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| **APPLICATION POSTDOCTORAL FELLOWSHIP (junior/senior) PROJECT OUTLINE (MAX. 10 pages)** |

**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 distribution** of plant and animal species is a longstanding question in ecology (Krebs, 1972). In land plants, the distribution of a species is simultaneously limited by climate, dispersal, and biotic interactions (Krebs, 1972). The most relevant climatic factors restricting plant species distribution globally are temperature and humidity. At local scales, microsite conditions such as light and substrate physicochemical quality play an important role as well (Krebs, 1972). Dispersal determines how far seeds are dispersed from mother plants and is limited by the presence of barriers that impede their dissemination (Nathan & Muller-landau, 2000). Biotic interactions can limit or increase species distributions. Antagonistic interactions such as interspecific competition will exclude some species from a given site, while mutualisms such as mycorrhizal symbioses can contribute to expand plant species distribution areas (Krebs, 1972; Dunn *et al.*, 2009; Afkhami *et al.*, 2014).

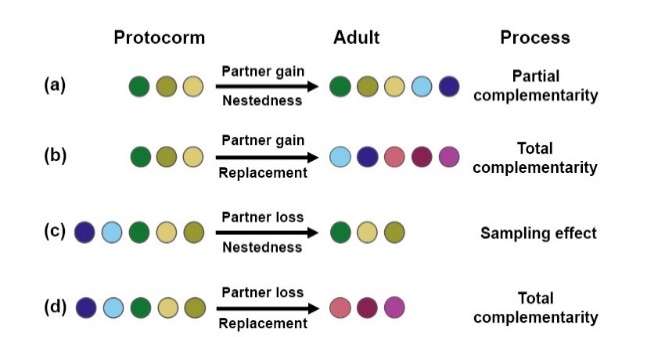
Whereas the effects of environmental conditions and dispersal on species distributions are relatively well understood, the ecological effects of obligate inter-specific interactions, such as symbioses, are far less understood (Dunn *et al.*, 2009; Afkhami *et al.*, 2014). Recent evidence suggests that the distribution ofspecies that rely on other species to complete their life cycle depends on the presence of suitable partners (partner availability)(Slatyer *et al.*, 2013) and on the level of specialization (partner breadth) (Batstone *et al.*, 2018). 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.

Studies addressing the determinants of species distribution are often focused on the biology of the adult individuals of the populations. 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 (Grubb, 2018)). The regeneration niche includes preferences for different substrates (Lusk, 1995), differential responses of seedlings to ecological gradients (Denslow, 1980), and the availability of microscopic mutualistic organisms (McCormick & Jacquemyn, 2014). Local availability of mutualists, particularly of symbiotic fungi, is one of the least studied aspect of 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 rapidly through the canopy of the forest (Benzing, 1990). To cope with these variable light conditions, epiphytes have specific adaptations to allow them …. Besides variable light conditions, two other factors will determine the regeneration niche of epiphytic orchids. First, seeds fully rely on **mycorrhizal fungi for germination**. Since these fungi are not inherited from maternal plants[[1]](#endnote-1),[[2]](#endnote-2), 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 (**partial mycoheterotrophy**), potentially reducing their dependency on mycorrhiza as compared to seedlings[[3]](#endnote-3). 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 species7-9  , 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 specialists10,11 to generalists12,13. For epiphytic orchids, we do not really know whether they are specialized or generalized14. The little evidence available suggests that epiphytic orchids may have a higher diversity of mycorrhizal interactions than terrestrial orchids15 and that they tend to be generalists16,17 , although highly specialized taxa have also been documented22,18. Studies over 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 distribution19. 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 fungi20 and that orchids with many mycorrhizal partners have more host tree species21. However, exceptions to this pattern have been reported as well29, suggesting that interactions of epiphytic orchids with their 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 light15. 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 development[[4]](#endnote-4),[[5]](#endnote-5), 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 species[[6]](#endnote-6). 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 fullfill those new needs19. Such ontogenetic partner turnover can result from complementarity or sampling effects in time19 (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. 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 common11, while losses are less well documented[[7]](#endnote-7). In epiphytic orchids, the role of mycorrhiza turnover on the successful transition to adulthood remains to be assessed9,15.



