CONRADO AUGUSTO VIEIRA

DIVERSITY OF MYCORRHIZAL FUNGI AND CARBON NUTRITION IN TROPICAL EPIPHYTIC ORCHIDS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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À minha vó Neném dedico

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BIOGRAFIA

CONRADO AUGUSTO VIEIRA é filho de Celci Maria Santos Vieira, nascido na cidade de Divinópolis, Minas Gerais, em 8 de abril de 1987. Graduou-se em Ciências Biológicas pelo Instituto Superior de Educação de Divinópolis, associado à Universidade Estadual de Minas Gerais no ano de 2009. Em fevereiro de 2012 ingressou como discente de mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa com conclusão em 26 de fevereiro de 2014. Em março de 2014 ingressou no doutorado do mesmo programa, com conclusão em fevereiro de 2018.

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RESUMO

VIEIRA, Conrado Augusto, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Diversidade de fungos micorrízicos e nutrição por carbono em orquídeas epífitas tropicais**. Orientadora: Maria Catarina Megumi Kasuya. Coorientadores: Denise Mara Soares Bazzolli, Olinto Liparini Pereira e Marc-André Selosse.

As orquídeas, no ambiente natural, dependem da interação com fungos micorrízicos para completar o ciclo de vida. No cerne da interação, estão envolvidos diferentes fungos que fazem a troca de compostos, principalmente carbono, com a orquídea hospedeira. Contudo, a diversidade de fungos micorrízicos, principalmente da família de fungos mais comumente encontrada em pesquisas com orquídeas brasileiras, a Tulasnellaceae, bem como o fluxo de carbono em orquídeas tropicais epífitas são ainda pouco conhecidos. Assim, investigamos (1) se há ocorrência dessa família na raiz de orquídeas tropicais epífitas e também na casca do forofito onde estão presentes e, se (2) ao longo dele, há diferença no fluxo de carbono e tipo de metabolismo (C3 ou CAM) entre o fungo e a orquídea. Grids foram utilizados para a amostragem em dois forófitos, contendo em cada, cinco indivíduos de *Isochilus linearis* (Jacq.) R.Br.. Foram coletadas amostras de todas as raízes das orquídeas e, ainda, da casca do forófito imediatamente abaixo das raízes das orquídeas, bem como da casca do forófito. Após a extração de DNA total, realizou-se a PCR com os pares de *primers* para fungos em geral (ITS86F+ITS4) e especificamente para Tulasnellaceae (ITS86F+ITS4-Tul). Os amplicons foram sequenciados por NGS (Next Generation Sequencing) pela plataforma Ion Torrent®. Os dados foram analisados e as sequencias identificadas em menor nível taxonômico possível. Também foram analisados pela ANOVA, índices de diversidade e pelas redes neurais artificiais (ANN). Para avaliar o fluxo de carbono, o metabolismo das orquídeas, bem como a estratégia de aquisição de carbono pela orquídea (micoheterotrófica - MH, autotrófica - AT ou mixotrófica – MT), foram feitas coletas de folhas de orquídeas, de espécies não pertencentes à família Orchidaceae e dos pelotons presentes nas raízes das orquídeas, crescendo em diferentes posições nos forófitos. As amostras foram secas até massa constante, moídas e a razão isotópica $^{13}C/^{12}C$ mensurada por espectrometria de massa de razão isotópica. Os dados obtidos foram submetidos à ANOVA, seguida por análise de agrupamento e de ANN. Observou-se a ocorrência de fungos em geral assim como da família Tulasnellaceae nos diferentes tipos de amostras, principalmente por uma espécie ainda não descrita, *Tulasnella* sp. 3. A família Tulasnellaceae (42,38 %) demonstrou ser a chave da diversidade em *I. lineares* bem como nos forófitos associados. Foi observada a presença de fungos previamente descritos em associações com liquens (*Pectenia* *plumbea*, *Biatora ligni-mollis* e *Bacidia laurocerasi*) o que sugere uma ponte para a entrada de N na associação. Além desses, os fungos *Candida smithsonii* e *Fusarium buharicum* apresentaram alta prevalência. Estes fungos já foram descritos em trato intestinais de coleóptero, sugerindo que, auxiliam na dispersão destes fungos quando ocorre a herbivoria das plantas hospedeiras. Não foi observado padrões de dependência do fungo micorrízico por parte da orquídea no gradiente de altimetria, apesar de se ter constatado o fluxo de carbono, e 11,11% das orquídeas apresentarem-se CAM. As orquídeas apresentaram-se como MH, AT e MT, com prevalência de 63,64%, 22,72% e 13,64% respectivamente. Dentre os três indivíduos MT, dois recebem mais C da fotossíntese do que do fungo micorrízico associado. As características fitofisionômicas da área de coleta, como a presença constante de névoa pode explicar a baixa prevalência de orquídeas do tipo CAM, e por se tratar de uma floresta densa, não foi observada diferença no fluxo de carbono, uma vez que se esperava que orquídeas em posições mais elevadas, receberiam mais luz e dependeriam menos do fungo micorrízico. Esse é o primeiro registro de Tulasnellaceae em casca de forófitos e a constatação do fluxo de carbono em orquídeas epífitas tropicais. As informações geradas neste estudo podem dar subsídio para a elaboração de planos de manejo mais eficientes, em que áreas com ocorrência dos fungos potencialmente formadores de micorriza de orquídeas pode ser capaz de sustentar a permanência das espécies.

ABSTRACT

VIEIRA, Conrado Augusto, D.Sc., Universidade Federal de Viçosa, February, 2018. **Diversity of mycorrhizal fungi and carbon nutrition in tropical epiphytic orchid**. Adviser: Maria Catarina Megumi Kasuya. Co-advisers: Denise Mara Soares Bazzolli, Olinto Liparini Pereira and Marc-André Selosse

Orchids, in the nature, depend on the interaction with mycorrhizal fungi to complete the life cycle. At the center of the interaction, different fungi are involved which promote the exchange of compounds, mainly carbon, with the host orchid. However, the diversity of mycorrhizal fungi, mainly those belong to Tulasnellaceae, the commonest family of orchid mycorrhizal fungus, as well as the carbon flux in tropical epiphytic orchids are still little known. We investigated if there is the occurrence of this fungal family in the root of epiphytic tropical orchids as well as in the phorophyte bark where they occur, and if along the phorophyte there is difference in the carbon flux between the fungus and the orchid. Grids were used for sampling in two phorophytes, with five individuals of *Isochilus linearis* (Jacq.) R.Br. in each. Samples were collected from all roots of the orchids and also from the bark of the phorophyte just below the orchids roots, as well as from the bark of the phorophytes. After extraction of total DNA, PCR was performed with the primers for fungi in general (ITS86F + ITS4) and specifically for Tulasnellaceae (ITS86F + ITS4-Tul). The amplicons were sequenced by NGS (Next Generation Sequencing) by the Ion Torrent^{TM} platform. The data were analyzed and the sequences identified in the lowest possible taxonomic level. The data were also submitted to ANOVA, and diversity indices and artificial neural networks (ANN) were analyzed. To evaluate the carbon flux, orchid metabolism (C3 or CAM), as well as the strategy of acquiring carbon by the orchid (mycoheterotrophic - MH, autotrophic - AT or mixotrophic - MT), we sampled leaves of orchid, non-orchids species and pelotons present in the roots of the orchids, growing in different height of phorophytes. The samples were dried to constant mass, ground and the $^{13}C/^{12}C$ isotopic ratio measured by isotope ratio mass spectrometer. The isotopic signatures data were submitted to ANOVA, cluster analysis and ANN. It was observed the general fungi and also of the family Tulasnellaceae (42.38%) in different types of samples, mainly a species not yet described, *Tulasnella* sp. 3. Tulasnellaceae has been shown to be the key to diversity in *I. linearis*, as well as in associated phytophytes. The presence of fungi previously described in associations with lichens (*Pectenia plumbea*, *Biatora ligni-mollis* and *Bacidia laurocerasi*) suggests a “bridge” for the entry of N in the mycorrhizal association. In addition, two fungi with high prevalence (*Candida smithsonii* and *Fusarium buharicum*) have been described in intestinal tract of Coleoptera. This fact suggests that herbivory can be a tool for dispersion of these fungi. No patterns of mycorrhizal fungus dependence were observed by the orchid and altimetry gradient, although carbon flow was observed, and 11.11% of the orchids presented CAM. The orchids were MH, AT and MT, with prevalence of 63.64%, 22.72% and 13.64% respectively. Among three MT samples, two received more C from photosynthesis than from the associated mycorrhizal fungus. The climatic characteristics of the collection site, such as the constant presence of fog, may explain the low prevalence of orchids with CAM type, and because it is a dense forest, no difference was observed in the carbon flux, since it was expected that orchids in higher positions, would receive more light and would less dependent on mycorrhizal fungi. This is the first record of Tulasnellaceae in phorophytes bark and the carbon flux in tropical epiphytic orchids. The information generated in this study can provide subsidy for the elaboration of more efficient management plans, in which areas with occurrence of fungi potentially forming orchid mycorrhiza may be able to sustain the permanence of the species.

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1. Introdução geral

A família Orchidaceae apresenta distribuição cosmopolita, com exceção dos polos (Roberts *et al.*, 2008). Estimativas apontam que existam 32 mil espécies de orquídeas em todo o mundo, além dos inúmeros indivíduos híbridos que ocorrem de forma natural ou por cruzamentos artificiais (Field *et al.*, 2017). As orquídeas exibem diferentes hábitos de colonização, podendo ser encontradas como terrestres, rupícolas, aquáticas, além de epífitas, essas que representam 69% de todas as espécies (Bone *et al.*, 2015). Os indivíduos dessa família apresentam diferentes padrões de cores, morfotipos e devido a isso, exibem um elevado valor comercial, por serem exploradas com finalidades ornamentais, culinárias e medicinais (Jiang *et al.*, 2015).

No Brasil, há registro de 2.419 espécies de orquídeas, das quais 1.620 distribuídas em 35 gêneros, são endêmicas (Forzza *et al.,* 2010). Embora o Brasil ocupe posição mundial de destaque em relação a diversidade, especialmente para Orchidaceae, planos de manejos específicos para essa família são ainda escassos (Mittermeier *et al.*, 2005; Forzza et al., 2010).

A fragmentação e/ou destruição de habitats, bem como da coleta ilegal e indiscriminada, tem proporcionado o decréscimo em número de indivíduos de orquídeas em cerca de 62% das espécies conhecidas (Zhang *et al.*, 2015), se considerar as populações ainda não reportadas, os valores podem ser ainda maiores. Fatores esses que podem causar a extinção de espécies, logo os fungos a elas associados, podem também sofrer consideráveis perdas (Zhang *et al.*, 2015; Reiter *et al.*, 2016).

As orquídeas são comumente observadas em associação com fungos. A interação fungo-planta é extremamente importante para que as orquídeas possam completar o seu ciclo de vida, principalmente nos estágios iniciais, por exemplo durante a germinação de suas diminutas sementes desprovidas de tecido de reserva (Kohout *et al.*, 2013; Sakamoto *et al.*, 2015). Em contra partida indivíduos adultos de orquídeas fotossinteticamente ativas fornece ao fungo recebe então em troca nutrientes, principalmente o carbono, caracterizando a simbiose do tipo micorrízica (Liebel *et al.*, 2010, 2015) .

Atualmente é bem estabelecido que as orquídeas dependem da associação com esses fungos, esses que penetram seus tecidos radiculares formando enovelados de hifas, com a formação de uma típica estrutura da associação do tipo micorriza orquidóide, os *pelotons* (Rasmussen, 2002). A nutrição da orquídea ocorre principalmente pela degradação desses *pelotons*, que são digeridos pela planta, promovendo a nutrição da mesma (Boldrini *et al.*, 2010).

