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A fine-scale spatial analysis of fungal communities on tropical tree bark unveils the epiphytic rhizosphere in orchids

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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; Counce *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

76 such aerial substrates (Lehnert *et al.*, 2017; Brundrett & Tedersoo, 2018; but see Rowe &
77 Pringle, 2005) and their fungal partners have thus so far largely been ignored. However, there
78 is now growing interest in the field of epiphytic fungal endophytes which could strongly
79 influence the dynamics of epiphyte plant populations (Leroy *et al.*, 2019). One symbiosis that
80 regularly occurs in the epiphytic habitats is the orchid mycorrhiza (Martos *et al.*, 2012; Herrera
81 *et al.*, 2018; Novotná *et al.*, 2018). Epiphytic orchids, representing no less than 80% of this
82 hyper-diverse plant family (with over 25 000 species (Givnish *et al.*, 2015)), harbor typical
83 hyphal coils within their root cortical cells, which are formed by the same families but different
84 species of saprotrophic basidiomycetes (Dearnaley *et al.*, 2012; Martos *et al.*, 2012; Xing *et al.*,
85 2019) compared to soil. The fungi are also required for germination of the minute, nutrient-
86 poor orchid seeds (Smith & Read, 2008). It was therefore hypothesized that the distribution of
87 orchids must be constrained by that of their mycorrhizal fungi (McCormick & Jacquemyn,
88 2014; McCormick *et al.*, 2018)

89

90 The distribution of orchid mycorrhizal fungi (OMF) has been investigated in soils (Jacquemyn
91 *et al.*, 2014, 2017; McCormick & Jacquemyn, 2014; McCormick *et al.*, 2016, 2018; Voyron *et*
92 *al.*, 2017), but only marginally on barks (Kartzinel *et al.*, 2013), perhaps because most studies
93 focus on temperate and Mediterranean ecosystems where orchids are strictly terrestrial. For
94 example, two recent studies (Waud *et al.*, 2016b,a) showed a decline in abundance and
95 similarity composition of OMF with distance from adult orchids, which likely explains the
96 patchy distribution of grassland orchids (Jacquemyn *et al.*, 2007, 2014). Still in grassland
97 habitats, Voyron *et al.*, (2017) found that communities of OMF are more similar in nearby soil,
98 *i.e.*, display spatial autocorrelation (Hanson *et al.*, 2012). As for the epiphytic environment,
99 very little is known on the spatial distribution of mycorrhizal fungi on bark [but see (Izuddin *et*
100 *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

season. The climate of the region is humid subtropical mesothermic, with temperatures ranging from 17 to 23°C and annual rainfall averaging 1300 mm (Rolim & Ribeiro, 2001). This forest is characterized by medium to large trees, and a high diversity of orchid species, the majority of which are epiphytic (Lana *et al.*, 2018).

2.2 Bark and root sampling

Two trees belonging to *Siparuna* sp. (Siparunaceae; tree 1) and *Himathanthus sucuuba* (Apocynaceae; tree 2) were selected in February 2015 and February 2016 (95 m away from each other) respectively because they had epiphytic orchids growing on their lower trunk, namely *Isochilus linearis* and *Epidendrum armeniacum*. Flexible plastic grids of regular mesh size (7 cm) were placed around these trunks covering the whole circumference of tree 1 (58 cm, Fig. 1) and tree 2 (23 cm, Fig. S1): grid 1 extended from 2 to 2.7 m above the ground and encompassed five individuals of *I. linearis*; whereas grid 2 extended from 0.7 to 2.4 m and encompassed five *I. linearis* and two *E. armeniacum* individuals. On each point of the grid, we sampled 100 mg of bark in the center of every pore of the grid using a sterilized scalpel without removing the covering lichens, mosses, or liverworts. The samples thus consisted in small amounts of surface bark (to the cambium, itself not included) and its cryptogamic cover, which was facilitated by the young age of trees and the thinness of these species' bark. We also sampled 3 mm root sections every 1.5 cm along each epiphytic orchid root starting from the stem (Fig. 1, S1). Bark was also collected under each root sample. On grid 1, we took additional bark samples in the close vicinity of orchid roots (Fig. 1). This resulted in a total of 482 samples for the two grids (*i.e.*, 167 on grid 1 and 315 on grid 2; Table S1) The rationale behind this dense sampling was that, (i) we expected high turnover of fungal communities at a scale of a few centimeters and (ii) we did not want to miss any important fungi on the bark, especially

