaf endophytes – thought to be highly diverse in tropical areas – the sapwood of tropical trees also harbors abundant and highly diverse endophytic communities. In this study alone, we detected 277 different species of fungal endophytes inhabiting the sapwood of *Hevea brasiliensis*. The sapwood of tropical trees therefore should be considered an additional frontier for discovery of fungal biodiversity. We also found that the population of *H. brasiliensis* distributed within its native range and growing under undisturbed primary forest presented a higher number of species, in comparison to the population of trees growing under managed conditions (169 vs. 132 species). Nevertheless, the fungal communities inhabiting trees under plantation also were diverse, sharing many taxonomic groups with their wild counterparts.

Even though the species accumulation curves were non-asymptotic, it remains possible to compare the diversity of the two communities, as long as the sampling effort is equal (Gotelli & Colwell, 2001; Hughes & Hellman, 2005). We found that our effective sampling effort was significantly different when endophytes were collected in the wild versus in managed habitats. Samples collected in the wild harbored a higher number of isolates than samples collected in plantations; therefore, their diversity measurement can be an artifact driven by their higher relative abundance. To explore this issue, we re-scaled diversity indices based on the number of isolates. The higher diversity recovered in endophyte communities inhabiting wild

trees was possibly due to the multiple layers of heterogeneity found in primary forests. These levels of heterogeneity can be differences in microhabitats, vegetation composition, or the genetic background of their host plants. Plantation settings, especially crops that are grown in monocultures, lack these levels of heterogeneity, which is manifested in their low number of microhabitats. Fungal endophytes of tropical trees are transmitted horizontally; therefore, surrounding vegetation can act as reservoirs for these organisms. In addition, the high diversity of plant species found in natural habitats creates many vertical layers within the forest, each one imposing distinct environmental factors to endophytes communities (i.e. difference in shading, temperature, and humidity). These different conditions create a series of environmental filters that result in the production of a heterogenic and taxonomically diverse source of fungal inoculum. As previously suggested for rainforest communities of herbivores (Novotný & Basset, 2005), we support the hypothesis that species richness and number of rare species in tropical endophytic communities are inflated by a continuous influx of fungal species from adjacent plants ("mass effect"; Shimida & Wilson, 1985). This migration might be particularly important for tropical endophytes distributed in undisturbed habitats (e.g. Amazonia) where many plant species grow in close proximity. For instance, up to 300 species of large (dbh > 10 cm) trees can be found in a one-hectare plot of Amazonian rainforest (Gentry, 1990).

Effect of sample size on diversity estimation

As previously mentioned, species accumulation curves did not show signs of stabilization for any of the complete datasets, revealing that sampling was insufficient for both habitats. On the other hand, species accumulation curves from truncated

datasets did show signs of stabilization, demonstrating the great influence that singletons can have on ecological analyses. The increase in the number of singleton species was directly related to the increase in sample size. As the samples were added, the number of species increased and the species accumulation curves became farther away from reaching an asymptote. Many investigators have opted not to consider singleton species in their ecological analyses (Arnold & Lutzoni, 2007; Davis & Shaw, 2008). Their decision of omitting singletons species is based on the possibility that these species are not permanent residents of the community (Magurran & Henderson, 2003). The present study stresses the influence of singleton species on the estimation of diversity and on the conclusions inferred from these values. For instance, if in this study only truncated datasets were used as input, we would have concluded that trees growing under monoculture plantations harbored an endophytic community more diverse than trees growing within their native habitat. The latter conclusion would conflict not only with the primary hypothesis of the present work but also against well established theories, such as the principle of multidimensional niche subdivision, resource allocation, and ecological functioning (Terborgh, 1985; Magurran & Henderson, 2003; Leigh et al., 2004; Silvertown, 2004; Gravel et al., 2006). Undisturbed forests are known and valued for their multiple layers of environmental heterogeneity that act as cradles of diversification. Even though the inclusion of singletons complicates the estimation of diversity, we suggest that their presence should be considered in endophyte studies, especially when the collection methods used (i.e., culture-based techniques) are known to mask the endophytes' real abundance (Arnold et al., 2007; Hyde & Soytong, 2008).

Diversity index performance

All the diversity indices used in this study were, in different degrees, affected by sample size and by the number of rare species. Hence, the goal should be to choose an index that depends less on these variables. Fisher's alpha and Shannon's are classic diversity indices that have been used for many years and are still popular in ecological studies. In our work, we found that Fisher's alpha was the most affected by the increase in sample size, especially for the Loreto complete dataset. Shannon's index was also found to be affected by sample size and as Fisher's α did not show signs of stabilization even when N = 50. Furthermore, Shannon's was the only diversity index that did not show a significant difference in diversity between Loreto and Tabasco complete datasets. We hypothesize that this was due to the non-significant difference in evenness found between the complete datasets. Therefore, we do not recommend using this index when dealing with similar datasets. Based on complete datasets, Chao 1 and ACE found Loreto dataset to be significantly more diverse than Tabasco. These indices were also affected by sample size, especially when complete datasets were used. When truncated datasets were used as input, we found the opposite results for all of the indices, but only significant results were obtained when using Species Observed (S_{obs}) and Shannon's diversity measurements. The latter again stresses the importance of choosing to include or exclude singletons from the ecological analyses. Because many of the indices are based on the number of rare species, truncated datasets were not affected by sample size when N > 25.

The quantification of a community's phylogenetic diversity (PD) has only been applied recently in ecological studies. This measurement resulted from the need

for prioritizing the selection of conservation areas that maximize the inclusion of genetic diversity (Faith, 1992). Later, PD has been used as a tool for comparing communities and to explain patterns of community structure (Swenson, 2009; Stegen & Hurlbert, 2011; Swenson, 2011). Classic diversity indices as well as the more modern ones (i.e. Chao, ACE) consider all species as units of equal value. However, if two communities have the identical number of species and equivalent patterns of species abundances but differ in the diversity of taxonomic levels to which they belong, it seems instinctive to state that the most taxonomically varied community is the most diverse (Magurran, 2008). When not included in the process of answering questions about community dynamics or evolution, diversity estimates represent just numbers. In contrast, differences in PD suggest that one of the communities might harbor members that have different colonization strategies, different symbiotic associations, or different production of secondary metabolites. Therefore, a comparison between communities should not conclude with the answer of which one has more species, but should investigate their composition and how the inhabiting species are distributed in a phylogenetic context. Tropical trees should be considered as chimeras assembled by multiple phylogenetic diverse fungal lineages. Unfortunately, we found that PD is greatly affected by sample size. When PD values are plotted against sample size or number of isolates, significantly different results are obtained (Table 3.6).

Projects involving fungal endophytes often compare communities (Higgins et al., 2007; U'Ren et al., 2009); therefore, similarity indices' performances should also be evaluated. We were unable to evaluate how these indices behave at different

sample sizes except that similarity indices such as Sorenson's were affected by the number of singletons (Table 3.4) and suggest that other similarity indices are influenced by sample size, in a similar way. When using truncated datasets, Sorenson's overestimates the similarity between the localities as a product of low sample size. The latter illustrates that low sample size not only affects directly alpha diversity but also its effects are carried on to larger scales such as beta diversity.

Sample representativeness and adequate sample size

Even though the number of independent samples (N = 50) used in this study was higher than the usual number included in previous studies, the estimated coverage was low for both localities. Due to the higher heterogeneity of habitats present in undisturbed areas, fungal endophyte diversity was lower in the locality of Loreto in comparison to Tabasco (40% vs. 50%). The estimated number of undetected species was high for both sites (252 for Loreto and 126 for Tabasco) and the estimated number of additional independent samples needed to cover the entire undetected diversity yielded unrealistic results (894 for Loreto and 614 for Tabasco). Although this estimation might not be representative of endophytic communities of other tropical trees species (see Suryanarayanan et al., 2011), results from previous research suggest that this would be the case for many (Evans et al., 2003; Arnold & Lutzoni, 2007; Vega et al., 2010).

Biodiversity is a comparative science and most of the questions about the structure and function of communities require relative comparisons; consequently, we only need to know the relative diversity among sites, over time, or under different treatments or regimes (Hughes et al., 2001; Hughes & Hellman, 2005). If one or more

accumulation curves fail to reach an asymptote, the curves themselves may be compared, after appropriate scaling (Gotelli & Colwell 2001; Hughes & Hellman, 2005). In theory, two habitats (sites, communities, samples, etc.) can be compared if: (1) their sampling effort is similar; if not, samples will need to be rarefied to the lowest common sample size; (2) samples are collected under standardized sampling protocols; if not, different kind of species may be over or under represented in different samples; and (3) sample size for each habitat is sufficient or representative of the community under the study (Hughes et al., 2001; Hughes & Hellman, 2005). Fungal endophyte researchers do not count with a standardized collection protocol that fulfills the mentioned requirements. There is no consensus on sampling effort or on the isolation techniques (i.e. subsample number and dimensions, type of artificial media). The latter problems can be solved with the design of a standard isolation protocol for fungal endophytes, including easily reproducible techniques. Projects usually fail in this last requirement (sample representativeness). Sample representativeness cannot be generalized along different habitats or ecosystems because it is affected by the heterogeneity in environmental factors unique to each of those areas. This issue is especially important for studies involving species-rich habitats, for which sample size may be low and species accumulation curves do not reach saturation. Even though achieving adequate sample size is unrealistic, it is important to estimate sample representativeness to determine what percent of the community is being covered by our sample size.

Results from this study emphasize the difficulty of reaching an agreement on a standardized sample collection protocol for fungal endophytes that can be shared

between research groups. This objective becomes more challenging due to the fact that each type of habitat possesses its own environmental factors dictating the diversity of their communities, and consequently, generalizations are inappropriate. However, it will be desirable to agree on a definition of "sample" and to be aware of the errors introduced by insufficient sampling. In addition, sample coverage should always be estimated as a way of evaluating how accurate or reliable are our conclusions.

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Chapter 4: Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of Pezizomycotina (Xylonomycetes)

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Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of Pezizomycotina (Xylonomycetes)

Abstract

Through a culture-based survey of living sapwood and leaves of rubber trees (*Hevea* spp.) in remote forests of Peru, we discovered a new major lineage of Ascomycota, equivalent to a class rank. Multilocus analyses reveal that this new lineage originated during the radiation of the Leotiomyceta, which resulted not only in the evolution of the Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Geoglossomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes, Sordariomycetes, but also of the majority of hyperdiverse foliar endophytes. Because its origin is nested within this major burst of fungal diversification, we could not recover strong support for its phylogenetic relationship within the Leotiomyceta. Congruent with their long phylogenetic history and distinctive preference for growing in sapwood, this new lineage displays unique morphological, physiological, and ecological traits relative to known endophytes and currently described members of the Leotiomyceta. In marked contrast to many foliar endophytes, the strains we isolated fail to degrade cellulose and lignin in vitro. Discovery of the new class, herein named Xylonomycetes and originally mis-identified by ITSrDNA sequencing alone, highlights the importance of inventorying tropical endophytes from unexplored regions, using multilocus data sets to evaluate the taxonomic placement of unknown strains, and exploring diverse plant tissues with traditional methods for evolutionarily, functionally, and taxonomically novel symbiotrophs.

Keywords: Ascomycota; endophytes; fungal phylogeny; *Hevea*; sapwood; tropical forest

Introduction

Fungal endophytes are a phylogenetically and ecologically diverse group united by the functional trait of residing within plant tissues without causing any apparent symptoms of disease (Petrini, 1991). Although the roles these symbionts play in their hosts are mostly unknown (Hyde and Soytong, 2008; Rodriguez et al., 2009), focal species of foliar endophytes – the most thoroughly studied group of endophytes – often provide ecologically important benefits to their hosts including herbivore deterrence, protection against pathogens, and the capacity to tolerate abiotic stress (e.g., Arnold et al., 2003; Alvarez et al., 2011; Rodriguez et al., 2009; Bittleston, 2011). The ubiquity and hyperdiversity of endophytes are now widely accepted (Hawksworth, 2001; Herre et al., 2005; Arnold, 2008; Arnold et al., 2009; U'Ren et al., 2010), as is their tendency to show tissue specificity in many cases: communities from woody tissues frequently differ from those in roots and leaves (Rodrigues et al., 2009; Gazis and Chaverri, 2010). Recent studies have revealed that endophyte communities demonstrate strong geographic structure (e.g., Arnold and Lutzoni, 2007; Davis and Shaw, 2008; Hoffman and Arnold, 2008), such that hosts form symbioses with distinctive communities in different sites across their ranges. The vast majority of known endophytes are Ascomycota in the Sordariomycetes, Dothideomycetes, Leotiomycetes, Pezizomycetes, and Eurotiomycetes (Arnold et al., 2009).

Fungi are recognized as one of the most diverse clades of life (Rossman et al., 1998; Muller et al., 2004). Current estimates for the global number of fungal species have risen from the long-lasting accepted 1.5 million (Hawksworth, 1991) to as many as 5.1 million species (O'Brien et al., 2005; Blackwell, 2011). Tropical endophytes, especially of foliage, are an important component of undescribed fungal diversity (Arnold et al., 2000; Arnold and Lutzoni, 2007; Smith et al., 2008). And, recent surveys have shown that the living sapwood of trees also harbors abundant and highly diverse assemblages of endophytic fungi (Verma et al., 2007; Thomas et al., 2008; Giordano et al., 2009; Hanada et al., 2010; Gazis and Chaverri, 2010; Parfitt et al., 2010; Tayung and Jha, 2010). Biochemical properties of leaves and sapwood (e.g., water content, lignin concentration) differ markedly; therefore, it is likely that sapwood hosts distinctive endophytic fungi relative to the fungal communities from leaves. However, sapwood remains extremely understudied for making general assumptions of their biodiversity patterns.

Through a survey of sapwood in wild rubber trees (*Hevea* spp.) in remote, forested regions of Peru, we isolated a collection of endophytes that we found to be highly divergent from all known fungal lineages. We used six loci from taxa representing all known classes of Pezizomycotina to infer the phylogenetic placement of these unusual strains. We also characterized the morphology of this new group of endophytes and evaluated their capacity to degrade lignin and cellulose *in vitro*. Our study reveals a new class of Ascomycota within the Leotiomyceta radiation with unique morphological and ecological characteristics.

Materials and Methods

As part of a project to characterize fungal diversity associated with rubber trees (*Hevea* spp., Euphorbiaceae), sapwood- and leaf endophytes were collected from *H. brasiliensis* and *H. guianensis* in Peru (Gazis and Chaverri, 2010; Gazis et al., 2011; Gazis et al. *in prep*.). Focal trees were in three localities within the Peruvian Amazon basin: (1) Los Amigos Biological Station, Madre de Dios: 12°30'43.20"S, 70° 3'34.09"W, (2) Amazon Conservatory of Tropical Studies (ACTS) Biological Station, Loreto: 3°14'57.60"S, 72°54'33.30"W, and (3) Tocache rubber plantations, San Martin: 8°11'51.50"S, 76°30'50.61"W. This study focuses on a group of fourteen strains isolated from sapwood and one from an asymptomatic leaflet.

2.1 Endophyte Isolation

We used a sterilized knife to cut three slivers of ca. 3 x 6 cm of dead bark from each tree at shoulder height and from three different parts of its circumference. After exposing the living sapwood (one section at the time), three pieces of *ca*. 5 mm of tissue were excised from each exposed area and quickly transferred to Petri plates containing CMD (BBL ™ corn-meal-agar + 2% dextrose), which contained 1% Neomycin–Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Leaf endophytes were collected through surface sterilization following Gazis and Chaverri (2010). Petri plates were kept at low temperature (ca. 4–8°C) until they were processed in the laboratory (Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, USA). Once in the laboratory, plates were incubated up to 2 months and emerging colonies were subcultured in Difco™ Potato Dextrose Agar (PDA) to obtain pure isolates.

2.2. DNA extraction, PCR, and sequencing

2.2.1. ITS data acquisition and analyses

Pure cultures of endophytes were grown in Difco™ Potato Dextrose Broth (PDB) at 25 °C for one week. Genomic DNA was extracted from the mycelial mat using Power Plant™ DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions with the following modifications: mycelial tissue was stored at -80 °C for several days prior to the extraction and the use of vortex was replaced by FastPrep®-24 (MP Biomedicals, Solon, OH, USA) to enhance tissue lysis.

The nuclear internal transcribed spacers and 5.8S gene (ITS) were amplified and sequenced following Gazis et al. (2011). Sequencher™ version 4.9 (Gene Codes©, MI, USA) was used to assemble bi-directional sequences into contigs and to confirm basecalls. ITS sequences were subjected to BLAST queries using the "blastn" algorithm implemented at NCBI to determine the putative identity of the strains (www.ncbi.nlm.nih.gov/BLAST). BLAST matched suggested affinity for lineage of lichen-forming fungi, which was surprising. Therefore, we used multi-locus analyses to confirm taxonomic placement.

