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The distribution of mycorrhizal fungi on tree barks correlates with the host preference of the tropical epiphytic orchid *Bulbophyllum variegatum* on La Réunion island.

Rémi Petrolli[1*], Rosa Kemmerling[1], Géromine Collobert[1], Eve Hellequin[1], Tony Robinet[2], Neil Lancaster[3], David L Roberts[4], Thierry Pailler[5], Claudine Ah-Peng[6,7], Marc-André Selosse[1,8,9], Florent Martos[1]

[1] Institut de Systématique, Évolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE-PSL, Université des Antilles, CP 39, 57 rue Cuvier, F-75005, Paris, France.

[2] Biologie des Organismes et Ecosystèmes Aquatiques (UMR 8067 BOREA) Muséum National d'Histoire Naturelle, CNRS, Sorbonne Université, IRD, UCN, UA, Station Marine de Concarneau, 29900 Concarneau, France.

[3] Royal Horticultural Society (RHS), Wisley, Woking, Surrey, UK.

[4] Durrell Institute of Conservation and Ecology, University of Kent, Canterbury, UK.

[5] Herbier Universitaire de La Réunion, UMR C53 Cirad-Université, Peuplements Végétaux et Bioagresseurs en Milieu Tropical, F-97410 St Pierre, La Réunion, France.

[6] UMR PVBMT, University of La Réunion, 97410 Saint-Pierre, La Réunion, France.

[7] Observatoire des Sciences de l'Univers de La Réunion (OSU-Réunion), UAR 3365, Université de La Réunion, CNRS, Météo France, IRD, Saint-Denis, France.

[8] Department of Plant Taxonomy and Nature Conservation, University of Gdańsk, Wita Stwosza 59, 80-308, Gdańsk, Poland.

[9] Institut Universitaire de France (IUF), Paris, France.

*Current address: Department of Environmental Sciences, University of Basel, Basel, Switzerland.

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Abstract: 165 words
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Materials and Methods: 2831 words
Results: 1240 words
Discussion: 2438 words
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Supp. figures and tables: 23 (15 + 8)
References: 67

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Abstract (165 words)

While host preference of tropical epiphytic plants is mainly driven by tree abundance and size, the distribution of mycorrhizal fungi among tree species is likely to influence host preference in orchids. Here, we investigated the host preference of ten epiphytic orchids in a one-hectare forest plot in La Réunion, revealing a strong preference in the orchid *Bulbophyllum varigatum*. Using an Illumina sequencing of the ITS-2 barcode of fungi, we identified fungal communities, including mycorrhizal Tulasnellaceae, both in the bark of host tree species in the area and in the roots of *B. variegatum* across the island. Bark-dwelling fungal and mycorrhizal communities varied between tree species, with fungal OTUs being specific to the host of *B. variegatum*. Three of them (one Serendipitaceae and two Tulasnellaceae) were found to be mycorrhizal at different stages of the orchid's life cycle. Our results suggest that the uneven distribution of mycorrhizal fungi between tree barks could influence the host preference of epiphytic orchids, a hypothesis that will require experimental testing.

Keywords

Agarista salicifolia, Bark fungi, Environmental DNA, Epiphytic host preference, Fungal spatial distribution, Orchid distribution, Orchid mycorrhizal fungi, Serendipitaceae, Tulasnellaceae.

Introduction (911 words)

Plant communities are shaped by the complex interplay between biotic and abiotic factors. By turning sums of individual species into complex networks of interactions, biotic interactions are particularly predominant in structuring these communities (Wisz *et al.*, 2013; Chomicki *et al.*, 2019; García-Girón *et al.*, 2020), including in the tropics (Callaway *et al.*, 2002; Schemske *et al.*, 2009; Chomicki *et al.*, 2019). In tropical wet forests, one of the most striking interactions involves the plants that do not root in the soil but rather anchor on the trunk or on the branches of trees as epiphytes (Zotz, 2016). Indeed, epiphytic plants are a major component of tropical rainforests and ca. 10% of vascular plant species are epiphytes (Zotz, 2013).

Epiphyte-tree relationships have been studied since the first works on the ecology of tropical forests, but the factors explaining these associations in communities are still poorly understood. Epiphytic plant species rooting on trees usually display a non-random distribution across tree species, meaning that they show varying degrees of preferences for few tree species and disregard others (Wagner *et al.*, 2015; Rasmussen & Rasmussen, 2018). While a significant fraction of these host preferences may be explained by structural biases mainly (*i.e.,* size, abundance and longevity of tree species; Wagner et al., 2015; Wagner & Zotz, 2020), true host preference might also be driven by biotic and/or abiotic factors in some epiphytic species. Beside abiotic factors (such as bark chemistry or structure), biotic interactions have been proposed to explain such uneven distributions of epiphytes on trees (*e.g.*, Chathurika Harshani *et al.*, 2014). Among these interactions, endophytic and mycorrhizal fungi may play a role in epiphytic orchids particularly (Pecoraro *et al.*, 2021).

The largest diversity of epiphytes is found in orchids which encompasses 70-80% of vascular epiphyte species. Epiphytic orchids are associated with mycorrhizal fungi mainly belonging to polyphyletic families in the Basidiomycota: the Tulasnellaceae and the Ceratobasidiaceae in the Cantharellales, and the Serendipitaceae in the Sebacinales (Dearnaley *et al.*, 2012; Weiß *et al.*, 2016; Cevallos *et al.*, 2017). Beside these groups, fungi in the Atractiellales are also frequently found in the roots of epiphytic orchids, especially in the Neotropics (Herrera *et al.*, 2019; Fernández *et al.*, 2023). Unlike the majority of plants, the mycorrhizal fungi of orchids are required throughout their lifecycle (Dearnaley *et al.*, 2012). In epiphytic orchids these fungi are supposed to live as saprotrophs on barks and, by supplying nutrients to their minute seeds, may allow the symbiotic germination of epiphytic orchids on trees. However, their importance for the germination of epiphytic orchid seeds is still subject to debate and awaits further investigation. During the subsequent development of the seeds into protocorms, seedlings and then adults, the mycorrhizal associates may vary or stay identical, depending on orchid species (Ventre-Lespiaucq *et al.*, 2021, Fernández *et al.*, 2023). In seedlings and adults, mycorrhizal fungi colonize both the roots of epiphytic orchids and the surrounding bark (Petrolli *et al.*, 2021) presumably improving the nutrition of their host.

