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Diversity of orchid root-associated fungi in montane forest of Southern Ecuador and impact of environmental factors on community composition

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List of abbreviations

AMF	Arbuscular Mycorrhizal Fungi/Fungus
ANOVA	Analysis of Variance
ARES	Académie de Recherche et d'Enseignement Supérieur
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CNP	Cajas National Park
DNA	Deoxyribonucleic Acid
ETAPA	Empresa Pública Municipal de Telecomunicaciones, agua potable, alcantarillado y saneamiento de Cuenca, Ecuador
HSD	Honest significance difference
ITS	Internal Transcribed Spacer
MH	Mycoheterotrophy
MUCL	Mycothèque de l'Université catholique de Louvain
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NMDS	Non-metric multidimensional scaling
nrDNA	Nuclear ribosomal deoxyribonucleic acid
OMF	Orchid Mycorrhizal Fungi
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PNP	Podocarpus National Park
PVC	Polyvinyl Chloride
Q5	Quebrada 5 (Waterfall 5)
RAF	Root Associated Fungi

SDS	Sodium Dodecyl Sulfate
T2	Transect 2
IUCN	International Union for Conservation of Nature
WCSP	World checklist of Selected Plant Families

GLOSSARY

Abiotic factor: *A non-living chemical or physical factor in the environment, such as soil, pH, forest fire, etc.* (https://www.biology-online.org/dictionary/abiotic_factor).

Biotic factor: *A factor created by a living thing or any living component within an environment in which the action of the organism affects the life of another organism* (https://www.biology-online.org/dictionary/Biotic_factor).

Co-evolution: *"the simultaneous evolution of entities and their environments, whether these entities be organisms or organizations"* (Porter, 2006).

Coexistence: Two species (or organisms) living in the same habitat in a way that one species is not eliminated by the other (Begon et al., 1999).

Colonization: The entry and propagation of one species in an area, habitat or population from which it was absent (Begon et al., 1999).

Community structure: The list of species and their relative abundance in a community (Begon et al., 1999).

Competition between species: The interaction between two organisms that fight for the same resource. Competition between two species is any interaction that affects adversely the growth and survival of two or more populations (Odum and Barret, 2006).

Competition: Interaction between two or more organisms or species. Both species suffers a reduction in birth rate or increment in mortality rate due to competition for resources (Begon et al., 1999).

Cost of mutualism: "*The metabolic and fitness costs associated with delivering benefit to a second species"* (Bever et al., 2012).

Dominant species: "*The species that predominates in an ecological community, particularly when they are most numerous*" (https://www.biology-online.org/dictionary/Dominant_species).

Endophyte: *"Either bacterial or fungal intracellular symbionts of plants that do not cause any visible signs of tissue damage or adverse effects on the host"* (Kageyama et al., 2008).

Endophytic fungi: Fungi that live in plants without causing symptoms (Brundrett, 2002).

Guild: Group of species that are assumed to exploit the same type of environmental resources in a similarly ecological relevant way (Nguyen et al., 2015).

Keystone mycorrhizal fungal: Species that have wide distribution across sites and plant species that probably favor the host local assembling and resilience (Cevallos et al., 2017).

Hotspot of biodiversity: Region irreplaceable and threatened that meets two criteria: at least 1500 endemic vascular plants and 30% loss of its primary vegetation (IUCN, 2017).

Microbial community: Groups of microorganisms that co-occur in a habitat because they are adapted to similar physical and chemical conditions (Konopka, 2009).

Mycorrhiza fungi: Specialized members of the vast population of microorganisms forming symbiosis with plants (Smith and Read, 2008).

Niche: The limits of all the important environmental characteristics with which the individuals of one species can survive, grow and reproduce (Begon et al., 1999).

Orchid mycorrhiza: Mutualistic interactions between fungi and members of the Orchidaceae including photosynthetic and achlorophyllous species (Dearnaley, 2007).

Peloton: Hyphae that form coiled masses in cells from cortical tissue (Clement, 1988).

Phylogenetic distance: The number of gene differences per locus between species or between populations within a species (Nei, 1972).

Protocorm: The germinated seed that grow into a mass of differentiated cells (Waterman and Bidartondo, 2008).

Resilience: Connotes the rate at which system functionality returns after an acute stress effect (Konopka, 2009).

Resource: Something consumed by an organism which is consequently no longer available for other organisms (Begon et al., 1999).

Root-associated fungi: Fungi that generally are considered benign intracellular inhabitants of plant roots that may control plant productivity and community assemblage (Jumpponen et al., 2017).

Saprotrophic: Organisms that have an external digestion of the death organic material and absorb the products across the cellular plasmic membrane (Begon et al., 1999).

Species richness: Number of species in a community (Begon et al., 1999).

Succession: The non-seasonal pattern, directional and continue of colonization and extinction of populations in one site or place (Begon et al., 1999).

Sympatric species: Two or more species that live in proximity (Begon et al., 1999) or occur together, which have identical or overlapping habitat ranges (Swarts and Dixon, 2009b).

SUMMARY

Context: Orchid roots harbor a wide fungal diversity, consisting mainly of species belonging to the Basidiomycota and Ascomycota phylum. These root-associated fungi (RAF) can be saprotrophs, latent pathogens or symbionts and fo the later can play important roles in the acquisition of minerals and carbon or as sources of bioactive compounds. To some extent the availability or distribution of RAF influences orchid spatial distribution and population dynamics.

Orchidaceae is one of the largest families of vascular plants. It is distributed in almost all terrestrial ecosystems with the exception of the poles. In Ecuador, the highest orchid diversity and endemism are found in the montane ecosystems. However, with the current rate of loss of diversity through natural processes or anthropogenic activities, many orchid species and probably their fungal associates (e.g. endophytes) are threatened with extinction. Orchids interact with fungi and pollinators to complete their life cycle and because of such associations, they are considered a unique model system for the study of biotic interactions and their co-evolutionary dynamics.

The studies evaluating the interaction between orchids and fungi have been focused mainly on taxonomic identification of the endophytic RAF. However, there is a knowledge gap on the abiotic or biotic factors that affect orchids and their fungal symbionts. Consequently, there is a need for data to understand the ecological implications of the ecosystem changes on the species dynamics. With the recent development and implementation of molecular tools (e.g. next generation sequencing (NGS), more in-deep ecological approaches are possible. In order to acquire new knowledge on the dynamics of orchids and fungi in tropical ecosystems, it is necessary to

identify the fungal endophytes associated with orchid roots and the environmental factors affecting their interactions with plants.

Objectives: In the present thesis, we aimed to evaluate (1) the diversity of endophytic RAF of epiphytic orchids and (2) the abiotic and biotic factors that could affect the orchid-fungi interactions. In order to fulfil these objectives, three main studies were conducted to assess (1) the effects of the orchid phylogeny and site where orchid populations were established, on the communities of endophytic RAF of *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* around the borders of the Podocarpus National Park (PNP) and *Epidendrum marsupiale* and *Cyrtochilum pardinum* in the Cajas National Park (CNP), (2) the mycorrhizal fungi communities in the co-existing orchids *C. flexuosum, C. myanthum* and *M. calantha* in the borders of the PNP, (3) the orchid mycorrhizal fungi communities associated with *Cyrtochilum retusum* and *Epidendrum macrum* along an altitudinal gradient and across a temporal variation by using trap plants systems under field conditions in the PNP.

Results and discussion: High-quality amplicons from the internal transcribed spacer 2 region (ITS2) were successfully obtained from the orchids sampled (177 adult plants and 35 seedlings). A large number of mycorrhizal and non-mycorrhizal fungi including members of Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla were detected. In the first study, we could observe in the PNP that orchid species (*C. flexuosum, C. myanthum* and *M. calantha*) and site are important factors that shape RAF communities. Conversely, in the CNP, no significant differences in RAF communities were detected between *E. marsupiale* and *C. pardinum* whatever the location site. Therefore, the possibility that the orchid phylogeny and location site of orchid distribution (abiotic and biotic factors) do not equally affect all orchid species cannot be ruled out. In the second study, we evaluated the orchid mycorrhizal fungal communities

associated with *C. flexuosum, C. myanthum* and *M. calantha*, and noticed divergent fungal communities between co-existing orchids as well as among sites/orchid populations. It is likely that different fungal patterns associated with inhabiting orchids represent a strategy to reduce niche competition. Moreover, the distinct fungal communities associated with the different orchid populations could be due to the particular environmental conditions of each study site. Despite the distinct mycorrhizal fungi communities observed, there was a set of mycorrhizal fungi overlapping among orchid populations. Finally, in the third study, conducted under field conditions with trap plants, we showed that orchid phylogenetic history, altitude and temporal variation influence the structure of the fungal endophytic communities. We demonstrated that mycorrhizal fungi are more widely distributed than orchids and that the environmental conditions prevailing at the sites where the orchids were established shape the interaction between orchid and fungi. Moreover, we noticed that a number of fungal species were present in almost all orchids and populations, suggesting a co-evolutionary process. Finally, we observed that mycorrhizal fungi richness did not decrease at higher altitudinal levels.

Conclusion: Our results suggests that epiphytic orchids in the PNP present differences in community structure of endophytic RAF that depend on abiotic and biotic factors such as host phylogeny, site, altitudinal levels or temporal variation. Contrastingly, endophytic RAF of orchids at CNP seem to harbor more homogenous communities. The endophytic RAF identified includes mycorrhizal and non-mycorrhizal fungi putatively classified as symbionts, saprophytes or pathogens. The potential benefits of the non-mycorrhizal fungi for orchid life remain unresolved. The interaction with multiple fungi could favor orchid site occurrence and co-existence among several orchid species. Additionally, our results suggest that populations of *C. flexuosum, C. myanthum* and *M. calantha* have site-adjusted mycorrhizal

communities structured around keystone fungal species. The combined use of field and laboratory approaches undoubtedly contributed to the understanding of the interactions that occurred between orchids and fungi, generating stronger ecological premises regarding to the environmental factors influencing such association and consequently in species conservation.

RESUMEN

Contexto: Las raíces de las orquídeas albergan una amplia diversidad de hongos, que consiste principalmente de especies que pertenecen a los filum Basidiomycota y Ascomycota. Los hongos asociados a raíces (RAF por sus siglas en inglés) pueden ser saprótrofos, patógenos latentes o simbiontes y pueden tener roles importantes para la adquisición de carbono o nutrientes, pero también pueden ser fuentes de compuestos bioactivos. Actualmente se debate si la disponibilidad y distribución de los RAF pueden influir en la distribución y dinámica de las poblaciones de orquídeas.

Orchidaceae es una de las familias de plantas vasculares con mayor número de espcies a nivel mundial. Las orquídeas son plantas cosmopolitas que están establecidas en casi todos los ecosistemas terrestres, con excepción de los polos. En Ecuador, es en los ecosistemas de montaña donde se encuentra concentrada la mayor diversidad y endemismo de orquídeas. Sin embargo, con la tasa actual de perdida de diversidad debido a procesos naturales o por actividades antrópicas, muchas especies de orquídeas están en peligro de extinción y probablemente también sus hongos endófitos asociados. Las orquídeas interactúan con hongos y polinizadores para completar su ciclo de vida, debido a las asociaciones que forman y estos son considerados como sistemas modelo para el estudio de las interacciones bióticas y su dinámica co-evolutiva.

Los estudios sobre la interacción entre las orquídeas y los hongos, se han enfocado principalmente en la identificación taxonómica de endófitos RAF. Existe un vacío en el conocimiento sobre los factores abióticos o bióticos que afectan la simbiosis orquídea-hongo. Consecuentemente, es necesario obtener datos para una mejor comprensión de las implicaciones ecológicas de los cambios en los ecosistemas en la dinámica de las especies.

Con el desarrollo e implementación de la secuenciación de nueva generación, es posible desarrollar estudios ecológicos más completos y fiables. Para adquirir nuevo conocimiento en la dinámica de las orquídeas y los hongos en ecosistemas tropicales, es necesario identificar los hongos endófitos asociados con raíces de orquídeas, así como también evaluar los factores ambientales que afectan su interacción con plantas.

Objetivos: En esta tesis, el objetivo fue evaluar (1) la diversidad de endófitos RAF de orquídeas epífitas y (2) los factores abióticos y bióticos que pueden afectar las interacciones orquídea-hongo. Para llevar a cabo estos objetivos, tres estudios se desarrollaron para evaluar (1) el efecto de la filogenia de la orquídea y del sitio donde las poblaciones de orquídeas se establecieron, en las comunidades de endófitos de *C. flexuosum, C. myanthum* y *M. calantha*, en áreas aledañas al PNP y *Epidendrum marsupiale* y *Cyrtochilum pardinum* en el CNP, (2) los hongos micorrízicos de las orquídeas coexistentes *C. flexuosum*, *C. myanthum* y *M. calantha*, en áreas aledañas al PNP, y (3) las comunidades de hongos micorrízicos asociados a *C. retusum* y *E. macrum* a lo largo de un gradiente altitudinal y a través de una variación temporal, usando sistemas de plantas trampa en condiciones de campo en el PNP.

Resultados y discusión: De todas las orquídeas muestreadas (177 plantas adultas y 35 plántulas trampa), se obtuvo amplicones de alta calidad de la región *internal transcribed spacer* (ITS2). Se identificó hongos micorrízicos y no micorrízicos, incluyendo miembros de los filum Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota y Zygomycota. En el primer estudio, observamos que en el PNP las especies de orquídeas (*C. flexuosum, C. myanthum* y *M. calantha*) y sitios son importantes factores que moldean la composición de las comunidades fúngicas. Contrariamente, en el CNP, no se detectó diferencias significativas en las comunidades de endófitos entre *E. marsupiale* y *C. pardinum* a pesar

de la ubicación. Existe la posibilidad de que la filogenia de las orquídeas y el sitio de ubicación de las orquídeas (factores abióticos y bióticos), no afecten de la misma manera todas las especies de orquídeas. En el segundo estudio, nosotros evaluamos las comunidades de hongos micorrízicos associadas a *C. flexuosum, C. myanthum* y *M. calantha*, y notamos diferentes comunidades fúngicas entre las orquídeas co-existentes y entre sitios/poblaciones de orquídeas. Además, las distintas comunidades de hongos micobiontes asociadas a las diferentes poblaciones de orquídeas pueden deberse a las condiciones ambientales particulares en cada sitio de estudio. A pesar de que se observó distintas comunidades de hongos micorrízicos, hubo un set de hongos micorrízicos traslapado en todas las poblaciones de orquídeas. Finalmente, en el tercer estudio, llevado a cabo en condiciones de campo usando plántulas trampa, nosotros revelamos que la historia filogenética de las orquídeas, la altitud y variaciones temporales influyen en la estructura de las comunidades de hongos endófitos. Se demostró que los hongos micorrízicos están más ampliamente distribuidos que las orquídeas y que las condiciones ambientales prevalentes en cada sitio donde las orquídeas se establecen pueden influir en la interacción entre hongos y orquideas. También notamos que algunos hongos están presentes en casi todas las orquídeas y poblaciones, lo que sugiere un proceso co-evolutivo entre orquídeas y hongos. Finalmente, observamos que la riqueza de los hongos micorrízicos de orquídeas no decrece a mayor altitud.

Conclusiones: Nuestros resultados sugieren que las orquídeas epífitas en el PNP tienen diferentes estructuras de las comunidades de los RAF endófitos, que dependen de factores abióticos y bióticos como la filogenia del huésped, el sitio, el nivel altitudinal y la variación temporal. Contrariamente, RAF de orquídeas del CNP albergan comunidades fúngicas más similares. Los RAF endófitos identificados, incluyen micorrizas y no micorrizas que han sido clasificados como presuntos simbiontes, saprófitos o

patógenos. No se puede descartar los beneficios potenciales que los hongos no micorrízicos tienen en la vida de las orquídeas. Es posible que la interacción con varios hongos favorezca la ocurrencia de las orquídeas en los sitios de estudio y también la coexistencia de varias especies de orquídeas. Adicionalmente, nuestros resultados sugieren que las poblaciones de *C. flexuosum, C. myanthum* y *M. calantha* tienen comunidades de hongos micorrízicos ajustadas de acuerdo al sitio, es decir que se estructuran alrededor de un núcleo de hongos que es contante en todas las poblaciones de orquídeas. Sin duda, el uso combinado de estudios de campo y de laboratorio contribuye al entendimiento de las interacciones que ocurren entre orquídeas y hongos, generando importantes premisas ecológicas sobre los factores ambientales que influyen en dicha asociación y consecuentemente en la conservación de las especies.

Outline of the thesis

Interactions between orchids and fungi are critical for the plant life cycle. Relationships with fungi can influence the orchid's ability to colonize and settle in a particular site. Therefore, to some extent, the interactions with fungi could explain the current orchid distribution. In Ecuador, orchids are found in almost all habitats from 0 to 4500 m a.s.l. and most of the endemic species are distributed across montane ecosystems. However, the environmental degradation, resulting from anthropogenic activities, is threatened the survival of orchid species and possibly the community stability of the endophytic species with which they interact. To understand the principles governing the symbiotic association between orchids and fungi, it is necessary to evaluate the orchid mycorrhizal fungi (OMF) diversity and the environmental factors that determine such relationship. However, there is limited information about how orchids and their fungal associates are interacting in tropical ecosystems, although these areas are recognized as biodiversity hotspot.

In this thesis, we aimed to evaluate the richness and diversity of endophytic root-associated fungal (RAF) and to elucidate the potential abiotic and biotic factors impacting the composition of orchid-fungi symbiosis. In order to address these objectives, three studies were conducted. In the first study, the objective was to assess the composition of endophytic RAF communities in *Cyrtochilum flexuosum, C. myanthum, Maxillaria calantha, Epidendrum marsupiale* and *C. pardinum* in function of the host phylogeny and the site, as main factors shaping the community composition. In the second study, the objective was to evaluate the diversity of mycorrhizal fungi associated with *C. flexuosum, C. myanthum* and *M. calantha* at two sites of montane rainforest. In the third study, the objective was to evaluate the patterns of mycorrhizal fungi communities associated with *E. macrum* and *C. retusum* along an altitudinal gradient and a temporal

scale. Studies in Chapters I and II were based on the sampling of natural orchid populations and the study in Chapter III was conducted using field-trap plant experiments with *in vitro*-produced orchid plantlets (Fig. 1).

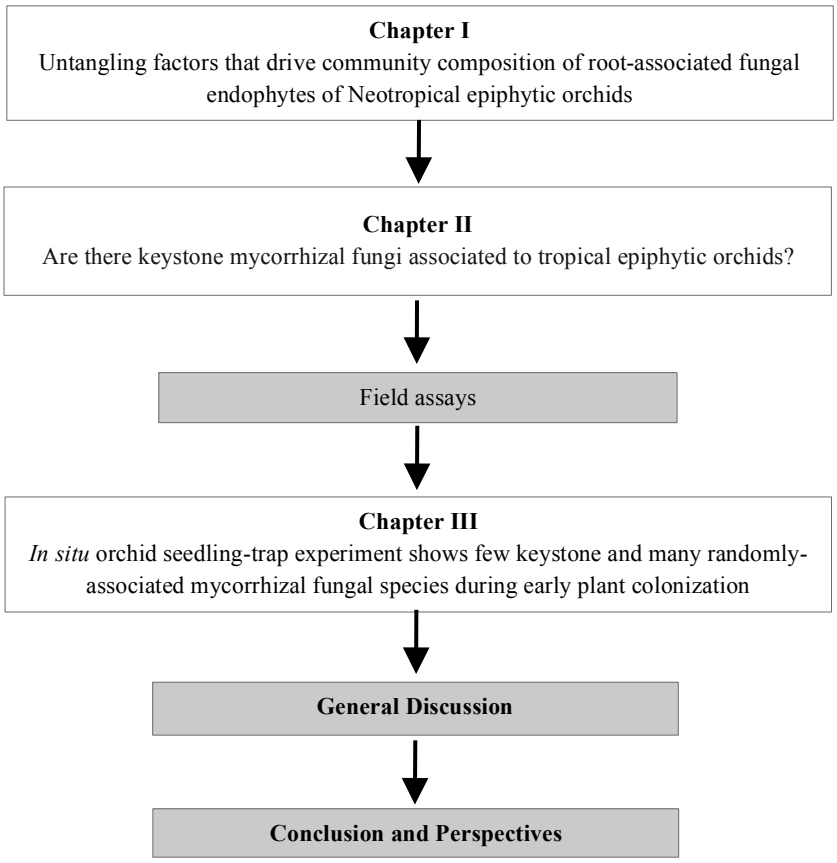

Fig. 1 Outline of the thesis

In the **INTRODUCTION** section, we presented the topic and the objectives of our research. In the **STATE OF THE ART** section, we reviewed the current literature on the endophytic RAF basic concepts as well as the orchid-mycobiont's ecological assemblage. We also reviewed the potential impact of environmental factors (e.g. site, altitude) on the composition of fungal endophytes communities and their implications for orchid occurrence. The **MATERIALS AND METHODS** section described

the study sites, the orchid species evaluated, the experimental details and analyses conducted throughout the thesis.

In the **RESULTS** section, the three objectives mention above where addressed in three successive chapters:

In **Chapter I**, five epiphytic orchids (three from PNP and two from CNP), were evaluated for their endophytic RAF composition and abiotic and biotic factors as potential factors shaping fungal community composition, using Illumina MiSeq. We found that orchid species at PNP had different endophytic RAF, determined by the host evolution and the site of sampling. Contrastingly, orchid species at CNP exhibited similar fungal endophyte communities independently of the orchid species or the site of orchid occurrence.

This work was published in "Fungal Ecology" (2018).

In **Chapter II**, the objective was to evaluate the diversity of orchid mycorrhizal fungi (OMF) associated with three epiphytic orchids at the two sites in the border of PNP. Results showed a polyphyletic group of mycorrhizal fungi. It was suggested that there is an effect of orchid host and sites on the composition of OMF communities. Co-existing orchids displayed a distinctive fungi association, but the phylogenetically closest orchids have more similar mycorrhizal communities. Orchid species was not a determining factor in the composition of mycorrhizal communities. Niche partitioning or micro-environmental conditions at each site could be the reason for the observed results.

This work was published in "Mycorrhiza" (2017).

In **Chapter III**, the objective was to evaluate the effect of altitudinal level, host phylogeny, site and temporal variation on the composition of OMF

community under field conditions using trap plants. The results suggested that host phylogeny, altitudinal levels, and temporal variation were determining factors to shape the composition of endophytic communities. The results also revealed that between sites, the OMF communities were no significantly different.

This work was submitted to Frontiers in Plant Science

The results of the three chapters were discussed in the **GENERAL DISCUSSION** section. In the **CONCLUSION AND PERSPECTIVES** section, the most remarkable ecological inferences and their potential implication for endophytic RAF conservation were considered.

Author's contribution

The work presented here was strictly realized during the time course of my PhD.

The sections: **Introduction, Context of the Study, General Discussion, Conclusion** and **Perspectives** were written by myself and have not been published elsewhere.

Chapter I is a research paper published in the journal Fungal Ecology. My contribution to this chapter was estimated to 70%. The orchid roots sampling in the Cajas National Park (CNP), Azuay province was done by Paulo Herrera Vargas, PhD. student at Rey Juan Carlos University (Spain). The sampling of orchid roots in the Podocarpus National Park (PNP), Zamora-Chinchipe province was done by myself. The molecular analyses were done by Paulo Herrera Vargas and myself. The bioinformatics and statistical analyses were performed by myself. The draft of the manuscript was done by myself and all the authors read and approved the manuscript.

Chapter II is a research paper published in the journal Mycorrhiza in 2017 (Cevallos et al., 2017). My contribution to this chapter was estimated to 70%. The orchid roots sampling, microscopic analyses and molecular work were performed by myself. The subsequently bioinformatics and statistical analyses were performed with the help of Prof. Aminael Sánchez-Rodríguez and Prof. Juan Pablo Suárez (UTPL, Ecuador). The writing of the paper was conducted by myself and all the authors read and approved the manuscript.

Chapter III is a research paper submitted to Frontiers in Plant Science. My contribution to this chapter was approximately 80%. Prof. Juan Pablo Suárez and me designed the experiment and conducted the fieldwork. Molecular and statistical analyses were conducted by myself. All co-authors wrote the manuscript.

I. INTRODUCTION

Diverse sets of fungal species with distinct ecological attributes such as saprotrophs, pathogens or symbionts have been identified in association with orchid roots (Kohout et al., 2013; Ma et al., 2015). It has been reported that members of Basidiomycota, Ascomycota (Dearnaley et al., 2012; Xing et al., 2015), Chytridiomycota, Glomeromycota and Zygomycota (Ma et al., 2015) are associated with terrestrial, epiphytic or lithophytic orchids. Fungi associated with orchid roots are assumed to be free-living organisms, capable of independent growth, meaning in the absence of orchids (McCormick and Jacquemyn, 2014). Although, recently it has been observed that fungal endophytes are abundant in close proximity to orchids (McCormick et al., 2018). In general, those fungi can adopt different life styles, from pathogen to saprotroph, non-mycorrhiza endophyte or mycorrhizal (see Pecoraro et al., 2013; Kohout et al., 2013). According to such ecological plasticity, many of these fungi are widely distributed even in harsh environments (Jacquemyn et al., 2012a).

Studies on endophytic RAF in orchids have included isolation-dependent and isolation-independent approaches (Kohout et al., 2013). Earlier investigations based on isolation-dependent methods have used taxonomic characters of OMF to define species (Warcup and Talbot, 1967, 1971, 1980). However, distinctive structures that allow appropriate taxonomic resolution are not always available for all the fungal species (Cruz et al., 2011). With the development of molecular techniques (isolation-independent methods), it is nowadays possible to identify a broader fungal diversity at a better systematic resolution (Selosse, 2014). In fact, the inclusion of next generation sequencing (NGS) technologies for mycological studies has facilitated ecological studies on RAF in orchid species (e.g. Esposito et al., 2016; Jacquemyn et al., 2016a).

Orchidaceae is considered as one of the largest and diverse families of flowering plants, which comprises above 27801 species (The Plant List,

2013). Orchids are highly valued in horticulture (Hew and Yong, 2004), food industries (Ordoñez et al., 2012) and even as medicinal plants (Gutiérrez, 2010). Furthermore, orchids have tangible benefits in water purification and as bioindicators of ecosystem health. Moreover, it is the intricacy of ecological associations that makes orchids optimal models to develop conservation strategies (Waterman and Bidartondo, 2008; Swarts and Dixon, 2009a).

More than any other group of plants, Orchidaceae are recognized as a family with unique strategies for pollination and nutrition, involving interactions with other organisms (Roberts and Dixon, 2008; Bronstein et al., 2014). The first group of organisms involves pollinators that favour sexual reproduction and the second group are root fungi that support early stages of orchid development. Both groups bring clear benefit to the orchids (Fray et al., 2015). While orchids need pollinators for a particular life stage, the association with fungal endophytes may occur from germination to the adult stages (Smith and Read, 2008, Swarts et al., 2010). Therefore, the availability of appropriate fungi can affect orchid fitness, more specifically their distribution and abundance (McCormick and Jacquemyn, 2014).

According to the red list of the International Union for Conservation of Nature (IUCN), Orchidaceae is the plant family with more species registered as in danger. The causes of orchid population decline and extinction are intrinsic factors such as natural biotic processes (e.g. demographic phenomena) and extrinsic factors, most frequently related with anthropogenic activities such as overexploitation, habitat loss and fragmentation, changes in abiotic conditions and the introduction of disease (Swarts and Dixon, 2009b). While intrinsic factors are part of natural dynamics in ecosystems, the extrinsic factors accelerate environmental changes and their impact on ecological interactions are thus more marked (Bailarote et al., 2012). It has been suggested that the study of the diversity

of orchids could give precise information on the current state of the environment and disturbances around the world (Bergman et al., 2006).

In Ecuador, the study of orchid endophytic fungi started only a decade ago with the pioneer study of Suárez et al. (2006). This study was followed by a number of other studies improving our understanding of the diversity of OMF in Ecuador and it is expected and even higher species richness in this hotspot of biodiversity (Herrera et al., 2010, 2018; Riofrío et al., 2013; Novotná et al., 2018; Suárez et al., 2008, 2016). In addition to the fungi richness description, some ecological inferences have been addressed, such as, the definition of ecological roles for some fungal endophyte groups, the determination of fungal dominant groups and orchid-fungi specificity (see Herrera et al., 2010; Kottke et al., 2010; Suárez et al., 2006, 2008, 2016). However, in Ecuador a large number of orchid species have never been studied for their fungal endophyte diversity. According to Rodriguez et al. (2009) fungi are key parts of the ecosystems, so the study of their biogeography and ecology could contribute to understand how mycorrhizal fungal communities and their host orchids are structured. The evaluation of abiotic and biotic factors affecting fungal endophyte distribution seems to be the best way to reach more accurate understanding of fungi biogeography and ecology (see Jacquemyn et al., 2016a; Oja et al., 2015; Mujica et al., 2016), thus helping in clarifying how the interactions between orchid and fungi are structured.

In this thesis, the general objective was to assess the diversity and community composition of endophytic RAF in epiphytic orchids and the effect of factors such as host phylogeny, site, altitude and temporal variation on the structure of fungal communities. Several studies support the fact that host phylogeny is a determining abiotic factor for the composition of endophytic RAF communities of terrestrial orchids (Jacquemyn et al., 2014; Pellegrino et al., 2014), however there is no information about the effect of

this factor on RAF communities of epiphytic orchids. Likewise, site, altitude and temporal variation have been recognized as abiotic factors that could shape plant-fungi interaction but such investigations evaluated the factors in a single or are few areas or orchid populations (Garnica et al., 2013; Geml et al., 2014; Jacquemyn et al., 2016a; Ma et al., 2015).

In order to understand the diversity and ecological dynamics between orchids and fungi, we first identified the whole diversity of RAF of *C. flexuosum, C. myanthum*, *M. calantha, Epidendrum marsupiale* and *C. pardinum* to evaluate the host phylogeny and orchid populations as potential factors affecting the fungal endophytes communities' composition. The OMF associated with *C. flexuosum, C. myanthum* and *M. calantha* were subsequently studied in function of abiotic and biotic factors. Finally, we determined the effect of altitudinal level, host phylogeny, site and temporal variation on OMF communities associated with *C. retusum* and *E. macrum,* through field experiments.

II. STATE OF THE ART

1. Orchid root-associated fungi

After the pioneer work on symbiotic germination (germination supported by mycobionts) by Bernard at the end of the 18th century, the orchid-fungi interaction has attracted the interest of the scientific community worldwide (Selosse et al., 2011). Nowadays, it is well known that under natural conditions, orchids establish particular interactions with fungi, through the colonization of the internal tissues of seeds or roots (Smith and Read, 2008). These interactions have important effects on the ecology, fitness, diversity and community structure of orchids (Brundrett, 2006). The fungi can supply carbon and inorganic nutrients to their host plants (Rasmussen, 1995) and are able to produce active compounds that confer protection against the attack of pathogens and herbivores (Ma et al., 2015).

Endophytic root-associated fungi (RAF) that are important for the industry (e.g. horticulture, fragrance, food) or with medicinal properties have been deeply studied (Ovando et al., 2005). In the recent years, around 200 orchid genera have been investigated to evaluate their RAF (Ma et al., 2015). Members of the phyla Basidiomycota, Ascomycota, Glomeromycota, Chytridiomycota and Zygomycota have been reported worldwide and according to their roles on orchid life cycle two groups of fungi were distinguished; the mycorrhizal and non-mycorrhizal ones (Dearnaley et al., 2012; Ma et al., 2015).

Some orchid endophytic RAF are assumed to grow independently of their host (McCormick and Jacquemyn, 2014). Consequently, fungi are more widely distributed than their orchid partners (Dearnaley et al., 2012; Oberwinkler et al., 2017). In the recent years, particular attention has been addressed to the role of abiotic and biotic factors on RAF diversity and composition in order to understand how fungal communities are structured at spatial and temporal scale (McCormick and Jacquemyn, 2014).

1.1 Colonization process

Root colonization by an orchid-fungus can occur at any development stage of the plant. However, orchids are crucially dependent on fungi at early development stages and such association can persist until adult stage, according to the distinctive nutritional demands of the orchids (autotrophic, mycoheterotrophic and mixotrophic) (Cameron et al., 2007; Rasmussen and Rasmussen, 2009). At early development stages, the seed germination is depending on the fungal colonization through the trichomes and suspensor cells (Fig. 2) (Smith and Read, 2008), while at protocorm to adult stages, the colonization occurs through the root hairs/velamen and further cortical cells (Clements, 1988).

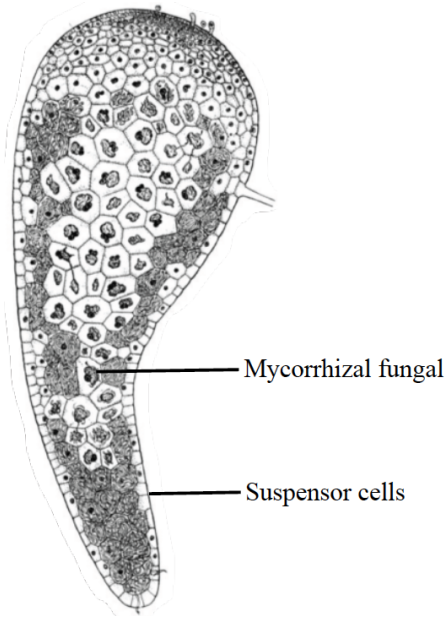

Fig. 2 Schematic representation of a protocorm colonized (Bernard, 1909).

