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Abstract

- Approximately 10% of vascular plants are epiphytes and, even though this has long been ignored in past research, can interact with a variety of fungi, including mycorrhizal ones. However, the structure of fungal communities on bark, as well as their relationship with epiphytic plants, is largely unknown.
- To fill this gap, we conducted environmental metabarcoding of ITS-2 region to understand the spatial structure of fungal communities of the bark of tropical trees, with a focus on epiphytic orchid mycorrhizal fungi, and tested the influence of root proximity.
- For all guilds, including orchid mycorrhizal fungi, fungal communities were more similar when spatially closed on bark, *i.e.*, displayed positive spatial autocorrelation. They also showed distance decay of similarity from epiphytic roots, meaning that their composition on bark increasingly differed, compared to roots, with distance from roots.
- We first showed that all the investigated fungal guilds presented a spatial structure at very small scales. This spatial structure was influenced by the roots of epiphytic plants, suggesting the existence of an epiphytic rhizosphere. Finally, we showed that orchid mycorrhizal fungi were aggregated around them, possibly resulting from a reciprocal influence between the mycorrhizal partners.

Key words

epiphytism; fungal guilds; metabarcoding; fungal spatial distribution; orchid mycorrhizal fungi; Tulasnellaceae

1. Introduction

Although globally distributed, microorganisms present a highly variable local richness and a spatial structure at every scale (from centimeters to thousands of kilometers), especially in soils (Green *et al.*, 2004; Green & Bohannan, 2006). Much of the soil microbial biodiversity appears to be intrinsically linked with plants in the rhizosphere and controls their community structure by monitoring soil-root interactions (Bever *et al.*, 2010). Reciprocally, soil microorganisms that develop nutritional and protective symbioses with roots are especially structured by host presence and diversity (Peay *et al.*, 2013) such as the mycorrhizal fungi that associate with approximately 90% of the vascular land flora (Van Der Heijden *et al.*, 2015; Brundrett & Tedersoo, 2018). Fungal metabarcoding studies in soils have shown that the mycorrhizal taxa are not randomly distributed, but exhibit spatial structure at rather fine scales, in temperate as in tropical systems (Anderson et al., 2014; Bahram et al., 2016; Coince et al., 2013; Pickles et al., 2010; Tedersoo et al., 2010; Zhang et al., 2017), *i.e.,* a patchiness due to host distribution, but also other factors such as spore dispersal and community interactions (Hanson *et al.*, 2012). However, the characterization of the underground distribution of soil fungi (mycorrhizal fungi, saprotrophs or pathogens) is complicated by the three-dimensional nature of soils, since differences may exist between soil horizons (Anderson et al., 2014; Bahram et al., 2015).

Unlike soils, tree barks can be easily investigated as their multiple layers can be sampled and sequenced at once, especially on young trees where the bark is usually thin. Thus, young barks can be seen as virtually two-dimensional and are ideal systems for surveying the spatial distribution of fungal communities and mycorrhizal taxa around their epiphytic plant hosts. Indeed, ca. 10% of vascular plant species root on barks in the tropical wet forests around the globe (Zotz, 2016). These plants have long been considered as essentially non-mycorrhizal in

such aerial substrates (Lehnert *et al.*, 2017; Brundrett & Tedersoo, 2018; but see Rowe & Pringle, 2005) and their fungal partners have thus so far largely been ignored. However, there is now growing interest in the field of epiphytic fungal endophytes which could strongly influence the dynamics of epiphyte plant populations (Leroy *et al.*, 2019). One symbiosis that regularly occurs in the epiphytic habitats is the orchid mycorrhiza (Martos *et al.*, 2012; Herrera *et al.*, 2018; Novotná *et al.*, 2018). Epiphytic orchids, representing no less than 80% of this hyper-diverse plant family (with over 25 000 species (Givnish *et al.*, 2015)), harbor typical hyphal coils within their root cortical cells, which are formed by the same families but different species of saprotrophic basidiomycetes (Dearnaley *et al.*, 2012; Martos *et al.*, 2012; Xing *et al.*, 2019) compared to soil. The fungi are also required for germination of the minute, nutrient-poor orchid seeds (Smith & Read, 2008). It was therefore hypothesized that the distribution of orchids must be constrained by that of their mycorrhizal fungi (McCormick & Jacquemyn, 2014; McCormick *et al.*, 2018)

The distribution of orchid mycorrhizal fungi (OMF) has been investigated in soils (Jacquemyn *et al.*, 2014, 2017; McCormick & Jacquemyn, 2014; McCormick *et al.*, 2016, 2018; Voyron *et al.*, 2017), but only marginally on barks (Kartzinel *et al.*, 2013), perhaps because most studies focus on temperate and Mediterranean ecosystems where orchids are strictly terrestrial. For example, two recent studies (Waud *et al.*, 2016b,a) showed a decline in abundance and similarity composition of OMF with distance from adult orchids, which likely explains the patchy distribution of grassland orchids (Jacquemyn *et al.*, 2007, 2014). Still in grassland habitats, Voyron et al., (2017) found that communities of OMF are more similar in nearby soil, *i.e.,* display spatial autocorrelation (Hanson *et al.*, 2012). As for the epiphytic environment, very little is known on the spatial distribution of mycorrhizal fungi on bark [but see (Izuddin *et al.*, 2019) for a first approach]. Similarly, the evolution of their community structure by distance

to epiphytic host roots is not yet resolved. We can only hypothesize that the low hydro-mineral supply (Yoder *et al.*, 2010; Izuddin *et al.*, 2019) and the particular structure of tree bark (*e.g.*, flaking bark, Rasmussen & Rasmussen, 2018) can constrain both orchids and fungi, and make the symbiosis even more necessary for the mycorrhizal partners.

Here, we aimed to bridge the gap in knowledge of epiphytic fungal and mycorrhizal fungal communities through an investigation of the fine-scale spatial distribution on trees of (i) all bark fungi, considering (ii) each fungal functional guild (including endophytes), and (iii) the OMF particularly. We investigated a low number of trees in order to design very dense sampling, especially in the vicinity of epiphytic roots, in order to get a first detailed view of the structure of fungal communities on bark and of their relationship to vascular epiphytic plants. We hypothesized that (i) as described in soils, these communities have no random distribution on the bark (patchiness). Due to the ability of many fungi to colonize plant roots, (ii) their distribution should be modulated by the distance to roots of vascular epiphytes. Particularly, (iii) communities of OMF should be aggregated around their orchid hosts.

2. Materials and Methods

2.1 Study area

The study site was situated in a protected fragment of the Atlantic Forest at “Parque Estadual Serra do Brigadeiro” (Rolim & Ribeiro, 2001), a secondary forest in transition between Dense Rain Forest and Semideciduous Forest (Fávaro, 2012), close to the city of Araponga, Minas Gerais state, Brazil (20°43’15.3”S; 42°28’54.0”W; elevation 1050 m ; IEF, 2007). The elevation provides frequent fogs throughout the year, and the humidity is around 80%, even in the dry

126 season. The climate of the region is humid subtropical mesothermic, with temperatures ranging
127 from 17 to 23°C and annual rainfall averaging 1300 mm (Rolim & Ribeiro, 2001). This forest
128 is characterized by medium to large trees, and a high diversity of orchid species, the majority
129 of which are epiphytic (Lana *et al.*, 2018).
130
131 **2.2 Bark and root sampling**
132
133 Two trees belonging to *Siparuna* sp. (Siparunaceae; tree 1) and *Himathanthus sucuuba*
134 (Apocynaceae; tree 2) were selected in February 2015 and February 2016 (95 m away from
135 each other) respectively because they had epiphytic orchids growing on their lower trunk,
136 namely *Isochilus linearis* and *Epidendrum armeniacum*. Flexible plastic grids of regular mesh
137 size (7 cm) were placed around these trunks covering the whole circumference of tree 1 (58 cm,
138 Fig. 1) and tree 2 (23 cm, Fig. S1): grid 1 extended from 2 to 2.7 m above the ground and
139 encompassed five individuals of *I. linearis*; whereas grid 2 extended from 0.7 to 2.4 m and
140 encompassed five *I. linearis* and two *E. armeniacum* individuals. On each point of the grid, we
141 sampled 100 mg of bark in the center of every pore of the grid using a sterilized scalpel without
142 removing the covering lichens, mosses, or liverworts. The samples thus consisted in small
143 amounts of surface bark (to the cambium, itself not included) and its cryptogamic cover, which
144 was facilitated by the young age of trees and the thinness of these species' bark. We also
145 sampled 3 mm root sections every 1.5 cm along each epiphytic orchid root starting from the
146 stem (Fig. 1, S1). Bark was also collected under each root sample. On grid 1, we took additional
147 bark samples in the close vicinity of orchid roots (Fig. 1). This resulted in a total of 482 samples
148 for the two grids (*i.e.,* 167 on grid 1 and 315 on grid 2; Table S1) The rationale behind this
149 dense sampling was that, (i) we expected high turnover of fungal communities at a scale of a
150 few centimeters and (ii) we did not want to miss any important fungi on the bark, especially