In general and despite substantial variations, orchids tend to interact with a limited number of mycorrhizal partners compared to other mycorrhizal plants (especially arbuscular; Põlme *et al.*, 2018, Li *et al.*, 2021). Since the germination and establishment of orchids is presumed to rely on the presence of one or several fungi in the environment, it was therefore hypothesized that the distribution of compatible mycorrhizal fungi could constrain that of the orchid species (McCormick & Jacquemyn, 2014; Kaur *et al.*, 2019). While investigated in soils since the late 2000's (Swarts *et al.*, 2010), studies have only recently questioned the role of mycorrhizal fungi in constraining the epiphytic orchids on certain tree species (Pecoraro *et al.*, 2021; Johnson *et al.*, 2023; Zhao *et al.*, 2024). In 2021, Pecoraro *et al.*, showed in a pilot study that the root-associated fungi of two epiphytic orchid species were correlated with the bark-dwelling fungal communities of their host tree species. However, the fungi responsible for the germination of seeds could not be identified, leading to an incomplete story. More recently, Zhao *et al.*, (2024) demonstrated that the distribution of the orchid *Dendrobium wangliangii* on a limited number of tree species was not explained by the distribution of their ubiquitous fungal associates in China, as suggested also by Johnson *et al.*, (2023) in *Dendrophylax lindenii* from Florida. Despite the limitations of existing studies, research into the role of mycorrhizal fungi in the host preference of epiphytic orchids has yielded contradictory results. This might be attributable to the absence of prior quantification of the host preferences studied or to alternative factors co-influencing these preferences.

In the tropical lowland rainforest of La Réunion island (Indian Ocean), the epiphytic orchid *Bulbophyllum variegatum* has been observed to occur almost exclusively on a single tree species, namely the Ericaceae *Agarista salicifolia*. However, this apparent host preference remains to be statistically evaluated or explained. In this study, we first conducted an epiphyte-tree interactions survey in a 1-ha forest plot in La Réunion island and statistically tested the hypothesis that (i) the epiphytic orchid *B. variegatum* exhibits strong host preference for the tree *A. salicifolia*. Secondly, we investigated through a metabarcoding approach fungal communities growing on the bark of several tree species in the forest, as well as those associated with the roots of *B. variegatum* in the whole island, with the hypothesis that (ii) *A. salicifolia* possesses distinct mycorrhizal and non-mycorrhizal bark fungal communities when compared to other tree species in the forest that may explain the orchid's host preference.

Materials and methods (2831 words)

Study species

Bulbophyllum variegatum Thouars is a large epiphytic orchid with sympodial growth living in the lowland tropical rainforest on La Réunion island (Indian Ocean). The species has been observed infrequently in Madagascar (Fischer *et al.*, 2007), but it flourishes in substantial populations on La Réunion where it has been observed to occur almost exclusively on one indigenous tree species, the Ericaceae *Agarista salicifolia* (Lam.) G. Don (hereafter referred as the focal species). The rare exceptions include a population of *B. variegatum* on trees of the exotic species *Eucalyptus robusta* (Myrtaceae) planted in rows on a single location (Fig. S1G-I). *E. robusta* is the only alternative tree species hosting a dynamic population of *B. variegatum*, but the few (< 10) trees involved are to put in contrast with the hundreds of *A. salicifolia* trees hosting *B. variegatum* on the island. *A. salicifolia* is a pioneer tree of young lava flows and grows as large trees in forests as old as 600 years (Albert *et al.*, 2020). The largest trees can have diameter at breast height (dbh) >100 cm in old-growth forest, but shrubs growing in pseudo-soils on other trees can also be found (Fig. S1F). The orchid *B. variegatum* has been observed to occur predominantly on the trunk (Fig. S1A-B), with less frequent observations on the roots (Fig. S1C) and the lower branches of *A. salicifolia* (Fig. S1F). Its vertical distribution on the tree is analogous to that of an orchid in the undergrowth, where it can form large clusters (Fig. S1A, H). An obligate C3 photosynthesis (data not shown) may prevent this orchid species from reaching the upper branches in its low-altitude forest habitat when exposed to water stress.

Host preferences of epiphytic orchids

Survey of epiphyte-tree interactions

An initial survey of the host preferences of epiphytic orchids was conducted in 2006 in a 1-ha forest plot in the Mare Longue (ML) Forest Reserve (Strasberg, 1996). It is a well-preserved lowland rainforest of the southeast part of the island (21°21’17” S 55°44’29” E; Fig. 1B), growing on a 400-y old basaltic lava flow and harboring > 40 native tree species.

To test the hypothesis of *B. variegatum*’s host preference for *A. salicifolia*, the presence or absence of all epiphytic orchid species was recorded using binoculars on all trees with dbh > 9.5 cm in this 1-ha forest plot. Trees with dbh < 9.5 cm host almost no epiphytic orchids in this forest. The relative abundance of epiphytic orchid species on trees was not recorded because, except for angraecoid orchids (*e.g.*, *Aeranthes*, *Angraecum*) which grow monopodially, many epiphytic orchids in this forest (*e.g.*, *Bulbophyllum*) are sympodial and colonial in growth. The height and dbh of each tree were also recorded at the same time. This survey resulted in an initial presence-absence matrix of 14 orchid species x 1,070 tree individuals (40 species, 23 families), with 1,818 observations. Four orchid species difficult to identify from the ground were subsequently removed from the data to avoid any species misidentification. Five tree species without any epiphytic orchid were also removed from the data.

Host preference statistical analyses

The binary matrix of interactions between epiphytic orchids (ten species remaining) and tree individuals was analyzed by three unrelated methods for consistency of the results (Wagner *et al.*, 2015): network analyses, chi-square goodness-of-fit, and random forest (RF) model. Because the strong host preference of *B. variegatum* for *A. salicifolia* would lead to the absence of this orchid on the other dominant host trees in this forest, the network description and analyses were performed using tree species having > 10 individuals in the forest plot (*i.e.*, 19 tree species, resulting in 1,667 observations of the 10 orchid species), while the eight most abundant tree species only were kept for goodness-of-fit and RF model analyses (*i.e.*, 1,494 observations of the 10 orchid species).