As primeiras observações da presença de estruturas fúngicas em tecidos vegetais de orquídeas foram realizadas em 1840, sendo descritas como grãos de amidos. Em 1853 houve o primeiro registro explicito da presença de hifas fúngicas no sistema radicular de uma espécie de orquídea deficiente em clorofila, *Corallorhiza innata* (Link 1840; Irmisch 1853 *input*: Andersen and Rasmussen 1996), então iniciou-se os trabalho nesse novo campo de pesquisa.

Na interação micorriza do tipo orquidóide, os fungos contribuem fornecendo água e elementos como carbono, nitrogênio, fósforo, vitaminas, de forma mais eficiente (Rasmussen, 2002). Esses fungos associados possuem maior capacidade de exploração do ambiente mediante a eficiência de produzir hifas com grande habilidade de colonização de microssítios (Roberts *et al.*, 2008; Heijden *et al.*, 2015). As orquídeas podem ser classificadas metabolicamente de acordo com a estratégia de aquisição de carbono, podendo ser: exclusivamente autotróficas, sendo o carbono presente somente pela atividade fotossintética, logo a fonte do carbono é a atmosfera; exclusivamente micoheterotrófica, em que a nutrição de carbono da planta é totalmente dependente do fungo micorrízico (ocorre principalmente nos estágios iniciais da associação e também em espécies aclorofiladas) e ainda; parcialmente mixotrófica, onde parte do carbono é fornecido pela fotossíntese e parte pelo fungo micorrízico associado (Dearnaley *et al.*, 2012).

Em relação a fonte de carbono, os fungos podem obter no ambiente e fornecer para a orquídea, através de duas estratégias: atividade saprofítica, em que o fungo irá degradar matéria orgânica do ambiente e fornecer para a orquídea como micorriza ou ainda como patógeno, fazendo uma ponte entre diferentes espécies vegetais (Andersen & Rasmussen, 1996; Wang *et al.*, 2006); adquirido da atmosfera pela atividade fotossintética da orquídea (tabela 1).

Tabela 1: Estratégias metabólicas de orquídeas para a aquisição de carbono (Silvera *et al.*, 2010)

Metabolismo	Fonte de Carbono	
Exclusivamente autotrófica	Atmosfera	
Exclusivamente micoheterotrófica	Atividade saprofítica do fungo	
	Rede entre espécies vegetais	Interação micorrízica
		Interação patogênica
Mixotrófica	Atmosfera	
	Atividade saprofítica do fungo	
	Rede entre espécies vegetais	Interação micorrízica
		Interação patogênica

Em estudos tróficos e de ecologia, o uso do isótopo ^{13}C pode auxiliar na elucidação de como as plantas se relacionam com o ambiente (Dawson *et al.*, 2002). Isótopos podem revelar mecanismos mais generalistas, como o aumento da quantidade de carbono liberado pela queima de combustíveis fósseis até mais específicos, no nosso caso de interações íntimas, entre diferentes organismos, como em associações micorrízicas (Martinelli *et al.*, 2009).

Para a determinação da origem do carbono e dinâmicas de fluxo na associação micorrízica de orquídeas, pode-se utilizar isótopos. Estudos primórdios com isótopos eram principalmente realizados com elementos radioativos, que apesar de serem menos abundantes, são mais facilmente detectados, contudo a legislação para a liberação e o risco do seu uso acabou-se por limitar o emprego dos radioisótopos (Griffith, 2004).

Com o advento e aprimoramento de equipamentos mais sensíveis à leitura dos isótopos estáveis, considerados como elementos marcados em sua abundância natural, proporcionaram uma nova perspectiva de estudos ecológicos pela assinatura isotópica (Dawson *et al.*, 2002). Essa marcação natural então pode ser empregada para definir transformações e rotas de determinados elementos nos mais diferentes campos da pesquisa científica pelo monitoramento dos elementos.

A assinatura isotópica é mensurada pela razão entre o elemento menos abundante sobre o mais abundante. Condições que afetam a aquisição de nutrientes como as que exercem influência sobre a fotossíntese, incluindo qualidade e quantidade de luz que chega aos tecidos vegetais, pressão de vapor nos estômatos, temperatura e outros, podem fazer que a razão isotópica seja mais ou menos próxima da atmosférica (Martin *et al.*, 2010; Dearnaley *et al.*, 2012) fazendo com que as assinaturas isotópicas dos fungos associados também sejam diretamente influenciadas por esses fatores.

O uso de isótopos em fungos associados com orquídeas ainda é pouco empregado, principalmente para ecologia, principalmente em combinação de estudos quando a riqueza e abundância desses fungos (McCormick *et al.*, 2016b). Além do melhor entendimento das dinâmicas ecológicas e evolutivas envolvidas com fungos micorrízicos de orquídeas, o mapeamento de espécies de quaisquer clados, incluindo fungos, são importantes para alimentar banco de dados que pode fornecer informações mais coesas sobre ecossistemas, incluindo a possibilidade de predizer efeitos de mudanças climáticas (Allen & Lendemer, 2016)

Fungos micorrízicos de orquídeas são de ocorrência cosmopolita e com estimativas de mais de 25 mil *taxa*, número que representa cerca de 2,5% de todos os fungos já descritos (Heijden *et al.*, 2015). Esses fungos podem formar micorrizas, logo fornecer nutrição, ou ainda estarem apenas presente nos tecidos como endofíticos (Oliveira *et al.*, 2014). A especificidade entre os parceiros (fungo – orquídea) são variadas, mas em orquídeas epífitas estão correlacionadas com a filogenia da orquídea hospedeira (Jacquemyn *et al.*, 2011; Martos *et al.*, 2012; Sakamoto *et al.*, 2015).

O principal grupo de fungos em associação com raízes de orquídeas são rizoctonióides (Martos *et al.*, 2012), um grupo polifilético que abrange diferentes *taxa,* do filo Basidiomycota (Ogoshi, 1996; Dearnaley *et al.*, 2012). Em rizoctonióides, ainda não foram descritos mecanismos claros de dispersão, e ainda há ausência de esporulação (Bonnardeaux *et al.*, 2007), o que promove dificuldades para a busca ativa visual em ambientes naturais.

Dentro dos rizoctonióides, Tulasnellaceae é uma das famílias mais comumente encontrada formando micorriza com orquídeas epífitas (Kohout *et al.*, 2013), essa família apresenta uma grande variação no espaço interno transcrito (ITS) e somado a falta de informações disponíveis em bancos de dados, promove uma dificuldade na identificação das espécies encontradas em associação com orquídeas (Taylor & McCormick, 2008). Membros da família Tulasnellaceae apresentam células diferenciadas, em que a sua gênese ocorre em condições de estresse, as células monilióides. Essas células apresentam parede espessa, são ancestrais a escleródios e são dadas como células de resistência (Moore *et al.*, 1996; Flor, 2009).

O número de espécies dentro de rizoctonia encontrados em associação com orquídeas demonstra a especificidade dos parceiros envolvidos (Andersen & Rasmussen, 1996). Nessas espécies epífitas, além de Tulasnellaceae, Sebacinaceae (majoritariamente da ordem

Sebacinales) e Ceratobasidiaceae também são frequentemente encontrados (Oliveira *et al.*, 2014). Desta forma, conhecer aspectos relacionados ao ciclo de vida, como o fluxo de nutrientes, principalmente de carbono; dinâmicas das interações com os organismos do ambiente; fungos presentes na associação e sua relação com as orquídeas são importantes fatores para a conservação da família Orchidaceae, principalmente para sustentar a elaboração de planos de manejos mais eficientes e que vão além da delimitação de áreas geográficas (Vieira *et al.*, 2011; Khan *et al.*, 2012; Seaton *et al.*, 2015).

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2. Tulasnellaceae is the key of fungi structure diversity in a tropical epiphytic orchid

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Abstract

Although the largest number of orchid species are epiphytes, efforts to study the diversity of mycorrhizal fungi have been concentrated on terrestrial ones. We focus on epiphytic orchids, very common in tropical forests, and the main family of fungi associated with it, the Tulasnellaceae. We investigated the diversity of total fungi and a specific Tulasnellaceae group in roots of *Isochilus linearis* (Orchidaceae), as well as in the bark of the phorophyte where the individuals of orchids occurred. After the extraction of total DNA and PCR with the pairs of primers ITS86F+ITS4 and ITS86F+ITS4-Tul, the samples were analyzed by NGS in Ion Torrent. The data were submitted to ANOVA, clustering analysis and the use of artificial neural networks. Tulasnellaceae was the commonest family, mainly of a species not yet described. We found indications of N entry in the fungus-orchid association by the strong presence of the fungus, *Pectenia plumbea*, *Biatora ligni-mollis* and *Bacidia laurocerasi,* previously described in lichens. We also observed the presence of fungus, *Candida smithsonii* and *Fusarium buharicum*, in the roots of *I. linearis* already described in the digestive tract of Coleoptera, which suggests the potential herbivory method for the dispersion of mycorrhizal fungi in epiphytic orchids. This is the first study to describe the presence of Tulasnellaceae in occurrence of epiphytic orchids, which may determine the success of the germination of new individuals of *I. linearis*.

Key-words: Orchidaceae, artificial neural network, Atlantic forest, hotspot, orchid conservation, orchid mycorrhizal fungi, orchid phorophyte

Introduction

The knowledge of the spatial distribution of mycorrhizal fungi in environments is fundamental to understand the ecological and evolutionary aspects between mycorrhizal fungi and hosts (Bahram *et al.*, 2015). Studies of spatial distribution have focus mainly in ectomycorrhiza (EcM) and arbuscular mycorrhiza (AM), however the Orchid mycorrhiza (OrM), more specifically for epiphytic orchids, are not know yet (Voyron *et al.*, 2017).

Orchids depends on the association with OrM fungi to complete their life cycle (Kohout *et al.*, 2013; Davis *et al.*, 2015). The presence of fungi able to associate with orchid can be a key for the establishment of new individuals of orchids, as well as populations resulting directly over orchid distribution (Voyron *et al.*, 2017). This aspect can limit the occurrence of orchids population due to the low fungi distribution (McCormick *et al.*, 2016a).

The fungi contributes to the orchid through a more efficient water absorption and exploitation of the environment by improving the acquisition of nutrients, such as C, N and P (Heijden *et al.*, 2015), mainly during seeds germination, as well as in the first stages (Sakamoto *et al.*, 2015). The peloton, a structure formed by a coil of mycorrhizal fungi hyphae is digested by the plant, which ensure plant nutrition (Boldrini *et al.*, 2010).

Most OrM fungi are saprophytic and can be found in the environment even in the absence of orchids. However, the orchid depends on OrM fungi for the establishment of populations (Davis *et al.*, 2015).

More than 25 thousand of fungi taxa are estimated to be in association with orchids and can potentially provide nutrients to plant. This number represent about 2.5% of all estimated fungi in the Earth (Heijden *et al.*, 2015), and the specificity in fungal partner is variable (Jacquemyn *et al.*, 2011; Kartzinel *et al.*, 2013) and are correlated with phylogeny of orchid hosts and area where these orchids are found (Davis *et al.*, 2015; Sakamoto *et al.*, 2015).

The main group of OrM fungus is rhizoctonia (Martos *et al.*, 2012), a polyphyletic group, which includes taxa distantly related (Dearnaley *et al.*, 2012). Rhizoctonia includes the families Tulasnellaceae, Sebacinales and Ceratobasidiaceae. These ones are often found associated with epiphytic orchids (Kohout *et al.*, 2013), but they do not form sexual spores (Bonnardeaux *et al.*, 2007), making the classical taxonomy difficult (see more in Jiang *et* *al*., 2015). It is estimated about 90 species of Tulasnellaceae (Linde *et al*., 2017), which family is the most commonly associated with root of epiphytic orchid in the Biome Atlantic Forest, Brazil (Oliveira *et al*., 2014).

