Wild trees in the Amazon basin harbor a great diversity of beneficial endosymbiotic fungi:

Is this evidence of protective mutualism?

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ABSTRACT:

It has been shown that the disappearance or drastic changes in ancestral and indigenous (or native) endosymbiotic microbiota can lead to many adverse health consequences. However, the effects of changes in beneficial endosymbionts in plants are poorly known (except for mycorrhizal and rhizobial associations). We sampled and compared endophytes from hundreds of trees belonging to the economically important genus *Hevea*, the source of natural rubber, in their native range in the Amazon basin and in plantations. We also conducted antagonism tests to

determine the potential some of these endophytes may have on selected plant pathogenic fungi. The natural and indigenous endosymbiotic mycota of the rubber tree (*Hevea*) contains a high diversity of beneficial fungi that may be causing a protective effect against pathogens (protective mutualism). In contrast, plantation trees have a reduced and different diversity of these beneficial fungi. We propose that abundance, and not just presence, of competitive fungal strains and species (i.e., *Trichoderma* and *Tolypocladium*) is creating a protective effect against pathogens in wild trees. This study provides support for the importance of mutualistic endosymbionts in plant health and ecosystem resilience, and calls for awareness of their potential loss by human-related activities.

Keywords: biodiversity, biological control, *Hevea*, natural rubber, tropical forest conservation

Introduction

Many studies have revealed that reduction or removal of natural endosymbiotic microbiota can adversely affect the health of their host. This protective mutualism conferred by the host's endosymbiotic community has been reported for many organisms, ranging from plants to humans. One example is the correlation between decrease in disease incidence and the diversity of existing symbionts within mammals' gut ecosystems (Round and Mazmanian, 2009). Similar improvement in host fitness has been reported for insects, in which endosymbiotic species of *Burkholderia* are acquired horizontally every generation (Kikuchi *et al.*, 2007). Even in humans it has been shown that the disappearance of ancestral indigenous commensal microbiota, which is intimately involved in human physiology, is not beneficial and may have many health consequences (Sears, 2005; Blaser and Falkow, 2009).

Protective mutualism conferred by horizontally transmitted microbes has been poorly studied in plants (except for mycorrhizal or rhizobial associations), especially in their native environments (Evans *et al.*, 2003; Berlec, 2012). Horizontal transmission refers to that which occurs among individuals through spores or other *ex planta* propagules; this is in comparison to vertical transmission, which occurs from parent to progeny (Rodriguez *et al.*, 2009). Even though an incredible diversity of horizontally transmitted endosymbiotic fungi (a.k.a. endophytes) has been reported for almost every plant studied, their functions are mostly

unknown. There is evidence that some of these endophytes provide benefits to the plant (e.g., drought resistance, induced resistance to pathogens, and antiherbivory, among others) while others may be detrimental (e.g., latent pathogens) (Saikkonen *et al.*, 1998; Arnold *et al.*, 2000; Rodriguez *et al.*, 2009). However, because most of these studies have characterized endophytes in plants away from their natural habitats (Arnold and Herre 2003, Rubini *et al.*, 2005, Saucedo-Garcia *et al.*, 2014), very little is known about their diversity and function in their native ecosystems, especially in tropical forests.

To begin to shed light on the potential effects of land-use changes (i.e., monoculture plantations) on beneficial fungal endosymbionts, we compared the diversity of endophytic fungi in plantations and in wild trees of the economically important tropical tree, *Hevea brasiliensis* (Euphorbiaceae) within and outside its native range. *Hevea brasiliensis* is the source of natural rubber and is native to the Amazon basin (Shultes, 1970) but planted extensively in Asia and Africa. Because horizontally transmitted endophytes in general are absent from seeds, once those seeds are transported from the wild and introduced to different habitats, they would likely be exposed to a different community of endophyte species, possibly excluding many coevolved or indigenous beneficial species (Evans *et al.*, 2003; Evans, 2008). The premises guiding our study were: (a) the endophyte diversity in wild rubber populations is different to that in plantation trees, because infection by horizontally transmitted endophytes depends on the availability and viability of fungal propagules (e.g., spores) in the surrounding environment; (b) wild trees will harbor a greater number of potentially mutualistic endophytes because these endophytes have coevolved or lived associated with their host longer than those in plantations; (c) trees growing in plantations will have more endophytes with potential to be pathogenic than wild trees because natural enemies (or antagonists) would be less frequent or absent; and (d) selected endophytic strains have antifungal properties against plant diseases. To test these premises, we compared the fungal endophytic community inhabiting the leaves and sapwood of wild and planted rubber trees within and outside its native range in the Amazon basin. We also conducted tests to determine the antagonistic potential that some of these endophytes would have against plant pathogenic fungi. This study provides support for the addition of mutualistic endosymbionts to the list of ecological services provided by natural forests, their importance in plant health and ecosystem resilience, and calls for awareness of their potential loss by human-related activities.