Network analyses. The strength of each orchid-tree interaction was studied by permutational analyses of the original network. The original matrix was randomized 10,000 times with the permutational algorithm *permatfull* of *vegan* (Oksanen *et al.*, 2022; R v4.1.0), keeping constant all marginal sums as to account for orchid species’ specificity as well as for poorness/goodness of trees as host for epiphytic plants. Original and randomized matrices were converted into percentages of trees within species colonized by each orchid species, and each frequency of the original matrix was then compared to the corresponding frequencies from the randomized matrices. P-values were computed as the rate at which original frequencies were larger (or smaller) than frequencies after randomization.

Chi-square goodness-of-fit. The chi-square goodness-of-fit test is used to compare observed proportions to expected probabilities, and was previously used to assess epiphyte preference for hosts based on presence/absence data (Wagner *et al.*, 2015). Here, we performed three tests under three different null hypotheses. The proportion of tree species colonized by each orchid species was compared to (i) equal probabilities of colonization among all tree species (equiprobable model), (ii) probabilities of colonization proportional to the number of tree individuals per species in the 1-ha forest plot (tree count-based model) and (iii) probabilities of colonization proportional to the total surface of the tree species in the forest plot (surface-based model, the surface being approximated by S = height x dbh x tree count). Residuals show the extent of variation between observed and expected probabilities and were extracted with the *chisq.test* function of R. Significant differences between observed and expected probabilities were assessed by the exact fisher test, *fisher.test* function of R following a Monte Carlo simulation.

Random forest model. RF analyses (Breiman, 2001) were used to assess the simultaneous roles of trees' surface, tree position inside the plot and tree species identity in controlling the colonization of epiphytic orchids. Analyses were performed using the *randomForest* R-package (Liaw & Wiener, 2002) using orchid species as entries and tree species surface, tree spatial position and identity as factors (Fig. S2). The *randomForest* function was used with default parameters for training/test data split (*i.e.*, bootstrap sampling; Liaw & Wiener, 2002), using 2,000 decision trees. Because RF modelling overpredicted the most abundant orchid species (*i.e., Bulbophyllum prismaticum* and *Angraecum mauritianum*, in addition with *B. variegatum*, data not shown), orchid individuals of each species (when possible) were sub-sampled 100-times providing average RF metrics. Orchid species entries for the RF predictions were either (model-i) all orchid species names or (model-ii) each orchid species *versus* all others. Residuals from confusion analysis in RF modeling (*i.e.,* successful prediction) were calculated with model-i and indicate the success in predicting orchid species identity based on the provided factors. Relative importance (RI) of factors in determining orchid species identity were calculated for the whole dataset (model-i) and for each orchid species separately (model-ii) based on the Gini coefficient method. Marginal effects, *i.e.*, mean response of the RF model for a given factor, were estimated for the prediction of *B. variegatum* alone (model-ii), using the *partialPlot* function which returns logit of probabilities of accurate species estimation (Liaw & Wiener, 2002).

Fungal DNA sampling and analyses

Sampling of bark-dwelling fungal communities.

The bark-dwelling fungal communities were investigated in the ML forest in February 2021 (Fig. 1B). A total of 61 trees distributed in 9 sub-plots in and around the 1-ha forest plot at ML, but in the same forest area, were selected to identify fungal communities associated to tree species including *A. salicifolia*. Each sub-plot was set up so as to have an *A. salicifolia* tree in the center, plus 5-6 large, nearby (generally within 15 m) trees belonging to other dominant host species in the forest (Fig. 1D), resulting in 6-7 sampled trees per plot. Each tree was spatially located in the forest, and its dbh recorded. As we aimed to identify the most comprehensive bark-dwelling fungal communities on each tree, the following sampling protocol was applied. On each trunk and for sampling purpose only, three height zones were defined as follow: Z1 (0–0.5 m), Z2 (0.5–2 m), and Z3 (2–5 m). On each zone, the bark was collected on multiple points (> 3) using sterile knives and combined in two samples, *i.e.*, a rhizospheric sample (R; bark collected within < 5 cm from an orchid root) and a non-rhizospheric sample (NR, bark collected at > 30 cm from orchid roots; Fig. 1C). Because orchid mycorrhizal fungi tend to colonize more frequently the bark in close proximity to roots (Petrolli *et al.*, 2021, 2022), the former were collected to characterize the fungal communities involved in the symbiosis directly, while the latter were collected to estimate the capacity of these fungi to colonize the bark at distance from the roots. Right after collection, these bark samples were quickly dried in silica gel until further processing. This resulted in 6 samples per tree (61 trees) covering a large part of their basal trunk (*i.e.*, from 0 to <5m), for a total of 366 bark samples in the forest (Table S2). Additionally, five younger *A. salicifolia* trees located out of the sub-plots (15 'NR' samples, 3 per tree, Table S2) were sampled for sequencing and analyzed as described below.

Sampling of root-associated fungal communities

As we wanted to assess *B. variegatum*'s mycorrhizal preference at the scale of the species' range (excluding Madagascar where the species is rare), its root-associated fungal communities were investigated not only in the ML forest, but also at six other sites (*i.e.*, populations) in lowland rainforest habitats along the east coast of the island, covering its whole distribution area (Fig. 1A).

The roots of 113 individuals of *B. variegatum* growing at 6 sites were collected in 2021. In addition to the individuals growing on *A. salicifolia* trees, we sampled the rare individuals growing on other hosts on the island. For each individual orchid, up to three small root portions were collected (328 samples in total) without uprooting the plant. Each root portion was carefully cleaned using sterile clamp and razor blade in sterile water under loupe microscope to remove epidermal cells and select sections with dense mycorrhizal colonization (ca. 0.5 cm long) which were dried in silica gel until further processing. Individuals without roots (*i.e.*, protocorms) were stored into 50% ethanol and processed entirely. For each plant, morphological characters (*i.e.*, leaf width and length, number of alive pseudobulbs and diameter of the largest one) as well as the height on the tree and the tree host identity were recorded on field. Additionally, we included for subsequent molecular analyses 40 and 19 samples collected during preliminary investigations in 2006 and 2020 respectively, which resulted in a total of 387 root samples (Table S1).