**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)

# Scientific research objectives

The major aim of this research is to understand **how multiple partners interacting with abiotic conditions influence the distribution of epiphytic orchids in hyper-diverse tropical forests**. 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: 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 occur and 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.

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

* how mycorrhizal fungi are distributed over the host tree trunk.
* how the vertical gradient of light within a host-tree affects epiphytic orchid germination.
* whether orchid mycorrhizal partners are replaced or retained over an individual's lifetime, and the underlying mechanisms.

**(3)** provide the **first comprehensive overview of the patterns and 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, and provide novel insights into:

* how 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 15-10 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.

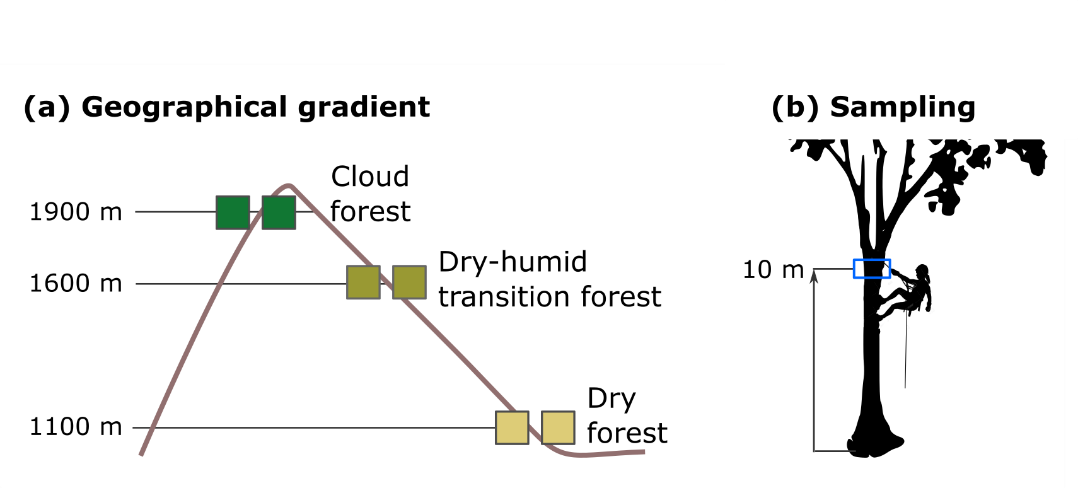
# Research methodology and work plan

The work will be structured in three work packages (WP). In WP 1, I will study how the tripartite interaction between orchid, host tree, and mycorrhizal fungi varies across a geographical gradient of temperature and humidity (H1). Therefore, I will sample in different forest types across the Colombian Andes (Fig. 2). 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 mycorrhizal fungi 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. [State the cohesion of the work packages in a phrase.]

**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** is probably an important determinant of orchid occurrence. 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 hand19,20. 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 have more chances of germinating in a variety of microsites than highly-specialized species.

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. 2a). 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 5 km apart and 10 host trees per plot. I will sample plants growing at a height of 10 m on tree trunks using the single-rope climbing method (Fig. 2b), 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.

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**Fig.** **2** Sampling design. **(a)** Geographical gradient including three forest types in the Andes Mountain Range, with two plots per altitude. **(b)** Sampling method.

Based on diversity inventories in 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 are expected to occur in at least two of them. I will register the **host tree species** where the different epiphytic orchid species occur (host-tree breadth), and the **mycorrhizal partners** in orchid roots (mycorrhizal fungi breadth). 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.

*Task 1.1 Characterizing abiotic conditions*

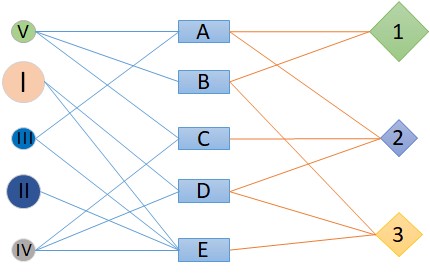
To characterize **regional climate** I will use climatic data from meteorological stations near the plots. To describe the **light environment of host trees** I will measure photosynthetically active radiation (PAR, mol·m-2·day-1) by taking three hemispheric photographs in each tree at 10 m, where orchid 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.