endophytic ones. All samples were frozen at -20°C within few hours in the nearby field laboratory of the Serra do Brigadeiro State Park headquarters for downstream molecular analyses. Two additional thin sections of orchid roots surrounding each sampled piece were collected to check for mycorrhizal fungal colonization on the following day under the microscope and all, without exception, displayed hyphal coils in at least one of each inspection section.

2.3 High-throughput sequencing of fungal communities

Bark and root samples were kept frozen and manually grinded in liquid nitrogen until a fine powder was obtained. Genomic DNA was extracted from this powder using the NucleoSpin Soil™ kit (Macherey-Nagel) following manufacturer's instructions and then each isolate was directly set to amplification trials using two primer pairs: ITS86-F/ITS4 (White et al., 1990; Turenne et al., 1999) amplifying the internal transcribed spacer 2 (ITS-2) of ribosomal DNA (Yang et al., 2018) of most fungi, and ITS86-F/ITS4-tul (Taylor & McCormick, 2008) amplifying the same region in Tulasnellaceae, since this OMF family requires specific primers for amplifying its ribosomal DNA (Martos et al., 2012). High sample multiplexing (up to 576 samples for each primer pair) was allowed through the use of a unique pair of barcoded primers during the PCRs, *i.e.*, 36 forward primers (ITS86-F barcodes 1-36) and 16 reverse primers (ITS4 barcodes 1-16 in fungi-specific, or ITS4-Tul barcodes 17-32 in Tulasnellaceae-specific, PCRs). Each barcode was a unique sequence of 8 bases added to the 5' end of the primer sequence, and the set of 36 barcodes was designed in order to have at least five differences between barcodes.

Tagging system negative controls were performed at this step (Hornung *et al.*, 2019; Zinger *et 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 μ L 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 μ L 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, $36\,477 \pm 31\,597$ sequences per sample) than the specific ITS86-F/ITS4-tul set of primers (41 OTUs, including 17 Tulasnellaceae, $15\,589 \pm 11\,583$ 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 ITS86-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, Sebaciniales 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: $F = 10.48$, $p = 0.002$), 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).

Here, the fungal communities growing on two trees were clearly distinct, both on the bark and in the roots of epiphytes (Fig. 2, S7). Although our sampling was done to characterize fungal diversity within rather than between trees, we can expect that the structure of epiphytic fungal communities is very complex (Kembel & Mueller, 2014; Vacher *et al.*, 2016) and far from understood. In both cases, they encompassed much more diverse guilds than the expected saprotrophic and lichenized fungi, showing the complexity of fungal communities as well as the presence in the environment of endophytic and symbiotrophic fungi. Furthermore, 377 fungal OTUs (8.6%) could not be identified at a lower taxonomic rank, suggesting that a high and previously unknown fungal diversity exists in such a tropical environment (Cevallos *et al.*, 2018).

As we chose to work on young trees, the thinness of their bark did not allow us to test for a possible differentiation of the fungal communities throughout the bark layers (*e.g.*, inner *versus* outer bark) but future studies could focus on this question using older trees with more differentiated barks. However, this thinness allowed us to exhaustively sample fungal communities at a given position. Whether these communities are spatially structured or are either homogeneously or randomly distributed remained an open question, which we investigated in this study.