To select strains for multilocus sequencing, we grouped them into operational taxonomic units (OTUs) based on 99% ITS sequence similarity, reflecting low predicted values of interspecific variability (Lieckfeldt and Seifert, 2000; Chaverri et al., 2003; Chaverri and Samuels, 2003; Cai et al., 2009; Pavlic et al., 2009; Rojas et al., 2010; Gazis et al., 2011). OTUs were assembled using the furthest neighbor

algorithm implemented in MOTHUR version 1.16.0 (Schloss et al., 2009: www.mothur.org), gaps were not considered as characters and sequences were reduced to a uniform length. Genetic variation was assessed among OTUs and isolates using MEGA version 5 (Tamura et al., 2011: www.megasoftware.net) to calculate nucleotide differences and the p-distances between and within the OTUs, using bootstrap sampling with 1,000 replicates.

2.2.2. Multilocus data acquisition

Three distinct ITS haplotype groups were detected based on 99% ITS similarity. One representative of each was selected for multilocus analyses, for which we sequenced five additional loci: three ribosomal RNA-coding genes, including the nuclear small subunit (nucSSU), nuclear large subunit (nucLSU), and the mitochondrial small subunit (mitSSU); and two protein-coding genes: the largest and second largest subunits of RNA polymerase II (*RPB1* and *RPB2*). All PCR reactions were assembled as follows: 2.5 µl PCR buffer (buffer IV with 15mM MgCl2; Abgene, Rochester, NY, USA), 2.5 µl dNTP (2mM), 2.5 µl bovine serum albumin (10 mgml-1; New England BioLabs Inc., Ipswich, MA, USA), 1.25 µl primers (10 mM), 0.15 µl Taq polymerase (5 U ml-1, Denville, South Plainfield, NJ, USA), 1 µl of a 1/10 dilution of genomic DNA, and double distilled sterile water to a total volume of 25 µl. To amplify *RPB1* and *RPB2*, we increased the volume of DNA to 3µl and the primer to 2µl. PCR was performed on a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA, USA). Products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA.) and both strands were sequenced at Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies on an ABI 3730xl DNA

analyzer (PE Applied Biosystems, Foster City, CA, USA). Sequencing reactions were prepared for 10 µl total volume using 1 µl primer, 1 µl purified PCR product, 0.75 µl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, CA, USA), 3.25 µl Big Dye buffer, and 4 µl double-distilled water. Data were edited as above and all sequences were subjected to BLAST queries of GenBank using "blastn" (ribosomal loci) or "blastx" (*RPB1* and *RPB2* sequences).

2.2.3. Multilocus data sets

The following data sets were assembled: a 2-locus (nucLSU+nucSSU) data set consisting of 362 taxa; 5-locus (nucLSU+nucSSU+5.8S+*RPB1*+*RPB2*) and 6-locus (5-locus+mitSSU) data sets consisting of 108 taxa; and a 6-locus data set consisting of 97-taxa. The 2-locus, 362-taxon dataset was prepared by adding three nucLSU and nucSSU endophyte sequences into the 359-taxon alignments (241 representative Ascomycota and 118 endophytic and endolichenic isolates) for each locus generated by Arnold et al. (2009). Bootstrap analysis on each data set with 1,000 bootstrap replicates and GTR+GAMMA were implemented in RAxML-VI-HPC (Stamatakis, 2006). All remaining datasets were derived from the 5-locus (nucLSU, nucSSU, 5.8S, *RPB1* and *RPB2*), 214-taxon matrix used in James et al. (2006). In that study, ten of the eleven recognized Pezizomycotina classes (*sensu* Weir and Blackwell, 2001) were represented (Laboulbeniomycetes was not included). For the 5-locus 108-taxon combined data set, taxa representing classes outside of Ascomycota, as well as members of Taphrinomycotina (109 taxa) were removed, and for the 5-locus 97-taxon combined dataset, all members of Saccharomycotina (11 taxa) also were removed. To

generate the 6-locus combined datasets (108-taxon and 97-taxon), the mitSSU sequences for all but 18 taxa present in our 108-taxon dataset were retrieved from the AFTOL (www.aftol.org) and GenBank (www.ncbi.nlm.nih.gov) databases.

All alignments were refined manually using MacClade 4.08 (Maddison and Maddison, 2005). For the nucSSU and nucLSU, the secondary structure model (Kjer, 1995) of *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen (Cannone et al., 2002) was followed. Ambiguously aligned regions (sensu Lutzoni et al., 2000) and introns were delimited and excluded from the analyses. A summary of alignment lengths and the number of included sites for each data set is shown in Table 4.1. The *RPB1* and *RPB2* genes provided the largest number of characters included in the phylogenetic analyses. Although the 5.8S region contained the second lowest proportion of ambiguously aligned characters (13%), most of the included characters were constant. Compared to the remaining ribosomal genes (except the 5.8S), *RPB1* and *RPB2* contained the lowest proportion of sites excluded from the analyses (11% and 17%, respectively versus 72-82%). By removing 11 taxa from the 108-taxon data set, the proportion of missing data decreased only by 2% and the number of included sites increased by 111.

2.2.4. Multilocus phylogenetic analyses

Because the mitSSU data set was not included in the phylogenetic study by James et al. (2006), and therefore was not tested for congruence against the other 5 loci (nucLSU, nucSSU, 5.8S, *RPB1* and *RPB2*), bootstrap analysis using RAxML (1,000 replicates, GTR+GAMMA model) was conducted separately on two partitions of the 108-taxon data set (mitSSU only, and the 5-locus, 108-taxon combined data

set) to detect topological incongruence. Conflict was assumed to be significant if a group of taxa was supported at ≥ 70% as monophyletic with one data set, but supported as non-monophyletic by another data set (reciprocal 70% ML bootstrap support criterion; Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). Conflict was detected for the placement of *Anisomeridium polypori*; therefore, this taxon was excluded from all analyses. The final 107- and 96-taxon combined data sets contained three representatives of rubber tree endophytes and 104 and 93 reference taxa from James et al. (2006), respectively.

JMODELTEST (Posada and Buckley, 2004; Posada, 2008) was run on each locus separately (ambiguously aligned regions and introns excluded) to select the models of nucleotide substitution for Bayesian analyses. The number of substitution schemes was set to 11, base frequencies + F, rate variation + I and + G, and the base tree for likelihood calculations was set to ML OPTIMIZED. Models were selected based on comparison of likelihood scores from 88 models according to the Akaike Information Criterion (AIC).

Phylogenetic relationships and internode robustness were estimated for the 6- and 5-locus, 107-taxon datasets, and the 6-locus 96-taxon data set, using maximum likelihood as implemented in RAxML-VI-HPC (Stamatakis, 2006) and using Bayesian Inference (BI) as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). In the ML analyses, the 6- and 5-locus combined data sets were divided into 10 partitions (nucSSU, nucLSU, 5.8S, mitSSU, *RPB1*/1st, 2nd, 3rd and *RPB2*/1st, 2nd, 3rd) and 9 partitions (all partitions as above except the mitSSU), respectively. The most likely tree and bootstrap support were estimated with 1,000

replicates using the general time-reversible (GTR; Rodriguez et al., 1990) evolutionary model with a Gamma distribution to account for rate heterogeneity among sites (GTRGAMMA).

One set of BI analyses was conducted on the same 10 and 9 partitions as the ML and another one on a reduced number of partitions with the first, second and third positions of the *RPB1* and *RPB2* combined (*RPB1*and *RPB2*/1st, *RPB1* and *RPB2*/2nd, and *RPB1*and *RPB2*/3rd) for a total of 7 partitions for the 6-locus data set, and 6 partitions for the 5-locus data set. BI analyses on each data set with different data partitions were completed with 8 chains and two independent runs for a total of 50,000,000 generations, with trees sampled every 500 generations. A GTR model with an estimated proportion of invariable sites (I) and a gamma distribution approximated with four categories to account for among site rate heterogeneity was used for all partitions, except for 5.8S, where a GTR + (Zharkikh, 1994) model was selected. Convergence of log likelihood scores (-Ln) was assessed with TRACER version 1.4 (Rambaud and Drummond, 2007) and stationarity was assumed when a stable equilibrium value was reached (Huelsenbeck and Ronquist, 2001). Average standard deviation of split frequencies between runs on the same data set but with different number of partitions were compared and the trees resulted from the runs with lower values (0.002 for the 5- and 6-gene 107-taxon data sets with 7 and 6 partitions versus 0.003 with 10 and 9 partitions, and 0.003 for the 6-gene 96-taxon data set with 10 partitions versus 0.008 with 7 partitions) were selected for the next step. A burn-in sample of 15,000 trees was discarded for each run. The remaining 20,000 trees (10,000 from each run) were used to estimate posterior probabilities (PP)

with the majority rule consensus tree command in PAUP* 4.0b10 (Swofford, 2002). Individual nodes were considered well supported when ML bootstrap values (BL) equal or greater than 70% and when PP values were equal or greater than 0.95. Saccharomycotina and Orbiliomycetes were used as outgroup for the 107-taxon and 96-taxon data sets analyses, respectively.

2.3 Morphological data

Isolates were grown on Difco™ Malt Extract Agar (MEA) and PDA for up to 1 month at 25 °C with alternating 12 h/12 h fluorescent light/darkness. Microscopic observations were made using an Olympus BX51 microscope. Measurements of continuous characters such as length and width were made using the Scion Image software beta 4.0.2 (Scion Corporation, Frederick, MD, USA). Continuous measurements (x1000 magnification) were based on at least 100 measured units and are reported as the extremes (minimum and maximum) in brackets separated by the mean plus and minus one standard deviation. Images were captured with an Olympus DP71 digital camera. Some composite images were made with Helicon Focus version 4.21.5 Pro (Helicon Soft, www.heliconfocus.com). Colors were described using the mycological color chart from Rayner (1970). A dried culture of the type specimen was deposited at the U.S. National Fungus Collections (BPI) and additional representative cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Attempts to obtain the teleomorph state in culture were made following Rooney-Latham et al. (2005) (with modifications). Different strains were grown in dual cultures (PDA and MEA) for 8 weeks at 25 °C with alternating 12h/12 h

fluorescent light/darkness. In addition, isolates were grown in additional media such as Synthetic Nutrient Agar (SNA, Niremberg, 1976) with a piece of sterile filter paper (Palm et al., 1995); oatmeal agar (OA, Difco™); and corn meal agar (CMA, Difco™).

2.4. Cellulase and ligninase assays

In-vitro assays were used to test if the strains were capable of degrading sapwood materials such as cellulose and lignin. To measure cellulase activity, 5 mm plugs of actively growing mycelium were transferred individually under sterile conditions from source cultures on PDA to carboxymethylcellulose medium (CMC-medium, prepared following Jeffries, 1987; see also Blume and Ennis, 1991) in 100 mm Petri plates (3 replicate plates per isolate). One plug from each isolate was transferred at the same time to PDA to confirm viability of the mycelium. Two cellulolytic strains (DC3368 and DC0448, representing foliar endophytes obtained from the Robert L. Gilbertson Mycological Herbarium, ARIZ) were cultivated concurrently on CMC-medium in triplicate as positive controls. Plates were incubated at 22 °C under 12 h light/dark conditions and assessed at three-day intervals over 21 d for growth and cellulase activity. On day 22, activity was assessed by flooding plates with a 0.2% w/v solution of Congo red, incubating at 22 °C for 30 min, destaining with successive washes of 1M NaCl, and measuring the diameter of cleared areas in the CMC-medium surrounding the plug.

A similar approach was used to assess ligninase activity, except that indulin medium, which contains a commercially available substitute for lignin, was used in place of CMC-medium (Nerud et al., 1991), and a known ligninolytic strain (foliar

endophyte DC3051 from ARIZ) was used as a positive control. Ligninase activity was measured by flooding plates with a 1% w/v solution of $FeCl_3$ and $K_3[Fe(CN)]_6$, incubating at 22 °C for 30 min, and rinsing with distilled water until cleared areas of the medium could be detected and scored for presence (activity) or absence (no activity).

Results

3.1. Preliminary identification of endophytes from sapwood using ITS

Top BLAST matches for the 15 ITS sequences from the unknown strains of sapwood endophytes from *Hevea* were all representatives of the Lecanoromycetes (based on "maximum identity"), a class of Ascomycota containing the majority of lichen-forming fungi. However, their maximum identity scores (83-92%) were relatively low, suggestive of the distinct nature of the sequences.

The ITS sequences showed little variation among these 15 strains. The distance analyses conducted with MOTHUR revealed 1 OTU based on 98% similarity, 3 OTU at 99%, and 9 unique OTU. Table S5 shows p-distances and number of base-pair (bp) differences between each of the 3 OTU groups at the 99% similarity level. No geographic clustering associated with the distance analysis was observed.

3.2. Phylogenetic placement of the endophytes

All three haplotypes of the sapwood endophytes (OTU 1-3, represented by TC269, 161, and 137, respectively) formed a monophyletic group with significant support in all analyses. According to the 5- and 6-locus 107-taxon phylogeny, this monophyletic

group is sister to the Lecanoromycetes+Lichinomycetes+Geoglossomycetes clade. This phylogenetic placement received significant support from all analyses except from BL on the 5-locus 107-taxon dataset (Fig. 4.1). Based on the 96-taxon data set, the phylogenetic placement of these sapwood isolates fall within the "Leotiomyceta" with high support, but their specific placement within that superclass is not significantly supported (Fig. 4.2).

3.3. Phylogenetic relationships within Ascomycota

Eight of ten classes currently recognized within the Pezizomycotina (Lecanoromycetes, Lichinomycetes, Geoglossomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes, Arthoniomycetes, Pezizomycetes) were delimited as monophyletic and highly supported by all phylogenetic analyses. The Dothideomycetes was recovered as monophyletic but with low support values, with the exception of the 6-locus, 96-taxon data set (PP=0.99; Fig. 4.2). However, the Arthoniomycetes-Dothideomycetes clade is associated with high support values from the four separate analyses of the 107-taxon data set. The Pezizomycetes were recovered mostly as non-monophyletic (Figs. 4.1 and 4.2), and received high support (ML and BI on the 6-locus 107-taxon data set) for Orbiliomycetes being more closely related to Pezizomycetes than *Peziza* (Fig. 4.1). Pezizomycetes were reconstructed as monophyletic with significant support (PP=0.98) based only on BI of the 6-locus 96 dataset (tree not shown). Only a few significantly supported phylogenetic relationships among classes were shared by both the 107- and 96-taxon phylogenies (Figs. 4.1 and 4.2): sister relationships between Sordariomycetes and Leotiomycetes;

Dothideomycetes and Arthoniomycetes; and the monophyletic delimitation of "Leotiomyceta."

Overall, the 107-taxon phylogeny has a more robust phylogenetic backbone and in addition to the highly supported clades present in the 96-taxon phylogeny, it includes several other highly supported monophyletic groups: Lecanoromycetes+Lichinomycetes+Geoglossomycetes (significant BL and PP values) + Eurotiomycetes (significant PP values) + Sordariomycetes+Leotiomycetes (significant PP value) + Arthoniomycetes-Dothideomycetes (significant BL and PP values) + Pezizomycetes (excluding Peziza; significant PP values) + Orbiliomycetes (significant BL and PP values from the 6-locus data set). The only conflicting relationship is the placement of Geoglossomycetes as the first split in the "Leotiomyceta" in the 96-taxon data set phylogeny (BL=74%; Fig. 4.2), but it is closely related to both Lichinomycetes and Lecanoromycetes in the 107-taxon phylogeny (BL and PP significant; Fig. 4.1).

3.4. Morphology

Macro- and microscopic descriptions are based on colonies grown on PDA and MEA after incubation at 25 °C for ca. 3-4 weeks. No teleomorph was observed in culture; therefore, descriptions are based only on the anamorph. Morphological features are shown in Fig. 4.3 (A – J). Conidiomata were pycnidial, astromatic, and composed of thin-walled cells forming tissue *textura angularis*. Conidial masses were liberated through the apical rupture of the pycnidia (ostiole absent). Conidiophores were absent. Conidiogenous cells were enteroblastic, phialidic, discrete, hyaline, and presented one collarette. Conidia were apically rounded with two lateral obtuse

projections appearing heart-shaped (narrower and truncated at base), hyaline when young, turning dark brick when mature, aseptate, thick-walled, smooth, and guttulated. After ca. 3 weeks, both types of agar acquired a vinaceous color diffusing pigment, especially at the margins of the growing colony. None of the structures reacted with 3% potassium hydroxide (KOH).

