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 (Sebacinales), 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 (Dearmaley 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 (Cantarellales), 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 loci 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 into two sister families: Sebacinaceae, previously Sebacinales subgroup A, and Serendipitaceae, previously Sebacinales subgroup B (Weiss et al. 2016). Members of Sebacinaeae 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 (Julu 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).

Cantarellales —. Members of Tulasnellaceae and Ceratobasidiaceae are placed in Cantarellales, a basal lineage in Agaricomycotina (Basidiomycota), which contains nearly exclusively saprotophs or mutualistic biotrophs (Hibbett et al. 2007, 2014; Veldre et al. 2013). Tulasnelloid fungi are worldwide 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 Anurellaceae (liverworts) thallus (Kottke et al. 2003; Krause et al. 2010) 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-5.8S 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
Psygmorchis 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 Atractiellomyces 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 Inundibura, 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 Seredipita 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 “phylotype 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 symbiont 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. Accordingly, Kottke *et al.* (2013) 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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