**The importance of light and mycorrhizal fungi in determining the distribution of epiphytic orchids in mega-diverse tropical forests**

# Rationale and positioning with regard to the state-of-the-art

Understanding the various **factors that limit the distribution** of plant and animal species is a longstanding question in ecology1. In land plants, the large-scale distribution of a species is simultaneously limited by climate and soil, dispersal, and biotic interactions1. The most relevant climatic factors restricting plant species distribution globally are temperature and precipitation. At local scales, microsite conditions such as light and substrate physicochemical quality play an important role as well1. Dispersal determines how far seeds are dispersed from mother plants and is limited by the presence of barriers that impede their dissemination2. Biotic interactions can limit or increase species distributions. Antagonistic interactions such as interspecific competition may exclude some species from a given site, while mutualisms such as mycorrhizal symbioses can contribute to expand plant species distribution areas1,3,4.

Whereas the effects of environmental conditions and dispersal on species distributions are relatively well understood, the ecological effects of **obligate inter-specific interactions**, such as symbioses, are far less understood3,4. Recent evidence suggests that the distribution ofspecies that rely on other species to complete their life cycle depends on the presence of suitable partners (partner availability)5 and on the level of specialization (partner breadth)6. Species that either associate with widespread partners or are generalist towards them are expected to have a broader spatial distribution, whereas species that associate with a limited number of partners or with partners that only sporadically occur in nature, are expected to have a narrow distribution. Furthermore, a species can interact with more than one type of partner at the same time, such as mycorrhiza, pollinators, or dispersal agents, which adds complexity to the determinants of species distribution.

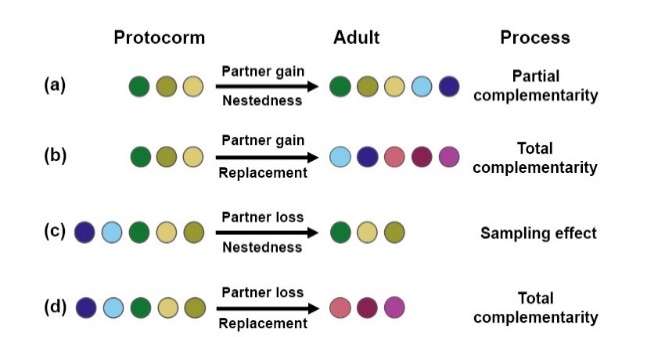
Current understanding of the determinants of species distribution is based mostly on the biology of the adult individuals. However, in sessile organisms such as plants, the distribution of adults can largely depend on the species' requirements during the earliest developmental life stages (the **regeneration niche**7). The regeneration niche includes preferences for different substrates8, differential responses of seedlings to ecological gradients9, and the availability of microscopic mutualistic organisms10. Local availability of mutualists, particularly of symbiotic fungi, is one of the least studied aspect of a plants' regeneration niche.

**Epiphytes** are plants that live non-parasitically on tree trunks and branches, and therefore need to find a **suitable host tree** for physical support. Because of their life style, epiphytes face strong ecological gradients at relatively short distances. Probably the most important environmental factor in tropical forest is the **availability of light**, which changes considerably through the canopy of the forest. To cope with these variable light conditions, epiphytes have specific adaptations to regulate their photosynthetic capacity and growth rate11. Nevertheless, whether light affects seed germination in epiphytes is an understudied issue, and the scarce information available is far from conclusive11. In contrast to epiphytes belonging to other families, the regeneration niche of **epiphytic orchids** depends on two additional factors. First, seeds fully rely on **mycorrhizal fungi for germination**. Since these fungi are not inherited from maternal plants12,13, the availability and spatial distribution of suitable mycorrhizal fungi are a key component determining the probability of germination and therefore the distribution of epiphytes in the forest canopy. Second, while the early life stages lack chlorophyll and fully depend on mycorrhiza for nutrition, adults of most species are capable of photosynthesis, potentially reducing their dependency on mycorrhiza as compared to seedlings (**partial mycoheterotrophy or even autotrophy**)14. Epiphytic orchids thus need to find suitable host trees and mycorrhizal fungi for germination, but also might experience changes in their environmental and nutritional requirements throughout development, meaning that the **regeneration and adult niches might differ substantially**.

