Generalism in the interaction of Tulasnellaceae mycobionts with orchids characterizes a biodiversity hotspot in the tropical Andes of Southern Ecuador

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Biotic interactions play an important role in the assembly and stability of communities. All orchids depend on mycobionts for early establishment, but whether individual orchid species depend on a specific or broad spectrum of mycobionts is still a matter of debate. Tulasnellaceae (Basidiomycota) is the richest and most widespread mycobiont worldwide. We assessed Tulasnellaceae richness in epiphytic and terrestrial orchids in different habitats, and evaluated the degree of generalism in orchid-Tulasnellaceae interactions and the robustness of this mutualistic system to the extinction of mycobiont partners. We sampled 114 orchid individuals including all common and rare species in 56 plots of 1 m² in 3 habitats: pristine forest, regenerating forest and a landslide site in a tropical montane rainforest in Southern Ecuador. We found 52 orchid and 29 Tulasnellaceae species. The composition of Tulasnellaceae OTUs was moderately to highly similar across habitats and between orchid growth forms. A significantly nested network architecture indicated the existence of a core of generalist Tulasnellaceae OTUs interacting with both rare and common orchids. Terrestrial and epiphytic orchids showed significant differences in robustness to the extinction of their Tulasnellaceae mycobionts. Thus, generalist mycobionts may be relevant for the preservation of hyperdiverse orchid communities in the tropics.

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1. Introduction

Biotic interactions such as mutualism play an important role in the assembly, persistence, and stability of communities (Fortuna et al., 2010). Fungi interact with over 80% of land plants, forming a mutualistic interaction known as mycorrhizae (Smith & Read, 2008). Mycorrhizal symbionts (mycobionts) are a vital component of plant ecosystems (Bonfante & Anca, 2009), and they improve plant growth by promoting nutrient uptake especially when the availability of soil nutrients is low (Yang et al., 2016; van der Heijden, Bardgett, & van Straalen, 2008). As orchids produce tiny seeds lacking stored nutrients, they require an obligate supplement of carbon and minerals supplied by mycobionts to establish protocorms in the wild (Rasmussen, 1995).

Recently, much attention has been given to documenting the diversity of the mycobionts associated with orchids and their degree of specificity (Jacquemyn, Honnay, Cammue, Brys, & Lievens, 2010; Kartzinel, Trapnell, & Shefferson, 2013; McCormick, Whigham, & O’Neill, 2004; Pellegrino, Luca, & Belluscì, 2014). Among these fungi, members of Tulasnellaceae (Basidiomycota) are one of the richest and most widespread mycobionts forming mycorrhizae with a broad spectrum of photosynthetic Orchidaceae in temperate and tropical ecosystems (Dearnaley, 2007; Dearnaley, Martos, & Selosse, 2012; Jacquemyn, Waud, Merckx, Lievens, & Brys, 2015a; Martos et al., 2012; Suárez et al., 2006). As Tulasnellaceae mycobionts are widespread on rotten wood and humus-rich substrates (Roche et al., 2010; Cruz, Suárez, Kottke, Piepenbring, & Oberwinkler, 2011, 2014), they are available to associate with orchids in terrestrial and epiphytic habitats of the tropics.