151 endophytic ones. All samples were frozen at -20°C within few hours in the nearby field
152 laboratory of the Serra do Brigadeiro State Park headquarters for downstream molecular
153 analyses. Two additional thin sections of orchid roots surrounding each sampled piece were
154 collected to check for mycorrhizal fungal colonization on the following day under the
155 microscope and all, without exception, displayed hyphal coils in at least one of each inspection
156 section.
157
158 **2.3 High-throughput sequencing of fungal communities**
159
160 Bark and root samples were kept frozen and manually grinded in liquid nitrogen until a fine
161 powder was obtained. Genomic DNA was extracted from this powder using the NucleoSpin
162 Soil™ kit (Macherey-Nagel) following manufacturer's instructions and then each isolate was
163 directly set to amplification trials using two primer pairs: ITS86-F/ITS4 (White et al., 1990;
164 Turenne et al., 1999) amplifying the internal transcribed spacer 2 (ITS-2) of ribosomal DNA
165 (Yang et al., 2018) of most fungi, and ITS86-F/ITS4-tul (Taylor & McCormick, 2008)
166 amplifying the same region in Tulasnellaceae, since this OMF family requires specific primers
167 for amplifying its ribosomal DNA (Martos et al., 2012). High sample multiplexing (up to 576
168 samples for each primer pair) was allowed through the use of a unique pair of barcoded primers
169 during the PCRs, *i.e.,* 36 forward primers (ITS86-F barcodes 1-36) and 16 reverse primers
170 (ITS4 barcodes 1-16 in fungi-specific, or ITS4-Tul barcodes 17-32 in Tulasnellaceae-specific,
171 PCRs). Each barcode was a unique sequence of 8 bases added to the 5' end of the primer
172 sequence, and the set of 36 barcodes was designed in order to have at least five differences
173 between barcodes.
174 Tagging system negative controls were performed at this step (Hornung *et al.*, 2019; Zinger *et*
175 *al.*, 2019), *i.e.,* pairs of barcoded primers were intentionally omitted in the final sequencing to

control for cross-contamination. PCR reactions were performed in 25 µL containing 0.2 mM each dNTP, 0.2 mM each primer, 1 unit AmpliTaq Gold® 360 DNA Polymerase (Life Technologies, Carlsbad, CA), 1X AmpliTaq Buffer supplied with $MgCl_2$ and 1.5 µL template DNA, using this program: initial denaturation 10 min at 95°C; 30 cycles of denaturation 30s at 95°C, annealing 30s at 56.5°C (ITS86-F/ITS4) or 55°C (ITS86-F/ITS4-tul), and elongation 30s at 72°C; final elongation 7 min at 72°C. Plate designs were randomized in order to avoid possible cross-contamination leading to misinterpretation in subsequent spatial analysis. After visualization on gel, the positive amplicons were purified with NucleoMag® NGS Clean-up and Size Select (Macherey-Nagel, GmbH & Co KG.), quantified by fluorescence with Qubit™ dsDNA High-Sensitivity (Invitrogen™), and pooled in equimolar ratios prior to library preparation and 2x250 bp paired-end sequencing on an Illumina MiSeq platform at Fasteris (Geneva, Switzerland). Three positive controls (mock community) and three negative controls (ultrapure water) were used per PCR trial (plate), resulting in a total of 36 positive and 36 negative controls in total. All of them were added to the amplicon pool for subsequent bioinformatic analysis (Hornung *et al.*, 2019; Zinger *et al.*, 2019). The mock community used in positive controls was a defined mixture of fungal DNAs of known concentration, including 4 Ascomycota and 21 Tulasnellaceae grown in pure cultures.

2.4 Sequencing data processing

Paired reads were merged using BBMerge (Bushnell *et al.*, 2017) and the resulting reads were demultiplexed using BBDuk (sourceforge.net/projects/bbmap/); mistagging (Zinger *et al.*, 2019) was also controlled but represented <0.2% of the reads. The reads were trimmed from their barcode+primer sequences allowing zero discrepancy, filtered for size >200 bp and quality ≥25 using CUTADAPT (Martin, 2011); sequences with "Ns" were removed at this step.

Operational taxonomic units (OTUs) were constructed using the SWARM 3.0 algorithm (Mahé *et al.*, 2015) with a resolution of 5 (Schneider-Maunoury *et al.*, 2018). Chimeras were removed using both the *de novo* search option of VSEARCH (Rognes *et al.*, 2016) and a reference-based search of UCHIME (Edgar *et al.*, 2011) against the reference dataset v7.2 (Nilsson *et al.*, 2015). The original (*i.e.,* trimmed) reads were then mapped on the filtered SWARM representative sequences using BLASTN (Altschul *et al.*, 1990) and a 97% threshold. This step deals with sequences of different size, especially when using two sets of primers amplifying different fragment lengths. OTUs represented by only one sequence (singletons) were removed. The most abundant sequence was chosen as representative of each OTU, and the final taxonomic assignment was performed using *assign_taxonomy.py* ('blast' assignment method, with default e-value of 0.001) in QIIME 1.9.1 (Caporaso *et al.*, 2010) against UNITE v8.0 ('All eukaryotes'; https://dx.doi.org/10.15156/BIO/786350). Raw sequences are available in Sequence Read Archive (SRA) under the BioProject accession no. PRJNA692353.

Two OTU tables were retrieved for each primer pair and merged into a single OTU table. OTUs were filtered with the DECONTAM package (Davis *et al.*, 2018) using the "frequency" method. We also discarded those found in the negative and positive PCR controls (Hornung *et al.*, 2019), and those of non-fungal origin. Moreover, we ran rarefaction curves using QIIME 1.9.1 (Caporaso *et al.*, 2010) (Fig. S2) and then rarefied the OTU table at 10^3 sequences (in order to both maximize the number of samples kept for subsequent analyses and the fungal community read coverage) (McKnight *et al.*, 2019) using the *rrarefy* function in the VEGAN package of R (Oksanen *et al.*, 2013).

2.5 Fungal functional guilds

OTUs found in at least one orchid root sample were considered as *endophytes*. Among them, those Basidiomycota belonging to Tulasnellaceae, Ceratobasidiaceae (Veldre *et al.*, 2013), Serendipitaceae (Weiß *et al.*, 2016), and Atractiellales (Kottke *et al.*, 2010) were recognized as *orchid mycorrhizal fungi* (OMF) (Dearnaley *et al.*, 2012). Besides, trophic guilds were assigned to all OTUs using FunGuild (Zanne *et al.*, 2019): we chose to keep those which were either exclusively *saprotrophs, symbiotrophs, plant pathogens,* or *lichenized fungi*. As a consequence of this filter, OTUs used for guild analyzes were mainly identified at least at the genus level (85.3%) or at the family level (97.2%). For the remaining OTUs, guilds provided by FunGuild were validated based on the author's expertise The OMF were kept in a separate category despite their saprotrophic and symbiotrophic ability (Dearnaley *et al.*, 2012; Selosse & Martos, 2014).

2.6 Statistical analyses

All statistical analyses (except Neutral Community Model which are conducted from abundance data) were conducted from presence-absence data because the specific primer pair biased the read counts of Tulasnellaceae relative to other fungi. We tested the effect of sample sources (root or bark, grid 1 or 2) on the composition of fungal communities with PERMANOVA (999 permutations; grids as "strata") using *adonis*, complemented with a *betadisper* test as indicated in the corresponding VEGAN package of R (Oksanen *et al.*, 2013). The data were visually assessed by nonmetric multidimensional scaling ordination (NMDS) using *metaMDS* in VEGAN. Additionally, we tested for each OTU the ecological dispersion from bark to orchid roots using a neutral community model (Sloan *et al.*, 2006) as detailed in Venkataraman *et al.* (2015) and Burns *et al.* (2016). Briefly, for each grid, the relationship between the abundance of an endophytic OTU (considering the OTUs shared between bark and

roots only) on the overall bark and the frequency detection of this OTU in roots was compared to a neutral model. This neutral model was computed using the *nlsM* function of the MINPACK.LM package of R. The variability of the model was assessed using 95% binomial proportion confidence interval (Wilson, 1927) using the HMISC package of R and the goodness of fit of the model was assessed using the coefficient of determination (R^2).

For fine-scale spatial analyses, we then calculated Euclidean distances, preferring the shortest distance between two samples on the cylindrical trunk, and the Jaccard index of ecological similarity using *vegdist* in VEGAN. Spatial autocorrelation of fungal communities was analyzed on each grid for (i) composition using Mantel in the ECODIST package of R, and (ii) richness using Moran's *I* in the SPDEP package of R; for the latter, the neighborhood matrix was computed considering a neighboring distance <10 cm. The significance of Mantel and Moran statistics was assessed by permutational tests (999 replicates). We further tested the spatial structures of single OTUs using the *joincount.test* in SPDEP.

Finally, we assessed the turnover of bark fungal communities with increasing distance from the roots, for OTU composition (*i.e.,* distance decay of similarity, based on Jaccard index, between root and bark samples) and OTU richness using a generalized linear model (*glm* function in the default package of R) specifying a log-link binomial (Millar *et al.*, 2011) and a negative binomial distribution respectively. As the similarities between samples are not independent of one another, coefficients of the binomial GLM were obtained using a leave-one-out Jackknife procedure as described in (Millar *et al.*, 2011). The significance of the distance decay of similarity was tested using a permutational Mantel test (Spearman method, 9999 permutations; Anderson et al., 2013), while the significance of the distance decay of richness was assessed by ANOVA (F-test).

3. Results

3.1 Roots and bark harbored distinct, but partially overlapping fungal communities

We retrieved a total of 22 554 729 reads after the molecular analyses and high-throughput sequencing. The primer pair ITS86-F/ITS4 yielded much more data and diversity (6 823 OTUs, 36 477 ± 31 597 sequences per sample) than the specific ITS86-F/ITS4-tul set of primers (41 OTUs, including 17 Tulasnellaceae, 15 589 ± 11 583 sequences per sample). Eighteen OTUs, including two Tulasnellaceae, were amplified by both sets of primers. Ascomycota from positive controls (mock community) were all retrieved after sequencing while Tulasnellaceae showing no mismatch with IT86-F and ITS4 or ITS4-tul primers were often but not always (56%) retrieved after sequencing, depending on their DNA quality and/or concentration.

After removing the controls, 3 121 888 passed our quality (removal of contaminants) and taxonomic filters (fungi only), resulting in a total of 4 390 fungal OTUs in the whole dataset (348 samples, Table S1), including Ascomycota (2 670) and Basidiomycota (1 303) mainly, but also Chytridiomycota (23), Mortiellomycota (9), Mucoromycota (7), Rozellomycota (2), Glomeromycota (1) and unidentified fungi (375). Overall, Agaricomycetes (Basidiomycota) were the most abundant (1 782 079 sequences; 57.08%), followed by Dothideomycetes (Ascomycota; 507 594 sequences; 16.26%; Fig. S3, Table S2). The list of all fungal families and genera detected in the dataset is presented in Table S3. Notably, Sebacinales OTUs all belonged to the clade of Serendipitaceae (data not shown; Weiß *et al.*, 2016).

Fungal richness (average number of OTUs per sample) was significantly different between bark

and roots within each grid, *i.e.,* grid 1 (116 ± 90 in bark, 42 ± 32 in roots) and grid 2 (125 ± 63 in bark, 255 ± 108 in roots; $p<0.001$ in both cases), as well as between roots ($p<0.001$) and between bark ($p=0.0081$) from the two grids.