Although specialized partnerships with host trees have been reported, most evidence so far indicates that most epiphytic orchids can grow on a broad range of tree species15–17, suggesting that tree availability may not be a major constraint to the distribution of epiphytic orchids. Less, however, is known about how mycorrhizal fungi determine the distribution of epiphytic orchids. In terrestrial orchids, there is a continuum from specialists18,19 to generalists20,21. For epiphytic orchids, we do not really know whether they are specialized or generalized22. The little evidence available suggests that epiphytic orchids may have a higher diversity of mycorrhizal interactions than terrestrial orchids23 and that they tend to be generalists24,25, although highly specialized taxa have also been documented22,26. Moreover, studies conducted across small spatial scales have shown that coexisting terrestrial orchids tend to have distinctive mycorrhizal communities and show strong spatial segregation, suggesting that mycorrhizal partners play a role in determining their distribution27. No such data are available for epiphytic orchids.

Little is also known about **how mycorrhizal communities vary** among host trees and over the host tree surface, and how this affects the distribution of epiphytic orchids. It has been proposed that epiphyte turnover among host trees may be mediated by mycorrhizal fungi28 and that orchids with many mycorrhizal partners have more host tree species29. However, exceptions to this pattern have been reported as well29, suggesting that interactions of epiphytic orchids with both trees and mycorrhizal fungi can be diverse and complex. Similarly, the vertical turnover in epiphytic orchids is known to relate to changes in bark characteristics as well as the epiphytes' requirements for light17. Yet, whether those factors influence the distribution of mycorrhizal partners remains to be assessed29. In general, solid evidence of how host trees and mycorrhiza affect epiphytic orchid distribution is still lacking, because **studies were conducted on a limited spatial scale and rarely included ecological gradients**.

Because seedlings often experience high mortality in later stages of their development30,31, several factors may affect the distribution of epiphytic orchids. These relate to seedling predation and pronounced differences between the *regeneration* and *adult niches* of the species32. Plant physiological needs often change over ontogeny, and the successful transition from seedling (protocorm) to adult might depend on acquiring new mycorrhizal partners that help fulfill those new needs6. Such ontogenetic partner turnover can result from complementarity or sampling effects in time6 (Fig. 1). **Complementarity** consists of a replacement of partners from the seedling to the adult stage, under the assumption that new partners potentially play complementary roles.



**Fig. 1** Hypothetical scenarios of ontogenetic partner turnover and their putative driving processes. Colours denote different partners. Text above arrows indicates changes in partner number; text below arrows indicates the prevailing component of composition turnover (nestedness or replacement).

On the other hand, **sampling effects** consist of adults retaining a subset of their partners from the seedling stage. Ontogenetic partner turnover through total complementarity is risky because a lack of suitable new partners can compromise survival to adulthood. Evidence from terrestrial orchids suggests that partner gains are common33, while total complementarity33 and partner losses19 have been less documented. In epiphytic orchids, the role of mycorrhiza turnover on the successful transition to adulthood remains to be assessed10,34.

# Scientific research objectives

The major aim of this research is to understand **how interactions with multiple partners influence the distribution of epiphytic orchids in hyper-diverse tropical forests and how these interactions are affected by abiotic conditions and the ontogenetic stage of the plant**. Specifically, the project aims at understanding how the availability of suitable mycorrhizal fungi affects germination and recruitment of epiphytic orchids along natural light gradients, and how this is affected by specific characteristics of the host tree. To this end, I will address three key aspects of the interaction between epiphytic orchids, host tree and mycorrhizal fungi: i) the availability of free-living fungi on the substrate as a key component of microsite quality; ii) changes in the interaction over the vertical gradient of light of the forest; and iii) ontogenetic turnover of mycorrhizal partners as a putative barrier to post-germination establishment. In particular, I will test the hypotheses that:

H1: the influence of partner availability on epiphytic orchid distribution depends on partner breadth and abiotic conditions.

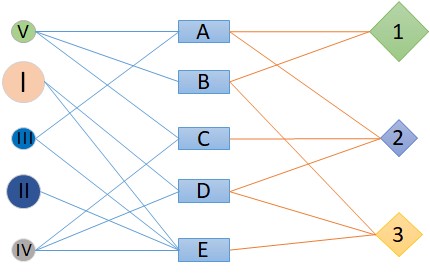
H2: the composition and the availability of free-living fungi changes over the vertical gradient of light and over the trunk of the host tree.