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For many years, there has been debate on whether individual orchid species depend on a specific or broad spectrum of mycobionts (Cozzolino & Widmer, 2006; Dearnaley, 2007; Shefferson et al., 2007; Wercup, 1981; Waud, Bussschaert, Lievens, & Jacquemyn, 2016). McCormick and Jacquemyn (2014) found specific orchid-mycobiont relationships with distinct mycorrhizal communities of coexisting orchids in temperate grasslands. Similarly, Jacquemyn, Brys, Waud, Bussschaert, and Liebens (2015b) found that groups of phylogenetically related orchid species were associated with specific groups of Tulasnellaceae in species-rich Mediterranean grasslands. When single orchid genera were studied, associated with specificity (Jacquemyn, 2016). McCormick and Jacquemyn (2014) found species-specific mycobiont-orchid interactions based on the network theory (Bascompte & Jordano, 2007) is gaining momentum in ecological research on complex and megadiverse systems (Jacquemyn et al., 2015b; Kottke et al., 2013; Martos et al., 2012). Understanding the processes that structure these networks will help explain if and how hyperdiverse sets of interacting species coevolve and are maintained in their habitats (Jordano, Vázquez, & Bascompte, 2009). This network approach provides a basic description of the general pattern of assembly and interactions (Jordano, Bascompte, & Olesen, 2003), such as nestedness (Bascue, Jordano, Melian, & Olesen, 2003) or modularity (Olesen, Bascompte, Dupont, & Jordano, 2007). Significant nestedness and modularity may indicate the importance of niche-based processes on community assembly, affecting the diversity, stability and coevolutionary dynamics between symbiotic species (Chagnon, Bradley, & Klironomos, 2012). For example, the network approach revealed contrasting nestedness and modularity of the orchid-symbiont relationship between a temperate system (Jacquemyn et al., 2011) versus a tropical (Martos et al., 2012) and a Mediterranean (Jacquemyn et al., 2015b) system. While the temperate European Orchis spp. and their mycobionts formed a significantly nested network, the Mediterranean and La Reunion Island networks were highly modular, epiphytic and terrestrial associations forming distinct modules in La Reunion. In a small area of a tropical mountain forest in Southern Ecuador, Kottke et al. (2013) found a significantly nested network architecture among the highly diverse orchids and 3 groups of diverse mycorrhizal fungi. However, they did not consider the effect of habitats, life forms or sites on the architecture of the fungi-orchids network based on Tulasnellaceae as mycobionts.

Orchidaceae are extraordinarily speciose and abundant, and form an essential part of the tropical mountain forest in the Southern Andes in Ecuador (Homeier & Werner, 2007). This area is considered a hotspot of plant biodiversity (Barthlott, Mutke, Rafiqpoor, Kier, & Kreft, 2005; Brehm et al., 2008), but the habitat is constantly threatened by anthropogenic activities, making its preservation a priority in global conservation. Most of its orchid species are endemic and rare (Homeier & Werner, 2007). Only 2 to 5 species are abundant (>2000 individuals in 0.1 ha), while most others are doubletons or singletons (Kottke et al., 2013). However, many species may still remain undescribed (Endara, 2000; Winkler, Hülber, & Hietz, 2009). This kind of asymmetric abundances is characteristic of extremely species-rich, mutually dependent communities (Medan et al., 2007). Orchid species richness in the tropical area may be supported by quite distinct habitats in the neighborhood such as pristine forests, young regenerating forests and frequent disturbance by landslides combined with a strong altitudinal gradient (Beck, Bendix, Kottke, Makeschin, & Mosandl, 2008). However, we are lacking the corresponding information on orchid mycobionts.

The main objective of this study was to assess the richness of Tulasnellaceae orchid mycobionts and the degree of specialization or generalism in orchid-Tulasnellaceae interactions in the montane rainforest area of Southern Andes, Ecuador. We specifically aimed to answer the following questions: (1) What is the richness of Tulasnellaceae mycobionts associated to epiphytic and terrestrial orchids in the tropical montane rainforest? (2) Does Tulasnellaceae richness and abundance differ among orchid life-forms (terrestrial versus epiphytic) or among sites (pristine forest, regenerating forest and regenerating landslide site)? (3) Are there any indications of generalism or specific partnerships between the species included in the mutualistic network? (4) Is the Tulasnellaceae-orchids network robust against the loss of Tulasnellaceae mycobiont species? If so, does it differ between epiphytic and terrestrial orchids?

2. Materials and methods

2.1. Study sites

This study was carried out in the Reserva Biológica San Francisco (RBSF) located half-way between Loja and Zamora, Zamora-Chinchipe Province, Ecuador (3°58'17''S, 79°04'33''W), adjacent to Podocarpus National Park on the steep eastern slope of the Cordillera El Consuelo, Southern Ecuadorian Andes. RBSF spans about 1000 ha over an altitudinal gradient of 1500 m–3400 m above sea level (a.s.l.). Permanent plots were established in 1997, and since then the montane rainforest has been comprehensively studied (Beck et al., 2008; Bendix et al., 2013), including the inventorying of mycorrhizal fungi (Haug, Setaro, & Suárez, 2013; Kottke et al., 2013, 2008). The evergreen forest is mostly composed of undisturbed, partly regenerating natural sites (Brehm et al., 2008; Weber, Günter, Aguirre, Stimm, & Mosandl, 2008), Average annual precipitation is about 2200 mm and mean temperature is 15.5 °C at 1900–2170 m a.s.l. (Bendix, Rollenbeck, Richter, Fabian, & Emck, 2008). Approximately 280 tree species, including 52 angiosperms and 1 gymnosperm, have been identified so far, and most of them are rare in the area (Homeier, Breckle, Günter, Rollenbeck, & Leuschner, 2010). Forest cover is close to 100% in the pristine forest and 73% in the 40-y-old regenerating forest included in this study. The relatively thin tree stems (5–25 cm diam) act as phorophytes for many orchids and other epiphytes, which is characteristic of the cloud forest (Homeier et al., 2013; Riofrío et al., 2007). The altitudinal gradient and the steepness of the forest-covered slopes further promote the frequent landslides caused by human activities like road construction, leading to high species turnover rates (Homeier, Werner, Gradstein, Breckle, & Richter, 2008; Osker, Daliz, Günter, Homeier, & Matezki, 2008).