The symbiosis is characterized by the production of coiled masses of fungal hyphae inside the cortical cells that are termed pelotons (Fig. 3) (Clements, 1988; Rasmussen, 1995; Brundrett et al., 2004). After colonization of the seeds, the formation of pelotons induces an increasing

meristematic activity, which triggers the development of the seed into a protocorm (Rasmussen, 1995). In achlorophyllous and some chlorophyllous orchids, the plants continue receiving organic and inorganic nutrients from the fungi (Dearnaley et al., 2012).

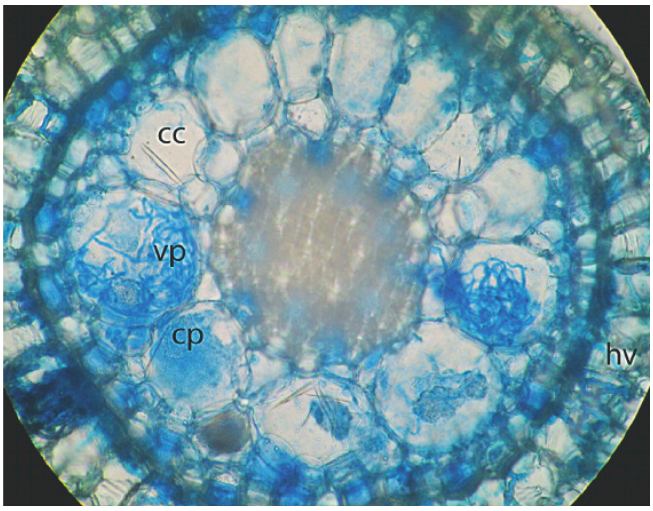

Fig. 3 Transversal section of a mycorrhizal *Stelis concinna* root. Vital pelotons (vp), collapsed pelotons (cp), cortical cells (cc), hyphae in velamen (hv) (from Suárez et al., 2009).

1.2 Fungal specificity

A long-standing question in studies of RAF has been the degree of specificity between orchids and fungi (Otero et al., 2007; Pecoraro et al., 2017). Multiples studies based on *in vitro* and *in situ* experiments have demonstrated that some specificity exist in the orchid-fungi interaction (Perkins and McGee, 1995; Bidartondo and Read, 2008), which could have implications on the distribution of orchid species and community structure. The level of specificity seems to be variable across orchid species (Barrett et al., 2010), habitats (Xing et al., 2013) and along orchid life cycle (Zettler et al., 2003; Herrera et al., 2017).

Studies in natural orchid populations showed that the variation in fungal communities between orchid species not always follow the same tendency. For example: distinctive fungal communities were found between species from the genus *Cephalanthera* (Pecoraro et al., 2017) and *Dendrobium* (Xing et al., 2013) but *Neottia* spp. exhibited preference for Sebacinales fungi (Těšitelová et al., 2015). Different degrees of specificity were also observed along the habitats of orchids. It has been shown that mediterranean orchid species exhibit low specificity for their fungal symbionts (Pellegrino et al., 2014), while tropical orchids like *Liparis japonica* showed high fungal specificity (Ding et al., 2014). Distinctive patterns of fungal specificity have also been observed at different stages of orchid development, for instance the seedlings versus adult plants of *Epipactis* species (Tešitelová et al., 2012).

2. Orchid mycorrhizal fungi

Mycorrhizal fungi have important functions in orchids. They are critical for seed germination and subsequently orchid growth (Rasmussen, 1995), but orchid seeds need to be associated with the appropriate fungi that provide the carbohydrates required to complete the germination (Rasmussen and Rasmussen, 2009). They also provide the plant with minerals and water (Yoder et al., 2000) through the hyphal coils (pelotons) (Smith and Read, 2008) and this process may continue until adulthood (Rasmussen, 2002).

Most fungi forming mycorrhizal associations belong to the Basidiomycetes phylum (Dearnaley et al., 2012). The first classifications of orchid mycorrhizal fungi determined a superficial group called *Rhizoctonia,* which included Sebacinaceae, Tulasnellales and Ceratobasidiales (Rasmussen, 1995; Roberts, 1999). It is widely accepted that the fungi from *Rhizoctonia* group are able to associate with other plants but also can switch

their ecological roles: from orchid mycorrhizal to saprotroph (Bidartondo et al., 2004).

3. Orchid non-mycorrhizal fungi

Non-mycorrhizal fungi refer to the fungi that colonize orchid's root tissues at some time in the plant's life, without forming any characteristic structure like the pelotons, but that do not cause pathogenic symptoms to the host (Kohout et al., 2015). Numerous non-mycorrhizal fungi have been associated to orchids. For instance, Ma et al. (2015) noticed the presence of over 110 fungal genera from the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla in orchids roots. Interestingly, some of the non-mycorrhizal fungi have no phylogenetic affinities with known mycorrhizal taxa (Stark et al., 2009). The non-mycorrhizal fungal species in free life (e.g. without orchids) could be found as pathogens, saprotrophs, parasites or symbionts of other plants (Dearnaley et al., 2012; Ma et al., 2015). Because of their ecological plasticity, non-mycorrhizal fungi have a wide distribution (Sudheep and Sridhar, 2012). In terms of dominant taxa, species from Xylariales are the major non-mycorrhizal fungi associated with tropical orchids (Yuan et al., 2009) and at temperate ecosystems, members of Helotiales dominate non-mycorrhizal communities (Kohout et al., 2013).

The evidence currently available about the evolutionary processes suggests that non-mycorrhizal fungi can have relevant roles on orchid life (Ma et al., 2015). It is widely recognized that non-mycorrhizal fungi produce bioactive compounds that potentially could favor the plant resistance to abiotic stresses, facilitating the plant adaptability to several environmental conditions (Ma et al., 2013). In fact, several fungi that have been screened for commercial purposes contributed important knowledge to clarify the roles of such non-mycorrhizal fungi (e.g. Vaz et al., 2009).

The community composition of non-mycorrhizal fungi has been studied in various orchid species and in highly diverse habitats but there is a lack of information on the environmental factors (e.g. climate, geography) that shape the structure of the communities (Kohout et al., 2013). The identification of non-mycorrhizal fungi using morphological and molecular approaches represents the baseline to understand the ecological dynamics between orchids and fungi. The first studies of non-mycorrhizal fungi were traditional performed using only culture-dependent methods and description of morphological characters (Ko Ko et al., 2011). However, isolation-dependent techniques fail for taxonomic placement for non-cultivable fungi or for fungi without sexual stages (e.g. spores, basidia) under *in vitro* conditions (Hyde and Soytong, 2008). The implementation of molecular tools in the recent decade has favored the capture of a wider range of non-mycorrhizal fungi that are not possible to detect using culture-dependent methods (Ko Ko et al., 2011). These days, the combination of morphological and molecular characterization in studies of non-mycorrhizal fungi is highly recommended (see Chutima et al., 2011; Jiang et al., 2011) to reach more accurate and comparative results (e.g. UNITE or GenBank) (Ma et al., 2015).

4. Orchidaceae family

Orchidaceae comprise 899 genera and 27801 species, representing about 10% of the Angiosperms diversity worldwide (The Plant List, 2013). Orchids are considered a cosmopolitan group, adapted to almost all types of ecosystem except the extreme deserts, glaciers and poles (Dressler, 1993). Consistent with reports that shown that the widest worldwide diversity is concentrated in Neotropical regions (Myers et al., 2000), especially in montane regions (Brundrett et al., 2001).

Orchids are unique with particular morphological attributes and biotic interactions. For instance, they produce dust-like seeds, have modified petals and have developed specific interactions with pollinators and mycorrhizal fungi for completing their life cycle (Arditti and Ghani, 2000; Roberts and Dixon, 2008). The peculiar structural adaptations are thought to have appeared as co-evolutionary processes with other organisms that to some extent favored the orchid diversification (Crane et al., 1995; Selosse, 2014). The one interaction formed with pollinators favor orchid reproduction (Fig. 4a) and the second one takes part with some fungal taxon to promote the orchid germination (Fig. 4b) and seedling establishment (Brundrett, 2002; Smith and Read, 2008; Waterman et al., 2011).

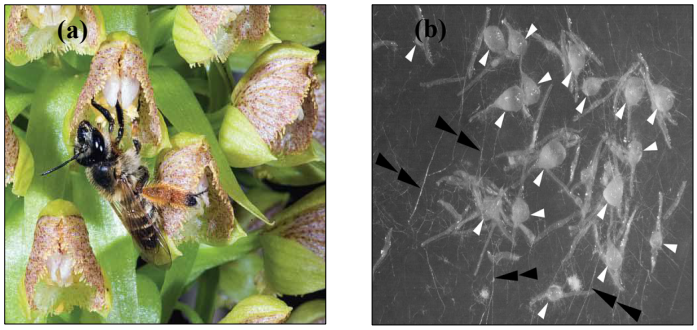

Fig. 4 Orchid interactions. (a) *Pterygodium magnum* attracts bees to support the pollination (from Waterman et al., 2011) and (b) protocorms of *Cremastra appendiculata* (white arrowheads) in symbiotic association with a mycobiont (black double arrowheads) (from Yagame et al., 2013).

The attraction of orchids in both Western and Eastern cultures has mainly been attributed to their exceptional beauty. Orchids are also valued in industries such as fragrance, horticulture, pharmacy or food (Roberts and Dixon, 2008). In 2000, the demand for orchids was estimated to 1598 million units of plants that in five years increased in 19% (Table 1). Although there is no information available of the current demand of orchids, companies selling orchids are present worldwide and the gross value generated contributes substantially to the economy of different countries

(Hew and Yong, 2004). At large-scale, the production of orchids is limited to a few numbers of orchid species with high economic value (Hew and Yong, 2004).

Table 1. World demand for orchids, estimated of the year 1995 and 2000 (adapted from Hew and Yong, 2004).

	Plant stock turnover (Million units)			Total estimated sales in 5 years (Millions of US$)
	1995	2000	Change in percentage	
Planting materials for cut-flower production	66	109	Increased by 39%	170
Planting material for potted plants	1220	1489	Increased by 18%	1891
Total	1286	1598	Increased by 19.5%	2061

4.1 Orchid diversity in Ecuador and the threats

In Ecuador, orchids are the major family of vascular plants with 3630 species identified (Jørgensen et al., 2011). According to the IUCN, 74% of orchid diversity was considered vulnerable and in Ecuador 11% was endangered or in critical danger already at the beginning of the century (Valencia, 2000). At present time, there is no quantitative information on the percentage of orchid species that are threatened, but it is generally admitted that there is a rapid loss of diversity (Mendoza, 2013). Orchids are distributed at all altitudinal levels, from 0 to 4500 m a.s.l. but most of the endemic orchids are hosted in mountain ecosystems between 1500-3000 m a.s.l. (Endara et al., 2009).

The exceptional beauty of orchids makes several species highly demanded worldwide for decorative purposes. The orchid trade has thus become an international activity (Simpson, 2006). In addition to trade, the

multiple human activities have caused disturbance or loss of natural ecosystems affecting as well orchid diversity (Dodson, 2002). For instance, the illegal collection of orchids is a major threat for the survival of endemic or native wild populations, which are exacerbated by the absence of quantitative information (e.g. the volume of orchids that are extracted each year) (Dodson and Escobar, 1993). It could be expected that orchid species with particular environmental requirements for their life cycle are prone to be affected by unexpected disturbances in their environment (Dodson and Escobar, 1993).

5. Classification of orchids

For centuries, the classification of orchid species has been based on their morphological characteristics (Rasmussen, 1999). However, some species have similar morphologies with convergent characteristics that lead to ambiguous classifications (see Hágsater et al., 2005). Currently, there are five recognized subfamilies: Apostasioideae, Cypripedioideae, Vanilloideae, Orchidoideae and Epidendriodeae (Fig. 5) (Hágsater et al., 2005; Pridgeon, 2009).

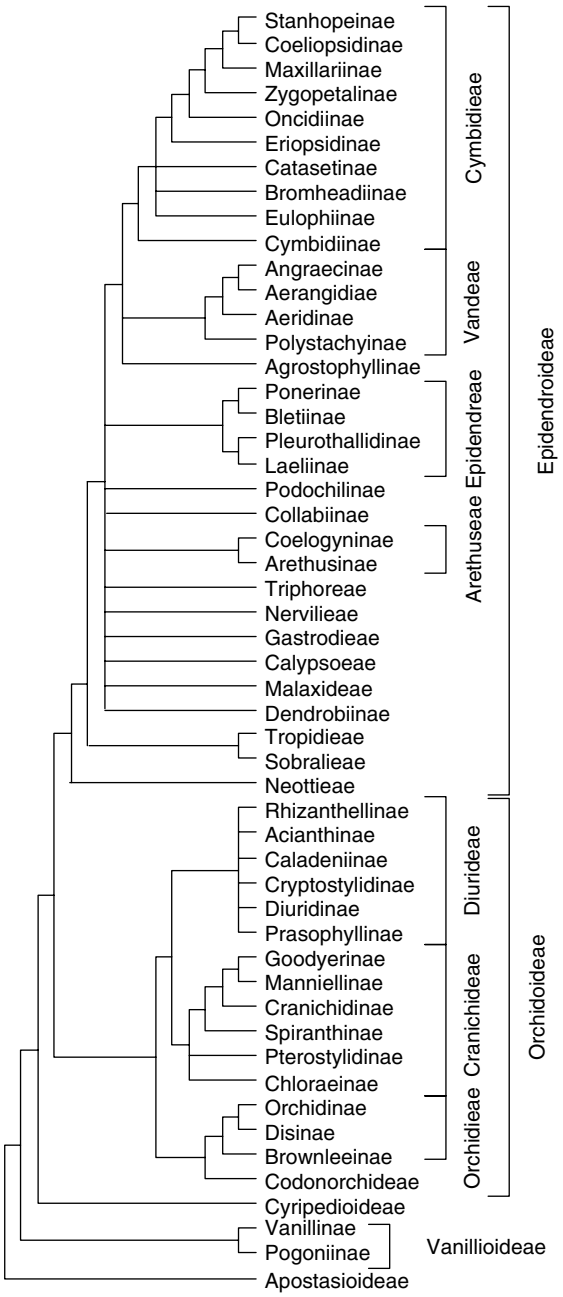

Fig. 5 Cladogram of orchid subfamilies relationships (from Chase et al., 2003).

According to the orchid lifestyle, species have been grouped in (1) terrestrial orchids growing on the soil, (2) epiphytic orchids growing under the canopy of trees on plant surfaces or in decaying wood and (3) lithophytic orchids growing on rock (Dearnaley et al., 2012).

Another classification based on the orchid's carbon nutrition at adult stage included three groups, (1) autotrophic, (2) mycoheterotrophic and (3) mixotrophic (Dearnaley et al., 2012). The autotrophic orchid species rely on fungi at the early stages of their development and their dependence decrease with the development of photosynthetic functions at the older stages. The mycoheterotrophic orchids are completely dependent on the carbon provided by the fungi throughout their life cycle. The mixotrophic orchids are able to obtain carbon compounds from photosynthesis as well as from the fungi, across their life cycle (Selosse and Roy, 2009).

6. Root-associated fungi and orchids are exceptional to study plant-microbial interactions

Orchids represent an essential element of the tropical diversity because of their distinguishing morphological (e.g. flower, seeds) and ecological (e.g. biotic interactions with pollinators and fungi) characteristics (Roberts and Dixon, 2008). In Ecuador, the orchid diversity recognized until now (3630 species) represent about 5.3% of the worldwide orchid diversity and one third is endemic (Suárez and Kottke, 2016). This endemism reaches its zenith in the mountain ecosystems between 1500 – 3000 m a.s.l. (Sierra, 1999). Moreover, it has been reported that from the entire orchid diversity, epiphytic species are dominant (Dodson, 1999).

Epiphytic orchids have specific roles in the functioning of the canopy ecosystem as part of the nutrient and water cycle (Nadkarni et al., 2004). Because of such specific characteristics, epiphytic orchids are

sensitive to environmental changes, caused by natural process or anthropogenic disturbances. They are thus considered as good indicators for changes in the ecosystem (Christenson, 2003).

Although, epiphytic orchid have been widely studied worldwide as important elements of the canopy, the particular interactions that occur with a diverse group of root-associated fungi are not fully understood. In natural conditions, orchids rely on a specific set of fungi for successful germination and seedling establishment (Swarts et al., 2010).

Thus, the interaction between epiphytic orchid and their RAF, represent to some extent a model system to assess the ecological interactions between plant and fungi but also to evaluate the health of ecosystems (Newman, 2009; Martos et al., 2012; Bronstein et al., 2014; Fay et al., 2015).

7. Environmental factors that could affect root-associated fungal communities in orchids

The composition of fungal communities associated with orchid roots has been evaluated in different temperate and tropical ecosystems. Several orchids such as *Epidendrum* spp. (Riofrío et al., 2013), *Orchis* spp. (Jacquemyn et al., 2012) or *Stelis* spp. (Herrera et al., 2010) have been analysed thoroughly. However, there is little information about the factors that could shape the composition of such fungal communities (Bunch et al., 2013).

Some efforts have been made to evaluate different abiotic and biotic factor such as site, orchid species, temporal patterns and orchid development stage. For instance, temporal patterns of RAF community composition were evaluated in *Paphiopedilum spicerianum* and no seasonal changes were observed. However, a single existing population did not allow the contrast

between orchid populations (Han et al., 2016). Environmental factors such as site of orchid occurrence and orchid species have been assessed almost all over the world. This concerned terrestrial, epiphytic and even achlorophyllous orchids (Martos et al., 2009; Jacquemyn et al., 2015b; Suárez et al., 2016). However, it is not always possible to evaluate multiple environment factors that could affect the RAF community composition, probably because of the contrasting climatic and geographic conditions at different ecosystems (pers. obs).

The distribution and abundance of fungal endophytes depend undoubtedly on many different ecological factors. Nevertheless, the identification of these communities and the comprehension on how they respond to changes in abiotic and biotic factors remains as a challenge (Varma, 2008).

III. RESEARCH OBJECTIVES

Orchids are distributed worldwide and represent one of the largest families of flowering plants. Members of Orchidaceae are recognized for their medicinal usages, industrial potential and ecological roles. They are important for horticulture, floriculture and as key element of ecosystems. These plants play crucial roles in ecosystem dynamics because they maintain complex interactions with other organisms such as pollinators and root-associated fungi (RAF). Thus, threats to orchids may threats the associated flora and fauna. Unfortunately in Ecuador the orchid diversity, especially in the montane forests, is endangered by disturbance of their environments caused by human activities.

Since fungi are fundamental for ecosystem functioning (plant nutrition, nutrient cycling and protections against pathogens), the rapid loss of biodiversity could lead to important environmental disturbances. Thus, the identification of endophytic RAF is a critical step to understand the ecology of orchid-fungi interactions. In the recent years, the implementation of next generation sequencing (NGS) technologies have helped in the detection/identification of fungi in the internal root tissues of orchids, increasing concomitantly the ecological understanding of orchid-fungi interactions. This has helped to understand how the endophyte communities are structured and which factors affect the symbiosis. However, most studies were conducted on terrestrial orchids of temperate ecosystems, while the highest orchid diversity is concentrated in tropical areas. The rare studies performed on tropical epiphytic orchids seemed to contradict some ecological patterns (e.g. dominant species, specificity) that have been found in terrestrial orchids (e.g. Beck et al., 2008). It is thus necessary to clarify and understand the ecological dynamics between orchid and fungi, by (1) identifying the endophytic RAF that interact with tropical orchids and (2) assessing the factors that likely affect the interaction.

In this thesis we aimed firstly to characterize the endophytic RAF present in epiphytic orchids and secondly to evaluate some abiotic and biotic factors (e.g. site, host phylogeny, altitude and temporal variation) potentially influencing the fungal communities and consequently the symbiosis. Specifically, in **Chapter I**, the objective was to identify the communities of endophytic RAF of five epiphytic orchids (*C. flexuosum, C. myanthum*, *M. calantha, E. marsupiale* and *C. pardinum*), but also to contrast the RAF communities between co-existing orchids and between orchid populations. Thus, we aimed to address the following question: **Do the orchid host species and site (*C. flexuosum, C. myanthum* and *M. calantha* at Podocarpus National Park and *Epidendrum marsupiale* and *C. pardinum* at Cajas National Park) influence the community composition of RAF?** . In **Chapter II**, the objective was to evaluate the mycorrhizal fungi community structure of three epiphytic orchids (*C. flexuosum, C. myanthum* and *M. calantha*). We aimed to address the following question: **Do mycorrhizal fungi communities differ between *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* that co-exist at two sites of evergreen upper montane forest?** In **Chapter III**, the objective was to evaluate experimentally, under field conditions, the mycorrhizal fungi communities associated with epiphytic orchids (*C. retusum* and *E. macrum*) along an altitudinal level and in a temporal variation. We aimed to address the following question: **Do the host phylogeny, altitudinal gradient, temporal variation and site of orchid occurrence affect the composition of mycorrhizal fungal communities associated with *Cyrtochilum retusum* and *Epidendrum macrum*?** The evaluation of endophytic RAF offered the possibility of better understanding the interaction between orchids and fungi and also helped to infer fungal traits and their potential implications for the stability of such associations in montane forest.

IV. MATERIALS AND METHODS

1. Orchid species

Seven epiphytic orchid species were selected. All are present in Southern Ecuador and included in the CITES list, Appendix 2 (https://cites.org/eng/disc/how.php). The studied orchid species (*Cyrtochilum flexuosum, C. myanthum, C. pardinum, E. marsupiale* and *Maxillaria calantha*) considered in Chapter I and II, were selected because they are distributed in at least two biotopes of montane forest at Southern Ecuador. Meanwhile for Chapter III, the orchid species (*C. retusum* and *E. macrum*) were chosen because they are distributed in montane forest of Ecuador and because *in vitro* seedlings were available in a local greenhouse (Ecuagenea, Azuay-Ecuador) for the seedling-trap experiment.

***Cyrtochilum flexuosum* Kunth:** This species belongs to the Cymbidieae tribe (Fig. 6). It was identified for the first time in 1816 by Kunth. This species is found in Brazil, Colombia and Ecuador. In Ecuador several populations are present on the north, center (www.tropicos.org; www. gbif.org) and south (surround area of Podocarpus National Park) between 1500 and 2700 m a.s.l. Flowering season occur between September and January (Mendoza, 2013). Further ecological information such pollinator or life cycle is not available.

Fig. 6 *Cyrtochilum flexuosum* (Photo by Eric Hunt, http://www.orchidspecies.com/orphotdir/cyrtcimiciferum.jpg).

***Cyrtochilum myanthum* (Lindl.) Kraenzl.:** This species belongs to the Cymbidieae tribe (Fig. 7). It was described for the first time in 1917 by Kraenzlin. This species is found in Southern Ecuador in the provinces of Loja and Zamora-Chinchipe between 2500 and 2700 m a.s.l. It is also recorded in the highlands of Bolivia, Colombia and Peru (www.tropicos.org). Flowering season occur between October and January (Mendoza, 2013). Further ecological information such pollinator or life cycle is not available.

Fig. 7 *Cyrtochilum myanthum* (Photo by Andreas Kay, https://www.flickr.com/photos/andreaskay/8144138190).

***Cyrtochilum pardinum* (Lindl.) Beer:** This species belongs to the Cymbidieae tribe (Fig. 8). It was described for the first time in 1838 by Lindley J. This species is found in Colombia, Peru and Ecuador between 1864 and 3600 m a.s.l. (www.tropicos.org). Ecological information such pollinator, life cycle or flowering season is not available.

Fig. 8 *Cyrtochilum pardinum* (Photo by Jay Pfahl, http://www.orchidspecies.com/cyrtpardinum.htm).

Cyrtochilum retusum* (Lindl.) Kraenzl.*: This species belongs to the Cymbidieae tribe (Fig. 9). It was documented for the first time in 1917 (WCSP, 2017) by Lindley. It is present in the province of Loja, Southern Ecuador at altitudes between 1700 and 3150 m a.s.l. (Dodson, 2002; Telenius and Shah, 2016; www.tropicos.org). Ecological information such pollinator, life cycle or flowering season is not available.

Fig. 9 *Cyrtochilum retusum* (Photo by Guilberto Merino, http://bluenanta.com/natural/54617/species_detail/).

***Epidendrum macrum* Dressler:** This species belongs to the Epidendreae tribe (Fig. 10). It was reported for the first time in 1967 (WCSP, 2017) by Dressler. This species is found in Ecuador and also in Peru at altitudes between 800 and 1400 m a.s.l. (Magill et al., 2016; www.tropicos.org). Ecological information such pollinator, life cycle, flowering season or phorophyte is not available.

Fig. 10 *Epidendrum macrum* (Photo by Ecuagenera, http://www.ecuagenera.com/Epidendrum-macrum/en).

***Epidendrum marsupiale* F. Lehm. & Kraenzl.:** This species belongs to the Epidendreae tribe (Fig. 11). It was recorded for the first time in 1899 (WCSP, 2017) by Lehmann and Kraenzlin. This species is present in the Andes of Colombia and Ecuador between 730 and 3200 m a.s.l. (www.tropicos.org). Ecological information such pollinator, life cycle, flowering season or phorophyte is not available.

Fig. 11 *Epidendrum marsupiale* (Photo by Ecuagenera, http://www.ecuagenera.com/Epidendrum-marsupiale/es).

***Maxillaria calantha* Schltr.:** This species belongs to the Cymbidieae tribe (Fig. 12). It was reported for the first time in 1921 (WCSP, 2017) by Schlechter. This species is present in Bolivia, Peru and Ecuador at altitudes above 2400 m a.s.l. (www.tropicos.org). In Southern Ecuador it is observed between 2050 to 2800 m a.s.l. (Mendoza pers. comm). Ecological information such pollinator, life cycle, flowering season or phorophyte is not available.

Fig. 12 *Maxillaria calantha* (Photo by Andreas Kay, https://www.flickr.com/photos/andreaskay/8216786410).

2. Sampling sites

2.1 Podocarpus National Park (PNP) and surrounding areas

The Podocarpus National Park covers 1463 km² and is located in Loja and Zamora-Chinchipe provinces, Southern Ecuador. Two sites of evergreen upper montane forest (Beck et al., 2008) were chosen for sampling *C. flexuosum* (species 1)*, C. myanthum* (species 2) and *M. calantha* (species 3) (Fig. 13). The first site is called "Curva misteriosa" (**site 1**; 3° 59′ S, 79° 08′ W) and the second site "El Tiro" (**site 2**; 3° 59′ S, 79° 06′ W). Distance between each site is four kilometers. Curva Misteriosa is characterized by a steep slope (51%), with trees 5-8 m high (Riofrío et al., 2007), a mean annual temperature of 20.8 °C and mean annual precipitation of 2193 mm (Bendix et al., 2008). El Tiro has the ridge covered with forests on the slope sides and open grass, bromeliad, or dwarf shrub formations along the crest line (Setaro et al., 2006), the mean annual temperature is 9.8 °C and mean annual precipitation is 3000 mm (Gradstein et al., 2008). The sampled area per site was approximately 700 m^2. At both sites, the characteristic vegetation included epiphytic plants such as orchids, ferns and bromeliads (Mandl et al., 2010). The climate is cool and prehumid while; the soil is poor and acidic with a pH from 4.7 to 3.2 (Gradstein et al., 2008).

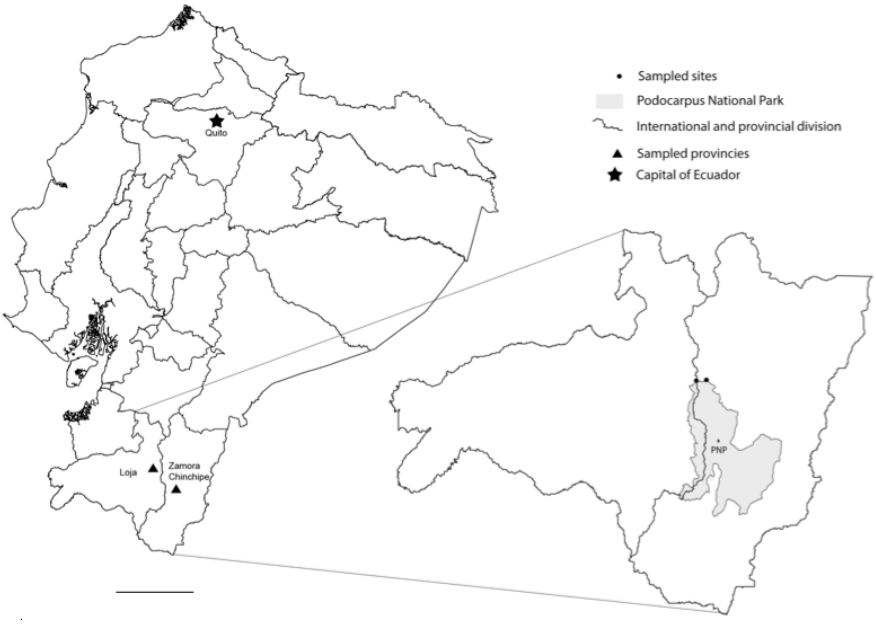

Fig. 13 Map of the sampled sites located on the surrounding areas of Podocarpus National Park. Sampled sites were identified with dots, Podocarpus National Park in gray, international and provincial division with continuous lines, sampled provinces with black triangles and the capital of Ecuador with the black star (Mendoza, 2013)**.** Scale bar = 80 km.

2.2 Cajas National Park (CNP)

Located in Azuay province, Southern Ecuador. This area of 285.4 km² is part of the Macizo del Cajas Biosphere Reserve. Four sites were selected: "High Mazán" (**site 3**; 2°52'13''S, 79°7'26''W), "Low Mazán" (**site 4**; 2°52'19''S, 79°7'8''W), "High Llaviucu" (**site 5**; 2°50'26''S, 79°10'29''W) and "Low Llaviucu" (**site 6**; 2°50'36''S, 79°8'37''W) to collect individuals of *E. marsupiale* (species 4) and *C. pardinum* (species 5) (Fig. 14). Sites are located between 3000 and 3500 m a.s.l. Sierra, (1999) classified these areas as evergreen higher montane forests. They host about 300 species of vascular plants (Montesinos, 1996) and Orchidaceae is the second most diverse family (ETAPA, 2005). The plant composition of these areas is very similar because of their vicinity and physio-climatic characteristics. The climate fluctuates between -2 ºC and 18

ºC, being December the warmest month and July the coldest (Minga et al., 2016). The annual precipitation is 1200 mm in average with hail and snow episodes (Ramsay and Oxley, 1997; ETAPA, 2005, Sklenár et al., 2011).

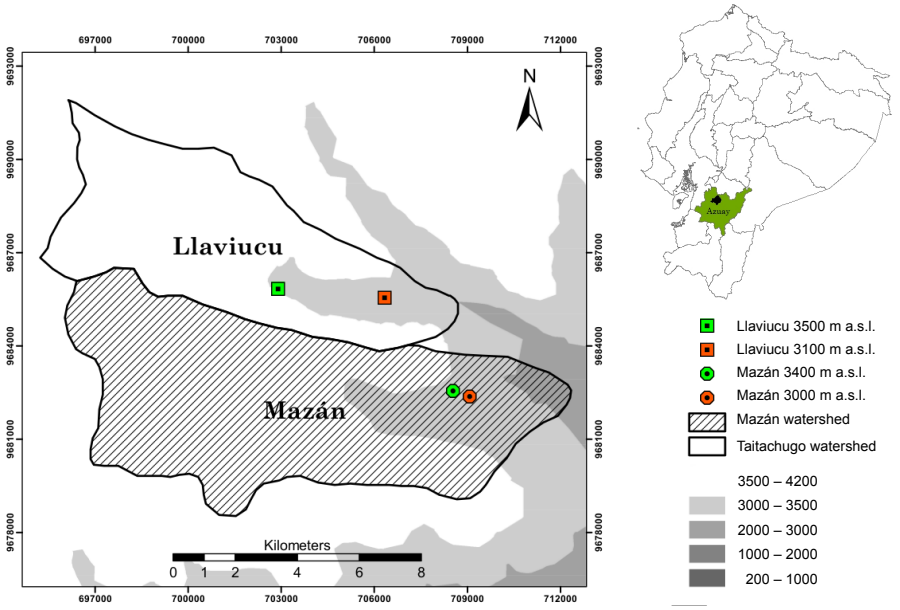

Fig. 14 Location of the study sites in El Cajas National Park (Azuay province) in Ecuador, South Ecuadorian Andes (Herrera et al., in press).

3. Seedling experiment setup

3.1 Selected sites for seedling assays

In order to establish the seedling experiments, two transects named T2 (Transect 2: 3º58.415'S, 79º04.516'W) and Q5 (Quebrada 5: 3º58.399'S, 79º04.243'W) were chosen in the Reserva Biológica San Francisco (RBSF) at the northern border of PNP. The area is located on the eastern slope of the Cordillera El Consuelo (3º58'S, 79º04'W), a recognized hotspot of biodiversity (Bussmann, 2005). The vegetation is characterized by a wide species diversity of ericads, liverworts, mosses and orchids (Parolly et al., 2004; Homeier et al., 2008). The rainy season is between April and August meanwhile the dry season is from September to March; the mean annual

temperature is 15.5 °C and the annual rainfall is 2000 mm (Table 8.7 in Beck et al., 2008).

3.2 Seedling-trap systems

To construct the seedling-trap, we used a polyvinyl chloride (PVC) cylinder (20 cm diameter and 15 cm height), 10 orchid seedlings from the same orchid species and a sun bag (Sigma-Aldrich, St. Louis-Missouri, USA). The orchids seedlings used for the field experiment were *C. retusum* (Fig. 9) and *E. macrum* (Fig. 10) obtained from Ecuagenera greenhouse (Azuay-Ecuador). The establishment of seedling-traps was as follows: the cylinder was settled on a tree branch (harboring at least one orchid, naturally established) and inside the cylinder. Finally, the cylinder was covered with a sun bag to avoid herbivory and leaf litter input. Seedling-trap systems were placed in direct contact with tree branches (Fig. 15). The seedling traps were installed in the transects T2 and Q5 at RBSF along 10 points established in a range between 1850 and 2150 m a.s.l. separated from each other ~30 m a.s.l. (Fig. 16). The field experiment was performed during May 2014 and April 2015.