Total fungal communities were significantly different between both grids (PERMANOVA: $F = 13.91$, $R^2 = 0.047$, $p = 0.001$, betadisper: $F = 12.69$, $p < 0.001$), and between roots and bark (PERMANOVA: $F = 4.57$, $R^2 = 0.016$, $p = 0.001$, betadisper: $F = 29.67$, $p < 0.001$), as shown by nested PERMANOVA. Additionally, grids and compartments (root *versus* bark) clearly segregated in NMDS analyzes (Fig. 2), despite significant heteroscedasticity which has been shown to poorly affect PERMANOVA test (Anderson & Walsh, 2013). However, 265 (18.8%) and 1,000 (38.4%) OTUs were shared between bark and roots in grids 1 and 2, respectively (Table S4). Neutral Community Model (NCM) analyses showed that, on both grid, endophytic fungal communities tended to differ from a neutral dispersal model 'from bark to roots' but were on the contrary largely overrepresented in the root compartment (Fig. S4-5). Only lichenized fungi on grid 2 were unambiguously less overrepresented in roots than other fungi. (Fig. S6). When considering roots only, fungal communities differed between orchid individuals (PERMANOVA: $F = 1.39$, $R^2 = 0.148$, $p = 0.001$, betadisper: $F = 98.47$, $p < 0.001$) and species (PERMANOVA: $F = 1.92$, $R^2 = 0.023$, $p = 0.001$, betadisper: 10.48, $p = 0.002$), but more strikingly between grids (PERMANOVA: $F = 4.91$, $R^2 = 0.058$, $p = 0.001$, betadisper: $F = 23.96$, $p < 0.001$; Fig. S7), with only 237 (10.36%) shared OTUs (Table S4).

Among the 31 OMF OTUs that were found, encompassing the four OMF families (see 2.5, Fig. 3), only five OTUs were shared between the two trees after rarefaction (Table S4) and only one OTU (Tulasnellaceae, TUL-1) when considering the roots only (Table S4, S5). However, the sharing of OMF between grids was not statistically different to that of other fungi, meaning that

the trees harbored different fungal communities overall. On grid 2, where two orchid species co-exist, OMF OTUs belonging to Ceratobasidiaceae (CER-1) and Serendipitaceae (SER-1) were shared between the two species when they were spatially close (Table S5, Fig. S1). Among the OMF found in roots, only 50% (2/4) and 47% (7/15) were also retrieved on bark on grid 1 and 2, respectively (Table S4), so that root communities did not appear to be only a subset of adjacent bark communities.

3.2 All fungal communities were spatially structured

All functional guilds, except lichenized fungi on grid 1 and symbiotrophs on both grids, showed significant spatial autocorrelation of OTU composition (Mantel test; Table 1). Among the different guilds, OMF on grid 2 showed the largest distance of positive spatial autocorrelation over approx. 30 cm (Fig. 4). The whole fungal community, as well as endophytes, also showed positive spatial autocorrelation over approx. 15 cm and 18 cm on grid 1 and 2, respectively. For the other guilds, *i.e.,* lichenized fungi, plant pathogens, saprotrophs, and symbiotrophs, spatial autocorrelation was more significant on grid 2, with positive autocorrelation between 0 and 10-20 cm, compared to grid 1 where it occurred within the first 10 cm only. The mean Jaccard index for adjacent points separated by 14 cm tended to be higher vertically than horizontally (comparison possible on grid 1 only; Fig. S8), but due to large variation in the values the difference was not statistically supported.

Regarding spatial autocorrelation of OTU richness, all fungal guilds, except symbiotrophs on grid 1, showed a significant autocorrelation (Moran's *I*; Table S7). OMF harbored the largest distance of positive autocorrelation on both grids (30 cm).

Spatial autocorrelation of single OTUs showed that only OMF on grid 2 tend to be more frequently spatially clustered than other fungi (Table S6). OMF families showed vertical stratification on grid 2 that covered a greater height on the tree (1.7 m), whereas this pattern was not obvious on grid 1 (covering 0.7 m only; Fig. S9).

3.3 Epiphytic roots influenced all fungal communities

The Jaccard similarity between roots and bark fungal compositions significantly decreased with increasing distance from the roots for the whole fungal community on both grids (Fig. 5). This was also observed for endophytes on both grids, for non-OMF symbiotrophs on grid 1 only, and for OMF, plant pathogens and saprotrophs on grid 2 only (Table 1, Fig. 5; see also Fig. S10 and Table S8 for details). The distance decay of bark fungal richness showed contrasting results with either non-significant or opposite results between grids (Fig. S12-14, Table S9).

The evolution of density (*i.e.,* spatial density based on occurrence data) of OMF on bark at a distance from colonized roots showed that they were spatially distributed in the close vicinity of the roots on both grids, but contrasting spatial structures were detected between OMF families on grid 2 (with Tulasnellaceae tending to picking at a longer distance from roots; Fig. S11). However, by comparing the density distribution of OMF *versus* endophytes (distance from roots beyond which 80% of the occurrences of a given OTU are limited), the OMF were not statistically closer to roots than other endophytes (Wilcox tests, $W = 370$, $p = 0.423$ and $W = 1142$, $p = 0.397$ for grid 1 and 2, respectively).

4. Discussion

4.1 Features of bark fungal communities compared to the soil's

The majority of fungal OTUs identified on the investigated tree barks belonged to Ascomycota (61%) and Basidiomycota (30%), even if the latter were the most abundant in reads. Other phyla that are usually associated with plants were largely missing in our dataset, such as arbuscular mycorrhizal fungi of the Glomeromycotina division (Spatafora *et al.*, 2017). These fungi poorly amplify with ITS primers (Berruti *et al.*, 2017) and, according to some observational studies (Lehnert *et al.*, 2017), they are less represented (but not absent, see for instance Rowe & Pringle, 2005) in epiphytic environments than in soils. Conversely, saprotrophic fungi are particularly expected on barks, including the ones known to be symbiotically associated with epiphytic orchids (Kottke *et al.*, 2010; Martos *et al.*, 2012; Herrera *et al.*, 2018; Novotná *et al.*, 2018). It is noteworthy that Tulasnellaceae were broadly retrieved from barks and roots on both trees, which shows the relevance of using specific primers (Tedersoo *et al.*, 2015), even though we cannot exclude that some Tulasnella species did not amplified with these primers.

Fungal metabarcoding has long been carried out in soils and in soil-dwelling plant roots (Schmidt *et al.*, 2013). Soils are complex three-dimensional environments where fungal species and functional groups occupy different horizons (Anderson et al., 2014; Bahram et al., 2015), and where mycorrhizal fungi may not be easily found outside the rhizosphere (Egidi *et al.*, 2018), perhaps due to insufficiently deep investigations. Conversely, bark, especially when thin, offers a nearly two-dimensional environment suitable for exhaustive sampling of microbial species in space and time. While bark has been commonly studied in the context of diseases (*e.g.* Arrigoni et al., 2020), it has only very recently been regarded as a niche for other fungal guilds (Izuddin *et al.*, 2019; Eskov *et al.*, 2020).

401 Here, the fungal communities growing on two trees were clearly distinct, both on the bark and
402 in the roots of epiphytes (Fig. 2, S7). Although our sampling was done to characterize fungal
403 diversity within rather than between trees, we can expect that the structure of epiphytic fungal
404 communities is very complex (Kembel & Mueller, 2014; Vacher *et al.*, 2016) and far from
405 understood. In both cases, they encompassed much more diverse guilds than the expected
406 saprotrophic and lichenized fungi, showing the complexity of fungal communities as well as
407 the presence in the environment of endophytic and symbiotrophic fungi. Furthermore, 377
408 fungal OTUs (8.6%) could not be identified at a lower taxonomic rank, suggesting that a high
409 and previously unknown fungal diversity exists in such a tropical environment (Cevallos *et al.*,
410 2018).
411 As we chose to work on young trees, the thinness of their bark did not allow us to test for a
412 possible differentiation of the fungal communities throughout the bark layers (*e.g.*, inner *versus*
413 outer bark) but future studies could focus on this question using older trees with more
414 differentiated barks. However, this thinness allowed us to exhaustively sample fungal
415 communities at a given position. Whether these communities are spatially structured or are
416 either homogeneously or randomly distributed remained an open question, which we
417 investigated in this study.
418
419 **4.2 Most OTUs tend to have an endophytic niche on bark**
420
421 In soils, there is growing evidence that fungal communities are spatially structured (Blaalid *et*
422 *al.*, 2012; Kadowaki *et al.*, 2014; Bahram *et al.*, 2016). In our study, total and endophytic
423 communities showed similar and strong spatial turnover on bark (Fig. 4, Table 1). This suggests
424 that there is no difference between fungi able to colonize living roots and those which are not.
425 This may be because most of these fungi regarded as endophytes only colonize roots

426 superficially (*e.g.* in orchid velamen; Herrera *et al.*, 2010) without any functional interaction
427 with the plants; yet many endophytic fungi are likely to extend away from plant tissues and
428 have a partially free-living lifestyle (*i.e.,* not into plant tissues; Selosse *et al.*, 2018). In our
429 study, most of the fungi were found as endophytes (34.63% and 78.25% for grids 1 and 2,
430 respectively; Table S4). These results reveal that on bark much of the fungal community is able
431 to colonize epiphytic roots, probably because it contains more carbon than the oligotrophic
432 surrounding bark. Accordingly, saprotrophs and plant pathogens (despite non-significantly for
433 the latter) tended to be slightly more present in roots than the rest of the community. On the
434 other hand, lichenized fungi, which can acquire their own carbon through photosynthesis, were
435 less present in roots than other fungi (Table S4).
436 Neutral community model analyses revealed that endophytes tended to be more frequent in
437 roots than expected by neutral dispersal processes (Fig. S4), which may suggest that root
438 colonization by endophytic fungi is not passive but is rather actively built by hyphal foraging.
439 Interestingly, lichenized fungi on grid 2 tended to be less frequent in roots than expected by
440 neutral dispersal processes (Fig. S6), suggesting that some fungi colonize (or contaminate) roots
441 accidentally. However, contrary to air dispersal in the context of which this model was initially
442 applied (Venkataraman *et al.*, 2015), fungi may rather disperse through hyphae growth (not
443 sporulation) at the small studied scales. Thus, the filtering of fungal communities by the root
444 compartment, although expected, may reflect the abundance of these fungi on adjacent bark.
445 The sampling procedure applied here successfully allowed us to investigate the very fine-scale
446 spatial structure of fungal guilds on the bark at proximity from epiphytic roots.
447
448 **4.3 All fungal communities harbor intrinsic spatial structures**
449
450 All trophic guilds harbored spatial structures at least on one tree (Fig. 4). Consistently with