Mycorrhizas of Orchidaceae were sampled at 4 sites which had different elevations and disturbance, from pristine forests to a landslide area. Sites 1 and 4 were located in pristine forests at 2000 m and 2170 m a.s.l., respectively. Site 3 was located in a 40-y-old regenerating forest at 2170 m a.s.l. close to Site 4, and Site 2 was located in an area at 1900 m a.s.l. where a human-caused landslide occurred 40 years ago (Supplementary Figs. S1 and S2).
The 40-y-old regenerating forest is a product of anthropogenic deforestation (Weber et al., 2008) and is structurally characterized by a lower proportion of trees (>10 cm diam at breast height (dbh)) and a greater proportion of bushes and tree ferns, thus appearing less homogeneous and more open than the primary forest surrounding it (Martinez, Mahecha, Lischeid, & Beck, 2008). The human-caused landslide produced a large gap which has vegetation in a young succession stage and is characterized by the growth of pioneer species that are not found in the primary forest (Bussmann, Wilcke, & Richter, 2008). Soil properties and fertility in landslide areas can vary considerably, providing less favorable conditions for plant growth than undisturbed soils (Bussmann et al., 2008). Ecological factors such as the amount of solar radiation, minimum and maximum temperatures at the forest ground and rainfall are higher in the regenerating forest and the landslide site than in the primary forest. In contrast, amount of litter fall, seed quantity and seed diversity are lower in these two habitats.

Permanent plots of 1 m² were randomly established at least 10 m apart at each site. Eight plots at each site were terrestrial and 8 plots were on tree stems (1 m in length, between 1 and 2 m above ground), except at Site 2 where only terrestrial plots could be established as there were no trees. Each plot included at least 5 orchid individuals of presumably different species. In 2008 the plots were visited several times throughout the flowering period to collect orchid samples to identify them at the species level.

2.2. Mycorrhizal sampling, fungal DNA isolation, PCR, cloning and sequencing

Roots were sampled from 2 individuals of randomly selected orchids at all plots except for two, where 3 individuals were sampled. Three root samples were collected per individual from a total of 114 orchid individuals. Roots considered to be mycorrhizal based on their appearance (color and health status) were collected from the pure humus layer in terrestrial orchids or in direct contact with the bark of tree stems for epiphytic orchids. At the landslide site, mycorrhizal roots were collected from the mineral soil. Samples were wrapped in aluminum foil, brought to the laboratory, and further handled within 24 h. Fungal colonization in root cortical tissue was verified by microscopic observation of fungal pelotons in thin sections stained by 0.05% methyl-blue (C.I. 42780) (Merck, Darmstadt, Germany) in lactic acid using an Axiosstar plus microscope (Carl Zeiss, Göttingen, Germany). Genomic DNA was extracted from 2 cm segments of well-colonized roots using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The internal transcribed spacers 1 and 2, including the 5.8 S region (nrDNA ITS-5.8 S) were targeted for PCR amplification using the universal eukaryotic primers ITS1 (5'-TCCGATGCTGAACTGCGG-3') (White, Bruns, Lee, & Taylor, 1990) and TW14 (5'-GCTATCTGAGGGAAACTTC-3') (Cullings, 1994) with the Phusion High-Fidelity PCR Mastermix (Finzymes, Espoo, Finland). PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, USA). Eight positive clones inserted with the targeted sequences were grown in liquid LB broth, Miller (Difco, Detroit, USA), purified with S.N.A.P. miniprep kit (Invitrogen, Carlsbad, USA) and sequenced bi-directionally with ABI 3730xl in Macrogen (Seoul, Korea) using universal primers M13F and M13R (For further details see Kottke et al., 2010).