Fig. 15 Set up of a seedling-trap system on a tree branch.

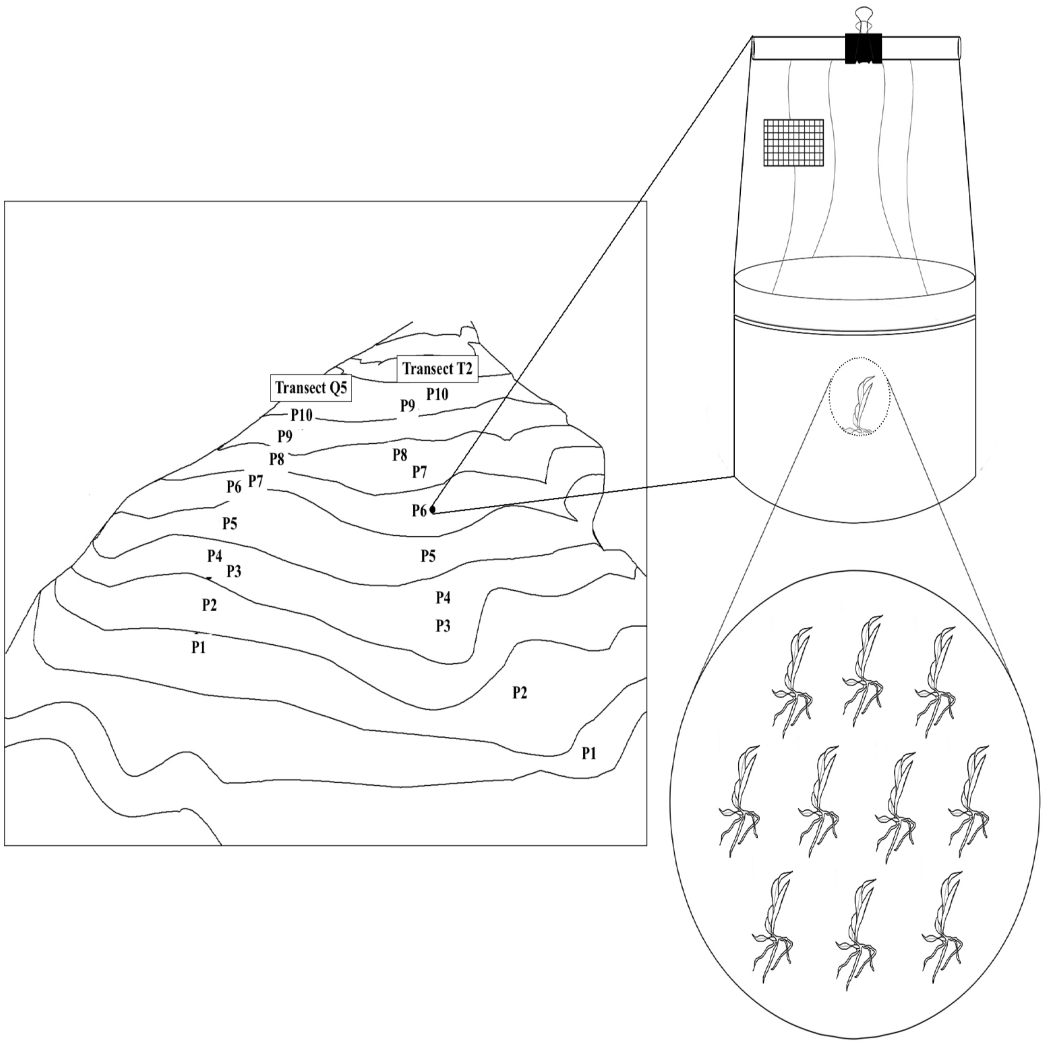

Fig. 16 Diagram of a seedling-trap system and the experimental design along the transect T2 and Q5 at 10 altitudinal levels established in a range between 1850 and 2150 m a.s.l. at the Reserva Biológica San Francisco. The coordinates were overlapped in the altitudinal map provided by the National Information System of Ecuador (http://www.sigtierras.gob.ec/). The GPS error could range between 3 to 15 meters.

3.3 Sampling of seedlings

The sampling of the seedling traps was performed twice, at 3 and 12 months after the start of the experiment. A number of seedlings did nor

survive until the first sampling and thus 1 to 4 seedlings were finally sampled per seedling-trap. All seedlings sampled from a single seedling-trap were considered as one sample. Only seedling-traps with more than 4 seedlings alive were sampled after nine additional months. Seedling-traps were codified as follows: transect name, altitudinal level, orchid species and sampling time (T2: transect T2, Q5: transect Q5; C: *C. retusum*, E: *E. macrum*; S1: first sampling and S2: second sampling and A1 - A10: altitudinal levels) e.g. T2CS1_A1, T2ES1_A1, Q5CS1_A1, Q5ES1_A1 and so on. All the seedling-traps from *C. retusum* or *E. macrum* established along T2 or Q5 and collected the first or second sampling were considered a treatment.

4. Orchid root sampling

4.1 Sampling of natural orchid populations

Adult orchids from *C. flexuosum, C. myanthum* and *M. calantha* were sampled during October 2012 and January 2013 at Curva Misteriosa (site 1) and El Tiro (site 2), close to the PNP. Eight to 12 individuals were sampled per population, depending on the orchid population size. In total 47 individuals were sampled and from each 3 to 5 roots, 1 to 2 cm long, were collected and kept in aluminum foil and transported to the lab the same day of sampling.

At CNP, during 2015 and 2016 roots from *E. marsupiale* and *C. pardinum* were collected in the sites “High Mazán” (site 3), “Low Mazán” (site 4), “High Llaviucu” (site 5) and “Low Llaviucu” (site 6). In total, 83 orchid individuals were sampled. Nine to 11 individuals were sampled per orchid population, depending on the orchid population. Samples were kept in ethanol 70% for further analyses.

4.2 Sampling from the seedling-traps

Roots of seedlings were sampled 3 and 12 months after set up of the trap plants. At first sampling (July 2014), 9 seedling-traps of *E. macrum* at T2 and 18 seedling-traps of *C. retusum* at T2 and Q5, were recovered. During the second sampling (April 2015), roots from 8 seedling-traps of *C. retusum* at T2 were collected. All the samples were stored in ethanol 70% for microscopic and molecular analyses.

5. Screening of orchid root-associated fungi

All orchid roots samples from site 1 to site 6 were evaluated under light microcopy to detect the occurrence of pelotons (hyphal coils) by transversal root sections. Roots were stained for 3 min with methyl blue solution (0.05%, Merck, Darmstadt, Germany) (Fig. 17). Only root fragments showing evidences of fungal colonization were kept at 4 °C and processed within the next 24 hours. The fragments without evidence of colonization were discarded. Meanwhile, due to scarcity of root material from the seedling-trap experiment, only 3 samples were randomly selected to analyse under microscope.

Fig. 17 Screening of *Cyrtochilum flexuosum* roots. Sections were stained with methyl blue 0.05%.

6. Molecular methods

6.1 External root disinfection

For molecular analysis of the endophytic fungi associated with epiphytic orchid species, the root surface was disinfected. Under aseptic conditions roots were rinsed using 1% sodium hypochlorite and 75% ethanol followed by 3 times rinsing with sterile distilled water during 1 min. The velamen (external root-tissue) was removed using forceps and scalpel to reduce contamination by external fungi. Remaining cortical tissue was kept in 2 µl microtubes for DNA isolation.

6.2 DNA isolation

6.2.1 DNA isolation from roots of adult orchids: Portions of 1 to 2 cm length of colonized roots, previously screened, were taken for each sample. The total DNA was recovered using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions but only the elution steps were adjusted. The samples were disrupted using the Qiagen TissueRuptor®. Four hundred µl of buffer AP1 and 4 µl of RNase A were added to the roots in the tube. Samples were briefly stirred on vortex and subsequently incubated at 65 °C for 10 min. During incubation the tubes were inverted every 2 min. In addition, 130 µl of P3 buffer (detergent provided by the Qiagen manufacturer) was added and mixed before being incubated for 5 min on ice. To pellet the debris of proteins and polysaccharides, the samples were centrifuged for 5 min (14000 x *g*). The supernatants were transferred into QIAshredder spin columns placed in 2 ml collection tubes. Another centrifugation (2 min, 14000 x *g*) was done and the flow-through was transferred into 1.5 ml new tubes. Afterwards, 1.5 volumes of the buffer AW1 (wash buffer) was added and integrated by pipetting to wash the DNA from residues. Further, 650 µl of the mixture was transferred into DNeasy

Mini spin columns that were placed in 2 ml collection tubes and centrifuged (1 min, ≥ 6000 x *g*). The collection tubes were emptied and the step repeated with the remaining sample. The spin columns were place into new 2 ml collection tubes. Five hundred µl of the buffer AW2 (wash buffer) was added for the second wash. Further, a centrifugation (1 min, ≥ 6000 x *g*) step was performed to dried spin columns. The flow-through from the collection tubes was discarded. Another 500 µl of buffer AW2 was added and then the tubes were centrifuged for 2 min at 20000 x *g*. To prevent the contact between the column and the flow-through, from each sample the spin column was carefully separated from the collection tube and the spin columns were transferred into a new 15 µl microcentrifuge tube. Finally, for the elution, 50 µl of buffer AE was added to each microcentrifuge tube and incubated for 5 min at room temperature (15-25 °C). Tubes were centrifuged for 1 min at ≥ 6000 x *g*. The step of elution was repeated once and finally the spin column was discarded and the DNA recovered was stored at -20 °C.

6.2.2 DNA isolation from the roots of orchid seedlings: The DNA was extracted from the root fragments using the PureLink® Genomic Plant DNA Purification Kit (Invitrogen) as described by the manufacturer. The lysate preparation per sample is described below. The samples were disrupted using the Qiagen TissueRuptor®. In the same microcentrifuge tube, 250 µl of resuspension buffer (R2) was added and homogenized by vortex to prepare the lysate. To isolate the DNA from the proteins and lipids, 15 µl 20% SDS and 15 µl of RNase (20 mg ml^{-1}) were used. The lysate was incubated for 15 min at 55 °C. Then, a centrifugation was carried out for 5 min at high speed to remove insoluble material. The supernatant was transferred to a new 1.5 µl microcentrifuge tube and 100 µl of precipitation buffer (N2) was integrated. To produce a clear lysate, the mix was incubated on ice for 5 min before another centrifugation for 5 min at maximum speed. Two hundred fifty µl of clear lysate was transferred into a new 1.5 µl

microcentrifuge tube and 375 µl of the binding buffer (B4) was supplemented. To purify the lysate, it was added into a PureLink® Spin Cartridge placed in a collection tube. To pellet the debris of proteins and polysaccharides, the sample was centrifuged (30 s, 10000 x *g*) at room temperature and the flow-through was discarded. The cartridge was placed into a wash tube and 500 µl of wash buffer (W4) was added before an additional centrifugation (30 s, 10000 x *g*). The collection tube was emptied and 500 µl of wash buffer (W5) was added. Afterwards, another centrifugation step (30 s, 10000 x *g*) was done and the resulting flow-through was discarded. Samples were washed twice with the wash buffer (W5). To remove residual of the buffer, one centrifugation step (2 min, maximum speed) was necessary. Carefully the spin column was placed in a new sterile 1.5 ml microcentrifuge tube. Finally, to elute the DNA, 50 µl of elution buffer (E1) was placed at the bottom of the spin column and incubated for 1 min. To recover the DNA, another centrifugation (1 min, maximum speed) was applied. The elution step was performed twice. The column was discarded and the recovered DNA stored at -20 °C until additional analyses.

6.3 PCR conditions

6.3.1 PCR for DNA isolated from roots of adult orchids: The fungal specific primer pair, ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′; Turenne et al., 1999) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990), was used to target the ITS2 region of the nrDNA (approximately 250 – 350 bp) (Fig. 18). The primer pair was chosen because it has been shown powerful and useful in characterizing diverse endophytic fungal communities using high throughput sequencing (Jacquemyn et al., 2014). The amplifications were performed in a total reaction volume of 25 µl that included 21.5 µl of Platinum PCR SuperMix (Invitrogen), 0.5 µl of each primer, 0.5 µl of BSA 1% and 2 µl of total DNA. Thermal cycling conditions

were as follows: initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation step for 30 s at 94 °C, annealing step for 30 s at 55 °C, extension step for 2 min at 72 °C and a final extension for 10 min at 72 °C. The amplicons were checked via electrophoresis (1 % agarose gel in 1 x SB buffer) to corroborate the amplification of the fragment. The expected size was ~250 – 350 bp.

6.3.2 PCR for DNA isolated from seedling assays: PCR reaction was carried out with two primer pairs: ITS86F (Turenne et al., 1999) combined with ITS4 (White et al., 1990) and ITS3 (5´-GCATCGATGAAGAACGCAGC-3'; White et al., 1990) combined with ITS4 (White et al., 1990). The primers amplify the ITS2 region of the nrDNA, approximately 250 – 350 bp (Fig. 18). The chosen PCR primer combination was chosen because in previous research it was shown that these primer pairs were highly complementary and outperformed other primer pairs to characterize endophytic fungal communities (Waud et al., 2016). One 20 µl PCR reaction contained 4 µl of 5X Phusion HF Buffer, 0.4 µl of dNTPs (10 mM), 0.4 µl of each primer, 0.8 µl BSA 10%, 0.2 µl of the Phusion DNA Polymerase (Thermo Fisher Scientific), 11.8 µl of ultrapure water and 2µl of total DNA. The cycling conditions were as follows: initial denaturation at 98°C for 30 s, 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. The PCR products were resolved by agarose gel electrophoresis (1% agarose gel in 1 x SB buffer) and the amplicons within a size range ~250 - 350 bp were kept.

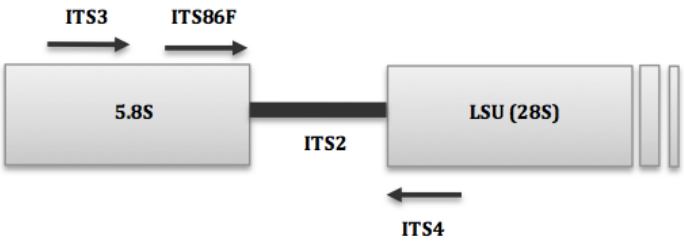

Fig. 18 Schematic representation of the nrDNA region targeted by the specific primers, forward and reverse (not at scale).

6.4 Purification and quantification

The amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's instructions. The amplicons obtained from the PCR pipeline for the seedling-trap experiment, obtained from the different primer pair combination (ITS86F/ITS4; ITS3/ITS4), were mixed as a single sample before purification. The quality of the purified products was determined through the evaluation of the AD260/280 ratio calculated using the Spectrophotometer NanoDrop® 2000c (Thermo Scientific, Wilmington, USA).

6.5 Sequencing

The purified amplicons were submitted to IMGM Laboratories GmbH (Martinsried, Germany) for indexing, library preparation, cluster generation and next generation sequencing on the MiSeq Illumina platform. According to the IMGM workflow, DNA was amplified together with the index and sequencing adapter. After several cycles of PCR reactions, PCR products with multiple sizes were obtained. The library pool was analysed with one run of the Illumina MiSeq® system. Using the index designated to each sample, the image and signal processing of the recorded signals as well as de-multiplexing of reads were performed. Finally, the quality control of the sequencing run and trimming of adapter were implemented.

7. Bioinformatics analysis

Analyses for OTU reconstruction were done with UPARSE software (Edgar, 2013). Following the USEARCH pipeline, all overlapping paired reads were merged into single sequences using the "fastq_mergepair" command with the–fastq_nostagger option to reduce errors while merging paired-end reads. Then, a quality filter with a maximum expected error threshold of 0.3 for single sequences was applied using the "fastq_filter" command. Read were globally cut off at 240 bp length and with the command "derep_fulllength", singletons were discarded. Additionally, through the "cluster_otus" command, OTUs were reconstructed using 97% homology. The taxonomic assignment to the highest possible taxonomic rank was performed through the PlutoF web-based sequence management workbench (Abarenkov et al., 2010b) based on the UNITE database (http://unite.ut.ee; Abarenkov et al., 2010a).

8. Statistical analysis

8.1 Data organization

According to the objectives of each chapter all the root-associated fungal endophytes or the OMF-OTUs were evaluated in order to identify which abiotic or biotic factors affect the structure of fungal communities. For instance, in the first chapter all reconstructed endophytic RAF-OTUs were analysed, meanwhile for the second and third chapter the efforts were focused on the analysis of OMF-OTUs. The OTU information was placed in a matrix that contained the sequence abundance for each sample. Before starting with the analysis, the matrices were converted into binary matrix on a per sample basis in order to make inferences on fungal community composition as a function of abiotic (sites, altitude and time) and biotic (host

phylogeny) factors. In the matrix, the columns corresponded to the individuals sampled and the rows to the OTUs identified.

8.2 Alpha diversity

The numbers of species compiled in the inventories correlated with the sampling effort were represented in accumulation curves (Jiménez and Hortal, 2003). To calculate fungal accumulation curves, binary matrixes of OTUs (RAF or OMF) identified at each site or transect were used as input data (e.g. Senés-Guerrero et al., 2014). Using EstimateS 9.1.1 software and a sample-based rarefaction method with 100 permutations, accumulation curves per site and transect were calculated (Colwell, 2013).

The inventory quality was assessed with the Clench equation: $Sn = a \cdot n/(1 + b \cdot n)$ where "*a"* is the rate of new species increment, "*n*" is the sampling effort, and "*b*" is a parameter related to the shape of the curve, for these calculations the Statistica software (StatSoft, Tulsa, OK, USA) was used. Furthermore, the variance explained by the Clench equation and the percentage of estimated fungal richness were also calculated following the premises suggested by Jiménez-Valverde and Hortal, (2003).

8.3 Beta diversity

Fungal communities were contrasted among individuals, either from co-existing orchid species or altitudinal gradients, in order to identify if there is an effect of the abiotic or biotic factors on the composition of fungal communities. Based on the binary matrices the differences of the communities were pairwise calculated using the Jaccard index. The Jaccard index compares members from two sets to see which members are shared and which are distinct, the measure of the similarity can be in a range from 0% to 100% where the higher percentage shows more similarity. To test if fungal communities differed, a one-way analysis of variance (ANOVA)

using the Jaccard distance (obtained from Jaccard index) as a dependent variable and with the Tukey's HSD test as post-hoc test was implemented in the statistical software SPSS 22 (IBM Corp., Somers, NY, USA). Differences between fungal communities were visualized by non-multidimensional scaling (NMDS) using the Jaccard similarity index as a distance measure in SPSS 22. Fungal communities between sites or collection times were tested using permutational analysis of variance (PERMANOVA) with 999 permutations, conducted with the adonis function in the vegan package (Oksanen et al., 2016) of R (R core team, 2015).

V. RESEARCH RESULTS

CHAPTER I

Untangling factors that drive community composition of root-associated fungal endophytes of Neotropical epiphytic orchids

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Preface

Numerous studies have identified a polyphyletic group of fungi associated with the roots of orchids, including members of Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla (Xing et al., 2013, 2015). Endophytic RAF communities include mycorrhizal and non-mycorrhizal fungi which are assumed to behave as benign intracellular inhabitants of plant roots that potentially favor the productivity and community assemblage (Dearnaley et al., 2012; Tedersoo, 2017). Mycorrhizal fungi are more frequently addressed because of their recognized ecological roles in orchid life cycle (Smith and Read, 2008). With the development of next generation sequencing technologies a wide group of non-mycorrhizal fungi have been identified (Smith and Peay, 2014). However, the community structure of the entire orchid endophytic RAF and the environment factors that could affect such community composition is not fully understood.

In **Chapter I,** five epiphytic orchids naturally distributed in a montane forest of Southern Ecuador were sampled to evaluate the entire endophytic RAF. Fungal diversity was analysed using nrDNA ITS2 with the Illumina MiSeq technology. In total 130 orchids individuals belonging to *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* at Podocarpus National Park and *Epidendrum marsupiale* and *Cyrtochilum pardinum* at Cajas National Park were sampled. Changes in the structure of fungal communities were established as a function of the host phylogeny and the site where the orchids grow.

Abstract

In orchids, most of the root-associated fungal endophytes remain undescribed as well as the drivers that affect their interactions with the plants. We characterized root-associated fungal endophytes of co-existing orchids across sites in two areas of montane rainforest in the Southern Ecuadorian Andes. We amplified the nrDNA ITS2 region of 130 orchid individuals with Illumina MiSeq technology and tested whether changes in the structure of fungal communities are associated with host phylogeny or the sites where the orchids grow. We identified 3492 OTUs corresponding to the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla. Fungal communities associated with orchids at the lower geographic areas (between 2050 and 2800 m a.s.l.) showed that host evolution and sites are drivers that could shape distinctive fungal communities, while at the highest geographic areas (between 3000 and 3500 m a.s.l.), no distinctive fungal communities were found neither between co-existing orchid species nor between sites. These results suggested that among orchid species, abiotic and biotic factors do not influence the composition of fungal communities in the same way.

Keywords:

Fungal community composition, Drivers of endophyte communities, Epiphytic orchids, Root-associated endophytes, Next generation sequencing, Fungal community analysis

1. Introduction

Orchid roots harbor a diverse set of fungal species with distinct ecological attributes such as saprobes, latent pathogens or symbionts (Kohout et al., 2013; Ma et al., 2015). Fungi located inside orchid roots in the velamen or in the cortical tissue are collectively termed root-associated

endophytes (Brundrett, 2002). However, studies focusing on root-associated endophytes frequently ignore the species present in the velamen because this thick corky epidermis is generally recognized as an adaptive structure for water and nutrient conservation (Zotz and Winkler, 2013). Fungi present in the velamen are thus most often considered as surface contaminants or opportunists associated to roots (e.g. Yuan et al., 2010). Thus, the root cortical tissue is the main target of multiple studies focusing on orchid fungal endophytes because it often harbors fungi that may interact with the plant such as symbionts (Brundrett, 2002; Smith and Read, 2008).

Fungal endophytes in root cortical tissues comprise a polyphyletic group (Smith and Read, 2008) that includes mycorrhizal and non-mycorrhizal fungi. While mycorrhizal fungi have been widely investigated across multiple orchid species and with recognized ecological roles in their life cycle (Dearnaley et al., 2012), non-mycorrhizal fungi have been less studied (Ma et al., 2015).

Most studies focusing on community composition of orchid root-associated endophytes (both mycorrhizal and non-mycorrhizal fungi) have been based on culture-dependent methods (Herrera et al., 2010; Novotná et al., 2018). However, the great majority of fungi cannot be grown in artificial conditions and thus culture-independent methods (sequencing and cloning) have been developed to increase the knowledge on fungal diversity (Kristiansen et al., 2001). With the development of powerful molecular methods in the recent years (e.g. next generation sequencing), the range of fungi identified to the species level has been increased markedly, making more accurate ecological inferences now possible (Smith and Peay, 2014).

Studies conducted on the orchid root-associated fungal endophytes, using culture-independent methods, mostly described fungal diversity and abundance (Kohout et al., 2013). However, how root-associated fungal

endophyte communities are assembled in orchids remains poorly investigated (for a review, see Dearnaley et al., 2012) and the information about the abiotic and biotic factors that drive such fungal community composition is even more limited.

Abiotic factors such as temperature and humidity are generally variable across sites but also in a same site, an altitudinal gradient could result in peculiar microclimates that shape plant-fungi interactions, as was observed in orchid mycorrhizal fungi (Garnica et al., 2013). Similarly, biotic factors such as the host phylogeny or the interactions between co-existing species were suggested to affect fungal distribution patterns (see Waterman et al., 2011). For instance, conform to the co-existence theory, species are able to cohabit because they use different niches (Götzenberger et al., 2012) determined by the host (Oliveira et al., 2014).

Nowadays, the information about the specific factors that drive the occurrence and variation of orchid root-associated fungal endophytes in natural environments is scarce (e.g. Bunch et al., 2013; Sudheep and Sridhar, 2012) probably because of the limited investigations of orchid species, populations or sites (Ma et al., 2015). Studies on orchid root-associated fungal endophytes were mostly focused on terrestrial orchids in temperate ecosystems (e.g. Jacquemyn et al., 2015a; Těšitelová et al., 2015), whereas only a few concerned epiphytic orchids in tropical areas (see Bayman and Otero, 2006; Herrera et al., 2010; Novotná et al., 2018; Oliveira et al., 2014). This fact contrasts with the higher orchid diversity concentrated in Neotropical ecosystems (Dressler, 1990; Pridgeon, 1995). In the Ecuadorian Andes, one of the world's hotspot of biodiversity (Beck et al., 2008), an important variety of Basidiomycetes and Ascomycetes has been identified associated with epiphytic orchid roots (e.g. Herrera et al., 2010; Herrera et al., 2018; Novotná et al., 2018; Riofrío et al., 2013; Suárez et al., 2006, 2008, 2016). However, it is likely that a large number of fungal endophytes

remain undescribed due to methodological biases (Kohout et al., 2013; Tedersoo et al., 2010a) and also because the target of each study was either mycorrhizal or non-mycorrhizal fungi but not both at the same time.

To fill this gap, the first critical step is to elucidate the diversity and community assemblage of orchids root-associated endophytes of cortical tissues. In the present study, we evaluated root-associated fungal endophytes colonizing the cortical tissues of native epiphytic orchid species: *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* co-occurring in the Podocarpus National Park (PNP) and *Epidendrum marsupiale* and *Cyrtochilum pardinum* co-occurring in the Cajas National Park (CNP). We used a metagenomic approach based on the analysis of the internal transcribed spacer 2 sequences via the Illumina MiSeq technology. Our objectives were to (i) characterize root fungal endophytes associated with the aforementioned epiphytic orchids; (ii) compare endophyte communities between co-existing orchid species, (iii) evaluate the root-associated fungal endophyte communities between orchid sites/populations and (iv) compare global orchid root-associated fungal endophytes between sites at PNP and CNP. We expected distinct endophyte communities between co-existing orchid species in case that the host orchid species display preferences for the root-associated fungal endophyte community composition. We also expected distinct root-associated fungal endophyte communities across sites/populations due to an effect of altitude or site over the community considering a habitat-dependent community composition hypothesis.

2. Materials and Methods

Sample collection

Roots of orchids were collected in 2012 and 2013, with a total of 130 individual plants sampled along six sites of evergreen upper montane forests in the Southern Ecuadorian Andes. Two sites close to the PNP in Zamora-Chinchipe province were chosen: the first one called 'Curva Misteriosa' (site 1) and the second one 'El Tiro' (site 2), both culminating between 2050 and 2800 m a.s.l. (see page **54** for coordinates, climatic, geographic, edaphic and vegetation information, section **2.1 Podocarpus National Park (PNP) and sunrounding areas**). The other four sites were 'High Mazán' (site 3), 'Low Mazán' (site 4), 'High Llaviucu' (site 5) and 'Low Llaviucu' (site 6), located in the CNP in Azuay province culminating between 3000 and 3500 m a.s.l. (see page **55** for coordinates, climatic, geographic, edaphic and vegetation information, section **2.2 Cajas National Park (CNP)**).

The study sites were selected based on the presence of common epiphytic orchid species. At site 1 and site 2, three epiphytic orchid species were sampled, belonging to the tribe Cymbidieae: *Cyrtochilum flexuosum* (species 1), *Cyrtochilum myanthum* (species 2) and *Maxillaria calantha* (species 3). At site 3, 4, 5 and 6, the common epiphytic orchid species were *Epidendrum marsupiale* (species 4) and *Cyrtochilum pardinum* (species 5), members of Epidendreae and Cymbidieae tribes, respectively. In total, 19, 28, 21, 21, 22 and 19 orchid individuals were collected from sites 1, 2, 3, 4, 5 and 6, respectively. Finally, transversal sections were obtained from each root sample (see page **60**, section **5. Screening of orchid root-associated fungi**).

Molecular analysis

The selected samples (root sections) were then surface-disinfected under sterile conditions (see page **61**, section **6.1 External root disinfection**). Since the fungi located in the velamen (dead tissue) are assumed to be surface contaminants (e.g. Yuan et al., 2010), the velamen was eliminated and only the cortical tissue (alive tissue) was kept for DNA isolation. For DNA extraction, two/three pieces of colonized roots (1–2 cm long) were used per plant individual. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer's instructions (see page **61**, section **6.2.1 DNA isolation from roots of adult orchids**). In order to amplify the fungal internal transcribed spacer 2 (ITS2) region, the primer pair ITS86F (Turenne et al., 1999) and ITS4 (White et al., 1990) was used. Each PCR reaction was performed with a negative control reaction without DNA template following the pipeline described in page 65. Amplicons within the appropriate size range (~ 250 – 350 bp) purified using Wizard® SV Gel and Clean-up System (Promega, Madison, WI, USA). Concentrations and purity of amplicons were determined using a 2000c Spectrophotometer NanoDrop® (Thermo Scientific, Wilmington, USA). Finally, the target amplicons were sequenced using Illumina MiSeq® technology (IMGM Laboratories GmbH, Martinsried, Germany) that generated 300 bp long paired-end reads.

Bioinformatics analysis

Operational taxonomic units (OTUs) from the raw Illumina data were reconstructed using the UPARSE software (Edgar, 2013) (see page **66,** section **7. Bioinformatics analysis**). Then taxonomic assignment of OTUs was performed using the BLASTN algorithm implemented in UNITE database (http://unite.ut.ee; Abarenkov et al., 2010a) through the PlutoF (Abarenkov et al., 2010b) web-based sequence management workbench

(2017-06-28 release). The taxonomically defined OTUs were parsed against the FunGuild v1.0 (http://www.stbates.org/guilds/app.php) database to designate putative trophic strategies.

Data analysis and statistics

Read counts of the root endophyte OTUs per sample were converted into presence/absence matrix to evaluate the relationships between root-associated fungal endophytes community and the host orchid species or study site. To do so accumulation curves and Clench equation were applied using EstimateS 9.1.1 software and Statistica software (see page **67**, section **8.2 Alpha diversity**). In addition, differences in endophytic RAF communities from co-existing orchid species were evaluated using permutational analysis of variance in R software and Jaccard index implemented in the statistical software SPSS 22 (see page **67** and **68**, section **8.3 Beta diversity**).

Analyses related to endophytic RAF community composition across study sites-orchid populations and across both areas of montane rainforest (PNP and CNP) were performed based on the binary data (presence/absence matrix) independent by species. Non-metric multidimensional scaling (NMDS) plots were generated using SPSS22 software and the effect of the site-population was also tested for significance using PERMANOVA analysis (see page **67** and **68**, section **8.3 Beta diversity**). Although, endophyte communities' comparison between both areas of montane rainforest is biased by local environment condition and by the orchid species, it could give us some insights about the factors that affect the endophyte community composition.

3. Results

Taxonomic coverage of root-associated fungal endophyte communities and putative life strategy

MiSeq sequencing of the 130 orchid individuals sampled at the six studied sites yielded a total of 76721 quality-filtered sequences with a length of 240 bp. During the OTU reconstruction from the quality-filtered sequences, singletons, as well as chimeric sequences (5.5% of all reconstructed sequences), were discarded to obtain 3413 OTUs (3% sequence dissimilarity cutoff) assigned to root-associated fungal endophytes. The data were deposited in GenBank (Bioproject PRJNA344001 and PRJNA417757). Endophyte communities identified in association with *C. flexuosum, C. myanthum, M. calantha, E. marsupiale* and *C. pardinum* included members of 103 orders (Table S1) into Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota. Summed over the two areas of montane rainforest (e.g. PNP and CNP), the highest number of OTUs (414) was assigned to the Agaricales, while considering the two areas of montane rainforest separately, Helotiales was the highest at PNP (156 OTUs) and Agaricales at CNP (385 OTUs). On average, individuals from *C. pardinum* harbored more OTUs (Fig. 19). The most frequent OTU was OTU1 belonging to the Xylariales. It was identified in all the individuals sampled at PNP and CNP.

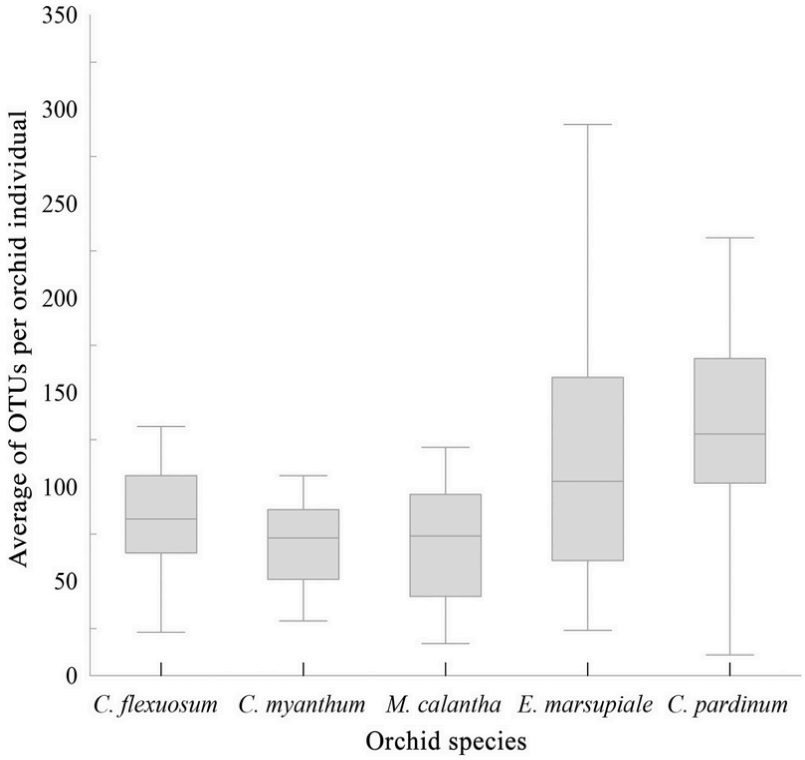

Fig. 19 Richness average of operational taxonomic units (OTUs) identified in association with *Cyrtochilum flexuosum*, *Cyrtochilum myanthum*, *Maxillaria calantha*, *Epidendrum marsupiale* and *Cyrtochilum pardinum*. The whiskers represented the higher and lower number of OTUs that an orchid individual had.

