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RESEARCH ARTICLE

Spatial turnover of fungi and partner choice shape mycorrhizal networks in epiphytic orchids

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Abstract

1. In soils, plants and fungi can form complex mycorrhizal networks allowing nutrient transfers between plant individuals and species. It is less clear, however, whether such networks exist on the bark of trees where epiphytic plant communities thrive in rainforests. Previous work showed that tropical epiphytic orchids especially, harbour symbiotic fungi in their roots, but the structure and determinants of the resulting networks remain unknown at the tree scale.
2. We tested the hypothesis that epiphytic orchids rooted in the same area on the bark share mycorrhizal fungi, regardless of their species (i.e. spatial determinant). For this purpose, we selected the trunk of six trees of two common species in a rainforest and sampled orchid roots, protocorms and surrounding bark. We identified mycorrhizal fungi including Tulasnellaceae using high-throughput sequencing of the ITS2 marker, and reconstructed orchid–fungus bipartite networks for each tree to analyse their structure and the spatial turnover of this symbiosis.
3. We found that epiphytic orchid communities form antinested and highly modular networks with mycorrhizal fungi spread on the bark. As expected, modules of interactions are explained by their spatial structure, with nearby roots sharing fungi, but also by the orchid species involved. These results reveal the presence of shared mycelial networks in epiphytic habitats, whose roles in the resilience and facilitation of epiphytic plant communities need to be assessed.
4. *Synthesis.* Tropical tree barks are densely colonized by certain mycorrhizal fungi that can form symbioses in nearby adult and young orchids simultaneously. These mycorrhizal networks may allow water and nutrient transfers to alleviate the stressful conditions of the epiphytic habitats.

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1 | INTRODUCTION

Most vascular plants harbour mutualistic fungi in their roots, forming associations called mycorrhizas (Brundrett & Tedersoo, 2018; Van Der Heijden et al., 2015). Mycorrhizas are chimeric organs, composed of both plant tissues and fungal hyphae, where nutrient exchanges can occur in both directions (Smith & Read, 2008). While plants provide photosynthetic acquired carbon to their fungal partners, the latter improve the hydro-mineral nutrition of their host and enhance their defence against both biotic and abiotic stresses. As mycorrhizal fungi can associate with plants of the same (Beiler et al., 2010) or different species (Richard et al., 2005; Selosse et al., 2002), they are known to form complex networks of interactions with roots in soils (e.g. Jacquemyn et al., 2015; Rog et al., 2020; Toju et al., 2014, 2016).

Plant-fungus networks can be classified into two categories: ecological networks, in which fungal species or operational taxonomic units (OTUs) interact with plant species that do not obligately co-occur, meaning that the fungi do not necessarily link different plant species in a given site, and physical networks, where single fungal mycelia can simultaneously interact with several plant individuals. Regarding mycorrhizas, physical networks have often been referred to as common mycorrhizal networks (CMNs; Simard & Durall, 2004; Van Der Heijden & Horton, 2009). Because they involve physical contacts between plant roots mediated by fungal hyphae, such networks can lead to fungal-mediated plant-plant interactions. First, two plants connected to the same fungus may asymmetrically contribute to its carbon need, resulting in a benefit for the less providing one (Walder et al., 2012). Second, both nutrients and information exchanges can occur between plants species (Klein et al., 2016; Selosse & Roy, 2009; Simard et al., 1997, 2015) even though their consequences are not always easy to experiment or observe (Booth, 2004; Liang et al., 2020).

Plant-fungus interaction networks have been mainly studied in soils, especially in temperate ecosystems. By contrast, tropical ecosystems have been largely overlooked, especially when considering the epiphytic habitat where plants grow on tree bark and twigs. Indeed, epiphytes represent 9% of vascular plants world-wide (Zotz, 2016), reaching up to 25% in tropical ecosystems (Nieder et al., 2001). Notably, these plants can undergo transient water and nutrient limitation between rainfall events (Yoder et al., 2010; Zotz, 2016). By mediating nutrients and/or water transfers between co-occurring plants, the networks formed by plants and fungal mycelia could thus be highly advantageous in the epiphytic habitat (Leake & Cameron, 2012) as also previously suggested in terrestrial mycorrhizal mutualisms (Bingham & Simard, 2011).

Epiphytic plants were long considered to lack mycorrhizal associations (Brundrett & Tedersoo, 2018) but many works are now demonstrating that mycorrhizas of the same or different types compared to their terrestrial counterparts, are also frequent in the epiphytic habitat (e.g. Kottke et al., 2010; Martos et al., 2012; Petrolli et al., 2021; Rowe & Pringle, 2005). Particularly, 70% of the epiphytic plant species are members of the Orchidaceae family (Zotz, 2016).

This family comprises no less than 28,000 species (Christenhusz & Byng, 2016), >80% of which are epiphytic (Givnish et al., 2015).

Orchids form one of the major types of mycorrhizas (Brundrett & Tedersoo, 2018), called orchid endomycorrhiza, which is constantly found throughout the family (Selosse et al., 2022; Smith & Read, 2008; Wang et al., 2021) in terrestrial as well as in epiphytic orchids (Dearnaley et al., 2012, 2016; Martos et al., 2012). The orchid-fungus symbiosis is atypical because it takes the form of intracellular hyphal structures called pelotons, and because fungal partners are required at each step of the plant life cycle from the symbiotic germination of the orchid dust seeds to the colonization of adult roots (Rasmussen, 1995; Smith & Read, 2008). Orchid mycorrhizal fungi (OMF) form a polyphyletic group of mostly Basidiomycota and, to a lesser extent, Ascomycota (Dearnaley et al., 2012; Selosse et al., 2022; Wang et al., 2021). In green photosynthetic orchids, the main fungal partners belong to the historically called 'rhizoctonias' (Dearnaley et al., 2012; Selosse et al., 2011) which include members of the Cantharellales (namely Ceratobasidiaceae and Tulasnellaceae; Veldre et al., 2013) and Sebaciniales (namely Serendipitaceae; Weiß et al., 2016).

On bark, orchids seem to associate with a limited range of fungal partners, including rhizoctonias (Herrera et al., 2019; Izuddin et al., 2019; Martos et al., 2012; Petrolli et al., 2021), as well as other fungal families such as *Mycena* (Zhang et al., 2012), *Fusarium* (Jiang et al., 2019) and some Pucciniomycota belonging to the Atractiellales (Herrera et al., 2019; Kottke et al., 2010).

Since they require a symbiotic germination, it is sometimes suggested that orchids 'take now and pay later', meaning that they first receive carbon from their fungal partners during germination and subsequently reward it at photosynthetic adult stage. Thus, CMNs in orchids may be particularly relevant when adults and germinating plants co-occur (Jacquemyn et al., 2012). Orchid-fungal networks have been investigated in several ecosystems (reviewed in Li, Shima, et al., 2021), but have often been limited to ecological networks, that is, at large scale and considering plant species instead of individuals, sometimes ignoring direct, physical interactions between plants mediated by fungi (e.g. Herrera et al., 2018; Kottke et al., 2008, 2013; Martos et al., 2012; Xing et al., 2020). This can be particularly limiting when considering epiphytic orchids because orchid individuals growing on different trees may hardly share single fungal hyphae. Yet, indirect plant interactions, such as nutrient or information transfers, can only occur at a scale at which fungal mycelia can be shared among plants.

In a recent investigation of epiphytic fungal communities in Brazilian orchids, we suggested that fungal communities, including OMF, extend away from the roots of their host to forage the surrounding bark in the epiphytic rhizosphere (Petrolli et al., 2021). More importantly, OMF fungi were found to be shared between different co-occurring orchid species, suggesting the existence of epiphytic CMNs among orchids of a single tree. However, these results were supported by only few individuals on a limited number of trees.

Additionally, as previous works have focused on ecological networks (merging interactions on different trees; see references

above), the factors shaping plant–fungal interactions at the scale of a single tree are still poorly understood. Particularly, whether these networks are shaped by species identity (i.e. orchids of the same species share mycorrhizal fungi), spatial distribution (i.e. orchids that are spatially close share similar mycorrhizal fungi regardless of their species) or stochastic determinants remains in question.

Using a spatially explicit sampling design, we investigated epiphytic plant–fungal networks, on six trunks of two common tree species in a tropical rainforest of La Réunion (French overseas island in the Indian Ocean) colonized by 10 epiphytic orchid species. Our working hypotheses were that, when spatially close, orchid individuals from (i) the same species and from (ii) different species shared mycorrhizal symbionts, meaning that the spatial distribution of the mycorrhizal partners should prevail in determining plant–fungus interactions in the stressing epiphytic habitat. Particularly, (iii) ‘seedlings facilitation’ may occur in the epiphytic orchid habitat, meaning that different growth stages of an orchid species share symbiotic fungi that may sustain the growth of adjacent seedlings.

