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MAIN FUNGAL PARTNERS AND DIFFERENT LEVELS OF SPECIFICITY OF ORCHID MYCORRHIZAE IN THE TROPICAL MOUNTAIN FORESTS OF ECUADOR

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ABSTRACT. Orchids are a main component of the diversity of vascular plants in Ecuador with approximately 4000 species representing about 5.3% of the orchid species described worldwide. More than a third of these species are endemics. As orchids, in contrast to other plants, depend on mycorrhizal fungi already for seed germination and early seedling establishment, availability of appropriate fungi may strongly influence distribution of orchid populations. It is currently debated if green orchids depend on specific mycobionts or may be equally promoted by a broad spectrum of mycorrhizal fungi, discussion mostly based on data from temperate regions. Here we summarize results obtained from broad scale investigations in the tropical mountain rain forest of Ecuador revealing associations with members of Serendipitaceae (Sebaciales), Tulasnellaceae, Ceratobasidiaceae (Cantharellales), and Atractiellales. Recent molecular data show that these worldwide spread fungal groups have broad ecological implications and are specifically suited as mycorrhizal fungi of green orchids. We found that main fungal partners and different levels of specificity among orchids and their mycobionts in the tropical mountain forests correspond to findings in other biomes despite the large ecological differences.

KEY WORDS: Atractiellales, Ceratobasidiaceae, epiphytic orchids, mycobionts, Serendipitaceae, Tulasnellaceae

Introduction. Interaction between orchids and their mycorrhizal fungi is characterized by production of thousands of tiny seeds lacking carbohydrate reserves. Colonization of a seed by a suitable mycorrhizal fungus is, thus, vital for successful germination, growth and establishment of orchids in nature. The associations with mycorrhizal fungi remain when orchids become photosynthetic (Dearnaley *et al.* 2012). The extent to which mycobionts support growth of adult orchids in nature is unknown, but the maintenance of the mycobionts is, without doubt, crucial for their juveniles. It was shown that success of seedling establishment is affected by occurrence of mature plants, despite the easy far-distance transport of orchid seeds (Jacquemyn *et al.* 2007; Jersáková & Malinová 2007; Riofrío *et al.* 2013). It is under current debate if orchid species depend on specific mycobionts or can equally or better be promoted by a broad spectrum of fungi (Kartzinel *et*

al. 2013; Kottke *et al.* 2013; McCormick & Jacquemyn 2014). Narrow, specific association might be more efficient (Kiers *et al.* 2011) and favor propagation of the respective plant and mycobiont against competing species, while a broader mutualistic interaction might be safer in long term survival and distribution. Thus, knowledge on identity of fungal mycobionts and degree of specificity in symbioses is of interest to understand life history and distribution of orchids and a precondition for conservation efforts in such diverse places like the mountain forests of Ecuador where orchids, with approximately 4000 species, constitute about 25% of native vascular plants (Dodson 2005; Neill 2012). Comprehensive molecular studies on the mycobionts of terrestrial and epiphytic orchids were therefore carried out in the tropical mountain forest of Southern Ecuador (Kottke *et al.* 2010, 2013; Riofrío *et al.* 2013; Suárez *et al.* 2006; Suárez *et al.* 2008; Suárez

et al. 2016). Results are summarized here and recent molecular phylogenetic and physiological data of the respective fungal groups are compiled to discuss the findings.

Main fungal partners associated with tropical orchids.