451 previous results on soil communities (Bahram *et al.*, 2016; Voyron *et al.*, 2017), OMF (despite
452 their low statistical power due to the limited number of OTUs), saprotrophs and plant pathogens
453 were the most spatially structured communities, as shown by Mantel (Table 1, Fig. 4) and
454 Moran (Table S7) tests, perhaps because they exploit resources that are more localized than,
455 *e.g.* lichenized fungi. As bark trees are subjected to water runoff, one could expect that fungal
456 communities would be more similar vertically than horizontally: this trend was observed, but
457 not statistically supported on grid 1 (Fig. S8). Yet, this analysis was limited by (i) the low
458 diameter of trunks, which limits horizontal measurements (especially on grid 2), and a limited
459 vertical range. Future sampling may investigate a possible axis-dependence of epiphytic fungal
460 communities.
461
462 Mycorrhizal fungi are particularly known to display spatial structures (Bahram *et al.*, 2015), for
463 example in ectomycorrhizal (Anderson et al., 2014; Coince et al., 2013; Pickles et al., 2010;
464 Tedersoo et al., 2010), arbuscular (Whitcomb & Stutz, 2007), ericoid (Toju *et al.*, 2016) or
465 orchid mycorrhizal fungi (Voyron *et al.*, 2017). OMF from grassland soils display spatial
466 autocorrelation up to several meters (Voyron *et al.*, 2017), whereas in our study spatial
467 autocorrelation was limited to a few tens of centimeters (Fig. 4). Although a weak spatial
468 structure is not excluded in soil (Oja *et al.*, 2016), this suggests that epiphytic OMF (or perhaps
469 epiphytic fungi as a whole) experience stronger competition for space on the limited bark
470 surface and thus are more segregated than in soils (Lekberg *et al.*, 2007; Mujic *et al.*, 2016). A
471 non-exclusive explanation may be the fact that the stressing and oligotrophic epiphytic
472 environment for the plant limits the availability of carbohydrates and, from there, the foraging
473 ability of the mycorrhizal mycelia. Alternatively, the turnover of the bark substrate by radial
474 growth, leading to flaking bark (Rasmussen & Rasmussen, 2018), could also disturb microbial
475 communities, preventing them from spreading throughout the bark surface.

In a recent study, Izuddin et al., (2019) showed that fungal communities vary between microsites (stem/branch/fork) on tropical trees. Our study highlights the fact that even without any apparent heterogeneity of substrate, bark fungal communities have a spatial structure and are neither randomly nor homogeneously distributed. As discussed below, vascular epiphytes, like orchids, also influence the diversity and spatial structure of fungal communities. Barks devoid of epiphytes would thus be of interest to compare the composition and spatial structure of fungal communities without epiphytic roots. Even though trees devoid of any epiphytes can be scarce in tropical rainforests (and often associated with altered habitat), we encourage such investigations for future studies as we do not exclude that contrasting patterns might be found. Here, as we tested the influence of these epiphytes on fungal communities, we chose trees colonized by orchids and showed for the first time that they can shape epiphytic fungal communities.

4.4 Roots influence all fungal communities in the epiphytic rhizosphere

Roots are generally colonized by numerous fungi which positively or negatively interact with the plants, *e.g.*, mycorrhizal fungi or fungal pathogens. It can thus be hypothesized that these fungal communities are influenced by the presence of roots (Goldmann *et al.*, 2016; Waud *et al.*, 2016a; Zhang *et al.*, 2017), and *vice versa*. For instance, it has been shown that saprotrophic, pathotrophic and symbiotrophic fungi were more abundant near the roots in soils (Zhang *et al.*, 2017). Our analyses of richness confirmed this tendency on grid 2 only (Fig. S12 and S14), showing that the distance decay of richness at a distance from roots is dependent upon the studied environments.

However, all the fungal guilds except lichenized fungi (see also section 4.2) were influenced

by the presence of epiphytic roots, at least on one grid. Thus, this significant decay of fungal community similarity with distance from the roots (Fig. 5, S10, Table S8) does suggest the presence of an epiphytic 'rhizosphere' similar to that classically described in soils. The mechanisms of this rhizosphere influence may differ from one guild to another. For instance, while the OMF should get direct or indirect nutrient and/or protection benefits from the orchid roots (Dearnaley *et al.*, 2012), plant pathogenic fungi should be attracted by the living plants (Morris *et al.*, 1998) and saprotrophic fungi should benefit from root exudates (Sun & Fries, 1992).

For OMF particularly, the strong decay of similarity observed on grid 2 (Waud *et al.*, 2016a), with short halving distance (Table S8), as well as the quasi absence of OMF at a distance higher than 60 cm from colonized roots (Fig. S11), suggests that the roots but also their close vicinity act as their main ecological niche (Dearnaley *et al.*, 2012). Although the latter observation and conclusion are often not reported from OMF analyses in soil (Egidi *et al.*, 2018), this may be due to problems of vertical depth of sampling which are alleviated by the thinness of the investigated bark environment.

4.5 Fungal communities could modulate epiphytic plant population dynamics

The clustered structure of the epiphytic fungal communities and the existence of an epiphytic root rhizosphere, although the two are intrinsically linked, should strongly influence plant establishment and dynamics. For orchids especially, whose seeds lack reserves and depend on OMF for germination (Dearnaley *et al.*, 2012), it is likely that the distribution of plants may be controlled by OMF distribution (McCormick & Jacquemyn, 2014; but see a balanced view in Kartzinel et al., 2013).

Here, the OMF were more spatially clustered than any other fungi (Table S6), reflected in the

526 vertical stratification on grid 2 (Fig. S9), which suggests that they could strongly constrain
527 orchid seed germination. In soil, it has also been proposed that the patchiness of orchid
528 individuals (Jacquemyn *et al.*, 2007) could be due to that of their mycorrhizal partners
529 (Jacquemyn *et al.*, 2012). Additionally, plant pathogens should also be involved in the
530 establishment of epiphytic plants by modulating the probability of a seedling to establish
531 (Sarmiento *et al.*, 2017). In the future, experimental approaches coupled to metabarcoding may
532 investigate, again with ease as the system is nearly two-dimensional, whether epiphytic orchid
533 seed germination can be predicted from the fungal community at the site of seed deposition
534 (Kartzinel *et al.*, 2013).
535
536 **4.6 Conclusion and perspectives**
537
538 While metabarcoding studies are increasingly used to describe microbial communities and their
539 spatial structure (Schmidt *et al.*, 2013), epiphytic habitats of tropical environments have been
540 largely overlooked in such research (McCormick *et al.*, 2018). We show for the first time that
541 the fungi and their trophic guilds, in accordance with our first hypothesis, are spatially
542 structured on barks colonized by vascular epiphytes, and that this structure is influenced by the
543 presence of their roots. We thus suggest that a rhizosphere effect also exists for epiphytic plants,
544 and particularly for OMF fungi, confirming our second and third hypotheses, respectively.
545 Additionally, some fungi, including OMF or pathogens, could also influence the presence of
546 the orchid roots (*e.g.*, through seed germination), and the mechanisms behind this epiphytic
547 rhizosphere are thus yet to be explored.
548 Since, although the two tree species were colonized by distinct fungal communities, results
549 were consistent between them, we expect that the observed features can be viewed as default
550 expectations for other bark fungal communities. Yet, future investigations in other epiphytic

environments, especially environments colonized by epiphytes with arbuscular mycorrhizal fungi, are needed to test this hypothesis and add further relief to the study of epiphytic fungal communities and their interactions with plants.

In this study, we observed a vertical niche differentiation for OMF communities, but not for other fungal guilds, probably because our sampling design was not appropriate to investigate such vertical gradients. Yet, a possible trend for lower vertical than horizontal structure was observed. As both biotic (*e.g.* interspecific competition) and abiotic (*e.g.* water runoff) factors should influence vertical niche segregation, future studies should focus on bark microbial communities of higher forest trees (Izuno *et al.*, 2016) and their role as potential drivers of epiphytic plant population structure and dynamics.

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Conflict of Interest

We declare no conflict of interest regarding this work.

Author Contributions

MAS and MCMK designed the study. CAV, MFB and EDSV performed the sampling and the molecular analyses. RP, FM, MJ and CV analyzed the data and wrote the manuscript which was edited by all co-authors.

Data Accessibility

Raw sequences are available in Sequence Read Archive (SRA) under the BioProject accession no. PRJNA692353.

The bioinformatic pipeline is provided in the GitHub page of the corresponding author (https://github.com/PetrolliR/MetaBarkCoding.git). Authors can provide initial table and metadata upon reasonable request.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. **1990**. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.

Anderson IC, Genney DR, Alexander IJ. **2014**. Fine-scale diversity and distribution of ectomycorrhizal fungal mycelium in a Scots pine forest. *New Phytologist* **201**: 1423–1430.

Anderson MJ, Tolimieri N, Millar RB. **2013**. Beta Diversity of Demersal Fish Assemblages in the North-Eastern Pacific: Interactions of Latitude and Depth. *PLoS ONE* **8**: 1–15.

Anderson MJ, Walsh DCI. **2013**. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecological Monographs* **83**: 557–574.

Arrigoni E, Albanese D, Longa CMO, Angeli D, Donati C, Ioriatti C, Pertot I, Perazzolli

M. **2020**. Tissue age, orchard location and disease management influence the composition of fungal and bacterial communities present on the bark of apple trees. *Environmental Microbiology* **22**: 2080–2093.

Bahram M, Kohout P, Anslan S, Harend H, Abarenkov K, Tedersoo L. **2016**. Stochastic distribution of small soil eukaryotes resulting from high dispersal and drift in a local environment. *ISME Journal* **10**: 885–896.