3.5. Cellulase and ligninase activity

All 15 sapwood isolates of this new lineage grew on PDA, and positive controls grown on the CMC- and indulin media demonstrated marked degradation of cellulose and lignin, respectively. However, none of the sapwood strains grew on the CMC- medium and no cellulolytic or ligninolytic activity was observed in proximity to the transferred plugs.

Discussion

In this study we used published molecular data sets with broad and, where possible, deep taxonomic representation (James et al., 2006 and Arnold et al., 2009) to assemble comprehensive datasets for the Pezizomycotina (*sensu* Schoch et al., 2009) to determine the phylogenetic affinities of endophytic strains discovered mainly from sapwood of Peruvian *Hevea* spp. Ten of the eleven accepted Pezizomycotina classes were represented in the main data set (Laboulbeniomycetes was not included). Maximum likelihood and Bayesian inference analyses of the 6 targeted loci (nucLSU, nucSSU, 5.8S, mitSSU, *RPB1* and *RPB2*) strongly supported the hypothesis that this group of sapwood endophytes represents a new monophyletic lineage of

ascomycetous fungi within the superclass Leotiomyceta (*sensu* Spatafora et al., 2006). The results from the phylogenetic analyses also suggested that this lineage should be designated as a new class, which we herein designate as the Xylonomycetes (type species, *Xylona heveae*).

As in many biodiversity surveys, the first attempt to identify this group of strains was by comparing their ITS sequences against the GenBank nucleotide data base. None of our strains matched closely with sequences already in GenBank (none showed more than 93% in similarity and ≥99% coverage), and highest top matches belonged to members of the Lecanoromycetes. Our phylogenetic analyses using multiple loci clearly demonstrate that the new lineage is not a member of the Lecanoromycetes. Interestingly, BLAST results did not include high quality matches to sequences obtained in environmental surveys of any kind (i.e., soil, wood decaying fungi, foliar endophytes), leading us to believe that this group has not been collected previously. We further compared our sequences against a database of 14,800 unpublished ITS sequences including leaf endophytes from boreal, temperate, tropical, and arid regions; endolichens from different biogeographic zones; coral and reef associated fungi; clone libraries from endophytes inhabiting tropical grasses; and tropical seed-associated fungi (Arnold, *unpubl. data*). No sequence in this database showed ≥95% similarity to the endophyte sequences obtained here.

The phylogenetic position of the main clades of Ascomycota (Fig.4.1) was similar to the results reported by James et al. (2006) and Schoch et al. (2009), among others (e.g., Lutzoni et al. 2004; Spatafora et al., 2006). As in these studies, we found significant support for the superclass Leotiomyceta (not accepted by Hibbet et al.,

2007) as a group encompassing Lecanoromycetes, Lichinomycetes, Geoglossomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes, Dothideomycetes and Arthoniomycetes. We also found strong support for a sister relationships between Arthoniomycetes and Dothideomycetes and between Sordariomycetes and Leotiomycetes (Fig.4.1). With the 5-locus 107-taxon data set the sister relationship of the Lichinomycetes with the Geoglossomycetes was well supported by bootstrap values and posterior probabilities. The same is true for the sister relationship of the Lecanoromycetes with the Lichinomycetes+Geoglossomycetes clade. As in previous studies, the backbone of the Leotiomyceta was never completely resolved with high confidence, even with the addition of protein-coding genes to ribosomal RNA-coding genes (Weir and Blackwell, 2001; Schoch et al., 2009).

We found strong support for phylogenetic placement of the putatively new lineage within Leotiomyceta, but outside all known classes within this superclass. When we used the data set composed by 6 loci and 107 OTUs, we obtained strong support (BL: 72, PP: 1; Fig.4.1) for this new lineage being sister to the Lecanoromycetes-Lichinomycetes-Geoglossomycetes clade. However, bootstrap support of this relationship decreased when only 5 loci were used to infer the phylogeny (BL: 60, PP: 1; Fig.4.1).

As for many groups of fungi (Rojas et al., 2008; Chaverri et al., 2010), this new lineage can only be described based on its anamorphic state in culture. To investigate whether it has been reported in the past, but has not been sequenced yet (hence, its sequence would not be available in public databases; Brock et al., 2009),

we examined the most comprehensive works involving "coelomycetous" fungi (the artificial group that includes all anamorphic fungi with sporulation occurring within conidiomata such as pycnidia and acervuli). Reviews of Morgan-Jones et al. (1972); Nag Raj (1978, 1980, and 1993); Nag Raj et al. 1989; and Sutton (1980) suggested that this anamorph has not been described previously. As for many fungi described on the basis of growth *in vitro*, morphological characters observed under laboratory conditions should be taken with caution since these might not represent the true characters in nature (Pellietier and Aubé, 1970).

Most of the morphological characters we found (see results) are shared by several classes within the Leotiomyceta (e.g., conidiomata with pycnidial anamorph, found in several classes; a non-ostiolate pycnidium with enteroblastic phialidic conidiogenesis, resembling anamorphs of Sordariomycetes and Dothideomycetes). However, the shape of the conidia produced by this new lineage appears to be one distinctive character. From the literature (Sutton, 1980; Crous et al., 2007; Crous et al., 2009), we found that only *Readeriella* (Capnodiales, Dothideomycetes) produces conidia with similar morphology. This new lineage has other similarities with *Readeriella* (e.g., in the conidiogenous cells); however, that genus produces dark mycelia and ostiolate pycnidia. Furthermore, the ITS regions for this genus is represented in the NCBI database, but it did not appear in the top 100 matches when the sequences from the new class were submitted to the query. The type species of *Readeriella (R. mirabilis)* and the type strain from *X. heveae* (TC161) have an ITS sequence similarity of 71% (S.E 0.03%).

From the visual examination of the 107 taxa-6 locus alignment, we found few molecular characters that distinguished the new species *X. heveae* from the rest of the strains. We define distinctive characters as the characters that are present in all the strains belonging to the new species but are not shared with the members of the other classes. When comparing the mitSSU, we only found one character exclusively present in the new species strains (character 140). On the other hand we found more differences when comparing the RPB1 and RPB2 regions. Based on RPB1 we found four codons that were only present in the new species strains, but only one (character 274-276) codifies for a distinctive amino acid (aspartic acid). In the case of RPB2, we found that the new species had 3 distinctive codons and, as in the case of RPB1, only one (character 919 – 921) codifies for a distinctive amino acid (threonine). We found no distinctive characters when comparing the nucSSU, nucLSU, and 5.8S. The detected distinctive characters can be used to distinguish the new species from the rest of species included in the present study; however, we do not know if these characters can be used to distinguish the newly introduced class, order, family, or genus (because we only have one species representing them, and more data about their within diversity is needed to establish if those molecular characters remain constant or conserved). The only way of improving our knowledge of the variation within the taxonomic levels encompassed in this new class is by increasing the sampling and adding more isolates that represent diverse lineages.

Fourteen of the focal strains were found as sapwood endophytes, and one was isolated from an asymptomatic leaflet. Foliar endophytes (Class 3, sensu Rodriguez et al., 2009) typically are horizontally transmitted and can enter the leaves through the

stomata or directly by piercing the leaf's cuticle (Arnold and Herre, 2003; Mejia et al., 2008). However, the mode of transmission of Xylonomycetes is not yet known. Endophytes occur frequently in sapwood (Verma et al., 2007; Thomas et al., 2008; Giordano et al., 2009; Hanada et al., 2010; Gazis and Chaverri, 2010; Parfitt et al., 2010; Tayung and Jha, 2010), and may colonize via lenticels or wounds in adults (Pearce, 1996). Alternatively, they may translocate to living sapwood through the vascular system of the host (Bailey et al., 2008; Bailey et al., 2009). Vertical transmission also is possible and cannot be ruled out based on the present study. Further work that evaluates infections in propagules of *Hevea* and careful studies of tissue preference will provide insight into the life cycle of the newly found lineage, including where and when in nature these fungi reproduce.

Isolates from the proposed class were isolated primarily from rubber plantations in San Martin (Tocache, central Peru), but also were found in wild populations of rubber trees in Loreto (ACTS, North East Peru) and Madre de Dios (Los Amigos, South East Peru). These sites differed in management, land use history, elevation, and other characteristics, suggesting that Xylonomycetes may occur over a wide range of conditions. To our knowledge, Xylonomycetes have not been isolated from any other species in the region (e.g., *Theobroma* spp.; Evans et al., 2003; Rubini et al., 2005; Thomas et al., 2008; Hanada et al., 2010). Thus strong conclusions about host breadth are premature in the absence of further sampling of sapwood and other tissues in co-occurring plants.

In terms of abundance, these fungi appear to be present at very low levels, in comparison to other rubber sapwood-endophytic species such as species of

Trichoderma (Gazis and Chaverri 2010; Gazis et al., 2011; Gazis and Chaverri, *in prep.*). For instance, at Los Amigos we obtained 247 isolates; only two isolates belonged to the new lineage, in comparison to 47 *Trichoderma* isolates. Similarly, we obtained 189 isolates from ACTS; 2 isolates belonged to the new lineage in comparison to 23 *Trichoderma* isolates. From Tocache, we obtained 253 isolates (from a total of 270 subsamples) of which 11 belonged to the new lineage in comparison to only 6 *Trichoderma* isolates. One important factor to consider is that the isolates from this study were obtained using culture-based methods. Strains that have rapid growth can mask the presence of slow growers (Arnold et al., 2007; Hyde and Sotong, 2008). Therefore, methods that do not depend on culturing, such as direct PCR from leaves and sapwood, will enhance our understanding of the ecology of this lineage.

Recent surveys of phylogenetically diverse fungal endophytes have shown their ability to degrade cellulose and lignin (Maria et al., 2005; Oses et al., 2006; Dai et al., 2010; Promputtha, et al., 2010). A study involving five angiosperm and coniferous host species revealed growth on CMC-medium by 89% of foliar endophytes (N = 35 species representing Sordariomycetes, Dothideomycetes, and Pezizomycetes), and measurable cellulase activity by 90% of the foliar endophyte species that grew on that medium (Orozco and Arnold, *in prep*.). Among xylem-inhabiting endophytes from the same host species, 100% of species grew on indulin medium, and measurable ligninase activity was detected in 67% of those species (N = 24 species; Orozco and Arnold, *in prep*.). In contrast we found that none of the isolates belonging to the new lineage grew in the CMC or in the indulin media,

suggesting that these strains do not have the enzymes necessary to breakdown lignin and cellulose (ligninase and cellulose, respectively). The latter can be a sign that this novel group of ascomycetous fungi live in symbiosis with other organisms, which facilitate their nutrient acquisition and potentially aid in the colonization of their hosts.

Taxonomy of the new lineage

Xylonomycetes R. Gazis & P. Chaverri, class. nov.

Etymology: from Greek "xylon" = wood; referring to the preferred substrate of the new lineage; Greek "mykes" = fungus.

Type genus: *Xylona* R. Gazis & P. Chaverri.

Diagnosis: Phylogenetically placed among Pezizomycotina, within "Leotiomyceta" (Schoch et al., 2009), differing from the other classes by its endophytic association with leaf and sapwood of living plants.

Reference Phylogeny: Figure 4.1

Phylogenetic notes: Strongly supported as a separate class within the Pezizomycotina (BP: 100%; PP: 1) and contained by the superclass "Leotiomyceta" (BP: 100%; PP: 1) sensu Schoch et al. (2009); based on 6 loci phylogeny (nucSSU, nucLSU, mitSSU, 5.8S, RBP1, and RPB2).

Xylonomycetales R. Gazis & P. Chaverri, ord. nov.

Type Genus: *Xylona* R. Gazis & P. Chaverri.

Xylonomycetaceae R. Gazis & P. Chaverri, fam. nov.

Type Genus: *Xylona* R. Gazis & P. Chaverri.

Xylona R. Gazis & P. Chaverri, gen. nov.

Etymology: From Greek Xylona -"from the forest."

Type species: *Xylona heveae* R. Gazis & P. Chaverri.

Description: Conidiomata pycnidial, astromatic; pycnidial wall composed of thin-walled cells forming tissue textura angularis; ostiole absent. Conidiophores absent. Conidiogenous cells enteroblastic, phialidic, discrete, hyaline, 1 collarette, smooth-walled. Conidia apically rounded with two lateral obtuse projections appearing heart-shaped, narrower and truncated at base, hyaline when young, turning dark brick when mature, aseptate.

Xylona heveae R. Gazis & P. Chaverri, sp. nov., Fig. 3A–3J

Etymology: The epithet refers to the name of the host (*Hevea*) from which the type species was isolated.

Description: Colonies on PDA and MEA effuse, at first white, later grayish sepia to fuscous black. Mycelium mostly superficial composed of branching sometimes anastomosing hyphae; hyphae septate, hyaline when young, turning pale brown to brown with age, smooth. Conidiomata pycnidial, astromatic; immersed when young, fuscous black, subspherical, (110) 170 (250) ± 40 µm x (120) 160 (180) ± 30 µm, entirely closed, pycnidial wall composed of thin-walled cells forming tissue textura angularis; when mature, superficial, unilocular, sometimes fusing with other pycnidia. Chlamydospores rarely formed, spherical, mostly terminal, single, and hyaline. Conidial masses liberated by apical rupture of pycnidia, ostiole absent. Conidiophores

absent. Conidiogenous cells lining inner wall of pycnidial cavity, enteroblastic, phialidic, discrete, hyaline, 1 collarette, smooth-walled, (5.1) 6.8 (8.9) ± 0.97 µm long, (1.9) 2.9 (3.6) ± 0.6 µm broad. Conidia abundantly produced, arising singly, apically rounded with two lateral obtuse projections appearing heart-shaped, narrower and truncated at base, hyaline when young, turning dark brick when mature, aseptate, thick-walled, smooth, guttulated, (2.4) 2.8 (3.5) ± 0.2 µm x (2.0) 2.9 (3.7) ± 0.3 µm. Colonies producing greater amounts of aerial mycelium when grown on PDA than on MEA; pycnidia produced earlier and in greater quantities when grown in MEA (after 2 weeks versus 3 weeks in PDA). After ca. 3 weeks, both types of agar acquired a vinaceous color diffusing pigment, especially at the margins of the growing colony. None of the structures reacted with 3% potassium hydroxide (KOH).

Diagnostic molecular characters: In comparison to the members of the classes included in this study, the new species *Xylona heveae* can be distinguished by: mitSSU: character 140, the new species *X. heveae* has a C while all the other members of the included classes have an A, G, or T. RPB1: characters 169-171 (GTT); characters 457-459 (AGT); character 997-999 (AGG); and characters 274-276 (GAT). The latter codes for the amino acid Aspartic acid unlike the other Ascomycota classes included in this study. RPB2: characters 616-618 (ATC); characters 1036-1038 (AAG), and characters 919-921 (ACT). The latter codes for the amino acid Threonine unlike the other Ascomycota classes included in this study.

Ecology: The majority (14 of 15) of the isolates belonging to the newly described lineage were isolated from the sapwood of living *Hevea* trees; one was recovered from a living, apparently healthy leaflet.

Known Distribution: Peru (Amazon basin).

Notes: Xylona heveae is distinct in having heart-shaped melanized conidia and in presenting an endophytic habit.

Conclusion

The recent discovery of the phylum-rank group "Cryptomycota" (Jones et al., 2011) emphasizes the need for further studies that help reduce the gaps in our knowledge of fungal diversity and stress the importance of elucidating the "missing fungi" for informing the fungal tree of life. The discovery of new fungal lineages can contribute not only to a better understanding of the phylogenetic history, evolution of morphological characters, and diversification of life styles of fungi, but also can shed light about the dynamics and ecology of the communities with which they interact. This study used explored an understudied substrate (sapwood of tropical trees) and discovered a unique and previously unknown class of Pezizomycotina that could not have been identified using methods that are currently in place for culture-free surveys of fungal biodiversity. Together our results reveal the importance of pairing traditional and modern methods in studies of fungal biology.

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Chapter 5: Amazonian natural forests are a reservoir of natural enemies against plant diseases: a comparison of wild and planted rubber trees (*Hevea* spp.)

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Amazonian forests are a reservoir of natural enemies against plant diseases: A comparison of fungi associated with wild and planted rubber trees (*Hevea* spp.).

Abstract

Fungal endosymbionts (i.e., endophytes) reside within living plants forming relationships that range from pathogenic to mutualistic. Studies have suggested their mutualistic role as plant protectors, revealing their potential application in biological control of crop diseases. We examined and compared the fungal endophytic communities inhabiting rubber trees distributed in the wild versus monoculture plantations to test the hypothesis that wild trees harbor a unique endophytic assemblage composed by a high number of natural enemies with biocontrol potential. Wild trees harbored a greater number of species regarded as natural enemies of plant pests and diseases, whereas species with plant pathogenic potential were more common in plantations. Wild trees were dominated by *Trichoderma* strains, a genus known and used commercially for its biocontrol properties. Results from this project raise the awareness of the potential loss, due to ongoing deforestation, of potentially mutualistic symbionts only found in wild habitats.