The autotrophic terrestrial and epiphytic orchids in the tropical mountain rain forest were found associated with a limited range of Basidiomycota in the Serendipitaceae (Sebacinales), Tulasnellaceae and Ceratobasidiaceae (Cantharellales), and Atractiellomycetes (Atractiellales) (Kottke *et al.* 2010, 2013; Otero *et al.* 2002; Otero *et al.* 2007; Suárez *et al.* 2006, 2008, 2016). Tulasnellaceae were the most species rich and most abundant group. All of these fungal groups produce cryptic fruiting structures, which hinder their taxonomic resolution by morphological characters, but molecular approach gives well supported insights at high resolution level (Oberwinkler *et al.* 2006, 2013a, Oberwinkler *et al.* 2013b, 2014; Cruz *et al.* 2016). The nuclear ribosomal RNA locus, in particular the highly variable internal transcribed spacer region including 5.8S (nrITS-5.8S) is among the most widely used locus for phylogenetic studies in fungi and was recently proposed as the universal barcode region for fungi (Schoch *et al.* 2012). The nrDNA are multi-copy genes and therefore easily amplified from environmental samples. Large numbers of sequences were thus obtained from our samples and analyzed by molecular phylogenetic models including all available data from gene bank. Considering also literature on molecular systematics and whole genome studies of Agaricomycotina (Basidiomycota) we may now better understand why just these fungal groups became orchid mycobionts (Hibbet & Matheny 2009; Kohler *et al.* 2015).

Sebacinales —. Sebacinales are a basal lineage in Agaricomycotina (Basidiomycota) and include root endophytes, saprotrophic species and species forming mycorrhizal associations (Weiss *et al.* 2004). Recently, Sebacinales were divided in two sister families: Sebacinaceae, previously Sebacinales subgroup A, and Serendipitaceae, previously Sebacinales subgroup B (Weiss *et al.* 2016). Members of Sebacinaceae form ectomycorrhizae with diverse tree species (Avis *et al.* 2003; Glen *et al.* 2002; Kennedy *et al.* 2003;

Shefferson *et al.* 2005; Tedersoo *et al.* 2006; Walker *et al.* 2005; Weiss *et al.* 2004) including tripartite associations with achlorophyllous and mixotrophic orchids (Julou *et al.* 2005; McKendrick *et al.* 2002; Selosse *et al.* 2004; Selosse *et al.* 2002; Taylor *et al.* 2003; Urban *et al.* 2003; Warcup 1971). Sebacinoid mycobionts from green orchids, including epiphytic orchids, appear in Serendipitaceae (Suárez *et al.* 2008). The same was found for Andean Ericaceae (Setaro *et al.* 2006a, 2006b).

Cantharellales —. Members of Tulasnellaceae and Ceratobasidiaceae are placed in Cantharellales, a basal lineage in Agaricomycotina (Basidiomycota), which contains nearly exclusively saprotrophs or mutualistic biotrophs (Hibbett *et al.* 2007, 2014; Veldre *et al.* 2013). Tulasnelloid fungi are world-wide spread, producing inconspicuous, resupinate basidiomata on rotten wood or bark of tree branches (Cruz *et al.* 2014; Cruz *et al.* 2016; Roberts 1999). Tulasnellaceae form mycorrhizae with a broad spectrum of plants, including mycorrhiza-like association with *Aneuraceae* (liverworts) thallus (Kottke *et al.* 2003; Krause *et al.* 2011) tripartite associations with the heterotrophic liverwort species *Cryptothallus mirabilis* and with surrounding trees as ectomycorrhizal partners (Bidartondo *et al.* 2003). *Tulasnella* was also found associated with *Graffenrieda emarginata* (Melastomataceae) forming a superficial layer on arbuscular mycorrhizas (Haug *et al.* 2004). Most importantly, however, *Tulasnella species* are the most frequent and widespread mycobionts of autotrophic orchids (reviewed by Kottke & Suárez 2009 and Dearnaley *et al.* 2012). While only few named *Tulasnella* species are reported as forming mycorrhiza with orchids, molecular phylogeny revealed a large number of genotypes, but also inconsistencies in species concepts and taxonomy challenging comparative ecological studies (Cruz *et al.* 2011, 2014, 2016). These recent studies on fresh samples of basidiomata showed however, that molecular approach using the ITS-5.8 region and a threshold of 4 % up to 8 % variability gives well supported clades considered as corresponding to species level.