Bahram M, Peay KG, Tedersoo L. **2015**. Local-scale biogeography and spatiotemporal variability in communities of mycorrhizal fungi. *New Phytologist* **205**: 1454–1463.

Berruti A, Desirò A, Visentin S, Zecca O, Bonfante P. **2017**. ITS fungal barcoding primers versus 18S AMF-specific primers reveal similar AMF-based diversity patterns in roots and soils of three mountain vineyards. *Environmental Microbiology Reports* **9**: 658–667.

Bever JD, Dickie IA, Facelli E, Facelli JM, Klironomos J, Moora M, Rillig MC, Stock WD, Tibbett M, Zobel M. **2010**. Rooting theories of plant community ecology in microbial interactions. *Trends in Ecology and Evolution* **25**: 468–478.

Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, Fontana G, Kauserud H. **2012**. Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. *Molecular Ecology* **21**: 1897–1908.

Brundrett MC, Tedersoo L. **2018**. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist* **220**: 1108–1115.

Burns AR, Stephens WZ, Stagaman K, Wong S, Rawls JF, Guillemin K, Bohannan BJM. **2016**. Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. *ISME Journal* **10**: 655–664.

Bushnell B, Rood J, Singer E. **2017**. BBMerge – Accurate paired shotgun read merging via overlap. *PLoS ONE* **12**: 1–15.

Caporaso G, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, *et al.* 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335–336.

Cevallos S, Herrera P, Sánchez-Rodríguez A, Declerck S, Suárez JP. **2018**. Untangling factors that drive community composition of root associated fungal endophytes of Neotropical epiphytic orchids. *Fungal Ecology* **34**: 67–75.

Coince A, Caël O, Bach C, Lengellé J, Cruaud C, Gavory F, Morin E, Murat C, Marçais B, Buée M. **2013**. Below-ground fine-scale distribution and soil versus fine root detection of fungal and soil oomycete communities in a French beech forest. *Fungal Ecology* **6**: 223–235.

Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. **2018**. Simple statistical

identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**: 1–14.

Dearnaley JDW, Martos F, Selosse MA. **2012**. Orchid Mycorrhizas : Molecular Ecology , Physiology , Evolution and Conservation Aspects. In: Fungal Associations, 2nd Edition The Mycota IX. B. Hock (Ed.). Springer Berlin Heidelberg, Germany, 207–230.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. **2011**. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194–2200.

Egidi E, May TW, Franks AE. **2018**. Seeking the needle in the haystack: Undetectability of mycorrhizal fungi outside of the plant rhizosphere associated with an endangered Australian orchid. *Fungal Ecology* **33**: 13–23.

Eskov AK, Voronina EY, Tedersoo L, Tiunov A V., Manh V, Prilepsky NG, Antipina VA, Elumeeva TG, Abakumov E V., Onipchenko VG. **2020**. Orchid epiphytes do not receive organic substances from living trees through fungi. *Mycorrhiza* **30**: 697–704.

Fávaro LB. **2012**. Estudo ecológico e econômico da palmeira Juçara (Euterpe edulis Mart.) no entorno do Parque Estadual Serra do Brigadeiro (PESB), MG.

Givnish TJ, Spalink D, Ames M, Lyon SP, Hunter SJ, Zuluaga A, Iles WJD, Clements MA, Arroyo MTK, Leebens-Mack J, *et al.* 2015. Orchid phylogenomics and multiple drivers of their extraordinary diversification. *Proc. R. Soc. B* **282**: 20151553.

Goldmann K, Schröter K, Pena R, Schöning I, Schrumpf M, Buscot F, Polle A, Wubet T. **2016**. Divergent habitat filtering of root and soil fungal communities in temperate beech forests. *Scientific Reports* **6**: 1–10.

Green J, Bohannan BJM. **2006**. Spatial scaling of microbial biodiversity. *Trends in Ecology and Evolution* **21**: 501–507.

Green J, Holmes A, Westoby M, Oliver I, Briscoe D, Dangerfield M, Gillings M, Beattie A. **2004**. Spatial scaling of microbial diversity. *Nature* **432**: 747–750.

Hanson CA, Fuhrman JA, Horner-Devine MC, Martiny JBH. **2012**. Beyond biogeographic patterns: Processes shaping the microbial landscape. *Nature Reviews Microbiology* **10**: 497–506.

Van Der Heijden MGA, Martin FM, Selosse MA, Sanders IR. **2015**. Mycorrhizal ecology and evolution: The past, the present, and the future. *New Phytologist* **205**: 1406–1423.

Herrera P, Suárez JP, Kottke I. **2010**. Orchids keep the ascomycetes outside: a highly diverse group of ascomycetes colonizing the velamen of epiphytic orchids from a tropical mountain rainforest in Southern Ecuador. *Mycology* **1**: 262–268.

Herrera P, Suárez JP, Kottke I, Méndez M, Molina MC, Méndez M, Suárez JP. **2018**.

674 Generalism in the interaction of Tulasnellaceae mycobionts with orchids characterizes a
675 biodiversity hotspot in the tropical Andes of Southern Ecuador. *Mycoscience* **59**: 38–48.
676 **Hornung BVH, Zwittink RD, Kuijper EJ**. **2019**. Issues and current standards of controls in
677 microbiome research. *FEMS Microbiology Ecology* **95**: 1–7.
678 **Izuddin M, Srivathsan A, Lee AL, Yam TW, Webb EL**. **2019**. Availability of orchid
679 mycorrhizal fungi on roadside trees in a tropical urban landscape. *Scientific Reports* **9**: 1–12.
680 **Izuno A, Kanzaki M, Artchawakom T, Wachrinrat C, Isagi Y**. **2016**. Vertical structure of
681 phyllosphere fungal communities in a tropical forest in Thailand uncovered by high-
682 throughput sequencing. *PLoS ONE* **11**: e0166669.
683 **Jacquemyn H, Brys R, Lievens B, Wiegand T**. **2012**. Spatial variation in below-ground
684 seed germination and divergent mycorrhizal associations correlate with spatial segregation of
685 three co-occurring orchid species. *Journal of Ecology* **100**: 1328–1337.
686 **Jacquemyn H, Brys R, Merckx VSFT, Waud M, Lievens B, Wiegand T**. **2014**. Coexisting
687 orchid species have distinct mycorrhizal communities and display strong spatial segregation.
688 *New Phytologist* **202**: 616–627.
689 **Jacquemyn H, Duffy KJ, Selosse M-A**. **2017**. Biogeography of Orchid Mycorrhizas. In:
690 Tedersoo L, ed. Biogeography of Mycorrhizal Symbiosis, Ecological Studies 230, L.
691 Tedersoo (ed.). Springer International Publishing, Cham, Switzerland, 159–177.
692 **Jacquemyn H, Rein B, Katrien V, Olivier H, Isabel R-R, Thorsten W**. **2007**. A spatially
693 of seedling recruitment in analysis explicit the terrestrial orchid Orchis purpurea. *New*
694 *Phytologist* **176**: 448–459.
695 **Kadowaki K, Sato H, Yamamoto S, Tanabe AS, Hidaka A, Toju H**. **2014**. Detection of
696 the horizontal spatial structure of soil fungal communities in a natural forest. *Population*
697 *Ecology* **56**: 301–310.
698 **Kartzinel TR, Trapnell DW, Shefferson RP**. **2013**. Critical importance of large native trees
699 for conservation of a rare neotropical epiphyte. *Journal of Ecology* **101**: 1429–1438.
700 **Kembel SW, Mueller RC**. **2014**. Plant traits and taxonomy drive host associations in tropical
701 Phyllosphere fungal communities1. *Botany* **92**: 303–311.
702 **Kottke I, Garnica S, Herrera P, Cruz D, Haug I, Suárez JP, Bauer R**. **2010**.
703 Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with
704 terrestrial and epiphytic neotropical orchids. *Proc. R. Soc. B* **277**: 1289–1298.
705 **Lana LG, Escobar TF, Godinho EM, Peluzio LE**. **2018**. Survey of Orchidaceae species of
706 Serra das Cabeças, in Parque Estadual da Serra do Brigadeiro, Araponga-MG, Brazil.
707 *Horticultura Brasileira* **36**: 156–166.

Lehnert M, Krug M, Kessler M. **2017**. A review of symbiotic fungal endophytes in lycophytes and ferns – a global phylogenetic and ecological perspective. *Symbiosis* **71**: 77–89.

Lekberg Y, Koide RT, Rohr JR, Aldrich-Wolfe L, Morton JB. **2007**. Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. *Journal of Ecology* **95**: 95–105.

Leroy C, Maes AQM, Louisanna E, Séjalon-Delmas N. **2019**. How significant are endophytic fungi in bromeliad seeds and seedlings? Effects on germination, survival and performance of two epiphytic plant species. *Fungal Ecology* **39**: 296–306.

Mahé F, Rognes T, Quince C, de Vargas C, Dunthorn M. **2015**. Swarmv2: highly-scalable and high-resolution amplicon clustering. *PeerJ* **3:e1420**.

Martin M. **2011**. Cutadapt removes adapter sequences from high-throuoghput sequencing reads. *EMBnet.journal* **17**: 10–12.

Martos F, Munoz F, Pailler T, Kottke I, Gonneau C, Selosse M-A. **2012**. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology* **21**: 5098–5109.

McCormick MK, Jacquemyn H. **2014**. What constrains the distribution of orchid populations ? *New Phytologist* **202**: 392–400.

McCormick MK, Taylor DL, Whigham DF, Burnett RK. **2016**. Germination patterns in three terrestrial orchids relate to abundance of mycorrhizal fungi. *Journal of Ecology* **104**: 744–754.

McCormick MK, Whigham DF, Canchani-Viruet A. **2018**. Mycorrhizal fungi affect orchid distribution and population dynamics. *New Phytologist* **219**: 1207–1215.

McKnight DT, Huerlimann R, Bower DS, Schwarzkopf L, Alford RA, Zenger KR. **2019**. Methods for normalizing microbiome data: An ecological perspective. *Methods in Ecology and Evolution* **10**: 389–400.