2.3. Fungal phylogenetic analysis and OTU delimitation

Sequences were edited and assembled into a consensus sequence using Sequencer 4.6 (Gene Codes, Ann Arbor, USA). Homology searches for each nucleotide sequence were carried out with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in GenBank database (https://www.ncbi.nlm.nih.gov) using the option mega-blast (Altschul et al., 1997). Only sequences of our strains similar to Tulanellaceae species were aligned using the G-INS-I option in MAFFT Ver. 6.602b (Katoh, Kuma, Toh, & Miyata, 2005). Alignments were checked to eliminate chimeric sequences using the software Bellerophon (Huber, Faulkner, & Hugenholtz, 2004) and also manually copying and comparing suspicious parts of sequences on BLAST (<200 bp by part).

A phylogenetic analysis was performed using the entire nrDNA ITS-5.8 S region. Due to the known difficulties in aligning Tulanellaceae sequences (Cruz, Suárez, Kottke, & Piepenbring, 2014; Suárez et al., 2006), we carried out a two-step procedure. Firstly, a neighbour-joining analysis was performed in PAUP* 4.0b10 (Swofford, 2002) using the BioNJ modification with Kimura 2-distances of the sole 5.8 S region (100 bp). The resulting tree displayed 4 prominent groups (Groups I–IV), and new alignments of the whole nrDNA ITS-5.8 S region were performed for each group (Group I = 604 bp, group II = 611 bp, group III = 576 bp and group IV = 927 bp). Phylogenetic reconstructions were carried out using a maximum likelihood (ML) analysis for each group in MEGA5 (Tamura et al., 2011) under the general time reversible DNA substitution model with 1000 bootstrap replicates, eliminating gaps and missing data. Additionally, a maximum parsimony (MP) analysis was performed for each group in MEGAS, using the closest-neighbour-interchange (CNI) on random trees method with 1000 bootstrap replicates. All trees were midpoint-rooted.

Operational taxonomic units (OTUs) were defined based on sequence similarity of the nrDNA ITS-5.8 S region. A table of p-distance was calculated in PAUP* 4.0b10 and sequences were grouped with OPTSIL (Göker, García-Blázquez, & Voglmayr, 2009) at a 95% and 97% threshold of sequence similarity and 0.5 of linkage fraction. For further analysis, we chose the 95% threshold, which was calibrated using molecular and morphological data of Tulanellaceae by Cruz et al. (2011, 2014). These OTUs were mapped into the ML trees.

2.4. Morphological criteria and DNA barcoding for orchid identification

The orchids in bloom at the time of sampling were subjected to morphological identification referring to Dodson et al. (unpublished work), Dodson and Escober (1993), Dodson (2001, 2002, 2003, 2004) and available web resources (e.g., http://www.pleurothallids.com).

Young leaves were collected from each individual sampled for mycorrhizas. DNA was extracted from fresh or dry (60 °C overnight) leaf segments using the DNeasy Plant Mini Kit following the manufacturer’s instructions. To identify orchids, we amplified 2 genes, i.e., the commonly used matK (Pridgeon, Solano, & Chase, 2001; Pridgeon & Chase, 2003; van den Berg et al., 2005) and ycf1, which according to Neubig et al. (2009) is phylogenetically informative at the species level in orchids. We also tried to amplify the nrDNA ITS-5.8 S gene but were unable due to the poor quality of the sequences. Firstly, the plastid gene matK was amplified using 2 primer combinations, i.e., 19F (5’-CGTTCTCATATTCGACTAG-3’) (Whitten, Williams, & Chase, 2000) and 881R (5’-TTMTCATGAAATAAGAGT-3’) (Pridgeon et al., 2001), and 731F (5’-TCTGAGCTTCTTTGAGG-3’) (Pridgeon et al., 2001) and trnk2R (5’-AATGTGCATGCATTGGT-3’) (Johnson & Soltis, 1995). PCR conditions were as follows: initial denaturation at 98 °C for 30 s followed by 30 cycles of 98 °C for 10 s, 52 °C for 20 s, and 72 °C for 30 s and then a final elongation step at 72 °C for 7 min. An annealing temperature of 58 °C was applied when the 731F and trnk2R primer combination was used. The PCR reaction volume was 25 µl with concentrations of 0.2 pmol of each primer, 10%
bovine serum albumin (BSA) (Sigma, St. Louis, USA) with a final concentration of 0.8 μg/μL (Iotti & Zambonelli, 2006) and 1U Phusion High-Fidelity PCR Mastermix. A negative control included PCR mix without DNA template.