The genera *Ceratobasidium* and *Thanatephorus* along with their *Rhizoctonia* anamorphs form a

group of closely related fungal taxa in the family Ceratobasidiaceae. A recent phylogenetic analysis revealed ten groups within the Ceratobasidiaceae (González *et al.* 2016). Species within the *Ceratobasidium-Thanatephorus* complex are known as crop pathogens but also as forming mycorrhizae with orchids and trees (Tedersoo *et al.* 2010). Delineating species in these groups has been problematic, efforts to solve this situation included anastomosis groups and molecular data (Oberwinkler *et al.* 2013b). Studies reporting the presence of members of Ceratobasidiaceae from tropical orchids remain scarce. Using isolation-dependent methods orchid species *Coppensia doniana*, *Tolumnia variegata*, *Ionopsis utricularioides* and *Psycmorchis pusilla* (tribe Cymbidieae) were shown to be predominantly associated to Ceratobasidiaceae (Valadares *et al.* 2015; Otero *et al.* 2002, 2004). However, using isolation-independent methods, members of Ceratobasidiaceae were absent in species of *Pleurothallis*, *Stelis* and *Epidendrum* (tribe Epidendreae) (Suárez *et al.* 2006, 2008; Kottke *et al.* 2010; Riofrío *et al.* 2013).

Atractiellales — Atractiellales belong to the subphylum Pucciniomycotina (Rust fungi), which comprises mainly parasites and to a lesser extent presumed saprophytes (Aime *et al.* 2006). The phylogenetic position of the mycobionts among potential saprophytes may indicate physiological flexibility from saprophytism to mutualism, as required for orchid mycobionts (Rasmussen & Rasmussen 2009). So far only three genotypes (operational taxonomic units, OTUs) of Atractiellomycetes were shown by combined ultrastructural and molecular investigations to form mycorrhiza with terrestrial and epiphytic orchids (Kottke *et al.* 2010; Riofrío *et al.* 2013; Suárez *et al.* 2016). Experimental proof for nutritional support of protocorms is, however, still lacking.

Orchid-mycobiont interaction with different levels of specificity in the tropical mountain forest.

Although a high number of orchid species have been recorded, studies on their mycorrhizal fungi are still scarce (Dearnaley *et al.* 2012), and in most cases, only few individuals per population have been sampled. Factors as orchid recruitment, seed dispersal limitations

and availability of suitable fungal partner are affecting the distribution of orchids at regional and local scales and may disguise specificities among plants and fungi. Our studies were concentrated on the conditions of tropical mountain rain forest where intensive sampling was carried out on epiphytic and terrestrial species. Molecular data revealed narrow preferences to broad sharing of partners.

In a pioneer study by Suárez *et al.* (2006) in the mountain forests of Zamora-Chinchipe of Ecuador, using a combination of fungal isolation and fungal-independent investigations, differences in the number of mycorrhizal fungal partners were found among three *Stelis* and one *Pleurothallis* species, two closely related genera (subtribe Pleurothallidinae, tribe Epidendreae). Preferences were evident in case of *S. concinna*, where the highest number of plant individuals was investigated, but the lowest number of fungi was detected. *Stelis concinna* was associated with only one member of Serendipitaceae and two members of Tulasnellaceae, two of these showing a wide elevation distribution range (Suárez *et al.* 2006). Beside these preferences, a broad sharing of mycobionts was observed among *Stelis* and *Pleurothallis* species (Suárez *et al.* 2008) potentially explained by their close phylogenetic relationship (Pridgeon, Solano & Chase 2001).

Riofrío *et al.* (2013) carried out a study in a nearby site, focusing on within-population variation of mycorrhizal associations of the epiphytic orchid *Epidendrum rhopalostele* (subtribe Laeliinae, tribe Epidendreae). Individuals were associated with only two different clades of closely related *Tulasnella*. The two clades were spatially randomly distributed showing no segregation patterns, both clades were related to the *Tulasnella* isolated from *Stelis* and *Pleurothallis* by Suárez *et al.* (2006), but corresponded to distinct genotypes.

Kottke *et al.* (2010) showed that three Atractiellales genotypes, closely related to *Infundibura*, formed mycorrhizas with many terrestrial and epiphytic orchid species in the Andean tropical forest and regenerating habitats. Similar broad sharing was found for *Tulasnella* and *Serendipita* genotypes from the same area (Kottke *et al.* 2013).