4.2 Most OTUs tend to have an endophytic niche on bark

In soils, there is growing evidence that fungal communities are spatially structured (Blaalid *et al.*, 2012; Kadowaki *et al.*, 2014; Bahram *et al.*, 2016). In our study, total and endophytic communities showed similar and strong spatial turnover on bark (Fig. 4, Table 1). This suggests that there is no difference between fungi able to colonize living roots and those which are not. This may be because most of these fungi regarded as endophytes only colonize roots

superficially (*e.g.* in orchid velamen; Herrera *et al.*, 2010) without any functional interaction with the plants; yet many endophytic fungi are likely to extend away from plant tissues and have a partially free-living lifestyle (*i.e.*, not into plant tissues; Selosse *et al.*, 2018). In our study, most of the fungi were found as endophytes (34.63% and 78.25% for grids 1 and 2, respectively; Table S4). These results reveal that on bark much of the fungal community is able to colonize epiphytic roots, probably because it contains more carbon than the oligotrophic surrounding bark. Accordingly, saprotrophs and plant pathogens (despite non-significantly for the latter) tended to be slightly more present in roots than the rest of the community. On the other hand, lichenized fungi, which can acquire their own carbon through photosynthesis, were less present in roots than other fungi (Table S4).

Neutral community model analyses revealed that endophytes tended to be more frequent in roots than expected by neutral dispersal processes (Fig. S4), which may suggest that root colonization by endophytic fungi is not passive but is rather actively built by hyphal foraging. Interestingly, lichenized fungi on grid 2 tended to be less frequent in roots than expected by neutral dispersal processes (Fig. S6), suggesting that some fungi colonize (or contaminate) roots accidentally. However, contrary to air dispersal in the context of which this model was initially applied (Venkataraman *et al.*, 2015), fungi may rather disperse through hyphae growth (not sporulation) at the small studied scales. Thus, the filtering of fungal communities by the root compartment, although expected, may reflect the abundance of these fungi on adjacent bark. The sampling procedure applied here successfully allowed us to investigate the very fine-scale spatial structure of fungal guilds on the bark at proximity from epiphytic roots.

4.3 All fungal communities harbor intrinsic spatial structures

All trophic guilds harbored spatial structures at least on one tree (Fig. 4). Consistently with

previous results on soil communities (Bahram *et al.*, 2016; Voyron *et al.*, 2017), OMF (despite their low statistical power due to the limited number of OTUs), saprotrophs and plant pathogens were the most spatially structured communities, as shown by Mantel (Table 1, Fig. 4) and Moran (Table S7) tests, perhaps because they exploit resources that are more localized than, *e.g.* lichenized fungi. As bark trees are subjected to water runoff, one could expect that fungal communities would be more similar vertically than horizontally: this trend was observed, but not statistically supported on grid 1 (Fig. S8). Yet, this analysis was limited by (i) the low diameter of trunks, which limits horizontal measurements (especially on grid 2), and a limited vertical range. Future sampling may investigate a possible axis-dependence of epiphytic fungal communities.

Mycorrhizal fungi are particularly known to display spatial structures (Bahram *et al.*, 2015), for example in ectomycorrhizal (Anderson *et al.*, 2014; Coince *et al.*, 2013; Pickles *et al.*, 2010; Tedersoo *et al.*, 2010), arbuscular (Whitcomb & Stutz, 2007), ericoid (Toju *et al.*, 2016) or orchid mycorrhizal fungi (Voyron *et al.*, 2017). OMF from grassland soils display spatial autocorrelation up to several meters (Voyron *et al.*, 2017), whereas in our study spatial autocorrelation was limited to a few tens of centimeters (Fig. 4). Although a weak spatial structure is not excluded in soil (Oja *et al.*, 2016), this suggests that epiphytic OMF (or perhaps epiphytic fungi as a whole) experience stronger competition for space on the limited bark surface and thus are more segregated than in soils (Lekberg *et al.*, 2007; Mujic *et al.*, 2016). A non-exclusive explanation may be the fact that the stressing and oligotrophic epiphytic environment for the plant limits the availability of carbohydrates and, from there, the foraging ability of the mycorrhizal mycelia. Alternatively, the turnover of the bark substrate by radial growth, leading to flaking bark (Rasmussen & Rasmussen, 2018), could also disturb microbial communities, preventing them from spreading throughout the bark surface.