Molecular analyses of bark and root samples

DNA was extracted from each bark or root sample (n = 768) and amplified by PCR through a double-tag metabarcoding approach as in Petrolli *et al.*, (2022), including PCR replicates as well as positive (homemade fungal mock community) and negative (H_20) PCR controls. Amplifications were performed using two sets of primers targeting the Internal Transcribed Spacer (ITS)-2 and replicated twice: ITS86-F/ITS4 targeting most of the Asco- and Basidiomycota (White *et al.*, 1990) and 5.8S-OF/ITS4-Tul specifically targeting the mycorrhizal Tulasnellaceae family (Dearnaley *et al.*, 2012; Vogt-Schilb *et al.*, 2020) which has been found in almost all orchids on La Réunion island (Martos *et al.*, 2012; Petrolli *et al.*, 2022) but fails to amplify with fungal universal primers (Rammitsu *et al.*, 2021). This resulted in 1322 PCR products, included all bark and root samples amplified with the ITS86-F/ITS4 primers, 139 bark (36.5%) and 301 root (77.8%) samples amplified with the 5.8S-OF/ITS4-Tul primers, and 114 positive/negative controls. After purification and quantification (see the details in Petrolli *et al.*, 2022), all these PCR products were pooled in 4 libraries, one per sample type x primer pair to account for the c. 50-bp difference in sequence length between the two pairs of primers. These four pools were sequenced in two 2*250 bp paired-end sequencing runs, one for the bark samples the other for the root samples, on an Illumina MiSeq platform at Fasteris (Geneva, Switzerland).

Raw sequencing data processing

Paired-reads were processed as in Petrolli *et al.*, (2022) using a customed pipeline based on VSEARCH (Rognes *et al.*, 2016). Briefly, assembled paired-end reads were demultiplexed and then clustered into Operational Taxonomic Units (OTUs) at 97% similarity levels, and chimeras, singletons and short OTUs (< 200bp) were not considered. OTUs were taxonomically assigned on the UNITE V8.3 (2021-05-10) eukaryote reference database (Nilsson *et al.*, 2015) and only fungal OTUs were further considered. Contaminant OTUs were discarded based on positive/negative PCR controls and *decontam* R-package (Davis *et al.*, 2018). False positive OTU detection was controlled by setting to zero OTUs that represented less than 0.8% of the sample reads, according to the estimated tag-jumps rate of this study (Zinger *et al.*, 2019). Samples with < 1000 reads were not considered in subsequent analyses. At the end 760 samples (of the initial 768 collected samples) amplified with one or both sets of primers were used in subsequent analyses.

Phylogenetic fungal relationships

Phylogenetic relationships between our OTUs (ITS-2) and sequences from GenBank (full ITS) were established using MAFFT (Katoh & Standley, 2013) in a two steps procedure: full-ITS sequences were first aligned with default parameters and OTUs representative sequences were then added to this constrained alignment using the -add option. Phylogenetic trees were built using IQTREE 2.1.3 (Minh et al., 2020) following the same two-step procedure with standard model selection and -g option at the second step. Bootstrap and sh-alRT values were computed using 1,000 replications.

Fungal diversity analyses

Since the two sets of primers used in this study display differential specificity, the metabarcoding dataset was analyzed considering both relative abundance data (each primer dataset separately) and presence/absence data (merged fungal universal and Tulasnellaceae-specific primer datasets). Functional diversity of fungal community was assessed using the

FungalTrait database (Põlme *et al.*, 2020). The putative mycorrhizal fungi of *B. variegatum* were defined as belonging to the main mycorrhizal families of epiphytic orchids on the island and worldwide, *i.e.*, Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and Atractiellales (Martos *et al.*, 2012; Petrolli *et al.*, 2022, Fernández *et al.*, 2023). Fungal community variations among samples were investigated through non-metric multidimensional scaling (NMDS) approaches using the *metaMDS* function of *vegan* (Oksanen *et al.*, 2022). Ecological dissimilarities were computed using the Bray-Curtis and the Jaccard indices for relative abundance and presence/absence data, respectively. Significant fungal community variations across sampled material (*i.e.*, roots *vs* bark) or tree species' bark were tested by permutational approaches using both global and pairwise *permanova* (*adonis2* function of *vegan*) complemented by multivariate homogeneity of group dispersion analyses (*betadisper* function of *vegan*).

Fungal turnover during orchid development

The leaf length of *B. variegatum* individuals was correlated to leaf width and pseudobulb's diameter (data not shown) and the former was used as a proxy for the age of each orchid individual. To test the turnover of mycorrhizal fungi during the development of *B. variegatum*, the relationship between main OTU's abundances in roots and leaf length of the sampled individuals was tested through a linear regression model followed by an anova test.

Spatial analyses of fungal communities

Spatial analyses of bark-dwelling fungal communities were conducted to differentiate tree host identity effect with spatial effects, and performed as in Petrolli *et al.*, (2022). Mantel tests, mantel correlograms (showing the maximum distance of positive autocorrelation, $\max_{dpa}$) and Principal Coordinates of Neighbour Matrices (PCNM, subsequently used in variance partitioning) were computed using the *vegan* R-package.

Differential abundance analyses of fungal OTUs

Since different methods yield different results (Nearing *et al.*, 2022), we combined two methods of differential analyses (DA) in order to produce a consistent evaluation of OTUs biased toward *A. salicifolia versus* other tree species. We used ALDEx2 (Fernandes *et al.*, 2013, 2014) and

ANCOM-BC (Lin & Peddada, 2020) because the two methods have been shown to minimize the false discovery rate and to only retain the most discriminatory OTUs among several other calculation methods (Nearing *et al.*, 2022). These two methods are well adapted to sequencing data, because they estimate the measurement error associated with high-throughput sequencing (Gloor & Reid, 2016). The ALDEx2 method first transforms the data using the log2-ratio transformation, which allows direct comparisons between OTUs' relative abundances in compositional datasets (such as ITS high-throughput sequencing), and then tests differential abundances of OTUs between conditions using the non-parametric Wilcox test, correcting p-values using the false discovery rate (FDR) approach of Benjamini & Hochberg, (1995). The calculation was performed with the *ALDEx2* package of R. The ANCOM-BC method specifically accounts for sampling fraction, *i.e.*, the fact that absolute abundances of OTUs in samples vary from their absolute abundances in the environment solely by sub-sampling effects (Lin & Peddada, 2020). As ALDEx2, it also deals with compositional datasets through log-ratio transformations of the data and additionally provides 95% confidence interval for differential abundances. The calculation was performed with the *ANCOMBC* package of R with default parameter, including the 'Holm' correction for p-values (Holm, 1979). Both methods were used on the two primer datasets (*i.e.*, fungal universal and Tulasnellaceae-specific) separately (using relative abundance data) and results were then combined in plot display.