H3: seedlings associate with a larger diversity of mycorrhizal fungi than adults allowing them to easily find a suitable partner in the complex canopy of the rainforest.

H4: ontogenetic changes in mycorrhizal partners result from sampling effects rather than total complementarity.

This study will provide **three innovative aspects to the field**. Specifically, it will:

**(1) address an unresolved question in plant ecology**: how more than one partner affects plant species' distribution. This is not trivial because a considerable proportion of tropical plant diversity relies on more than one partner for successful establishment. In epiphytic orchids in particular, most studies have focused on bipartite interactions, *i.e.*, epiphyte-host tree or epiphyte-fungi interactions, while a tripartite network approach (epiphyte-mycorrhiza-host tree) better reflects the actual situation (Fig. 2).



**Fig. 2** Schematic representation of a tripartite interaction network between orchids (circles), mycorrhiza (squares) and host trees (rhombi). Characters depict different species; lines depict inter-specific interactions.

**(2)** provide the **first experimental test** of how tripartite interactions affect orchid distribution within a vertical light gradient. Combining **careful field observations and seed germination experiments with cutting-edge molecular analyses**, I will be able to:

* assess how mycorrhizal fungi are distributed over the trunk of the host tree.
* investigate how changes in light availability through the canopy of a host-tree affects germination and establishment of epiphytic orchids.
* determine whether orchid mycorrhizal partners are replaced or retained over an individual's lifetime, and to assess the underlying mechanisms.

**(3)** provide the **first comprehensive overview of the potential drivers of tropical epiphytic orchid distribution**. This research **will push the state-of-the-art forward**, moving from local studies of focal orchid species towards an integrative approach over larger scales, yielding novel insights into:

* how environmental and climatic factors influence the interaction network and community structure of epiphytic orchids.
* how orchid partner breadth and partner availability influence the large-scale distribution of epiphytic orchids.

To test the proposed research hypotheses, I will build and analyse tripartite interaction networks to infer changes in the orchid-mycorrhiza-host tree interactions over a geographical gradient of temperature and moisture. I expect to find a continuum of orchid-partner interactions, from strict generalists to strict specialists. Forests with more stressful abiotic conditions (e.g., warm and dry) will harbour orchid species that depend more strongly on their partners, while moist forests will host a wider variety of strategies. Solid evidence of how light gradients affect fungi availability and mycorrhizal symbioses in epiphytic orchids is still lacking. This project will address for the first time the effect of two partners on the distribution of epiphytic orchids in megadiverse communities across geographical and local ecological gradients.

Our knowledge on how seedling and adult niche requirements ultimately affect epiphytic orchid distribution is only fragmentary since the few studies available deal mainly with adult plants, but neglect germination or transitions between ontogenetic stages. Such knowledge, however, is key to design effective, evidence-based orchid conservation actions. In addition, the technology for massive DNA sequencing of microscopic fungi has been developed only in the last 10-15 years, which now allows to quantify the molecular diversity of fungal partners with an unprecedented resolution. A major current need in the field is an integrated approach that addresses (i) how host trees and mycorrhizal fungi limit epiphytic orchid distribution across geographical ecological gradients, and (ii) how the regeneration niche as defined by mycorrhizal fungi and the abiotic environment, and the transition to the adult stage determine individual establishment in epiphytic orchid populations.

# Research methodology and work plan

The proposed work plan combines extensive field sampling and *in-situ* germination experiments with cutting-edge molecular and statistical analyses. I will conduct fieldwork in Colombia, where I will collaborate with Dr. Nicola Flanagan, professor at Pontificia Universidad Javeriana, Cali. I will undertake laboratory and computing tasks in Belgium, in the research group of Dr. Hans Jacquemyn, professor at KU Leuven.

The envisaged work has been structured into three work packages (WP). In WP 1, I will investigate how the tripartite interaction between orchid, host tree, and mycorrhizal fungi varies across a geographical gradient of temperature and humidity (H1). To this end, I will work in different forest types across the Colombian Andes (Fig. 3). In WP 2, I will investigate the distribution of free-living mycorrhizal fungi over a local gradient of light (H2) represented by three trunk heights. Field sampling for this WP will be conducted in dry-humid transition forests. In WP 3, I will assess whether orchid mycorrhizal communities change between the seedling and the adult stages of epiphytic orchids (H3) and infer potential underlying ecological mechanisms (H4). To this end, I will perform seed germination experiments in dry-humid transition forest plots. Each WP will produce a research paper (*Deliverables 1, 2, and 3, respectively*) that will be submitted to high-impact scientific journals such as New Phytologist, Molecular Ecology or Ecology Letters.