In studies with orchid mycorrhizal fungi are common to use isolation methods and germination tests, but these strategies restrict the real diversity of OrM fungi present in habit and also in orchid root (Stark *et al*., 2009). Therefore, the molecular approaches can provide more information about the community present in the environment and as well as in the orchid roots. However the primer available in the literature can estimate different communities information and complementary strategies can be adopted (McCormick *et al*., 2016a).

Spatial distribution of OrM fungi, including host tissues, can be analyzed by next-generation sequencing (NGS). This approach can provides information about the microbial community diversity (Mccormick & Jacquemyn, 2014). NGS can provide information about all DNA presents in the samples. The primers used will guide the taxa more correlated with orchid. However, one pair of primers cannot have amplicons of all fungi community present, and the use of more than one primer set can be more efficient (see more in Taylor & McCormick, 2008). There is few studies focusing on orchid mycorrhizal fungi communities (Bahram *et al*., 2015), approach which can provide more complex information and can be a key for the orchid conservation.

Orchids have a high commercial value, being used for ornamentation, cooking and as having medicinal properties (Jiang *et al*., 2015). For that, around 62% for all known orchids have been the population decreased (Zhang *et al*., 2015). So, knowing the aspects related to the life cycle and dynamic interactions are important to create efficient conservation plans for Orchidaceae (Khan *et al*., 2012; Pereira *et al*., 2014) (see more in Seaton *et al*., 2015). Managements actions for conservation are scarce in Brazil, although Brazil is considered one of the source of biodiversity for all organisms (Mittermeier *et al*., 2005), especially Orchidaceae, with 2,419 species, being endemic 35 genera and 1,620 species (Forzza *et al*., 2010).

The human activities, such as plants over-collecting, habitat fragmentation and deforestation are the major factors driving an important number of orchid species for extinction (Zhang *et al*., 2015), which may include associated fungi.

We hypothesized that although many OrM fungi, the *Tulasnella* group is commoner to be associated in epiphytic orchid roots. Then, the objective of this study was to investigate if Tulasnellaceae family group is present on orchid root and phorophyte bark where epiphytic orchid occurs, and to establish a map of distribution of Tulasnellaceae in epiphytic orchid environment.

Methods

Sampling site

The sampling site was a fragment of the Atlantic Forest at "Parque Estadual Serra do Brigadeiro" – PESB in the city of Araponga, Minas Gerais state, Brazil (figure 1), with total area 149,84 km^2 at 1,050 m (IEF, 2007). It is a rock formation and is a protected area. The high altimetry of the region provides a frequently fog throughout the year, where the humidity range is 80%, even in the dry season. This area is a secondary forest with transition between Dense Rain Forest and Semideciduous Forest (Fávaro, 2012), and some exotic trees are present, such as eucalypt and coffee.

The annual means of precipitation is 1,185 mm and temperature varying between 16.38 and 27.05 °C (INMET, 2017). The average density of plant species is 642 individuals per hectare (Fávaro, 2012).

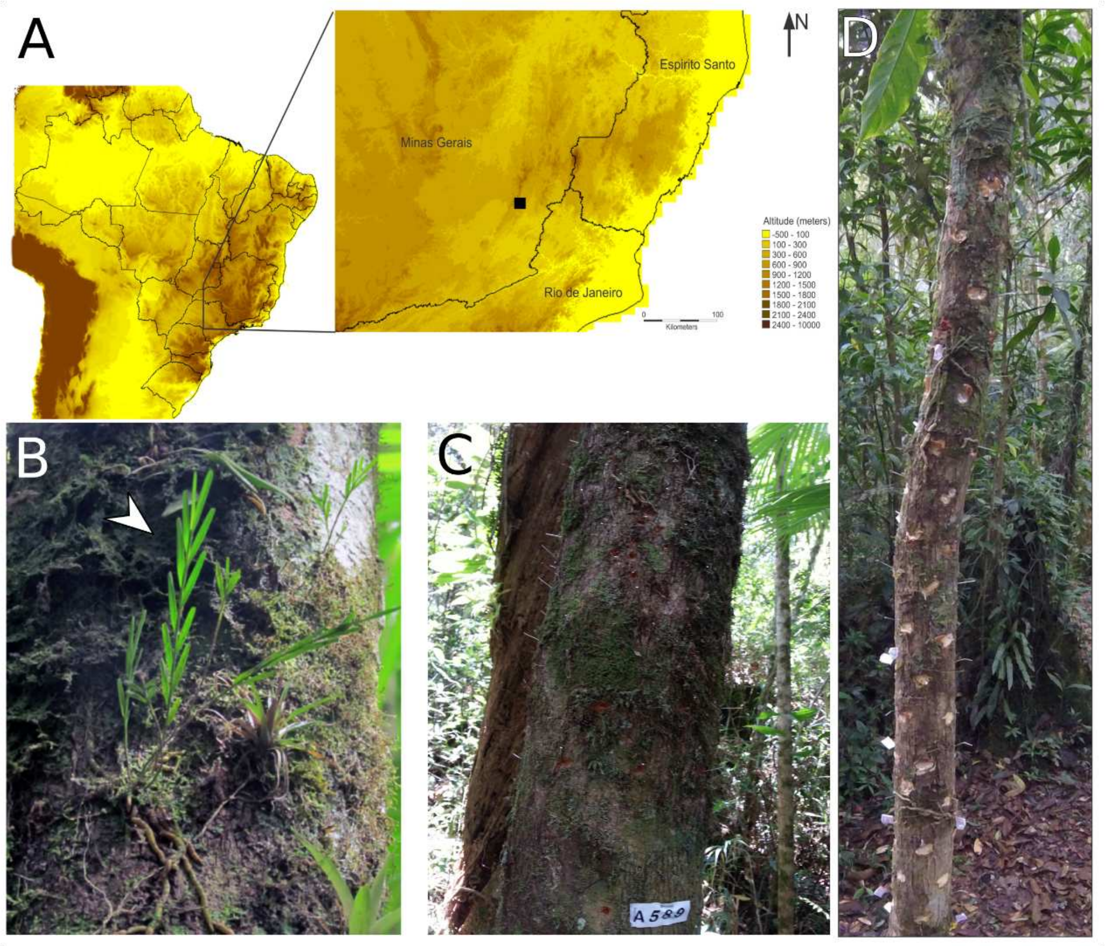

Figure 1: Sampling site, orchids species and phorophytes. A) ■ Sampling site, Serra do Brigadeiro State Park, Araponga city, Minas Gerais State, Brazil (S20°43’ W 042°28’) B) *Isochilus linearis* (arrow) growing on *Siparuna* sp phorophyte., C) *Siparuna* sp. phorophyte, and D) *Himatanthus articulatus* phorophyte.

Mapping OrM fungi

For this purpose, it was stablished a grid experimental design strategy. We searched for two phorophytes that housed more than one orchid individual in a small distribution band, so that the grid used for standardization was not very large and distributed above 2 m height, for become the sampling easier. The orchid species was chosen, *Isochilus linearis*, by root biology, which are thick compared to other species, which facilitated the sampling and grow on the bark of the tree in a scattered way without overlapping roots.

The first phorophyte used was *Siparuna* sp. – Siparunaceae, with five individual of *Isochilus linearis* (Jacq.) R.Br.; the second phorophyte was *Himatanthus articulatus* (Vahl) Woodson (Apocynaceae) with five individuals of *I. lineares* and two of *Epidendrum armeniacum* Lindl. The phorophytes were 21 m distance from each other. Three and four orchids of *I. linearis* were sampled, respectively, from *Siparuna* sp. and *Himatanthus articulatus* (figure 1).

A grid of 30 x 50 cm with squares of 7 cm was used for standardization the points of collect. Fragments of 3 mm each 1.5 cm over root of orchid were collected. The roots were washed in running water and surface disinfested by immersion in 70% alcohol solution for one minute and immersion in 20% sodium hypochlorite solution for six minutes. Subsequently, they were washed three times in sterile distilled water. All root fragments were checked for the presence of peloton, under stereomicroscope (40x). At each point of the grid, the samples of bark, about 100 mg, were also collected using a sterilized scalpel. The number of the samples depended on the diameter of the tree and orchids distribution. All samples were placed into the microtube with 200 µl of SDS buffer and stored at -20 ºC for conservation, until analysis.

Molecular approach

The total DNA of the collected samples: orchid root (OR), phorophyte bark (PB) and bark under the root (BUR) were extracted using NucleoSpin Soil^{TM} kit (Macherey-Nagel), according to the manufacturer's instructions. For PCR, we selected samples randomly: Ten from OR (five samples from each phorophyte), eight from PB and BUR (four samples for each phorophyte). The quantity and quality DNA were evaluated by Nonadrop TM (Thermo Fisher Scientific) and agarose gel electrophoresis.

The primer pairs used were: i) ITS3 + ITS4-OF (White *et al*., 1990; Taylor & McCormick, 2008), ii) ITSOF-T + ITS4-ngsUni (Tedersoo *et al*., 2010; Tedersoo & Lindahl, 2016), iii) ITS86F + ITS4 (White *et al*., 1990; Turenne *et al*., 1999) and iv) ITS86F + ITS4-Tul (Turenne *et al*., 1999; Taylor & McCormick, 2008).

The pair of primers iii, for estimating the total diversity of fungi (TFSP), was used for DNA of OR samples, and specifically for DNA from OR, PB and BUE the primer set iv, for the focus on Tulasnellaceae (TSP) were used, for obtaining fragments around 400 pb long.

The confirmation of the PCR products was performed by electrophoresis in 1.5% agarose gel stained with ethidium bromide solution and observed under UV light. The amplicons obtained were purified with magnetic beads from NucleoMag™ NGS Clean-up and Size Select by Macherey Nagel, sequenced by Ion Torrent™ (Ion PGM Hi-Q Sequencing Kit – Thermo Fisher Scientific), 400bp kit with the chip Ion 316™ .

Bioinformatics analysis

All the sequences were edited using Qiime (Caporaso *et al.*, 2010). The steps were: i) making the trimmed files – by removing the sequences of primers used and adaptors of Ion Torrent™, ii) checking the quality of fragments sequences from Ion Torrent™, using the software FastQC v. 3 (Andrews, 2010) and removing the sequences unreliable and with a quality less than 27 and length size of 150bp; iii) removing the chimeras; iv) transforming the sequences in an operational taxonomic units (OTU); v) removing singletons, artificial sequences and with less abundance (less than 10 OTUs); and vi) signing the OTUs in taxa at similarity of 97% for the BLAST algorithm from data source of 2017, in the UNITE (Koljalg *et al.*, 2014). We chose to use the UNITE database because of the high reliability of the data.

The taxa found in each group of samples were set in a table as percentage for better visualization and absolute values were in submitted to ANOVA and for evaluation of significant values they were tested by LSDB using R software v.3.4.3 (R Core Team, 2017).

The taxa data from Qiime were used for calculate diversity index and clustering analysis (UPGMA, Euclidean, 9999 bootstrap), using PAST software v.3.12 (Hammer *et al.*, 2001), and for rarefaction and extrapolation in “R” software with iNEXT package v. 2.0.12 (Hsieh *et al.*, 2016).