The 3’ end of ycf1 plastid gene was amplified using the primers 3720F (5’-TACGTATGTAAGGGAATGG-3’) and 5500R (5’-GCTGTATTGCGATCAAACGAGG-3’) (Neubig et al., 2009). PCR conditions were as follows: initial denaturation at 98 °C for 3 min followed by 35 cycles of 98 °C for 30 s, 58 °C for 1 min, and 72 °C for 3 min, ending with a final elongation step at 72 °C for 3 min. PCR products were sequenced bi-directionally by ABI 3730xl in Macrogen using the same primers as for PCR amplification. Sequence chromatograms were edited using the software Sequencer 4.6.

BLAST search (Altschul et al., 1997) was used to find published sequences with a high similarity to the named orchid species. As most of our orchid species have not been sequenced before, naming was generally only successful at the genus level (Supplementary Table S1).

2.5. Estimating richness and evaluating similarity of Tulasnellaceae OTUs across orchid life forms and sites

Potential richness and inventory completeness of Tulasnellaceae OTUs were evaluated using the program EstimateS Ver. 8.2.0 (Colwell et al., 2012). Individual-based species accumulation curves were calculated for the epiphytic and terrestrial life-form orchids, as well as for the sites. The obtained curves and the curves describing their 95% confidence intervals were fitted to a Clench curve (Soberón & Llorente, 1993) using Statistica Ver. 7.0.61.0 (StatSoft, Tulsa, Oklahoma, USA) following Jiménez and Hortal (2003). The asymptote of each curve was calculated as a/b, where a and b are the two parameters of the Clench curve. The sampling effort needed to record 95% of the estimated proportion of OTUs was calculated according to Jiménez and Hortal (2003).

Similarity in the composition of Tulasnellaceae OTUs between epiphytic and terrestrial orchids and across sites was evaluated by Chao-Jaccard and Chao-Sørensen similarity indices based on incidence and abundance (Chao, Chazdon, Colwell, & Shen, 2005) using the program EstimateS Ver. 8.2.0. These indices account for shared, but unobserved species among samples.

2.6. Description of the mutualistic network

Qualitative interactions based on presence/absence data between orchid species and Tulasnellaceae OTUs were assembled in an ordered binary matrix (Fig. 1). Degree distributions, i.e., the number of links per Tulasnellaceae OTU and orchid species, were displayed and network connectance was calculated to further characterize the structure of the mutualistic network using R software Ver. 3.1.1 (R Core Team, Vienna, Austria) following Jiménez and Hortal (2003) in the software R. Three scenarios of species extinction were simulated: (1) random extinction, (2) ordered extinction from the most-to the least-linked species, and (3) ordered extinction from the least-to the most-linked species. We also partitioned the network into groups to assess the relative robustness of epiphytic or terrestrial orchids to Tulasnellaceae OTUs extinction, following Santamaría et al. (2014). The possibility of secondary extinction of symbiotic fungi in response to the primary extinction of orchids was not simulated due to the asymmetry in dependence in this mutualistic system. Based on the bipartite pollination networks suggested by Santamaría et al. (2014), R50 ranged from 1 (maximum robustness) to 1 divided by the number of pollinators or, in our case, Tulasnellaceae fungi (minimum robustness to partner extinction).

Differences in the robustness of the network to the simulated Tulasnellaceae extinction scenarios were evaluated in R with a one-way general linear model (GLM) using the 300 iterations as replicates. The orchid life forms (epiphytic and terrestrial orchids) were considered factors in the GLM analyses. Significance of the differences in robustness was calculated by means of a posteriori Tukey’s honesty significant difference test according to Santamaría et al. (2014).