Results (1240 words)

Epiphyte-tree interactions

In the forest plot, the proportions of individuals of each orchid species growing on a given tree species ranged from 0 to 42% (in percentage of individuals) except from *Bulbophyllum variegatum* whose 91% of individuals grew on *Agarista salicifolia* (Fig. 2A). Permutational null model analysis revealed that 8.9% and 8.4% of these epiphyte-tree interactions were respectively stronger and weaker than expected by chance (Fig. S3C), including *B. variegatum* on *A. salicifolia* (stronger interaction, $p < 0.001$). This latter interaction appeared to be the most consistent with 93.2% of *A. salicifolia* trees ($n = 44$) being colonized by *B. variegatum* (Fig. S3D). In comparison, the second strongest interaction concerned *Angraecum pectinatum* whose

42% of individuals grew on *Nuxia verticillata* (Fig. 2A) while only 50.8% of this tree species individuals hosted *A. pectinatum* (Fig. S3D).

The proportions of orchid individuals per species on tree species significantly differed from those expected by chance (Chi^2 test, $p < 0.05$) for most orchids when considering equal probabilities to colonize each tree species (Fig. 2B). When considering tree counts and sizes (*i.e.*, total trunk surface in the forest-plot), only four orchid species significantly differed from null colonization probabilities in both models, including *B. variegatum* on *A. salicifolia* (Fig. 2B).

We built Random Forest (RF)-based models in order to predict epiphyte identities considering both tree's surface, spatial position and species. RF models mostly failed to predict orchid species (*i.e.*, successful prediction from RF $\leq$ 50%) except for *B. variegatum* which was successfully predicted in 89.1% of its decision trees (Fig. 2C). The two second most successfully predicted orchids, *i.e.*, *Angraecum pectinatum* and *Aeranthes arachnites*, were successfully predicted in only 34.6% and 22.4% of their decision trees, respectively. While the two latter were mostly predicted based on tree sizes (relative importance, RI_{DBH*H} ca. 50%, Fig. 2C), the most important predicting factor in the whole model (Fig. S3E), *B. variegatum* was predicted based on host tree identity (*i.e.*, *A. salicifolia* or not, $RI_{AgaSal} = 57.4\%$), consistently with marginal effects of tree species on *B. variegatum*'s prediction ($logit(p)_{AgaSal} > 0$; Fig. S3F).

Bark-dwelling fungal community variations

The sequencing of bark samples led to a total of 21,272,462 raw reads which were clustered at a 97% identity threshold, leading to a final set of 1,290 bark-dwelling fungal OTUs after data filtering (see methods). The fungal richness varied across tree species at similar sampling depth and none of the accumulation curve reached a plateau, even when considering the whole bark-dwelling community (Figs. 3A, S4A-C). Fungal community was mainly composed of Asco- (47.3%) and Basidiomycota (40.8%), mainly Helotiales (10.3%) and Cantharelalles (10.0%), respectively (Fig. 3B). Overall, these fungi were mainly classified as saprotrophs and plant pathogens according to FungalTraits (Fig. S5). Bark-dwelling fungal communities differed between tree species (PERMANOVA, $p < 0.001$, Table S4), even though along with true centroid effects (visualized in NMDS analysis, Fig. S6B-C), variations in sample's dispersion were also observed (BETADISPER, $p < 0.001$, Table S4). Bark-dwelling fungal communities also differed between tree species when considering saprotrophs or pathogens only (Tables S7-8, Fig. S7). While *A. salicifolia* seemed to differ the most from other tree species in NMDS (Figs. 3C, S6C), pairwise comparisons between main tree species showed that all tree species' bark fungal compositions significantly differed from each other's (Tables S5-S6). Considering all tree species, the global fungal OTU richness was not statistically different between rhizospheric and non-rhizospheric bark samples (Wilcoxon' test, $p > 0.05$, Figure S8) while rhizospheric bark samples hosted more OMF OTUs than non-rhizopheric samples ($p < 0.001$). Fungal communities differed between rhizospheric and non-rhizospheric bark samples in our PERMANOVA analyses ($p < 0.001$), but this result was very poorly reflected in NMDS analyses (Fig. S9). Spatial autocorrelation of bark-dwelling fungal communities in the whole forest was significant (Mantel test, $r = 0.055$, $p < 0.001$) but low ($max_{dpa} < 50$m), and spatial distribution of trees explained less of the fungal community variations than tree species identity according to variance partitioning (Fig. S10).

Biased fungal communities on focal tree species

To more precisely assess the specific fungal composition of the bark of *A. salicifolia*, we conducted differential abundance (DA) analyses to detect OTUs significantly biased toward this tree species. The two methods ANCOM-BC and ALDEx2 were complementary in their results and together detected 8 and 6 significantly biased toward and against *A. salicifolia*, respectively (ANCOM-BC, Holm-corrected p-values < 0.05; ALDEx2, FDR < 0.05, Figs. 3D-E). In the Tulasnellaceae, TUL1 and TUL3 were the most significantly biased toward *A. salicifolia* while TUL2 was specifically biased against *A. salicifolia* (*i.e.*, ANCOM-BC log-fold change > 0.2, ALDEx2 log2-fold change > 10, Figs. 3D-E). These results were corroborated by the distribution of these OTUs on tree species (Fig. 3F), showing that TUL1 and TUL3 were largely more abundant on *A. salicifolia* than on other tree species, while TUL2 mainly colonized other tree species. On the bark, these OTUs were more abundant in (but not restricted to) the rhizospheric compared to the non-rhizopheric bark samples (Fig. 3F), while they were retrieved multiple times in each of the three defined sampling height (*i.e.*, Z1-3).

Endophytic fungal communities of *B. variegatum* roots

Sequencing the root samples of *B. variegatum* across the whole island led to a total of 23,406,692 reads belonging to 723 root-endophytic fungal OTUs after data filtering (see methods). Fungal richness varied between orchid populations and no plateau could be recovered in accumulation curve analyses (Figs. 3A, S4A-B) even when considering mycorrhizal fungi only (Fig. S4C). As for the bark, root endophytic fungal communities were mainly composed of Asco- (56.2%, with 21.7% Helotiales) and Basidiomycota (35.3%, with 13.0% Cantharellales) fungi (Fig. 3B) but significantly differed from bark-dwelling communities (PERMANOVA < 0.001; BETADISPER < 0.001; Table S4, Figs. 3C, S6A). Overall fungal communities associated to roots also varied between sites and/or host trees (Fig. S6D-E, Table S4, see also Fig. S13).