Using FunGuild database, the putative life strategy was assigned only to OTUs with taxonomic assignment at 'species' level (1200 OTUs). Analysed OTUs were assigned to saprotroph, pathotroph-saprotroph, saprotroph-symbiotroph, pathotroph-saprotroph-symbiotroph, pathogen-saprotroph-symbiotroph, symbiotroph, pathotrophs or pathotroph-symbiotroph guilds (Fig. 20). Finally, evaluation of the endophyte communities using accumulation curves showed that in none of the study site the fungal communities reached a plateau (Fig. 21). It was calculated that 48%, 51%, 48%, 46%, 43% and 45% of the estimated richness was identified at site 1, 2, 3, 4, 5 and 6, respectively.

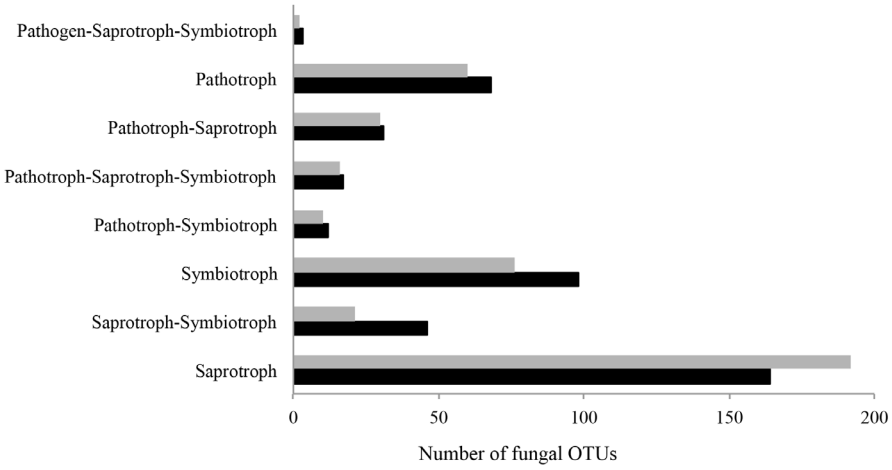

Fig. 20 Frequency distribution displaying the number of operational taxonomic units (OTUs) belonging to the different trophic guilds identified at Podocarpus National Park (gray bars) and Cajas National Park (black bars).

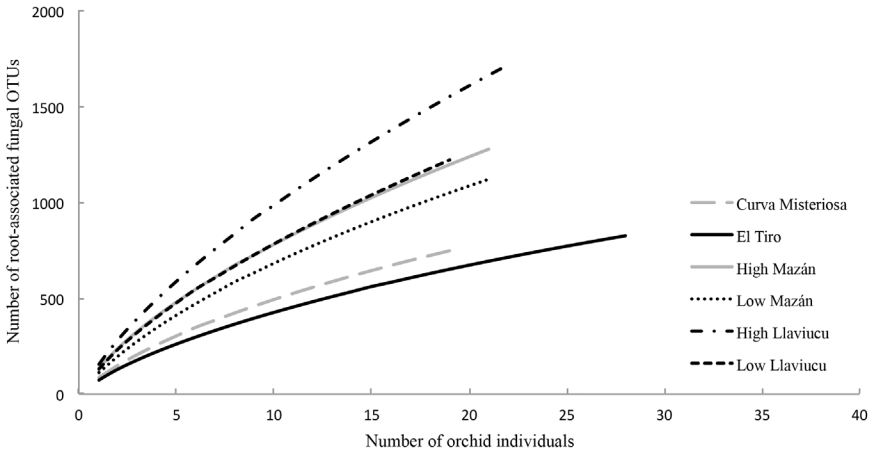

Fig. 21 Species accumulation curves describing the identified number of operational taxonomic units (OTUs) of root-associated fungal as a function of the sampled orchids per studied sites.

Root-associated fungal endophyte communities between co-existing orchid species

PERMANOVA analysis revealed that *C. flexuosum, C. myanthum* and *M. calantha,* co-existing at site 1 and site 2 located at PNP, harbored

significantly different fungal endophyte communities ($P < 0.0001$ and 0.0003, respectively). Conversely, no significant differences in the fungal endophyte communities were found for *E. marsupiale* and *C. pardinum* that co-exist at CNP, specifically at sites 3, 4, 5 and 6 ($P = 0.2521$, 0.6999, 0.4662 and 0.6936, respectively). In addition, the similarity between fungal endophyte communities was assessed in co-existing orchid species pairwisely using Jaccard indexes calculated by contrasting binary vectors that represent the presence/absence of OTUs associated to individuals of each species pair. This analysis could be conducted at PNP. Analysing the variance of Jaccard indexes (dependent variable) in function of the host phylogenic distance (factor) by means of a one-way ANOVA, we found that at site 1, the similarity was significantly higher between fungal endophyte communities associated with *C. flexuosum* and *C. myanthum* than endophyte communities associated with either species and with *M. calantha*. In contrast, at site 2 the similarity between the fungal endophyte communities associated with *C. flexuosum* and *M. calantha* were significantly higher than the similarity between endophyte communities associated with the close relatives Cyrtochilum species (Table 2).

Table 2 Analysis of variance of the similarity between root-associated fungal endophytic communities associated with *Cyrtochilum flexuosum, Cyrtochilum myanthum, and Maxillaria calantha*. Similarity between fungal communities is expressed as a Jaccard index.

Comparison	Site 1		Site 2	
	Jaccard indexes mean difference ± std. error	*P*-value	Jaccard indexes mean difference ± std. error	*P*-value
C. flexuosum-C. myanthum vs. *C. myanthum-M. calantha*	0.04279* ± 0.02314	0.001	-0.00428 ± 0.01602	0.815
C. flexuosum-C. myanthum vs. *C. flexuosum-M. calantha*	0.03200*± 0.01981	0.001	-0.03839* ± 0.01754	0.001
C. myanthum-M. calantha vs. *C. flexuosum-C. myanthum*	-0.04279* ± 0.02314	0.001	0.00428 ± 0.01654	0.815
C. myanthum-M. calantha vs. *C. flexuosum-M. calantha*	-0.01079± 0.02069	0.434	-0.03411* ± 0.01939	0.001
C. flexuosum-M. calantha vs. *C. flexuosum-C. myanthum*	-0.03200*± 0.01982	0.001	0.03839* ± 0.01754	0.001
C. flexuosum-M. calantha vs. *C. myanthum-M. calantha*	0.01079± 0.0207	0.434	0.03411* ± 0.01939	0.001

*Difference between Jaccard indexes considered statistically significant (*P*-value ≤0.05).

Root-associated fungal endophytes between orchid populations

The NMDS ordinations did not show clear distinctive patterns in fungal endophyte communities between orchid populations (Fig. S1). However, the PERMANOVA analysis revealed that endophytes associated with *C. flexuosum, C. myanthum* and *M. calantha* (orchids distributed at PNP) differed significantly between orchid populations (P = 0.0031, 0.0029 and 0.0303, respectively). Meanwhile, endophyte communities associated with *E. marsupiale* and *C. pardinum* (orchids distributed at CNP) were not significantly different between orchid populations (P =0.6992 and 0.4948,

respectively). The subsequent pairwise Tukey test performed for comparing the means showed that the endophyte community associated with *E. marsupiale* at site 3 was less similar to the endophyte communities at sites 4, 5 and 6 (Table S2), whereas, the root endophyte community associated with *C. pardinum* at site 4 was the most divergent.

Finally, we identified a set of root-associated fungal endophytes that overlapped across populations of all evaluated orchid populations and another set of endophytes identified only at a particular site-population combination (Table S3 and Fig. S2). Within the overlapped OTUs, members of Helotiales were the more abundant on populations of *C. flexuosum, C. myanthum* and *M. calantha* while Glomerales and Hypocreales were the most abundant on the populations of *E. marsupiale* and *C. pardinum*, respectively.

Differences in root-associated fungal endophyte communities between the two areas of montane rainforest (PNP vs CNP)

The NMDS ordination of the root-associated fungal endophytes communities between orchid species at PNP versus orchid species at CNP, yielded distinct endophyte communities (Fig. 22). This was confirmed by the PERMANOVA analysis ($P< 0.0001$). However, 45 similar OTUs were found at both areas of montane rainforest and identified in at least one individual per sampled orchid population. The order with the higher number of OTUs was Helotiales with six OTUs, followed by Cantharellales and Sebacinales with four OTUs each; Xylariales, Thelephorales, Polyporales, Pleosporales with three OTUs each; Atractiellales, Chaetothyriales, Agaricales with two OTUs each; Sordariales, Hymenochaetales, Capnodiales, Pezizales, Glomerellales, Lecanorales, Hypocreales, Diaporthales, Gloeophyllales, Ostropales, Malasseziales, Mucorales with one OTU and one undefined Ascomycota OTU.

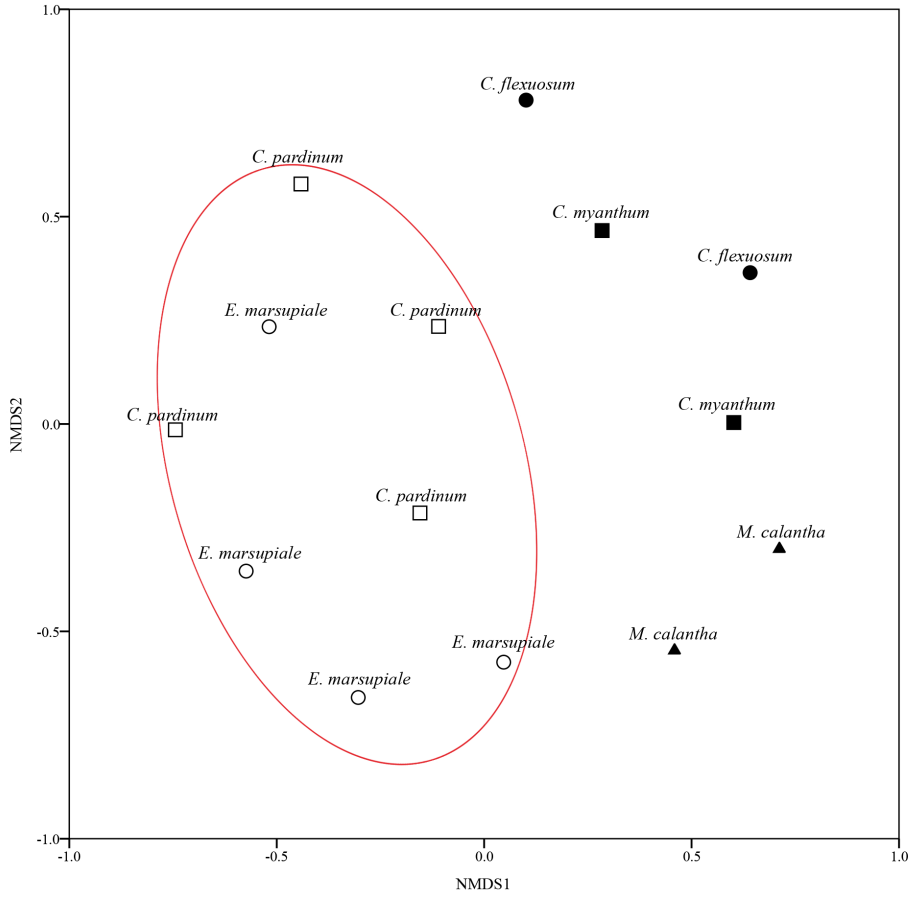

Fig. 22 Fungal endophytes communities detected in *Cyrtochilum flexuosum* (species 1, black dots), *Cyrtochilum myanthum* (species 2, black squares), *Maxillaria calantha* (species 3, black triangles), *Epidendrum marsupiale* (species 4, white dots) and *Cyrtochilum pardinum* (species 5, white squares), distributed in Podocarpus National Park (black figures) and Cajas National Park (white figures). Stress value = 0.109211.

4. Discussion

Diversity of root-associated fungal endophytes

Root fungal endophytes are generally considered as favorable inhabitants of plants that may contribute to their productivity and eventually to the maintenance of ecosystem functions (Jumpponen et al., 2017). In orchids, although the ecological implications of these fungal communities

are not yet clarified, a polyphyletic fungal group has been reported (Kohout et al., 2013). In this study, we applied Illumina MiSeq sequencing to characterize the diversity of root-associated fungal endophytes of five epiphytic orchid species (e.g. *C. flexuosum, C. myanthum, M. calantha, E. marsupiale* and *C. pardinum*) distributed in montane forests of Southern Ecuador. Out of the 130 collected individuals, 3413 OTUs of endophytic fungi were detected and up to 1718 different OTUs per orchid species. Although an important number of OTUs here identified, contrasting with earlier studies performed with traditional sequencing methods (e.g. Herrera et al., 2010; Kottke et al., 2013), the inventories at all sites were still incomplete. However, the use of Illumina MiSeq technology represents a powerful platform for the identification of the microbial communities, including rare species (Tedersoo et al., 2010a) than traditional sequencing methods (e.g. Sanger sequencing).

The detected OTUs belonged to the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla. Based on culture-dependent studies conducted in the same area, Herrera et al. (2010) and Novotná et al. (2018) identified much less endophytes. Indeed, Novotná et al. (2018) isolated 49 OTUs (13 orders) from three orchid species, including *C. myanthum,* while Herrera et al. (2010) identified 58 isolates (12 orders) from four orchid species belonging to Pleurothallidinae subtribe. Although, culture-dependent methods are necessary either to preserve the fungal diversity in culture collections and to perform *in vitro* studies focused on orchid-fungi interactions (Novotná et al., 2018), these methods largely underestimate the diversity (Waterman et al., 2011) mainly because not all fungi are able to grow under *in vitro* conditions (Kageyama et al., 2008). The culture-independent method used in the present study (e.g. NGS) yielded a much higher diversity, likely increasing our comprehension of fungal endophytes communities associated with orchids. However, this method is

destructive and no fungi could be preserved in collection. Both approaches should thus be considered as complementary and useful.

Our results revealed that the Agaricales was the most OTU-rich order (414 OTUs) summed over the six sampling sites. Members of Agaricales have been described as mycobionts of achlorophylous and green orchids (Bidartondo et al., 2004; Martos et al., 2009) but also as mycorrhizal symbionts of epiphytic orchids (Kartzinel et al., 2013b). Albeit, the most frequent OTU was OTU1 (identified in the 130 individuals), taxonomically assigned to *Hypoxylon griseobrunneum*, a member of Xylariales. Similar results were obtained in previous studies conducted in Southern Ecuador where Suárez et al. (2006) and Novotná et al. (2018) found that most of the isolates from the epiphytic orchids *Stelis* spp. belonged to the genus *Hypoxylon*. Moreover, in tropical latitudes, members of Xylariales have been reported as common, diverse and occasionally dominant root-associated endophyte (Yuan et al., 2009), present with different host plants, denoting preference and selectively to some extent (Chen et al., 2013). In other tropical regions from Central America and La Réunion, Cantharellales was the order with the highest species richness (Kartzinel et al., 2013b; Martos et al., 2012).

Fungal endophytes are probably part of a trade-off association that is still not fully understood (Kia et al., 2016). Undoubtedly, different fungal taxa impact differently orchid-fungi interactions through their contrasting set of traits (Kia et al., 2016). Consequently they may have an effect on plant productivity (Bever et al., 2012) and resistance to pathogen damages (e.g. Chen et al., 2013). According to their trophic mode, root-associated endophytes that inhabit in a plant roots could be symbiotroph, saprotroph, pathotroph-saprotroph, saprotroph-symbiotroph, pathotroph-symbiotroph or pathotroph (Kia et al., 2016; Ma et al., 2015). This was also clearly illustrated in the present study. Indeed, from the 1200 OTUs assigned to a

putative life strategy, most belonged to saprotrophs (544 OTUs), followed by symbiotrophs (297 OTUs) and to a lesser extend to pathotrophs (152 OTUs). There were also 79, 50, 47, 24 and 7 fungal endophytes OTUs assigned as pathotroph-saprotroph, saprotroph-symbiotroph, pathotroph-saprotroph-symbiotroph, pathotroph-symbiotroph and pathotroph-saprotroph-symbiotroph, respectively. The putative ecological roles of endophytic fungi, obtained in this study, and their effect on orchids needs to be further evaluated in order to comprehend the root-fungal Orchidobiome, which is then the diversity and community structure of fungi of the roots of orchids. Oliveira et al. (2014) suggested the necessity to clarify the effects of endophytic fungi on endangered orchids in Brazil, mainly because fungal communities potentially contribute to orchid adaptation to changing environmental conditions. It is thus necessary either to isolate fungi via culture-dependent techniques to investigate their potential role on orchids and to evaluate the total diversity and community structure to augment our knowledge on the potential organisms present that may have functional attributes. Nowadays, endophytic fungi on orchids remain poorly characterized because the proportion of OTUs identified may not be assigned to a species in public database (Ma et al., 2015; Oliveira et al., 2014). Thus, for fungal taxonomic work, it is of the highest priority to increase the DNA databases (Abarenkov et al., 2010a) that will allow a more comprehensive characterization of fungal communities associated to orchids but also to other plants and ecosystems. In addition, the use of transmission electron microscopy (e.g. Suárez et al., 2006, 2008) or the evaluation of nutrient flow (e.g. Cameron et al., 2006) could provide new evidence on the functional roles of root-associated fungal endophytes.

Communities of fungal endophyte associated with co-existing orchid species

The few studies on root-associated fungal endophytes conducted so far were mostly focused on species identification (e.g. Bayman and Otero, 2006; Boddington and Dearnaley, 2008). The potential drivers and how such drivers determine the assembly of fungal endophyte communities assembly have most often not been considered. In the present study, the root-associated fungal endophytes were evaluated in function of co-existing orchid species, considered as a potential driver for endophyte communities. In natural ecosystems co-existing species, in theory, do not compete for the same resources (Tilman, 1982) unless small-scale habitat heterogeneity is present and consequently a segregation of niches could be expected (Selosse, 2014). Studies on sympatric orchids reported distinctive mycorrhizal communities that could represent niche partitioning which may contribute to orchid co-existence (Cevallos et al., 2017; Jacquemyn et al., 2014). Our results at PNP corroborates these observations. Indeed, distinct endophyte communities were found between co-existing orchid species at both study sites. For instance, we found mycorrhizal fungi that promote nutrient uptake (Rasmussen, 2002) but also non-mycorrhizal fungi that are thought to be sources of bioactive compounds (Xing et al., 2015). Such diversity in fungal functional attributes could help orchids to adapt to the changing environmental conditions (Oliveira et al., 2014). Considering the mutualism-parasitism continuum hypothesis (Johnson et al., 1997), changes in ecological context could cause a transition from mutualism to parasitism or vice versa. In orchid communities, species form complex networks of orchid-fungi interactions (Martos et al., 2012), although such associations do not always have benefits for both partners. For instance, at the early developmental stages of the orchid, the associated fungi do not receive any reward from the orchid seeds (Smith and Read, 2008), which has been

considered parasitism to some extent (Dearnaley et al., 2007). Meanwhile, the interaction between fungi and adult orchids, where both partners have benefits (or at least a transitory backflow), is recognized as mutualism (Cameron et al., 2006; Fochi et al., 2017). In some interactions, fungi that facilitate seed germination could also associate with orchids at the adult stage (Cameron et al., 2008). In this case, the transition of the relationship, from parasitism to mutualism, could be given in function of the orchid life cycle (van der Heijden et al., 2015).

In addition, host phylogeny seems to drive fungal communities assemblages. This was evidenced by Jacquemyn et al. (2011b) who identified similar mycorrhizal fungi associated to different *Orchis* species. Likewise, in the Angraecoid orchid subtribe the reported mycorrhizal fungi were closely related species (Martos et al., 2012). Interestingly, in the present study we found (in site 1) that closely related *Cyrtochilum* species shared more similar endophytic fungal communities among each other than with *M. calantha*, in accordance with the results of Chapter II. This observation was, however, not repeated at site 2. Therefore, increasing the number of individuals, at both sites, should contribute to corroborate if the host phylogeny is a driver for endophyte community composition.

In contrast to the findings at sites 1 and 2 (PNP), co-existing orchids *E. marsupiale* and *C. pardinum*, at CNP (sites 3, 4, 5 and 6) showed similar fungal endophyte communities. Although *E. marsupiale* and *C. pardinum* belong to distinct tribes (Epidendreae and Cymbidieae, respectively), host phylogeny in this case apparently is not a determining driver for endophyte community structure. Phylogenetic distances within the Epidendroideae subfamily have been recently evaluated and it was reported that Epidendreae and Cymbidieae tribes were closely related (Freudenstein and Chase, 2015).

Apart from host phylogeny, fungal endophyte community composition could be influenced by many abiotic and biotic factors (Barnes et al., 2016). For instance, the altitude is widely recognized as an important factor that determines the fungal communities in soil, especially in the Andes (Geml et al., 2014). Although microorganisms are considered globally cosmopolitan, abiotic factors probably impact the fungal diversity, especially in habitats with harsh environmental conditions (Jumpponen et al., 2017). However, the 45 OTUs identified at both PNP and CNP, seem not to be restricted by abiotic factors. It is likely that the fungi that was found in all studied orchids in both areas of montane rainforest, have exceptional characteristics and are prone to develop under a more wider gradient of abiotic conditions (Cray et al., 2013).

Root-associated endophyte fungi across orchid population

Factors that could drive fungal endophyte community composition across orchid population are still no fully described or assessed. Environment and geography have been shown to affect the diversity and composition of some root-associated fungal communities but the effect of each factor could be variable or specific across ecosystems (Barnes et al., 2016). Here we showed that root endophyte communities associated with *C. flexuosum, C. myanthum* and *M. calantha,* evaluated independently per orchid species, varied substantially across orchid population, suggesting that host phylogeny is not a determining factor for endophyte community composition. Our results corroborate previous observation of Jacquemyn et al. (2016a) on mycorrhizal fungi, revealing that fungi can be characteristic of a specific site as a result of local conditions. This supported the theory that orchid endophytic fungi are subjected to local selection because of particular environmental conditions (Ma et al., 2015) at each sample site. At PNP, the study sites have similar forest structure and are classified as evergreen upper montane forests (Beck et al., 2008), but with peculiar temperature and rain

conditions. Oliveira et al. (2014) reported similar results in an early study in a Neotropical region in Brazil, where *Hadrolaelia jongheana, Hoffmannseggella caulescens,* and *H. cinnabarina* displayed different endophyte community compositions with few overlapping fungal endophytes as consequence of local factors (soil conditions, vegetation and climate).

Our results at CNP showed that populations of *E. marsupiale* and *C. pardinum* had similar endophyte communities. This was probably because the sites at CNP are located in the same mountain foothill under similar temperature and rainfall conditions (ETAPA, 2005), corroborating the effect of the site under fungal endophyte communities. Moreover, it is likely that proximity between sites could facilitate fungal dispersal (Jumpponen et al., 2017). As a consequence, overlapping endophytic fungi are more probable. Notwithstanding that very little is known about the restrictions of fungal endophyte distribution (Queloz et al., 2011), the possibility that our results were somewhat the consequence of geographic proximity between sites or an effect of contrasting environmental condition due to high altitudinal levels at montane areas, cannot be ruled out. Moreover, it is not excluded that orchid species in CNP present an ancestral ecological conservatism in endophyte preferences. Because, identified fungi are not specifically associated with certain orchid species, probably orchids have a preference for such widely distributed fungi (Otero et al., 2007).

In conclusion, the fungal endophyte communities assessed on epiphytic orchids seem to follow different strategies of assembly. On the one hand, fungal endophytes at PNP appears to be impacted by abiotic factors, while the results at CNP suggest that the orchid's evolutionary history is likely the major determinant for the composition of fungal communities. Either way, the assessment of additional orchid species and sites could help

elucidating which factors are determinant for root-associated fungal endophytes.

Endophyte communities between the two areas of montane rainforest (PNP vs CNP)

Sampling approaches at both areas of montane rainforest were not totally comparable because each one has specific environmental conditions that resulted in particular ecosystem structure (Baquero, 2004) in addition to the specific orchid species sampled per area. Thus, a thorough analysis combining data from both areas of montane rainforest to make inferences about the effect of biotic or abiotic factors on OMF communities was beyond the scope of this study. However, the contrast of the all the fungal endophytes associated with epiphytic orchids (independently of orchid species) between the two areas of montane rainforest could give some insights about the fungal community structure at a larger scale. We showed that orchid root-associated fungal endophyte communities were highly different between PNP and CNP. Our results corroborate the hypothesis of Baas Becking (1934) that local environmental condition could configure fungal endophyte communities, assuming the hypothesis that microorganisms are widely distributed and the environment is shaping which ones are able to grow. In addition to the distinct fungal endophyte communities' composition at PNP and CNP, we also identified a set of endophytic fungi that were present in both areas of montane rainforest. Albeit the distance between both areas of montane rainforest is approximately 125 km, 45 OTUs-fungi core-species (21 orders) were found in both situations. These fungi were mycorrhizal, saprobes or latent pathogens. Following the premise that endophytes are cosmopolitan (Baas Becking, 1934), it is likely that overlapping endophytic fungi have large population distribution (Fitter, 2005; Jumpponen et al., 2017) but may not be very specialized. Overlapping endophytes probably have ecological

plasticity and are able to play different roles in the interaction (Pecoraro et al., 2017). We hypothesize that the fungal core-species could represent and advantage to fill some physiological demands (e.g. nutritional or pathogen defense) when other fungi are not available. Clarifying the potential roles of the fungal endophytes in the orchid life cycle needs to be explored to better understand the ecological dynamics of plant-fungi interactions (Oliveira et al., 2014).

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CHAPTER II

Are there keystone mycorrhizal fungi associated to tropical epiphytic orchids?

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Preface

In **Chapter I,** we characterized the root-associated fungal endophytes associated with the sympatric orchids *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* at two sites located close to the Podocarpus National Park (PNP) and *Epidendrum marsupiale* and *Cyrtochilum pardinum* at Cajas National Park (CNP). A polyphyletic group of fungi putatively assigned to mycorrhizal and non-mycorrhizal fungi were identified. We demonstrated that host phylogeny and site of orchid occurrence are determinant factors of the fungal endophyte communities associated with orchids at PNP, while this was not true at CNP.

From the whole diversity of endophytic fungi associated with orchid roots, it is recognized that mycorrhizal fungi provide carbon and inorganic nutrients for germination and subsequent development (Smith and Read, 2008). The current available information is mostly on the identity of the fungi that are involved in the symbiosis but little is known about the factors that affect the spatial distribution of these mycorrhizal fungi (Jacquemyn et al., 2012a). With the increasing use of molecular techniques, a wider group of fungi could be investigated. In studies conducted on terrestrial orchids, it was shown that next generation sequencing allowed to identify a higher diversity of orchid mycorrhizal fungi (OMF) than using first generation sequencing, thus offering valuable information to understand the potential impact of environmental factors on orchid-fungi symbiosis (Jacquemyn et al., 2016a; Oja et al., 2015). Thus, in **Chapter II,** using Illumina MiSeq technology we characterized the OMF of *Cyrtochilum flexuosum, C. myanthum* and *M. calantha*). We identified significant differences of mycorrhizal communities between orchid species and across orchid population but there was a core of mycorrhizal fungal species that are widely identified along all orchid species and populations.

Abstract

In epiphytic orchids, distinctive groups of fungi are involved in the symbiotic association. However, little is known about the factors that determine the mycorrhizal community structure. Here we analysed the orchid mycorrhizal fungi communities associated with three sympatric Cymbidieae epiphytic tropical orchids (*Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha*) at two sites located within the montane rainforest of Southern Ecuador. To characterize these communities at each orchid population, the ITS2 region was analysed by Illumina MiSeq technology. Fifty-five mycorrhizal fungi operational taxonomic units (OTUs) putatively attributed to members of Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae were identified. Significant differences in mycorrhizal communities were detected between the three sympatric orchid species as well as among sites/populations. Interestingly, some mycorrhizal OTUs overlapped among orchid populations. Our results suggested that populations of studied epiphytic orchids have site-adjusted mycorrhizal communities structured around keystone fungal species. Interaction with multiple mycorrhizal fungi could favor orchid site occurrence and co-existence among several orchid species.

Keywords*:*

Epiphytic tropical orchids, Co-existing species, Illumina sequencing, ITS2, Microbial community assembly, OTU reconstruction

1. Introduction

In natural environments, most orchid species depend on mycorrhizal fungi for seed germination and subsequent seedling development (Smith and Read, 2008). This association often remains effective until orchids reach the photosynthetic stage (Dearnaley et al.,

2012). In green orchids, the mycorrhizal fungi include a polyphyletic group of basidiomycetes (Dearnaley et al., 2012) and in the specific case of epiphytic orchids, members of Ceratobasidiaceae, Serendipitaceae, Tulasnellaceae and Atractiellomycetes (Suárez et al., 2006, 2008; Kottke et al., 2010; Valadares et al., 2015) are generally reported. The association with appropriate mycorrhizal fungi is critical for the survival of epiphytic orchids that often develop under harsh conditions characterized for instance by water shortages and a lack of organic and inorganic nutrients (Zotz and Hietz, 2001; Martos et al., 2012).

There are a number of aspects of the orchid mycorrhizal fungi (OMF) association not yet fully understood. A standing question in this regard is whether orchid species depend on a specific set of mycorrhizal fungi or can be equally or better promoted by a broad spectrum of fungi (Kartzinel et al., 2013b; McCormick and Jacquemyn 2014). There is evidence indicating that the lack of suitable mycorrhizal fungi could represent a limiting factor for epiphytic orchid species in terms of spatial distributions and population fitness (McCormick and Jacquemyn, 2014). Other studies dealing with terrestrial orchid populations have shown changes in their mycorrhizal associations across different sites (Jacquemyn et al., 2012b; Těšitelová et al., 2015) and between co-existing species (Waterman et al., 2011; Jacquemyn et al., 2014). Recently, it has been suggested that the distribution of OMF is, to some extent, variable across sites depending on the local environmental conditions (Jacquemyn et al., 2014).

In sympatric orchid species, the differences in the associated OMF communities might contribute to species co-existence by reducing competition for nutrients and thereby leading to niche partitioning (McCormick and Jacquemyn, 2014). However, few overlaps between OMF communities have been observed in sympatric orchid species to

date (Jacquemyn et al., 2014; Pellegrino et al., 2014). It could be expected that sympatric orchids share some generalist mycorrhizal fungi, frequently available and well integrated in OMF networks that contribute to community stability in stressful situations (Kottke et al., 2013; Suárez et al., 2016). The high diversity of orchid species present in the Andean montane forest makes it relevant to address whether plants depend on specific fungal species. So far, investigations on OMF in Andean montane rainforests have concentrated on only one orchid population per study and therefore do not provide strong clues on OMF community structure variation across orchid distribution sites. Studies conducted in Southern Ecuador described the OMF communities associated to epiphytic species of *Pleurothallis, Stelis* and *Epidendrum* (Suárez et al., 2006, 2008; Kottke et al., 2010; Riofrío et al., 2013). *Tulasnella spp.* appeared as the most frequent mycorrhizal group followed by members of *Atractielalles* and *Serendipitaceae* (Suárez and Kottke, 2016). Investigating whether the site has an effect on the structure of associated mycorrhizal communities can improve our understanding of orchid fungal associations in a highly diverse scenario like the tropical montane forest of Ecuador.

In the present study we investigated the OMF communities associated to three epiphytic orchid species in two different sites of the montane rainforest in Southern Ecuador. We sampled two populations of the species *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* and assessed the structure of the associated OMF by means of metagenomics of the internal transcribed spacer 2 sequenced using the Illumina MiSeq technology. With this experimental setup, we aimed at addressing the following questions: (i) How does mycorrhizal community differ among co-existing orchid species? and (ii) Does mycorrhizal community differ between the two sites?

Since the studied orchid species are sympatric, we expected OMF communities to overlap to some extent between co-existing species. We also expect similar OMF preference patterns across study sites, assuming that orchid distribution could be limited by the presence of distinctive mycorrhizal fungi.

2. Materials and Methods

Study areas and sampling scheme

The study was conducted at two sites of the evergreen upper montane forest (Beck et al., 2008) close to the Podocarpus National Park, in the Southern Ecuadorian Andes. The first site, Curva Misteriosa (site 1), is located between 2050-2250 m a.s.l. and the second site, El Tiro (site 2) is located between 2600-2800 m a.s.l. (see page **54**, section **2.1 Podocarpus National Park (PNP) and surrounding areas**). The sites were chosen after monitoring the distribution of the epiphytic orchids *Cyrtochilum flexuosum* (species 1, Fig. 6)*, Cyrtochilum myanthum* (Lindl.) (species 2, Fig. 7) and *Maxillaria calantha* (species 3, Fig. 12). In total, 68 orchid individuals were sampled (see page **59**, section **4.1 Sampling of natural orchid populations**) and microscopically analysed to verify the mycorrhizal colonization (see page **60**, section **5. Screening of orchid root-associated fungi**). Root sections with evidence of mycorrhizal colonization were surface disinfected (see page **61**, section **6.1. External root disinfection**) meanwhile no further analysis was performed for the remaining non-mycorrhizal root sections.