Materials and methods

Tree data and endophyte collection

A total of 190 *Hevea* trees were located in eight different localities, in seven of the localities populations of *H. brasiliensis* were sampled, and in one locality (Los Amigos, LA) the sister species *H. guianensis* was sampled. Data for each tree was annotated, i.e., GPS coordinates, elevation, diameter at breast height (dbh) and height. 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, Table S2 shows environmental data for each site, and Table S3 shows the height and diameter for the wild populations of trees. Fungal endophytes were collected from living leaves and sapwood tissue. Collection protocols are fully described in Gazis and Chaverri (2010), but a summarized description follows. Three segments of *ca.* 3 × 3 mm from each leaflet were surface-sterilized through sequential immersion in 2% (v/v) sodium hypochlorite (bleach) solution, 70% (v/v) ethanol, and sterilized water, and placed onto CMD+ (BBL™ corn-meal-agar + 2% dextrose + antibiotic). To isolate endophytes from stems, living sapwood was exposed on three areas of the stem and in each of those areas three pieces of *ca.* 5 × 2 mm of living sapwood tissue were excised and transferred immediately to Petri plates containing CMD+. An antibiotic solution (1% solution of Neomycin–Penicillin–Streptomycin, Sigma-Aldrich, St. Louis, MO, USA) was added to the media to eliminate bacterial endophytes. The plates were incubated for several days (up to 2 months) and the emerging colonies were subcultured to obtain pure isolates.

DNA extraction, amplification, and sequencing

Individual isolates were grown in Difco™ potato-dextrose-broth (PDB) at 25 °C for up to 2 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) of the nuclear ribosomal DNA were amplified in one reaction, using the ITS5 and LR5 primers (White *et al.*, 1990). Even though the ITS can give an approximate species identification (at least to the level of a species complex), it has been shown that it is not appropriate for *Trichoderma* and that introns of translation elongation factor 1 α locus (*tef1*) are a better tool for identifying species (Chaverri *et al.*, 2015). Due to the importance of this genus for this project, we additionally sequenced the *tef1* region (~500 bp), using the *ef728M* and *ef2* primers (Carbone and Kohn 1999;

Rehner 2001). Refinement in the definition of other fungal species found here could be done in future studies. PCR reactions and conditions for the ITS marker and for the *tefl* are fully described in Gazis and Chaverri (2010) and in Chaverri *et al.* (2015). PCR products were cleaned using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA) and sequenced at MCLAB laboratories (www.mclab.com).

DNA sequence processing, grouping, and taxonomic attribution

Sequencher™ ver. 4.9 (Gene Codes©, MI, USA) was used to assess the quality of sequence chromatograms and to edit them when necessary. Sequences were aligned using MAFFT version 6 with the E-INS-i strategy (Kato and Hiroyuki, 2008). Alignments were visualized using MESQUITE version 7.2 (Maddison and Maddison, 2009) 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 (Schloss *et al.*, 2009). Sequences with a 99 % similarity in ITS sequence were assigned 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 (Rojas *et al.*, 2010; Gazis *et al.*, 2011). Isolates were identified to the genus rank using the BLASTn algorithm implemented in GenBank (www.ncbi.nlm.nih.gov/BLAST). The average sequence length submitted as query was ~500–600 bp (ITS1 + 5.8 + ITS2), and the minimum sequence coverage was set to 90 %. Matches that had 95 % or more ITS similarity, belonging to ex-type cultures or published studies, were considered reliable. *Trichoderma* isolates were identified using the *tefl* region. Sequences for representative OTUs were deposited in GenBank (Table S4).