A study carried out near the town of Baños, Tungurahua province, far from Zamora-Chinchipe

area, revealed that *Teagueia* morphospecies (subtribe Pleurothallidinae, tribe Epidendreae) were associated with members of Tulasnellaceae and Atractiellales. The phylogenetic analysis of ITS-5.8S sequences of members of Tulasnellaceae showed sequences in four clades. Sequences from the previous study by Suárez *et al.* (2006) are closely related to these clades. The obtained Atractiellales sequences were identical to “phylo type I” as found by Kottke *et al.* (2010) from the previous study in Zamora-Chinchipe, Ecuador. Results show up to three different phylogenetic species of mycobionts associated to one *Teagueia* species suggesting high potential for sharing mycobionts among *Teagueia* spp. All the detected mycobionts had wide geographical distribution.

Discussion. The molecular phylogenetic based investigations of orchid mycobionts in the tropical mountain rain forest area of Southern Ecuador revealed the well established fungal groups, Serendipidaceae, Tulasnellaceae and Ceratobasidiaceae. Additionally, Atractiellales were found as widespread mycobionts. The latter can be found in some data sets of previous investigations, but were not seriously considered before. Although we cannot definitely exclude that further fungal groups may be detected in future, we may ask why just members of these few fungal families are suitable mycobionts of green orchid. The vast majority of plants form mycorrhizae with Glomeromycota, obligate symbionts with no access to extraradical sugars. Thus, these mycobionts are unsuitable to feed the orchid protocorm. Mycobionts in Agaricomycetes, however, derived from saprotrophic relatives multiple times and the orchid mycorrhizal groups, in basal position of Agaricomycetes, preserved some genes for decay enzymes (Hibbett *et al.* 2007; Kohler *et al.* 2015). Preservation of the respective genes means sufficient organic matter decay for acquiring carbon and nitrogen to feed the orchid protocorm without attacking living cells in a parasitic manner. According with these capabilities, these fungi are prepared to grow on tree bark (Kartzinel *et al.* 2013) and may additionally profit from the water-retention capacity of the velamen covering roots of epiphytic orchids. The particular conditions in the epiphytic habitat may have potentially supported adaptations to the specific groups of mycorrhizal fungi. The life history sets

Orchidaceae apart from all other Monocotyledonae and was obviously facilitated by switching from Glomeromycota to mainly these Agaricomycetes in Basidiomycota as mycorrhizal fungi. We may consider this switch as a synapomorphy of the family (Yukawa, Ogura-Tsujita, Shefferson & Yokoyama. 2009).

Another question under debate concerns about potential specificity among orchids and mycobionts. Most investigations focused on rather species poor orchid populations in temperate areas (McCormick & Jacquemyn 2014). It was generally found that orchid rarity was unrelated to specificity, but coexisting, terrestrial orchid populations were associated with distinct mycobiont communities. In case of tropical epiphytic orchids, availability of appropriate fungi may be especially critical for plant survival under the restricted conditions of shortage of water or organic and inorganic nutrients (Zotz & Hietz 2001; Martos *et al.* 2012; Dearnaley *et al.* 2012). Fungi may be rare to meet on tree branches and stems, potentially restricted to species adapted to these niches. Accordingly, Martos *et al.* (2012) found significant differences among epiphytic and terrestrial orchid mycobionts in old world tropical Reunion island. We carried out a comparatively large-scale survey in the new world tropical forest of the Ecuadorian Andes on epiphytic and terrestrial orchids. Our results showed different levels of specificity, ranging from unspecific, multi-species networks to narrow orchid clades associated with few world-wide spread *Tulasnella* genotypes, and to narrow species-species associations along an elevation gradient or among dense populations of an epiphytic orchid. Species richness, environmental conditions but also sampling efforts and methods of fungal identification may still bias a clear statement on narrow specificity in the wet tropical habitat. More likely, sharing of mycobionts will improve the rate of successful germination of orchid seeds and also promote co-existence of closely related species as observed in the tropical montane rain forest (Kottke *et al.* 2013).

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