2 | MATERIALS AND METHODS

2.1 | Study site and species

The study was conducted in February 2020 in a well-preserved fragment of lowland rainforest on La Réunion island (Mare-longue, 21°21′14″S 55°44′29″E, elevation 205–560m; Strasberg, 1994) under the permission number DIR-I-2020-013. Six trees, three of each *Labourdonnaisia calophylloides* Bojer (Sapotaceae) or *Nuxia verticillata* Lam. (Stilbaceae; Table 1), were selected according to their diameter (approx. 20cm) at breast height and the coexistence of at least three orchid species on their trunk. *L. calophylloides*, the dominant tree species of this forest, has a straight trunk with cracked bark, whereas *N. verticillata* (also common) has a twisted, crevassed trunk with leafy exfoliating bark (Figure S1). Both tree species are endemic to the Mascarene islands and were shown to host at least 15 and 14 orchid species respectively in a survey of 1-ha plot in this forest (Lancaster, 2004). The selected trunks (labelled A–F), harboured a total of 10 epidendroid orchid species in six genera that were morphologically identified (Table 1; Table S1), except protocorms on trunk F that were later identified by DNA analysis (see below). All the tree and orchid species studied are listed as ‘Least Concern’ or ‘Data Deficient’ according to the IUCN, except *Beclardia macrostachya* (Thouars) A. Rich which is ‘Near Threatened’ (IUCN France, CBNM, FCBN & MNHN, 2013).

2.2 | Sampling procedure

On each trunk, the position of each sample (roots, protocorms and bark) was precisely determined in a coordinate system comprising the height on the trunk between c. 1 and 3 m and the horizontal distance on the trunk from a vertical reference line, using measuring

tape (Figure S1). Yellow–orange portions (c. 2 cm) of roots in contact with the bark, that is, where mycorrhizal colonization is known to occur (Martos et al., 2012), were sampled using sterile razor blades leaving the orchids alive on their trunk. Their surface was sterilized in 70% ethanol, then quickly dried in silica gel. Each root sample was assigned to an orchid species and growth stage: protocorm, no leaf; stage 2, one leaf; stage 3, young seedling (not in age of flowering); stage 4, adult (Figure S2). Plant individuals were not counted given that half of these orchids have sympodial growth allowing vegetative multiplication. In addition to the roots of adults and seedlings, orchid protocorms found on trunk F were sampled and preserved in 50% ethanol at 4°C and later identified by DNA analysis (see below). Because the OMF also distribute on the bark around the roots of epiphytic orchids (Petrolli et al., 2021), we also collected c. 50mg outer bark samples at regular intervals in the coordinate system using a sterilized blade. These bark samples were immediately dried in individual bags of silica gel, preventing any cross-contamination between samples. In the end, 341 root sections, 7 protocorms, and 105 pieces of bark, that is, 453 samples, were collected and used for molecular analyses.

2.3 | Molecular analyses of fungi and plants

The root or bark samples were powdered in a TissueLyser II (Qiagen) with two sterilized tungsten beads per tube, and genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen) or the NucleoSpin Soil Mini Kit (Macherey-Nagel, GmbH & Co KG.) respectively. Polyvinylpyrrolidone (PVPP) was added to the bark samples during cell lysis to precipitate the tannins. Due to their small size, the protocorms were ground manually in the lysis buffer using sterilized pellet pestles, and their DNA was extracted as for the roots. Each sample was then amplified by PCR using two sets of primers targeting the internal transcribed spacer 2 (ITS2) of nuclear ribosomal DNA which provides more suitable results than the ITS1 (Yang et al., 2018), namely the widely used ITS86-F/ITS4 (White et al., 1990) amplifying both Asco- and Basidiomycota except the OMF family Tulasnellaceae (Rammitsu et al., 2021), and the more recent 5.8S-OF/ITS4-Tul (Vogt-Schilb et al., 2020) to specifically amplify Tulasnellaceae. Each PCR amplification was conducted with both forward and reverse tagged primers as in (Petrolli et al., 2021). The PCR experimental design also included three negative (ultrapure water) and three mock community controls per plate, resulting in 36 positive and 36 negative controls in total. We also included tagging system negative controls to assess cross-contamination during PCR trials, library preparation or sequencing (Hornung et al., 2019; Zinger et al., 2019). The mock community consisted in a defined mixture of 10 fungal strains, including both Asco- and Basidiomycota (Table S2). The organization of samples in PCR plates was randomized. PCR were performed in 25 µl containing 0.2mM each dNTP, 0.4 µM each primer, 1 unit AmpliTaq Gold® 360 DNA Polymerase (Thermo Fisher Scientific), 1X AmpliTaq Buffer supplied with MgCl₂ and 1.5 µl template DNA, using the following program:

TABLE 1 Sampling design. Of the 453 sequenced samples (tree bark and orchid roots), 368 harboured orchid mycorrhizal fungi (OMF) sequences and were thus kept for subsequent analyses. Concerning protocorms, this represented one of seven sampled protocorms. N samples, number of sampled orchid roots. Additional details regarding the orchid tribes and sub-tribes are given in Table S1

| Tree ID | N samples (inc. protocorms) | Sample type | Species | Family | Coordinates | Alt. (m) |
|---------|-----------------------------|-------------|-------------------------------------|-------------|----------------------------|----------|
| A | 17 | Bark | <i>Labourdonnaia calophylloides</i> | Sapotaceae | 21°21'06"S 55°44'31" E | 237 |
| | 8 | Roots | <i>Oberonia disticha</i> | Orchidaceae | — | — |
| | 20 | Roots | <i>Aeranthes arachnites</i> | Orchidaceae | — | — |
| | 15 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |
| B | 16 | Bark | <i>Labourdonnaia calophylloides</i> | Sapotaceae | 21°20'57.7"S 55°44'38.6" E | 338 |
| | 7 | Roots | <i>Polystacchia concreta</i> | Orchidaceae | — | — |
| | 26 | Roots | <i>Angraecum mauritianum</i> | Orchidaceae | — | — |
| | 3 | Roots | <i>Angraecum pectinatum</i> | Orchidaceae | — | — |
| | 10 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |
| C | 17 | Bark | <i>Labourdonnaia calophylloides</i> | Sapotaceae | 21°21'20"S 55°44'20"E | 205 |
| | 15 | Roots | <i>Angraecum pectinatum</i> | Orchidaceae | — | — |
| | 5 | Roots | <i>Polystacchia concreta</i> | Orchidaceae | — | — |
| | 11 | Roots | <i>Angraecum mauritianum</i> | Orchidaceae | — | — |
| | 7 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |
| | 8 | Roots | <i>Bulbophyllum occultum</i> | Orchidaceae | — | — |
| D | 17 | Bark | <i>Nuxia verticillata</i> | Stilbaceae | 21°20'28"S 55°44'14"E | 553 |
| | 6 | Roots | <i>Beclardia macrostachya</i> | Orchidaceae | — | — |
| | 45 | Roots | <i>Angraecum cucullatum</i> | Orchidaceae | — | — |
| | 9 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |
| | 8 | Roots | <i>Polystacchia concreta</i> | Orchidaceae | — | — |
| E | 17 | Bark | <i>Nuxia verticillata</i> | Stilbaceae | 21°20'30"S 55°44'08"E | 556 |
| | 13 | Roots | <i>Angraecum ramosum</i> | Orchidaceae | — | — |
| | 14 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |
| | 23 | Roots | <i>Angraecum cucullatum</i> | Orchidaceae | — | — |
| F | 21 | Bark | <i>Nuxia verticillata</i> | Stilbaceae | 21°20'29"S 55°44'07"E | 560 |
| | 23 | Roots | <i>Angraecum ramosum</i> | Orchidaceae | — | — |
| | 21 | Roots | <i>Beclardia macrostachya</i> | Orchidaceae | — | — |
| | 43 (7) | Roots | <i>Angraecum cucullatum</i> | Orchidaceae | — | — |
| | 1 | Roots | <i>Polystacchia concreta</i> | Orchidaceae | — | — |
| | 7 | Roots | <i>Bulbophyllum prismaticum</i> | Orchidaceae | — | — |

initial denaturation 10 min at 95°C; 35 cycles of denaturation 30s at 95°C, annealing 30s at 56.5°C (ITS86-F/ITS4) or 52°C (5.8S-OF/ITS4-tul), and elongation 30s at 72°C; final elongation 12min at 72°C. Whenever samples did not amplify with the more universal primer set (47 samples), we used a nested PCR protocol with ITS1/

TW13 as follows—initial denaturation 10 min at 95°C; 35 cycles of denaturation 30s at 95°C, annealing 30s at 55°C and elongation 1 min at 72°C; final elongation 12min at 72°C—then ITS86-F/ITS4 as above. Each PCR trial was replicated twice, pooled and loaded on agarose gel for visualization of the amplicons. Each amplicon was

then purified with the NucleoMag® NGS Clean-up and Size Select (Macherey-Nagel), quantified by fluorescence using the Qubit™ dsDNA High-Sensitivity (Invitrogen™), and then mixed equimolarly with other amplicons in four pools. The four pools were sequenced in two 2×250bp paired-end sequencing runs on an Illumina MiSeq platform at FASTERIS (Geneva, Switzerland).