Keywords

Biological control, co-evolution, ecosystem services, forest conservation, fungal conservation, *Microcyclus ulei*, mycoparasitism, natural enemies, South American Leaf Blight, *Trichoderma*

Introduction

Each year, over 6,000 km^2 of Amazonian forest are destroyed by human activities including land-use change, logging, and gold mining (Almeyda et al. 2010) With these vanishing areas, we lose not only valuable endemic species, but also the chance of discovering, bioprospecting, and later exploiting an uncountable number of species unknown to science. Many of these undescribed species have been overlooked by scientists because of their microscopic size, microhabitat specialization, or perhaps due to an obligate symbiosis with their hosts that precludes their collection through classical techniques (Rosling et al. 2011). The kingdom Fungi has the highest projected number of unknown species, with an estimated 80% of their diversity remaining to be characterized (Blackwell 2011). Furthermore, tropical regions are believed to encompass the majority of the undescribed species of fungi, many of which are endemic (Mueller and Schmit 2007). Consequently, their loss to deforestation must be of relevant proportions.

Fungi are the most important agents causing ailment in plants, where more than 80% of the reported disease epidemics are caused by these organisms (Agrios 2005). Fungal diseases affect many economically important crops, including *Hevea* spp. (Euphorbiaceae), the source of natural rubber (NR). NR has been a crucial commodity for the past 100 years. Even though NR is synthesized by several plant species (Mooibroek and Cornish 2000) *H. brasiliensis* alone accounts for 90% of the world's NR production, representing more than 40% of the global rubber consumption (Information Center for Natural Rubber). In 2010, worldwide production reached more than 10 million metric tons and due to its unique physicochemical

properties is forecasted to rise 4.3% by 2013 (International Rubber Study Group). The rubber tree is native to the Amazon basin (Shultes 1970). Currently, rubber is primarily produced in large-scale plantations in Southeast Asia and Africa because in the Americas the crop is affected by the deadly "South American Leaf Blight" (SALB). SALB is caused by the fungus *Microcyclus ulei*, which is endemic to the Americas (Lieberei 2007).

The agricultural range expansion of *H. brasiliensis* has been extensive, reaching biomes distant from its native habitat (Onokpise 2004). With globalization and the increasing rates of intercontinental trade (Evans 2010), many are concerned about the spread of SALB and its potential introduction to Asia and Africa where the disease is absent and could result in significant negative economic and societal impacts (Evans 2010, FAO 2011). These losses would not only affect the economies of the rubber-producing countries, but also those of consumer regions such as the United States and Europe. The potential negative consequences of the proliferation and impact of SALB on NR production has prompted the United Nations Institute for Disarmament Research to label SALB as a potential "biological weapon of mass destruction" (Tulliu and Schmalberger 2003). Because chemical fungicides have largely failed to control SALB (Le Guen et al. 2007), an integrated pest management strategy that includes biological control is of great urgency to prepare for a potential introduction of SALB into Asia and Africa (Evans 2010). In addition to SALB, trees in plantations are constantly jeopardized by the possible invasion of more virulent strains of common rubber diseases (e.g., *Corynespora cassiicola*, *Colletotrichum acutatum,* and *Rigidosporus lignosus*). Asian plantations would be especially

susceptible because of the narrow genetic diversity of their high-yielding and susceptible cultivars (Pryyadarshan 2003, Onokpise 2004).

Classical biocontrol aims to restore ecological balance and reduce pathogen levels by introducing coevolved natural enemies or biocontrol agents. These agents are selected for their high specificity and their presence in the native geographic range of their hosts (Thomas et al. 2008). As examples, endophytic strains of *Trichoderma harzianum*, *T. koningiopsis*, *T. ovalisporum*, and *T. theobromicola* were collected from wild cacao (*Theobroma* spp.) and were proven to have biocontrol properties against important *Theobroma* diseases (Holmes et al. 2004, Samuels et al. 2006). The only known areas with resistance or low incidence to SALB were reported in natural forests within the Amazon basin (Le Guen et al. 2002). This supports the premise that natural enemies (i.e., endophytes with biocontrol properties) might be found in the native habitat of *Hevea*. Therefore, an assessment of the endophytic community inhabiting wild *Hevea* populations could reveal potentially useful fungal strains.

Endophytic microorganisms reside within living plant tissues forming symbiotic relationships that range from pathogenic to mutualistic (Bacon and White 2000). The ubiquity of endophytic fungi among and within plants and the premise that endophytic fungi have been associated with plants since they first colonized land implies that plants and endophytes likely share an intimate relationship (Krings et al. 2012). Evidence suggests a mutualistic relationship in which fungal endophytes increase resistance to plant pathogens by producing antifungal and antiherbivory compounds (Crawford et al. 2010), by direct parasitism[18], and by triggering plant-

wide defense mechanisms (Harman et al. 2004). Furthermore, studies hypothesize that fungal endophytes have coevolved with host plants to protect them from pests and diseases but clear coevolution patterns have only been studied for the vertically transmitted fungus *Neotyphodium* (=*Epichloë*) and their monocotyledonous plant hosts (Clay and Schardl 2002). Coevolutionary patterns have not been examined for horizontally transmitted endophytes, such as the ones inhabiting tropical trees. Because horizontally transmitted endophytes in general are absent from reproductive propagules (e.g., seeds) obtained from the wild, once those seeds are transported and introduced to different habitats, they would likely be exposed to a different community of endophyte species, possibly excluding many beneficial or biocontrol agents (Evans et al. 2003).

The premises guiding our study were: (1) the endophyte diversity in wild rubber populations is different than in plantations trees because infection of horizontally transmitted endophytes depend on the availability and viability of fungal propagules in the surrounding environment; (2) wild trees harbor more endophytic biocontrol agents than plantations trees because the endophytes have possibly coevolved and lived within the host longer than those in plantations; and (3) trees growing in plantations will have more endophytes with potential to be pathogenic than wild trees because natural enemies (biocontrol agents) would be less frequent or absent. To test these premises, we compared the fungal endophytic community inhabiting the leaves and sapwood of wild and planted rubber trees. We collected numerous independent samples (n = 190 trees) from an extensive range of wild populations (including those North and Southeast of the Peruvian Amazon) as well as

from several broadly distributed plantations (Cameroon, Mexico, and Peru). Strains were indentified using molecular techniques and localities were grouped under wild or plantations for comparative purposes. The abundance of characterized strains was compared between treatments and ecological groups that displayed significant results or interactions were further segregated by tissue (sapwood versus leaf). This study provides support for the addition of mutualistic symbionts to the list of ecological services provided by natural forests, and calls for awareness of their potential loss by human-related activities.

Materials and Methods

Endophyte Collection

Fungal endophytes were collected from a total of 190 *Hevea brasiliensis* trees. Trees were distributed under two types of management: wild or plantations. Wild trees refer to *Hevea* trees found growing naturally within its native habitat whereas planted (=managed) trees refer to *Hevea* trees growing under monoculture conditions. Table S1 shows the collections sites and their respective number of samples. Collection protocols are fully described in Gazis and Chaverri (2010). Tree diameter at breast height (dbh) and height were measured for each tree to determine if there was an effect on the endophyte community.

DNA Extraction, Amplification, and Sequencing

Individual isolates were grown in Difco™ potato-dextrose-broth (PDB) under 25°C for up to two weeks, depending on their growth rate. Genomic DNA was extracted from the mycelial mat using a Power Plant™ DNA isolation kit (MO BIO

Laboratories Inc., Solana Beach, CA, USA). The Internal Transcribed Spacer (ITS) and a region of the Large Subunit (LSU) were amplified in one reaction, using the ITS5 and LR5 primers. PCR reactions of 25 µl were set up using the following ingredients: 12.5 µl of GoTaq® Green Master Mix (Promega Corporation, WI, USA), 1.25 µl of 10 µM reverse primer, 1.25 µl of 10 µM of forward primer, 1 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), a maximum of 25 ng/µl of genomic DNA, and double distilled water to complete the total volume. The PCR reaction conditions for the ITS+LSU amplification were as follow: 94°C for 2 min followed by 15 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and primer extension at 74°C for 1 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and primer extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA.) and sequenced at MCLAB laboratories (www.mclab.com).

DNA Sequence Processing, Grouping, and Taxonomic Attribution

We used Sequencher™ ver. 4.9 (Gene Codes©, MI, USA) to assess the quality of sequence chromatograms and to edit them if necessary. Sequences were aligned using MAFFT version 6 with the E-INS-i strategy. Alignments were visualized using MESQUITE version 7.2 and the 5’ and 3’ ends were trimmed to a uniform length. Complete ITS sequences were clustered into putative species or “Operational Taxonomic Units” (OTUs) using the furthest neighbor algorithm implemented in MOTHUR. Sequences with a 99% in ITS sequence similarity were assigned as belonging to the same OTU. We used a 99% similarity threshold based on previous

studies that demonstrated a low ITS sequence variability in many fungal endophyte groups. Isolates were indentified to the genus rank using the BLASTn algorithm implemented in Genbank (www.ncbi.nlm.nih.gov/BLAST). Matches that had 95% or more in similarity (ITS) belonging to ex-type cultures or published studies were considered reliable.

Statistical Analyses

Once all sequences were grouped into OTUs, their frequency at each locality was measured. We compared the number of species and isolate abundance in relation to their management type (wild or planted) and tissue origin (sapwood or leaf). The first step was to assess if our data were normally distributed. For this, we applied the normality test, Kolmogorov-Smirnov, implemented in SPSS® ver. 13.0 for windows. The data were found to significantly deviate from normality; therefore, only non-parametric tests were used for all comparisons. For the first set of comparisons, replicates (individual trees) were segregated into two groups according to their management type (group 1 = wild and group 2 = plantation). To assess if there was a significant difference between management types, in the number of species, the overall abundance, and in the abundance of specific genera, a non-parametric equivalent of the t-test (Mann-Whitney U test) was performed. To avoid the increase of familywise error, due to a high number of multiple comparisons, only genera with an isolate abundance of more than 40 were used. For the groups of endophytes that showed significant results, we further assessed if the difference was present in the leaf or sapwood community or in both. For the latter, we performed multiple comparisons through a non-parametric equivalent of the ANOVA (Kruskal-Wallis test). Sample

units were segregated into subsamples coming from leaves (wild or planted) or sapwood (wild or planted). To investigate if there was a correlation between tree height and diameter and the number of species and abundance, we used field data collected in wild localities. Correlation tests between *Trichoderma* and selected plant pathogenic genera were conducted using a two-tailed Sperman's test. All statistical tests were performed in SPSS ver. 13 and the *p*-value was corrected for multiple comparisons (Bonferronis' correction).

Species accumulation curves and Sample size coverage

Species accumulation curves for each locality were created in EstimateS ver. 7.5 (http://viceroy.eeb.uconn.edu/estimates) using MOTHUR 99% ITS sequence similarity OTU groups. Species accumulation curves were randomized (using 1000 randomizations without replacement) to create "smooth curves". To avoid any influence of density on the comparisons between localities, we checked for significant differences on the number of isolates obtained by each sample size. Graphics were created using SPSS. Sample size coverage was estimated following Chao et al. 2009.

Ecological Analyses

In this study, we define ecological role as the nutritional mode and potential relationship that an organism has with its host or with the other members of the endophytic community. To investigate if there was a trend in the abundance of strains of specific ecological roles in relation to the type of management, we assigned a potential ecological role to each of the OTUs that were identified to genus. This assignment was based on published literature and on the USDA-ARS database (Farr

& Rossman et al. 2012, http://nt.ars-grin.gov/fungaldatabases). Genera were assigned to one of the following ecological roles: (1) plant pathogen, (2) saprobe, (3) fungicole, or (4) entomopathogen. In cases where isolates belonged to genera for which more than one ecological role have been reported (e.g., *Trichoderma* have been reported to contain saprobic and fungicolous species), ecological role was assigned based on the closest species match using the BLAST algorithm from GenBank. For instance, all *Trichoderma* isolates found in this study are more closely related to clades reported to be fungicolous. Therefore, the isolates belonging to *Trichoderma* have been classified under the fungicole ecological role. There were cases in which isolates belonged to clades with different ecological roles (e.g., *Elaphocordyceps* and *Fusarium*). In the case of *Elaphocordyceps*, strains were classified into two different *Elaphocordyceps* groups, i.e. fungicole and entomopathogen. In the case of *Fusarium*, for which species belonging to all four ecological roles have been reported, isolates were segregated into four groups based on their closest species match. *Purpureocillium* represented a challenging case because all isolates (based in ITS) were close to *P. lilacinus*, and this species has been reported to be both, saprobe and entomopathogen. This genus was eliminated from the ecological analyses (their abundance was not significantly different between management types, so it was not expected to bias the results). The abundance of strains belonging to each of the ecological roles was compared based on their type of management; using similar statistical techniques applied to compare the abundance of each genus (see Statistical Analyses). EcoSim ver. 7 was used to investigate if the endophytic community under

study is non-randomly structured, which can suggest interspecific competition or other antagonistic interactions among its components.

Phylogenetic Diversity Analyses

To explore if the fungal endophytic communities inhabiting trees in the wild and managed habitats not only vary in the number of OTUs but also in the harbored phylogenetic diversity (PD), we estimated the PD, as defined by Faith (1992), for each management type. For the latter, we used the Phylogenetic Diversity Analyzer (PDA) version 0.5 (http://www.cibiv.at/software/pda). PDA uses as input phylogenetic trees; therefore, ITS phylogenetic trees with bootstrap analysis were constructed using the Bayesian algorithm implemented in MrBayes version 3.1. All OTU-representative sequences were used in the analysis (Ascomycota, Basidiomycota, and Zygomycota). Alignments were exported into MrBayes format using the default settings implemented in MESQUITE version 7.2. Nexus files were submitted into the Cipres portal for computing (www.phylo.org; CIPRES Science Gateway V 3.1.). The last one hundred trees (50 from each run) were uploaded into the PDA online server for PD calculation. Statistic parameters and comparison between management types were performed using SPPS. In addition, ITS phylogenies were inferred under maximum likelihood using GARLI (Genetic Algorithm for Rapid Likelihood Inference) through the web service available at www.molecularevolution.org.

Results

Wild rubber trees have an endophytic assemblage with a higher diversity and different composition than those trees in plantations

Even though species accumulation curves did not reach a plateau (Fig. 5.1) and only ~35% of the estimated diversity was revealed for each habitat, the species accumulation curve in wild plants showed a steeper slope indicating a potential higher overall diversity. In agreement with other studies that have compared the endophyte community of hosts distributed within versus outside their native habitat (Bacon and White 2001) a greater number of projected species was estimated for the community inhabiting wild population of trees (Fig. 5.1). The higher diversity recovered in wild trees could be a result of the multiple layers of heterogeneity found in natural forests, which act as environmental filters shaping the community's species composition. These levels of heterogeneity refer to differences in microhabitats, vegetation composition, or genetic background of their host plants, all of which are absent in monoculture plantations (Mariau 2001, Alexander 2010).

Endophytic infection was present in 100% of the samples and in 74.27% of the subsamples. We found a significant difference in sampling effort, influenced by the great number of strains harbored in plantation trees. A total of 2,346 strains were recovered and 710 species were detected based on the 99% ITS sequence similarity threshold used to delimit the working units or species. Even though the total number of species was higher in the wild (411 versus 404), plantation trees harbored a significantly greater number of species per tree reflecting the overdispersion of species characteristic of tropical forests (Alexander 2010).Comparable phylogenetic

diversity (PD) was found between management regimes (P=0.22; 132.96±7.8 and 134.36±8.2 for wild and plantations, respectively). Nevertheless, the species accumulation curves for the wild habitat showed a steeper curve indicating that many more individual trees need to be sampled for its real diversity to be revealed.

This study also shows that the taxonomic composition of the fungal endophytic community harbored within rubber trees varies significantly between management types. Trees from wild habitats were dominated by *Trichoderma* strains whereas the majority of strains obtained from plantations trees belonged to *Colletotrichum* (results are shown in Table 5.1 and Fig. 5.2). Composition differences in endophytic communities inhabiting hosts distributed within versus outside their native range have been previously reported (Bacon and White 2000). In concordance with these studies we found that genera known to be generalists and cosmopolitans were more common in introduced areas whereas wild trees presented a greater abundance of genera considered to be host-specific. Differential taxonomic structure of horizontally transmitted endophytic communities is directly related to the composition of the surrounding available inoculum (Wright 2002). Therefore, the presence or absence of a particular taxon is dictated by the abiotic and biotic variables (i.e., humidity, temperature, competition) affecting the survival, growth, and dispersal of particular fungal species composing this pool. Even though, *Hevea* is cultivated in tropical regions, differences in species composition of the available inoculum and the low diversity of the surrounding vegetation (which act as reservoirs of fungal species) are important variables that create a low species overlap between wild and plantation habitats.