Artificial neural networks (ANN) was based in artificial intelligence. For that, using software Qiime, the NGS fragment data calculates the correlation between all the samples and taxa from the absolute number of times, which each fungus appeared in each analyzed sample. These data were then analyzed by Gephi v. 0.9.1 software (Bastian *et al.*, 2009). The distance among the dots represented the correlation distance, and the wider is the line thickness shows that the correlation is stronger. Tree different ANN were designed: first for general fungal primers set, second for Tulasnellaceae in orchid's roots and the third for all amplicons.

Results

Pelotons were observed in all OR samples, and them fungi identify by using the specific primers. The DNA were also successfully extracted from PB and BUR, but the amount varied depend on the sample (*p-value*: 2.2345e-08 of X^2), and the DNA from PB was twice higher than OR and BUR (table 1).

The primers set (i) ITS3 + ITS4-OF and (ii) ITSOF-T + ITS4-ngsUni were discarded (were not used) because they were not stable after analyzed by NGS. In the first, the amplicons length is very variable, and the last because the amplicons length are too large for Ion Torrent™ sequencing (Supplementary material 1, figure S1)

Taxa of fungi belonging to Tulasnellaceae was observed in OR and Biatriosporaceae and Ceratobasidiaceae in OR, PB and BUR (Supplementary material table S1). Species of these fungi have also been described as OrM fungi for epiphytic orchid (Taylor & McCormick, 2008; Martos *et al.*, 2012; Koljalg *et al.*, 2014; Oliveira *et al.*, 2014).

In OR (Supplementary material table S1) the occurrence of an Hysteriales (Peter *et al.*, 2016) and the genera *Pisolithus* (de Freitas Pereira *et al.*, 2017), *Trechispora* (Rosenthal *et al.*, 2017), known as ectomycorrhizal fungi were detected. Fungi described as endophytic, *Aureobadium pullulans* (Musetti *et al.*, 2007), *Pyrenochaetopsis leptospora* (Koljalg *et al.*, 2014), *Paraphaeospharia pilleata* (Kohlmeyer *et al.*, 1995), *Cadophora* (Glassman *et al.*, 2017) and *Chalara* (Benucci *et al.*, 2016), were also observed. Fungi associated with liquen species were also present: *Bacidia laurocerasi* (Lõhmus *et al.*, 2012), *Biatora ligni-mollis* (Spribille *et al.*, 2009)and *Pectenia plumbea* (Ekman & Jørgensen, 2002). Many other fungi taxa described as saprophyte were observed: *Microcera larvarum*, *Cryptococcus* and *Clvaria zollingeri*, both from soil and the order GS29 – Agaricomycetes (Koljalg *et al.*, 2014;

Triest *et al.*, 2016; Tedersoo *et al.*, 2017). Some others have been found associated with eucalyptus leaves: *Antennariella placitae* (Cheewangkoon *et al.*, 2009), *Cladosporium exasperatum* (Bensch *et al.*, 2010) and Mycosphaerellaceae (Crous *et al.*, 2009). *Candida smithsonii* and *Fusarium buharicum* were found in OR, which have been described in the digestive tract of coleopteran (Suh & Blackwell, 2004; Koljalg *et al.*, 2014), see the complete data in supplementary material table S1.

The Shannon and Simpson indices showed to be three times and 12% higher, respectively, using TFSP than TSP (Table 1). The rarefaction and extrapolations curves for ITS86F + ITS4 showed that the root sampling was enough (figure 3A). However, for ITS86F + ITS4-Tul, obtained from the diversity index showed that root and bark samples were properly sampled, however for BUR it is need greater sampling (figure. 3B).

The amplicons for TSP which "No blast" were more common in BUR samples. The Tulasnellaceae were very common (*p*-value = 0.0162) in all samples of OR, PB and BUR). Some taxa were only found in OR and PB, like Russulaceae, while other like Trechisporales was only found in OR (figure 4C).

Table 1: Extracted DNA, sequencing data r two set of pair of primers., and diversity indices for orchid root (OR), bark under root (BUR) and phorophyte bark (PB)

Description	OR		BUR	PB
	DNA (ng/µL)			
Average	24.22		22.90	49.53
Standard deviation	35.31		35.23	31.59
Coefficient of variation	1.45		1.53	0.63
Variance	5.94		5.93	5.75
Amplitude	1-209.03		1.36-222	3.2-172
	Primers set			
	ITS86F+ITS4	ITS86F+ITS4-Tul		
	OR	OR	BUR	PB
Fragments (number)	118541	11087	1183	32314
OTUs (number)	14740	3917	153	5367
GC %*	51	52	49	48
	Diversity Indices			
Simpson_1-D	0,9118	0,7799	0,7695	0,8656
Shannon_H	3,014	1,667	1,568	2,061

*GC% is from one specifically partial fragment of ITS region. This do not represent for all fungi genome.

The Shannon diversity index of fungi of TSP show that the sampling of root and bark were enough, however, for BUR more samples have to be done, further investigation is needed to reveal the total diversity. For TGSP the h' the sampling in root was enough (figure 2A). The root and bark samples are closer than BUR (figure 2B).

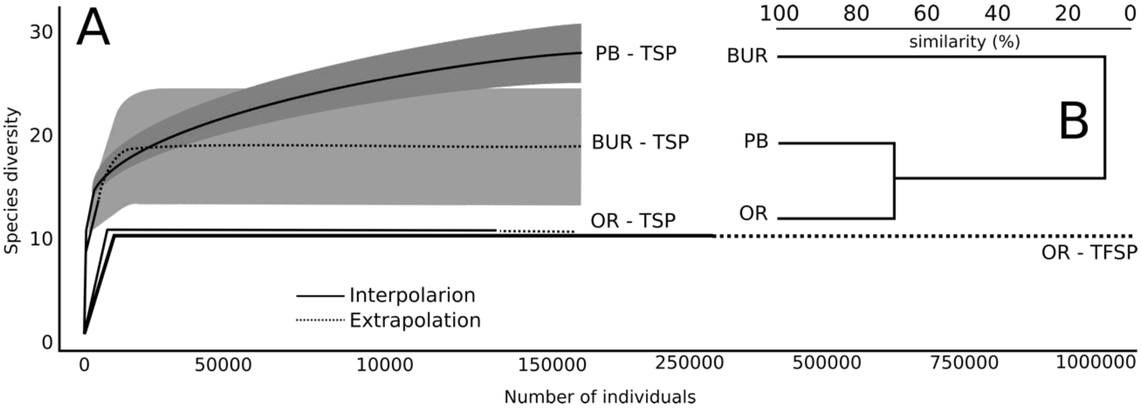

Figure 2. Rarefaction and extrapolation curve with Shannon diversity (A). Clustering analysis of Tulasnellaceae (TSP) (B), being TFSP: ITS86F + ITS4 TSP: ITS86F + ITS4-Tul, OR: Orchid root; BUR: Bark under root and PB: Phorophyte bark.

Using TFSP, *Cladosporium fusiform*, *Tremellales* genus and *Pectenia plumbea* showed to be the key in ANN structure for OR (figure 3A). Other taxa known as OrM, like *Rhizoctonia*, as well as Ceratobasidiaceae were observed, but with little contribution to ANN structure (figure 3A). Pannareaceae and Capnodiales are prevailing in OR (figure 4A). No blast fragments were very low, only 0,16% (figure 3A).

Using TSP, Tulasnellaceae is structuring ANN (figure 4A, B). It's possible to observe some taxa more correlated with Cantharellales, for example, are stronger linked with PB, however, it is weakly associated with BUR and OR. Three different species, *Tulasnella irregularis*, *Tulasnella* sp. 1 and *Tulasnella* sp. 2 are only correlated with OR. *Lactarius saponaceus* (Russulaceae) correlated with OR and PB. The order Trechisporales were found in OR, but not strongly. The presence of "No blast" reads are more associates with BUR samples (figure 4C).

The Tulasnellaceae provides the main support for the ANN structure, even when the data from both primer sets (TFSP and TSP) were analyzed mixed (figure 5). Other OrM genus, belonging to Ceratobasidiaceae and rhizoctonia were observed, however, they did show a strong correlation between samples.

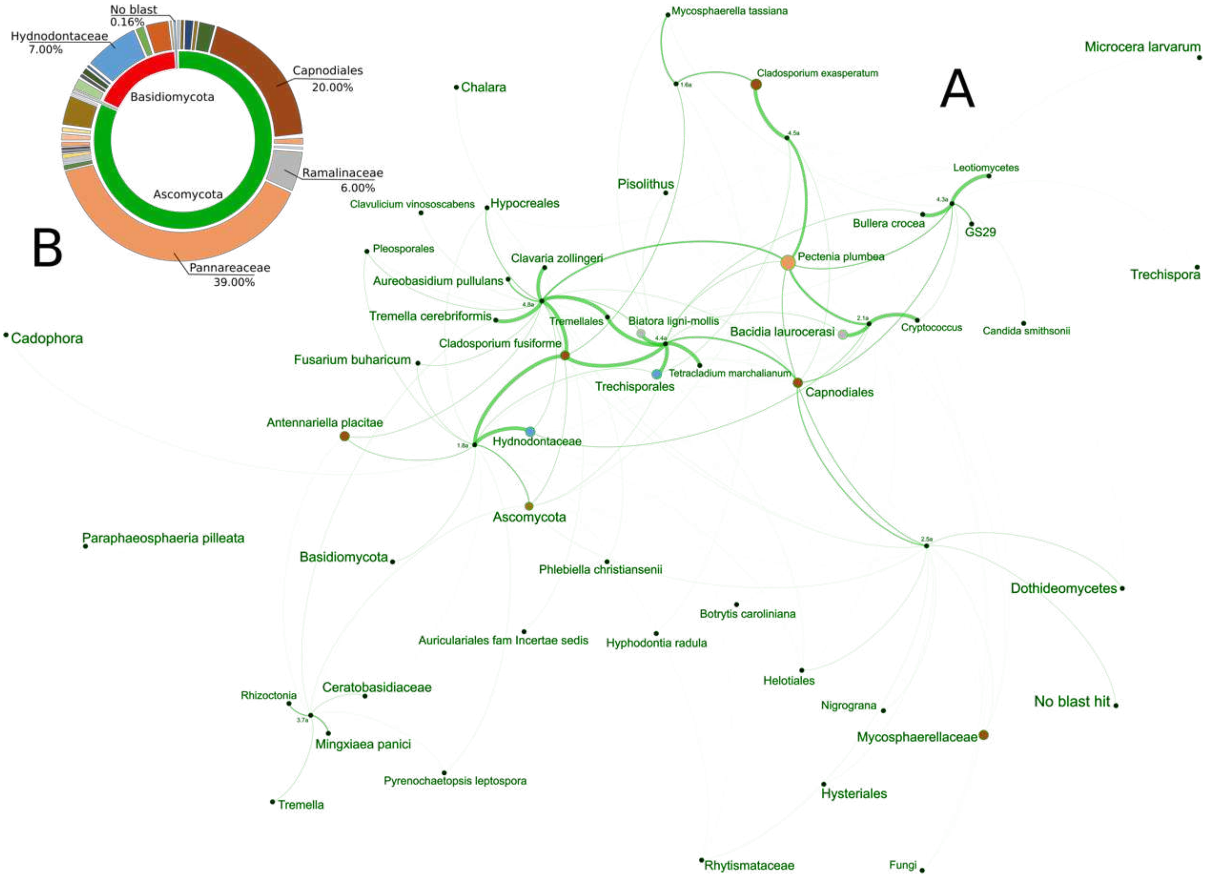

Figure 3. Structure of the mycorrhizal fungi of orchids and percentage of occurrence from ITS86F + ITS4 NGS amplicons from root samples. A) Artificial neural network analysis indicates few species of mycorrhizal fungi as of great relevance for the root diversity of Isochilus linearis. The thickness of the line represents the correlation between samples and taxonomic group. B) Compilation taxa percentage found in orchid roots show Pannaeacae like the most important family for I. linearis mycorrhizal fungi diversity.