For the species identification of protocorms, we performed seminested PCRs with first ITS1P/ITS4 (Ridgway et al., 2003) then ITS1/ITS4 (White et al., 1990), and sequenced these amplicons with the Sanger method at Eurofins Genomics (Germany). The obtained sequences were aligned with sequences of orchids from La Réunion retrieved on GenBank using MAFFT (Katoh et al., 2002), and a phylogenetic inference was performed by ML using IQTREE 2.1.3 (Minh et al., 2020) for phylogenetic comparison, using default parameters.

2.4 | Bioinformatics and OTU filtering

Paired reads from 2×250bp Illumina sequencing were processed using a custom pipeline based on VSEARCH (Rognes et al., 2016) as in Perez-Lamarque, Krehenwinkel, et al. (2022). Briefly, paired-end reads were assembled using the *fastq_mergepairs* function with default parameters and assembled reads with more than two errors in the alignment were discarded. The resulting assembled reads were assigned to samples according to their tag+primer sequences using CUTADAPT (Martin, 2011) allowing zero discrepancies. Tag jumps (Zinger et al., 2019) was estimated at this step and represented 0.04% of the reads. Operational taxonomic units (OTUs) were clustered using a 97% similarity threshold with VSEARCH algorithm. Chimeras were detected at this step using the *uchime_de-novo* option of VSEARCH and removed from the dataset. OTUs were taxonomically assigned using the *usearch_global* option of VSEARCH with default parameters on the UNITE V8.3 (2021-05-10) eukaryotes reference database (Nilsson et al., 2015).

Singletons and short OTUs (<200bp) were removed before OTU table building. Nonfungal OTUs and OTUs abnormally detected in the positive and negative controls were also discarded from the dataset, as well as OTUs identified as contaminants by the DECONTAM R-package with frequency method (Davis et al., 2018). Finally, based on the previously estimated tag-jumps rate, OTUs that represented <0.5% of the sample reads (hereafter referenced as the bioinformatics threshold) were set to 0, hence minimizing the impact of artefactual sequences and tag jumps. Samples with <1000 reads or without OMF were not considered in subsequent analyses. Because we used two pairs of primers of varying specificity, the read counts obtained with ITS86-F/ITS4 and 5.8S-OF/ITS4-Tul were not comparable and therefore, only the presence-absence (1/0) of the OMF OTUs colonizing the 368 remaining samples was considered in following statistical analyses (except in Figure 1; Figures S3, S15 where all fungi were analysed). As OTUs with low abundances can be over-represented in statistical analyses using presence-absence data, we repeated the main analyses by excluding OTUs whose per-sample relative abundance was ≤10% (instead of the 0.5% mentioned

above), to confirm the robustness of our results to potential mid-to-low-abundance errors.

Amplicon sequence variant (ASV) construction methods have been developed to improve the delineation of microbial taxa and the reproducibility of metabarcoding data, although their relevance in comparison to OTUs has been questioned when using ITS regions and Dikarya fungi (Tedersoo et al., 2022). We therefore repeated some of the analyses, that is, the network analyses (see below), using the most abundant unique sequences (up to five) inside each OMF OTU. This finer delineation of OMF, that is, 100%-similarity OTUs, aimed at determining whether the same fungal haplotype was found in several adjacent roots or protocorms of the same or of different orchid species.

2.5 | Diversity analyses

The following beta diversity analyses were conducted with the VEGAN R-package (Oksanen et al., 2013), using the Jaccard index as implemented in the *vegdist* function. To assess differences in OMF composition between substrates (i.e. root vs. bark), orchid species and tree individuals and species in the overall dataset, we used a non-metric multidimensional scaling (NMDS) approach using the *meta-MDS* function. The differences were tested using a PERMANOVA, with 999 permutations, using the *adonis* function. As the studied epiphytic orchids grew on different trunks in the same forest area, the permutations were constrained to each tree individual using the *strata* option in *adonis*. To test the effect of tree individuals on fungal communities within orchid species growing on several trees we conducted a PERMANOVA using the tree identity (A–F) as explanatory variable and orchid species as *strata*.

2.6 | Network analyses

Bipartite interaction networks have been used to describe and analyse the patterns of interactions between species belonging to two groups of organisms, for example, plants and their mycorrhizal fungi (Jacquemyn et al., 2011; Martos et al., 2012). These networks usually have a nonrandom, rather nested or modular structure (Chagnon et al., 2012). In a nested network, interactions are between generalist species or between specialist and generalist species, with little or no reciprocal specificity (Bascompte et al., 2003). Nestedness is a common feature of mutualistic networks where it is supposed to confer stability (Thébault & Fontaine, 2010), but it can also result from neutral processes (Krishna et al., 2008). In a modular network, however, interactions are mainly within modules with little or no interaction between modules (Olesen et al., 2007). Modularity may indicate the presence of reciprocal specialization (e.g. in parasitic co-evolution), but also environmental constraints (Martos et al., 2012). However, the study scale and the individual- versus species-level scale considered both have large influence on a given network's structure (e.g. Olesen et al., 2007).