Plantations harbor more endophytes with potential to become plant pathogens

In this study, plantation trees were dominated by endophytes with pathogenic potential ($P \leq 0.001$). The most common genus, *Colletotrichum*, is a common pathogen of many plant species including species of *Hevea* (Farr and Rossman 2012). Other genera present in significantly higher numbers in plantations were *Fusarium*, *Guignardia*, and *Lasiodiplodia*, all of which contain many plant pathogenic species (Gilbert 2002). Furthermore, species from all these genera have been reported as pathogens of *H. brasiliensis*. DNA sequences from most of the reported rubber pathogens are not represented in public databases; therefore, in many cases we were not able to confirm that the isolates codified under "plant pathogen" have indeed been isolated from diseased *Hevea* material. Nevertheless, all the obtained sequences belonging to *Lasiodiplodia* and *Colletotrichum* genera showed highest similarity with *L. theobromae* and *C. gloeosporioides* respectively, two pathogenic species of *H. brasiliensis*. *Fusarium* species can present different ecological roles (see methods), but the majority present in plantations showed high similarity with the species *F. oxyxporum* and *F. solani*, both reported as pathogens of *H. brasiliensis*. Fungicolous *Trichoderma* species were also present at high frequency in plantations (second to *Colletotrichum*), although their abundance was less than half when compared to wild trees.

Populations of tropical plants growing in their native environments are rarely decimated by pathogen outbreaks. The results from this study support various hypotheses on the causes behind the absence of severe epiphytotics in natural forests. One of the most accepted hypotheses is based on the complex forest structure

produced by the low population density of most tropical tree species, which reduces opportunities for disease transmission (Gilbert 2002, Valencia et al. 2004). As a consequence of competition or seed dispersal strategies, individuals of the same plant species tend to grow distantly, limiting the spread of host specific pathogens (Mariau 2001). This is especially true in tropical areas where the density of tree species can be very low (Evans and Turnbull 2004). In contrast, plants under management are usually grown as monocultures (Bicalho et al. 2008), eliminating the barriers for pathogen dispersal. The apparently insignificant effects that pathogens have on wild plant populations could also be a consequence of their high genetic diversity (Silva et al. 2001, Le Guen et al. 2009). Populations living close to their native habitats hold high genetic diversity including genetic resistance against pathogens (Priyadarshan 2003).

The lower incidence of diseases outbreaks in natural habitats can also be explained through evolutionary theories, such as the "arms race" and the "mutualist facilitation" hypotheses. According to the "arms race" hypothesis, hosts and pathogens are in a constant coevolutionary cycle in which resistance is acquired by the host and overcome by the pathogen through time. This cycle creates a fluctuation in both host and pathogen populations that act as a balancing force for their coexistence (Van Valen 1973, Maor 2005). The "mutualist facilitation" hypothesis argues that the replacement of lost mutualists/commensals from plants' native ranges with new mutualists/commensals in their introduced ranges is important for the establishment and success of introduced plants (Mitchell et al. 2006). Changes in mutualistic species composition may limit success or even prevent establishment and

naturalization of a plant in a new area if the pool of mutualists available to it does not include species with which it coevolved (Parker 2005). In addition, differences in species composition between plants' native and introduced ranges may be biased either towards or against successful introduction of the plant to the new area (Kliromonos 2003, Parker 2005, Mitchell et al. 2006). Such biases arise because a plant species shares a longer co-evolutionary history with a specific assemblage of mutualists/commensals in its native range than in its introduced range.

Wild trees harbor more endophytes with potential for biological control

Our study showed that wild trees harbor more fungicolous strains than trees in plantations ($P \leq 0.001$), thus, more strains with potential for biological control. Wild trees were dominated by strains belonging to *Trichoderma*, a genus known and used commercially for its biocontrol properties (Harman et al. 2004). The latter contrast the higher abundance of plant pathogenic strains found in plantations trees. These findings stress the difference not only in the taxonomic composition of the fungal endophytic communities hosted by *Hevea* trees under different management regimes, but also their distinctive ecology. Furthermore, the number of fungicolous species was higher in wild plants, whereas plant pathogens were more diverse in plantations (Table 5.2). The latter displayed a difference in abundance as well as in genetic diversity.

This study has insufficient data to determine if there is a cause-and-effect relationship between the state of the forest (i.e., natural versus plantation) and the total number of potentially beneficial endophytic species/taxa. The latter is due to the many variables affecting the composition and abundance of fungal endophytic communities that would need to be accounted for in a regression analysis, including temperature,

humidity, soil type, wind currents, light exposure, and especially the composition of the surrounding vegetation that can serve as a reservoir of fungal species, all of which can influence the composition and abundance of the fungal inocula that become endophytic (Bacon and White 2000, Arnold et al. 2003). Nevertheless, we reveal a pattern in which a high number of strains closely related to fungicolous species exist as part of the fungal endophytic community of wild trees. This pattern suggests that these endosymbionts may play a role in the balance between hosts and their pathogens.

We also analyzed the correlation between the abundance of *Trichoderma* and the abundance of selected pathogenic genera to test the premise that the presence of endophytes with antifungal properties may be limiting the presence of plant pathogens. We found a significant negative correlation between the abundance of strains belonging to *Trichoderma* and *Phomopsis* (P=0.01), which would in part support our premise since *Phomopsis* is a genus that mainly includes plant pathogenic species (Farr and Rossman 2012). From the data collected in this study, the mechanisms by which *Trichoderma* may be protecting the rubber trees from the pathogens are not clear. Previous studies have shown the ability *Trichoderma* has to directly parasitize fungal pathogens, produce secondary metabolites that have a negative effect on plant diseases, outcompete fungal pathogens, promote host growth, and enhance disease resistance in inoculated plants (Hermosa 2012). Hence, we consider wild strains of these *Trichoderma* species as promising candidates for future biocontrol *in vitro* and *in planta* studies.

To further tests if the assemblage of the community under study is influence by the interaction between its components, we compared our data against a simulated

community created under the null model. Results from the analysis suggests that the structure of the endophytic community inhabiting *Hevea* significantly diverge from a random pattern and that further ecological mechanisms such as competition or other antagonistic interactions may be shaping the species composition and abundance (P=0.02, C-score and Combo). These values also suggests that the community contain species-pairs that co-occur less frequently than expected, which can be a result of one species having an antagonistic effect on the other one.

Implications for the conservation of natural tropical forests

The present research emphasizes the importance of the ecological services that natural forests provide to human needs. Previous studies have highlighted some ecological services such as their ability to sequester carbon, regulate weather patterns, preserve soil integrity, filter water, and serve as a reservoir of biodiversity and natural products, among many others (Balmford et al. 2002). Our results add an additional motivation to protect native forests, which is to preserve beneficial endosymbionts that can aid in the fight against crop diseases and optimize the exploitation of renewable natural resources. Furthermore, results obtained here demonstrate a positive correlation between tree diameter and the number of species harbored within its sapwood, which encourage the preservation of old growth forests.

Conclusions

Amazonian natural old-growth forests are a rich reservoir of potentially useful fungi, including those that can be used in biological control of plant diseases. Results from this study demonstrate that rubber trees distributed within their native range harbor

endosymbiotic fungi that may be involved in maintaining the health of the trees, more so than those in plantations. These results support previous suggestions that trees in the wild should harbor more beneficial fungi than those in artificial settings (i.e., plantations; Webber 1981, Rubini et al. 2005). We found a significant negative correlation between the abundance of strains belonging to fungicolous genus *Trichoderma* and the plant pathogenic *Phomopsis* that further supports this hypothesis. Host protection by endophytes could be occurring through various mechanisms: direct mycoparasitism, antimicrobial chemical production, competition for space and nutrients, induction of plant growth-promoting compounds, or enhancement of the host's natural immune system (Pal 2006).

We believe that multiple factors act in concert to create a complex scenario in which pathogens and their plant hosts coexist in balance. Environmentally compatible and sustainable disease control practices should attempt to recreate this scenario. One strategy would be the introduction of natural enemies into artificial environments. These species should provide evidence for high specificity to their hosts or prey, supported by a long-shared evolutionary history. Endosymbionts with antagonistic properties against rubber tree diseases could be inoculated in seedlings before they are grown in plantations (Bailey et al. 2008). The latter, in addition to other practices such as resistance breeding programs, promoting stands composed by individuals with heterogeneous genetic background, and quarantine regulation that prevent the spread of endemic pests should be undertaken as an integrated pest control management for natural rubber production.

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TABLES AND FIGURES

Table 1. Symbiotic criteria use to characterize fungal endophyte classes. Adapted from Rodriguez et al. 2009.

	Clavicipitaceous	**Non-Clavicipitaceous**		
Criteria	**Class 1**	**Class 2**	**Class 3**	**Class 4**
Host Range	Narrow	Broad	Broad	Broad
Tissue colonized	Shoot and rhizome	Shoot, root, and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefit	Non-Habitat adapted	Non-habitat adapted and habitat adapted	Non-habitat adapted	Non-habitat adapted

Table 2. Type of inter-species antagonisms leading to biological control of plant pathogens. Adapted from Pal and McSpadden 2006.

Type	**Mechanisms**	**Examples**
Direct antagonism	Hyperparasitism/predation	*Trichoderma* spp., *Nectria* spp.
Mixed-path antagonism	Antibiotics	2,4-diacetylphloroglucinol Phenazines, Cyclic lipopeptides
	Lytic enzymes	Chitinases, Glucanases, Proteases
	Waste products	Ammonia, Carbon dioxide, Hydrogen cyanide
	Physical/chemical interference	Blockage of soil pores, Germination signals consumption, Molecular cross-talk confused
Indirect antagonism	Competition	Exudates/leachates consumption Siderophore scavenging Physical niche occupation
	Induction of host resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormone-mediated induction

Table 1.1 List of identified OTUs, their abundances and origin.

Endophyte species	**Origin**		**Total Abundance**
	Sapwood	**Leaf**	
Pestalotiopsis aff. *palmarum*	7	32	39
Trichoderma harzianum	19	13	32
Penicillium paxilli	7	3	10
Penicillium aff. *sclerotiorum*	3	6	9
Trichoderma koningiopsis	7	0	7
Penicillium brevicompactum	5	1	6
Cladosporium cladosporioides	2	2	4
Perisporiopsis aff. *melioloides*	1	2	3
Alternaria alternata	1	2	3
Penicillium sclerotiorum	3	0	3
Arthrinium sp.1	0	2	2
Endomelanconiopsis endophytica	0	2	2
Endomelanconiopsis microspora	1	1	2
Entonaema pallida	1	1	2
Fimetariella rabenhorstii	2	0	2
Fusarium polyphialidicum	2	0	2
Guignardia aff. *heveae*	0	2	2
Leptosphaereaceae sp.1	1	1	2
Nectriaceae sp. 1	2	0	2
Khuskia sp.1	1	1	2
Umbelopsis sp.1	1	1	2
Arthrinium sp.2	0	1	1
Biscogniauxia sp.1	0	1	1
Annulohypoxylon sp.1	1	0	1
Cochliobolus sp.1	1	0	1
Colletotrichum gloeosporioides	0	1	1
Colletotrichum sp.1	1	0	1
Colletotrichum sp.2	0	1	1
Colletotrichum sp.3	0	1	1
Colletotrichum sp.4	0	1	1
Coprinus aff. *radians*	1	0	1
Daldinia sp. 1	0	1	1
Dothidiomycetes sp.1	1	0	1
Epicoccum nigrum	1	0	1
Fusarium proliferatum	0	1	1
Hypocreales sp.1	1	0	1
Lasiodiplodia theobromae	1	0	1
Leptosphaerulina chartarum	1	0	1
Mycosphaerellaceae aff. *Epicoccum*	0	1	1
Penicillium aculeatum	1	0	1
Penicillium aff. *glabrum*	0	1	1
Penicillium aff. *spinulosum*	1	0	1
Penicillium chrysogenum	0	1	1
Penicillium meleagrinum	1	0	1
Phoma glomerata	1	0	1
Phoma pinodella	0	1	1
Phomopsis aff. *theicola*	0	1	1
Pleospora sp.1	1	0	1
Pochonia suchlasporia	1	0	1
Rubrinectria olivacea	1	0	1
Trametes gibbosa	1	0	1
Umbelopsis sp.2	0	1	1
Xylaria allantoidea	0	1	1
Xylaria sp.1	0	1	1
Xylariaceae sp.1	0	1	1

Xylariaceae sp.2	0	1	1
Morphospecies 1	1	0	1
Morphospecies 2	1	0	1

Table 1.2. Endophyte diversity indices and their estimates by site and origin.

	Species Richness	Evenness	Abundance	Fisher's α	Shannon's (H')	Simpson's* (λ)	Chao 1	Chao 2
Site 1	27	0.79	67	18.92	2.69	0.14	110.62	252.76
Site 2	23	0.8	52	25.23	3	0.14	84.54	81.48
Site 3	30	0.9	56	30.32	3.15	0.07	113.5	124.88
Leaf	35	0.75	90	22.41	2.85	0.16	120.22	135.88
Sapwood	36	0.85	85	30.32	3.15	0.08	100.96	341.14
Total Tambopata	58	0.78	175	-	3.16	0.1	120	117

Table 1.3. Comparison of the different similarity indices estimates among collection sites and plant parts: combined and partitioned data (by plant part) are shown.

	SS	MH^1	MH^2	MH^3	BC^1	BC^2	BC^3	J^1	J^2	J^3	SC^1	SC^2	SC^3
S1 vs. S2	7	0.897	0.069	0.924	0.504	0.024	0.548	0.161	0.293	0.130	0.28	0.453	0.230
S1 vs. S3	11	0.592	0.147	0.702	0.373	0.023	0.387	0.239	0.241	0.2	0.415	0.388	0.33
S2 vs. S3	11	0.674	0.756	0.674	0.481	0.421	0.357	0.261	0.192	0.142	0.385	0.322	0.25
Leaf vs. Sapwood	13	0.544	-	-	0.402	-	-	0.224	-	-	0.366	-	-

SS: Species shared; MH: Morisita-Horn similarity index; BC: Bray-Curtis similarity index; J: Classical Jaccard's similarity index; SC: Classical Sorensen's similarity index; 1: Combined estimate (leaves and sapwood); 2: Estimate only considering sapwood isolates; 3: Estimate only considering leaf isolates

Table 3.1. References of published studies on horizontally transmitted fungal endophytes, from 2000 through 2010. Their sampling design information is provided.

	Reference	Number of sites	Number of hosts	Number of independent samples
1	Arnold et al., 2000	2	2	Species 1: 9 individuals Species 2: >14 individuals
2	Frölich et al., 2000	2	2	3 individuals/species
3	Müller and Hallaksela, 2000	1	1	1 individual
4	Arnold et al., 2001	1	9	3 individuals/species
5	Gamboa and Bayman, 2001	2	1	7 individuals /locality
6	Müller et al. 2001	3	1	11 individuals (3:2:6)
7	Cannon and Simmons, 2002	2	12	1 individual/species
8	Gamboa et al., 2002	3	5	2 individuals/species
9	Hata et al., 2002	2	1	Site1: 1 individual Site2: 10 individuals
10	Suryanarayanan et al., 2002	4	23	2 individuals/species
11	Arnold et al., 2003	5	1	3 individuals /site
12	Evans et al., 2003	2	1	20 / site
13	Ragazzi et al., 2003	3	3	3 individuals /species/site
14	Guozhong et al., 2004	2	12	NS
15	Suryanarayanan and Thennarasan 2004	1	1	1 individual
16	Murali et al., 2005	2	1	5 individuals / site
17	Rubini et al., 2005		1	5 individuals
18	Santamaria and Bayman, 2005	6	1	3 individuals / site
19	Tejesvi et al., 2005	3	1	Site 1: 3 individuals Site 2: 1 individual Site 3: 1 individual
20	Helander et al., 2006	3	1	35 trees / site
21	Arnold and Lutzoni, 2007	8	21	3 – 9 individuals /species
22	Gond et al., 2007	Several	1	3 individuals
23	Helander et al., 2007	19	2	1 – 7 individuals /species/site
24	Higgins et al. 2007	2	3	15 individuals /species
25	Murali et al., 2007	2	15	3 individuals /species /site
26	Promputtha et al., 2007	5	1	10 individuals
27	Unterseher et al., 2007	1	4	2 individuals
28	Verma et al., 2007	3	1	1 individual /site
29	Hoffman and Arnold, 2008	2	3	4 individuals /species
30	Huang et al., 2008	NS	29	1 individual /species
31	JianQiu et al., 2008	1	6	1 individual /species
32	Shipunov et al., 2008	Several	1	5-16 individuals /site
33	Krishnamurthy et al., 2009	Several	33	2 individuals /species
34	Tejesvi et al., 2009	2	4	NS
35	Unterseher et al. 2009	1	1	20 individuals
36	Gazis and Chaverri, 2010	1	1	15 individuals
37	Jiun-Horng et al., 2010	1	2	3 individuals /species
38	Rosa et al., 2010	6	1	15 – 60 individuals /site Total = 180 individuals
39	Unterseher and Schnittler, 2010	3	1	NS
40	Vega et al., 2010	Several	7	NS

Table 3.2. Summary of the complete datasets.