Statistical analyses

Once all sequences were grouped into OTUs, their frequency (= abundance) 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 of origin (sapwood or leaf). The Kolmogorov-Smirnov test revealed that the data were not normally distributed, non-parametric tests were used for all comparisons. For the first set of comparisons, replicates (individual trees) were separated into two groups: group 1 = wild, 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 family-wise error due to multiple comparisons, only

genera with an isolate abundance of more than 40 were used. For the groups of endophytes that showed significant results, differences in the leaf or the sapwood community or in both were also tested for. For the latter, multiple comparisons were performed 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). Finally, correlation between tree height and diameter and the number of species and abundance, was tested using field data collected in wild localities and the non-parametric test, Spearman's rho. All statistical tests were performed in SPSS ver. 13.0 and the *p*-value was corrected for multiple comparisons (Bonferroni's correction).

Analysis of similarities (ANOSIM) based on 1000 permutations was used to check for management type difference in respect to community composition. Additionally, plots based on Non-metric Multidimensional Scaling (NMDS) and Principal Coordinate Analysis (PCoA), were produced to visualize the aggregation of the trees based on their generic and ecological composition. Four datasets were constructed: (1) complete dataset from all eight localities, (2) all localities minus data from Cameroon, (3) all localities minus data from Cameroon and Mexico, and (4) only data from *H. brasiliensis* distributed within its native range. These datasets were constructed to evaluate the robustness of the results at different geographical scales. The three mentioned statistical analyses were run using the software PAST ver. 3.04 with Bray-Curtis distance (Hammer *et al.*, 2001). Differences in community composition were also evaluated based on phylogenetic information. Fast UniFrac (<http://unifrac.colorado.edu>) was used to determine if the two management types (wild vs. plantation) had significantly different endophytic communities (Hamady *et al.*, 2010). A phylogenetic tree containing all endophyte sequences was used as the input. Due to the high number of sequences, a distance tree (Neighbor Joining) was built with MAFFT using the complete amplicon (ITS1+5.8S+ITS2+partial LSU). A weighted UniFrac significance was run with 1000 permutations and normalized abundances.

Species accumulation curves and sample size coverage

Species accumulation curves for each locality were created in EstimateS ver. 7.5 (Colwell, 2005) 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, significant differences on the number of isolates obtained by each sample size were checked for (Colwell *et al.*, 2004).

Graphics were created using SPSS ver. 13.0. Sample size coverage was estimated following Chao *et al.* (2009).

Ecological analyses

To investigate if there was a trend in the abundance of strains of specific ecological roles in relation to the type of management, a putative ecological role was assigned to each of the OTUs that were identified to genus. “Ecological role” was defined as the nutritional mode and possible or hypothesized relationship that an organism has with its host or with the other members of the endophytic community. This assignment was based on published literature and on the USDA-ARS database (Farr and Rossman, 2012). Genera were assigned to one of the following putative ecological roles: (1) plant pathogen, (2) saprobe, (3) mycotroph (feeds on or parasitizes other fungi), or (4) entomopathogen (feeds on or parasitizes insects) (Table S5). The putatively plant pathogenic species may include those that are also facultative pathogens with a saprotrophic stage. Those labeled as “saprobes” included species that have never been reported as plant pathogens. In cases where isolates belonged to genera for which more than one ecological role has been reported (e.g., *Trichoderma* spp. have been reported as saprobes and mycotrophs), ecological role was assigned based on the closest species match using the BLAST algorithm from GenBank. For instance, most *Trichoderma* isolates found in this study are more closely related to clades reported to be mycotrophs, i.e., *T. harzianum* and *T. koningiopsis* species complexes (Chaverri and Samuels, 2013; Chaverri *et al.*, 2015). Therefore, the isolates belonging to *Trichoderma* were classified as mycotrophs. There were cases in which isolates belonged to clades with different ecological roles (e.g., *Fusarium* and *Purpureocillium*). 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 a saprobe and an entomopathogen (Luangsa-ard *et al.*, 2011). 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 (wild vs. plantation); using similar statistical techniques applied to compare the abundance of each genus (see Statistical Analyses).