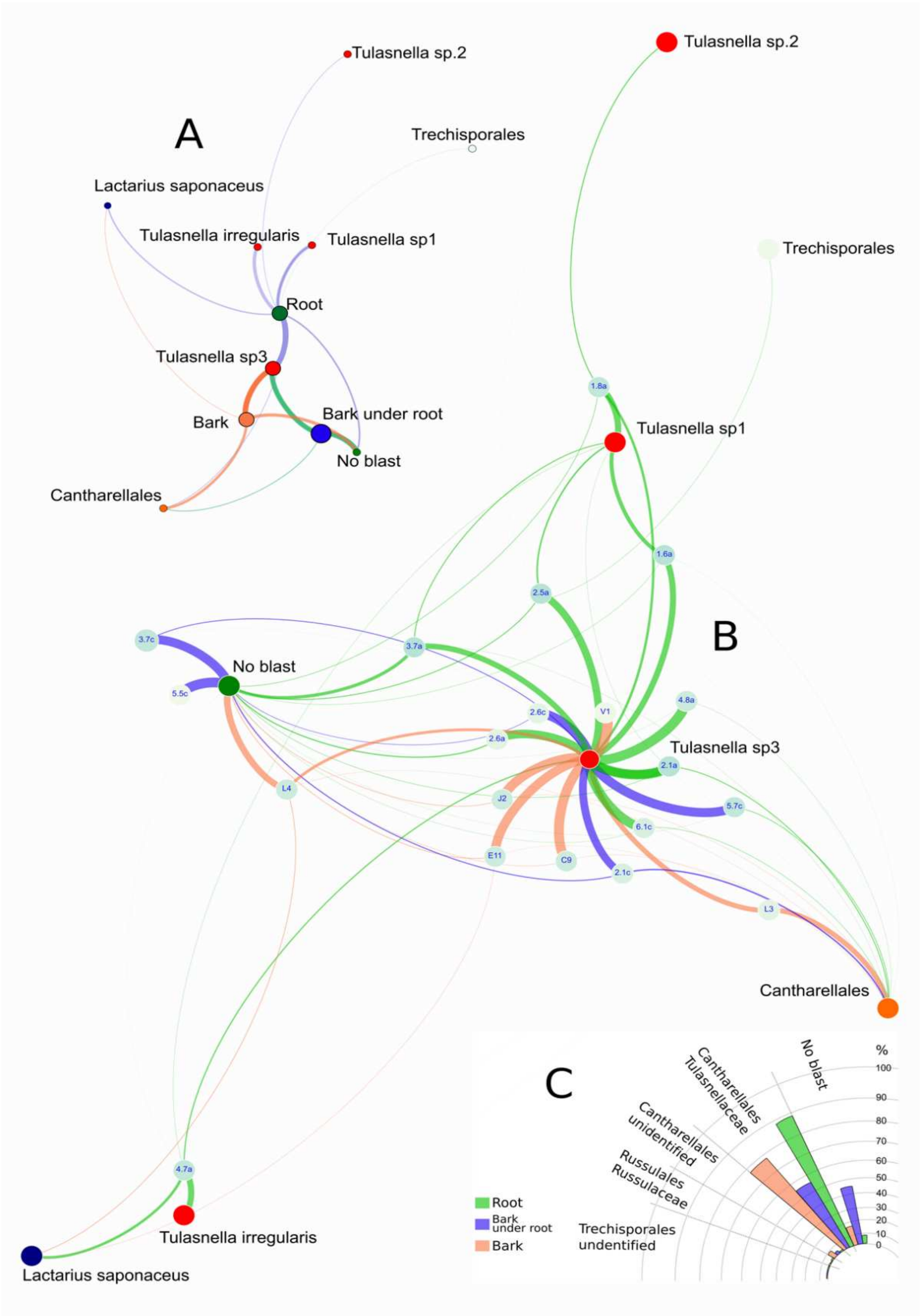

Figure 4: Artificial neural network analysis from ITS86F + ITS4-Tul, NGS amplicons. A) Samples type (root, bark, bark under root) are fixing, B) from each sample type. The thickness of the line represents the correlation between samples and taxonomic group. The *Tulasnella* sp. 3 was the strong relation with all kind of samples (Root, Bark under root and Bark). The taxa *L. saponaceus*, *T. irregularis*, *Trechisporales*, *Tulasnella* sp.1, *Tulasnella* sp. 2 are more correlation with root samples. C) Compilation taxon proportion (%) found in root from orchids, bark under root and bark from phorophyte.

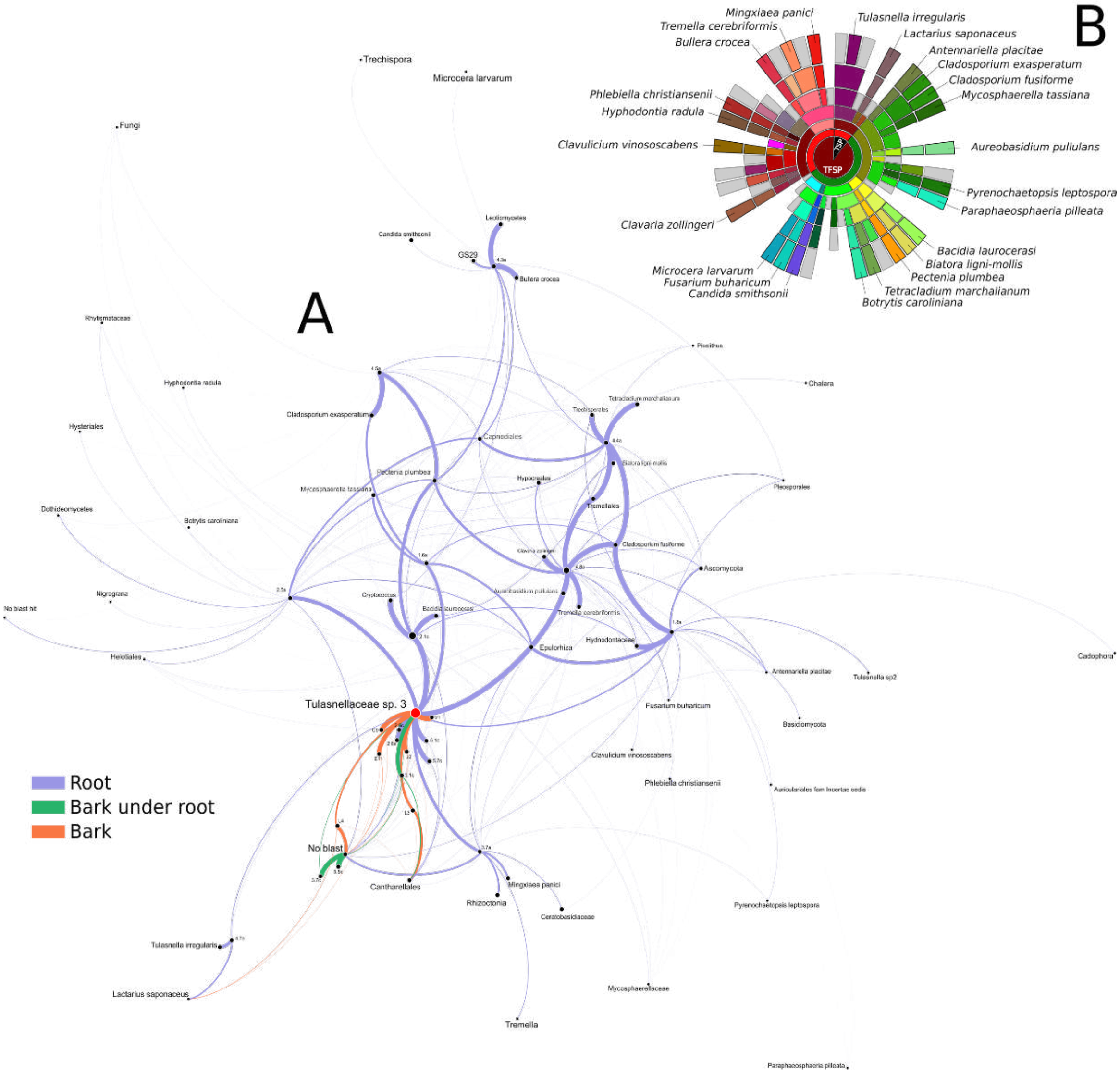

Figure 5. Structure of orchid mycorrhizal fungi in *Isochilus linearis* root. A) Artificial neural network analysis of ITS86F + ITS4-Tul and ITS86F + ITS4 amplicons compiled mixed and B) Presence of fungi data from ITS86F + ITS4-Tul and ITS86F+ITS4 amplicon. In the center of graph: TFSP, total fungi set primer; TSP, Tulasnellaceae set primers; Gray box, unedified taxa.

Discussion

We addressed the ecological interaction between fungi and epiphytic orchids, an important group in tropical habitats, mainly in the hotspot as Atlantic Forest. Taxonomic studies have already been done (Pereira *et al*., 2005, 2009, 2014; Oliveira *et al*., 2014), nevertheless with ecological approach. Therefore, this is the first study in this forest on OrM fungi, investigating not only these fungi in the orchids, but also in the phorophyte where it is growing. Using the orchid *I. linearis* growing in two phorophytes, the NGS data showed the total diversity and the interaction between orchid and environment. These information can help us to understand the factors related to evolution, since the associated fungus can guide evolutionary processes (Stark *et al*., 2009).

The barcoding sequence and primer choice can determinate the diversity of organisms. We used as OrM barcoding the rDNA ITS2, with about 350 bp, fragment size efficiently sequenced by IonTorrent™. The first primer set, ITS86F + ITS4, is more indicated for demonstrating the total diversity of fungi associate with orchid (Waud *et al*., 2014) and the second, ITS86F + ITS4-Tul, for Tulasnellaceae, the most common family found in epiphytic orchids (Taylor & McCormick, 2008).

The community of fungi associate with orchid as OrM and, or endophytic are highly diverse, mainly for species with restrict distribution, which usually is associated with a generalist fungus to increase the success of the species persistence (Martos *et al*., 2012). The most common fungi in the OR was *Pectenia plumbe* (14%), which is also described in association with liquen in Norway (Ekman & Jørgensen, 2002). This fungus is may be a connection between orchid and liquen growing on the phorophyte bark. Similarly, the presence of *Lactarius saponaceus*, one species of Trechisporales and *Pisolithus*, can be another network with orchid, since these fungi are described as EcM fungi (Benucci *et al*., 2016; de Freitas Pereira *et al*., 2017; Rosenthal *et al*., 2017) .

Surprisingly, we have found two fungi in the OR that have already been described in the digestive tract of beetles. Although they are not described as mycorrhizal fungi, the herbivory action of coleopteran species may be putative strong indication that this insect can also disperse OrM fungi, besides wind, rain, birds (Bahram *et al*., 2015).

The higher number of fragments and OTUs in OR from TFSP than TSP amplicons was expected, once the first primer set is more generalist (Taylor & McCormick, 2008; Waud *et al*., 2014). However, the use of TSP was very important to show the high diversity of Tulasnellaceae, and also the necessity to use specific primer to determinate some target species (Oja *et al*., 2015).

Since TFSP have high capacity to amplify the mycorrhizal fungi, and the Shannon-Wiener index with this amplicon is greater than observed in *Hoffmannseggella cinnabarina* root also in the PESB, but using library of clone sequencing (Oliveira *et al*., 2014), showed that the technic used to evaluate the diversity can determine the group of fungi detected. Furthermore, different orchid species can display different fungal community (Dearnaley *et al*., 2012), even geographical distance can affect the fungal community of terrestrial orchids (Stark *et al*., 2009), more than epiphytic species, in which a phylogenetic correlation have been observed (Martos *et al*., 2012).