Molecular analysis and Illumina sequencing

Total DNA was extracted from 1 to 2 cm-long portions of colonized roots using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol (see page **61**, section **6.2.1 DNA isolation from root of adult orchids**). The parameters for the amplification of the

internal transcribed spacer 2 region (ITS2) are described in page **63**, section **6.3.1 PCR for DNA isolated from roots of adult orchids**. The samples were further analysed using the Illumina MiSeq® technology (see page **65**, section **6.5 Sequencing**).

Bioinformatics analysis

The raw Illumina data obtained from the 47 samples was used for OTUs reconstruction with the UPARSE software (Edgar, 2013) (see page **66**, section **7. Bioinformatics analysis**).

Data analysis

OTUs matching a taxonomical assignment consistent with known OMF associated to tropical orchids were selected for further analysis (Suárez et al., 2006, 2008; Kottke et al., 2010; Martos et al., 2012; Riofrío et al., 2013). OMF-OTUs information was converted into a presence-absence matrix on a per-sample basis to make inferences on OMF composition as a function of host orchid species and ecological sites. The presence/absence matrix was built in a way that the columns corresponded to each of the six sampled species/site pools e.g. species 1 site 1, species 2 site 1, species 3 site 1 and so on. Binary matrix of the OMF-OTUs identified at each site was used as input data to calculate fungal accumulation curves and richness (e.g. Senés-Guerrero et al., 2014) (see page **67**, section **8.2 Alpha diversity**). Furthermore, differences in mycorrhizal communities among individuals of co-existing species were calculated pairwise using the Jaccard similarity index (Jacquemyn et al., 2015a) (see page **67** and **68**, section **8.3 Beta diversity**). Additionally, variation in mycorrhizal communities between orchid species was evaluated by one-way analysis of variance (ANOVA) for each orchid species with Tukey's HSD test and to test whether mycorrhizal communities differed between the two sites using the binary

data from the orchid populations independently by species a permutational analysis of variance was conducted (see page **67** and **68**, section **8.3 Beta diversity**).

3. Results

Fungal OTUs

After verifying the extent of mycorrhizal colonization in root cortical cells (which was observed in 54 of the 68 sampled orchids), high quality amplicons for the internal transcribed spacer 2 region (ITS2) were successfully obtained from the DNA of 47 root samples. Amplicons were distributed as follows: 19 samples from site 1 and 28 from site 2. Illumina sequencing of these amplicons yielded 965160 raw paired-end 300 bp long reads. OTUs reconstruction from quality-filtered Illumina reads resulted in 2957 fungal OTUs after singleton and chimeric sequences removal. OTUs were defined based on a 97% sequence similarity cutoff. During the OTUs reconstruction process, singletons represented 2.5% of all the reconstructed sequences while chimeric sequences corresponded to 5.4%. As mentioned before, both types of low confidence results were removed from the final OTUs set. BLAST analysis of the representative sequences of each OTU against the UNITE database revealed the presence of sequences corresponding to both mycorrhizal and non-mycorrhizal fungi including Ascomycetes and other generalist soil fungi (data shown in Chapter I).

Overall mycorrhizal fungi diversity

A total of 55 OTUs were assigned to mycorrhizal fungi that are known to be associated with green orchids (which will be regarded from now on as OMF-OTUs). Of these, 32 OTUs corresponding to members of Serendipitaceae, 14 OTUs to Ceratobasidiaceae and nine OTUs to Tulasnellaceae were identified. The representative sequences for

mycorrhizal OTUs were deposited in GenBank (Bioproject PRJNA344001). In almost all orchids, OTUs belonging to more than one OMF genus were found to inhabit the same root. Of the 55 OMF-OTUs, 36 were detected at site 1 and 39 at site 2. Twenty-three OMF-OTUs were found in association to a single orchid species at a particular site (Table S4), 24 OMF-OTUs appeared to be shared among a subset of species (Fig. 23 a, b), and eight OMF-OTUs were present at both sites shared by all orchid species (Table S5). The per site number of identified OMF-OTUs is representative of the expected diversity and accounted for the 70% and 71% of expected diversity in site 1 and site 2 respectively. However, based on our sampling efforts OMF-OTUs accumulation curves did not reach the asymptotes (Fig. 24).

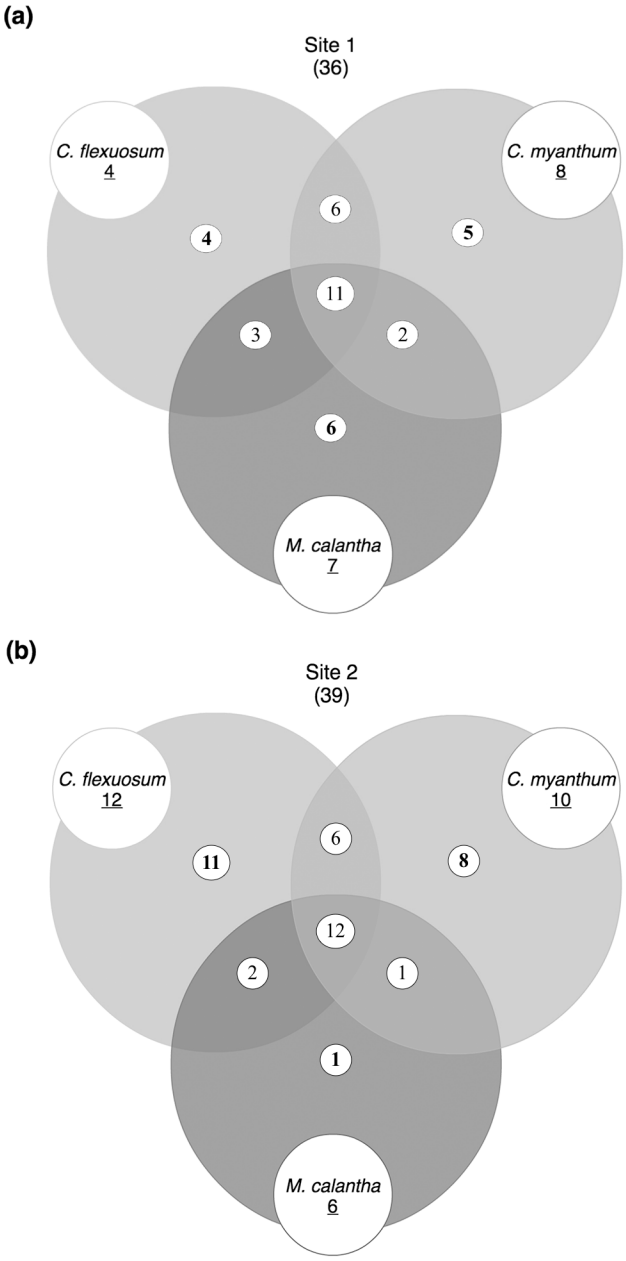

Fig. 23 Per-site and per-orchid species distribution of OMF-OTUs. The total number of identified OMF-OTUs is shown within brackets below the name of the corresponding site. Numbers of plant replicates (successfully amplified) at each site are also shown with underlined numbers below the name of the corresponding orchid species. The rest of the numbers represent OMF-OTUs (a, b).

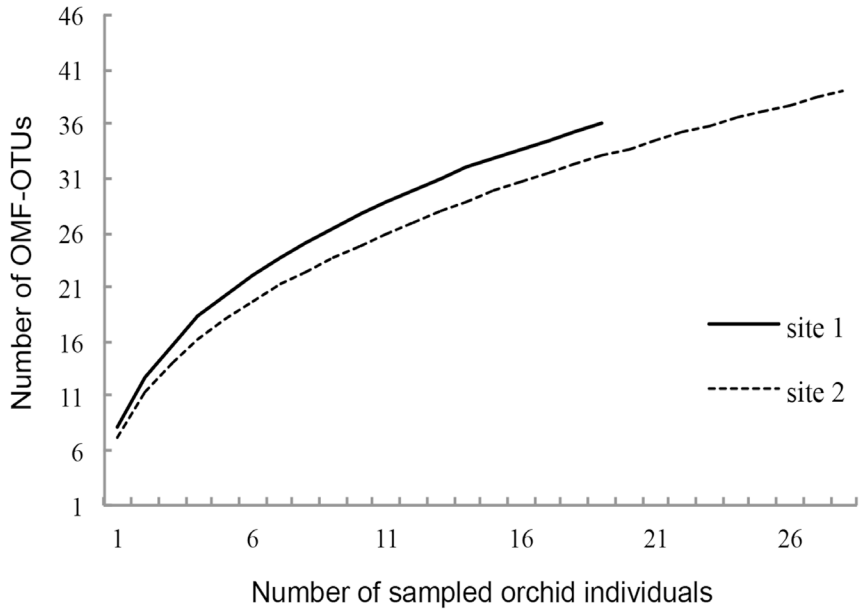

Fig. 24 Rarefaction analysis performed on the OMF-OTUs richness obtained from orchid individuals of the three studied species (C. *flexuosum, C. myanthum*, and *M. calantha*) at Curva Misteriosa (site 1) and El Tiro (site 2) sites.

Per-site mycorrhizal communities shared among co-existing orchid species

The similarity between associated OMF communities was assessed in pairs of co-existing orchids species *C. flexuosum*, *C. myanthum* and *M. calantha* at each site. For each possible comparison, Jaccard indexes were obtained by contrasting binary vectors that describe the presence/absence of OMF-OTUs associated to individuals of each species pair. While analysing the variance of such Jaccard indexes (dependent variable) in function of the phylogenetic distance (factor) across co-existing orchid species by means of a one-way ANOVA, we obtained the results shown in Table 3. At both sites, the similarity of associated OMF communities varied significantly as a function of the phylogenetic distance that separates the three orchid species (p=0.001 for site 1 and p=0.012 for site 2). At site 1, the

similarity between OMF communities associated to *C. flexuosum* and *C. myanthum* was significantly higher than the similarity between OMF communities associated to either species and to *M. calantha*. Whereas at site 2 the similarity between OMF communities associated to *C. myanthum* and *M. calantha* was significantly higher than the similarity between OMF communities associated to the close relatives in *C. flexuosum* and *C. myanthum*

Table 3 Analysis of variance of the similarity between OMF communities associated to individuals of pairs of co-existing orchid species *C. flexuosum, C. myanthum,* and *M. calantha* at both study sites. The similarity between associated OMF communities is expressed as a Jaccard index.

Comparison	Site 1		Site 2	
	Jaccard indexes mean difference ± std. error	*P*-value	Jaccard indexes mean difference ± std. error	*P*-value
C. flexuosum-C. myanthum vs. *C. myanthum-M. calantha*	0.147677*± 043	0.003	-0.090233*± 0.030	0.009
C. flexuosum-C. myanthum vs. *C. flexuosum-M. calantha*	0.148025*± 0.043	0.003	-0.032100± 0.030	0.533
C. myanthum-M. calantha vs. *C. flexuosum-C. myanthum*	0.1476778* ± 0.044	0.003	0.090233* ± 0.030	0.009
C. myanthum-M. calantha vs. *C. flexuosum-M. calantha*	0.000347 ± 0.043	1.000	0.058133 ± 0.030	0.030
C. flexuosum-M. calantha vs. *C. flexuosum-C. myanthum*	-0.148025* ± 0.043	0.003	0.032100 ± 0.030	0.533
C. flexuosum-M. calantha vs. *C. myanthum-M. calantha*	0.000347 ± 0.043	1.000	-0.058133 ± 0.030	0.133

*The difference between Jaccard indexes is considered statistically significant if *P*-value ≤0.05.

Between-sites and per species mycorrhizal communities

PERMANOVA analysis conducted to test whether the site influenced the OMF communities associated to each of the studied orchid species showed that the OMF communities associated to individuals of *C. myanthum* as well as those associated to individuals of *M. calantha* changed significantly across study sites (*P*-value = 0.008 and *P*-value = 0.028, respectively). No significant change was observed across sites in the OMF communities associated to individuals of *C. flexuosum* (*P*-value = 0.17). This result should be taken with caution since the analysis is skewed by the unequal number of sampled individuals of *C. flexuosum* at each site (4 individuals at site 1 and 12 individuals at site 2).

4. Discussion

Overall mycorrhizal fungi diversity

Mycorrhizal fungi communities associated to the epiphytic tropical species *C. flexuosum*, *C. myanthum* and *M. calantha* (Cymbidieae tribe) were assessed for the first time. Using an Illumina ITS2 targeted sequencing approach, a large diversity of OMF was recovered, representing 32 Serendipitaceae OTUs, 14 Ceratobasidiaceae OTUs and nine Tulasnellaceae OTUs. These three families have been identified as the prime mycorrhizal partners in a wide number of tropical and temperate orchids (Dearnaley et al., 2012). Our results supported the large diversity of OMF associated to tropical epiphytic orchids in the montane forests previously reported (e.g. Suárez et al., 2006, 2008; Kottke et al., 2010; Riofrío et al., 2013). Although the use of different PCR primer pairs makes studies not directly comparable, previous studies carried out on orchid species from the Epidendreae tribe e.g. *Pleurothallis*, *Stelis* (Suárez et al., 2006, 2008), *Epidendrum* (Riofrío et al., 2013) and *Teagueia* (Suárez et al., 2016), have shown Tulasnellaceae as the

main fungal group, followed by Serendipitaceae and Atractiellales. OTUs from Ceratobasidiaceae were not reported in these studies (Kottke et al., 2013, Suárez et al., 2016). Other studies, which are comparable to ours in terms of primer pair used (ITS86F and ITS4), and which addressed OMF of *Anacamptis, Gymnadenia* and *Orchis* genera (tribe Orchideae), showed Ceratobasidiaceae as their main fungal partner. The latter studies also showed that the predominant fungal group tends to vary across orchid species and even sites (Jacquemyn et al., 2016a, Waud et al., 2016).

Per-site mycorrhizal communities among co-existing orchid species

The presence of appropriate fungal partners is indispensable to an orchid's life (Dearnaley et al., 2012). Our findings are in line with this premise demonstrating that co-existing orchid species at a given site tend to associate with different OMF communities. Similar results have been observed by Jacquemyn et al. (2014, 2016a) and Pellegrino et al. (2014) who reported that co-existing terrestrial orchid species (tribe Orchideae) were associated to different OMF communities. In situations of sympatry, the association with distinct mycorrhizal communities is likely an advantage, decreasing competition (McCormick and Jacquemyn, 2014). In fact, different fungal lineages are expected to rely on the access to different nutrient resources (Pellegrino et al., 2014). Conversely, associations with the same fungal community members could represent a disadvantage for orchids when obtaining resources from the environment.

At site 1, the observed mycorrhizal communities associated to individuals of both *Cyrtochilum* species exhibited higher similarities to each other than with the community associated to individuals of *M. calantha.* These results are in agreement with previous studies showing that the similarity among mycorrhizal communities associated to terrestrial *Orchis* species follow a trend that is significantly correlated with the phylogenetic

distances among orchid species (Jacquemyn et al., 2011a, 2012b). Another previous study with epiphytic orchid species revealed that closely related species were associated preferentially with the same or related fungi (Martos et al., 2012). We thus interpreted that the observed high similarity between OMF communities associated to *Cyrtochilum* species could be due to their phylogenetic relatedness, the same for the lower similarity with *M. calantha*. The phylogenetic distance between *Cyrtochilum* and *Maxillaria* genera has been recently confirmed by Freudenstein and Chase, (2015). Similar composition of OMF communities among related orchid species could represent part of an ancestral ecological niche (Losos, 2008) persistent along a co-evolution process as shown in the recently evolved *Teagueia* species where the same subset of OMF was broadly associated with related species (Suárez et al., 2016).

In contrast to site 1, at site 2 the OMF communities were not significantly more similar between *Cyrtochilum* species when compared to the community associated to *M. calantha*. Instead of this, OMF communities were less divergent between *C. myanthum* and *M. calantha.* This apparent absence of correlation between OMF communities' structure with the phylogenetic relationship of their hosts might be due to unequal micro-environmental conditions across study sites.

Mycorrhizal communities between sites

When assessing whether there is a "site" effect on the composition of mycorrhizal communities associated to each of the three orchid species by a PERMANOVA analysis, we observed that only *C. flexuosum* has consistent similar OMF communities at both sites/populations. As mentioned before, this result must be interpreted with caution due to the unequal number of individuals sampled between populations. In contrast, each population of *C. myanthum* and *M. calantha* were associated to a distinctive

mycorrhizal community at each site. In line with our observation, a recent study carried out in Costa Rica showed divergent OMF communities among eleven populations of *Epidendrum firmum* from different cordilleras, with little correlation to elevation, temperature and precipitation seasonality (Kartzinel et al., 2013b) After phylogenetic OMF diversity analysis across orchid populations, these authors suggested that distinctive mycorrhizal associations could represent a symbiotic assurance for orchid persistence, when OMF are stochastically available in disturbed or dynamic habitats (Kartzinel et al., 2013b). Similar results have been obtained in the tropical orchid species *Dendrobium officinale* and *D. fimbriatum,* evaluated at two sites within a subtropical climate (Xing et al., 2013). Different associated OMF were observed for two *D. officinale* populations, with only one mycorrhizal fungal species shared. In contrast, *D. fimbriatum* populations have almost complete overlapping between associated fungi communities with the exception of a unique OMF identified exclusively at one study site (Xing et al., 2013).

In our study, although OMF communities were significantly different between sites/populations, patterns of association were consistent in terms of the frequency distribution of mycorrhizal OTUs identified per mycorrhizal group between sites/populations. This implies that at both sites/populations the OMF genera's frequency increase in the order *Tulasnella*<*Ceratobasidium*<*Serendipita*. Although, genera frequency patterns were similar between orchid populations, each one has a specific composition of OMF-OTUs.

This observation supported the concept that the composition of mycorrhizal communities can be site-adjusted as has been observed in populations of Mediterranean orchids (Jacquemyn et al., 2014). Interestingly, our results also showed mycorrhizal overlap between orchid populations of the same species across sites. *C. myanthum* populations

shared 15 OMF-OTUs and *M. calantha* populations shared 10 OMF-OTUs (data not shown). Thereby, we suspected that mycorrhizal communities could be site-adjusted around a core of keystone species that are globally distributed (Table S5). Persistent keystone partners apparently occurred in some photosynthetic orchids with variable degrees of specificity among orchid species (Jacquemyn et al., 2010; Xing et al., 2013), sometimes considered as generalist with wide distribution in various ecological conditions (Bailarote et al., 2012). It is fair to say that we are still far from starting to understand how OMF communities are built and the factors, which determine their specificity.

Orchids-mycorrhizal symbiosis involved multiple fungal species that, in the ecological context, could play divergent roles but in a synergistic fashion to ensure orchids adaptability (Table S4, Table S5). On the one hand, species that appeared like keystone core of OMF communities likely have a central role in local mycorrhizal assembling and species resilience as has been observed in AMF (see Guimarães et al., 2011). Less frequent OMF associated with a specific site/community might be contributing to orchid species resilience (Pellegrino et al., 2014) by reducing the competitive interaction among the members of the OMF community and consequently a diverse mycorrhizal community could improve plant's adaptability (see Hart et al., 2013).

Conclusions

Illumina-based ITS2 region barcoding analysis showed that mycorrhizal communities associated to co-existing epiphytic orchid species from two Andean study sites vary in accordance to their host phylogenetic relatedness. This suggests that studied co-existing orchid species occupy distinctive fungal micro-niches. Further, our results taken from these sites showed that particular site/population OMF communities are built around

keystone mycorrhizal fungi that might represent an ancestral fungal niche. Further approaches are necessary to confirm if this tendency occurs widely among sites of orchid occurrence and between species. Such studies will be crucial to elucidate which are the key mycorrhizal fungi that potentially contribute to the distribution of orchid species. We foresee that studies achieving a greater phylogenetic resolution of both orchids and associated fungi could contribute to reveal other features of their association.

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CHAPTER III

In situ orchid seedling-trap experiment shows few keystone and many randomly-associated mycorrhizal fungal species during early plant colonization

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Preface

In **Chapter I,** we observed that root-associated fungal endophytes associated with five epiphytic orchids distributed at Podocarpus National Park and Cajas National Park, were differently affected by abiotic and biotic factors. However, a set of overlapping fungi was identified at all sites and species. We also found that phylogenetic imprints and local site environmental conditions could shape the structure of root-associated fungal endophyte communities. In C**hapter II,** we further found that *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* are associated with distinct mycorrhizal fungi communities across populations but, similarly as in **Chapter I**, there was a group of overlapping fungi. It is likely that populations of studied orchids have site-adjusted mycorrhizal communities.

The factors that affect the composition of fungi communities associated with epiphytic orchids are not always possible to evaluate because not all orchids have wide distribution. Thus, in **Chapter III,** field experiments were conducted to test the effects of abiotic and biotic factors on mycorrhizal communities associated with *Cyrtochilum retusum* and *Epidendrum macrum.* With the analysis of the Illumina-based ITS2 region barcoding we evaluated specifically whether orchid species, site of orchid distribution, altitudinal level or temporal variation affected the composition of orchid mycorrhizal fungi communities.

Abstract

Orchids are known for their vast diversity and interdependency with mycorrhizal fungi. Under *in situ* conditions, the biotic and abiotic factors determining the composition and distribution of mycorrhizal fungal communities in orchids remain largely unexplored. Therefore *in situ* experiments are needed to better understand the interactions between orchids and fungi. A seedling-trap experiment was conducted in the Andes of Southern Ecuador in the Reserva Biológica San Francisco, a well-known biodiversity hotspot, to investigate the effect of orchid species, site, altitude or temporal variation on orchid mycorrhizal fungi (OMF) associated with *Cyrtochilum retusum* and *Epidendrum macrum*. Illumina MiSeq sequencing of the internal transcribed spacer (ITS2) region was used to determine the OMF community composition. The results exhibited 83 OMF operational taxonomic units belonging to Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and Atractiellales. The OMF community compositions were different between orchid species, altitudinal level and temporal variation but were not different between sites. The combination of using in situ trap plants and Illumina-based ITS2 analysis provided unique insights on OMF community composition, supporting that orchid seedlings have a core of keystone OMF that are ubiquitously distributed and stable across temporal change while the majority are randomly associated.

Keywords:

Biotic and abiotic factors, Keystone mycorrhizal, Mycorrhizal fungi, Seedling-trap experiment, Temporal variation

1. Introduction

In nature, orchids rely on particular interactions with their pollinators and root fungal associates (e.g. the mycorrhizal fungi) for

completing their life cycle (Selosse, 2014). Orchid mycorrhizal fungi (OMF) influence plant development at different life stages (Cameron et al., 2006). For instance, the tiny seeds of orchids lack carbohydrate reserves making them dependent on mycorrhizal fungi for germination and subsequent development into protocorms (Smith and Read, 2008).

It is widely accepted that local abundance and population dynamics of orchids is largely dependent on mycorrhizal fungi (McCormick and Jacquemyn, 2014). However, even if an increasing number of OMF have been identified (Kartzinel et al., 2013b; Kohout et al., 2013), their community structures and the factors affecting their spatial distribution in natural environments, remains poorly explored (McCormick and Jacquemyn, 2014).

Across the last three decades, our knowledge on the diversity and community composition of OMF has increased markedly with the development of powerful sequencing technologies (Merckx, 2013). With Sanger sequencing technology, fungi within the Tulasnellaceae (Suárez et al., 2006, 2016; Riofrío et al., 2013; Herrera et al., 2018), Serendipitaceae (Suárez et al., 2008), Ceratobasidiaceae (Otero et al., 2004) and Atractiellales (Kottke et al., 2010) were reported as dominant associates of tropical orchids. The development of next generation sequencing (NGS) technologies (e.g. 454 and Illumina), has further helped to improve the characterization of fungal communities and assess their ecological dynamics (Cardenas and Tiedje, 2008). For instance, using Illumina MiSeq amplicon sequencing, Herrera et al. (2018) demonstrated that OMF communities are composed of a core of widely distributed generalist fungal OTUs (Tulasnella-OTUs), interacting with rare and common orchid species. In Chapter II and Herrera et al. (in press) further noticed that OMF communities were site-adjusted, consisting of a core of generalists and ubiquitous keystone OMF-OTUs (stable component) plus OTUs identified at

specific site orchid-populations (dynamic component). This suggests that the structure of the dynamic component of the OMF communities are determined by local environmental conditions and host`s evolutionary history and that keystone species occur irrespective of the orchid species or site. Conversely, in temperate regions, significant differences in OMF communities were reported across orchid populations (Jacquemyn et al., 2012b) or species (Těšitelová et al., 2015) and through temporal variation (Oja et al., 2015), without a core of generalist and overlapped OMF communities.

A number of biotic and abiotic factors (e.g. orchid species, site, temporal variation) have been reported to impact the structure of OMF communities (Jacquemyn et al., 2012b; Ercole et al., 2015; Esposito et al., 2016; Cevallos et al., 2017). However, it is often difficult to generalize the ecological premises to all orchid species or geographic regions, mainly because particular combination of environmental factors may markedly influence the local OMF community structure (Peay et al., 2010). Most data on the orchid-fungi assemblages comes from *in vitro* experiments (e.g. Těšitelová et al., 2012; Fracchia et al., 2016) and from the collection of orchid roots sampled from their natural habitats at a specific spatial or temporal scale (e.g. Oja et al., 2015; Mujica et al., 2016). In contrast, field experiments to study the orchid-fungal symbiosis are less numerous, probably because of the morphological and biological characteristics of orchids (e.g. minute seeds, nutritional requirements) that make it difficult to conduct *in situ* investigations (Bayman et al., 2002). Compared to *in vitro* studies, it seems obvious that field experiments could provide more realistic insights on the ecology and evolutionary patterns of orchid-fungal interactions (Bidartondo and Read, 2008; Phillips et al., 2011; Waterman et al., 2011; McCormick et al., 2012; Tešitelová et al., 2012).

The evolutionary history of orchids is considered as one of the key factors that shapes the structure of the OMF community, with more closely related orchid species it is predicted to have more similar OMF communities (Těšitelová et al., 2015; Cevallos et al., 2017). In co-existence, the related orchid species tend to be associated with similar mycorrhizal fungi (Suárez et al., 2016), although the entire OMF community structure could be different (McCormick and Jacquemyn, 2014). In addition, the site (where each orchid population is present) has also been considered as a driver of the composition of OMF communities. Depending of the spatial scales, the combination of fungal dispersal limitations, biogeographic history and adaptive evolution create a unique fungal assemblage (Peay et al., 2010). The altitude also affects the OMF communities. Although little information is available, it has been reported that OMF communities change with increasing elevation and that the peak of OMF richness and abundance occurs at the mid-elevation in the montane forest (Jacquemyn et al., 2005; Geml, 2017). In temporal scale, OMF communities are suggested to change because of the orchid nutritional demands across the lifecycle (Smith and Read, 2008; Ercole et al., 2015). The temporal dynamic of the orchid-fungi symbiosis has been evaluated across seasonal, environmental conditions and orchid developmental stages (e.g. fruiting, flowering) (Rasmussen and Whighan, 2002; Shefferson et al., 2005; Ercole et al., 2015). However, it remains unclear whether the temporal shifts of OMF partners are a consequence of the succession or represent opportunistic associations due to the extrinsic conditions (Otero et al., 2007; Oja et al., 2015). The evidence available thus far shows that it is unlikely that a single factor is responsible for the structure and composition of OMF communities (McCormick and Jacquemyn, 2014).

In natural orchid populations, the evaluation of the influence of multiple biotic or abiotic factors (e.g. altitudinal gradient, temporal

variation) on OMF communities is not always possible, probably because of the particular distribution and life cycle that each orchid species had. When orchids are more largely distributed in specific areas, the implementation of field experiments provides opportunities to study the effect of multiple environmental factors on the communities of OMF (Phillips et al., 2011). Here, a field experiment was conducted using *in vitro* seedlings of *Cyrtochilum retusum* (Lindl.) Kraenzl. and *Epidendrum macrum* Dressler, two epiphytic orchid species present in Southern Ecuador. The orchid species were established along an altitudinal gradient in two sites of the Podocarpus National Park (Southern Ecuador), and the OMF communities of the *in vitro* seedling were detrmined by Illumina MiSeq amplicon sequencing analysis. This provided a unique opportunity to elucidate whether co-existing orchid species, site, altitudinal level and temporal variation have an effect on the assembly and structure of mycorrhizal communities *in situ*.

2. Materials and Methods

Study site

The study was conducted in the eastern Andes of Southern Ecuador in the Reserva Biológica San Francisco (RBSF), a well-known biodiversity hotspot (Myers, 2000) classified as tropical montane forest (see page **56**, section **3.1 Selected sites for seedling assays** for climate data) (Beck et al., 2008). The vegetation is characterized by an exceptional richness of plant families such as Orchidaceae (337 spp.) and Lauraceae (40 spp.) (Homeier et al., 2008). The field experiment was set up in the lower area of the RBSF, between 1850 and 2150 m a.s.l. in two transects known as T2 and Q5 (see page **56**, section **3.1 Selected sites for seedling assays**) (Bussmann, 2003). The distance between T2 and Q5 is about 775m. These transects were

selected because of the data available on the environment (e.g. climate, flora) (Beck et al., 2008).

A field experiment was established from May 2014 to April 2015, using *in vitro* produced seedlings of the epiphytic orchids *Cyrtochilum retusum* **(**Lindl.) Kraenzl. (Fig. 9) (identified as C in the treatments coding) and *Epidendrum macrum* Dressler (Fig. 10) (identified as E in the treatments coding). Both species are naturally distributed in Southern Ecuador (see page **49**, section **1. Orchid species**) but thus far were never recorded in the RBSF.

Set up of the seedling-trap experiment and sampling

Seedling-trap systems, consisted of a polyvinyl chloride cylinder (20 cm diameter and 15 cm height) with 10 orchid seedlings inside (same species) and covered with a 0.02 µm pore size sun bag (Sigma-Aldrich, St. Louis-Missouri, USA) to avoid herbivory and litter input (Fig. 16). Only tree branches supporting at least one orchid naturally established were selected. No peculiar attention was given to the height of the tree branches. In both transects (T2 and Q5), seedling-traps were installed as described in page **57**, section **3.2 Seedling-trap systems**. All seedling-traps installed along one transect and harboring the same orchid species were considered as one treatment, resulting in four treatments. In total, 40 seedling-trap systems (2 transect x 10 altitudinal levels x 2 orchid species) were established that included 10 replicates per treatment.

Seedling-trap systems were sampled three (S1) and twelve (S2) months after their installation (see page **58** and **59**, section **3.3 Sampling of seedlings**). The number of seedlings collected from each seedling-trap at three and 12 months varied between one and eight, depending on the number of surviving seedlings per system. In both samplings, all the roots collected from the plants in one seedling-trap system were pooled as a single sample

and stored in 70% ethanol for molecular analysis. Mycorrhizal colonization was confirmed as is described in page **60**, section **5. Screening of orchid root-associated fungi.**

Molecular analysis

Total DNA from seedling roots was extracted using the PureLink® Genomic Plant DNA Purification Kit (Invitrogen) according to the manufacturer's protocol (see page **62**, section **6.2.2 DNA isolation from the roots of orchid seedlings**). To analyse the ITS2 region of nuclear ribosomal DNA, two primer pairs were used for PCR amplification (see Waud et al., 2014): ITS86F (Turenne et al., 1999) combined to ITS4 (White et al., 1990); and ITS3 (White et al., 1990) combined to ITS4 (White et al., 1990) (see page **64**, section **6.3.2 PCR for DNA isolated from seedling assays**). After resolving the amplicons by agarose gel electrophoresis, only amplicons within the expected size range (~250-350 bp) were kept. Amplicons obtained from the same DNA template with different primer pairs were mixed as a single sample, subsequently purified and sequenced (see page **65**, sections **6.4 Purification and quantification, and 6.5 Sequencing**).

Bioinformatics analysis of sequenced data

Raw Illumina data was processed using the UPARSE software (Edgar, 2013) (see page **66,** section **7. Bioinformatics analysis**). Then OTUs with taxonomic identity attributed to a member of OMF (Suárez et al., 2006, 2008; Kottke et al., 2010; Valadares et al., 2015) were retained for further analyses. Raw Illumina data was submitted to GenBank under the Accession Number PRJNA396957.

Statistical analysis

The OMF-OTUs frequency of occurrence was transformed into binary matrix on a per sample basis as input data to make inferences on OMF diversity and community composition as a function of the orchids host, sites, altitudinal levels or the temporal variation. In the matrix, the columns corresponding to each of the recovered seedling-trap systems were codified as follows: transect name, orchid species, sampling time and altitudinal level (T2= transect T2, Q5= transect Q5; C= *C. retusum*, E= *E. macrum*; S1= first sampling and S2= second sampling and A1 - A10= altitudinal levels) e.g. T2CS1_A1, T2ES1_A1, Q5CS1_A1, Q5ES1_A1 and so on. To evaluate the entire OMF richness per treatment, the presence/absence matrix was used as input data for the construction of accumulation curves (Jiménez-Valverde and Hortal, 2003) (see page **67,** section **8.2 Alpha diversity**.

To test differences in OMF communities among co-existing orchid species (T2CS1 vs T2ES1) and temporal variation (T2CS1 vs T2CS2), individually per each altitudinal level, beta diversity determined as the ratio of the number of OMF-OTUs shared and the total number of OTUs in samples (Orgiazzi et al., 2013) was calculated by similarity index of Chao-Jaccard (see page **67** and **68**, section **8.3 Beta diversity**). Similarly, to evaluate the effect of the altitude on the mycorrhizal community per treatment (T2CS1, T2ES1, T2CS2, Q5CS1), the beta diversity was calculated by pairwise comparison between altitudinal levels of the same treatment.