Temporal turnover of specific root endophytes

The most frequent endophytic fungi retrieved in the roots of *B. variegatum* were TUL1 and TUL3 in the Tulasnellaceae (Fig. S14), SER4 in the Serendipitaceae (Fig. S15) and HEL7 in the Helotiales (Fig. 4A, see also Fig. S11). TUL3 and SER4 were mostly associated with the youngest plant developmental stages of *B. variegatum* (Fig. 4B). In seedlings, they were the most abundant OTUs detected with each set of primers, representing > 90% (TUL3) and > 50% of sequences (SER4) retrieved with the Tulasnellaceae-specific and universal primers, respectively (Fig. 4B), but their abundance decreased in the roots of adult plants (ANOVA F-test $p < 0.005$; Fig. 4C). Importantly while TUL3 was found to colonize sporadically the roots of individuals (all adults) growing on alternative host species, SER4 only colonized the roots of individuals growing on the exotic tree *Eucalyptus robusta* (Fig. S13). Meanwhile, TUL1 in the Tulasnellaceae was neither abundant nor frequent in roots of seedlings (Fig. 4B), but its abundance increased in the roots of adult plants (ANOVA F-test $p < 0.005$; Fig. 4C) where it became the most abundant OTU retrieved with the Tulasnellaceae-specific primers (Figs. 4B, S12B). Notably, TUL1 was by far the most abundant OTU in the Tulasnellaceae colonizing the roots of *B. variegatum* individuals growing on the five alternative host tree species encountered (Fig. S13B). HEL7 in the Helotiales was not abundant and not limited to any plant developmental stage (Fig. 4C).

Discussion (2438 words)

Host preferences of epiphytic orchids

As most epiphytes, orchid species usually colonize several host tree species (Zotz, 2016). According to this general tendency, we found that the ten epiphytic orchid species studied here were growing on several tree species in their lowland rainforest habitat of La Réunion island (Indian Ocean). However, statistical analyses revealed that most of the orchid species displayed a non-random distribution on trees in the 1-ha forest plot investigated. The most consistent association involved *B. variegatum* and *A. salicifolia* with 91% of the orchid individuals growing on this tree species. The second most consistent association involved *A. pectinatum* whose only 42% of individuals were found growing on the tree species *N. verticillata*.

In the past, various degrees of host preference have been documented in epiphytic orchids (Wagner et al., 2015; Rasmussen & Rasmussen, 2018). Yet the underlying evidence are often based on observational data or do not take into account structural biases (such as tree size or longevity, Wagner *et al.*, 2015; Wagner & Zotz, 2020). Spatially limited observations of rare species can also lead to erroneous strong host preference, as documented by Crain & Tremblay, (2012) (see also Tremblay *et al.*, 1998).

In this study, the strong host preference of *B. variegatum* for *A. salicifolia* was first assessed in a 1-ha plot of well-preserved natural forest where the two species are abundant. Goodness-of-fit and random forest analyses were used to discriminate true biased distributions from tree abundance- or size-related distributions (Wagner *et al.*, 2015) and showed that this association is not driven by the abundance or the size of the host tree in the forest. Tree size was the most important determining factor in the random forest model for all orchid species (including *A. pectinatum*) except *B. variegatum* which was mostly explained by the identity of the host tree (*i.e.*, *A. salicifolia*). Meanwhile, > 90% of *A. salicifolia* trees were colonized by *B. variegatum* individuals, indicating a strong reciprocal association. As a comparison, *A. pectinatum* also displayed some degree of host preference for *N. verticillata* but it could be explained by the abundance and large size of this tree species in the forest plot (Fig. S3B). *N. verticillata* is a common 'good host' in the forest and is frequently colonized by numerous orchid species, as can be seen in Fig. 2A. In contrast, *A. salicifolia* could host several orchid species but was mainly colonized by *B. variegatum* in the forest plot investigated.

The strong association between *B. variegatum* and *A. salicifolia* could not result from false negative detections during survey because (i) *B. variegatum* is a large epiphyte forming dense colonies easily discernable from ground (sometimes covering *A. salicifolia* but not surrounding trees; Fig. S1A-B) and (ii) recent investigations of orchid communities in the canopy at the same site confirmed that it is adapted to the undergrowth of *A. salicifolia* (pers. com.). Conversely, many alternative hosts (not including the exotic tree *E. robusta*) are in fact branches intermingled with those of *A. salicifolia* on which *B. variegatum* probably established and started growing (Fig. S1F-G). During our investigations of root endophytic fungal communities associated with *B. variegatum* in seven populations along the east coast (see below), we found individuals growing almost exclusively on *A. salicifolia*, which confirms the strong host preference of this species on the island scale. This preference, here coupled with reciprocity, is thus uncommon and to our knowledge, undocumented. In Madagascar, *B. variegatum* has been rarely observed (Fischer *et al.*, 2007) and its hosts are currently unreported, deserving further investigations.

Bark-dwelling fungal communities

The strong host preference of *B. variegatum* is so far unexplained and could result from both biotic or abiotic factors (or a combination of both). Here, we compared the bark-dwelling fungal communities of *A. salicifolia versus* surrounding trees to identify unique fungi and/or fungal communities that could constrain the preferential if not specific establishment on this host tree. Bark-dwelling fungal communities were rich and mainly composed of Asco- and Basidiomycota. Resulting fungal guilds were dominated by saprotrophs and plant pathogens, in accordance with previous studies in the same (Petrolli *et al.*, 2022) or different (Cook *et al.*, 2022) tropical regions. As already reported by Pellitier *et al.*, (2019) in temperate forests, tree species mostly influenced fungal community composition in our tropical system, especially compared to spatial effects. Differential abundance (DA) analyses revealed that only few OTUs were statistically differentially abundant between *A. salicifolia* and other tree species, including few of the most abundant OTUs in the dataset. One mycorrhizal OTU in the Tulasnellaceae was significantly less abundant on *A. salicifolia*'s trunks compared to surrounding trees (*i.e.*, TUL2). Based on DNA sequence comparison with previous studies, this fungus is commonly retrieved in the roots of epiphytic orchids in ML (Martos *et al.*, 2012, see also Fig. S14), which is in accordance with its wide distribution on trees. In contrast, few mycorrhizal OTUs in the Tulasnellaceae (*i.e.*, TUL1 and TUL3) and endophytic OTUs in the Helotiales (*i.e.*, HEL7) showed a statistical preference for the bark of *A. salicifolia*. These OTUs were therefore designated as good candidates for a putative role in constraining the epiphyte's distribution on *A. salicifolia*.