The samplings effort were sufficient of OR and PB, showing the importance to sequence many fragments of the same orchid, since many fungi can stay in the same fragment of OR (Oja *et al*., 2015). However, for BUR, our methods were not able to estimate the total diversity, needing about three times more samples for providing results closer to real diversity, which was calculated by iNext. This can have affected the clustering OR with PB, then with BUR.

Rhizoctonia represents a polyphyletic group and hardly a single primer pair can be able to efficiently detect species diversity (Taylor & McCormick, 2008), and Tulasnellaceae, belonging to this group, was the most important family for *I. linearis* (see supplementary data, table S1). *Tulasnella* species is very common as OrM fungi in Atlantic Forest (Pereira *et al*., 2005, 2014; Oliveira *et al*., 2014). Results from TSP amplicon confirm the importance of this family, since the values of "no blast" were low. One unidentified *Tulasnella* species represent 37% of all taxa (see supplementary data, table S1), and was a key of *I. linearis* OrMF. Investigation can be done for isolating to confirm the morphological and molecular features, and maybe to describe the new species. This *Tulasnella* species also plays a key role in the structure of fungal diversity, in all collected samples (OR, PB and BUR). This result was expected, since we used a specific primer (TSP), but if another group was determining the dot of "No Blast" could be the center of ANN.

The Tulasnellaceae have been recognized to be associated with others terrestre orchids, such as *Cypripedium* genera (Shefferson *et al.*, 2007) *Chiloglottis* (Roche *et al.*, 2010), and are the key of structure of network in species of genus *Orchis* (Jacquemyn *et al.*, 2011), which is also observed for *I. linearis* analyzing our TSP data. However, analyzing TFSP data it was not possible to observe Tulasnellaceae in network structure, but Ceratobasidiaceae, a very common OrM fungi, but with little contribution in the structure of ANN. In some orchids like *Goodyera*, the Ceratobasidiaceae (Shefferson *et al.*, 2010) are more important than observed for us in *I. linearis*. However no Ceratobasidiaceae was found in association with *Cypripedium japonicum* (Gang *et al.*, 2017).

The TSP were able to amplify Tulasnellaceae in 88.48% of root samples, value closer to obtained for *Orchis* sp., using the primers ITS3 and ITS4OF, which was 84.33% of samples (Jacquemyn *et al.*, 2011). This results are expected since Tulasnellaceae are common taxa in orchids (Shefferson *et al.*, 2005, 2010) and more important for epiphytic ones (Pereira *et al.*, 2005; Martos *et al.*, 2012; Oliveira *et al.*, 2014).

Plants in general, including orchids, can exudate products from photosynthesis that can drive microorganisms to be present close to the roots (Cameron *et al.*, 2006). The exudate profile of *I. linearis* may have selected specific microorganisms that we were not able to identify with the methodology used, so the percentage of unidentified OTUs is higher in BUR.

By TFSP amplicons, the percentage of *Cladosporium* corresponded to 20% of total diversity, representing a key in the architecture of fungi associated with root in *I. linearis*. Although using another the pair of primers, ITS1 + ITS4, This fungus was also detected as an endophytic in *Cypripedium japonicum* (Gang *et al.*, 2017).

In *I. linearis* roots, *Pectenia plumbea*, belonging to Peltigerales, is the most representative order, twice more than Capnodiales, and had already been described as a new lichen species, commonly associated with *Nostoc* (Otálora *et al.*, 2017), a cyanobacteria, a nitrogen-fixing bacteria, what can be suggesting that *P. plumbea* can transfer nitrogen, fixed by *Nostoc* to the orchid. These mechanism is already described in arbuscular mycorrhiza, between *Geosiphon pyriformis* (Glomeromycota) and *Nostoc punctiforme* (cyanobacteria), where the nitrogen fixed in heterocyst cells are shared with fungi by endosymbiont and with host plant mycorrhizal activity (Schußler, 2009).

We found two species of Ramalinaceae family which have already been described previously in liquen association, with cosmopolitan distribution, including in bark tree at south American (Gasparyan *et al*., 2017), although it showed lower importance than Pannareacae and Capnodiales, Ramalinaceae. The last fungi showed a strong correlation with our root samples, being in the core of the ANN from TFSP amplicon.

The results of ANN using the both pairs of primers showed that Tulasnellaceae remained key in the architecture of fungal diversity. Other groups, which were important, like Pannareaceae and Capnodiales, did not exhibit strong influence about architecture of ANN. The *Tulasnella* sp. 3 seems to be the "link" between the diversity observed by the two amplicons (TSP and TFSP). Groups of fungi that are commonly described as OrM, such as Ceratobasidiaceae, did not show strong correlation with our samples, being detected only in root samples, but not in the BUR and PB, showing the importance of *Tulasnella* to *I. linearis*.

Not only mycorrhizal fungi are found in association with orchids roots, but also endophytic ones, that revealed a different profile of fungi community over the seasons and in different ecosystems (Kohout *et al*., 2013, Ma *et al*., 2015). Many taxa are unidentified, showing there are many taxa are yet unknown. The use of different pair of primer, such as ITS4-Tul2 (Oja *et al*., 2015), could identify more species of Tulasnellaceae. The non-standardization of diversity among samples showed that even in a small scale, microsites exist and can determine which fungi will be present (Mccormick & Jacquemyn, 2014).

Conclusion

- Tulasnellaceae is present in the orchid root and bark of phorophyte. This family of fungi seems to be a very diverse group and is key of diversity structure of OrM fungi in *I. linearis*.
- There is a great diversity of fungi associated with *Isochillus linearis*, including mycorrhizal and endophytic ones.

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Author contributions

CA Vieira developed the research and wrote the paper; CA Vieira, MF Bocayuva, TRG Veloso, ES Cruz, DMS Bazzolli, e MA Selosse collected and analyzed the material; CA Vieira, J Lambourdière e R Debruyne did the sequencing. MA Selosse collaborated to the research design and proposal; MA Selosse and MCM Kasuya supervised the research reviewed the entire manuscript.

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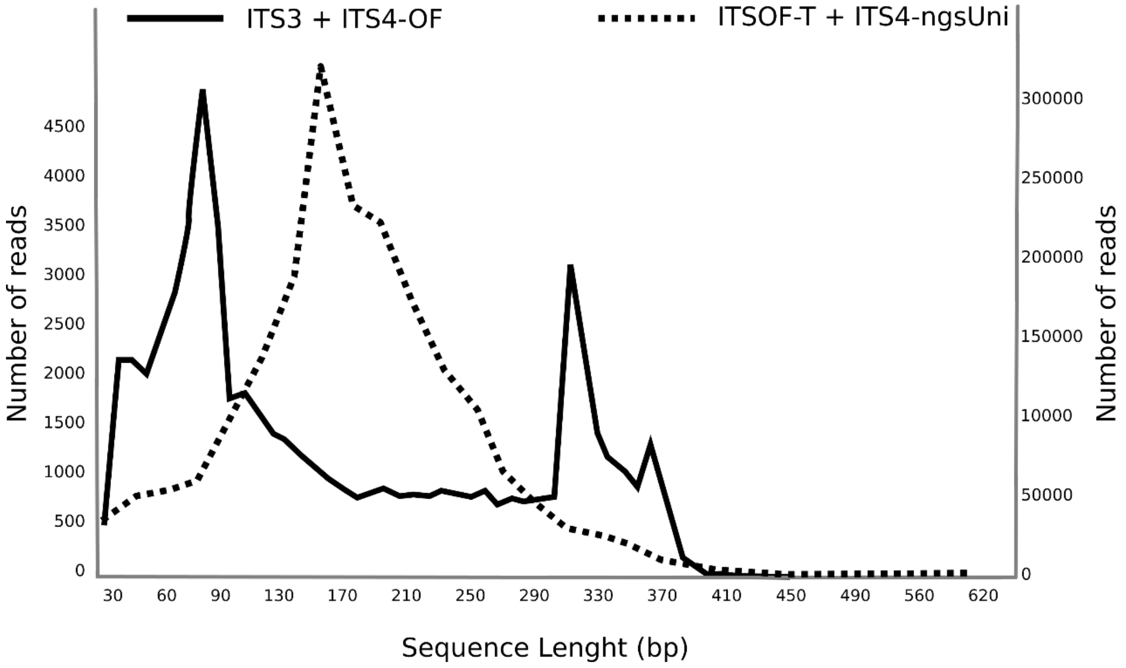
Supplementary material 1

To investigate the diversity of orchid mycorrhizal fungi (OrMF) associated with *Isochilus linearis* and the bark of the phorophyte where it grows and to determinate the better for the estimated total diversity of OrMF from the Ion Torren™ (Ion PGM Hi-Q Sequencing Kit – Thermo Fisher Scientific), we did the test of the pair of primer. Eight root samples and eight bark samples were sequencing from the total DNA opted with NucleoSpin Soil™ kit (Macherey-Nagel), according to the manufacturer's instructions.

The PCR were performing with the pair of primers set: PCR 1; ITS3 – 5' – GTCGATGAAGAACGAAGC - 3' (White *et al.*, 1990) with ITS4-OF – 5'-GTTACTAGGGGAATCCTTGTT- 3' (Taylor & McCormick, 2008) and PCR 2: ITSOF-T 5' –ACTTGGTCATTTAGAGGAAGT – 3' (Tedersoo *et al.*, 2010) with ITS4ngsUni – 5'–CCTSCSCTTANTDATATGC – 3' (Tedersoo & Lindahl, 2016). The products of PCR 2 set are too large to Ion Torrent sequencing kit, the samples are submitted to fragmentation with NEBNext™ Fast DNA Fragmentation & Library Prep Set for Ion Torrent™.

The sequencing results reveled the products of both pair of primers (PCR 1 and 2) but they were not satisfactory. The PCR 1 products showed instable fragment length (Figure S1 – red line), with the major three different length fragments, with the highest number of reads was 75bp, followed by 315bp and 360bp. The fragmentation step adopted for the products of the PCR 2, produced many small fragments (Figure S1 – blue line). During the bioinformatics analysis with the FASTQc software v.3 (Andrews, 2010), the high number of small fragments can promote errors in the assembly of fragments with sizes closer than expected, 400 and 600 (for PCR1 and 2, respectively). In addition, mycorrhizal fungi of orchids have a very variable ITS region (Taylor & McCormick, 2008), so small or artificially assembled fragments may provide non-true information about diversity in the *I. linearis* root samples. Thus, we discarded the products of these two primers, since the molecular strategies adopted, and the sequencing equipment were not able to provide reliable STI fragments for the use of identification and diversity estimates.



Sequecing data	PCR 1	PCR 2*
Amplification size (average size)	400bp	600bp
Number of reads	62046	1974332

Figure S1: Sequence Length and number of reads of two pair of primers tested for sampling diversity of orchid mycorrhizal fungi of *Isochilus linearis* root after sequencing on the Ion Torrent^{TM} with 400bp kit. PCR 1; ITS3 + ITS4-OF and PCR 2: ITSOF-T + ITS4ngsUni, these PCR products was fragmented before library construction.