Antagonism or competition tests and analyses

EcoSim ver. 7.72 (Gotelli and Entsminger, 2012) was used to investigate if the endophytic community under study was non-randomly structured, which may suggest interspecific competition or other antagonistic interactions among its components as well as differential use of habitats between taxa. Only non-singleton taxa were used in this analysis. Three community-structure indices were calculated: C-score, number of checkerboards species pairs, and number of species combinations (= Combo). The C-score measures species segregation (Stone and Roberts, 1990): if the resulting C-score is significantly larger than the one produced by the null-distribution, then at least some pairs co-occur less often than expected by chance (i.e., taxa are segregated). The reciprocal scenario is also true: if the C-score is less than the one produced by the null model, then more species are co-occurring than expected by chance. An assemblage that is dominated by competition would have taxa that are significantly segregated (i.e., large C-score); on the contrary, an assemblage dominated by mutualistic interactions would have taxa that are significantly aggregated (i.e., small C-score). The number of checkerboards is defined as the number of species pairs in the matrix that form perfect checkerboards and never co-occur. In a competitively structured community, the observed number of checkerboard species pairs should be significantly larger than expected by chance. The third community structure index, Combo, is the number of unique combinations found between pairs of sites. In a competitively structured community, there should be fewer unique species combinations than by chance (Horner-Devine *et al.*, 2007). Default settings and five thousand iterations were used in the EcoSim analysis. In addition, Pairs ver. 1.0 (Ulrich, 2008) was used to investigate if there were pairs of genera that co-occur more or less frequently than expected by chance. To avoid multiple comparisons, only genera with an abundance of 40 and more were included. One thousand iterations were used in this analysis. Because *Trichoderma*, a common mycotroph, was the most abundant group in wild trees, correlation tests between its abundance and that of selected plant pathogenic genera were conducted using a two-tailed Spearman's test in SPSS.

Preliminary *in vitro* antagonism tests were also conducted using 70 *Trichoderma* cf. *harzianum* strains against two pathogens of *Hevea*: *Colletotrichum acutatum* (strain AF1 from Nigeria) and *C. cf. gloeosporioides* (strain 'Colle-Mex' from Mexico) (Holliday, 1995; Jayasinghe *et al.*, 1997; Ogbemor *et al.*, 2007). *Trichoderma* cf. *harzianum*, a species complex, was selected for the biocontrol experiments because of its well-known antifungal activities (El-Hasan *et al.*, 2009; Chen *et al.*, 2012). All the strains that formed a clade and were identified as

T. cf. harzianum, based on their ITS sequence, were tested. These strains were collected in the wild and in plantations (59 and 11, respectively). Plantations were distributed in Peru (IB), Mexico (MX) and Cameroon (CM); and wild habitats in various locations within Peru (MS, IQ, H, LA, T). For the *in vitro* antagonism tests, similar methods to Mejía *et al.* (2008) were used. Briefly, *Colletotrichum* strains were confronted, in dual plate assays, against *Trichoderma* strains. Hyphal plugs (2mm Ø) of pathogens and endophytes were placed on opposite sides of Petri dishes containing potato dextrose agar. *Colletotrichum* strains were plated 3 d earlier due to their slower growth rate. Plates were incubated at constant temperature (25 °C) and under a 12 hr light/dark regime. Evaluation of interactions began 48 hr after endophytes were placed into assay plates. The radius of the colony of each pathogenic strain was measured during the 3d after incubation started. Three control plates containing each pathogenic strain alone were kept for reference, and their average colony radius was used in the comparisons. For *C. acutatum*-*T. cf. harzianum* tests, three types of activity were evaluated: (1) growth inhibition of the pathogen by the *Trichoderma* strain; (2) competition for substrate: overgrowth of one organism by another; and (3) mycoparasitism: direct parasitism on the hyphae of the pathogen generally observed as hyphal coiling. If the *Trichoderma* strain inhibited the growth of the pathogen, showed more radial growth than the pathogen, or parasitized the pathogen, then it is considered preliminary evidence of antagonism. For *C. cf. gloeosporioides*, only growth inhibition tests were done. *C. acutatum* was focused on because of its economic importance and more significant impact in plantations in Asia (Jayasinghe *et al.*, 1997) than those of *C. cf. gloeosporioides*.

Results

A total of 190 *Hevea* trees were surveyed for fungal endophytes; 95 from natural forests in various areas in the Peruvian Amazon and 95 from plantations in Cameroon, Mexico, and Peru. The 2,346 strains collected were classified in 710 putative species (based on 99% ITS nuclear ribosomal DNA sequence similarity clustering) corresponding to 455 genera; 411 and 404 species in wild and plantation trees, respectively. Fifteen percent of the OTUs were shared between management types, representing 105 species (Table S4 and S6). None of the species accumulation curves reached an asymptote, suggesting that there are still many more endophytic species to be found. However, the curve belonging to the plantation dataset was less steeply inclined and the projected number of species was less than the one predicted for the wild community (Fig 1). Leaves and sapwood tissue harbored different fungal communities, in terms

of abundance and species composition, with some genera overlap (Table 1). Diversity was higher in sapwood; whereas abundance was higher in leaves (Fig S1 a-b). Nevertheless, the ecological patterns for the combined datasets (leaves + sapwood), which are described later, were consistent when communities were partitioned by host tissue. Therefore, results presented hereafter refer to the endophytic assemblage inhabiting rubber trees as a whole (leaves + sapwood). Significant results (positive correlation) were only found between tree diameter and the number of OTUs inhabiting the sapwood ($p=0.02$). There was no correlation between tree diameter or height and the abundance of particular genera.