Root endophytes of B. variegatum

The analysis of the roots of > 100 individuals of *B. variegatum* across its full distribution range (*i.e.*, La Réunion island) showed that their endophytic fungal communities were mainly represented by the same OTUs that were significantly biased toward the bark of *A. salicifolia* (*i.e.*, Tulasnellaceae and Helotiales). Along with these OTUs, one mycorrhizal fungus in the Sebacinales (*i.e.*, SER4 in the Serendipitaceae family) was also frequent and abundant in the roots of *B. variegatum*. Tulasnellaceae and Serendipitaceae form two of the most common groups of mycorrhizal fungi in epiphytic orchids (Martos *et al.*, 2012, Wang *et al.*, 2021). Other mycorrhizal groups frequently associated with epiphytic orchids (*i.e.*, Ceratobasidiaceae and Atractiellales) were almost absent from the roots of *B. variegatum*, confirming previously documented mycorrhizal associations in the genus *Bulbophyllum* (Martos *et al.*, 2012, Calvert *et al.*, 2017, Xing *et al.*, 2019, Liang *et al.*, 2022). Helotiales are part of the Dark Septate Endophyte (DSE) complex which encompasses endophytic fungi beneficial for plants, including epiphytes (Leroy *et al.*, 2019). Recent work showed that Helotiales may improve orchid's responses to environmental stresses (Liu *et al.*, 2022) but there is no evidence so far and to our knowledge that they can be involved in orchid seed germination. Furthermore, given that Helotiales were frequent but not abundant in the roots of *B. variegatum*, their presence in the roots studied here might reflect mere endophytism rather than functional mycorrhizal association. At the contrary, Tulasnellaceae and Sebacinales, which are known to promote the germination of numerous epiphytic orchids species across tropical forest (*e.g.*, Meng *et al.*, 2019; Shao *et al.*, 2020) could be involved in the germination of *B. variegatum*'s seeds on *A. salicifolia*.

Endophytic fungi and host preference

Fungi forming mycorrhizas in the minute seeds of orchids (leading to the development of subsequent embryo) may be retained or not in later developmental stages of the plant (Ventre-Lespiaucq *et al.*, 2021; Fernández *et al.*, 2023). Therefore, bark-dwelling fungi involved in the germination of epiphytic orchid seeds may be found at least in the early developmental stages of the plants, and possibly in the roots of fully grown adults. In this study, two mycorrhizal OTUs (TUL3 in the Tulasnellaceae and SER4 in the Serendipitaceae) were mainly associated with the earliest growth stages of *B. variegatum* (Fig. 5). Notably, SER4 represented nearly 100% of sequences retrieved from the youngest growth stages studied (one site) with the fungal universal primers. The high frequency and abundance of these fungi in young seedlings make them good candidates for constraining the distribution of the epiphytic orchid on its host through germination. These two OTUs were found to be associated only with *B. variegatum* in a large survey of orchid root endophytic fungi on the whole island (Martos *et al.*, 2012, see also Figs. S14-S15), suggesting a specific interaction between the plant and the fungi. Importantly, these fungi were absent from alternative tree hosts with the notable exception of the exotic *Eucalyptus robusta* (see Fig. S13), the only alternative tree species known to host dynamic populations (*i.e.*, with renewing) of *B. variegatum* on the island. The other investigated alternative tree species only hosted adults of *B. variegatum* and for the majority of them, they were - or used to be, in contact with a standing or already dead *A. salicifolia* (see for instance Fig. S1F-G).

Meanwhile, adults of *B. variegatum* were mainly associated with one OTU of mycorrhizal Tulasnellaceae (*i.e.*, TUL1) which was barely associated with young seedlings. This fungus was found preferentially on the bark of *A. salicifolia* in the forest but was also able to colonize more sporadically and alone, the bark of other trees (Petrolli *et al.*, 2022 and this study). Therefore, the ability of *B. variegatum* to survive (but not germinate) on alternative tree species might be explained by the ability of its late-developmental stage associated fungus to colonize these trees (Fig. 5). However, the plants growing on other trees did not form dynamic colonies (except on the exotic *E. robusta*, see above) perhaps because of the low efficiency of this mycorrhizal fungus to degrade organic compounds from the bark or other organic debris on these trunks, a hypothesis that needs to be tested in the future.

The role of biotic interactions in constraining orchid's distribution

Previous studies have hypothesized that at local scale, orchids may be limited in their distribution by their saprotrophic fungal symbionts (McCormick & Jacquemyn, 2014). This mycorrhiza-mediated constraint has been documented in rare terrestrial orchid species, such as *Caladenia huegelii* (Swarts *et al.*, 2010) or *Platanthera praeclara* (Kaur *et al.*, 2019) which both associate with a very narrow range of spatially limited orchid mycorrhizal fungi. The efficiency of the fungal partners to form mycorrhizas with germinating seeds, rather than their mere presence, could also constrain the distribution of orchids as exemplified in the *Drakaea* genus by Philips *et al.*, (2011). In the tropical epiphytic environment however, Zhao *et al.*, (2024) recently demonstrated that the host preference of the epiphytic orchid *Dendrobium wangliangii* was not fully explained by the broad distribution of its main fungal partners (*Fusarium* spp.) in the habitat (see another example in Johnson *et al.*, 2023). As highlighted by these contrasted results, the role of mycorrhizal fungi in constraining the distribution of their orchid host might be highly dependent on the orchid species considered, but also on its mycorrhizal specificity and on the distribution (limited or not) of its fungal partners. Notably, a narrow mycorrhizal specificity is not necessarily linked with a constrained distribution of the orchid as long as the mycorrhizal partner is ubiquitous in the environment (*e.g.*, Rammitsu et al., 2019).

In our study, all the epiphytic orchid species studied but *B. variegatum* displayed a non-specific distribution across tree species in the forest. Previous studies in the same habitat have shown that these species are associated with numerous and/or generalist fungal partners (Martos *et al.*, 2012; Petrolli *et al.*, 2022), which could explain such weak or null host-preferences. As an example, the second most host-specific orchid studied here, *i.e.*, *A. pectinatum*, is known to associate with several mycorrhizal fungi including the TUL2 in this study (Martos *et al.*, 2012; Petrolli *et al.*, 2022), which was largely distributed on tree species in the forest.