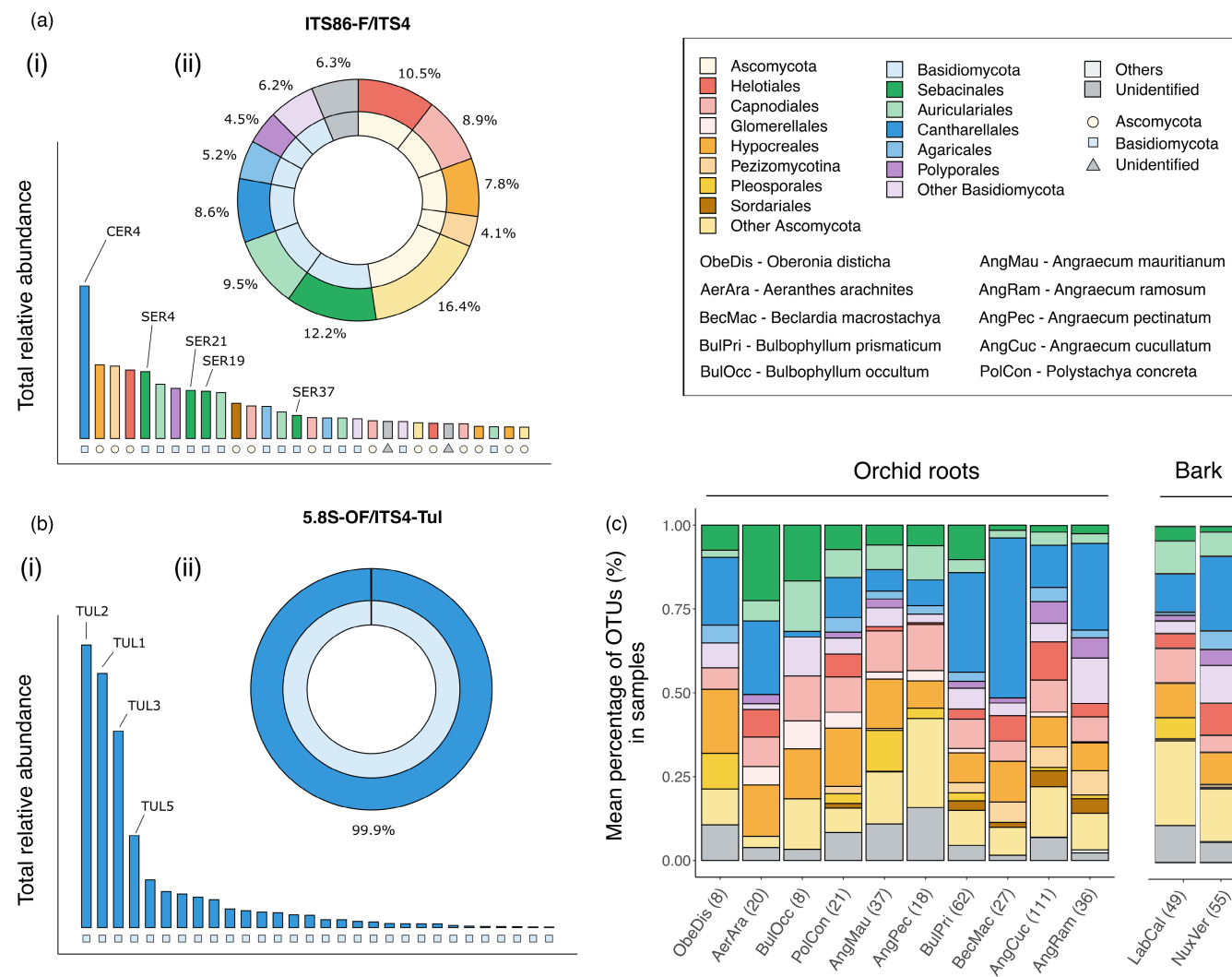


FIGURE 1 Overview of fungal community composition in the epiphytic environment colonized by orchid species identified with the primers (a) ITS86-F/ITS4 or (b) 5.8S-OF/ITS4-Tul across the whole dataset. (i) Ranked relative abundances of the first 30 OTUs. OTUs are coloured according to their taxonomy and main OMF OTUs are named. (ii) Relative abundance of the main identified fungal clades. (c) Main fungal clades occurring in epiphytic orchid roots and in the surrounding bark with the two set of primers using presence/absence data. LabCal, *Labourea calophylloides*; NuxVer, *Nuxia verticillata*. The number of samples considered is given in brackets.

In order to study interactions at the scale of fungal mycelia, all network analyses were performed using the six interaction matrices independently, corresponding to the six trunks studied. Binary matrices of interactions between fungal OTUs (rows) and plant root samples (columns; i.e. bipartite networks), retrieved after the low-count occurrences filtering described above, were used as inputs for the following analyses. From each matrix, we evaluated both the nestedness and the modularity in the observed network and tested their significance against 1000 null models reconstructed using the *permatswap* function of the VEGAN R-package. Nestedness was calculated using the *nested* function of bipartite (Dormann et al., 2008), considering the 'NODF2' method. The modularity 'Q' was calculated using the *computeModule* function of the same package using default method (Newman) and 1000 steps. As module detection may slightly vary during calculation due to randomized procedure, modules were first calculated 100

times and then the most frequent set of modules was kept for subsequent analyses. To assess the reliability of each obtained module, we then searched how many times these modules were found in 1000 additional modularity calculation, and calculated p-values accordingly for each one of them.

2.7 | Spatial analyses

The spatial distances between samples were calculated from their coordinates taken in the field, considering the smallest distance between them on the cylindric trunks. The trunk of the tree F separated into two vertical branches and therefore, the spatial distances between samples located on different branches were calculated by considering the smallest distance between these samples passing through the fork. To assess for the presence of a spatial structure in

the orchid–OMF interaction, spatial autocorrelations of OMF communities in orchid roots were analysed using mantel correlograms in vegan, with the 'spearman' method, and 999 permutations. To corroborate the presence of an epiphytic rhizosphere as previously observed in an Atlantic Forest (Petrolli et al., 2021), we calculated the distance decay of similarity between orchid roots and surrounding bark as in the above reference.

In order to include the full spatial component in statistical analyses (i.e. not only the height on tree trunk which was only used as a proxy of the spatial component in illustrations), we used a principal coordinates of neighbour matrices (PCNM) approach. PCNM allows the reduction of a complex system of spatial coordinates into a limited number of vectors called eigenvectors. PCNM eigenvectors were computed using the *pcnm* function of the *VEGAN* package. Eigenvectors (i.e. PCNM axes) associated with positive eigenvalues were submitted to a forward selection (Blanchet et al., 2008) using the code provided in (Bauman et al., 2018). Variance partitioning was then performed using the *varpart* function in the *VEGAN* R-package, using the orchid species identity as first explanatory variable and the sets of selected PCNM vectors as second explanatory variable. Associated p-values were computed after redundancy analyses (RDAs) of the previously cited variables, followed by an 'anova' test considering either orchid species or selected PCNM vectors as explanatory variables of the OMF communities.

Additionally, for each module in each reconstructed network (see above), we tested if the module was determined (i) by the spatial distribution of orchid roots and/or (ii) by the orchid species composition inside the module. To do so, we randomized 10,000 times the sample names in the interaction matrix and compared (i) the mean spatial distance (mD) between samples within module and (ii) the species composition (sC) within module, between the original and randomized matrices. For each module, p-values were computed as the proportion of (i) $mD_{\text{randomized}} < mD_{\text{original}}$ or (ii) $sC_{\text{randomized}} = sC_{\text{original}}$.

3 | RESULTS

3.1 | Data quality and fungal community description

A total of 25,806,753 and 24,989,711 raw sequencing reads were obtained with primer pairs ITS86-F/ITS4 and 5.8S-OF/ITS4-Tul respectively. The remaining 32,593,936 reads obtained after the selective bioinformatics process clustered into 4564 OTUs in total. The expected fungal species were all identified in the 36 mock community positive controls. After filtering OTUs' occurrences representing <0.5% of reads from each sample, the remaining reads in positive controls all belonged to fungal species of the mock community. Applying this stringent curation of the data led to 794 OTUs (i.e. 27,595,918 reads). The 5.8S-OF/ITS4-Tul primer pair detected 57 OTUs of Tulasnellaceae (Figure 1), among which only one OTU was amplified with the more universal pair ITS86-F/ITS4. Despite our fine sampling of roots and bark on each tree between 1 and 3 m

in height, the rarefaction curves did not reach a plateau, neither for all fungal OTUs nor only OMF OTUs (Figure S3).

Ninety-one of 794 OTUs (11.5%) were assigned to families of OMF, namely Tulasnellaceae (57 OTUs) and Ceratobasidiaceae (3 OTUs) in the Cantharellales and Serendipitaceae (31 OTUs) in the Sebaciales, representing in average between 12% and 50% of the orchid root-associated fungi (Figure 1C). Other putative OMF, for example, Atractiellomycetes (two OTUs), *Fusarium* (two OTUs) or *Mycena* (one OTU), were too occasional to be considered as key OMF here and were not kept in our analyses. Regarding other fungal guilds, the 703 fungal OTUs included non-OMF saprotrophs (13.6%), plant pathogens (7.7%) and lichenized fungi (4.8%; Figure S4A). Remaining OTUs (62.5%) could not be unambiguously assigned to a unique trophic guild due to insufficiently taxonomic resolution.

Tulasnellaceae were the most frequent OMF in almost all orchid species (Figure S4B). While Tulasnellaceae were equally distributed between both tree species, Serendipitaceae were more frequent on *L. calophylloides* than on *N. verticillata* (Figure 2). Conversely, the most abundant Ceratobasidiaceae was found only on *N. verticillata* where it was notably retrieved in all the roots of the epiphytic orchid *Beclardia macrostachya*.

3.2 | Communities of OMF in orchid roots and on bark

On each tree trunk, OMF communities significantly differed between bark and orchid roots (nested PERMANOVA; $F = 4.53$, $R^2 = 0.05$, $p = 0.001$; Figure S5). The overall OMF community varied more across tree species ($F = 47.27$, $R^2 = 0.11$, $p = 0.001$) and even more across tree individuals ($F = 35.87$, $R^2 = 0.32$, $p = 0.001$, Figure S5).