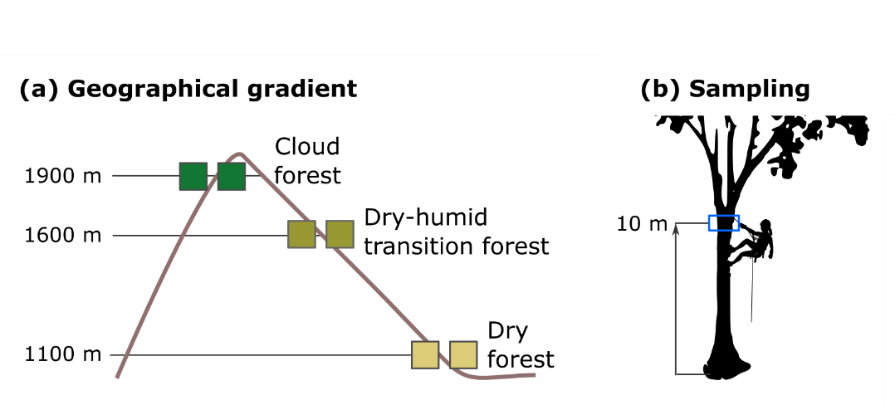
**WP1 Assessing the influence of partner availability on epiphytic orchid distribution.**

It is reasonable to assume that the frequency of seed germination will be higher in orchid species that associate selectively with common instead of rare fungal partners, implying that **partner availability in the substrate** plays an important role in determining orchid distribution. Yet, orchid species differ in their degree of specialization in the interaction (**partner breadth**), meaning that they can associate selectively with a few fungal partners (*specialist species*), or indistinctively with many of them (*generalist species*). Theory predicts that natural selection favors highly specialized interactions as a way to avoid cheaters. This view has been recently challenged on the basis that generalist interactions can be advantageous under environmental heterogeneity, where species should not be very choosy in order to associate with the partners at hand6,35. In this way, generalist species can broaden their distribution by shifting partners (**partner turnover**) when the preferred partner is not locally available. Species exhibiting such ability can be expected to have higher chances of germinating in a variety of microsites than highly-specialized species.

*Task 1.1 Collecting epiphytic orchid roots in natural forests*

The study will be conducted in natural, mostly undisturbed tropical forest ecosystems. I will sample communities of epiphytic orchids in three forest types along a humidity-altitude gradient in the western mountain range of the Colombian Andes (Fig. 3a). The forest types encompass dry forests in the Cauca River Valley, dry-humid transition forests (DHTF) in the valley slope, and cloud forests in the Pacific slope. In each forest type I will choose two plots of 50 x 50 m that are at least 5 km apart and contain >10 different host trees per plot. I will sample orchids growing at a height of 10 m on tree trunks using the single-rope climbing method (Fig. 3b), collecting three 2-cm root fragments in up to five individuals per orchid species of all the species found. Sampling will not destroy the plants.

*Identifying species -* Based on diversity inventories conducted at the study locations, I expect to find approximately 120 orchid species in total (García-Revelo, S. unpubl.; Ospina, NH. unpubl.). Some orchid species are expected to be unique to a particular forest type, while others occur in at least two of them. For each sampled individual, I will record the **host tree species** on which it was growing and will assess host-tree breadth. I will **identify orchids and host trees** with the help of a local taxonomist (Nhora Ospina, Universidad del Valle, Cali) and in case of doubt will confirm the identity of ambiguous tree and orchid species using genetic barcoding.



**Fig.** **3** Sampling design. **(a)** Geographical gradient including three forest types in the Andes Mountain Range, with two plots per altitude. **(b)** Sampling method.

*Characterizing abiotic conditions -* To characterize **regional climate** conditions, I will use climatic data from meteorological stations near the plots. To describe the **light environment of host trees** I will measure photosynthetically active radiation (PAR, mol·m-2·day-1) by taking three hemispheric photographs in each tree at 10 m, where orchid roots will be sampled. To improve PAR estimation accuracy, I will measure the diffuse to direct PAR ratio with BF5 diffuse PAR sensors (Delta-T Devices, UK) in three randomly chosen host trees. The completion of this task is a milestone (M1). Potential delays (*e.g.*, due to bad weather) have been considered in the task schedule (Table 1).