ANOSIM showed a significant difference in community composition among the management types when all the localities were included ($p \leq 0.0001$, $R=0.11$), when Cameroon was excluded ($p \leq 0.0001$, $R=0.10$), when Cameroon and Mexico were excluded ($p \leq 0.0003$, $R=0.17$), and when only data from *H. brasiliensis* distributed within native range was included ($p \leq 0.0001$, $R=0.26$). ANOSIM results using the ecological groups were also significant when all the localities were included ($p \leq 0.0001$, $R=0.13$) and when Cameroon was excluded ($p \leq 0.0001$, $R=0.11$), but not when Cameroon and Mexico were excluded ($p \leq 0.20$, $R=0.04$) nor when only data from *H. brasiliensis* distributed within native range was included ($p \leq 0.06$, $R=0.07$). NMDS ordination based on genera composition did not show evidence for aggregation of trees based on management type, when all the localities were included (stress=0.38), when Cameroon was excluded (stress=0.38), when Cameroon and Mexico were excluded (stress=0.33), nor when only data from *H. brasiliensis* distributed within native range was included (stress=0.32) (Fig S2 a-d). However, when genera were pooled into ecological groups, NMDS showed evidence for aggregation of trees based on management type, when all the localities were included (stress=0.25), when Cameroon was excluded (stress=0.25), when Cameroon and Mexico were excluded (stress=0.19), and when only data from *H. brasiliensis* distributed within native range was included (stress=0.18) (Fig S3 a-d). PCoA biplots showed the aggregation of trees based on management type when using generic composition (Fig S4 a-d) and ecological grouping (Fig S5 a-d). Differences in community composition between management types were confirmed by the Fast UniFrac analysis ($p \leq 0.001$), indicating that each community presents a distinct phylogenetic signature.

Significant differences were found in the diversity (richness and abundance) of potentially beneficial endophytic fungi in wild versus plantations trees. Strains of species known

as mycotrophs or fungal antagonists (e.g., *Trichoderma*; Harman *et al.*, 2004; Hermosa *et al.*, 2012) and insect pathogens (e.g., *Tolypocladium*; Samson and Soares, 1984) were significantly more abundant in trees distributed in the wild (*Trichoderma* Wild = 2.67/tree vs. Plantation = 1.24/tree; *Tolypocladium* Wild = 0.33/tree vs. Plantation = 0.13/tree; $p \leq 0.001$) and were positively correlated with that management type ($p \leq 0.001$; Fig 2 and 3 and Table 1). Overall, the antagonistic fungal genus *Trichoderma* was the most abundant in wild trees, and not so much in plantation trees (Table 1). In contrast, strains belonging to potentially plant pathogenic species (e.g., *Colletotrichum*, *Fusarium*, *Guignardia*, *Lasiodiplodia*, and *Pestalotiopsis*, among others; Holliday, 1995; Jayasinghe *et al.*, 1997; Fernando *et al.*, 2001; Mariau, 2001; Farr and Rossman, 2012) were significantly more abundant in trees in plantations ($p \leq 0.001$; Fig 2, Table 1, and Table S7 and S8), having a positive correlation with that management type ($p \leq 0.01$).

The two community-structure indices, C-score and Combo, suggest that further ecological mechanisms may be shaping the community's species composition and abundance (e.g., competition and antagonism, among others). C-score analyses show that the structure of the endophytic community inhabiting *Hevea* significantly diverges from a random pattern, producing an index larger than expected by chance ($p \leq 0.001$). The Combo index (number of species combinations) shows that the observed number of species combinations was significantly smaller than expected by chance, presumably because of competition ($p \leq 0.04$). No significant results were obtained for the number of checkerboard species pairs index ($p = 0.52$). Pairs ver. 1.0 analysis showed that the pair comprising *Trichoderma*-*Guignardia* (mycotroph-plant pathogen, respectively) occurred significantly less often than under a null model ($p \leq 0.02$). Spearman's rho revealed that the abundance of *Trichoderma* was negatively correlated with the abundance of the plant pathogenic genus *Diaporthe* ($p = 0.02$).