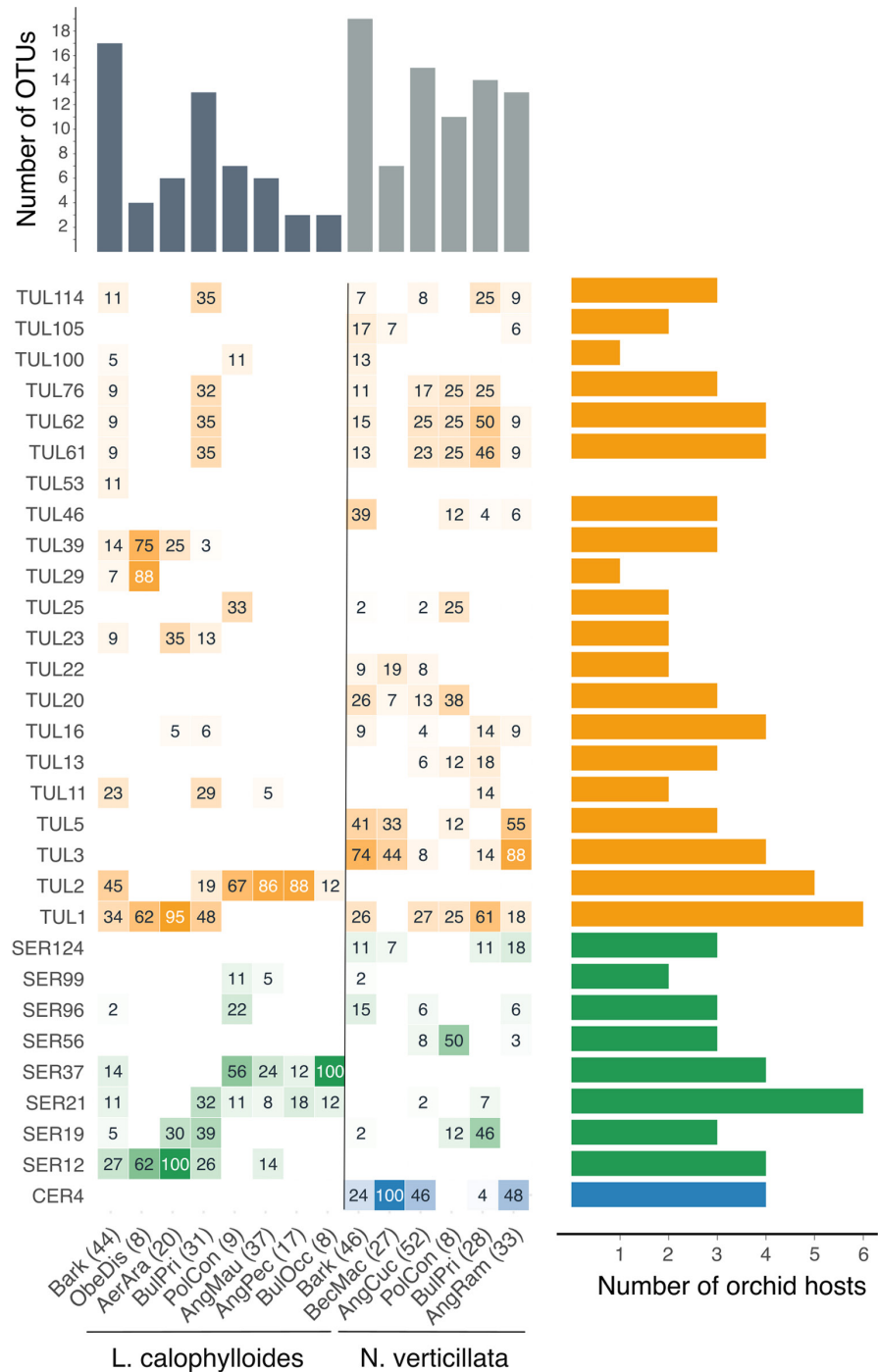
Considering orchid roots only, the OMF communities also varied across tree individuals ($F = 38.18$, $R^2 = 0.33$, $p = 0.001$; Figure S6) and tree species ($F = 52.45$, $R^2 = 0.12$, $p = 0.001$). They also differed between orchid species when controlling for tree individuals ($F = 7.99$, $R^2 = 0.25$, $p = 0.001$). We also found an effect of tree individuals on OMF communities while controlling for orchid species ($F = 25.98$, $R^2 = 0.33$, $p = 0.001$; see e.g. *Polystachya concreta* on Figure S6).

3.3 | Plant-fungus network analyses

OMF sharing between orchids was variable among OTUs and orchid species, although a high level of OTU's sharing was clearly visible (Figure 2). The two most shared OTUs were TUL1 and SER21 (six orchid species each) while CER4 was largely shared between orchid species growing on *N. verticillata* (Figures 2 and 6).

The orchid–fungus networks were very consistent in their structure among the six trees, showing significant modularity and antinestedness (Figure S8). Using unique sequences within each OTU instead of the 97% OTUs did not change these results

FIGURE 2 Communities of orchid mycorrhizal fungi (OMF) in 10 epiphytic orchid species growing on two tree species (three individuals each). Only the 30 most abundant OTUs are represented. Numbers in the table indicate the % of colonized roots. The number of sampled roots or bark is given in brackets. *L. calophylloides*, *Labourdonnaisia calophylloides*; *N. verticillata*, *Nuxia verticillata*. TUL, Tulasnellaceae; SER, Serendipitaceae; CER, Ceratobasidiaceae. Right, number of orchid host species for each OMF OTU. Top, number of different OTUs colonizing each compartment (bark or orchid species).

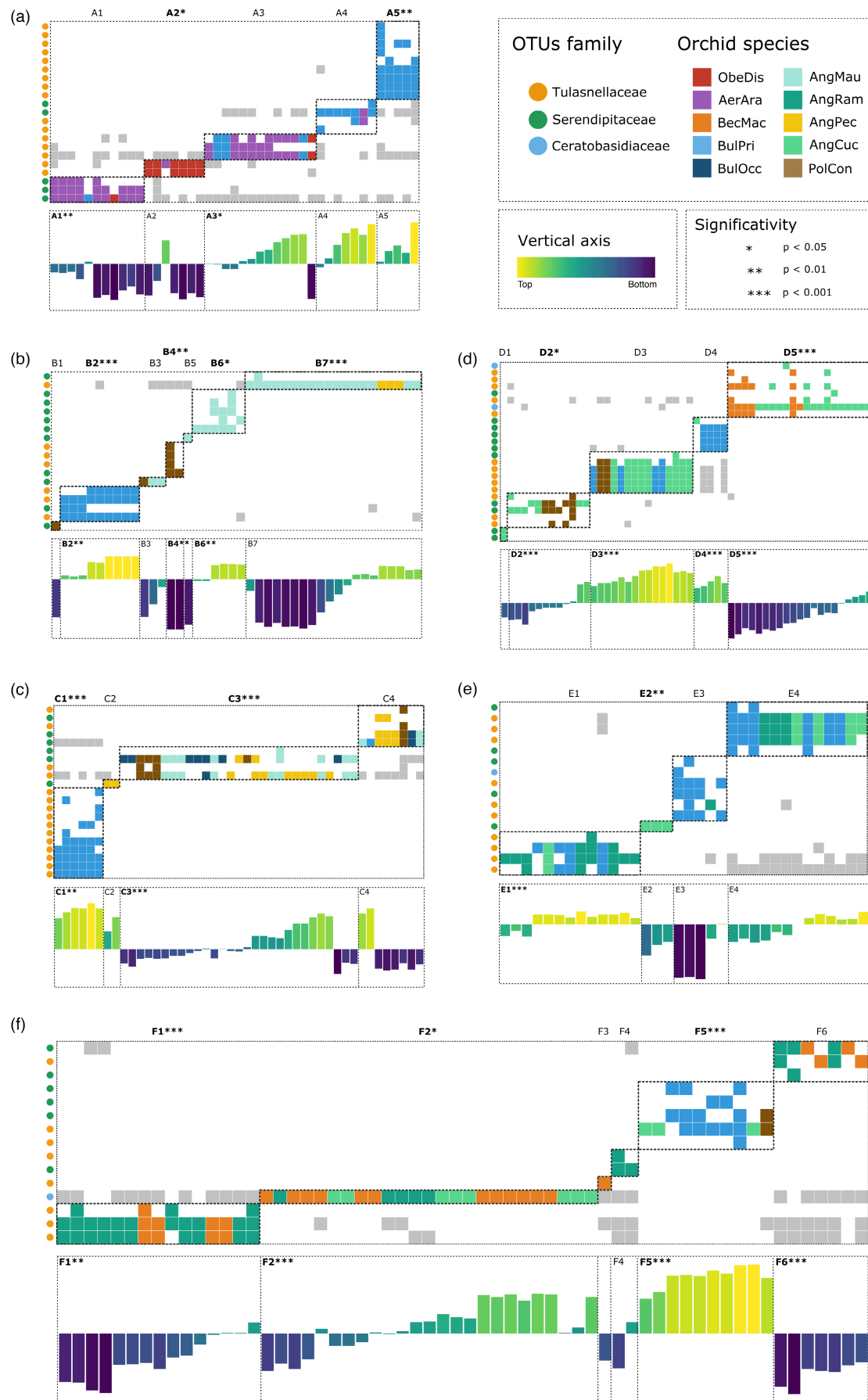


(Figure S9). Modules of interactions between OMFs and orchid roots involved either one (29.6%), two (37.0%), three (25.9%) or >4 (7.4%) orchid species (Figure 3, see also Figure S10). Similarly, they also included one (41.7%), two (50.0%) or three (8.3%) OMF families.