PERMANOVA was performed (see page **67** and **68**, section **8.3 Beta diversity**) to evaluate whether OMF communities differed significantly as a function of host phylogeny and the sites of orchid occurrence. Finally, to visualize the differences among sites (T2CS1 vs Q5CS1) and temporal

variation (T2CS1 vs T2CS2) we constructed non-metric multidimensional scaling (see page **67** and **68**, section **8.3 Beta diversity**).

3. Results

In the first sampling (e.g. at month 3), we recovered nine seedling-trap systems from each treatment: T2CS1, T2ES1 and Q5CS1. The two seedling-trap systems recovered from Q5ES1 were not considered due to low number of samples. In the second sampling (e.g. at month 12), we recovered samples from eight seedling-trap systems from T2CS2, and as in the first sampling; we did not consider Q5CS2 due to low number of samples (Table S6). The number of seedling recovered per seedling-trap was not considered because sampling unit was each seedling-trap.

Fungal OTUs

In the 35 samples assessed, 865071 high-quality sequences of ITS2 (~300 bp) were retrieved after chimeric sequences were discarded (11.2% of total number of sequences). Operational taxonomic unit reconstruction based on a 97% sequence similarity cutoff resulted in 1757 fungal OTUs. BLAST analysis of the representative sequences from reconstructed OTUs showed the presence of sequences matching both mycorrhizal and non-mycorrhizal fungi, the later were not considered in subsequent analyses. Putative mycorrhizal fungi were ascribed to 83 OTUs belonging to Cantharellales (53 OTUs), Sebacinales (28 OTUs) and Atractiellales (2 OTUs) orders (Fig. 25) and Table S7).

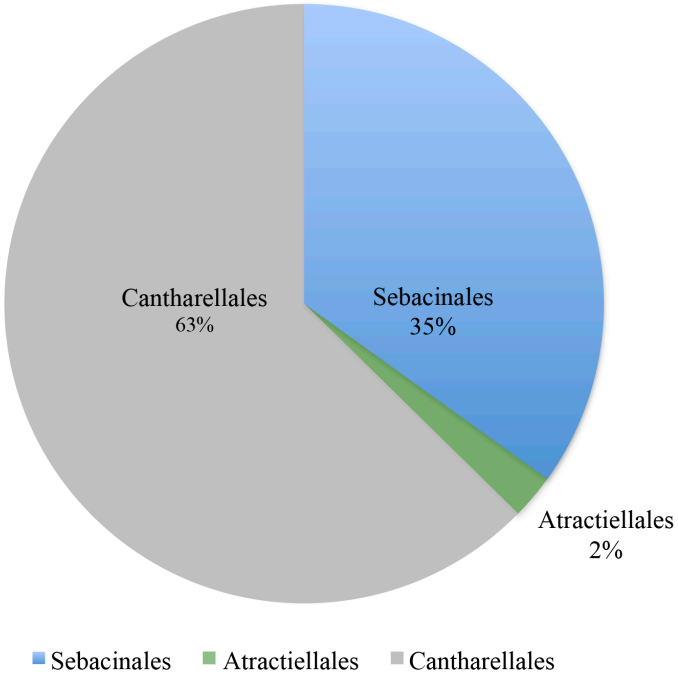

Fig. 25 Frequency distribution of the identified fungal orders of the orchid mycorrhizal fungi identified in association with *Cyrtochilum retusum* and *Epidendrum macrum*.

Rarefaction curves of OMF-OTUs showed that the plateau levels did not reach an asymptote in any of the treatments (Fig. 26). In total, 39, 23, 35 and 52 OTUs were found in the T2CS1, T2ES1, Q5CS1 and T2CS2 treatments, respectively. Six OTUs (OTU19, OTU24, OTU144, OTU225, OTU358 and OTU4582) were identified in all treatments in at least one altitudinal level. Only OTU138 was identified at all altitudinal levels in the treatment T2CS2.

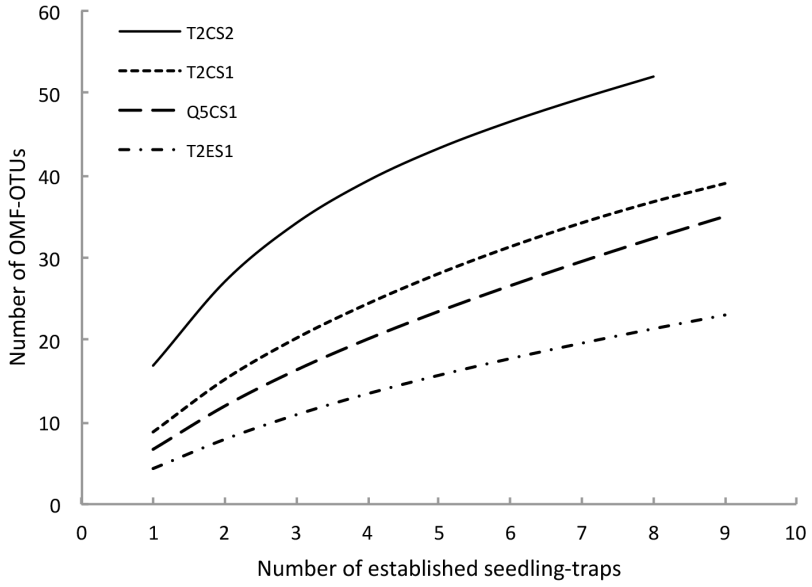

Fig. 26 Rarefaction curves of orchid mycorrhizal fungi OTU (operational taxonomic unit) richness in four treatments of the seedling-trap experiment. T2: transect T2; Q5: transect 5; C: *Cyrtochilum retusum*; E: *Epidendrum macrum*; S1: 1st sampling and S2: 2nd sampling.

Mycorrhizal communities as a function of the orchid species, site, altitude and temporal variation

Mycorrhizal communities evaluated as a function of the orchid species (T2CS1 vs T2ES1) showed that the higher number of OMF-OTUs occurred in *C. retusum* but at both species the highest richness occur between altitudes A5, A6, A8 and A9 (Table 4). Chao-Jaccard index revealed low similarity in the composition of OMF communities between *C. retusum* and *E. macrum* (Table 4). The index was no possible to obtain for transect Q5 because the recovered seedling-traps corresponded only to *C. retusum*. PERMANOVA analysis performed to contrast the diversity among co-existing orchid species showed significant difference in the OMF communities (*P*-value = 0.046).

Table 4. Number of orchid mycorrhizal fungi (OMF) OTUs and the similarity index for the co-occuring orchids *Cyrtochilum retusum* and *Epidendrum macrum* at the different altitudinal levels (A1-A10).

Altitudinal level	M a.s.l.	*Cyrtochilum retusum*	*Epidendrum macrum*	Shared OTUs	Chao-Jaccard
A1	~ 1850	6	2	0	0
A2	~ 1883	7	2	2	0.286
A4	~ 1949	7	2	1	0.125
A5	~ 1982	10	3	2	0.182
A6	~ 2015	18	4	2	0.1
A8	~ 2081	10	7	1	0.063
A9	~ 2114	6	5	3	0.375
A10	~2147	2	3	0	0

Assessing the influence of the site on OMF communities (T2CS1 vs Q5CS1), the NMDS ordination showed distinctive OMF communities but with a substantial overlap (Fig. 27). In total, 39 and 35 OTUs were identified in treatments T2CS1 and Q5CS1, respectively and from these, 17 OMF-OTUs were overlapped at both sites-transects. Analysis by PERMANOVA provided evidence to confirm that the community composition of OMF associated with *C. retusum* was not significantly different between the sites-transects (*P*-value = 0.242).

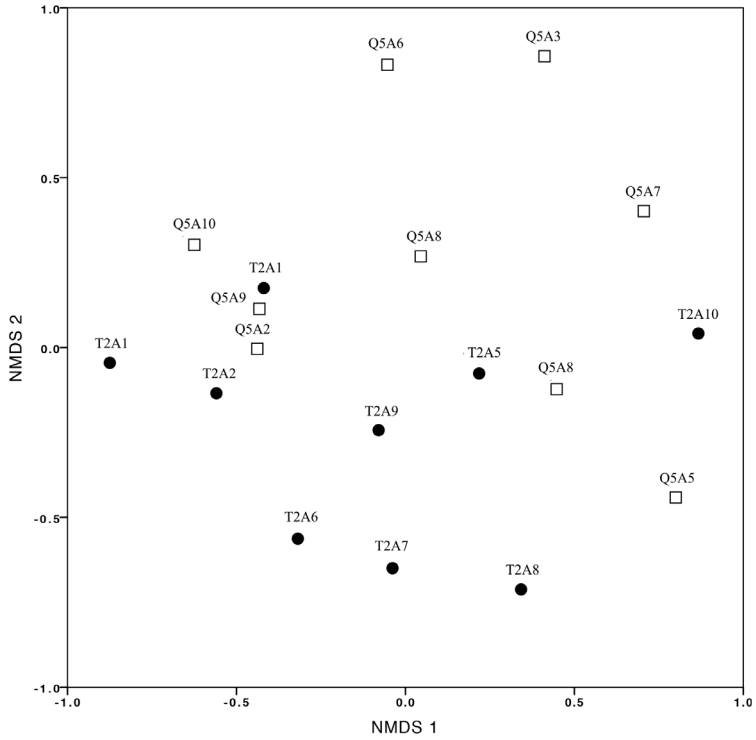

Fig. 27 Non-multidimensional scaling (NMDS) plot of orchid mycorrhizal fungal communities associated with *Cyrtochilum retusum* at T2 (black dots) and Q5 (white squares) transects across altitudinal level (A1-A10). Stress value = 0.070.

Moreover, along altitudinal levels the Chao-Jaccard index, calculated independently for each treatment (T2CS1, T2ES1 and Q5CS1), showed low similarity in the composition of OMF communities in almost all the pairwise comparisons (Table S8). The altitudinal levels with greater similarity of the OMF communities were not necessarily the closest.

Finally, evaluating the temporal variation (T2CS1 vs T2CS2), the number of OMF-OTUs detected per seedling-trap system varied between six and 18 after three months (1st sampling, T2CS1) and between 5 and 22 after 12 months (2nd sampling, T2CS2) (Table S9). Chao-Jaccard index calculated by contrasting T2CS1 and T2CS2, independently per each altitudinal level,

showed low similarity in the OMF communities (Table S9), as evident in the NMDS (Fig. 28).

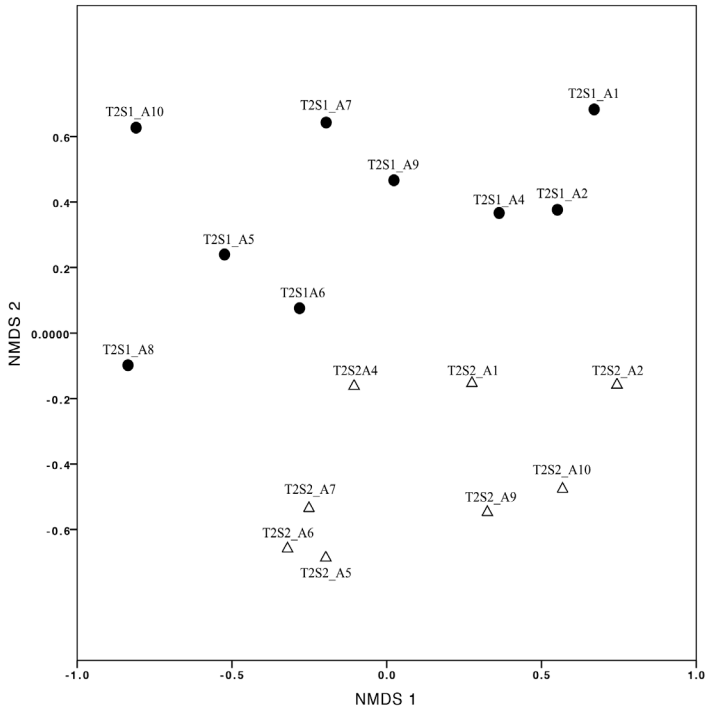

Fig. 28 Non-multidimensional scaling (NMDS) plot of mycorrhizal fungi detected in *Cyrtochilum macrum* sampled at two colonization times. The black dots correspond to samples obtained from the first sampling (T2CS1 treatment) and the white triangles corresponded to the second sampling (T2CS2 treatment) across altitudinal levels (A1-A10), after three months and one year, respectively, of the essay was established (stress value = 0.058).

4. Discussion

Fungal diversity

In this study, we assessed the OMF communities associated with the epiphytic orchids *C. retusum* and *E. macrum* along an altitudinal gradient in the RBSF (Southern Ecuador) using a seedling-trap experiment. Although field experiments are infrequently considered, it represents a more realistic approach to evaluate the orchid-fungi associations. The seedling-trap system developed in our study could thus help decipher the effect of environmental

factors (e.g. sites, altitudinal level) on OMF communities. For instance, with the use of seed packets (field experiments) it was demonstrated that habitat conditions had little influence on seed germination in four species of the genus *Epipactis* (Těšitelová et al., 2012). Moreover, several studies that evaluated seedling and adult orchids from the same species, demonstrated the occurrence of distinct OMF communities (Oja et al., 2017; Waud et al., 2017). Thus, the seedling-trap system could contribute to identify the factors that shape the ecological specialization between orchids and fungi, along the orchid ontogeny to reach more precise ecological conclusions.

In accordance with previous studies performed in tropical areas (Kottke et al., 2010; Cevallos et al., 2017), our results revealed that epiphytic orchids were associated with a highly diverse group of mycorrhizal fungi (materialized by 83 OTUs), comprising members of Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and Atractiellales. Members of the Tulasnellaceae appear to be globally distributed, as they have been more frequently reported to associate with epiphytic orchids in many forest (i.e. Martos et al., 2012; Riofrío et al., 2013; Suárez et al., 2016; Oberwinkler et al., 2017). Consistently, in our study the dominant fungal group associated with *C. retusum* and *E. macrum* was Tulasnellaceae, representing 41% of the entire mycorrhizal diversity identified. Serendipitaceae was the second more frequent group identified in our study (35%), supporting earlier studies that recognized Serendipitaceae as a persistent orchid partner worldwide (e.g. Suárez et al., 2008; Martos et al., 2012). In a recent study performed in the same geographical location (Cevallos et al., 2017), fungi putatively assigned to Serendipitaceae were the most frequent taxa identified in association with epiphytic adult orchids of the Cymbidieae tribe. However, in our study, we added a second set of complementary primers (ITS86F/ITS4 combined with ITS3/ITS4) to reduce the biases, and reach a more accurate description of fungal communities (Waud et al., 2014). Since the present study evaluated

mycorrhizal fungi of seedlings, our results supported the hypothesis that the partners involved in the symbiosis depend on the orchid life stage as was recently reported by Waud et al. (2017). These authors reported distinct OMF communities associated with seedling and adult individuals of *Liparis loeselii* (L.) Rich. We suggest that OMF communities have successional dynamic along the life cycle of the plant.

We detected six OMF-OTUs (OTU19, OTU24, OTU144, OTU225, OTU358 and OTU4582) in all treatments. Among these, OTU19 (Ceratobasidiaceae), OTU24 (Serendipitaceae) and OTU144 (Serendipitaceae) were phylogenetically congruent (≥ 97%) with OTU33 (Ceratobasidium), OTU2 (Serendipitaceae) and OTU13 (Serendipitaceae), respectively, reported by Cevallos et al. (2017). These OTUs identified by Cevallos et al. (2017) were broadly identified in two populations of *Cyrtochilum flexuosum, C. myanthum* and *Maxillaria calantha*, distributed in the surrounding areas of Podocarpus National Park. This finding supports the hypothesis that a core of generalist fungi, widely distributed, could be an essential component of the OMF communities (Pandey et al., 2013; Xing et al., 2017).

The effect of orchid species, site, altitude and temporal variation on mycorrhizal communities composition

The exact factors driving variation in the composition and structure of OMF communities are still unclear (Jacquemyn et al., 2016b). There is growing evidence that mycorrhizal variation is influenced by factors such as seasonal dynamics (Oja et al., 2015), biotope (Han et al., 2016), orchid species (Jacquemyn et al., 2010) or orchid life cycle (Bidartondo and Read, 2008). Our results seem to confirm these observations because clear differences in OMF communities were observed between co-existing orchid species, along an altitudinal gradient and during a temporal variation.

The distinct OMF communities observed between co-existing orchid species strongly support previous results reporting divergent mycorrhizal communities between terrestrial orchids from the genera *Anacamptis, Neotinea, Orchis, Ophrys* and *Serapias* co-occurring at a given site (Jacquemyn et al., 2014). Theoretically, two species are not able to co-exist when they are feeding on the same resources (Tilman, 1982) unless there is small-scale habitat heterogeneity that conducts niche differentiation through segregation of mycorrhizal fungi (Jacquemyn et al., 2014; Voyron et al., 2017). The preference for different mycorrhizal fungi can represent part of a niche partition that may contribute to orchid co-existence (McCormick and Jacquemyn, 2014). More specifically, the distinct OMF communities potentially favor changes in the realized niche among inhabiting species and might have considerable implications for community dynamics, as a result of limiting factors of the habitat (Gerz et al., 2018). The distinctive mycorrhizal partners among orchid species might represent part of an ancestral niche prevalent throughout co-evolutionary processes (Losos, 2008; Selosse, 2014) shaping the realized niche of orchid species. Consequently, it is not excluded that the orchid evolutionary history could be the factor that drives the composition of OMF communities (Davis et al., 2015; Cevallos et al., 2017). Therefore, we suggested that although orchids are not associated with a specific set of mycorrhizal fungi, the niche partitioning and the orchid-fungi co-evolution are filtering the effective fungi associated with orchids.

Similarly to orchid species, the site of orchid occurrence has been frequently reported as a determining factor of OMF communities (Kartzinel et al., 2013b; Xing et al., 2013; Cevallos et al., 2017). In our study, no significant differences were observed in OMF communities between the two transects. This result contrasted with a recent study by Kartzinel et al. (2013b) showing distinctive mycorrhizal communities across 11 populations of *E. firmum* an epiphytic orchid naturally distributes in Costa Rica. These

differences were mostly attributed to the divergent climatic of the sites. Moreover, different OMF communities have also been observed in association with the terrestrial orchid *Anacamptis morio*, suggesting a role for environmental variation (Voyron et al., 2017). The similarity between our study sites (e.g. T2 versus Q5) could probably been attributed to the fact that the sites-transects were closely related regarding their environmental characteristics (e.g. rainfall, temperature). For instance, both sites were located in the eastern slope of the Cordillera El Consuelo and in a general framework with similar climatic condition (Beck et al., 2008). Identical results were obtained in a recent study evaluating OMF associated with epiphytic orchids distributed in four sites of the Cajas National Park (Herrera et al., in press).

We observed that the OMF communities associated with *C. retusum* and *E. macrum* consisted of a stable component that include keystone species recorded in both transects and a dynamic component comprising a number of fungi exclusively identified at specific sites-transects. Similar results were observed in two populations of *Dendrobium officinale,* where the OMF communities included a set of fungi widely identified across orchid populations and a set of fungi specifically identified at one of the two orchid populations but not both (Xing et al., 2013). Overall, our findings support the hypothesis that OMF communities are structured around keystone fungal species as was observed in the OMF communities associated with *C. flexuosum, C. myanthum* and *M. calantha* (Chapter II). However, further analyses that include more sites are needed to confirm this hypothesis.

It is generally accepted that the altitude is a factor that drive the structure of communities (e.g. plants and animals) (McCain, 2005; Grytnes and Beaman, 2006). However, the effect of the altitude on the OMF communities is almost unknown. Suárez et al. (2006) reported a decrease of the OMF diversity with increasing altitude. Curiously, in our study, the

fungal diversity did not decrease with increasing altitude. Moreover, orchid mycorrhizal communities were significantly different along the altitude level. The orchids seem to be mycorrhizal specific and although *C. retusum* and *E. macrum* are not naturally distributed at study sites, we suggest that the identified fungi could represent a potential mycorrhizal.

In the Andean region, the characteristic steep slopes (Montgomery et al., 2001), especially in Southern Ecuador, contribute to the ecosystem heterogeneity at small scale (Beck et al., 2008) and could favor the occurrence of certain fungi (McCormick and Jacquemyn, 2014). Even though distinctive OMF communities were found, the fungi widely detected (keystone species) across altitudinal levels could represent mycorrhizal fungi with exceptional ecological plasticity, able to persist under stress conditions (Selbmann et al., 2013). For instance, *Serendipita vermifera* (previously *Sebacina vermifera*) has a metabolic plasticity that likely improves plant productivity and stress tolerance (Ray et al., 2016). The association with stress resistant mycorrhizal fungi may support the orchid survival in epiphytic niches, characterized by environment stress (e.g. water shortage, low nutrient availability) (Martos et al., 2012). However, it still remains to be elucidated whether the changes in the OMF detected along the altitudinal gradient are due to an opportunistic association (Dearnaley et al., 2012) or if the altitude induced changes in the OMF community.

In addition, our results suggested a significant temporal variation on the composition of OMF communities. We found that OTUs-OMF richness increased across time. Changes on mycorrhizal communities have been observed in relation to the development stages of hosts and season (Bidartondo and Read, 2008; Kohout et al., 2013; Oja et al., 2014; Ercole et al., 2015). For instance, Bidartondo and Read, (2008) reported that *Cephalanthera damasonium* and *Cephalanthera longifolia* had different OMF communities across orchid development but with a subset of fungi

permanently identified. Further studies are necessary to validate if these differences in OMF communities are due to the orchid developmental stage and the concomitant changes in physiological processes (e.g. distinctive nutrient uptake) (Rasmussen and Rasmussen 2009; Kohout et al., 2013).

We suggest that OMF communities experience a successional process of colonization along the development stages of the plant, as our results suggest that the OMF communities were structured by keystone species (OMF identified either after three or twelve months) and a dynamic component (OMF changing over time). *In vitro* experiments have demonstrated that keystone species of OMF, that were isolated from adult plants, were able to promote orchid seed germination (Fracchia et al., 2013). Furthermore, in field experiments, orchids at early development stages were shown to form associations with a set of available OMF (Bidartondo and Read, 2008), but these OMF may not necessarily be present in the roots of the orchid throughout its entire lifecycle (Shimura et al., 2009). Information on the temporal dynamics of OMF communities is currently (Ercole et al., 2015), and periodic samplings across the entire orchid lifecycle is required to determine if keystone OMF remin throughout orchid development. We speculate that the OMF keystone species here identified, represent a permanent core of OMF, as they were found across sampling sites and orchid species (Cevallos et al., 2017).

Conclusions

Our results show that the combination of orchid seedling-trap experiments and Illumina MiSeq ITS2 sequencing analysis is an adequate approach for determining the composition of OMF communities and the abiotic and biotic factors which structure these communities. Our results support that OMF communities are composed of a dynamic and a core set of keystone species, and that the orchid-fungi symbiosis is a dynamic

interaction governed by temporal variations, environmental filtering, and co-evolutionary history.

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VI. GENERAL DISCUSSION

Ecosystems worldwide face disturbances, some caused by natural processes but most of them being occasioned by human activities (Running, 2008). The rising demand for natural resources (e.g. oil, metals, food) has increased the pressure on ecosystems and accelerated the loss of diversity that, in some cases, is impossible to restore (Chazdon, 2003). For instance, the FAO reported that during the 2010-2015 period, the net annual loss of forests in South America was 2 million hectares. The conservation of ecosystems has thus become a challenge for numerous countries (Watson et al., 2014) and various conservation initiatives have been implemented. For example, the establishment of biodiversity hotspots, corresponding to areas with at least 1500 endemic vascular plants and less than 30% of it original natural vegetation, has been set up (Myers, 2000). Unfortunately, these hotspots, although being priority for conservation and investigation, are not completely protected (Myers, 2000).

One of the most diverse and threatened families of vascular plants is Orchidaceae. Orchids are distributed almost everywhere with the exception of the poles and extreme deserts (Conservation International, 2017). In natural ecosystems, orchids have complex life cycles involving root fungal partners and specific pollinators (Smith and Read, 2008; Waterman et al., 2011), becoming a model system to study the interactions between plant, fungi and animals (Bronstein et al., 2014; Selosse, 2014; Fay et al., 2015). Some orchid species are considered as indicators of ecosystem health because of their sensitivity to environmental changes (Newman, 2009).

Orchids rely on fungi for germination and early seedling development (Smith and Read, 2008; Rasmussen and Rasmussen, 2009). Therefore, the presence of specific and appropriate fungi strongly influences orchid distribution (McCormick and Jacquemyn, 2014). Numerous studies have been conducted on the fungal species involved in the orchid-fungi association (Dearnaley et al., 2012) and their specificity (Riofrío et al., 2013;

Pellegrino et al., 2014). However, it is currently debated which are the environment factors that are affecting the structuration of RAF communities (McCormick et al., 2012). Based on data from temperate regions, it was shown that some orchid species exhibited specificity for certain fungal groups (Jacquemyn et al., 2016a, Waud et al., 2016), while this was not the case for other species (Jacquemyn et al., 2010), suggesting that environmental factors such as habitat or geography have important influences on the fungi distribution and consequently on the orchid-fungus interactions (McCormick and Jacquemyn, 2014).

In tropical areas, there is limited information about the influence of environment factors on the association between orchids and fungi, although numerous studies reported a polyphylogenetic set of taxa associated with terrestrial and epiphytic orchids (Otero et al., 2002; Kottke et al., 2010; Suárez and Kottke, 2016; Xing et al., 2017). From the orchid endophytic RAF, those with mycorrhizal life style are more frequently described, even though the most abundant are the non-mycorrhizal fungi from which the role in the orchid life cycle is still unclear (Ma et al., 2015). The study of the specificity and distribution of the orchid endophytic RAF will probably provide a better understanding of the ecology and life history of the orchid-fungi interactions. In fact, the assessment of endophytic RAF could represent a baseline for conservation efforts, especially in biodiversity's hotspots such as the montane forests of Ecuador (Suárez and Kottke, 2016).

Root-associated fungal endophytes have been mostly identified/characterized in terrestrial orchids (Dearnaley et al., 2012; Ma et al., 2015). The extrapolation of information about RAF of terrestrial orchids to epiphytic species is not always possible (e.g. distinctive dominant groups), probably because of the orchid different morphological, anatomical and physiological traits (Moreira and Isaias, 2008). It is thus necessary to increase our knowledge on the endophytic RAF of tropical epiphytic orchids.

The implementation of molecular tools and especially high-throughput barcoding methods (isolation-independent) has become a must for ecological studies (e.g. Tešitelová et al., 2015; Jacquemyn et al., 2017a, b). Thus, in the present thesis the diversity and community composition of endophytic RAF of epiphytic orchids of Southern Ecuador was evaluated using Illumina MiSeq. In **Chapter I**, the diversity and community composition of endophytic RAF of *C. flexuosum, C. myanthum*, *M. calantha, E. marsupiale* and *C. pardinum*, were evaluated at six sites (two closes to the PNP and four at CNP) of montane forest in Southern Ecuador. The assessment of the whole fungal endophytes diversity showed that host phylogeny and sites are factors that shape the structure of the fungal communities. In **Chapter II**, the orchid mycorrhizal communities associated with *C. flexuosum, C. myanthum* and *M. calantha,* three inhabiting orchid species of two surrounding areas of the PNP (El Tiro and Curva Misteriosa), were assessed. The results showed that mycorrhizal communities differed between inhabiting orchid species and study sites. This suggested that the mycorrhizal communities are structured depending on the host phylogeny and the habitat. Since in Ecuador the conjunction of climatic and geographic conditions represent, to some extent, a barrier for species distribution and ecological interactions (Dodson, 2003), in **Chapter III**, the effects of abiotic and biotic factors (orchid species, altitudinal level, site and time) on the mycorrhizal fungi communities associated with *C. retusum* and *E. macrum* were evaluated through field experiment using *vitro* plants. With the implementation of field experiments we aimed to encompass more study sites, altitudinal levels and temporal scales, than the orchids have in natural populations. We noticed that the orchid species, the altitudinal level and the temporal variation shaped distinctive mycorrhizal fungal communities. Meanwhile, the site of orchid occurrence did not influence the composition of mycorrhizal fungal communities.

The functions of all the endophytic RAF in the plant life cycle are not yet fully understood although they are believed to play key roles in plant productivity or at least are considered not to cause any pathogenic symptoms on the host (Kohout et al., 2013; Jumpponen et al., 2017). Although, there are few studies focused on the whole diversity of endophytic RAF in orchid species the implementation in the last decade of high-throughput sequencing technologies (e.g. NGS) have undoubtedly increased our knowledge on the community composition and thus ecological roles of orchid RAF (Kohout et al., 2013; Nilsson et al., 2013). In our study, mycorrhizal and non-mycorrhizal fungi were detected in the roots of all orchids assessed. Fungal endophytes OTUs identified belonged to Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla and were putative assign as (decrease in the order of OTU abundance) saprotrophs > symbiotrophs > pathotrophs > pathotroph-saprotroph > saprotroph-symbiotroph > pathotroph-saprotroph-symbiotroph > pathotroph-symbiotroph > pathotroph-saprotroph-symbiotroph (see **Chapter I**). Meanwhile, specifically evaluating OMF members of Serendipitaceae, Ceratobasidiaceae and Tulasnellaceae were observed (see **Chapter I** and **Chapter III**), supporting previous studies (Dearnaley et al., 2012; Pandey et al., 2013; Těšitelová et al., 2015; Herrera et al., 2018).

The most abundant fungal family, in terms of number of OTUs, was Serendipitaceae (see **Chapter II**). However, in earlier studies performed close to our study sites, Tulasnellaceae was the dominant group associated with orchid species from Epidendreae tribe (e.g. Suárez et al., 2006, 2008; Kottke et al., 2010; Riofrío et al., 2013). These differences could possibly be related to different molecular pipelines used in earlier study because in our field experiment we observed that Tulasnellaceae was the dominant group (see **Chapter III**). According to Oberwinkler et al. (2017) species from Tulasnellaceae are worldwide distributed. It is probable that such

cosmopolitan fungal distribution justifies that several orchid species in tropical and temperate areas are frequently associated with members of Tulasnellaceae (Dearnaley et al., 2012). Comparing with several studies conducted worldwide, the dominant mycorrhizal group seems to vary also according to the orchid phylogenetic history. For instance, orchids from Orchideae tribe were mostly associated with members of Ceratobasidiaceae (Pecoraro et al., 2015; Waud et al., 2016) while species from Neottieae were predominantly associated with fungi from Sebacinales (Jacquemyn et al., 2015b; Těšitelová et al., 2015).

Despite an increasing literature describing OMF, it remains largely unknown which and how abiotic or biotic factors are affecting mycorrhizal communities (e.g. Jacquemyn et al., 2010, 2012a; Oja et al., 2015; Han et al., 2016).

Distinct fungal endophyte community compositions were detected at PNP between the co-existing orchid species *C. flexuosum, C. myanthum* and *M. calantha* (see **Chapter I, II** and **III**). This contrasted with the results obtained at CNP, where *E. marsupiale* and *C. pardinum* showed similar fungal endophyte communities among inhabiting orchids and between orchid populations. Interestingly, a set of fungal endophytes (mycorrhizal, saprobes and latent pathogens) was present at all sites and in each orchid species, representing the core fungal microbiome.

The analysis of the communities of orchid endophytic RAF is not frequently addressed mainly because most of these fungi are cryptic, and poorly defined at taxonomic and functional levels (Ma et al., 2015) (see **Chapter I**). In contrast, mycorrhizal fungi have been studied more frequently for decades using isolation-dependent and isolation independent methods (Dearnaley et al., 2012) (see **Chapter II**).

Some studies have characterized the orchid endophytic RAF in tropical (Herrera et al., 2010; Oliveira et al., 2014; Novotná et al 2018) and terrestrial (Jiang et al., 2011; Kohout et al., 2013) orchids. However, their role in the life cycle of orchids were not considered and thus remained unclear. The large diversity of RAF with contrasting life history strategies (e.g. symbionts, pathogen, saprophyte) makes the evaluation of the patterns of orchid-fungi interaction difficult. It is probable that the roles that endophytic RAF are playing in the orchid life are no perceptible in the time scale during which the studies are conducted (van der Heijden et al., 2015). Nevertheless, studies on the ecophysiology, exploring for instance nutritional or pathogen defense processes, are need to better understand the orchid-fungal interactions (Oliveira et al., 2014) (see **Chapter I**).

The results shown that endophytic RAF communities are affected by the host phylogeny (see **Chapter I, II** and **III**) and site (see **Chapter I** and **II**), supported the theory that fungi are not stochastically distributed and in some way contribute to the orchid adaptability to changing environmental conditions (Oliveira et al., 2014; Ma et al., 2015). Thus, the interaction between orchids and fungi may be affected by abiotic factors, such as local environmental conditions (site, altitude) but also biotic factor, such as orchid phylogenetic history (orchid species) (Martos et al., 2012).

In co-existence, theoretically, species are able to inhabit together if they occupy different niches (Tilman, 1982). The inhabiting orchids *C. flexuosum, C. myanthum* and *M. calantha* (Chapter II) tend to interact with different endophytic RAF (mycorrhizal and non-mycorrhizal) communities. Similar patterns have been observed in terrestrial orchids from temperate ecosystems evaluating OMF (Jacquemyn et al., 2014; Pellegrino et al., 2014). Corroborating our earlier observations, our field experiment showed that the two studied epiphytic orchids, *C. pardinum* and *E. macrum*, exhibited distinctive mycorrhizal communities (Chapter III). The presence of

different OMF could represent a niche partition that in theory, favor the species co-existence (McCormick and Jacquemyn, 2014). Orchid species that co-exist could probably access different nutrient resources if they are associated with different fungal lineages (Pellegrino et al., 2014). Interestingly, the endophytic RAF communities of both *Cyrtochilum* species displayed higher similarities than with the OMF community of *M. calantha* (see **Chapter I** and **II**). Martos et al. (2012) found that close related terrestrial and epiphytic orchids were associated to similar fungal taxa. We suggest that the orchid phylogenetic relatedness (Freudenstein and Chase, 2015) could shape the RAFcommunity (e.g. Shefferson et al., 2007; Valadares et al., 2015) as a result of an evolutionary history (Losos, 2008).