	LORETO	**TABASCO**
Number of samples	50	50
Number of sub-samples	450	450
Number of sub-samples that showed growth	320 (71%)	307 (68%)
Number of Isolates recovered	360	342
Number of OTUs (99% ITS)	169	132
Number of singletons/no singletons	120/49 (71%)	87/49 (64%)
OTUs belonging to Ascomycota	133 (79%)	100 (77%)
OTUs belonging to Basidiomycota	34 (20%)	32 (23%)
OTUs belonging to Zygomycota	2 (1%)	0

Table 3.3. Diversity indices estimation for Tabasco and Loreto datasets. Datasets were further partitioned into complete (singletons included) and truncated (singletons excluded). Significance was calculated in SPSS using data from 100 randomizations extracted from EstimateS and values are indicated by bold font.

	Loreto complete	Tabasco complete	Loreto truncated	Tabasco truncated
Number of Species	169	132	50	50
Species Observed (CI, SD)	**169 (169 - 169, 0)**	99.74 (98.46 - 101.02, 6.5)	44.38 (43.84 - 44.92, 2.7)	**54.63** (54.51 - 54.75, 0.6)
Fisher's α (SD)	**127 (108 - 146, 11.35)**	79.5 (66.5 - 92.5, 6.65)	19.5 (15.5 - 23.5, 2.05)	20 (16 - 29, 1.98)
Shannon (CI,SD)	4.31 (4.13 - 4.48, 0.09)	4.20 (4.05 - 4.34, 0.08)	3.10 (3 - 3.2, 0.1)	**3.47** (3.4 - 3.5, 0.07)
Chao-1 (CI, SD)	**422** (319 - 595, 65)	262 (206 - 361, 37)	50 (50 - 52, 0.68)	**55** (55 - 56, 0.57)
ACE (CI, SD)	**411** (389 - 543, 77)	255 (253 - 349, 48)	50 (50 - 50,0)	**55** (55 - 55,0)
Evenness (CI,SD)	0.44 (0.41 - 0.54, 0.03)	0.49 (0.48 - 0.59, 0.03)	0.45 (0.37 - 0.51, 0.04)	**0.58** (0.50 - 0.64, 0.04)

*Shannon index (H') = 1.5 – 5; E = e^{H}/S: 0 – 1; CI = confidence intervals; (SD) = standard deviation

Table 3.4. Similarity analyses between datasets. Results from the complete datasets are followed by the results based on their truncated versions. Values in parenthesis correspond to the Standard Error (S.E.) obtained from 1000 randomizations using SPADE software.

	Loreto	**Tabasco**
Total number of OTUs	277	
Ascomycota unique OTUs	113	80
Basdiomycota unique OTUs	30	28
Zygomycota unique OTUs	2	0
Ascomycota shared OTUs	20 (11)	
Basidiomycota shared OTUs	4 (02)	
Sorenson's classic	0.17 (0.02) – 0.27 (0.02)	
Morisita - Horn	0.13 (0.03) – 0.11 (0.03)	
Bray - Curtis	0.16 (0.01) – 0.17 (0.02)	
Chao-shared estimated	46.65 (20.6) – 13 (0)	

Table 3.5. Mean phylogenetic diversity (PD) at different sample sizes for the localities of Loreto and Tabasco (complete datasets). The number of taxa refers to the number of strains included in the phylogenetic analysis. One hundred phylogenetic trees, inferred using Bayesian algorithm, were produced for each sample size and used to calculate the PD. Mean, confidence interval (CI), standard deviation (SD), and significance was calculated using SPSS. Significant results are denoted by bold font. Only members of the Ascomycota were used in the analysis.

	LORETO		**TABASCO**	
N	**Taxa**	**PD (CI, ±SD)**	**Taxa**	**PD (CI, ±SD)**
05	26	3.73 (3.69-3.77, ±0.20)	22	**4.10 (4.04-4.15, ±0.28)**
10	58	5.52 (5.47-5.56, ±0.24)	45	**6.73 (6.64-6.82, ±0.45)**
15	73	7.48 (7.42-7.54, ±0.31)	70	**8.53 (8.45-8.61, ±0.42)**
20	100	8.80 (8.72-8.87, ±0.38)	92	**12.87 (12.70-13.04, ±0.86)**
25	131	11.75 (11.65-11.85, ±0.49)	109	**12.26 (12.17-12.35, ±0.46)**
30	155	15.23 (15.08-15.38, ±0.75)	135	**15.88 (15.78-15.99, ±0.53)**
35	186	**23.58 (23.34-23.81, ±1.19)**	159	16.87 (16.73-17.00, ±0.69)
40	216	**27.24 (27.03-27.46, ±1.08)**	175	21.17 (20.96-21.39, ±1.10)
45	244	**32.16 (31.86-32.46, ±1.53)**	205	24.59 (24.50-24.67, ±0.44)
50	269	**40.52 (39.87-41.17, ±3.26)**	229	29.93 (29.75-30.11, ±0.92)

Abbreviations: N, number of independent samples used in the analysis; PD= Phylogenetic Diversity un-rooted.

Table 3.6. Estimated sample effort for Tabasco and Loreto complete datasets, using abundance data. Shaded column indicate the coverage reached by the actual sample size used in this study.

Locality	N	S_{obs}	S_{est}	f0	f1	f2	q0	g	mg (g=1)	mg (g=0.99)	mg (g=0.95)	mg (g=0.90)	mg (g=0.80)	mg (g=0.7)	mg (g=0.6)	mg (g=0.5)
Tabasco	50	132	262	125	87	27	1.55	**0.52**	614	311	181	126	70	37	14	-
Loreto	50	169	422	252	120	28	2.38	**0.40**	894	435	264	190	117	74	43	19

Abbreviations are: N, number of independent samples collected; S_{obs} = species observed; S_{est} = estimated asymptotic species richness (based on Chao1 estimator; f1, the number of species represented by exactly one individual ("singletons"); f2, the number of species represented by exactly two individuals ("doubletons"); q0, the probability to detect a previously undetected species; g, target fraction of S_{est} that is to be reached; mg, represents the number of additional samples needed to reach the target coverage 100% (g=1), 99% (g=0.99), 95% (g=0.95), 90% (g=0.90), 80% (g=0.80), 70% (g=0.70), 60% (g=0.60), and g=50% (g=0.50). The values in "g" column represent the fraction of coverage reached by our sampling.

Table 4.1. Summary of datasets, including number of OTUs, length of alignments and number of characters that were included in the analyses, for each gene separately and when combined after removal of ambiguous regions. Regions that could not be aligned with confidence (ambiguous) and introns were excluded from phylogenetic searches.

Dataset	Number of OTUs	Number of Characters			
		Total length	Ambiguous and/or introns	Constant	Total Included
5.8S (ITS)	96	162	13	78	149
	107	162	13	76	149
nucSSU	96	5601	4520	569	1081
	107	5612	4590	546	1022
nucLSU	96	3670	2580	569	1090
	107	3695	2663	557	1032
mitSSU	96	2410	1980	159	430
	107	2531	2101	126	430
RPB1 (A-F)	96	3108	318	812	2790
	107	3111	345	738	2766
RPB2 (7-11)	96	2247	276	628	1971
	107	2235	375	605	1860
Combined data	96	17198	9687	2815	7511
	107	17346	10087	2648	7259

Table 5.1. Abundance of the genera that were present in high frequency (more than 40 isolates) and their comparison based on management regime. Significance was calculated for the estimates mean per tree, using the non-parametric t-test implemented in SPSS (Mann-Whitney U). N represents the number of independent samples used in each analysis. Four localities were sampled for each management regime, encompassing a total of 190 individual trees. Significance was evaluated based on a $P \leq 0.005$ (after Bonferroni correction) and it is indicated by bold font. Groups with significant differences were further segregated into tissue of origin.

	Abundance (N = 4)			Mean / per tree (N = 95)		Mean / per tree / sapwood (N=95)		Mean / per tree / leaf (N=60)	
	T	W	P	W	P	W	P	W	P
Bionectria	43	20	23	0.21	0.24	-	-	-	-
Colletotrichum	209	61	148	0.64	**1.56**	0.05	0.09	1.08	**2.35**
*Fusarium**	68	11	57	0.12	**0.60**	0.08	**0.45**	0.05	0.23
Guignardia	80	14	66	0.15	**0.69**	0.03	0.05	0.24	**1.03**
Lasiodiplodia	46	10	36	0.11	**0.38**	0.04	**0.33**	0.10	0.05
Penicillium	149	91	58	0.96	0.61	-	-	-	-
Pestalotiopsis	152	66	86	0.69	0.91	0.26	0.79	0.80	0.21
Phomopsis	154	67	87	0.71	0.92	-	-	-	-
Purpureocillium	135	80	55	0.84	0.58	-	-	-	-
Trichoderma	372	254	118	**2.67**	1.24	**2.32**	1.05	**0.75**	0.27

**Fusarium* strains belonging to the plant pathogenic group

Abbreviations: T= total (wild + plantations); W = wild; P = plantations.

Table 5.2. Results from the assignment of identified Ascomycota OTUs to potential ecological roles. OTUs were first identified to genus (based on a 95% ITS sequence similarity in relation to NCBI database) and later assigned to an ecological role based on published reports. Significance tests were performed based on their incidence per tree. Significance was based on a $P \leq 0.01$ (corrected for multiple comparisons), results are denoted by bold font. Confidence intervals are given within parenthesis.

	Sum Abundance (N = 4)			OTU number (99% ITS)			Genera number (95% ITS)			Mean / per tree (N = 95)	
Ecological role	T	W	P	W	P	W&P	W	P	W&P	W	P
Plant Pathogen	528	159	369	83	101	41	10	14	14	1.67 (1.31-2.04)	**3.88** (3.20-4.57)
Saprobe	536	261	275	44	36	18	2	5	12	2.75 (2.22-3.28)	2.89 (2.36-3.43)
Fungicole	427	282	145	63	19	19	0	2	4	2.97 (2.53-3.41)	**1.53** (1.25-1.80)
Entomopathogen	53	31	22	13	5	4	3	1	4	0.33 (0.21-0.44)	0.23 (0.10-0.36)

*Number of OTUs identified: 455 from a total of 570.

W= wild

P = plantations

Figure 1. Natural distribution of species from the genus *Hevea*. The center of origin of the genus is considered to be the upper region of the Amazon Basin.

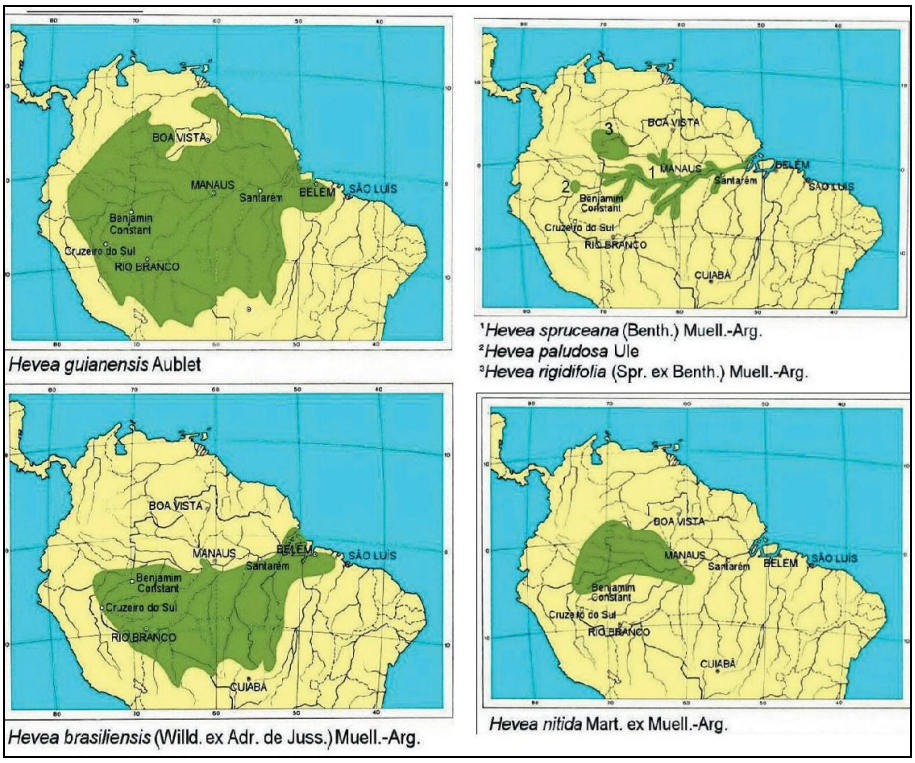


Figure 1.1 Frequency of OTUs in relation to abundance. Only the OTUs with more 3 or more isolates have been included.

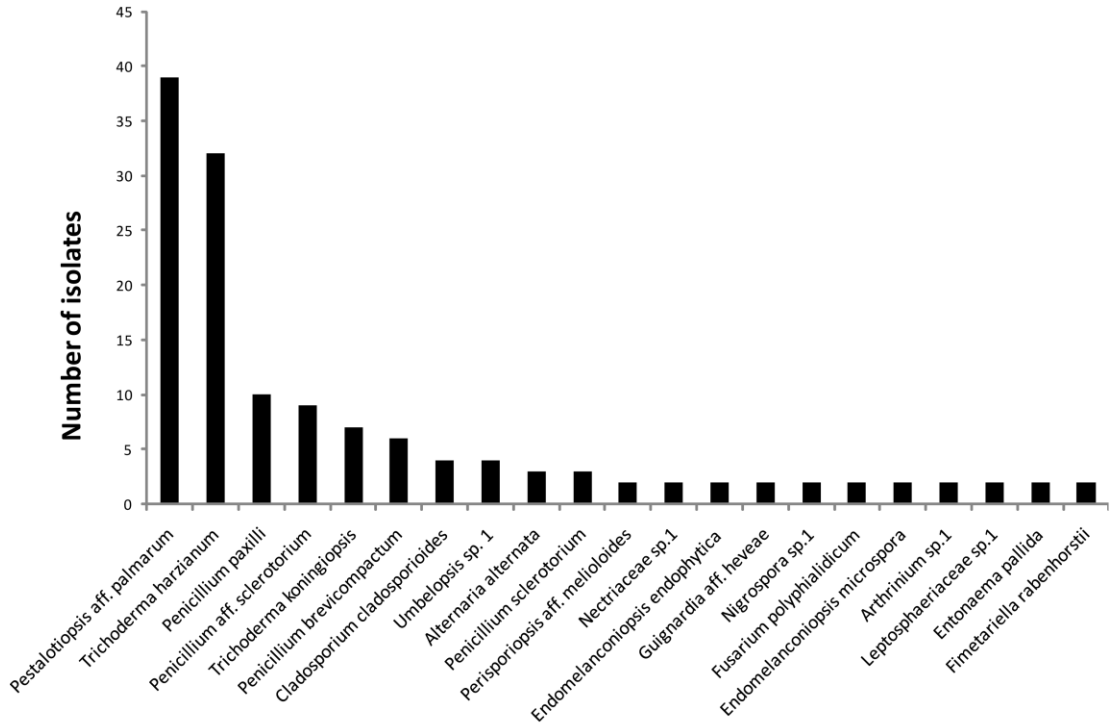


Figure 1.2 Phylogenetic relationships among 129 isolates of endophytic fungi obtained in culture from asymptomatic foliage and sapwood of *Hevea brasiliensis* and 52 representatives of the Ascomycota.The tree was produced using Maximum Likelihood (ML) method based on combined data from ITS and a partial segment of the LSU region. Ln = -32444.3439. Bar indicates the nucleotide substitutions per site. Thickened lines indicate a bootstrap value >70%.