3. Results

3.1. Fungi sampling

All collected roots were colonized by mycobionts. Staining showed coils of young hyphae and structures that denoted collapsed or degenerating pelotons in the cortical tissue. DNA was isolated and sequenced from the colonized roots of all 114 sampled orchid individuals. A total of 418 mycorrhizal fungal DNA sequences were obtained from the complete nrDNA ITS-5.8 S and partial nrDNA 28 S region (approximately 1500 bp length). The most frequently amplified mycorrhizal fungi belonged to members of Tulasnellaceae (310 sequences) and were obtained from 89 orchid individuals (4.13 sequences on average per orchid species). Members of Atracteliellae (3 OTUs) and Serendipitaceae (12 OTUs) were also obtained from 32 to 19 orchid individuals, respectively. Serendipitaceae and Atracteliellae sequences were removed from further analyses in this study. A total of 100 of the original 310 Tulasnellaceae sequences were discarded due to poor sequence quality, inappropriate length or chimera formation (14 chimeras...
Thus, we used 210 high-quality Tulasnellaceae sequences for phylogenetic analysis and OTU definition. All sequences were deposited in GenBank (accession numbers are shown in Supplementary Table S1).

### 3.2. Phylogenetic analysis and OTU delimitation of Tulasnellaceae

The neighbour-joining analysis of the sole 5.8 S region obtained 4 well-supported bootstrap groups (Groups I–IV). Group I had the highest number of sequences with a total of 78 sequences, while Groups II, III and IV had 56, 32 and 44 sequences, respectively. Alignments of the complete nrDNA ITS-5.8 S region within these 4 groups resulted in well-resolved clades with high support values for ML and MP analyses (Supplementary Figs. S3–S6). When a fraction linkage of 0.5 was used, we obtained 29 OTUs based on a threshold of 95% sequence similarity and 33 OTUs were obtained at a threshold of 97%. The OTUs obtained at the 95% threshold are mapped in the 4 phylogenetic trees of Tulasnellaceae (Supplementary Figs. S3–S6). As the results of ML and MP analyses were consistent with the monophyly of most of the 29 delimited OTUs (Supplementary Figs. S3–S6), OTU delimitation was phylogenetically supported.

### 3.3. Orchid identity and occurrence

High-quality sequences were obtained for 87 of 114 sampled orchid individuals which were BLAST-searched to confirm the morphological identification of the orchids at the generic level. A total of 89 orchid individuals containing Tulasnellaceae as mycohciobionts were delimited to 52 orchid species by matK and ycf1 barcoding and morphotyping. A few species could be named when they were in flower or when sequences were available, but most of these species were not successfully sequenced. Orchids belonged to 9 genera (Supplementary Table S1) in subfamily Epidendroideae. *Stelis* (18 spp.), *Pleurothallis* (13 spp.), *Maxillaria* (9 spp.), *Epidendrum* (5 spp.) and *Elephantus* (3 spp.) were represented by multiple species, while *Artorima*, *Prosthechea*, *Sobralia* and *Oncidium* were only represented by a single species each. Individual species were mostly present as singletons (25 spp.), a few as doubletons (12 spp.) and a few were encountered 3 or more times (15 spp.) (Fig. 1).

Seven orchid species were found at the landslide plots, while 45 species were found on the 48 epiphytic and terrestrial forest plots. Species composition was quite different between the landslide and the forest sites as well as between terrestrials and epiphytes. *Artorima*, *Prosthechea*, *Sobralia*, *Epidendrum* sp. 2, *Maxillaria* sp. 4 and *Maxillaria* sp. 7 only occurred at the landslide site, while the Pleurothallidinae genera *Pleurothallis* and *Stelis* were restricted to the forest and were the most commonly sampled individuals in the pristine (Sites 1 and 4) and in the regenerating forest (Site 3). *Maxillaria* sp. 5 was present both in the forest and landslide plots. Only *Maxillaria* sp. 22, *Pleurothallis coriaceardia* and 4 species of *Stelis* (*S. superbiens* and *Stelis* sp. 3, 11 and 30) were found in both epiphytic and terrestrial forest plots.
habitats (Supplementary Table S1).