*Task 1.2 Molecular analyses and bioinformatics*

Mycorrhizal DNA extraction and sequencing analyses will be performed at KU Leuven (Belgium). After acquiring the necessary licenses, root samples will be exported to Belgium. Licenses for research purposes are usually granted within 1-3 months (N Ospina, pers. comm.). In case of delays or problems with acquiring the licenses, I will extract mycorrhizal DNA in the molecular biology lab of Dr. Flanagan and export the DNA products. DNA will be extracted from 0.5 g mycorrhizal root fragments using UltraClean Plant DNA Isolation Kit (Mo Bio Laboratories Inc., CA, USA). Amplicon libraries will be created for Illumina MiSeq sequencing using two primer combinations amplifying the ITS-2 region of eukaryotic rDNA, ITS3 / ITS4OF36,37 and ITS86F / ITS436,38. ITS3 / ITS4OF has previously been shown to favour the amplification of basidiomycete fungi and detection of orchid-associating families of the Basidiomycota, while ITS86F / ITS4 provides amplification of a broad spectrum of fungal taxa. All samples will be assigned a unique combination of forward index (i5) and reverse index (i7) sequences following the dual-index strategy outlined by Kozich et al.39 for paired-end Illumina MiSeq sequencing. Amplicons generated using indexed ITS3 / ITS4OF or ITS86F / ITS4 primers will be pooled into their own separate amplicon libraries, which will be sequenced using the 500-cycle MiSeq Reagent Kit v2 (Illumina).

*Bio-informatics analysis:* De-multiplexed paired-end reads from either the ITS3 / ITS4OF or ITS86F / ITS4 library will be aligned and merged using the USEARCH *fastq\_mergepairs* command. Combined taxonomic units definition and chimera removal will be performed using both the *cluster\_otus* command (UPARSE) generating OTUs clustered at 97% sequence homology or *unoise3* command (UNOISE) generating denoised ZOTUs, followed by mapping of reads to the final (Z)OTUs list using *otutab* with default cut-off setting of 97% for OTUs command to assign abundances to each (Z)OTU and construct both UPARSE OTU and UNOISE ZOTU tables. Finally, (Z)OTUs will be assigned taxonomic identities using the *sintax* command in conjunction with the UNITE USEARCH reference dataset v.7.2 (UNITE, 2017).

*Task 1.3 Assessing inter-specific interactions and network analyses*

Network analysis captures patterns of interactions between species40. I will build **tripartite networks** comprised of three types of nodes: i) epiphytic orchids, ii) mycorrhizal OTUs, and iii) host trees (Fig. 2). Tripartite network analysis provides information on the *number of links per species* (which species are more connected) and *layer interdependence* (a measure of how much information about one layer predicts information in another layer)41, and allows to *detect communities within the network* ('blocks', a metric similar to modularity of bipartite networks)41–43. I will calculate these metrics using muxViz, a tool for analysing multiplex networks44. Tripartite network analysis is only recently being used to address multiple interactions and, despite the field evolves rapidly42, networks involving more than two partners have to be decomposed into bipartite networks to obtain other relevant metrics45, such as *modularity*45, *nestedness*46, and *specialization*47. In the context of network analysis, a high modularity indicates there are subsets of strongly connected species interlinked through a few interactions45. A high nestedness indicates that there is a core of the most generalist species interacting among them46. For each network, I will calculate the degree of nestedness and modularity. In addition, I will also quantify the partner breadth of a species by assessing the degree of interaction specialization at the species (*d*') and network (*H*') level47. All analyses will be performed using the bipartite package48 in R49.

With this information, I will be able to test H1, which states that the influence of partner availability on epiphytic orchid distribution depends on partner breadth and abiotic conditions. In particular, I expect network nestedness and specialization to vary between forests. In cloud forests, where abiotic stress is lowest, epiphyte abundance is high and competition for space is strong12, there will be a wide array of orchid-mycorrhiza-host tree interactions, from generalists to specialists. This is because generalization often allows plants to use a wider variety of microsites6 and specialization potentially reduces competition among coexisting orchids27. In dry forests, where abiotic conditions are harsher, physiological adaptation will be a strong determinant of orchid distribution, and interaction networks will probably be more generalized to increase the probability of successful establishment.