Millar RB, Anderson MJ, Tolimieri N. **2011**. Much ado about nothings: using zero similarity points in distance-decay curves. *Ecology* **92**: 1717–1722.

Morris PF, Bone E, Tyler BM. **1998**. Chemotropic and contact responses of Phytophthora sojae hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiology* **117**: 1171–1178.

Mujic AB, Durall DM, Spatafora JW, Kennedy PG. **2016**. Competitive avoidance not edaphic specialization drives vertical niche partitioning among sister species of ectomycorrhizal fungi. *New Phytologist* **209**: 1174–1183.

Nilsson RH, Tedersoo L, Ryberg M, Kristiansson E, Hartmann M, Unterseher M,

Porter TM, Bengtsson-Palme J, Walker DM, De Sousa F, *et al.* 2015. A comprehensive, automatically updated fungal ITS sequence dataset for reference-based chimera control in environmental sequencing efforts. *Microbes and Environments* **30**: 145–150.

Novotná A, Benítez Á, Herrera P, Cruz D, Filipczyková E, Suárez JP. **2018**. High diversity of root-associated fungi isolated from three epiphytic orchids in southern Ecuador. *Mycoscience* **59**: 24–32.

Oja J, Vahtra J, Bahram M, Kohout P, Kull T, Rannap R, Kõljalg U, Tedersoo L. **2016**. Local-scale spatial structure and community composition of orchid mycorrhizal fungi in semi-natural grasslands. *Mycorrhiza* **27**: 355–367.

Oksanen J, F. Guillaume Blanchet RK, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, M. Henry H. Stevens HW. **2013**. Vegan: Community Ecology Package. *[WWWdocument] URL http://cran.r- project.org/package=vegan [access in February 2018]*.

Peay KG, Baraloto C, Fine PVA. **2013**. Strong coupling of plant and fungal community structure across western Amazonian rainforests. *ISME Journal* **7**: 1852–1861.

Pickles BJ, Genney DR, Potts JM, Lennon JJ, Anderson IC, Alexander IJ. **2010**. Spatial and temporal ecology of Scots pine ectomycorrhizas. *New Phytologist* **186**: 755–768.

Rasmussen HN, Rasmussen FN. **2018**. The epiphytic habitat on a living host : reflections on the orchid – tree relationship. *Botanical Journal of the Linnean Society* **186**: 456–472.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F. **2016**. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4:e2584**.

Rolim FA, Ribeiro GA. **2001**. *Levantamento do potencial turistico do parque estadual da Serra do Brigadeiro - PESB com o Suporte em Geoprocessamento*.

Rowe AR, Pringle A. **2005**. Morphological and molecular evidence of arbuscular mycorrhizal fungal associations in Costa Rican epiphytic bromeliads. *Biotropica* **37**: 245–250.

Sarmiento C, Zalamea PC, Dalling JW, Davis AS, Stump SM, U'Ren JM, Arnold AE. **2017**. Soilborne fungi have host affinity and host-specific effects on seed germination and survival in a lowland tropical forest. *PNAS* **114**: 11458–11463.

Schmidt PA, Bálint M, Greshake B, Bandow C, Römbke J, Schmitt I. **2013**. Illumina metabarcoding of a soil fungal community. *Soil Biology and Biochemistry* **65**: 128–132.

Schneider-Maunoury L, Leclercq S, Clément C, Covès H, Lambourdière J, Sauve M, Richard F, Selosse MA, Taschen E. **2018**. Is Tuber melanosporum colonizing the roots of herbaceous, non-ectomycorrhizal plants? *Fungal Ecology* **31**: 59–68.

Selosse MA, Martos F. **2014**. Do chlorophyllous orchids heterotrophically use mycorrhizal

776 fungal carbon? *Trends in Plant Science* **19**: 683–685.
777 **Selosse MA, Schneider-Maunoury L, Martos F**. **2018**. Time to re-think fungal ecology?
778 Fungal ecological niches are often prejudged. *New Phytologist* **217**: 968–972.
779 **Sloan WT, Lunn M, Woodcock S, Head IM, Nee S, Curtis TP**. **2006**. Quantifying the roles
780 of immigration and chance in shaping prokaryote community structure. *Environmental*
781 *Microbiology* **8**: 732–740.
782 **Smith SE, Read DJ**. **2008**. *Mycorrhizal Symbiosis*. Cambridge, Great Britain: Elsevier Ltd.,
783 2008.
784 **Spatafora JW, Aime MC, Grigoriev I V., Martin F, Stajich JE, Blackwell M**. **2017**. The
785 fungal tree of life: from molecular systematics to genome-scale phylogenies. *Microbiology*
786 *Spectrum* **5**: 3–34.
787 **Sun YP, Fries N**. **1992**. The effect of tree-root exudates on the growth rate of
788 ectomycorrhizal and saprotrophic fungi. *Mycorrhiza* **1**: 63–69.
789 **Taylor DL, McCormick MK**. **2008**. Internal transcribed spacer primers and sequences for
790 improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist* **177**:
791 1020–1033.
792 **Tedersoo L, Anslan S, Bahram M, Põlme S, Riit T, Liiv I, Kõljalg U, Kisand V, Nilsson**
793 **RH, Hildebrand F, *et al.* 2015**. Shotgun metagenomes and multiple primer pair-barcode
794 combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycoKeys* **10**:
795 1–43.
796 **Tedersoo L, Sadam A, Zambrano M, Valencia R, Bahram M**. **2010**. Low diversity and
797 high host preference of ectomycorrhizal fungi in Western Amazonia, a neotropical
798 biodiversity hotspot. *ISME Journal* **4**: 465–471.
799 **Toju H, Tanabe AS, Ishii HS**. **2016**. Ericaceous plant-fungus network in a harsh alpine-
800 subalpine environment. *Molecular Ecology* **25**: 3242–3257.
801 **Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani AM**. **1999**. Rapid
802 identification of fungi by using the ITS2 genetic region and an automated fluorescent
803 capillary electrophoresis system. *Journal of Clinical Microbiology* **37**: 1846–1851.
804 **Vacher C, Cordier T, Vallance J**. **2016**. Phyllosphere fungal communities differentiate more
805 thoroughly than bacterial communities along an elevation gradient. *Microbial Ecology* **72**: 1–
806 3.
807 **Veldre V, Abarenkov K, Bahram M, Martos F, Selosse M-A, Tamm H, Kõljalg U,**
808 **Tedersoo L**. **2013**. Evolution of nutritional modes of Ceratobasidiaceae (Cantharellales,
809 Basidiomycota) as revealed from publicly available ITS sequences. *Fungal Ecology* **6**: 256–

268.

Venkataraman A, Bassis CM, Beck JM, Young VB, Curtis JL, Huffnagle GB, Schmidt TM. 2015. Application of a neutral community model to assess structuring of the human lung microbiome. *mBio* **6**: 1–9.

Voyron S, Ercole E, Ghignone S, Perotto S, Girlanda M. 2017. Fine-scale spatial distribution of orchid mycorrhizal fungi in the soil of host-rich grasslands. *New Phytologist* **213**: 1428–1439.

Waud M, Busschaert P, Lievens B, Jacquemyn H. 2016a. Specificity and localised distribution of mycorrhizal fungi in the soil may contribute to co-existence of orchid species. *Fungal Ecology* **20**: 155–165.

Waud M, Wiegand T, Brys R, Lievens B, Jacquemyn H. 2016b. Nonrandom seedling establishment corresponds with distance-dependent decline in mycorrhizal abundance in two terrestrial orchids. *New Phytologist* **211**: 255–264.

Weiß M, Waller F, Zuccaro A, Selosse M-A. 2016. Sebacinales - one thousand and one interactions with land plants. *New Phytologist* **211**: 20–40.

Whitcomb S, Stutz JC. 2007. Assessing diversity of arbuscular mycorrhizal fungi in a local community: role of sampling effort and spatial heterogeneity. *Mycorrhiza* **17**: 429–437.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes fr phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ, eds. PCR Protocols : A Guide to Methods and Applications. London, Great Britain: Academic Press, 315–322.

Wilson EB. 1927. Probable inference, the law of succession, and statistical inference. *Journal of the American Statistical Association* **22**: 209–212.

Xing X, Jacquemyn H, Gai X, Gao Y, Liu Q, Zhao Z, Guo S. 2019. The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest. *Oikos* **128**: 1254–1264.

Yang RH, Su JH, Shang JJ, Wu YY, Li Y, Bao DP, Yao YJ. 2018. Evaluation of the ribosomal DNA internal transcribed spacer (ITS), specifically ITS1 and ITS2, for the analysis of fungal diversity by deep sequencing. *PLoS ONE* **13**: 1–17.

Yoder JA, Imfeld SM, Heydinger DJ, Hart CE, Collier MH, Gribbins KM, Zettler LW. 2010. Comparative water balance profiles of Orchidaceae seeds for epiphytic and terrestrial taxa endemic to North America. *Plant Ecology* **211**: 7–17.

Zanne AE, Abarenkov K, Afkhami ME, Aguilar-Trigueros CA, Bates S, Bhatnagar JM, Busby PE, Christian N, Cornwell WK, Crowther TW, *et al.* 2019. Fungal functional

ecology: bringing a trait-based approach to plant-associated fungi. *Biological Reviews* **95**: 409–433.

Zhang K, Adams JM, Shi Y, Yang T, Sun R, He D, Ni Y, Chu H. **2017**. Environment and geographic distance differ in relative importance for determining fungal community of rhizosphere and bulk soil. *Environmental Microbiology* **19**: 3649–3659.

Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE, *et al.* 2019. DNA metabarcoding - Need for robust experimental designs to draw sound ecological conclusions. *Molecular Ecology* **28**: 1857–1862.

Zotz G. **2016**. *Plants on Plants – The Biology of Vascular Epiphytes*. Heidelberg, Berlin, Germany: Springer International Publishing, 2016.

Supporting Information

Fig. S1: Sampling design of tree 2.

Fig. S2: Rarefaction curves.

Fig. S3: Global fungal composition.

Fig. S4: Application of Neutral Community Model (NCM) to fungal endophytes.

Fig. S5: OTUs deviation from Neutral Community Model (NCM).

Fig. S6: Fungal guilds deviation from Neutral Community Model (NCM)

Fig. S7: NMDS on total fungal community, considering root samples only.