Endophytic *Trichoderma* was negatively correlated with the abundance of endophytic potentially pathogenic genera. The antagonism experiments showed that all *Trichoderma* strains tested inhibited the growth of both pathogenic *Colletotrichum*, ranging from 6.3% to 50% (Fig 4 and 5 and Table 2). There was no difference in growth inhibition of *Colletotrichum* spp. by *Trichoderma* strains from the wild when compared to those from plantations. However, only one strain from plantations reduced the growth of *C. acutatum* by about 30 % (31.3%) (Table S9). In contrast, 10 strains from the wild inhibited the growth of the pathogen by 30% or more (Table S9). In the tests against *C. cf. gloeosporioides*, 11 *Trichoderma* strains from plantation inhibited

the growth of the pathogen by 30–40%; and 52 strains from wild trees inhibited growth by 30–40% and 2 strains by 40–50% (Table S10).

There was evidence of competition (overlap or overgrowth of *Trichoderma* over *Colletotrichum*), antibiosis (formation of inhibition zone or halo), and mycoparasitism (*Trichoderma* hyphae coiling around *Colletotrichum* hyphae)(Fig 6 A-D). 40.7 % and 36.4% of *Trichoderma* strains from the wild and plantation, respectively, overlapped or overgrew *Colletotrichum*, suggesting that competition is the main antagonistic mechanism (Tables S9 and S10). Sixteen (27.1%) and four (36.4%) of the confrontations with *Trichoderma* strains from wild and plantations, respectively, showed a combination of antibiosis and competition. In only 18.6% of the confrontations with strains from wild trees (none from plantation), was an inhibition zone observed. Mycoparasitism was only clearly observed in three confrontations, one and two *Trichoderma* strains from plantation and wild, respectively (Fig 6B and C). Even though there was growth inhibition in a few of the tests, neither antibiosis, competition nor mycoparasitism could be clearly determined.

Discussion

We found that the natural and indigenous endosymbiotic mycota of the rubber tree (*Hevea*) contains a high diversity of beneficial fungal species that may be causing a protective effect against pathogenic fungi (protective mutualism). In contrast, plantation trees lacked most of these beneficial fungi, thus adding an additional hypothesis behind the causes of disease outbreaks in plantations (or other plant crops). Wild *Hevea* trees were dominated by strains of the mycotrophic or antagonistic genus *Trichoderma* and the entomopathogenic genus *Tolepocladium* (Table 1). A similar trend was also reported by Evans *et al.* (2003) in wild *Theobroma* spp. trees distributed in the Amazon basin. Even though *Hevea* is cultivated in tropical regions, additional factors other than temperature and rainfall likely influence the differences observed between management types. Differences in available *inocula* and surrounding vegetation (which act as reservoirs of fungal species) are important variables that create a low species overlap between wild and plantation habitats (Helander *et al.*, 2007). The latter confounding factors have not been taken into account in this study as we are solely describing a pattern observed when comparing wild and plantation habitats outside and within the native range of the targeted plant host. Moreover, patterns described here are mainly based

on hypothesized functional groups, as the OTUs have been assigned an ecological role based on experimental knowledge about their closest relatives. Nevertheless, this research serves as baseline for future testing of ecological hypotheses and encourages the search for natural enemies in native habitats.

We found a significant negative correlation between the abundance of *Trichoderma* and potentially pathogenic endophytic species (i.e., latent pathogens). Previous studies have shown that *Trichoderma* can parasitize fungal pathogens, produce antifungal secondary metabolites, outcompete fungal pathogens, promote host growth, and enhance disease resistance in plants (Harman *et al.*, 2004; Hermosa *et al.*, 2012). We, thus, propose that *Trichoderma* is an important or even essential component in the protection against pathogens in natural forests. We hypothesize that abundance, and not just presence, of competitive *Trichoderma* strains and species is creating a protective effect against pathogens in wild trees. In our study, trees in the wild had *ca.* 2.5× more *Trichoderma* strains than trees in plantations. This supports a “multiple defender effect” hypothesis, which was first described for corals and their mutualistic crustaceans (McKeon *et al.*, 2012). The multiple defender effect hypothesis states that the presence of multiple mutualists creates a more synergistic, additive and protective effect than just the presence of one of them. Another study concluded that the combined effects of arbuscular mycorrhizal fungi and fungal endophytes on plant growth were additive (Larimer *et al.*, 2012). In our antagonism tests, even though there were no differences in average growth inhibition by *T. harzianum* against *C. acutatum* and *C. gloeosporioides*, the majority of the strains from wild trees had a much stronger inhibitory effect (30–50%).