The site of orchid occurrence also can affect the composition of RAF communities (Jacquemyn et al., 2012a, b). Consistently, most of the study orchid species (*C. myanthum* and *M.* calantha) had particular OMF community at each study site (see **Chapter I** and **Chapter II**). Similar results have been reported by Kartzinel et al. (2013b) who showed that populations of the tropical orchid *Epidendrum firmum* distributed along contrasting ecosystems in Costa Rica, had site-dependent OMF communities. The possibility that our observations were the result of a site-adjustment when mycorrhizal fungi are stochastically available should be considered (Kartzinel et al., 2013b) because the composition of OMF communities was different at each site. Furthermore, we observed that RAF community was structured by a set of fungi identified only in a specific orchid population (dynamic component) and a set of fungi widely identified at several orchid populations (stable component). The fungi specifically identified at an orchid population could support the spatial distribution of orchid while the fungi widely distributed might contribute to the resilience of orchids (Pellegrino et al., 2014). Meanwhile, the fungi identified at several orchid species and populations could represent keystone species and likely

have a central role in local fungi assembling and species resilience as has been observed in AMF (see Guimarães et al. 2011).

The field experiment performed to evaluate the site as a factor affecting the OMF communities contrasted previous results because similar fungal communities were identified across two populations of *C. pardinum* (see **Chapter III**). Probably some environmental characteristics were similar between study sites because of the proximity (Beck et al., 2008). However, it is also probable that the orchid species is not a deteminant factor of the OMF communities as has been observed in a recent study evaluating epiphytic orchids in the Cajas National Park (Ecuador) (Herrera et al., in press).

Additionally, when we compared exclusively the mycorrhizal communities across altitudinal levels, almost no similar OTUs were observed. It is suggested that the ecosystem heterogeneity occurring at different altitudes favors the occurrence of distinct OMF (Jacquemyn et al., 2005; Geml, 2017). Even it is still unclear if orchid-fungi interactions represent an opportunistic association driven by the altitudinal gradient (see **Chapter III**).

Another abiotic factor that could potentially affect the communities of fungi associated with orchid roots is the temporal variation. The information about temporal variations was obtained across orchid life stages and seasons reporting significant changes of OMF communities across time (Bidartondo and Read, 2008; Kohout et al., 2013; Oja et al., 2014; Ercole et al., 2015). Likewise, temporal variations of the OMF communities were observed with the field assay. The mycorrhizal fungi diversity identified after one year of the assay was higher than the diversity after 3 months. The orchid development stage could determine the fungal mycorrhizal communities (Bidartondo and Read, 2008; Oja et al., 2015). It is admitted that the nutritional needs of orchids may vary with life stage (Rasmussen and

Rasmussen, 2009). Kohout et al. (2013) demonstrated that the orchid-fungi interaction changed between the pre-flowering and flowering stages. However, the OMF communities have overlapped OTUs (26%), supporting the hypothesis that the OMF communities are structured around a set of keystone fungi, determined by the host phylogeny (Herrera et al., 2017) that can remain in time (Shimura et al., 2009) (see **Chapter III**).

The combination of currently available technologies offers powerful alternatives for ecological analysis (Bálint et al., 2016). For instance, the use of both taxonomic and molecular tools is more and more recommended for delimitation of fungal species (Cruz et al., 2014) or for fungal inventories combined with germination assays to evaluate their symbiotic potential (Fracchia et al., 2013). Similarly, the dynamics of orchid-mycorrhizal interactions are frequently evaluated under *in vitro* conditions in order to have the basic knowledge of such associations (Stewart and Kane, 2007). However, the combinations of fungal isolation and physiological analysis present important biases for interpretation because most of the fungi are not able to grow under *in vitro* conditions and some may even loose their symbiotic attributes under axenic culture (Stewart and Kane, 2007). Contrasting to *in vitro* studies, experimental studies within field are not frequently considered because of their complexity to evaluate extrinsic variables (environmental conditions). However, they offer major potentials to contextualize more accurately the ecological dynamics of fungi (e.g. Waterman et al., 2011; McCormick et al., 2012).

VII. CONCLUSIONS AND PERSPECTIVES

Since orchids rely on fungi to provide inorganic nutrients throughout their life cycle, the understanding of how such interactions are structured is highly desired for ecosystem conservation (Boddington and Dearnaley, 2008; Waterman et al., 2008). Characterizing the fungi associated with orchids represent a prior objective for ecological studies. The development in the last decade of high-throughput sequencing techniques has dramatically improved our knowledge on the microbiome of animals and plants (Di Bella et al., 2013). The application of these techniques to orchid-fungi may provide crucial information on the fungal diversity and communities structures associated with these plants (Těšitelová et al., 2012; Jacquemyn et al., 2014, 2016a, b; Waud et al., 2016). However, fungal endophyte communities are more complex than what was previously reported with the occurrence large polyphyletic fungal groups. Currently, the understanding of the evolutionary history and ecological dynamics of orchid-fungi interactions is one priority objective of numerous research teams. Undoubtedly, the description of the fungal endophytes and the environmental factors that could affect the orchid-fungi interaction will help to clarify such ecological issues.

Orchids are important plants in horticulture, food, fragrance and pharmaceutical industries (Hew et al., 2004). The highest species diversity of orchids is concentrated in tropical areas (Hinsley et al., 2017). In Ecuador specifically, most of the orchid species are found in premontane and montane forests at elevations of 300 – 3000 m a.s.l (Dodson and Escobar, 1993).

Here, we identified and evaluated the endophytic RAF diversity (mycorrhizal and non-mycorrhizal fungi) of *C. flexuosum, C. myanthum* and *M. calantha* at two sites close to Podocarpus National Park (Southern Ecuador) and the endophytic RAF diversity of *E. marsupiale* and *C. pardinum* in four sites at Cajas National Park. We evaluated if site and host

phylogenic history had any effect on fungal community composition associated with *C. flexuosum, C. myanthum* and *M. calantha*. We also investigated the effects of abiotic and biotic factors (host phylogeny, site, altitude and time) on the mycorrhizal community composition associated with seedling of *E. macrum* and *C. retusum*, through a field experiment with trap plants.

The following questions were addressed in the thesis:

Do the orchid host species and site (*C. flexuosum, C. myanthum* and *M. calantha* at Podocarpus National Park and *Epidendrum marsupiale* and *C. pardinum* at Cajas National Park) influence the community composition of root-associated fungi?

The results of **Chapter I** revealed that the abiotic and biotic factors, host species and site, did not impact similarly the composition of root-associated fungal endophyte communities. The host phylogeny and the site of orchid occurrence affected the structure of endophytic RAF communities at Podocarpus National Park, while these factors did not influence the community structures at Cajas National Park. Fungi belonging to Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota phyla were detected in the roots of *C. flexuosum, C. myanthum* and *M. calantha.* These results also suggested that the culture-independent methods are more efficient to estimate the RAF diversity than the culture-dependent methods (Waterman et al., 2011). However, the combination of both methods is necessary to assess and complement the information on the richness, diversity and roles that the endophyte have in orchid life. On one hand, culture-dependent methods are necessary to establish the biological entity of the mycobionts and to ascertain their functional roles in the symbiosis (Sathiyadash et al., 2012). Although not all the mycobionts are susceptible to isolation, the fungi growing *in vitro* might represent sources

for the further utilizing in orchids conservation (Cameron et al., 2008; Ding et al., 2014). On the other hand, culture-independent methods reduce the time necessary for detection and identification of microbial communities and enables simultaneous detection of multiple fungi in a single individual plant (Jacquemyn et al., 2012b). In our study, we were able to propose for 1200 endophytic fungi their putative life strategy, based on the available literature in FunGuild database (Nguyen et al., 2015). However, to determine the exact role (trophic mode) of endophytic fungi in the orchid life, it is necessary to carry out experimental assays using fungal isolates. In future studies, the implementation of experiments to clarify the effect of the endophytes on orchids life could favor the conservation of some endangered species (Oliveira et al., 2014).

We observed that the host phylogeny could be a determining factor not only for OMF communities but also to the whole orchid endophytic RAF. However, fungal diversity differed between the two locations. At PNP the inhabiting orchid species harbored distinct endophytic RAF, while at CNP, the orchid species had similar fungal communities, even though they belong to distinct tribes.

The site of orchid occurrence shapes the community composition of root-associated fungal endophytes. Although in general terms our study sites at PNP have similar vegetation structure, there are local environmental conditions (annual temperature, annual precipitation) that could explain the distinct fungal community composition. In contrast, for the study sites at CNP more similar environmental conditions have been registered (temperature, rainfall) and consequently similar fungal endophyte communities have been reported. It cannot be ruled out that the orchid species at CNP present an ancestral ecological conservatism in endophyte preferences.

The assemblage strategies between orchids and fungi are determined by abiotic and biotic factor but not all affect equally all the interactions. The assessment of more orchid species with different phylogenetic relatedness could give more clear insight about the effect of the host phylogeny on the endophytic RAF as was evaluated for OMF (e.g. Shefferson et al., 2005; Jacquemyn et al., 2010, 2012b).

Do mycorrhizal fungi communities differ between *Cyrtochilum flexuosum, Cyrtochilum myanthum* and *Maxillaria calantha* that co-exist at two sites of evergreen upper montane forest?

In **Chapter II,** the results showed that mycorrhizal fungi communities differed between co-existing orchid species and across sites. The Illumina-based ITS2 region barcoding analysis of the orchid roots showed a diverse community of OMF that included members of Atractiellales, Cantharellales and Sebacinales. The dominant order was Sebacinales. The results suggested that co-existing orchid species likely occupied distinct fungal micro-niches. Fungal communities were structured with a set of OMF locally available combined with a set of OMF widely distributed. The last could represent a keystone of mycorrhizal fungal communities, permanent across orchid species and sites.

Sympatric orchid species associated with distinct mycorrhizal communities likely represent an advantage for the orchid species co-existence because of decreasing competition (McCormick and Jacquemyn, 2014). The dissimilarity of mycorrhizal fungal communities depended on the phylogenetic relationships among co-existing orchid species: at greater phylogenetic distance, greater divergence in OMF but also RAF (**Chapter I**) communities was found (Jacquemyn et al., 2011a, 2012b). The hypothesis that the structure of OMF communities depends on the orchid phylogeny as

result of the co-evolutionary process (Losos, 2008) needs further clarifications.

The success of orchid species depends largely on their ecological plasticity, defined as the ability of species to adapt to the variation of environmental conditions (Pigliucci, 2005). It has been suggested that species widely distributed have larger plasticity (Niinemets and Valladares, 2004). It is probable that the OMF-OTUs identified at all sites/populations have exceptional ecological plasticity as well as the widely distributed no-mycorrhizal OTUs reported in **Chapter I**.

Since the interaction between orchid and mycorrhizal fungi are necessary for seed germination and successful establishment of seedlings, the association with the fungi locally available could be a symbiotic assurance for orchid persistence (Kartzinel et al., 2013a). These patterns have been evidenced in orchids with variable degree of mycorrhizal specificity (Xing et al., 2013). Mycorrhizal communities are site-adjusted around a group of "keystone" fungal species broadly distributed with exceptional plasticity and a set of fungi available locally that likely contribute to orchid spatial distribution.

Future approaches, focused on the elucidation of the specific roles that fungi are playing in orchid life will help to understand how the mycorrhizal communities are structured and if they have synergistic action. The implementation of gene expression studies could contribute to understand how the different OMF are interacting with orchids (Perotto et al., 2013). To do so, it is fundamental to preserve fungi in axenic cultures (Novotná et al., 2018).

Do the host phylogeny, altitudinal gradient, temporal variation and site of orchid occurrence affect the composition of mycorrhizal fungal communities associated with *Cyrtochilum retusum* and *Epidendrum macrum*?

The identification of the mycorrhizal fungi that are part of the orchid-fungi symbiosis has been extensively evaluated across the world. Earlier studies based on isolation-dependent methodologies only described a fraction of the fungal diversity. With the development of high-throughput molecular techniques (isolation-independent), a wider group of mycorrhizal fungi could be described. Most of the information available until now has been obtained from the evaluation of natural orchid populations and there is almost no complementary data using field experiments. In **Chapter III**, the structure of the OMF communities associated with *C. retusum* and *E. macrum* was dependent on the orchid species, altitudinal level and also temporal variations, while the site of orchid occurrence seems not to drive significant differences in mycorrhizal communities associated with both orchid species. The use of field experiments in combination with molecular techniques can give exceptional ecological insights that are not always possible in a spatial or temporal scale (e.g. McCormick et al., 2012). To better understand the obtained resulted it is necessary to implement studies involving several orchid species, more contrasting study sites and at larger temporal scales.

The OMF diversity is only partially described, mainly due to methodological limitations. For instance, our field experiment was carried out using seedlings, so it cannot be ruled out that the patterns of orchid-mycorrhizal interaction are due to the stage of orchid development (Waud et al., 2017). The implementation of field experiments could support the evaluation of OMF communities along temporal scales involving multiple stages of orchid life.

The identification of the abiotic and biotic factors shaping the mycorrhizal community composition represents fundamental information for species conservation (orchids and fungi). Until now, there is increasing evidence that support the hypothesis that the orchid evolutionary history is one biotic filter that affects the mycorrhizal community composition (see **Chapter II**). However, further analyses are necessary to evaluate if the orchid-fungi interaction is determined by co-evolutionary processes as happened between orchids and pollinators (Waterman and Bidartondo, 2008, Davis et al., 2015).

The results in **Chapter III** supported our earlier findings in **Chapter II** regarding the structure of mycorrhizal fungal communities. All the mycorrhizal fungal communities had a set of fungi widely distributed and a set of fungi exclusively identified at certain site, altitudinal level and orchid species. Because our observations were based on orchid seedlings without natural distribution at study sites, the fungi associated with *C. retusum* and *E. macrum* represent a potential mycorrhizal niche.

Along the orchid life cycle the nutritional requirements change because of the different physiological process (e.g. germination, seedling establishment, reproduction) and as Ercole et al. (2015) suggested, the mycorrhizal association between orchid and fungi is dynamic. Studies evaluating the OMF across different stages of orchid life represent an opportunity to clarify if the orchid-fungi symbiosis is a temporal process or if there is a succession along the orchid life.

For conservation purposes, the recognition of the partners involved in the orchid-fungi interaction represent the backbone. However, how such interactions are structured and which are the drivers, are still pending questions. Current investigations evaluating the ecological dynamics of the

orchid-fungi interaction show better and more accurate observation with the combination of *in vitro,* field and molecular techniques.

VIII. OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

1. Scientific publications

1.1 Research Article Published

- **Cevallos S,** Sánchez-Rodríguez A, Decock C, Declerck S, Suárez JP (2017). Are there keystone mycorrhizal fungi associated to tropical epiphytic orchids? Mycorrhiza 27:225–232. DOI 10.1007/s00572-016-0746-8

- **Cevallos S,** Herrera P, Sánchez-Rodríguez A, Declerck S, Suárez JP (2018). Untangling factors that drive community composition of root associated fungal endophytes of Neotropical epiphytic orchids. Fungal Ecology 34: 67-75. DOI 10.1016/j.funeco.2018.05.002

1.2 Research Article submitted

- **Cevallos S,** Declerck S, Suárez JP (Submitted to Frontiers in Plant Science). *In situ* orchid seedling-trap experiment shows few keystone and many randomly-associated mycorrhizal fungal species during early plant colonization.

2. Conference Participation

- Gordillo A, **Cevallos S,** Garcés M, Decock C (2015) Poster. Reinforcement of the fungal expertise in Ecuador via case studies of fungal plants interactions in selected ecosystems and the development of biotechnological-oriented fungal resources centers. Symposium Biodiversity and Development, a global heritage. Brussels-Belgium 26th November 2015.

- **Cevallos S,** Gordillo A (2015) Oral Presentation. Reinforcement of the fungal expertise in Ecuador via case studies of fungal

plants interactions in selected ecosystems and the development of biotechnological-oriented fungal resources centers (Contribución al estudio de la diversidad fúngica en el Ecuador). Conference at the Jornadas de Micologia UTPL-Ecuador. Loja-Ecuador 25-27th March 2015.

- **Cevallos S,** Sánchez-Rodríguez A, Decock C, Declerck S, Suárez JP (2015) Poster. Changes in epiphytic orchid mycorrhizal fungi communities between two sites in tropical mountain forest in Southern Ecuador. 36th New Phytologist Symposium. Munich-Germany 29 November–01st December 2015.

- **Cevallos S,** Sánchez-Rodríguez A, Decock C, Declerck S, Suárez JP (2016) Oral Presentation. Diversidad y ecología de hongos micorrízicos de orquídeas: identificación molecular y eco-fisiología. Symposium Diversidad fúngica en ecosistemas naturales y contaminados y colecciones de cultivos de microorganismos. Quito-Ecuador 28th September 2016.

- **Cevallos S,** Sánchez-Rodríguez A, Decock C, Declerck S, Suárez JP (2016) Oral Presentation. Are there keystone mycorrhizal fungi associated to tropical epiphytic orchids? International symposium of the "Plataforma de Investigación y monitoreo de la Biodiversidad y de los Ecosistemas". Loja-Ecuador 1st December 2016.

- **Cevallos S,** Herrera P, Suárez JP (2017) Oral presentation. Cambios en la estructura de las comunidades de hongos endófitos asociados a orquídeas epífitas comunes en dos bosques montanos aledaños al Parque Nacional Podocarpus. Conference at the VI Investiga UTPL. Loja-Ecuador 31st May 2017.

3. Student supervision

- Supervision in Ecuador of one student (Anabel de los Ángeles Cueva Agila) from the Biology faculty (UTPL), who performed during 2014 her bachelor thesis at the mycorrhizal laboratory at UTPL. The thesis was entitled “Caracterización molecular de hongos micorrízicos aislados a partir de cuatro especies de orquídeas epífitas, en dos pisos altitudinales de bosque montano”.

X. SUPPORTING INFORMATION

SUPPORTING TABLES

Research Results

Chapter I

Table S1 Putative taxonomic assignment of fungal operational taxonomic units (OTUs) identified in association with: *Cyrtochilum flexuosum, Cyrtochilum myanthum, Maxillaria calantha, Epidendrum marsupiale* and *Cyrtochilum pardinum*. The putatively taxonomic category in few cases was possible at a kingdom level.

Taxonomic category	Number of OTUs	Ecological function of some species within the order
Agaricales*	414	Mycorrhizal fungi associated with orchids of the Genus *Orchis* (Jacquemyn et al., 2010). Endophyte associated with *Wullschlaegelia aphylla* and *Gastrodia similis* (Martos et al., 2009).
Helotiales*	282	Orchid endophyte of *Epidendrum firmun* (Kartzinel et al., 2013b).
Pleosporales*	188	Fungi associated with protocorms of *Epidendrum firmum* (Kartzinel et al., 2013b). Endophyte associated with ericoid roots (Smith and Read 2008).
Chaetothyriales*	181	Root-associated fungi identified in *Pleurothallis lilijae* (Herrera et al., 2010).
Glomerales	181	Arbuscular mycorrhizal associated with liverworts (Russell and Bulman, 2005).
Hypocreales*	165	Endophyte and saprotrophic associated with Endophyte associated with *Wullschlaegelia aphylla* and *Gastrodia similis* (Martos et al., 2009). Root-associated fungi identified in *Stelis superbiens, Pleurothallis lilijae* and *Stelis* sp. (Herrera et al., 2010)
Cantharellales*	150	Mycorrhizal fungi associated with orchids of the Genus *Orchis* (Jacquemyn et al., 2010).
Capnodiales*	126	Orchid endophyte with potential for beneficial or deleterious associations (Kartzinel et al., 2013b).

		Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Xylariales*	119	Endophytic fungi associated with *Dendrobium nobile* (Yuan et al., 2009). Root-associated fungi identified in *Stelis superbiens, Stelis hallii, Stelis concinna, Pleurothallis lilijae* and *Stelis* sp. (Herrera et al., 2010).
Polyporales*	108	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Thelephorales*	87	Mycorrhizal fungi associated with orchids of the Genus *Orchis* (Jacquemyn et al., 2010).
Hymenochaetales*	76	Saprotrophic identified in *Wullschlaegelia aphylla* and *Gastrodia similis* roots (Martos et al., 2009). Mycorrhizal fungi identified in epiphytic orchid species from Reunion Island (Martos et al., 2012).
Pezizales*	78	Mycorrhizal partners (Tuber sp.) in *Epipactis microphylla* (Dearnaley et al., 2012). Orchid root-associated non-*Rhizoctonia* fungi associated with *Bipinnula* spp. (Mujica et al., 2016)
Sebacinales*	75	Mycorrhizal fungi associated with the epiphytic orchid *Stelis hallii, S. superbiens, S. concinna and Pleurothallis lilijae* (Suárez et al., 2008).
Lecanorales*	75	Mycorrhizal fungi identified in epiphytic orchid species from Reunion Island (Martos et al., 2012).
Diaporthales*	73	Non-mycorrhizal fungal endophytes of *Dendrobium nobile* (Yuan et al., 2009). Orchid root-associated non-*Rhizoctonia* fungi associated with *Bipinnula* spp. (Mujica et al., 2016)
Russulales	70	Endophyte of *Dipodium hamiltonianum* (Dearnaley and Le Brocque, 2006). Saprotrophic identified in *Wullschlaegelia aphylla* and *Gastrodia similis* roots (Martos et al., 2009).
Mortierellales	67	Fungi associated with *Pseudorchis albida (*Kohout et al., 2013).
Ascomycota*	43	Root-associated fungi of with *Pseudorchis albida* (Kohout et al., 2013).

Saccharomycetales	64	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Tremellales	64	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Trechisporales	56	Endophyte associated with *Wullschlaegelia aphylla* and *Gastrodia similis* (Martos et al., 2009).
Auriculariales	52	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Boletales	49	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Septobasidiales	42	-
Eurotiales	33	Endophyte of *Dipodium hamiltonianum* (Dearnaley and Le Brocque, 2006).
Leotiomycetes	27	Endophytic fungi, associated with roots of orchids (Oliveira et al., 2014).
Sordariales*	26	Fungi associated with *Pseudorchis albida (*Kohout et al., 2013). Endophytic fungi with mycorrhizal potential associated with *Hadrolaelia* spp. (Oliveira et al., 2014).
Diversisporales	25	Mycorrhizal fungi associated with *Apteria* sp. (Merckx 2013).
Orbiliales	25	Fungi associated with *Pseudorchis albida (*Kohout et al., 2013).
Atheliales	22	-
Ostropales*	21	-
Atractiellales*	17	Mycorrhizal fungi with tropical orchids (Kottke et al., 2010).
Glomerellales*	16	Endophytic fungi associated with *Ginkgo biloba* (Zheng et al., 2013). Root endophytic fungi of *Holcoglossum* spp. (Tan et al., 2012).
Pyrenulales	15	-
Trichosporonales	15	-
Corticiales	14	-

Helicobasidiales	14	-
Sporidiobolales	14	-
Dothideomycetes	13	Fungi associated (pathogenic) with *Pseudorchis albida (*Kohout et al., 2013).
Mucorales*	13	Orchid non-mycorrhizal fungi related (Ma et al., 2015).
Verrucariales	13	-
Chaetosphaeriales	10	-
Endogonales	9	-
Fungi	9	-
Gloeophyllales*	9	-
Dacrymycetales	8	-
Tubeufiales	8	-
Agaricostilbales	7	-
Chytridiomycota	7	-
Geoglossales	6	-
Gomphales	6	-
Hysteriales	5	-
Archaeorhizomycetales	5	-
Chytridiales	4	-
Cystofilobasidiales	4	Endophyte associated with *Wullschlaegelia aphylla* and *Gastrodia similis* (Martos et al., 2009).
Exobasidiomycetes	4	-
Kickxellales	4	-
Leucosporidiales	4	-
Microbotryales	4	-
Taphrinales	4	-
Teloschistales	4	-
Venturiales	4	-
Wallemiales	4	-
Amphisphaeriales	3	Orchid non-mycorrhizal endophytic fungi (Ma et al., 2015).
Botryosphaeriales	3	Non-mycorrhizal fungal endophytes inhabiting *Dendrobium nobile* (Yuan et al., 2009).
Exobasidiales	3	-
Glomeromycota	3	-
Malasseziales*	3	Mycorrhizal fungi identified in epiphytic orchid

		species from Reunion Island (Martos et al., 2012).
Phallales	3	-
Pucciniales	3	-
Rhizophydiales	3	-
Thelebolales	3	-
Acarosporales	2	-
Archaeosporales	2	-
Dothideales	2	-
Filobasidiales	2	-
Geastrales	2	-
Hysterangiales	2	-
Microstromatales	2	-
Myriangiales	2	-
Onygenales	2	-
Pertusariales	2	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Agaricomycetes	1	Ericoid mycorrhizal fungi (Wurzburger et al., 2012).
Aphyllophorales	1	Potential mycorrhizal (Umata 1995).
Basidiobolales	1	-
Basidiomycota	1	-
Blastocladiales	1	-
Coniochaetales	1	-
Cystobasidiales	1	-
Elaphomycetales	1	-
Entorrhizales	1	-
Erythrobasidiales	1	-
Gyalectales	1	-
Heterogastridiales	1	-
Holtermanniales	1	-
Kriegeriales	1	-
Lobulomycetales	1	-
Magnaporthales	1	-
Microascales	1	-
Monoblepharidales	1	-
Mycocaliciales	1	-

Naohideales	1	-
Neocallimastigales	1	-
Ophiostomatales	1	-
Patellariales	1	-
Peltigerales	1	-
Rhytismatales	1	-
Sordariomycetes	1	Fungi associated (pathogenic) with *Pseudorchis albida* (Kohout et al., 2013).
Togniniales	1	-
Trichosphaeriales	1	Root-associated fungi identified in *Epidendrum firmum* associates (Kartzinel et al., 2013b).
Umbilicariales	1	-

* Orders with overlapped OTUs between the Podocarpus National Park and Cajas National Park.

Table S2 Comparison of the variance between fungal endophyte communities associated with *Epidendrum marsupiale* (Sp4) and *Cyrtochilum pardinum* (Sp5). High Mazán (HM): site 3, Low Mazán (LM): site 4, High LLaviucu (HLL): site 5 and Low LLaviucu (LLL): site 6.

Comparison			Sp4		Sp5	
			Jaccard indexes mean difference ± std. error	*P*-value	Jaccard indexes mean difference ± std. error	*P*-value
HM - LM	vs.	HLL - LLL	0.003 ± 0.018	0.997	0.006 ± 0.023	0.981
HM - LM	vs.	HM - LLL	-0.008 ± 0.018	0.842	-0.011 ± 0.023	0.734
HM - LM	vs.	LM - LLL	-0.003 ± 0.018	0.997	0.008 ± 0.024	0.92
HM - LM	vs.	HM - HLL	0.005 ± 0.017	0.97	-0.009 ± 0.023	0.899
HM - LM	vs.	LM - HLL	0.013 ± 0.017	0.289	0.017 ± 0.023	0.242
HLL - LLL	vs.	HM - LLL	-0.011 ± 0.019	0.615	-0.017 ± 0.022	0.275
HLL - LLL	vs.	LM - LLL	-0.006 ± 0.019	0.952	0.003 ± 0.023	0.999
HLL - LLL	vs.	HM - HLL	0.002 ± 0.018	1	-0.014 ± 0.022	0.465
HLL - LLL	vs.	LM - HLL	0.010 ± 0.018	0.645	0.012 ± 0.022	0.648
HM - LLL	vs.	LM - LLL	0.005 ± 0.019	0.982	0.020 ± 0.024	0.178
HM - LLL	vs.	HM - HLL	0.012 ± 0.018	0.38	0.003 ± 0.023	0.999
HM - LLL	vs.	LM - HLL	0.020* ± 0.018	0.019	0.029* ± 0.282	0.004
HM - LLL	vs.	HM - HLL	0.008 ± 0.018	0.834	-0.017 ± 0.023	0.322
HM - LLL	vs.	LM - HLL	0.016 ± 0.018	0.141	0.009 ± 0.023	0.874
HM - HLL	vs.	LM - HLL	0.008 ± 0.017	0.779	0.026* ± 0.255	0.012

* Difference between Jaccard indexes statistically significant (*P*-value ≤0.05)

Table S3 Overview of the identified root-associated fungal (RAF) endophytes OTUs of *Cyrtochilum flexuosum, C. myanthum* and *Maxillaria calantha*.

Orchid species	Site	Total OTUs-RAF identified	OTUs-RAF identified in a specific site	Overlapped OTUs- RAF between sites
Cyrtochilum flexuosum	Site 1	336	202	124
	Site 2	491	124	
Cyrtochilum myanthum	Site 1	318	199	119
	Site 2	318	199	
Maxillaria calantha	Site 1	345	218	127
	Site 2	336	209	

Table S4. Mycorrhizal operational taxonomic units (OTUs) specific to a particular site/orchid population

Orchid species	OTU ID	Inferred taxonomy	Known host species/family or source of origin (Reference)	Known features of the plant-mycorrhizal interaction
C. flexuosum-Site 1	OTU 1	*Serendipita* sp.	- Spruce stumps (Kubart et al., 2016) - Soil (Stone et al., unpublished)	- Fungal community variable along latitudinal gradient.
	OTU 32	*Serendipita* sp.	- *Neottia cordata*/Orchidaceae (Těšitelová et al., 2015) - *Hoffmannseggella cinnabarina*/Orchidaceae (Oliveira et al., 2014)	- Orchid mycorrhizal. - Habitat type could influences mycorrhizal occurrence and community structure.
	OTU 46	*Ceratobasidium* sp.	- *Epidendrum firmum*/Orchidaceae (Kartzinel et al., 2013b)	- Orchid mycorrhizal. - Orchids exhibited broad mycorrhizal specificity. - Mycorrhizal community variable among neighboring orchid populations.
C. myanthum-Site 1	OTU 23	*Serendipita* sp.	- *Coccoloba* sp./Polygonaceae (Tedersoo et al., 2010b) - *Pakaraimaea dipterocarpacea*/Dipterocarpaceae	- Spatial factors could influence the fungal occurrence.

			(Moyersoen et al., 2014) - *Dicymbe corymbosa*/Caesalpinioideae (Smith et al., 2011)	
	OTU 27	*Serendipita* sp.	- Soil lowland tropical rainforest (McGuire et al., 2010) - *Dactylorhiza sambucina*/Orchidaceae (Jersáková et al., 2015)	- Saprotrophic fungal occurring in a specific tropical forest driven variable nutrient cycling patterns over small spatial scales. -Orchid mycorrhizal co-occurring with multiple fungal in same orchid individual.
	OTU 28	*Serendipita* sp.	- *Disterigma microphyllum* and *Gaultheria erecta*/Ericaceae (Setaro and Kron 2011) - *Coelogyne viscosa/*Orchidaceae (Xing et al., 2015)	- Close related mycorrhizal along geographic areas in America. - Orchid mycorrhizal occurring in depending on the orchid growth habitat.
	OTU 29	*Serendipita* sp.	- *Dactylorhiza sambucina*/Orchidaceae (Jersáková et al., 2015) - Soil lowland tropical rainforest (McGuire et al., 2010).	- Orchid mycorrhizal co-occurring with multiple fungal in same orchid individual. - Saprotrophic fungal occurring in a specific tropical forest driven variable nutrient cycling

				patterns over small spatial scales.
M. calantha-Site 1	OTU 17	*Serendipita* sp.	- Soil (Rosling et al., 2016) - Forest soil (Sui, unpublished)	- Fungal contribute to P cycling.
	OTU 22	*Serendipita* sp.	- Orchid sp. (Setaro and Kron 2011) - Orchidaceae (Martos et al., 2012) - *Hoffmannseggella caulescens*/Orchidaceae (Oliveira et al., 2014)	- Orchid mycorrhizal. - Stressful life conditions driven orchid-fungi specialization. - Habitat type could influences mycorrhizal occurrence and community structure.
	OTU 30	*Serendipita* sp.	- Orchid sp. (Setaro and Kron 2011) - *Cavendishia bracteata*/Ericaceae (Setaro and Kron 2011)	- Orchid mycorrhizal. - Stressful life conditions driven orchid-fungi specialization. - Fungal in close related to Sebacina in different geographic areas.
	OTU 31	*Serendipita* sp.	- Orchid sp. (Setaro and Kron 2011) - *Hoffmannseggella caulescens* and *Hadrolaelia jongheana*/Orchidaceae/Orchidaceae (Oliveira et al., 2014)	- Orchid mycorrhizal. - Stressful life conditions driven orchid-fungi specialization. - Habitat type could influences mycorrhizal occurrence and community

				structure.
C. flexuosum-Site 2	OTU 10	*Serendipita* sp.	- *Homogyne alpina*/Asteraceae (Garnica et al., 2013) - *Sphyrospermum buxifolium* and *Gaultheria erecta*/Ericaceae (Setaro and Kron 2011)	- Fungal incidence and structure of fungal community influenced by environment. - Fungal in close related to Sebacina in different geographic areas.
	OTU 11	*Serendipita* sp.	- Spruce stumps (Kubart et al., 2016) - Soil (Stone et al., unpublished)	- Fungal community change along the latitudinal gradient.
	OTU 20	*Serendipita* sp.	- Soil (Wurzburger et al., 2012) - *Daphne striata*/Thymelaeaceae (Garnica et al., 2013) - *Globularia nudicaulis*/Plantagegenaceae (Garnica et al., 2013)	- Saprotrophs in organic soil horizons. - Fungal incidence and structure of fungal community influenced by environment.
	OTU 40	*Ceratobasidium* sp.	- *Brassica napus*/Brassicaceae (Zhou et al, 2014) - Corn crops (Ohkura et al., 2009) - Orchid sp./Orchidaceae (Waud et al., 2014)	- Infecting corn crops. - Orchid mycorrhizal.
	OTU 41	*Ceratobasidium* sp.	- Orchid sp./Orchidaceae (Waud et al., 2014) - Orchid sp./Orchidaceae (Jacquemyn et al., 2015a)	- Orchid mycorrhizal. - Specifically associated with an orchid species. Mycorrhizal community

			- *Anacamptis morio*/Orchidaceae (Ercole et al., 2015)	partitioned in subsets across co-occurring orchid species with little overlap between subsets. - Orchid-fungi symbiosis remains constant over the time.
	OTU 42	*Ceratobasidium* sp.	- *Neottia ovata*/Orchidaceae (Jacquemyn et al., 2015b) - *Neottia cordata*/Orchidaceae (Těšitelová et al., 2015) - Soil organic horizon (Taylor et al., 2014)	- Orchid mycorrhizal not constant across orchid populations. - Mycorrhizal occurrence influenced by the habitat type.
C. myanthum-Site 2	OTU 14	*Serendipita* sp.	- Orchid sp./Orchidaceae (Jacquemyn et al., 2015a) - *Anthyllis vulneraria*/Fabaceae (Weiß et al., 2011)	- Orchid mycorrhizal. - Specifically associated with an orchid species. Mycorrhizal community partitioned in subsets across co-occurring orchid species with little overlap between subsets. - Beneficial on plant growth.
	OTU 16	*Serendipita* sp.	- *Riccardia palmata*/Aneuraceae (Krause et al., 2011) - *Neottia cordata*/Orchidaceae (Těšitelová et al., 2015) - Soil (Kluber et al., 2011)	- Endophyte. Identified in different geographic regions in Europe. - Mycorrhizal occurring influenced by the habitat

				type. - In soil, stable fungal composition through time.
	OTU 18	*Serendipita* sp.	- *Cavendishia nobilis*, *Disterigma microphyllum* and Disterigma alaternoides/Ericaceae (Setaro and Kron 2011)	- Fungal in close related to Sebacina in different geographic areas
	OTU 26	*Serendipita* sp.	- Soil (Stone *et al.,* unpublished)	No information
	OTU 44	*Ceratobasidium* sp.	- *Neottia ovata*/Orchidaceae (Jacquemyn et al., 2015b) - Tolumnia variegata./Orchidaceae (Otero et al., 2004)	- Orchid mycorrhizal not constant across orchid populations. - Orchids are generalists in the association.
M. calantha-Site 2	OTU 24	*Serendipita* sp.	- Soil (Kluber et al., 2011) - *Neottia cordata*/Orchidaceae (Těšitelová et al., 2015)	- Stable fungal composition through time. - Mycorrhizal occurring influenced by the habitat type.