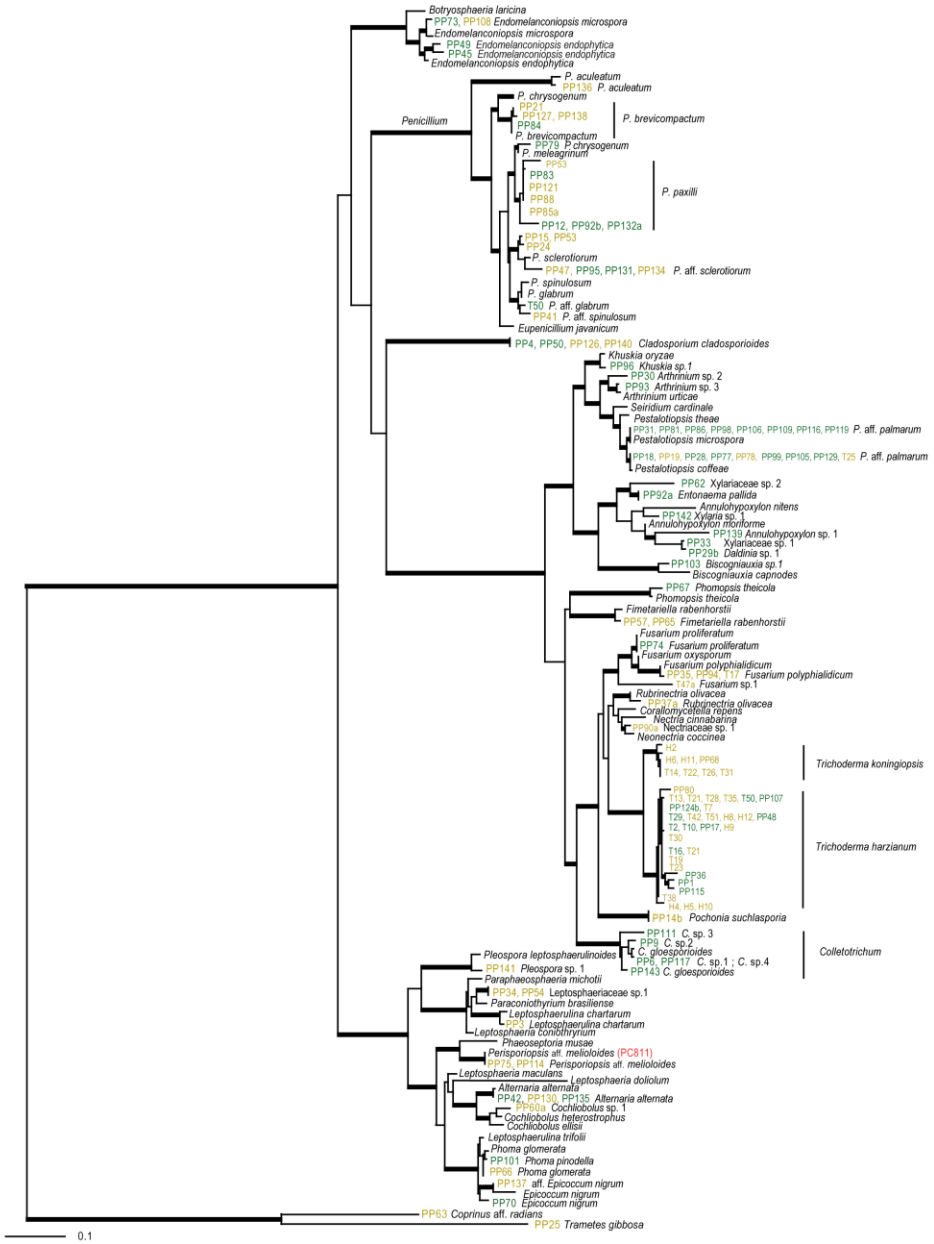


Figure 1.3 Cumulative number of fungal endophytes OTUs as function of the number of collections.The solid black line indicates the OTU accumulation curve for the sapwood and leaf community combined; and the black dashed lines indicate the OTU accumulation curves for each of the communities separately.

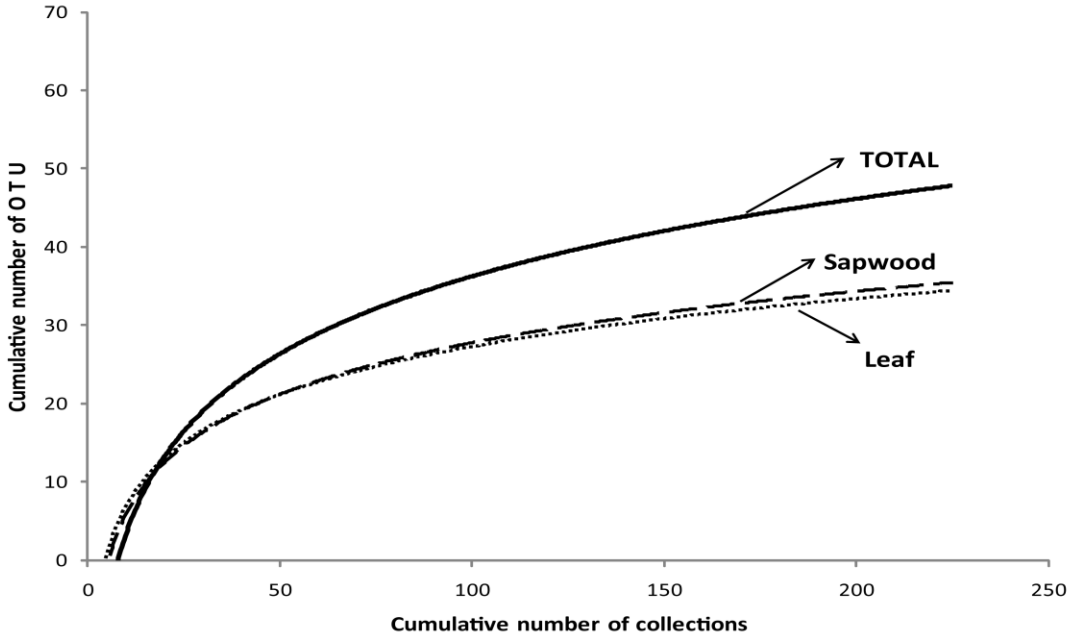


Figure 2.1 Phylogenies resulting from Maximum Parsimony analyses of the ITS nrDNA (a) *Pestalotiopsis* cf. *microspora*, (b) *Colletotrichum* cf. *gloeosporioides,* and (c) *Trichoderma* cf. *harzianum*. Nuclear locus included ITS1, ITS2, and 5.8 S. Node support values are indicated as follows: bootstrap support in Maximum Parsimony / bootstrap support in Maximum Likelihood / Bayesian Posterior Probability = (MP/ML/BPP). Branches that have been shortened are denoted by (\). Terminal labels denote the following geographic origin, management type and host species: LA (Los Amigos – Peru, wild, *H. guianensis*), IB (Iberia – Peru, plantation, *H. brasiliensis*), IQ (Iquitos – Peru, wild, *H. brasiliensis*), CM (Cameroon, plantation, *H. brasiliensis*).

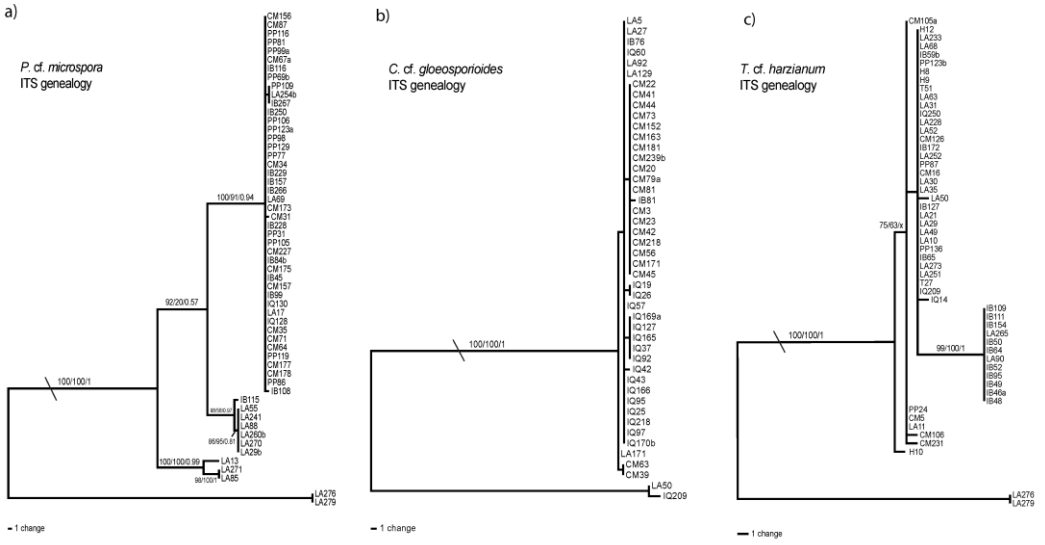


Figure 2.2 Phylogenies of 55 *Pestalotiopsis* cf. *microspora* (a), 56 *Colletotrichum* cf. *gloeosporioides* (b), and 61 *Trichoderma* cf. *harzianum* (c) isolates resulting from Maximum Parsimony analysis of the concatenated 3 nuclear regions (complete gpd, ITS, and tef1). Node support values are indicated as follows: bootstrap support in Maximum Parsimony / bootstrap support in Maximum Likelihood / Bayesian Posterior Probability / genealogical concordance value = (MP/ML/BPP/gsi). Branches that have been shortened are denoted by (\). Distribution within the tree are indicated by (S) = sapwood and (L) = leaves.

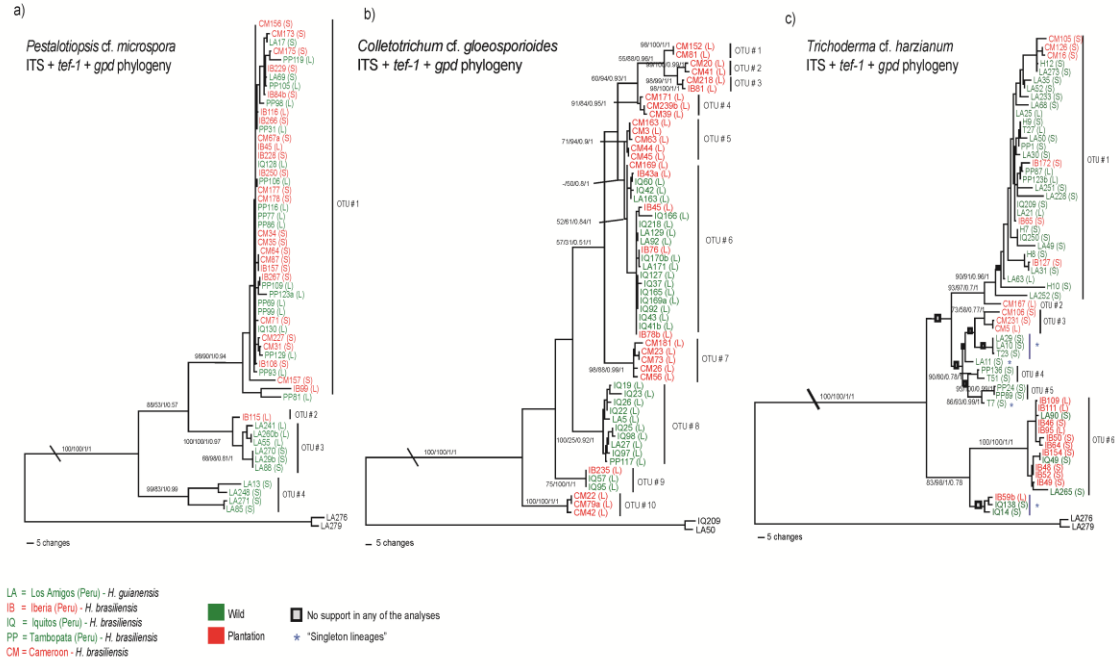


Figure 2.3 Recombination network determined by SplitsTree (GTR character transformation and reticulate network split transformation) from the concatenated data (gpd, ITS, and tef1). a) *Pestalotiopsis* cf. *microspora*, b) *Colletotrichum* cf. *gloeosporioides*, and c) *Trichoderma* cf. *harzianum*. Split bootstrap support higher than 70% is represented by the bold lines. For each dataset, putative phylogenetic species are enclosed within dotted lines.

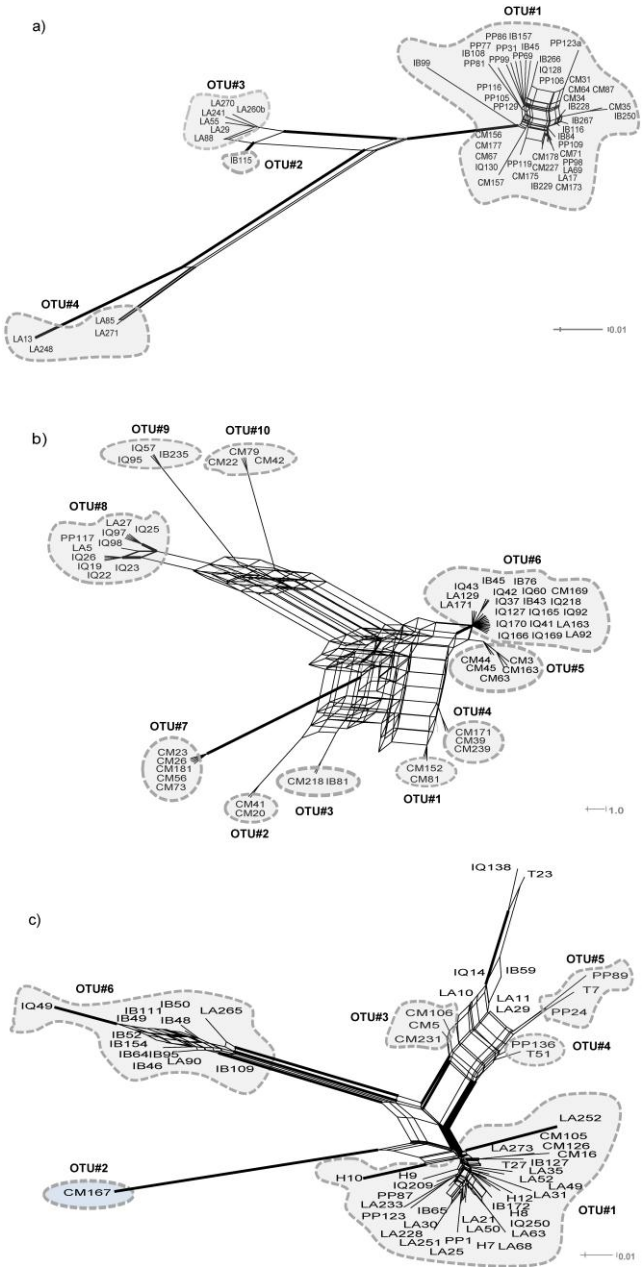


Figure 3.1 Graphs showing the species accumulation curves for each dataset, based on Species Observed (Sobs). Data was retrieved from Estimates using 1000 randomizations with replacement and plotted in SPSS. (A) based on sample number and (B) based on number of isolates. Error bars represent 95% confidence intervals (CI) of the mean Sobs.

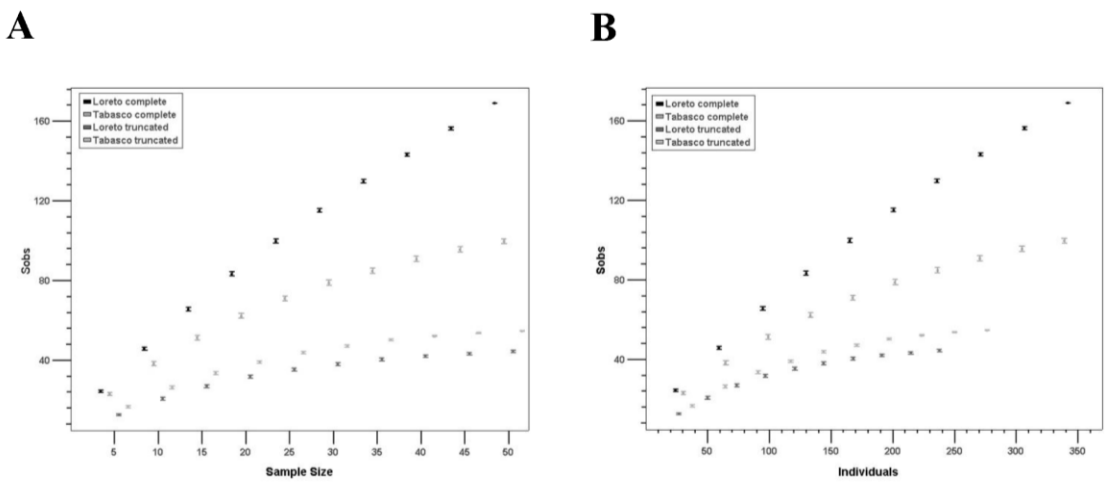


Figure.4.1 Phylogenetic placement of rubber-tree endophytes isolated from their sapwood (as Mycetes novum Xylonomycetes shown in gray oval) in the Pezizomycotina phylogeny (Saccharomycotina used as outgroup) based on a combined 6-locus (nucLSU, nucSSU, 5.8S, mitSSU, RPB1 and RPB2) data set for 107 taxa. Values and boxes associated with internodes represent BL (before slash) and PP (after slash) support from the 5-locus 107 taxon data set (upper row) and the 6-locus 107-taxon data set (lower row). Black boxes indicate significant support (BL $\geq$ 70% and PP $\geq$ 0.95), white boxes indicate non-significant support, and gray boxes (and dashes) indicate significantly supported conflicting relationships.