476

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

489

490 **4.4 Roots influence all fungal communities in the epiphytic rhizosphere**

491

492 Roots are generally colonized by numerous fungi which positively or negatively interact with
493 the plants, *e.g.*, mycorrhizal fungi or fungal pathogens. It can thus be hypothesized that these
494 fungal communities are influenced by the presence of roots (Goldmann *et al.*, 2016; Waud *et*
495 *al.*, 2016a; Zhang *et al.*, 2017), and *vice versa*. For instance, it has been shown that saprotrophic,
496 pathotrophic and symbiotrophic fungi were more abundant near the roots in soils (Zhang *et al.*,
497 2017). Our analyses of richness confirmed this tendency on grid 2 only (Fig. S12 and S14),
498 showing that the distance decay of richness at a distance from roots is dependent upon the
499 studied environments.

500 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

vertical stratification on grid 2 (Fig. S9), which suggests that they could strongly constrain orchid seed germination. In soil, it has also been proposed that the patchiness of orchid individuals (Jacquemyn *et al.*, 2007) could be due to that of their mycorrhizal partners (Jacquemyn *et al.*, 2012). Additionally, plant pathogens should also be involved in the establishment of epiphytic plants by modulating the probability of a seedling to establish (Sarmiento *et al.*, 2017). In the future, experimental approaches coupled to metabarcoding may investigate, again with ease as the system is nearly two-dimensional, whether epiphytic orchid seed germination can be predicted from the fungal community at the site of seed deposition (Kartzinel *et al.*, 2013).

4.6 Conclusion and perspectives

While metabarcoding studies are increasingly used to describe microbial communities and their spatial structure (Schmidt *et al.*, 2013), epiphytic habitats of tropical environments have been largely overlooked in such research (McCormick *et al.*, 2018). We show for the first time that the fungi and their trophic guilds, in accordance with our first hypothesis, are spatially structured on barks colonized by vascular epiphytes, and that this structure is influenced by the presence of their roots. We thus suggest that a rhizosphere effect also exists for epiphytic plants, and particularly for OMF fungi, confirming our second and third hypotheses, respectively. Additionally, some fungi, including OMF or pathogens, could also influence the presence of the orchid roots (*e.g.*, through seed germination), and the mechanisms behind this epiphytic rhizosphere are thus yet to be explored.

Since, although the two tree species were colonized by distinct fungal communities, results were consistent between them, we expect that the observed features can be viewed as default 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.

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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*.

972 **Figure 4. Spatial autocorrelation of fungal community composition.** Mantel correlograms
 973 show spatial autocorrelation based on presence/absence of (A-B) total fungal community,
 974 fungal endophytes and orchid mycorrhizal fungi (OMF) and (C-D) lichenized, plant pathogen,
 975 saprotroph and symbiotroph fungal guilds (Jaccard index) for (A and C) grid 1 and (B and D)
 976 grid 2. A positive value corresponds to a positive autocorrelation and conversely. Filled circles
 977 indicate a significant value. Thick lines in the bottom of each graph indicate the distance of
 978 significant spatial autocorrelation for each guild represented. Total and endophyte curves are
 979 confounded on panel B.
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981 **Figure 5. Distance-decay of similarity** (based on Jaccard index) between roots and bark for
 982 total (large panel, blue) and orchid mycorrhizal fungi (OMF; small panel, orange) communities
 983 for (A) grid 1 and (B) grid 2. Colored lines show the regressions of a binomial GLM (log link
 984 function) and the colored areas represent the associated 95% confidence interval. A significant
 985 tendency (Mantel test) is indicated by an asterisk (* $p < 0.05$, *** $p < 0.001$, n.s, non-
 986 significant). See Table S8 for details.