Table S5. Mycorrhizal OTUs that showed overlap across sites/orchid populations.

Orchid Species	OTU ID	Inferred taxonomy	Known host species/family or origin source (Reference)	Known features of the plant-mycorrhizal interaction
C. flexuosum *C. myanthum* *M. calantha*	OTU 2	*Serendipita* sp.	- Soil (Kluber et al., 2011) - *Neottia ovata*/Orchidaceae (Těšitelová et al., 2015)	- Stable fungal composition through time - Orchid mycorrhizal occurring independently of habitat type.
C. flexuosum *C. myanthum* *M. calantha*	OTU 4	*Serendipita* sp.	- *Stelis* sp./ Orchidaceae (Setaro et al., 2013) - Orchid sp. 6 (Setaro et al., 2013)	- Orchid mycorrhizal. -Close related fungi from same group distinctive associated to epiphytic and terrestrial orchid in the tropical rainforest in south Ecuador.
C. flexuosum *C. myanthum* *M. calantha*	OTU 12	*Serendipita* sp.	- *Stelis* sp./ Orchidaceae (Setaro et al., 2013) - Soil (Kluber et al., 2011)	- Orchid mycorrhizal. -Close related fungi from same group distinctive associated to epiphytic and terrestrial orchid in the tropical rainforest in south Ecuador. - In soil, stable fungal composition through time
C. flexuosum *C. myanthum* *M. calantha*	OTU 33	*Ceratobasidium* sp.	- *Piperia yadonii*/Orchidaceae (Pandey et al., 2013)	- Orchid mycorrhizal. - Dominant fungal families remain constant among habitats.
C. flexuosum *C. myanthum* *M. calantha*	OTU 34	*Ceratobasidium* sp.	- Orchid sp./Orchidaceae (Jacquemyn et al., 2015a) - *Tolumnia variegata*/Orchidaceae (Otero et al., 2004)	- Populations partitioned in subsets across co-occurring orchid species with little overlap between subsets. - Orchids are generalists in the association.
C. flexuosum *C. myanthum* *M. calantha*	OTU 35	*Ceratobasidium* sp.	- *Piperia yadonii*/Orchidaceae (Pandey et al., 2013)	- Dominant fungal families remain constant among habitats.
C. flexuosum *C. myanthum* *M. calantha*	OTU 39	*Ceratobasidium* sp.	- *Epidendrum firmum*/Orchidaceae (Kartzinel et al., 2013b)	- Orchid mycorrhizal. - Orchids exhibited broad mycorrhizal specificity.

			- lawn (Yu and Li unpublished)	
C. flexuosum *C. myanthum* *M. calantha*	OTU 54	*Tulasnella* sp.	- Orchid sp. (Herrera et al., submitted)	- Orchid mycorrhizal.
C. flexuosum *C. myanthum*	OTU 3	*Serendipita* sp.	- *Dactylorhiza sambucina*/Orchidaceae (Jersáková et al., 2015) - Soil lowland tropical rainforest (McGuire et al., 2010). - Soil, high nitrogen soil (Vineis et al., unpublished)	- Orchid mycorrhizal co-occurring with multiple fungal in same orchid individual. - Saprotrophic fungal occurring in a specific tropical forest driven variable nutrient cycling patterns over small spatial scales.
C. flexuosum *C. myanthum*	OTU 5	*Serendipita* sp.	- *Hoffmannseggella cinnabarina*/Orchidaceae (Oliveira et al., 2014) - *Coelogyne viscosa*/Orchidaceae (Xing et al., 2015) - *Pterygodium magnum*/Orchidaceae (Waterman et al., 2011)	- Orchid mycorrhizal. - Stressful life conditions driven orchid-fungi specialization. - Habitat type and orchid growth habit could influences mycorrhizal occurrence and community structure. - Fungal partner are conserved at different areas.
C. flexuosum *C. myanthum*	OTU 7	*Serendipita* sp.	- *Neottia ovata*/Orchidaceae (Těšitelová et al., 2015) - *Anthyllis vulneraria*/Fabaceae (Weiß et al., 2011)	- Orchid mycorrhizal occurring independently of habitat type. - Beneficial on plant growth.
C. flexuosum *C. myanthum*	OTU 36	*Ceratobasidium* sp.	- *Anoectochilus formosanus*/Orchidaceae (Jiang et al., 2015)	- Orchid mycorrhizal. - In orchids, increase seed germination and promoted protocorm growth.
C. flexuosum *C. myanthum*	OTU 38	*Ceratobasidium* sp.	- *Piperia yadonii*/Orchidaceae (Pandey et al., 2013) - Air filter sample (Frohlich et al., unpublished)	- Orchid mycorrhizal. - Dominant fungal families remain constant among habitats.
C. flexuosum *M. calantha*	OTU 47	*Tulasnella* sp.	- *Cypripedium tibeticum*/Orchidaceae (Yuan et al., 2010)	- Orchid mycorrhizal. - Mycorrhizal group is dominant and common

			- *Orchis mascula*/Orchidaceae (Waud et al., 2014) - *Anacamptis morio*/Orchidaceae (Bailarote et al., 2012)	among orchid species from same genus.
C. flexuosum	OTU 51	*Tulasnella* sp.	- Orchid sp. (Herrera et al., submitted) - *Cypripedium* spp./Orchidaceae (Shefferson et al., 2007)	- Orchid mycorrhizal. - Association with particular fungi is phylogenetically conserved.
C. myanthum	OTU 48	*Tulasnella* sp.	- Orchid sp. (Herrera et al., submitted) - *Cypripedium* spp./Orchidaceae (Shefferson et al., 2007)	- Orchid mycorrhizal. - Association with particular fungi is phylogenetically conserved.
M. calantha	OTU 13	*Serendipita* sp.	- *Disterigma alaternoides*/Ericaceae (Setaro and Kron 2011) - *Cavendishia nobilis*/Ericaceae (Setaro et al., 2013) - *Ceratostema reginaldii*/Ericaceae (Kottke et al., 2008)	- Fungal in close related to Sebacina in different geographic areas. - Fungal overlapped between ericad species in tropical montane rain forest area.

Chapter III

Table S6 Collected seedling-traps and number of identified OTUs. T2: Transect T2, Q5: Transect Q5; C: *Cyrtochilum retusum,* E: *Epidendrum macrum*; S1: 1st sampling, S2: 2nd sampling and A1-A10: Altitudinal levels.

Sampled seedling-trap	Altitude m a.s.l	Mycorrhizal OTUs	Non-mycorrhizal OTUs
T2CS1_A1	~ 1850	6	63
T2CS1_A2	~ 1883	7	56
T2CS1_A3	~ 1916	-	-
T2CS1_A4	~ 1949	7	76
T2CS1_A5	~ 1982	10	88
T2CS1_A6	~ 2015	18	99
T2CS1_A7	~ 2048	8	152
T2CS1_A8	~ 2081	10	144
T2CS1_A9	~ 2114	11	73
T2CS1_A10	~2147	2	52
T2ES1_ A1	~ 1850	2	107
T2ES1_ A2	~ 1883	2	72
T2ES1_ A3	~ 1916	3	68
T2ES1_ A4	~ 1949	2	32
T2ES1_ A5	~ 1982	3	99
T2ES1_ A6	~ 2015	4	33
T2ES1_A7	~ 2048	-	-
T2ES1_A8	~ 2081	7	34
T2ES1_ A9	~ 2114	13	77
T2ES1_ A10	~2147	3	56
Q5CS1_ A1	~ 1850	-	-
Q5CS1_ A2	~ 1883	5	46
Q5CS1_ A3	~ 1916	4	92
Q5CS1_ A4	~ 1949	21	161
Q5CS1_ A5	~ 1982	5	91
Q5CS1_ A6	~ 2015	5	69
Q5CS1_ A7	~ 2048	2	74
Q5CS1_ A8	~ 2081	8	131
Q5CS1_ A9	~ 2114	5	85
Q5CS1_ A10	~2147	5	69
T2CS2_ A1	~ 1850	14	148
T2CS2_ A2	~ 1883	5	136
T2CS2_ A3	~ 1916	-	-
T2CS2_ A4	~ 1949	11	163
T2CS2_ A5	~ 1982	22	114
T2CS2_ A6	~ 2015	19	228
T2CS2_ A7	~ 2048	19	111
T2CS2_ A8	~ 2081	-	-
T2CS2_ A9	~ 2114	25	140
T2CS2_ A10	~2147	19	150

Table S7. Operational taxonomic units putatively assigned to mycorrhizal fungi using UNITE database.
* Sequences identity lower than 90%.

OTU Id	Number of sequences	Order	Family/Genus	Length (bp)	Score	E-value	Sequence identity %
OTU_19	26844	Cantharellales	Ceratobasidiaceae	360	568.1	0	99.12
OTU_24	3256	Sebacinales	-	329	413.6	0	89.86
OTU_27	8757	Cantharellales	Cantharellales	362	523.5	0	94.25
OTU_34	7526	Sebacinales	Serendipitaceae	317	509.7	0	97.79
OTU_46	6628	Cantharellales	Ceratobasidium	443	652.2	0	94.87
OTU_60	2186	Sebacinales	-	348	468.5	0	92.57
OTU_138	2023	Cantharellales	-	382	152.6	0	74.41
OTU_144	293	Sebacinales	-	335	566.4	0	99.4
OTU_153	1524	Sebacinales	-	336	477.1	0	94.07
OTU_178	370	Cantharellales	-	347	94.3	0	92.75
OTU_191	1051	Cantharellales	Ceratobasidium	428	638.5	0	95.56
OTU_196	376	Sebacinales	-	341	506.3	0	95.56
OTU_204	474	Sebacinales	Serendipita	459	130.3	0	94.44
OTU_210	409	Sebacinales	-	395	612.7	0	94.44
OTU_223	1138	Cantharellales	-	410	595.6	0	94.87
OTU_225	4722	Cantharellales	Ceratobasidiaceae	353	542.4	0	99.38
OTU_298	406	Sebacinales	-	328	509.7	0	96.65
OTU_325*	167	Cantharellales	-	383	116.6	0	72.51
OTU_358	102	Cantharellales	-	331	80.5	0	91.8
OTU_402	131	Sebacinales	-	334	549.2	0	98.5
OTU_418	95	Atractiellales	-	354	609.3	0	100
OTU_426	98	Cantharellales	Ceratobasidium	363	559.5	0	96.43
OTU_473	75	Cantharellales	Ceratobasidium	358	502.9	0	93.85
OTU_492	53	Sebacinales	-	334	569.8	0	99.7
OTU_548	51	Sebacinales	-	340	544.1	0	97.65
OTU_566	138	Sebacinales	Sebacinaceae	339	521.7	0	96.46
OTU_579	178	Cantharellales	-	403	638.5	0	97.51
OTU_634	147	Cantharellales	-	358	478.8	0	95.62
OTU_699	215	Cantharellales	-	347	94.3	0	92.75
OTU_715*	49	Cantharellales	-	383	84	0	70.83
OTU_763	70	Cantharellales	Ceratobasidiaceae	357	542.4	0	96.08
OTU_830	57	Cantharellales	Ceratobasidium	416	700.3	0	99.28
OTU_907*	51	Cantharellales	-	381	99.4	0	71.69
OTU_933	205	Cantharellales	Ceratobasidium	364	569.8	0	96.98
OTU_1047	34	Sebacinales	-	384	587	0	97.81
OTU_1089	20	Sebacinales	-	334	494.3	0	96.01
OTU_1103*	194	Cantharellales	-	382	128.6	0	73.21
OTU_1168	39	Sebacinales	-	383	518.3	0	92.53
OTU_1176	65	Cantharellales	-	331	90.8	0	95.08
OTU_1226	15	Atractiellales	-	346	585.3	0	99.42
OTU_1259	90	Cantharellales	Ceratobasidium	411	650.5	0	97.32

OTU_1305*	114	Cantharellales	-	382	111.4	0	72.34
OTU_1308*	41	Sebacinales	Sebacinaceae	427	473.7	0	87.94
OTU_1408*	27	Sebacinales	Sebacinaceae	339	265.9	0	81.38
OTU_1410	24	Sebacinales	-	340	580.1	0	99.71
OTU_1417*	6	Cantharellales	-	309	326	0	85.25
OTU_1442*	128	Cantharellales	-	382	138.9	0	73.74
OTU_1451*	22	Cantharellales	-	382	104.6	0	76.33
OTU_1470	41	Sebacinales	-	366	609.3	0	98.91
OTU_1527	35	Cantharellales	-	334	554.4	0	98.8
OTU_1592*	101	Cantharellales	-	383	108	0	72.15
OTU_1628	25	Cantharellales	-	382	89.1	0	91.3
OTU_1863	36	Cantharellales	-	330	85.7	0	93.44
OTU_1912	25	Cantharellales	Ceratobasidium	445	743.2	0	98.88
OTU_2038*	14	Cantharellales	-	433	401.6	0	87.6
OTU_2180	4	Cantharellales	Ceratobasidiaceae	358	585.3	0	98.32
OTU_2416*	63	Cantharellales	-	405	118.3	0	72.32
OTU_2462	132	Cantharellales	-	370	116.6	0	91.21
OTU_2514*	6	Cantharellales	Tulasnellaceae	431	398.1	0	84.13
OTU_2661*	13	Cantharellales	-	382	109.7	0	72.22
OTU_2870	37	Cantharellales	-	340	94.3	0	92.75
OTU_2877*	8	Cantharellales	-	382	111.4	0	72.3
OTU_3176	5	Sebacinales	-	371	581.8	0	97.04
OTU_3440	2	Sebacinales	Serendipitaceae	317	417	0	92.11
OTU_3477	6	Sebacinales	-	364	513.2	0	93.73
OTU_3539	7	Cantharellales	Ceratobasidiaceae	390	604.2	0	96.67
OTU_3753*	2	Cantharellales	Ceratobasidium	370	343.2	0	84.31
OTU_3843*	6	Cantharellales	-	436	278	0	81.02
OTU_3866	80	Cantharellales	-	340	94.3	0	92.75
OTU_3927	2	Cantharellales	-	416	616.2	0	94.99
OTU_3928	3	Sebacinales	Serendipitaceae	386	576.7	0	95.6
OTU_4003	2	Cantharellales	Ceratobasidiaceae	445	611	0	93.26
OTU_4038	2	Cantharellales	-	469	604.2	0	91.51
OTU_4049*	5	Cantharellales	Ceratobasidiaceae	395	372.4	0	84.58
OTU_4105	8	Cantharellales	Ceratobasidiaceae	445	623	0	93.72
OTU_4199	8	Cantharellales	-	328	535.5	0	99.37
OTU_4217	3	Cantharellales	Ceratobasidium	358	473.7	0	91.99
OTU_4568	17	Sebacinales	-	328	530.3	0	99.05
OTU_4582*	553	Sebacinales	-	352	423.9	0	89.64
OTU_4657	5	Sebacinales	-	340	520	0	96.19
OTU_4825*	11	Cantharellales	-	412	145.8	0	73.48
OTU_4850	5	Sebacinales	-	373	458.2	0	90.19
OTU_4899	5	Sebacinales	Serendipitaceae	317	509.7	0	97.79

Table S8 Similarity index of mycorrhizal communities, estimated pairwise between the altitudinal levels of each treatment (T2: Transect T2, Q5: Transect Q5; C: *Cyrtochilum retusum,* E: *Epidendrum macrum*).

Sampling		T2C				T2E				Q5C			
1st sample (1s)	2nd sample (2s)	1s OTUs	2s OTUs	Shared	Chao-Jaccard	1s OTUs	2s OTUs	Shared	Chao-Jaccard	1s OTUs	2s OTUs	Shared	Chao-Jaccard
1	**2**	6	7	3	0.214	2	2	0	0	5	4	0	0
1	**3**	-	-	-	-	2	3	1	0.839	5	21	5	1
1	**4**	6	7	2	0.41	2	2	0	0	5	5	0	0
1	**5**	6	10	0	0	2	3	0	0	5	5	1	0
1	**6**	6	18	2	0.27	2	4	0	0	5	2	0	0
1	**7**	6	8	2	0.664	-	-	-	-	5	8	2	0.049
1	**8**	6	10	0	0	2	7	0	0	5	5	4	0.917
1	**9**	6	11	2	0.177	2	13	0*	0	5	5	3	0.278
1	**10**	6	2	0	0	2	3	0	0	-	-	-	-
2	**3**	-	-	-	-	2	3	0	0	4	21	1	0.002
2	**4**	7	7	4	0.687	2	2	0	0	4	5	0	0
2	**5**	7	10	2	0.469	2	3	2	0.057	4	5	1	0.001
2	**6**	7	18	4	0.438	2	4	0	0	4	2	1	0.013
2	**7**	7	8	2	0.192	-	-	-	-	4	8	0	0
2	**8**	7	10	1	0.034	2	7	2	0.055	4	5	0	0
2	**9**	7	11	4	0.353	2	13	1	0	4	5	0	0
2	**10**	7	2	0	0	2	3	2	0.351	-	-	-	-
3	**4**	-	-	-	-	3	2	1	0.01	21	5	1	0
3	**5**	-	-	-	-	3	3	0	0	21	5	3	0.001
3	**6**	-	-	-	-	3	4	0	0	21	2	2	0.002
3	**7**	-	-	-	-	-	-	-	-	21	8	4	0.062
3	**8**	-	-	-	-	3	7	1	0.002	21	5	5	0.901
3	**9**	-	-	-	-	3	13	0	0	21	5	4	0.45

3	**10**	-	-	-	-	3	3	0	0	-	-	-	-
4	**5**	7	10	3	0.277	2	3	0	0	5	5	0	0
4	**6**	7	18	5	0.465	2	4	0	0	5	2	1	0
4	**7**	7	8	2	0.34	-	-	-	-	5	8	1	0.001
4	**8**	7	10	0	0	2	7	1	0.002	5	5	0	0
4	**9**	7	11	4	0.325	2	13	0	0	5	5	0	0
4	**10**	7	2	0	0	2	3	0	0	-	-	-	-
5	**6**	10	18	7	0.399	3	4	0	0	5	2	1	0.001
5	**7**	10	8	2	0.012	-	-	-	-	5	8	1	0
5	**8**	10	10	3	0.022	3	7	2	0.029	5	5	1	0
5	**9**	10	11	4	0.186	3	13		0	5	5	1	0
5	**10**	10	2	1	0.002	3	3	2	0.055	-	-	-	-
6	**7**	18	8	4	0.435	-	-	-	-	2	8	1	0.005
6	**8**	18	10	2	0.047	4	7	2	0.013	2	5	0	0
6	**9**	18	11	5	0.299	4	13	3	0.984	2	5	0	0
6	**10**	18	2	1	0.002	4	3	0	0	-	-	-	-
7	**8**	8	10	4	0.183	-	-	-	-	8	5	2	0.038
7	**9**	8	11	4	0.183	-	-	-	-	8	5	1	0.007
7	**10**	8	2	1	0.002	-	-	-	-	-	-	-	-
8	**9**	10	11	4	0.02	7	13	4	1	5	5	3	0.277
8	**10**	10	2	1	0.002	7	3	2	0.055	-	-	-	-
9	**10**	11	2	1	0.002	13	3	1	0	-	-	-	-

Table S9 Number of operational taxonomic units (OTUs) identified in T2CS1 and T2CS2 treatments (T2: Transect 2; C: *Cyrtochilum macrum*; S1: 1st sampling, S2: 2nd sampling) and similarity index of mycorrhizal fungal communities identified in function of the temporal variation.

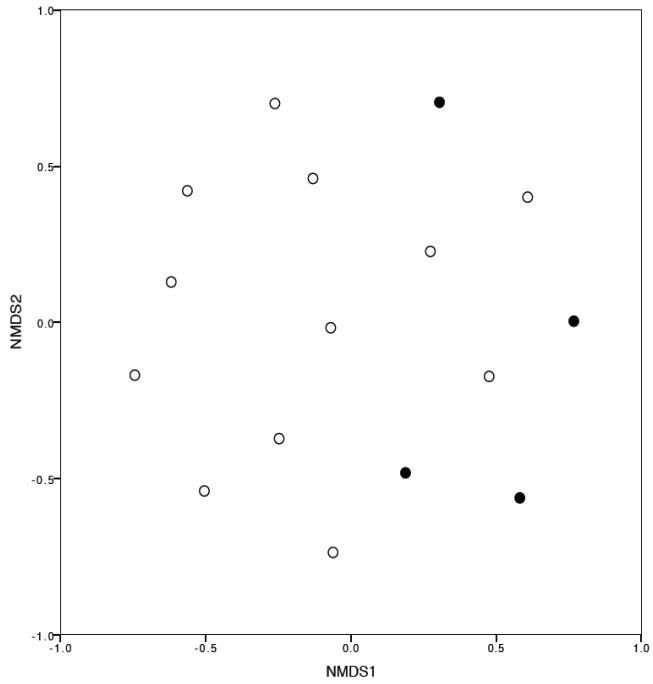
Altitudinal point	T2CS1	T2CS2	Shared OTUs	Chao-Jaccard	*P-value*
A1	6	14	2	0.2	0.006
A2	7	5	2	0.182	0.346
A3	-	-	-	-	-
A4	7	11	1	0.118	0.180
A5	10	22	5	0.143	0.001
A6	18	19	6	0.08	0.753
A7	8	19	4	0	0.001
A8	10	-	-	-	-
A9	11	25	7	0.129	0
A10	2	19	1	0.16	0

SUPPORTING FIGURES

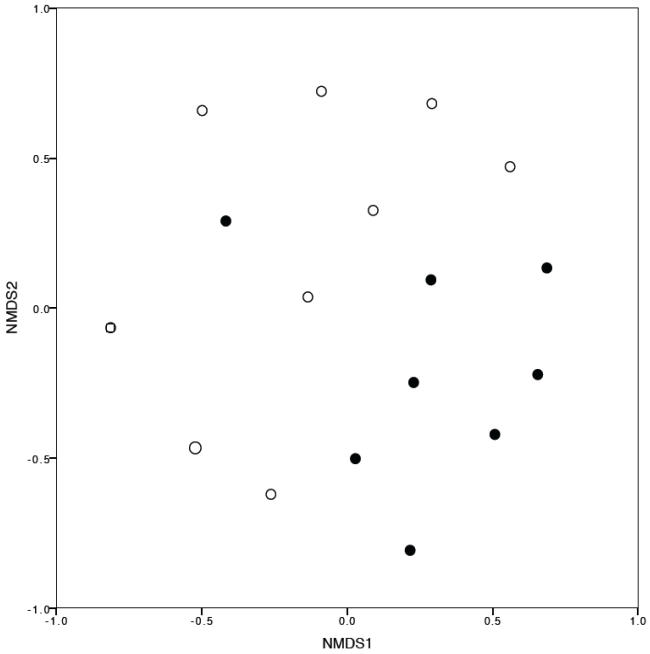
Research Results

Chapter II

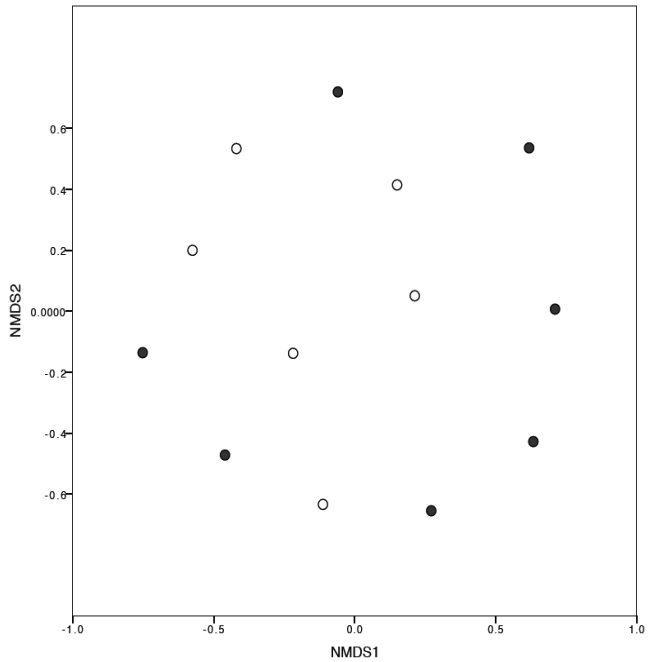
Fig. S1 Non-metric multidimensional scaling (NMDS) plot of root-associated endophyte fungi communities detected in association with a) *Cyrtochilum flexuosum,* b) *Cyrtochilum myanthum,* c) *Maxillaria calantha* d) *Epidendrum marsupiale* and e) *Cyrtochilum pardinum,* at the different orchid populations.



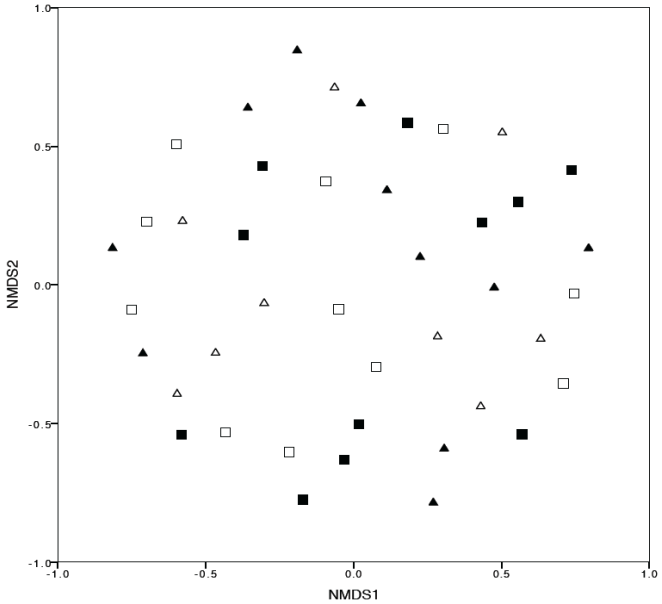
a) *Cyrtochilum flexuosum* (stress value = 0.123). Black dots corresponded to Curva Misteriosa and white dots to El Tiro.



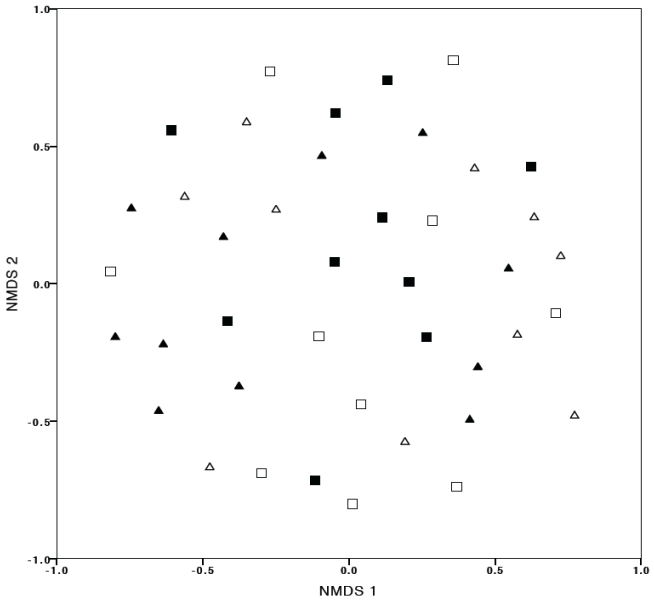
b) *Cyrtochilum myanthum* (stress value = 0.114). Black dots corresponded to Curva Misteriosa and white dots to El Tiro.



c) *Maxillaria calantha* (stress value = 0.108). Black dots corresponded to Curva Misteriosa and white dots to El Tiro.



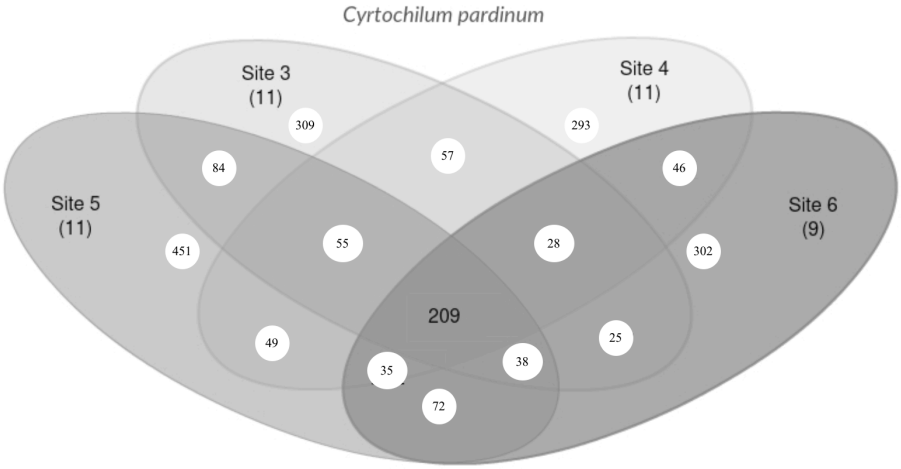
d) *Epidendrum marsupiale* (stress value = 0.142). Black squares to High Mazán, white squares to Low Mazán, black triangles to High Llaviucu and white triangles to Low Llaviucu.



e) *Cyrtochilum pardinum* (stress value = 0.143). Black squares to High Mazán, white squares to Low Mazán, black triangles to High Llaviucu and white triangles to Low Llaviucu.

Fig. S2

(a)



(b)

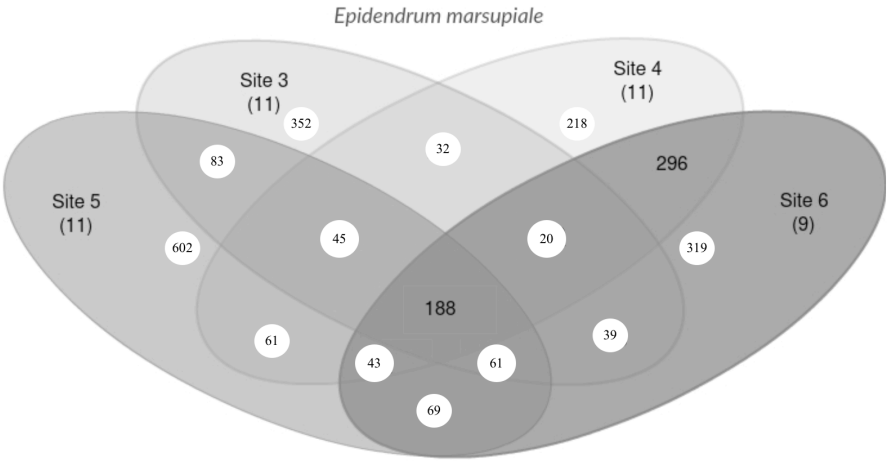


Fig. S2 Differences in fungal community composition between orchid populations of *Epidendrum marsupiale* and *Cyrtochilum pardinum* represented in Venn Diagrams. Total orchid sampled per study site are in brackets, number of OTUs per site or overlapped between sites shown in white circles (**a, b**).

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