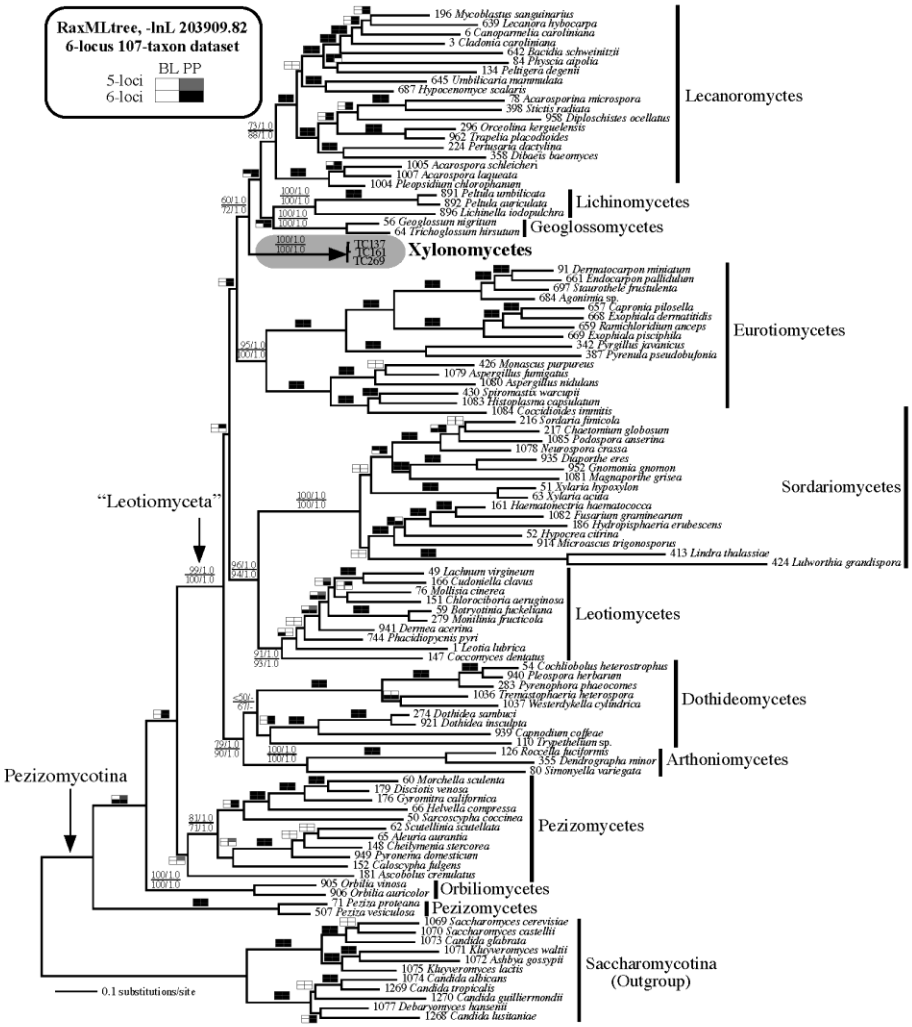


Figure 4.2 Phylogenetic placement of rubber endophytes (as Mycetes novum indicated by a horizontal arrow) in the Pezizomycotina phylogeny (Orbiliomycetes used as outgroup) based on a combined 6-locus (nucLSU, nucSSU, 5.8S, mitSSU, *RPB1* and *RPB2*) data set for 96 taxa, partially derived from James et al. (2006). Clades at the class level were collapsed. Values associated with internodes represent significant BL (≥ 70%; before slash) and PP (≥ 0.95; after slash) support. Dash indicates a significantly supported conflicting relationship.

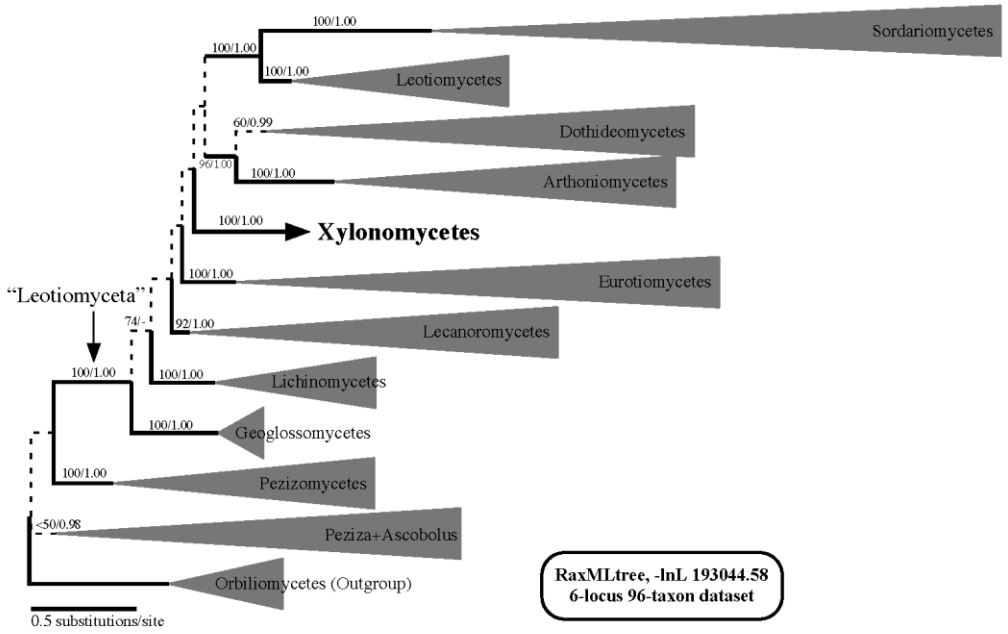


Figure 4.3 *Xylona heveae* (type material). (A) Two-week old culture on MEA with profuse pycnidia, note the vinaceous pigmentation of the agar at the margin of the colony. (B-C) Four-week old culture on MEA and PDA showing mature pycnidia. (D) Pycnidia covered by large masses of conidia (arrow). (E) Young pycnidium. (F) Chlamydospores. (G) Conidiogenous cell with conidia attached. (I - J) Conidia, note the truncated base and the guttulate characters. Scale bars: (C) 200µm, (D) 100µm, (E) (H) (I) 20µm, (G) 250µm.

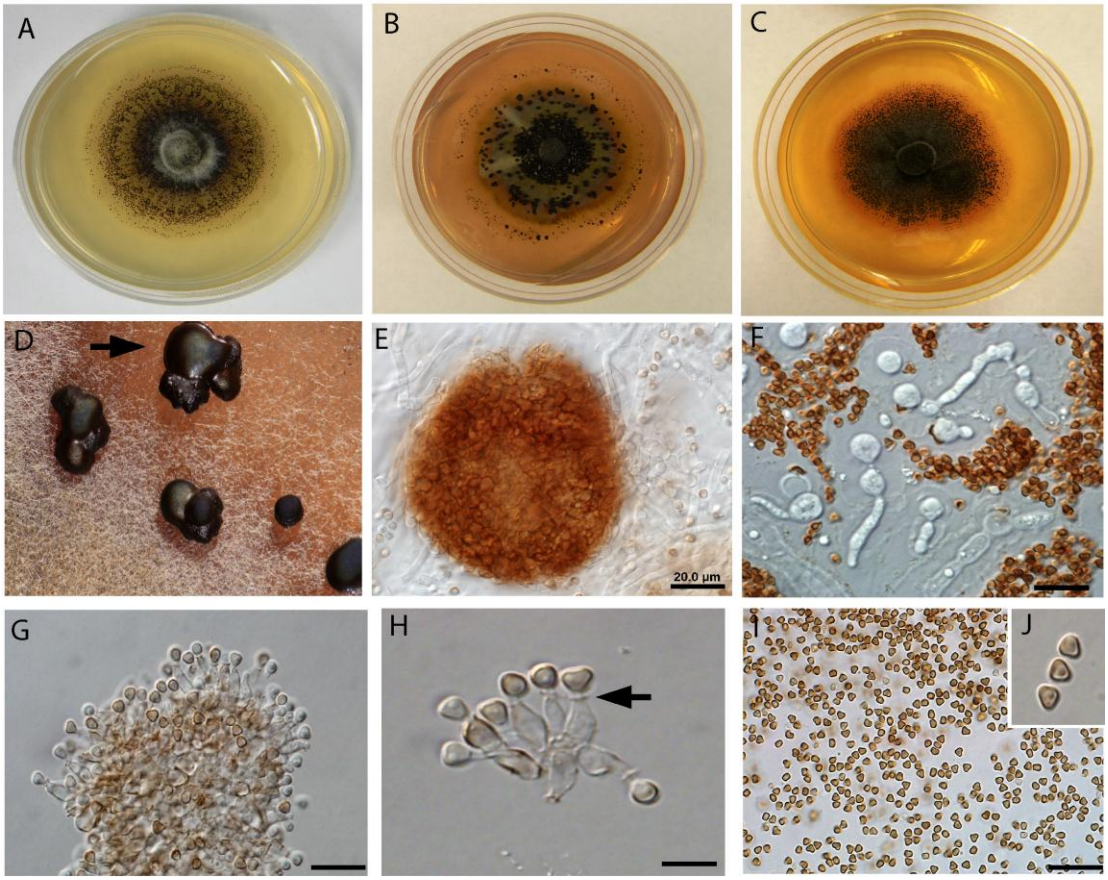
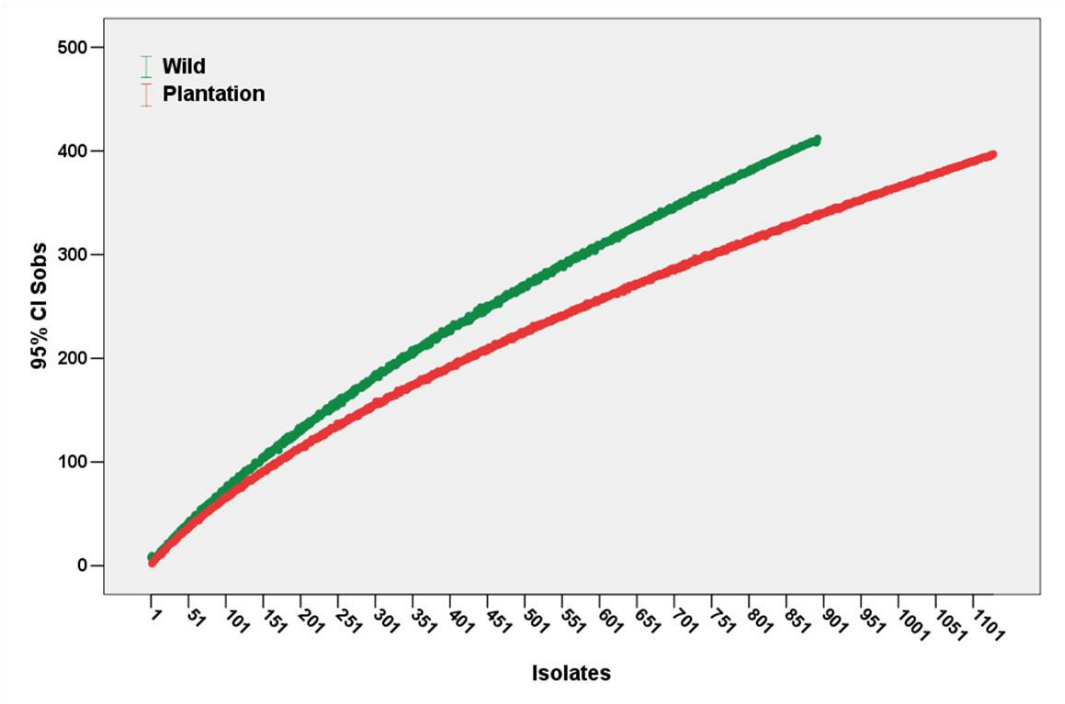
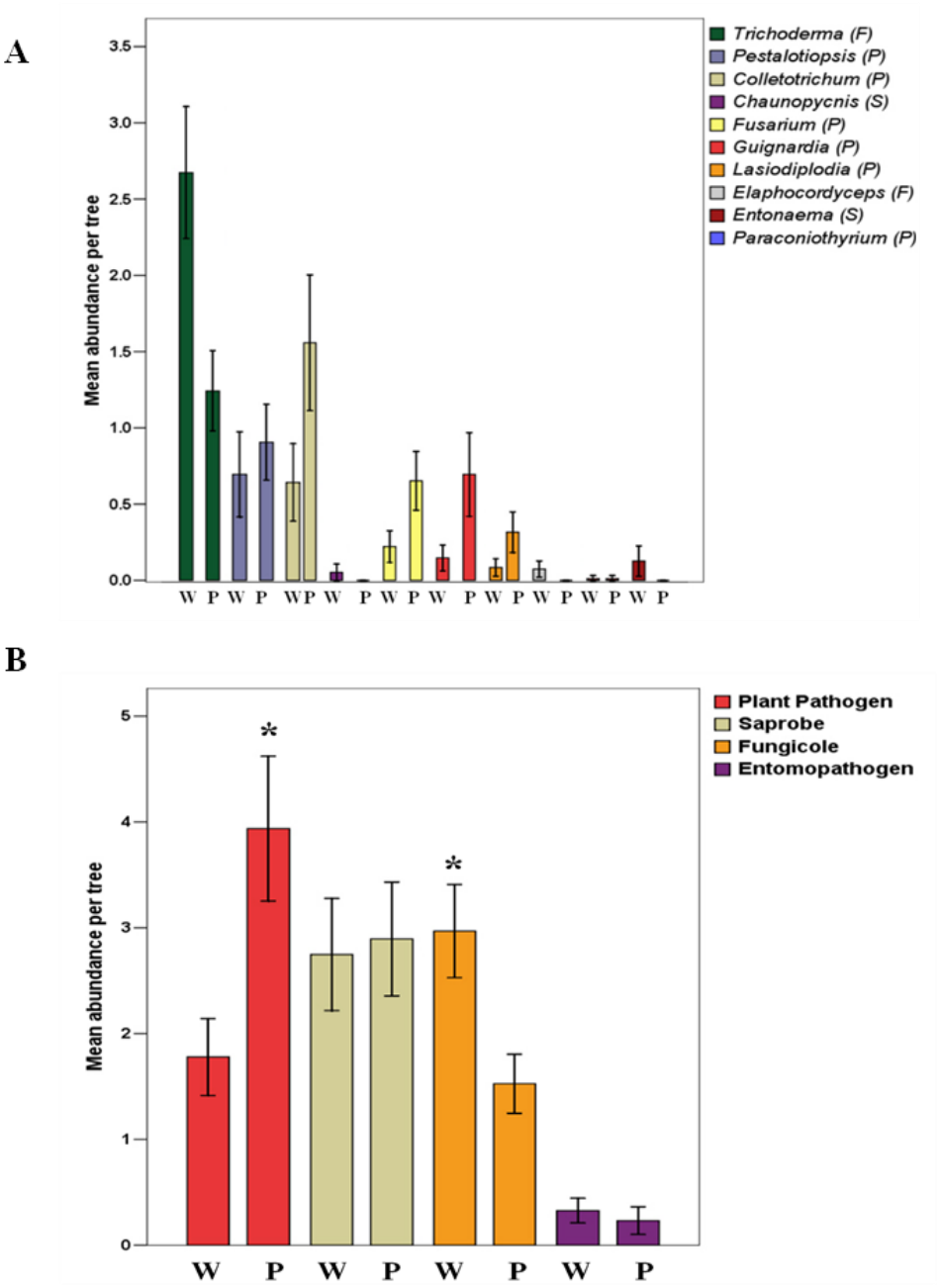


Figure 5.1 Species accumulation curves based on the number of strains due to the significant difference in sampling effort. Y axis shows the 95% confidence interval (CI) of the Species Observed (Sobs). The projected number of species was estimated based on CHAO 1 and was calculated for the lowest common amount of strains (883). The projected total number of species for the wild dataset is 1060±19.47, whereas for the plantation dataset is 932±82.37. Curves were made in EstimateS and plotted in SPSS.



*Projected number of species

Figure 5.2 (A) Bar graph comparing the mean abundance per tree for few fungal genera. Only genera that showed significant differences are included in the figure. **(B)** Bar graph comparing the mean abundance per tree, of isolates belonging to each ecological role. Significant results are marked with an asterisk.



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