Fig. S8: Turnover of fungal community on vertical *versus* horizontal axis.

Fig. S9: Vertical density distribution of OMF families.

Fig. S10: Distance-decay of similarity from roots for other fungal guilds.

Fig. S11: Density distribution of OMF at a distance from roots.

Fig. S12: Distance-decay of bark richness from roots for total fungal community.

Fig. S13: Distance-decay of bark richness from roots for OMF community.

Fig. S14: Distance-decay of bark richness from roots for other fungal guilds.

Table S1: Details of the sampling depth.

Table S2: Global fungal composition.

Table S3: Fungal composition at family and genera levels *(.xlsx file*).

Table S4: Number of OTUs shared between roots and bark compartments in each grid.

Table S5: OMF composition of orchid roots (*.xlsx file*).

Table S6: Spatial autocorrelation of OTUs.

Table S7: Spatial autocorrelation of richness based on Moran calculation.

878 **Table S8:** GLM result parameters for distance-decay of similarity.

879 **Table S9:** ANOVA results for distance-decay of bark richness from roots.

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Table 1. General spatial features for each fungal guild. The number of OTUs in each guild is given prior to rarefaction. Mantel R index and Sim. Dec. β slope (slope of similarity decay between bark and roots, based on Jaccard index, and associated r value for Mantel test; see Table S6 for details) are calculated after rarefaction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s, non-significant.

Guild	Grid	Number OTUs	Mantel R	Sim. Dec. β slope ; r
Total	1	2445	0.238***	0.013 ; 0.231*
	2	3724	0.215***	0.004 ; 0.235***
Endophyte	1	1816	0.234***	0.012 ; 0.201*
	2	3233	0.191***	0.004 ; 0.235***
OMF	1	14	0.206***	0.005 ; $-0.291^{n.s}$
	2	31	0.560***	0.035 ; 0.604***
Lichenized	1	196	0.005^{ns}	0.043 ; 0.123^{ns}
	2	263	0.072**	0.006 ; 0.032^{ns}
Plant Pathogen	1	333	0.098**	0.013 ; 0.012^{ns}
	2	493	0.091**	0.002 ; 0.144**
Saprotroph	1	637	0.125**	-0.002 ; 0.082^{ns}
	2	956	0.070**	0.002 ; 0.092*
Symbiotroph	1	26	-0.005^{ns}	0.009 ; 0.392*
	2	55	0.094^{ns}	0.003 ; -0.041^{ns}

Figure 1. Sampling design of tree 1. (A) Sampling design of the first tree (*Siparuna sp.*) containing five individuals of the epiphytic orchid *Isochilus linearis* (orange, 1-5). Bark was sampled regularly (black filled circles) along the grid, and additionally several points were randomly sampled around orchid individuals 1 and 4 (black filled stars). The grid covered the whole circumference of the tree, and grey filled circles indicate the position of sampling points from the opposite side of the grid (*e.g.* points designated by an arrow are at the same position on the trunk). Orchid roots were regularly sampled along each root (orange filled rectangles). The picture (B) shows a detail of orchid individual 3 before sampling, showing that orchid roots were easily traceable along the trunk, allowing precise spatial analyses. Scale: 3 cm. The sampling design of tree 2 is presented in Fig. S1.

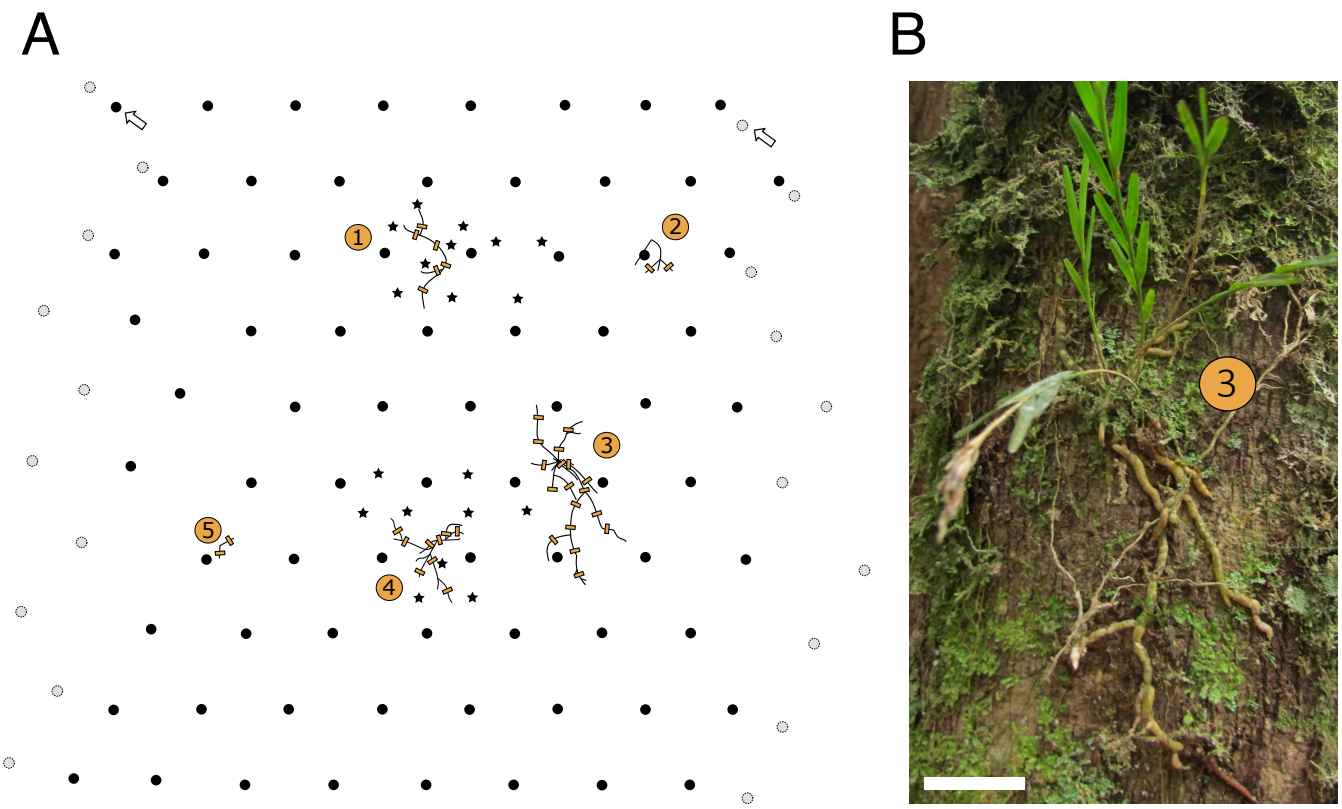

Figure 2. Non-metric multidimensional scaling (NMDS) on total fungal community, considering all samples and presence/absence data. Stress = 0.2098. Nested PERMANOVA revealed significant differences between both grids ($F = 13.91$, $R^2 = 0.047$, $p = 0.001$, betadisper: $F = 12.69$, $p < 0.001$), sample type (root *versus* bark; $F = 4.57$, $R^2 = 0.016$, $p = 0.001$, betadisper: $F = 29.67$, $p < 0.001$) and their interaction ($F = 3.07$, $R^2 = 0.011$, $p = 0.001$).

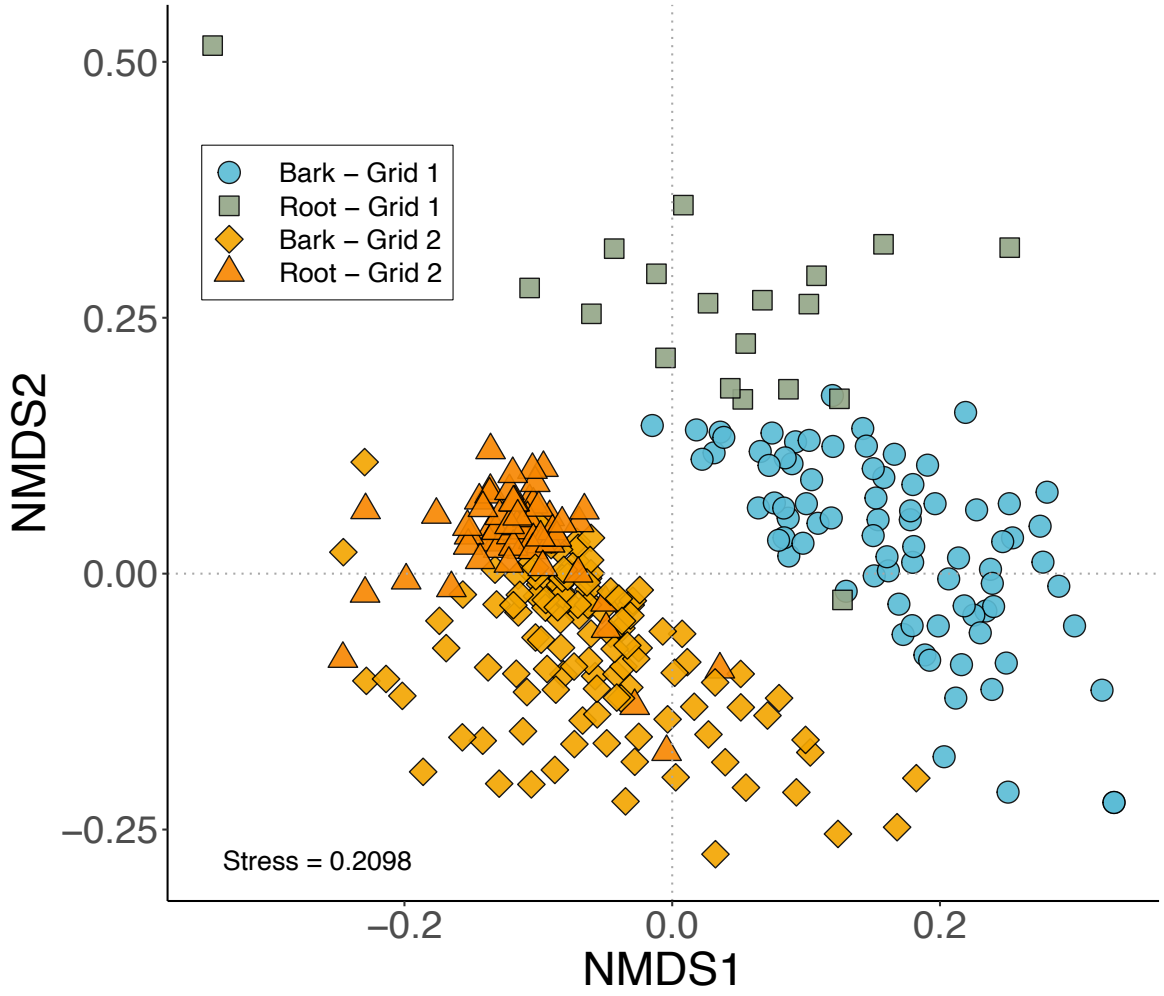

Figure 3. OTU composition of the orchid mycorrhizal fungi (OMF) communities in orchid roots. Bars show the cumulative proportion of sequences (in %) for each OTU in each orchid individual. One *Isochilus linearis* individual on grid 1 has been removed because of the absence of detected OMF in its roots. Bar colors indicate the OTU family. The number of samples available for each individual ($N_{samples}$) as well as the number of OMF OTUs (N_{OTUs}) found in them are given above each bar. The orchid individual numbers at the bottom of the graph refer to the sampling designs illustrated in Fig. 1 and Fig. S1. See Table S5 for further details concerning these communities. I. linearis: *Isochilus linearis*; E. armeniacum: *Epidendrum armeniacum*.