3.4 | Spatial versus orchid species determinants of the network structures

On each tree individual, we observed a positive spatial autocorrelation of OMF communities in roots ranging from 15 to 90 cm (Figure 4;

FIGURE 3 Interaction networks between orchid mycorrhizal fungi (OMF, rows) and their orchid hosts (columns) and associated structures. Networks are represented separately for each tree (a–f). Top; modules of interactions are delimited by grey-dashed lines. Squares inside modules are coloured according to the orchid species. Full circles at the left of each network indicate the OMF family of each OTU. Modules are named above each graph and those that are significantly shaped by the orchid species composition are bolded and associated with their significance code (*, <0.05; **, <0.01; ***, <0.001). Bottom; spatial distribution of orchid roots. The coordinate of each orchid root in the vertical axis on the coordinate system was plotted and coloured according to its value. Modules that are significantly shaped by the spatial distribution of orchid roots are bolded and associated with their significance code (see above). See Table S3 for significance details.



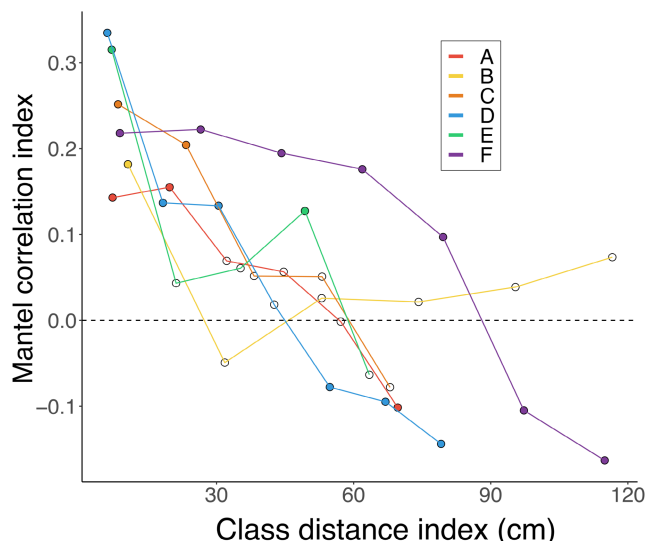


FIGURE 4 Mantel correlogram showing spatial autocorrelation of the interactions between orchid mycorrhizal fungi (OMF) and orchid roots for each tree. Full circles indicate significant ($p < 0.05$) autocorrelation (positive or negative).

Figure S11). This suggests that, even when not considering the orchid species, the interactions between OMF and the roots were spatially structured, at least over short distances (~20 cm). In addition, network visualizations suggested that OMF OTUs were mostly shared between spatially close orchid roots (Figure 3; Figure S10).

We used two approaches to quantify these tendencies. First, at the network scale, variance partitioning revealed that, on each tree, the identity of orchid species, the spatial distribution of samples (as assessed with PCNMs) and their interaction, both had a significant influence on OMF communities (Figure 5; Figure S11). Second, we quantified the contribution of each of these two determinants in the networks' structures at the module scale by comparing the modules observed in Figure 3 to similar sized modules in randomized matrices. Results (synthesized in Table S3) showed that the spatial component or the species identity component alone, explained respectively 22.2% and 11.1% of the observed modules. The interplay between these two components explained 37.0% of the modules. Accordingly, roots sharing OMF on a single tree were spatially closer to each other than roots that did not share any OMF OTU, even when considering different orchid species (Figure S12).

In addition, OTUs that were frequent in the roots of an orchid species on a tree tended to be also detected in other orchid species from the same tree (Figure S13) suggesting that OTUs that intensively colonized an orchid species had more chance to colonize other spatially close species.

We finally observed that OMF OTUs retrieved in roots were more frequent in the surrounding bark than at greater distances, as suggested by the distance decay of similarity of OMF communities between orchid roots and bark (Figure S14). This reveals that fungi could effectively link orchid roots of different species by their expansion from roots to the epiphytic rhizosphere.

3.5 | OMF turnover from protocorms to adult orchids in *Angraecum cucullatum*

On tree F all the protocorms and all but one of the stage 2 seedlings successfully sequenced by Sanger method belonged to the orchid species *A. cucullatum*. The remaining seedling could not be identified and was not kept for analyses. These protocorms and seedlings were mostly colonized by non-OMF fungi, but four seedlings and one protocorm were also colonized by a single OMF OTU, that is, CER4 (Figure S15), which was also detected in all growth stages of *A. cucullatum* on other trees (Table 2). We additionally found that this OTU was shared between different orchid species and different growth stages of *A. cucullatum* (Figure 6). When looking at the distribution of unique sequences within this OTU as a proxy of fungal haplotypes, we found mainly two sequences (Figure 6; Figure S16) that were also shared between the roots of different but spatially close orchid species, including protocorm and seedlings of *A. cucullatum* and surrounding adults of *A. ramosum*.

4 | DISCUSSION

4.1 | Diversity of OMF in bark-dwelling communities

After filtering low-count OTU's occurrences (i.e. <0.5% of reads in each sample), we retrieved 794 fungal OTUs in the whole dataset, 91 (11.46%) of which were considered as OMF. Perhaps because of the use of an additional specific primer pair, Tulasnellaceae were the most abundant OMF in nearly all orchid species. However, the need to use specific primers to amplify this family is largely acknowledged and has been recently demonstrated by Rammitu et al. (2021). Tulasnellaceae and Serendipitaceae were both represented by numerous OTUs whereas only three OTUs of Ceratobasidiaceae were detected, only one being frequent, suggesting that Ceratobasidiaceae might be rarer in these epiphytic rainforest habitats (Martos et al., 2012).

4.2 | Mycorrhizal specificity in epiphytic orchids and consequences for fungal networks

The question of whether orchid species are mycorrhizal generalists or specialists has long triggered research in terrestrial (Jacquemyn et al., 2011) as well as in epiphytic (Herrera et al., 2019) orchids (reviewed by Li, Yang, et al., 2021). Here, no orchid species associated with less than three OTUs from at least two OMF families. Some species were opportunistic in their mycorrhizal interactions, as illustrated by *Bulbophyllum prismaticum* which was growing on both tree species and associated with a richer guild of OMF than any other orchid species. *B. prismaticum* could therefore be considered as a *true generalist* sensu Shefferson et al. (2019).

Conversely, all investigated roots from *Beclardia macrostachya* or *Aeranthus arachnites* were associated with CER4 and SER12