**WP2 Assessing how mycorrhizal fungi are distributed over the host tree trunk.**

The interaction between orchids and mycorrhizal fungi is asymmetric. Orchid seeds have to find suitable mycorrhizal fungi for germination, while fungi do not necessarily depend on the orchid for their survival. However, it is reasonable to assume that the spatial distribution of fungi themselves depends on characteristics of the environment. Spatial variation in the composition of mycorrhizal fungi communities is likely to affect seed germination and consequently the distribution of epiphytic orchids. If the composition of fungal communities is heterogeneous over the substrate, orchid species that specialize on a limited number of fungi, may suffer from establishment limitation. In contrast, generalist orchid species that associate with a variety of mycorrhizal fungi are expected to be less limited by the presence of mycorrhizal and therefore will occupy a broader range of microhabitats.

*Task 2.1 Sampling the host-tree bark for mycorrhizal fungi*

To investigate variation in mycorrhizal communities between and within host tree species, I will sample 20 trees from the dry-humid transition forest plots from WP 1. I will establish sampling points at heights of 10, 6 and 2 m in the trunk, representing a vertical light gradient (Fig. 4). At each height, I will measure light using hemispheric photography as described in WP 1. I will collect three bark samples per height to obtain the fungal communities that occur on the bark of trees and perform fungal DNA extraction and sequencing analyses as described in WP 1. This will provide a picture of the pool of mycorrhizal fungi locally available at different heights of the trunk. The proportion of mycorrhizal fungi actually interacting with orchids will be identified by combining two pieces of information: 1) OTU lists derived from the regional scanning in WP 1, and 2) the mycorrhizal fungi associated to the adult orchids present nearby.

*Task 2.2.* *Molecular analyses and bioinformatics*

I will extract fungal DNA from the bark as described in *Task 1.2* (WP1). Subsequently, Illumina sequencing and bio-informatics analyses will be performed as outlined in Task 1.3 (WP1).

*Task 2.3 Data analysis*

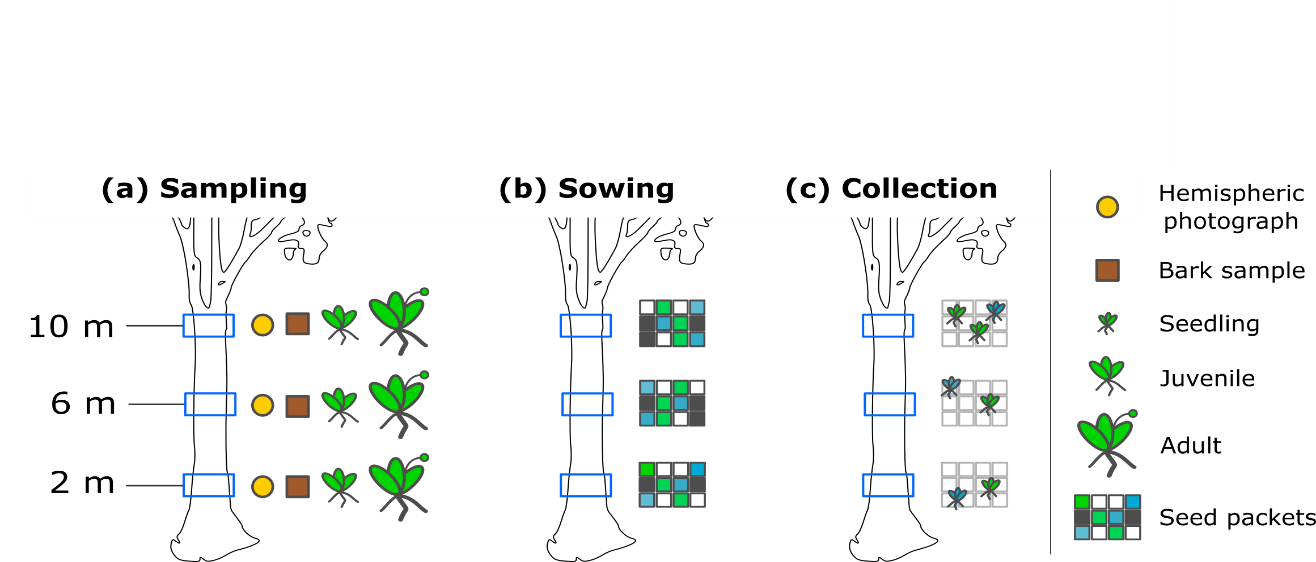
I will determine which bark fungi correspond to mycorrhizal taxa. With this information, I will be able to test H2, which states that the availability of free-living fungi changes over the vertical gradient of light. To this end, I will first describe mycorrhiza community composition, richness and turnover across tree heights. I will perform non-metric multidimensional scaling (NMDS) analysis over a mycorrhizal OTU presence-absence matrix and assess differences in species composition between host trees and heights with PERMANOVA, estimate richness with rarefaction curves50, and turnover using Jaccard index51. Second, I will build linear models in R49 to test whether mycorrhizal fungi richness and composition (response variables) vary over trunk heights and among host tree species (fixed factors).