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Supplementary material 2

Table S1. The taxonomic fungi from each primer set, for general fungi (ITS86F + ITS4) and for Tulasnellaceae (ITS86F + ITS4-Tul)

Primer set	Phylum	Class	ordem	family	genus	specie	% of colonization	behavior	Substrate	Source	reference	site
ITS86F + ITS4-Tul	Basidiomycota	Agaricomycetes	Cantharellales	Tulasnellaceae	Tulasnella	unidentified	3.36	OrM	-	-	Oliveira et al 2014	-
						Tulasnella irregularis	1.54	OrM	-	-	Oliveira et al 2014	-
						unidentified	0.27	OrM	-	-	Oliveira et al 2014	-
					unidentified	unidentified	37.21	OrM	-	-	Oliveira et al 2014	-
				unidentified	unidentified	unidentified	2.48	OrM	-	-	Oliveira et al 2014	-
			Russulales	Russulaceae	Lactarius	Lactarius saponaceus	0.93	EcM	-	-	Verbeken1996 / Benucci et al 2016	-
			Trechisporales	unidentified	unidentified	unidentified	0.05	EcM	-	-	RoSenthal et al 2017	-
ITS86F + ITS4	Ascomycota	Dothideomycetes	Capnodiales	Antennulariellacea	Antennariella	Antennariella placitae	1.54	-	Leaf spots	-	Cheewangkoon 2009	Australia
				Cladosporiaceae	Cladosporium	Cladosporium exasperatum	2.31	-	Leaf eucalyptus	-	Bensch 2010	Australia
						Cladosporium fusiforme	0.93	-	-	chicken food	Zalar et al 2007	Canada
				Mycosphaerellacea	Mycosphaerella	Mycosphaerella tassiana	2.75	-	-	CCA-treated Douglas- r pole	Zalar et al 2007	USA
					unidentified	unidentified	0.33	-	Leaf spots eucalyptus	-	Crous et al 2009	cosmopolitan
				unidentified	unidentified	unidentified	7.05	OrM/ErM	-	-	Martos et al 2012 /Baba et al 2016	cosmopolitan
			Dothideales	Dothioraceae	Aureobasidium	Aureobasidium pullulans	2.26	Endophytic	-	-	Musetti et al 2007	Asia
			Hysteriales	unidentified	unidentified	unidentified	0.16	EcM	-	-	Peter et al 2016	cosmopolitan
			Pleosporales	Biatriosporaceae	Nigrograna	unidentified	0.05	OrM	Oorchid root	-	UNITE	Amerila latina
				Cucurbitariaceae	Pyrenochaetopsis	Pyrenochaetopsis leptospora	0.27	Endophytic/soil	-	-	UNITE	cosmopolitan
				Montagnulaceae	Paraphaeosphaeria	Paraphaeosphaeria pilleata	0.05	Endophytic	-	-	kohlmeyer1995	USA/BR
				unidentified	unidentified	unidentified	1.26	Endophytic	-	-	kohlmeyer1995	USA/BR
			unidentified	unidentified	unidentified	unidentified	0.55	OrM, EcM, Endophytic	-	-	-	cosmopolitan
		Lecanoromycetes	Lecanorales	Ramalinaceae	Bacidia	Bacidia laurocerasi	0.49	Liquen	Bark	-	lohmus et al 2012	Estonia
					Biatora	Biatora ligni-mollis	0.88	Liquen	Bark	-	Spribille et al 2009	Canada
			Peltigerales	Pannariaceae	Pectenia	Pectenia plumbea	14.00	Liquen	Bark	-	Ekman Jergensen 2002	Norway
		Leotiomycetes	Helotiales	Helotiales_fam_Inc	Cadophora	unidentified	0.16	Endophytic/soil	-	-	Glassman et al 2017	-
				Helotiales_fam_Inc	Tetracladium	Tetracladium marchalianum	0.11	OrM/Soil/endo	-	-	UNITE/Benucci et al 2016	cosmopolitan
				Sclerotiniaceae	Botrytis	Botrytis caroliniana	0.05	patogenus	Commercial strawberry fields	-	Dowling et al 2017	US
				unidentified	unidentified	unidentified	0.49	OrM/Soil/patogenus	-	-	-	-
			Rhytismatales	Rhytismataceae	unidentified	unidentified	0.27	-	Root	-	Toju et al 2013	Japan
			unidentified	unidentified	unidentified	unidentified	0.11	OrM, EcM, Endophytic	-	-	-	-
		Pezizomycotina*	Pezizomycotina*	Pezizomycotina*	Chalara	unidentified	0.11	Endophytic	Root	-	Benucci et al 2016	USA
		Saccharomycetes	Saccharomycetales	Saccharomycetales	Candida	Candida smithsonii	0.16	-	Digestive tract of coleptera	-	Suh & Blackwell 2004	Panama
			Hypocreales	Nectriaceae	Fusarium	Fusarium buharicum	1.10	-	Digestive tract of coleptera	-	UNITE	Spain
					Microcera	Microcera larvarum	0.05	-	Environment	-	Triest et al 2016	Belgium
				unidentified	unidentified	unidentified	1.65	-	Environment	-	-	cosmopolitan
		unidentified	unidentified	unidentified	unidentified	unidentified	3.08	-	Environment	-	-	cosmopolitan
	Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria	Clavaria zollingeri	0.11	-	Soil	-	UNITE	USA
			Auriculariales	Auriculariales*	unidentified	unidentified	0.16	OrM	-	-	Taylor & McCormick 2008	-
			Boletales	Sclerodermataceae	Pisolithus	unidentified	0.27	EcM	-	-	Freitas Pereira et al 2017	Brazil
			Cantharellales	Ceratobasidiaceae	Rhizoctonia	unidentified	1.81	OrM	-	-	Oliveira et al 2014	Cosmopolitan
					unidentified	unidentified	0.55	OrM	-	-	Oliveira et al 2014	Cosmopolitan
				Clavulinaceae	Clavulicium	Clavulicium vinososcabens	0.16	-	-	Sporocarp	UNITE	Sweden
			GS29	unidentified	unidentified	unidentified	1.98	-	Environment	-	Tedersoo et al 2017	-
			Hymenochaetales	Schizoporaceae	Hyphodontia	Hyphodontia radula	0.11	-	Hebarium	Sporocarp	Chen et al 2017	Cosmopolitan
			Polyporales	Polyporales*	Phlebiella	Phlebiella christiansenii	0.11	-	-	Sporocarp	UNITE	Finland / Sweden
			Trechisporales	Hydnodontaceae	Trechispora	unidentified	0.05	EcM	Root	Pine	RoSenthal et al 2017	USA
					unidentified	unidentified	1.43	EcM	-	-	-	-
				unidentified	unidentified	unidentified	0.99	EcM	-	-	-	-
		Tremellomycetes	Tremellales	Bulleraceae	Bullera	Bullera crocea	0.60	-	Root	-	UNITE	cosmopolitan
				Tremellaceae	Cryptococcus	unidentified	0.33	-	Environment	Soil	UNITE	USA
					Tremella	Tremella cerebriformis	0.27	-	-	-	UNITE	Russian
						unidentified	0.55	-	Root	-	Lorberau et al 2017	-
				Tremellales*	Mingxiaea	Mingxiaea panici	2.09	-	-	plant	UNITE	Thailand
				unidentified	unidentified	unidentified	0.16	-	Root	-	-	

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3. Mycorrhizal fungi receive more carbon from the orchids growing in the top of phorophyte than those in the bottom

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Abstract

In the classic concept of symbiosis, the multi-beneficiary is the key of interaction. Mycorrhizae are notoriously described as a symbiosis where the exchange of compounds, especially C, dictates the persistence of intimate relationships. We evaluated in epiphytic orchids, which represent the majority of Orchidaceae species, if the position at different heights in phorophyte influences the carbon flow between fungus-orchid. Two phorophytes present in a secondary forest of Atlantic forest, with average height of 22 meters were sampled. To evaluate the carbon flux, orchid metabolism (C3 or CAM), as well as the strategy of acquiring carbon by the orchid (mycoheterotrophic - MH, autotrophic - AT or mixotrophic - MT), we sampled leaves of orchid and non-orchids species and pelotons present in the roots of the orchids, growing in different height of phorophytes. The samples were dried to constant mass, ground and the 13C/12C isotopic ratio measured by isotope ratio mass spectrometer. We performed regression analysis, clustering test and artificial neural networks to determine the presence of carbon flux in the evaluated species. There was no standardizing of C flux along the altimetry gradient, 11.11% of the orchids were classified as CAM. The orchids presented metabolism MH, AT and MT with prevalence of 63.64%, 22.72% and 13.64%, respectively. Among the three MT samples, two received more C from photosynthesis than from the associated mycorrhizal fungus. This is the first work evaluating C nutrition in tropical epiphytic orchids. In this study C flux was the key to the interaction, showing its importance in the interaction fungus-orchid and there is a dependence. In tropical forest mycorrhizal fungi receive more carbon from the orchids growing in the top of phorophyte than those in the bottom.

Key-words: Carbon-13; natural isotope abundance; Orchidaceae, artificial neural network, carbon flux

Introduction

Orchidaceae is a cosmopolitan family, with exception of poles and extremely dry deserts (Roberts *et al.*, 2008). Orchids can be terrestrial, epiphytic or lithophytic, but approximately 73% are epiphytic or lithophytic, using respectively, other plants or rock as support for their growth (Roberts *et al.*, 2008). Brazil has been registered 2650 orchids species, being 1800 endemic, promoting this country as prominent in the World in the number of species (Giulietti *et al.*, 2005). All orchid, in natural condition, depends on orchid mycorrhizal fungi (OrMF) for seed germination, providing mainly carbon (Gebauer *et al.*, 2016), since the orchids seeds is a very small dust-seed, without endosperm (Rasmussen & Rasmussen, 2014). Besides carbon, OrMF are essential to ensure an efficient water absorption and exploitation of the environment by improving the acquisition of mineral nutrients, such as N and P (Heijden *et al.*, 2015).

This physiological network is very important for orchids not only during seeds germination, but also in the first stages of growth (Sakamoto *et al.*, 2015). In the orchid-fungi symbiosis, the pelotons are present inside of plant root tissue, a structure formed by a coil of hyphae, which is digested by the plant, which ensure plant nutrition (Boldrini *et al.*, 2010). The nutrients offered to orchid by symbiont fungus, including C and N (Hobbie *et al.*, 2001) is result from it activities as saprophyte, or by network with other plant species, as pathogenic or mycorrhizal association (Liebel *et al.*, 2010, 2015). Nevertheless, after orchid has grown, the fungus can receive carbon from the photosynthesis, as well as vitamins and a safety place to live (Roberts *et al.*, 2008; Liebel *et al.*, 2015).

The evolutionary approach indicates that epiphytic species derived from terrestrial species with root climbers from dark humid forests (Johansson, 1974). The fungi association improve orchids to colonize habitat with low nutrient availability, as well as low light incidence, like dark forest (Suetsugu *et al.*, 2017).

The ability OrMF for searching nutrient, mainly carbon, depend on the capacity of explore the environment, such as rock, litter, wood, leaves and, more important for epiphytic orchids, the phorophyte bark (Mehra *et al.*, 2017), where the nutrient availability is generally very low in epiphytic habitats.

The OrMF of tropical orchids are mainly Ascomycota or Basidiomycota (Oliveira *et al*., 2014). The dominant group is rhizoctonia, a polyphyletic filamentous fungi with similar features in their anamorphic states (Bonnardeaux *et al*., 2007). Furthermore, the family Tulasnellaceae is the most common in Brazilian neotropical orchids, like in *Epidendrum secundum* (Pereira *et al*., 2005, 2014).

Environmental factors like water availability, environmental humidity, including relative humidity, vapor pressure, temperature, seasonality, composition of bark and light intensities select which epiphytic species can grow in the host tree (Johansson, 1974; Li & Zhang, 2016). In the same way, the OrMF present in theses environment can determine which species of orchids can settle in the habitat.