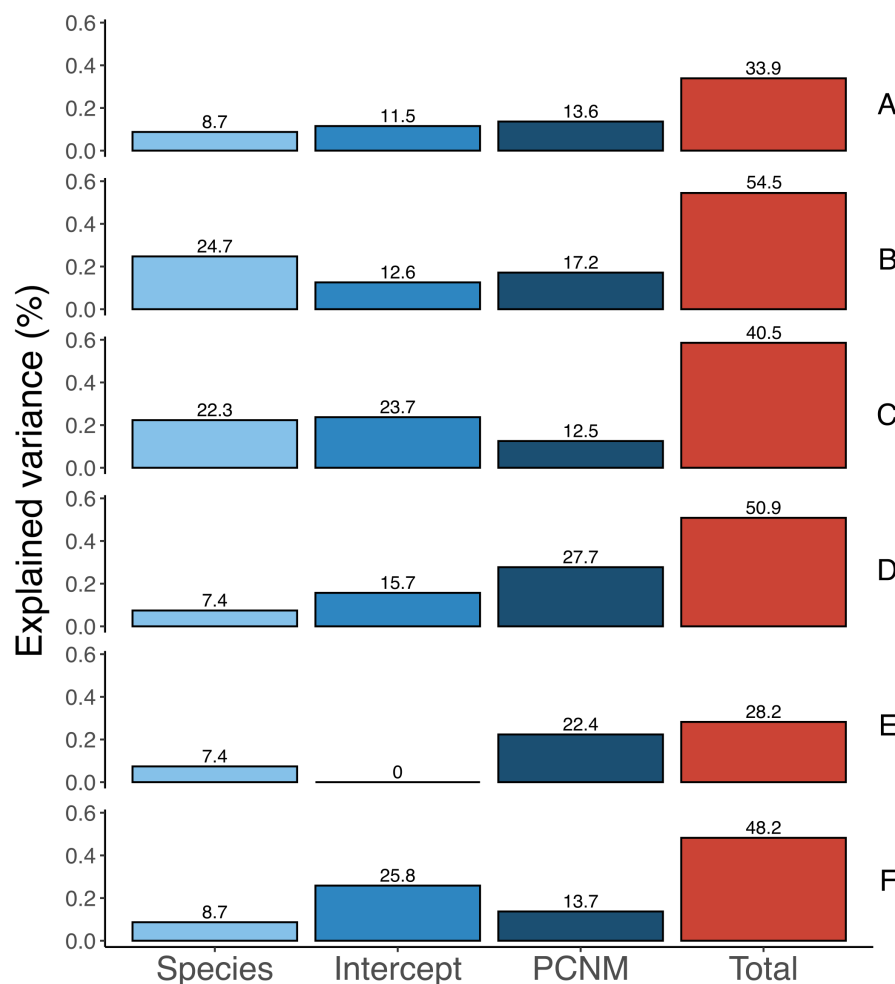


FIGURE 5 Drivers of the orchid mycorrhizal fungi (OMF)–orchids networks, using variance partitioning for each tree of the OMF β -diversities between orchid species identity ('species'), spatial component ('PCNM' i.e. set of PCNM vectors retained by) and their interaction ('intercept'). The 'Total' represents the total variance explained by these two explanatory variables and their interaction. The two variables explained a highly significant ($p < 0.001$) part of the total variance for each tree except 'Species' on grid E ($p = 0.033$).

respectively. While these interactions were observed on a limited number of individuals, they were consistent with a previous study conducted at the scale of the whole island (Martos et al., 2012). This suggests that, as conceptualized by Shefferson et al. (2019), these species show signs of *apparent generalism*, meaning that in addition with few specific interactions, they also interact, more inconsistently, with a broader range of mycorrhizal partners (Herrera et al., 2019; Selosse et al., 2022; Shefferson et al., 2019; Xing et al., 2019). Moreover, CER4 was only detected in the roots of other species spatially close to *B. macrostachya*. This could imply that some orchids could promote their mycorrhizal symbiont on certain habitats (Li, Yang, et al., 2021; McCormick et al., 2018) enable them to interact with other species in their vicinity.

Given that some orchid species (e.g. *B. prismaticum*) seem to weakly filter their mycorrhizal partners from the environment, these observations question the role of the spatial component in determining mycorrhizal associations and plant–fungus networks in epiphytic orchids.

4.3 | Fungal networks in the epiphytic habitat

Orchid species can be terrestrial, lithophytic (growing on rocks) or epiphytic (Dearnaley et al., 2012). Root endophytic fungi, including OMF, tend to vary across these habitats (Martos et al., 2012;

Xing et al., 2014, 2019). Thus, for both strictly epiphytic orchids and their mycorrhizal partners, the tree can be viewed as an island and the forest as an archipelago. Consequently, each tree and its bark-inhabiting communities constitute an island of physical interactions.

Network properties are difficult to compare between studies as they highly depend on the sampling design (Pölme et al., 2018). For instance, ecological networks (i.e. considering plant species rather than individuals, as in Cevallos et al., 2017; Jacquemyn et al., 2011, 2016; Martos et al., 2012; Kottke et al., 2013; Herrera et al., 2018; Xing et al., 2019; Perez-Lamarque, Petrolli, et al., 2022) cannot be directly compared with common mycorrhizal networks (CMNs; see references below) where physical links by fungal mycelium are likely to occur. As we worked on coexisting orchids with putative mycelial connections, we focus here on the latter category and analysed separately the orchid–OMF network of each tree.

In our study, networks on each tree showed a modular structure. The majority of modules comprised the roots of several orchid species, indicating that OMF are shared among co-occurring epiphytic orchid species. Modules comprising several orchid species have also been observed in co-occurring meadow's orchids by Jacquemyn et al. (2015), while studies conducted in other sites in close Mediterranean (Jacquemyn et al., 2014) or temperate grasslands (Jacquemyn et al., 2012) had previously shown that co-occurring orchid species tended to segregate their fungal communities. However, in

TABLE 2 Orchid mycorrhizal fungi (OMF) community composition (in % of colonized roots) of *Angraecum cucullatum*. Individuals were sampled at various growth stages (see Figure S2). The number of samples (N samples) are indicated for all trees (colonized by *A. cucullatum*, i.e. D, E and F) and for tree F only because the latter was the only one to host all growth stages. For each OMF OTU, the % of colonized roots are given by numbers in italic; numbers in brackets indicate the same % for grid F only. The underlined OTUs CER4 and TUL13 were the only two OMF OTUs detected on grid F

| | Protocorm | Stage 2 | Stage 3 | Stage 4 |
|--------------------|-----------|---------|---------|---------|
| N samples (Total) | 7 | 19 | 37 | 49 |
| N samples (grid F) | 7 | 19 | 14 | 4 |
| Ceratobasidiaceae | | | | |
| CER4 | 14 (14) | 21 (21) | 27 (21) | 18 (0) |
| CER1708 | — | — | 3 | — |
| Tulasnellaceae | | | | |
| TUL1 | — | — | 3 | 27 |
| <u>TUL13</u> | — | — | 3 (13) | 4 (50) |
| TUL22 | — | — | 3 | 6 |
| TUL61 | — | — | 3 | 22 |
| TUL62 | — | — | 3 | 24 |
| TUL76 | — | — | 3 | 16 |
| TUL265 | — | — | 3 | 14 |
| TUL3 | — | — | — | 8 |
| TUL16 | — | — | — | 4 |
| TUL20 | — | — | — | 14 |
| TUL25 | — | — | — | 2 |
| TUL114 | — | — | — | 8 |
| TUL253 | — | — | — | 2 |
| TUL390 | — | — | — | 2 |
| Serendipitaceae | | | | |
| SER56 | — | — | 5 | 4 |
| SER96 | — | — | 5 | 2 |
| SER164 | — | — | 5 | 6 |
| SER21 | — | — | 3 | — |
| SER629 | — | — | — | 2 |
| SER666 | — | — | — | 2 |
| SER702 | — | — | — | 2 |
| SER1559 | — | — | — | 2 |

the above-cited references, orchids were coexisting in plots of several dozens of metres and in soil their root systems may not have been in vicinity, contrary to the small areas of bark where root systems of coexisting orchids often overlap. Thus, in the systems studied by Jacquemyn et al. (2012, 2014, 2015), where orchids had very patchy distributed communities (a common property of terrestrial orchids in grasslands; Jacquemyn et al., 2012, and references therein), fungal sharing might be

less likely to occur. On bark, however, several epiphytic orchid species often coexist even at very fine scale (Petrollo et al., 2021). The limited available surface for both orchids and fungi on bark could then favour symbiont sharing between coexisting epiphytes compared to soil.

4.4 | Explanatory factors for the orchid–fungus network modularity

In addition to modularity, networks all displayed antinestedness, a topology already observed in other mycorrhizal types such as ectomycorrhizal (Bahram et al., 2014) or ericaceous mycorrhizal networks (Toju et al., 2016). Almeida-Neto et al. (2007) related antinestedness to several mechanisms, including modularity or high community turnover. When organizing the networks according to the spatial distribution of the samples (Figure S7), we found a high fungal turnover indicating that the modules could be explained by the spatial turnover of the fungal communities.

Indeed, the modules of orchids–OMF interactions could either be controlled by the orchid species identity, by the spatial assemblage of the orchid community (e.g. Toju et al., 2014) or by stochastic effects (Encinas-Viso et al., 2016). We found here that most modules could be explained by nonstochastic effects as also revealed by the large variance ($\geq 30\%$) explained by species and/or spatial effects in the variance partitioning. Regarding modules, 37.0% of them were explained both by the orchid species identity and root spatial distribution, which mirrors the fact that orchid roots were not randomly distributed on bark. Some modules were, however, explained by only one of the two factors, namely spatial assemblage (22.2%) or species identity (11.1%), suggesting that these factors effectively shaped the fungal interactions among roots. Strikingly, variance partitioning revealed that most of the variance in *L. calophylloides* (tree A–C) was explained by the orchid species composition, whereas on *N. verticillata* (tree D–F) it was mostly explained by spatial effects. It suggests that the environment (e.g. the structure or chemical composition of the bark type which may influence mycelial growth) can modulate orchid preferences for fungi.