*Task 1.2 Assessing partner breadth*

Since mycorrhizal fungi are microscopic and may form complex interactions with both host trees and orchids, I will combine **meta-barcoding of mycorrhizal DNA** with **network analysis tools**[[8]](#footnote-1) to assess mycorrhizal partner breadth[[9]](#footnote-2),[[10]](#footnote-3). An individual orchid can host 1-30 different fungal partners[[11]](#footnote-4). To accurately describe **mycorrhizal diversity** associated with each individual plant I will extract DNA from 0.5 g mycorrhizal root fragments using UltraClean Plant DNA Isolation Kit (Mo Bio Laboratories Inc., CA, USA). I will use two complementary primer pairs (ITS3/ITS4OF and ITS86F/ITS4) for detailed characterization of diverse orchid mycorrhizal communities[[12]](#footnote-5). I will use Illumina sequencing to obtain mycorrhizal operational taxonomic units (OTUs), which are the commonly used units of microbial diversity. I will **identify orchids and host trees** with the help of a local taxonomist (Nhora Ospina, Universidad del Valle, Cali) and confirm ambiguous tree and orchid records using genetic barcoding.

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

Network analysis captures patterns of interactions between species[[13]](#footnote-6). I will build **tripartite networks** comprised of three types of nodes: i) epiphytic orchids, ii) mycorrhizal fungi, and iii) host trees (Fig. 3). 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)[[14]](#footnote-7), and allows to *detect communities within the network* ('blocks', a metric similar to modularity of bipartite networks)42,[[15]](#footnote-8),[[16]](#footnote-9). Tripartite network analysis is only recently being used to address multiple interactions and, despite the field evolves rapidly43, networks involving more than two partners have to be decomposed into bipartite networks to obtain other relevant metrics[[17]](#footnote-10), such as *modularity*[[18]](#footnote-11), *nestedness*[[19]](#footnote-12), and *specialization*[[20]](#footnote-13). A high modularity indicates there are subsets of strongly connected species interlinked through a few interactions46. A high nestedness indicates that there is a core of the most generalist species interacting among them47. Partner breadth of a species can be quantified with the degree of interaction specialization at the species (d') and network (H') level48.



**Fig. 3** 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.

I expect nestedness and specialization to vary between forests. In cloud forests, where abiotic stress is lowest, epiphyte abundance is high and competition for resources is strong9, orchid-mycorrhiza-host tree interactions will be weaker and orchids will establish generalist associations with the most abundant partner species39. In dry forests, where the conditions are harsher, physiological adaptation of the orchid will be a strong determinant of its distribution and also interaction networks will probably be more specialized9,17.

**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 likely 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 Variation in mycorrhizal communities*

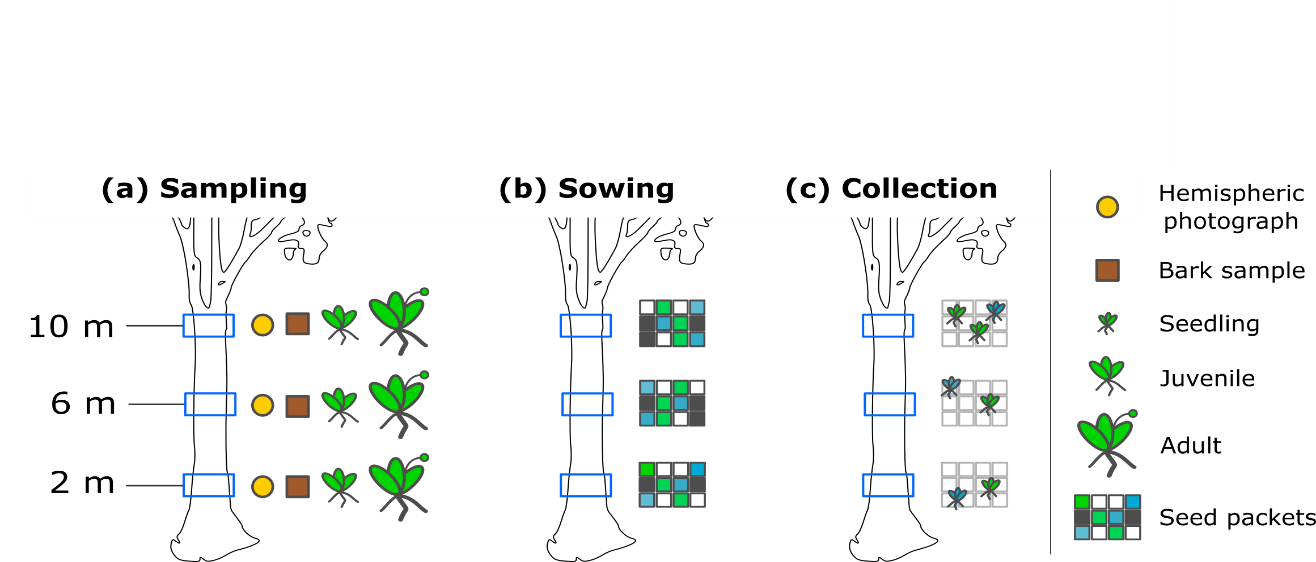
To investigate variation in mycorrhizal communities, I will sample 20 trees from the dry-humid transition forest plots from WP 1. I will establish sampling points at heights of 10, 6 and 2 m in the trunk, representing a vertical light gradient (Fig. 4). At each height, I will measure light using hemispheric photography as described in WP 1. I will collect three bark samples per height to obtain bark fungi 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 in different regions of the trunk. The proportion of mycorrhizal fungi actually interacting with orchids will be identified by combining three pieces of information: 1) OTUs lists derived from the regional scanning in WP 1, 2) the mycorrhizal fungi associated to the adult orchids present nearby, and 3) the mycorrhizal fungi identified from seedlings derived from *in-situ* germination assays.