3.4. Richness and similarity of Tulasnellaceae OTUs across life forms and sites

The higher number of Tulasnellaceae OTUs occurred in terrestrial orchids (Fig. 2; Supplementary Tables S2) and 10 OTUs were common to both life forms. The accumulation curves of Tulasnellaceae OTUs in epiphytic and terrestrial orchids indicated an inventory completeness of 66% and from 38 to 71% at the different sites (Fig. 2; Supplementary Table S2). Richness of Tulasnellaceae OTUs was considered significantly different across life forms or sites when the 95% confidence intervals of the corresponding accumulation curves did not overlap. Broad overlap between the 95% confidence intervals of the accumulation curves indicated no differences in OTU richness across habitats or sites (Fig. 2; Supplementary Table S2) (Ellison & Gotelli, 2004). Chao-Jaccard and Chao-Sørensen indices showed high similarity in the composition of Tulasnellaceae OTUs between epiphytic and terrestrial habitats (Table 1). However, similarity was lower

Fig. 2. Accumulation curves of Tulasnellaceae OTUs obtained by accumulating orchid samples across (A) orchid growth forms (epiphytic, dashed line; terrestrial, continuous line) or (B) sites (Site 1, continuous black line; Site 2, dashed black; Site 3, continuous grey; Site 4, dashed grey lines), of a tropical mountain rain forest located in the Reserva Biológica San Francisco, Zamora-Chinchipe Province, Southern Ecuador. Dots show the asymptotic values for each accumulation curve and vertical bars indicate their confidence intervals at 95% (with the same color and type of line).
between the landslide (Site 2) and the regenerating forest (Site 3) (Table 1).

3.5. Structure of the orchid-Tulasnellaceae network and stability against species loss

The bipartite network formed by the 29 Tulasnellaceae OTUs and the 52 orchid species (Supplementary Figs. S7 and S8) had a connectance of 7.2% and was significantly nested (NODF = 16.2; *p* < 0.05 for both ER and CE null-models). NODF values were 18.2 for orchids and 9.9 for Tulasnellaceae. The number of singletons and doubletons was high for Tulasnellaceae and orchids (55% and 71%, respectively) (Fig. 1). The network had a few generalist fungi (OTUs T1, T9, T13, T16) frequently linked with a few common orchid species (*S. superbiens*, *Stelis* sp. 11, *Pleurothallis* sp. 25). OTU T1 was the most common phylogenetic species associated with 26 individuals and 19 orchid species, followed by 3 others frequently linked with OTUs T16, T13 and T9 (Fig. 1). Fungi and orchids with few links were mainly linked to partners with many links, with one exception (T21 linked to *Maxillaria* sp. 7). No significant modularity was found for the bipartite network (*M* = 0.6, *p* = 0.9).

Index R50, indicating the robustness of the network to Tulasnellaceae OTUs extinction, ranged from 0.45 when the loss of Tulasnellaceae proceeded from the most-to the least-connected, to 0.75 when extinction was random and 0.93 when extinction proceeded from the least-to most-linked Tulasnellaceae. The robustness of epiphytic and terrestrial orchids to Tulasnellaceae extinction showed R50 values between 0.32 and 1.00 (Fig. 3). One-way GLM showed a significant effect of orchid life form on robustness to Tulasnellaceae OTUs extinction (Fig. 3). In the random extinction sequence, terrestrial orchids were more robust than epiphytic orchids, while the opposite was found in the most-to least-connected sequence (Fig. 3).

4. Discussion

We found that the interactions between orchids and Tulasnellaceae fungi in the tropical forest of Southern Ecuador were characterized by a high richness of Tulasnellaceae, shared between epiphytic and terrestrial orchids across sites (landslide, pristine and regenerating forest). The structure of this mutualistic network was nested, and its robustness differed only slightly between the orchid life-forms (epiphytic or terrestrial) of this network.