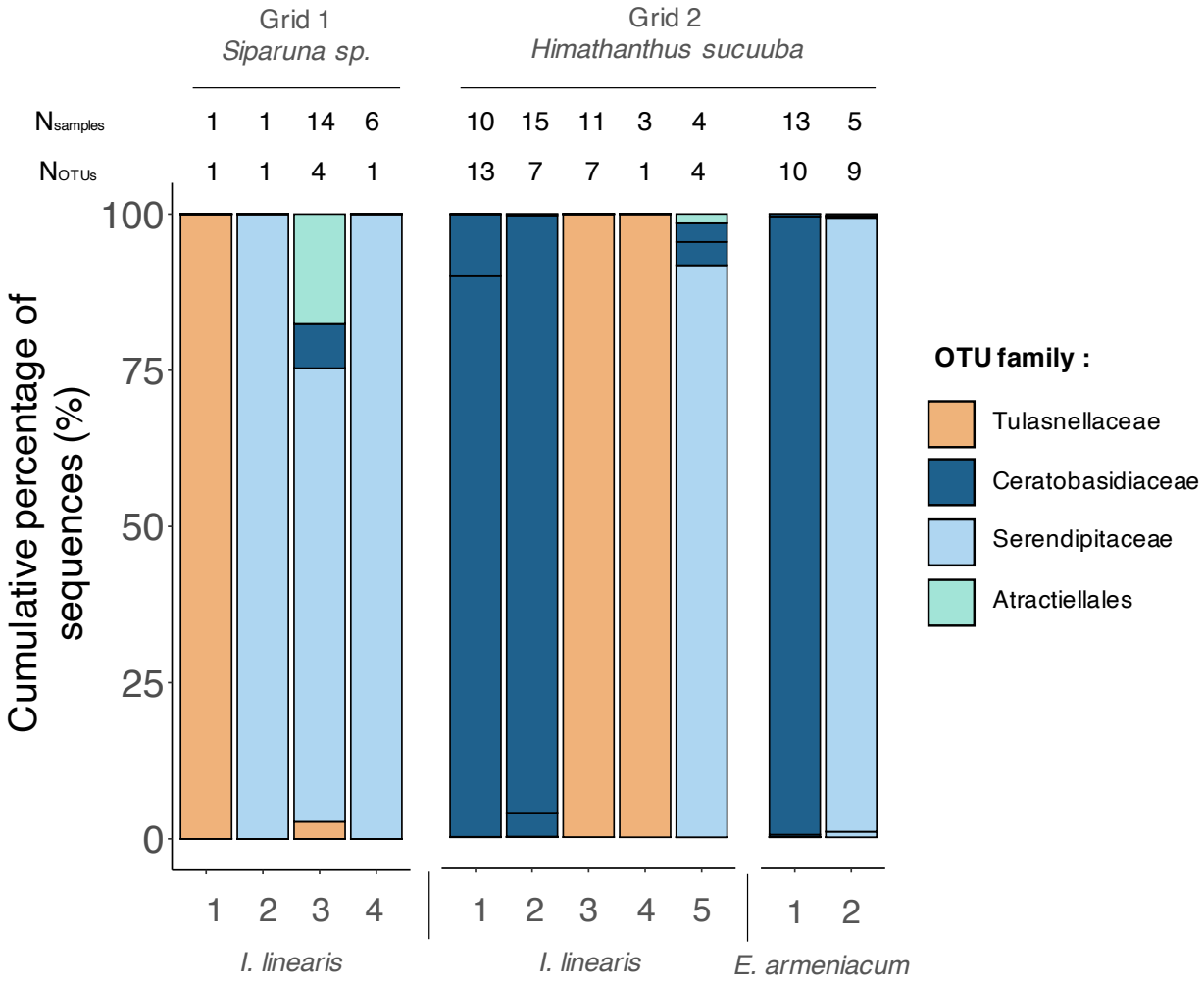

Figure 4. Spatial autocorrelation of fungal community composition. Mantel correlograms show spatial autocorrelation based on presence/absence of (A-B) total fungal community, fungal endophytes and orchid mycorrhizal fungi (OMF) and (C-D) lichenized, plant pathogen, saprotroph and symbiotroph fungal guilds (Jaccard index) for (A and C) grid 1 and (B and D) grid 2. A positive value corresponds to a positive autocorrelation and conversely. Filled circles indicate a significant value. Thick lines in the bottom of each graph indicate the distance of significant spatial autocorrelation for each guild represented. Total and endophyte curves are confounded on panel B.

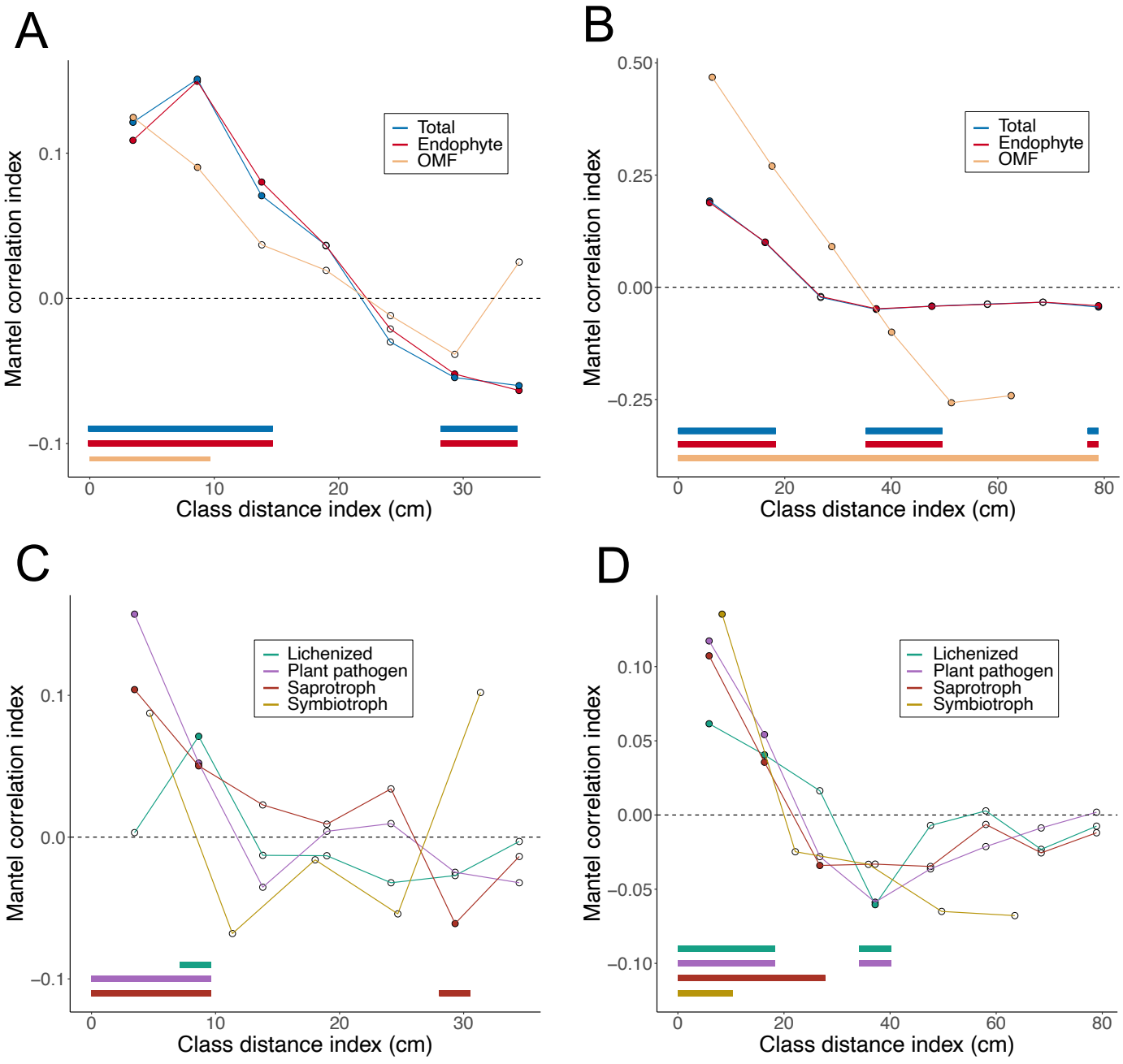

Figure 5. Distance-decay of similarity (based on Jaccard index) between roots and bark for total (large panel, blue) and orchid mycorrhizal fungi (OMF; small panel, orange) communities for (A) grid 1 and (B) grid 2. Colored lines show the regressions of a binomial GLM (log link function) and the colored areas represent the associated 95% confidence interval. A significant tendency (Mantel test) is indicated by an asterisk (* $p < 0.05$, *** $p < 0.001$, n.s, non-significant). See Table S8 for details.