*Task 2.2 Mycorrhizal fungi associated to adult orchids*

I will extract fungal DNA and analyze the sequences as described in WP 1. During this sampling, I will also collect ripe capsules of these species in order to obtain the seeds for setting the germination assays (Task 2.3).

*Task 2.3 In-situ germination assays*

I will extract the seeds from the capsules to prepare seed packets[[21]](#endnote-8). I will use plastic wraps31 to attach three seed packets per orchid species at the three heights in 10 trees per plot (720 seed packets in total) (Fig. 4b). This experiment will be monitored every three months by a member of Dr. Flanagan's lab. Eight to ten months after sowing I will harvest the seedlings (Fig. 4c) to collect protocorm fragments for mycorrhizal DNA extraction and sequencing, which will be performed as described in WP 1.



**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 2.4 Data analysis*

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 with PERMANOVA, estimate richness with rarefaction curves[[22]](#footnote-14), and turnover using Jaccard index[[23]](#footnote-15). Second, I will test whether mycorrhizal fungi communities vary over the tree trunk by building linear models controlled by the host tree species.

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

To assess turnover in mycorrhizal communities across different life stages, I will use the same study species and experimental design as in WP2. I will compare the diversity and composition of mycorrhizal fungi communities extracted from seedling and adult roots. In particular, to test the hypothesis that seedlings will have a greater diversity of mycorrhizal fungi than adults (**H3**), I will calculate mycorrhizal OTU diversity in seedlings and adults for each species, and test whether seedlings associate with a higher diversity of fungi. 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.

*Sampling*

I will use the data collected in WP2: OTUs obtained from adult orchid roots in Task 2.2, and OTUs obtained from seedling roots in Task 2.3.

Task 3.1 Data analysis

To test the hypothesis that ontogenetic partner turnover occurs through sampling effects rather than complementarity (**H4**), I will calculate OTU turnover from seedlings to adults for each species, and partition total OTU turnover into its nestedness and replacement components[[24]](#endnote-9). I expect nestedness to represent a greater proportion of OTU turnover (Fig. 1c). This result would support the general hypothesis that switching partners over ontogeny is a risky strategy.

Task 3.2 Data analysis

**WORK PLAN**

The proposal consists of three work packages (**WP**), nine milestones (**M**) and four deliverables (**D**) (*Table 1*). Ideal start date is October 1st, 2019.

**Table 1** Gantt chart showing work packages (WP), milestones (M) and deliverables (D) of the project. Blue: research packages; yellow: training; green: seminars. The chart is not exhaustive.



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*Give an overview of the bibliographical references that are relevant for your research proposal.*

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    [↑](#endnote-ref-9)