Tulasnella richness in European temperate habitats has reached asymptotic values near 10^e12 OTUs (Jacquemyn & Deja, 2012; Jacquemyn et al., 2011). Not surprisingly, this diversity is higher in tropical forests, as indicated by previous studies in La Reunion (58 OTUs) (Martos et al., 2012) and the preliminary results for our study area (33 OTUs at 3% sequence divergence in Kottke et al., 2013). Here, we obtained 29 Tulasnella OTUs, and the non-saturated accumulation of fungal OTUs suggests that more locally-distributed rare species may be present at our study sites.
If differences in sampling effort among studies are not considered, *Tulasnella* richness may depend on the threshold (95% in this study, 97% in Martos et al., 2012) of sequence similarity for OTU delimitation. Thresholds of 95–97% sequence similarity (of ITS region) have been widely used to delimitate Tulasnellaceae species (Cruz et al., 2014, 2011; Gilranda et al., 2011; Jacquemyn & Deja, 2012, 2014; Jacquemyn et al., 2011; Linde et al., 2017). Furthermore, Linde, Phillips, Crisp, and Peakall (2014) supported the use of our chosen threshold as well as the use of the ITS region to congruently delimitate species within Tulasnellaceae based on an extensive multi-locus analysis. However, even when a more conservative threshold (99%) was used to delimitate OTUs, results remained consistent with our main conclusions (Supplementary Table S3). On the other hand, *Tulasnella* richness may also depend on the primers selected to amplify fungal DNA. To increase the possibility of identifying all fungi diversity in the root, we used a pair of universal eukaryotic primers (ITS1 and TW14) which target highly-conserved sites in the SSU and LSU, respectively (Lindahl et al., 2013). Although we detected a broad diversity of Tulasnellaceae OTUs, including the most frequent ones. These results were unexpected and contrast with the findings for *La Reunion* (Martos et al., 2012), where significant differences were found in mycobiont composition between epiphytic and terrestrial orchids. These contrasting findings may be due to several factors, such as climatic and edaphic niche differences (Beck & Richter, 2008), the phylogenetic and biogeographic history of these regions, or simply to different sampling strategies (plots versus taxonomy-based sampling, and small areas versus large landscapes).

The use of complex network tools in our analysis allowed us to disentangle the structure of this orchid-mycobiont system dominated by rare species. The network showed significant nestedness but no modularity, contrary to findings in another tropical system (Martos et al., 2012). A nested structure, which is widespread in pollinator and seed-disperser webs (Bascompte & Jordano, 2007), is of fundamental importance for community formation and biodiversity maintenance (Bastolla et al., 2009; Gómez, Perfectti, & Jordano, 2011). A significantly nested mutualistic network indicates that rare orchids associate with common mycobionts and rare mycobionts associate with generalist orchids (Bascompte, Jordano, & Olesen, 2003, 2006; Okuyama & Holland, 2008), resulting in marked asymmetry of interactions (Bascompte et al., 2003). A nested structure also means that there is a core of generalist orchids and mycobionts, as in other non-tropical mycorrhizal interactions (Vályi, Rillig, & Hempel, 2015). Specialization can sometimes be overestimated by low sampling effort, particularly in small ecological networks (Fründ, Mccann, & Williams, 2016). Although our network was undersampled and caution is needed in interpreting these results, we consider that the nested structure is reliable because this network feature has been found to be robust to undersampling (Nielsen & Bascompte, 2007).

In the Tulasnellaceae–orchids network, robustness progressively decreased from the least-to-most-linked extinction scenario to the random and the most-to least-linked extinction scenarios. This pattern has also been described in pollination networks (Burgos et al., 2007; Memmott, Waser, & Price, 2004; Santamaría et al., 2014). In our network, this reveals that generalist fungi and orchids play an important role in the stability of the mutualistic system. Terrestrial and epiphytic orchids showed similar robustness to the extinction of their mycobionts, and although differences were statistically significant, the effect was minor. This means that mycobionts are equally important for the persistence of both subsets of orchids. A broad generalization of interactions is important for network robustness (Pocock, Evans, & Memmott, 2012). Loss of generalist keystone species (most-connected species) may drive cascades of biodiversity loss. Furthermore, high specificity is unlikely in mutualistic interactions, as species that can associate with a greater variety of species have a wider range of ecological options for survival (Timms & Read, 1999).

Our results indicate a high diversity of Tulasnellaceae mycobionts and at least a core of generalist Tulasnellaceae OTUs. Irregular distribution of mycorhizal fungi is considered a key factor for the persistence of small orchid population sizes as found in tropical forests (Otero & Flanagan, 2006). Our findings suggest that the availability of common mycorhizal fungi supports the persistence of a wide range of orchids in the area, by at least two mechanisms: (1) simultaneous association with several mycobionts may maximize nutrient absorption, especially for epiphytic habitats where nutrients are limited (Jacquemyn et al., 2010), and (2) obligate mutualisms are stabilized by the simultaneous association with facultative mutualists (Takimoto & Suzuki, 2016). These conditions of Tulasnellaceae may support the development and coexistence of the high orchid richness in the tropical montane rainforest of Southern Ecuador.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.myc.2017.08.003.

References