**WP3 Assessing turnover in mycorrhizal fungi over epiphytic orchid ontogeny.**

To assess turnover in mycorrhizal communities across different life stages, I will conduct *in-situ* seed germination assays to obtain seedlings. This WP will be conducted in dry-humid transition forest plots, using the seeds of four local orchid species. More specifically, I will collect ripe capsules of four orchid species to obtain the seeds for setting the germination assays (Task 3.1). I will test hypotheses 3 and 4 by comparing the **richness** (H3) and **composition** (H4) of mycorrhizal fungi communities extracted from **seedling** and **adult** orchid roots.

*Task 3.1 Setting up* in-situ *germination assays*

I will collect seeds from fruit capsules to prepare seed packets52. I will use plastic wraps52 to attach three seed packets per orchid species at the three heights in 10 trees per plot (720 seed packets in total) (Fig. 4b). The completion of this task is a milestone (M2). A potential risk is obtaining a low germination success (medium likelihood). I will implement two preventive measures to increase the probability of germination: (i) the use of an effective sowing method52, and (ii) a high number of replicates. In addition, this experiment will be monitored every two months by a member of Dr. Flanagan's lab. This will ensure that I do not miss the germination period for not being physically there.



**Fig.** **4** Study design. **(a)** Bark and adult sampling at 3 trunk heights, **(b)** *in-situ* germination assays (each colour denotes a different species), **(c)** collection of seedlings.

*Task 3.2 Collecting seedlings from germination assays*

Eight to ten months after sowing, I will harvest the seedlings (Fig. 4c) to collect the protocorms. Germination success will be assessed by recording the number of seedlings per species and height. At the same time, I will also collect roots of the four orchid species for assessment of the mycorrhizal fungi in the adult plants.

*Task 3.3 Molecular analyses and bioinformatics*

I will extract fungal DNA from the roots of adult individuals and seedlings as described in *Task 1.2* (WP1). Subsequently, Illumina sequencing and bio-informatics analyses will be performed as outlined in Task 1.3 (WP1).

*Task 3.4 Data analysis*

I will estimate **mycorrhizal OTU richness in seedlings and adults** for each species, and I will test whether seedlings have a greater richness of mycorrhizal fungi than adults (**H3**). To this end, I will build a linear model in R, with mycorrhizal richness as the response variable and the developmental stage (seedling and adult) as a fixed factor. I expect seedlings to associate with a greater number of mycorrhizal OTUs. A confirmation of this hypothesis would indicate that seedlings are more generalist than adults, suggesting that the establishment of mycorrhizal associations is opportunistic during early life stages. To test the hypothesis that ontogenetic partner turnover occurs through sampling effects rather than complementarity (**H4**), I will calculate **OTU composition turnover from seedlings to adults**. I will assess OTU turnover between both developmental stages, and partition total turnover into its nestedness and replacement components51. High nestedness values indicate that adults associate with a subset of seedlings' OTUs, whereas high replacement values indicate that adults associate with a set of OTUs that are absent in seedlings. I expect nestedness to represent a greater proportion of OTU turnover than replacement (Fig. 1c). This result would indicate that mycorrhizal turnover over the ontogeny is due to sampling effects instead of total complementarity, thereby supporting the general hypothesis that switching partners over ontogeny is a risky strategy.

**Work plan (see the last page)**

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**Work plan**

The proposal consists of three work packages (**WP**) (*Table 1*). Ideal start date is October 1st, 2019.

**Table 1** Work packages (WP) and tasks of the project. Blue: fieldwork; yellow: laboratory work; green: data analyses and manuscript writing.