In contrast, the two fungi associated with the earliest developmental stages of *B. variegatum* were limited to the bark of *A. salicifolia*. This result suggests that in this system, the limited distribution of the fungal partners could constrain (through symbiotic germination) the distribution of their orchid host on certain trees.

The influence of the bark on the dynamic of mycorrhizal fungi has been studied in the past, and Chathurika Harshani *et al.*, (2014) showed for example that the symbiotic germination of the epiphytic species *Dendrobium aphyllum* was inhibited by the bark extracts of non-host trees. In our system, chemical compounds from non-host barks could inhibit the growth of the mycorrhizal partners of *B. variegatum* (or seed germination directly, Frei *et al.*, 1972) but given the very narrow range of compatible tree species, it seems more likely that the particular bark of *A. salicifolia* promotes the growth of mycorrhizal fungi involved in the germination process. Bark structure and water retention capacity have also been shown to influence the distribution of epiphytic plants on trees (Zarate-García *et al.*, 2020; da Silva *et al.*, 2023) and could also constrain the distribution of these mycorrhizal fungi. Interestingly, old trees of *A. salicifolia* and *E. robusta* display similar bark features on the field (*i.e.*, thick and spongious bark; Fig. S1) which could be analyzed in the future to test this hypothesis.

Like the other root endophytes of *B. variegatum* (Fig. S8C-D), the fungi associated with the earliest developmental stages were especially retrieved in the bark samples collected at the close vicinity of this orchid's roots. This suggests that their ability to colonize the bark is limited and that their growth on the trunk may be promoted by the presence of the orchid. This dynamic model where the orchid first benefits from its mycorrhizal fungi at the early growth stages and then promotes in return their survival and/or growth on the substrate, has been suggested in the past in the terrestrial orchid *Tipularia discolor* (McCormick *et al.*, 2021). In our system, we hypothesize that the fungal partners associated with the early-developmental stages of *B. variegatum* (*i.e.*, TUL3 and SER4) have the ability to grow on the trunk of *A. salicifolia* due to certain bark physical and/or chemical properties, but that this growth is constrained in space, perhaps through competitive interactions with other fungi. Their expansion on the substrate could yet be favored by the orchid subsequent development, a hypothesis that needs further investigations.

Conclusions

Host preference in epiphytic plants is often debated, and its causes remain unclear in most cases (Wagner *et al.*, 2015). In this study, we demonstrated a strong host preference of the epiphytic orchid *B. variegatum* for its tree host *A. salicifolia*, and we tested for the presence of a particular mycorrhizal assemblage on the bark of this tree. Our results show that the mycorrhizal partners associated with young developmental stages of the orchid are limited to the compatible host tree species and could therefore constrain the germination of their orchid host on these trees. The mechanisms involved remain unknown and could involve chemical and/or physical constraints of the bark for the fungal partners alone or in association with the orchid. Further experiments, including *in situ* symbiotic germination tests, are needed to test these hypotheses in the future.

Supporting information

Figure S1 Features of *Bulbophyllum variegatum*'s distribution on trees.
Figure S2 Random Forest (RF) workflow.
Figure S3 Additional assessment of epiphytes' preference for tree species.
Figure S4 Fungal and mycorrhizal accumulation curves.
Figure S5 Fungal guild composition.
Figure S6 Fungal beta-diversities in the dataset.
Figure S7 Fungal beta-diversities for fungal guilds.
Figure S8 Variation of bark fungal richness with root proximity
Figure S9 Bark fungal beta-diversities between rhizospheric and non-rhizospheric samples
Figure S10 Influencing factors shaping bark-dwelling fungal communities in the forest.
Figure S11 Root endophytic fungal communities of *Bulbophyllum variegatum*.
Figure S12 Details of fungal communities in the roots of *B. variegatum* in adults.
Figure S13 Details of fungal communities in the roots of *B. variegatum* on non-focal trees.
Figure S14 Phylogenetic tree of Tulasnellaceae (ITS marker).
Figure S15 Phylogenetic tree of Sebacinales (ITS marker).

Table S1 Detailed root sampling of *Bulbophyllum variegatum*.
Table S2 Detailed sampling of bark samples in the Mare Longue forest.
Table S3 Correspondence between plant's names and abbreviations.
Table S4 Beta-diversity analyses (bark-dwelling and root-associated fungi).
Table S5 Pairwise beta-diversity analyses on bark-dwelling fungal communities (p/a).
Table S6 Pairwise beta-diversity analyses on bark-dwelling fungal communities (abundance).
Table S7 Beta-diversity analyses (bark-dwelling saprotrophic fungi).
Table S8 Beta-diversity analyses (bark-dwelling pathogenic fungi).

Competing interests

We declare that we have non conflicts of interest regarding this work.

Author contributions

Rémi Petrolli: Conceptualization (environmental DNA survey), Investigation (data collection), Data curation, Formal analysis, Visualization, Writing – Original Draft. **Rosa Kemmerling:** Data curation, Formal analysis, Writing – review & editing. **Géromine Collobert:** Investigation (data collection), Data curation, Writing – review & editing. **Eve Hellequin:** Data curation, Formal analysis, Writing – review & editing. **Tony Robinet**: Data curation, Formal analysis, Writing – review & editing. **Neil Lancaster:** Conceptualization (epiphytic vegetation survey), Investigation, Data curation, Writing – review & editing. **David L Roberts:** Conceptualization (epiphytic vegetation survey), Investigation, Writing – review & editing. **Thierry Pailler:** Conceptualization (epiphytic vegetation survey), Investigation, Writing – review & editing. **Claudine Ah-Peng:** Resources, Writing – review & editing. **Marc-André Selosse:** Conceptualization (environmental DNA survey), Funding acquisition, Writing – review & editing. **Florent Martos:** Conceptualization (environmental DNA survey), Investigation (data collection), Data curation, Formal analysis, Funding acquisition, Writing – Original Draft.

Data availability

Raw data will be deposited in the Sequence Read Archive (SRA) after acceptance. Full bioinformatics code (R and bash) is available at https://github.com/PetrolliR/Uneven_Distributions_Orchid-Bark_Fungi.

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