Although with lower incidence than in sapwood (Table 1), *Trichoderma* isolates were also recovered from living leaf tissue. The latter contradicts studies targeting *Coffea arabica*, for which *Trichoderma* was shown to inhabit its rhizosphere but was not recovered as a leaf endophyte (Mulaw *et al.*, 2010, Vega *et al.*, 2010, Mulaw *et al.*, 2013). However, these other studies were conducted in different geographic regions and following different protocols. On the other hand, other studies targeting tropical trees such as *Guarea guidonia* (Gamboa and Bayman, 2001) and *Manilkara bidentata* (Lodge *et al.*, 1996), support our findings. Furthermore, experimental assays have demonstrated that certain *Trichoderma* species can enter and migrate from the soil to the above ground tissue (including leaves) through the root system (Bailey *et al.*, 2008) or from the air to trichomes to the leaf tissue (Bailey *et al.*, 2009).

Strains with a predicted entomopathogenic ecological role were also significantly more abundant in wild trees ($p \leq 0.001$, Table 1), almost double than in plantations. Most of these strains belonged to the genus *Tolypocladium*, which is known to deter and parasitize diverse groups of insects (Samson and Soares, 1984), especially those that feed on plant roots (e.g., cicada nymphs and scarab beetle larvae). Previous research has shown that entomopathogenic fungi can become endophytic (i.e., *Beauveria*, *Metarhizium*, *Purpureocillium*) (Evans *et al.*, 2003; Vega *et al.*, 2008) but their potential role as mutualists has not been widely tested. *Tolypocladium* species have been screened for their potential applications in biological control of crop pests, showing promise in the control of some cacao fungal diseases and pest aphids (Hanada *et al.*, 2010; Scorsetti *et al.*, 2012). Even though we did not test the *Tolypocladium* strains isolated in this study, based on their close phylogenetic relationship to species with known entomopathogenic properties (Gazis *et al.*, 2014), we considered them as potential candidates for future biocontrol assays against *Hevea* pests (e.g., the litter beetle *Luprops tristis*, mites of the family Eriophyidae and *Tenuipalpus*, and the lace bug *Leptopharsa hevae*) (Sabu and Vinod, 2009; Santos, 2010). It is believed that the primary mechanism of insect deterrence is through the production of secondary metabolites (Vega *et al.*, 2008; Ownley *et al.*, 2010).

Besides the multiple defender hypothesis explained earlier, the causes behind the absence of severe epiphytotics in tropical forests can also be explained through two other evolutionary hypotheses. The “arms race” hypothesis states that 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 and Shirazu, 2005). The “mutualist facilitation” hypothesis also supports our results. This hypothesis contends that the replacement of lost mutualists/commensals (e.g., *Trichoderma* and *Tolypocladium*) 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 (Klironomos, 2003). 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 (Parker and Hay, 2005). 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.

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 plant disease control practices should attempt to recreate this setting. One strategy is to introduce multiple protective endosymbionts, such as fungi (Jones, 2013) but also bacteria (Compant *et al.*, 2010), from a host's native habitat, analogous to the use of probiotics to improve health in humans or other animals (Fraga *et al.*, 2011; Berlec, 2012; Alonso and Guarner, 2013). These species should have high specificity to their hosts or prey that is supported by a long-shared evolutionary history. Endosymbionts with antagonistic properties against pathogens that affect rubber trees could be inoculated in seedlings before they are grown in plantations (Bailey *et al.*, 2008). This strategy, in addition to other practices such as resistance breeding programs, promoting stands composed by individuals with heterogeneous genetic background and quarantine regulations to prevent the spread of endemic pests, should be undertaken as an integrated pest control management for natural rubber production.