Importantly, OTUs that were frequent in the roots of one species tended to be also present in the roots of other surrounding species. Although such a pattern may evoke mere physical contact with the root surface or endophytism (i.e. extraradical mycelium foraging the bark and colonizing other roots without forming mycorrhizas; Wilson, 1995), it was also retrieved when only considering strong (i.e. high-count) interactions. This is unlikely to reflect contamination or endophytism and supports that such OTUs effectively form mycorrhizas on both species. Although this should be tested on field, it raises the issue of functional cooperation between co-occurring plant species.

4.5 | Towards common mycorrhizal networks and nursery effect in the epiphytic habitats?

The results described above were consistent when considering fungal OTUs or haplotype (i.e. intra-OTU polymorphism), suggesting

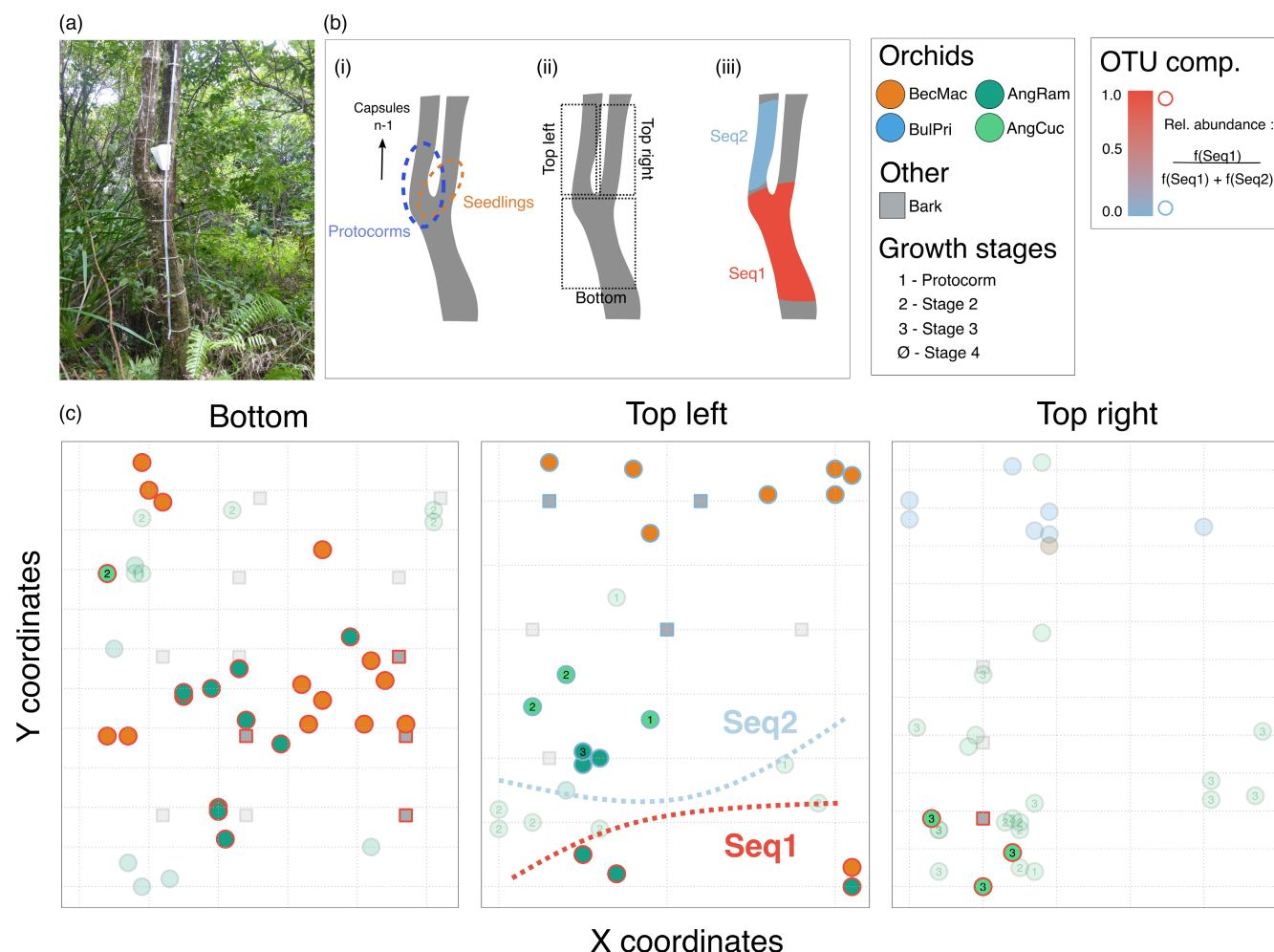


FIGURE 6 Distribution of CER4 on tree F. (a) Tree F during sampling. The trunk of tree F split into two branches but was included for analyses because of its bark-inhabiting orchid richness. (b–c) As tree F had two branches, the spatial distribution of OTUs cannot be plotted on a single graph, and the distributions were thus split on three panels as indicated in panel b-ii. Protocorms and seedlings of *Angraecum cucullatum* were found close to the fork as indicated in panel b-i. (c) Distribution of CER4 in orchid roots (circles) or bark (squares); the distance between two parallel grey lines is 10 cm. Coloured symbols indicate that the OTU has been detected in the sample. For *A. cucullatum*, symbols are labelled according to their growth stage (see Figure S2). The 100 most abundant unique sequences composing the OTU CER4 were analysed (see Figure S16) and two of them (Seq1 and Seq2) mainly composed the OTU. Their relative abundance is indicated by the colour of the symbol's stroke revealing that the two sequences exclude each other and most probably represent two fungal individuals. Their observed distributions on the tree bark are represented on panel b-iii and delimited in panel c (top left) as dotted lines.

that OTU's sharing mirrors that of fungal mycelium (e.g. CER4 on tree F). Although these results should be confirmed by other molecular methods revealing individual polymorphism (Beiler et al., 2010, 2015), they most probably show that some fungal mycelia are shared by several co-occurring epiphytic orchids (McCormick et al., 2016).

Notably, we studied protocorms and seedlings of *A. cucullatum* on a tree colonized by several other orchid species. These naturally established germinations give key information about their early mycorrhizal needs. Although OMF colonization inside those protocorms was scarce, an OMF OTU retrieved in few young seedlings of *A. cucullatum* (stages 2 and 3) was also present in the roots of co-occurring adults, both from the same and different species. Such a fungal sharing between adults and seedlings has been linked with seedling's facilitation in ectomycorrhizal symbiosis (e.g. Diédhiou

et al., 2010; Liang et al., 2020), and further studies are needed to test for facilitation in epiphytic orchids. Notably, OTUs were frequently found on bark in the vicinity of colonized roots, which shows that a *continuum* of mycelia exists on the bark between roots in the so called epiphytic rhizosphere (Petrollo et al., 2021). Yet, contrary to soils (Jacquemyn et al., 2012), seeds of epiphytic orchids have not always been found to germinate more efficiently on bark in the vicinity of adults (Kartzin et al., 2013; but see Yang et al., 2017). Here, all protocorms were already green which could suggest that mycorrhizal dependence of epiphytic orchids (at least in *A. cucullatum*) occurs after germination, especially for water and nitrogen supply.

We hypothesize that the sharing of mycelium between orchid individuals of the same or different species could help sustaining the whole colony and allow a nursery effect, by sharing nutrients such as

nitrogen or phosphorus whose inputs on bark are occasional, localized and ephemeral (Zotz, 2016). Following this hypothesis, limited hydro-mineral supply in epiphytic conditions (Yoder et al., 2010) may also promote fungal sharing, a form of reciprocal help for nutrition. Indeed, stressing conditions tend to select for more reciprocally beneficial interactions between plants (e.g. Callaway et al., 2002; see also Brooker et al., 2008). From a fungal point of view particularly, rerouting resources among different partners increases sustainability and resilience by creating a diverse host set (Selosse et al., 2006). Thus, isotopic labelling should be performed in the epiphytic habitats to reveal whether the CMNs revealed in this study are truly functional or not (Klein et al., 2016; Selosse & Roy, 2009; Simard et al., 1997, 2015).

AUTHOR CONTRIBUTIONS

Florent Martos, Marc-André Selosse, Lucie Zinger and Rémi Petrolli designed the study and performed the sampling additionally with Géromine Collobert and Chantal Griveau. Rémi Petrolli performed the molecular, bioinformatics and statistical analyses. Rémi Petrolli, Florent Martos, Benoît Perez-Lamarque, Lucie Zinger and Marc-André Selosse wrote the manuscript which was edited by all coauthors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this work.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw data have been deposited in the Sequence Read Archive (SRA) under the BioProject accession no. PRJNA692353.

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