This research emphasizes the importance of endosymbionts as an ecological service derived from natural forests. Each year, extensive areas of Amazonian forest are destroyed as a consequence of human activities (Almeyda Zambrano *et al.*, 2010). Deforestation results in the loss of valuable endemic species from all Kingdoms of life, of which many have the potential for providing valuable ecosystem services. With these vanishing areas, we are losing the chance of discovering, bioprospecting and later exploiting an uncountable number of species unknown to science. Studies that aim to uncover the value and utility of species that are of microscopic size, have a microhabitat specialization and act as obligate symbionts with their hosts, promote the value of sustaining native tropical forests.

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Figure Legends

Figure 1. Species accumulation curves. 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.

Figure 2. Comparison of the mean abundance per tree for strains that showed significant differences between management regimes (M = mycotroph, P= plant pathogen, E=entomopathogen). Only genera that showed abundance higher than 40 individuals were used in this analysis.

Figure 3. Species accumulation curves for (A) mycotrophs, (B) entomopathogens and (C) plant pathogens.

Figure 4. Results from antagonistic assays. (A) Growth inhibition results from the confrontation assays using *Trichoderma* strains against *Colletotrichum acutatum* (AF1).

Figure 5. Growth inhibition in the confrontations assays using *Trichoderma* strains against *Colletotrichum cf. gloeosporoides* ("Colle-Mex").

Figure 6. Examples of antagonism. (A) A 7d culture of *Colletotrichum acutatum* (strain AF1) growing in PDA; (B) Dual culture of *Trichoderma* strain IQ214 against *C. acutatum* (strain

AF1), showing the formation of inhibition halo; (C) Dual culture of *Trichoderma* strain MS173 against *C. acutatum* (strain AF1), showing *Trichoderma* overgrowing *C. acutatum* and sporulating over its colony; and (D) Micrograph of a section from a dual culture of *Trichoderma* MS173 against *C. acutatum* (strain AF1), showing the coiling of the *Trichoderma* hyphae around *C. acutatum* hyphae.

Table 1. Genera that showed significant differences in their abundance per tree, based on their type of management. Significance was only calculated for the estimates per tree, using the non-parametric Mann-Whitney implemented in SPSS (U), and is denoted by bold font. N represents the number of independent samples used in each analysis. Four localities were sampled for each management type, encompassing a total of 190 individual trees (95 trees in each management type).

	Abundance (N = 4)			Mean / per tree (N = 95)		Mean / per tree / sapwood (N=95)		Mean / per tree / leaf (N=60)	
	T	W	M	W	M	W	M	W	M
<i>Colletotrichum</i>	209	61	148	0.64	<u>1.56</u>	0.05	0.09	1.08	<u>2.35</u>
<i>Entonaema</i>	13	1	12	0.01	<u>0.13</u>	0.00	0.00	0.02	<u>0.20</u>
<i>Fusarium</i>	108	36	72	0.38	<u>0.76</u>	0.25	<u>0.54</u>	0.24	0.37
<i>Guignardia</i>	80	14	66	0.15	<u>0.69</u>	0.03	0.05	0.24	<u>1.03</u>
<i>Lasiodiplodia</i>	38	8	30	0.08	<u>0.32</u>	0.03	<u>0.28</u>	0.10	0.05
<i>Paraconiothyrium</i>	6	6	0	<u>0.06</u>	0.00	<u>0.04</u>	0.00	0.02	0.00
<i>Pestalotiopsis</i>	152	66	86	0.69	<u>0.91</u>	0.26	<u>0.79</u>	<u>0.80</u>	0.21
<i>Tolypocladium</i>	43	31	12	<u>0.33</u>	0.13	<u>0.33</u>	0.13	0.00	0.00
<i>Trichoderma</i>	372	254	118	<u>2.67</u>	1.24	<u>2.32</u>	1.05	<u>0.75</u>	0.27

Abbreviations: T= total (wild + managed); W = wild; M = managed.

Table 2. Number (#) and percentage of *T. cf. harzianum* strains with antagonistic activities (antibiosis, competition, growth inhibition, and mycoparasitism) against *C. acutatum*.

	Origin of <i>Trichoderma</i> strain	
	Wild	Plantation
Activity	# (%)	#(%)
Growth inhibition	59 (100)	11 (100)
Only competition (overlap)	24 (40.7)	4 (36.4)
Only antibiosis (inhibition zone)	11 (18.6)	0
Antibiosis and competition	16 (27.1)	4 (36.4)
Antibiosis, competition, and mycoparasitism	2 (3.4)	1 (9.1)
No antibiosis, competition or mycoparasitism observed	12 (20.3)	2 (18.2)
TOTAL	59 (100)	11 (100)

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