The orchids can be classified into three physiological types related to carbon (C) nutrition: i) Fully autotrophic species, which are photosynthetic active at adulthood; ii) Fully mycoheterotrophic, which species are dependent on fungal carbon throughout their life cycle; and iii) partial mixotrophic, which take carbon from photosynthetic way and also fungal carbon (Dearnaley *et al*., 2012; Liebel *et al*., 2014).) (Hobbie *et al*., 2001).

Analysis for distinguish of different physiological types of orchid and flux between orchid to OrMF or OrMF to orchid can be measured from natural stable isotope abundance of ^{13}C (Liebel *et al*., 2015). To study the fungal ecology, the use of ^{13}C is very helpful as a tool for analysis the dynamics of interaction (Griffith, 2004). It is very common to use ^{13}C in laboratory condition, but it can also be used in natural environment for determinate the real change of C between fungi and plant.

The use of isotopes is more common in arbuscular mycorrhiza and ectomycorrhizal association because the economic importance (Bago *et al.*, 2000; Hobbie *et al.*, 2001). Recently studies were investigating ecological and physiology with isotopes as important tool (Slavikova *et al.*, 2016).

With the use of $^{13}CO_2$-enriched source, it has been estimated that 3 to 50% photosynthates products are allocated to ectomycorrhiza and ericoid mycorrhizal fungi (Smith & Read, 2008; Heijden *et al.*, 2015). Thought C-enriched can provide stronger data of mensuration, but we used ^{13}C natural signatures for results closer of reality. The use $^{13}CO_2$-enriched in orchid epiphytic could provide information for the proportion of photoassimilates from saprophytic or photosynthesis activity can be found in orchids (Liebel *et al.*, 2015), in epiphytic orchid this information is not available yet. In Arbuscular Mycorrhiza, the glucose from photosynthetic plant activity is rapidly are converted by fungi into trehalose and polyols that are largely unavailable for plant uptake (Smith & Read, 2008), but in OrM the peloton are digested and the complete understand mechanisms are unknown yet.

Knowing that epiphytic orchids are highly dependent on mycorrhizal fungi, due to the low availability of nutrient in the bark, and there is no information related to carbon flux in epiphytic orchids, we evaluated (i) the distribution of CAM (crassulacean acid metabolism) *versus* C3 orchids along a height gradient on phorophyte, and (ii) based on ^{13}C enrichment of the fungal pelotons recovered from the roots, whether the fungus received plants photosynthates. This is the first study in Carbon flux in epiphytic tropical orchids and the results of this study contribute for better understanding about the ecological mycorrhizal dynamics in epiphytic tropical orchids.

Methods

Study site

The sampling site was a fragment of the Atlantic Forest, localized in a protected area of a State Park "Parque Estadual Serra do Brigadeiro" – PESB, in the city of Araponga, Minas Gerais state, Brazil (figure 1), with total area of 149,84 km^2 at 1,050 m (IEF, 2007). It is a rock formation and the high altimetry of the region provides a frequently fog throughout the year, where the humidity range is 80%, even in the dry season. This area is a secondary forest with transition between Dense Rain Forest and Semideciduous Forest (Fávaro, 2012), and some exotic trees are present, such as eucalypt and coffee.

The annual means of precipitation is 1,185 mm and temperature varying from 16.38 to 27.05 °C (INMET, 2017). The average density of plant species is 642 individuals per hectare (Fávaro, 2012).

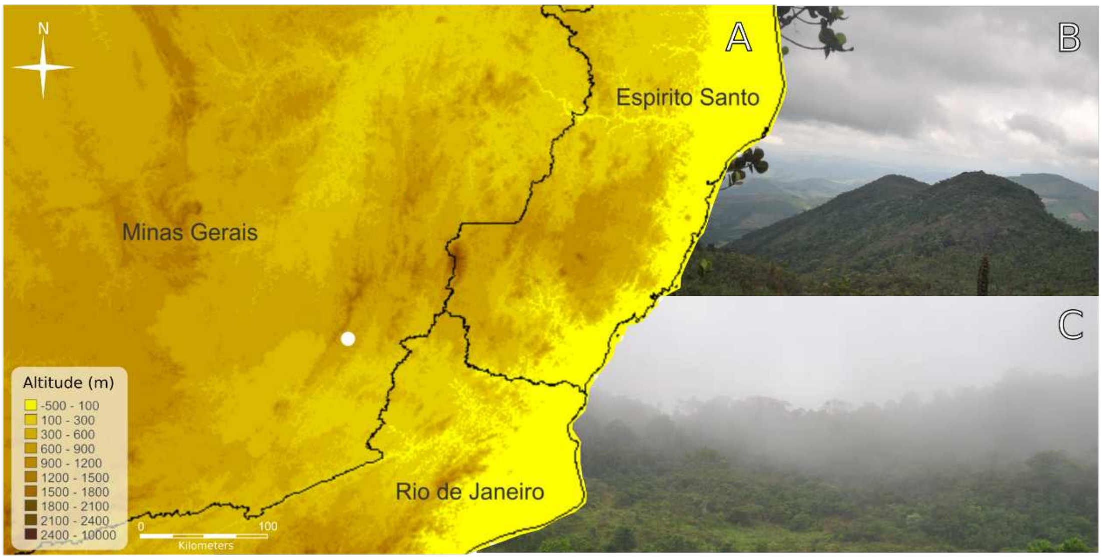

Figure 1: Localization of Parque Estadual Serra do Brigadeiro, Araponga/MG, Brazil (S20°43' W 042°28'). (A) O - sampling site position (white cicle) and (B) the PESB topography of inselberg and (C) the fog of PESB Atlantic Forest.

Designer of sampling

We seek for tree with about 25 m height, rich in epiphytic plants, including orchids and non-orchids plants. We find two different phorophyte, one individual Myrtaceae, collected in February (rainy station) and one Moraceae (*Ficus* sp.), collected in August (dry station). The Myrtaceae (Berry, 1915) as well as Moraceae (Rohwer, 1993) are cosmopolitan and very common in Atlantic forest biome.

Leaves samples of epiphytic species, orchids and non-orchids, were collected, trying always to take at approximately the same height, since different factors, as light and water vapor pressure can affect the stomatal regulation, change the CO_2 uptake as well as the ^{13}C signature (Liebel & Gebauer, 2011).

Collected leaves were placed between filter papers and storage in plastic bags, containing silica gel, and transported to the laboratory. In the field, under stereosmicroscope, pelotons were picked up from orchid roots, of 19 individuals growing on Myrtaceae and 21 individuals growing on Moraceae, using sterile needles and storage in microtubes. Some orchids showed very small root, with few cortex cell, therefore it was not possible to pick up peloton to $\delta^{13}C$ analysis.

From Myrtaceae, a total of 22 orchids and 23 non-orchids were sampled, ranged from 2 to 18 m height from the ground. From Moraceae, a total of 25 orchids and 29 non-orchids were sampled, ranged from 3.5 to 19 m height from de ground. Some non-orchids species were collected from neighbor phorophyte or sometimes growing direct from the ground.

$^{13}C/^{12}C$ carbon ratio measure

All samples, leaves of orchids and non-orchid leaves, and pelotons of OrMF for $\delta^{13}C$ analysis were deposited in drying oven at 65 °C up to constant weight (Bidartondo *et al.*, 2004). The leaves were grounded in ball mill to obtain a fine powder (Liebel & Gebauer, 2011). All tools were cleaned with absolute alcohol.

For analysis, 1 mg of leaf sample or 0.1 to 1 mg for OrMF (peloton) were used. All samples were deposited in tin capsule and the $^{13}C/^{12}C$ carbon ratio were analyzed by Flash 2000 and $\delta^{13}C$ by Thermo Scientific DeltaV advantage IRMS (maximum variation of $\delta^{13}C$ = 0.15 ‰).

Carbon flux and strategies of metabolism

The data of $\delta^{13}C$ measurement were submitted to analysis of variance (ANOVA), and the media compared by Test t of Bonferroni (0.05). The software "R", version 3.2.2 (R Core Team, 2017) with ExpDes package version 1.1.2 (Ferreira *et al.*, 2015) was used to compare the difference of $\delta^{13}C$ signatures among orchids, non-orchids species and OrMF.

The determination of carbon flux from $\delta^{13}C$ signature and height, the cluster analysis (CA) was done using software PAST v. 3.12 (Hammer *et al.*, 2001) classical group, with UPGMA algorithm and Euclidean similarity index with 10.000 bootstrap. The formation of CA groups was interpreted by ^{13}C signature source: (i) when the orchid and its OrMF was grouped, we adopted that the C in orchid is from saprophytic fungi activity and the orchid is mycoheterotrophic -MH (Gebauer *et al.*, 2016); (ii) when orchid, OrMF and non-orchid species are grouped, we adopted that the C in OrMF is from orchid photosynthesis or from saprophytic fungi activity, and orchid is mixotrophic - MT; and (iii) when orchid and non-orchid are groped, the C in orchid is from photosynthesis, and there is no carbon flux from and to OrMF, so orchid has autotrophic metabolism AT.

To determinate the metabolism, when the signature of $\delta^{13}C$ are between -12 and -22 are considered CAM and between -22.1 and -33 as C3 (Silvera *et al.*, 2010).

The isotope dilution was done to determinate the percentage of carbon ($\%x_{dF}$) found in the orchid comes from the OrMF and how much comes from the photosynthetic orchid activity by equation 1 adapted from Gebauer & Meyer (2013).

$$\%x_{dF} = \frac{\delta x_{MT} - \delta x_R}{\delta x_{OrMF} - \delta x_R} \qquad Eqn\,1$$

This was calculated using a linear two-source isotopic mixing model based on individual δ-values of partial mixotrophic orchid (δx_{MT}), mean δ-values of reference, non-orchid plants of each height (δx_R) and the OrMF (δx_{OrMF}). The data were analyzed only for mixotrophic orchid.

To determinate the correlation between the $\delta^{13}C$ signature of orchids and non-orchids leaves and the OrMF by pelotons and height in the different samples, it was used the regression analysis using "R" software, v. 3.2.2 (R Core Team, 2017). This $\delta^{13}C$ signature were also used for analysis in artificial neural network (ANN), based in artificial intelligence for determination the $\delta^{13}C$ signature how closer were theses samples, using Gephi software, v. 0.9.1 (Bastian *et al.*, 2009), where the distance among the dots represented the correlation distance.

Results

We observe orchid growing over the all phorophyte trunk, from 2 to 19 m, which contradicts the theory that low luminosity near the ground limits the growth of orchids (Johansson, 1974). All orchids presented OrMF, which was confirmed by the pelotons present in cortex cells, even as in the orchid that was not possible to collected pelotons for C analysis.

Orchids exhibit $\delta^{13}C$ ranging from -15.13 to -31.57, with average of -26.52, where 11.11% orchids presented the CAM metabolism. For non-orchids species, $\delta^{13}C$ ranged from -14.11 to -32.89, average of -26.69, where 9.43% presented CAM metabolisms. The OrMF showed $\delta^{13}C$ ranging from -12.90 to -31.28, average -25.43 (Table 1, figure 2).

No difference ($p > 0.05$) for ^{13}C signatures among the samples (cv = 14.6%). By PCA, the samples distribution explained 40.78%, but it was random, and it was not possible to determinate a correlation among samples. MH orchids (63.64%) showed means values of ^{13}C greater than autotrophic (22.72%) and mixotrophic ones (13.64%), with -23.554, -27.575 and – 28.606 respectively.

Among mixotrophic orchids (Table 2), Orchid sp.3 received 1.19x more C from OrMF than photosynthesis activity ($\%x_{dF}$ = - 1.19), while for *Octomeria*, 97% of C is from photosynthesis ($\%x_{dF}$ = 0.097) and for *Maxillaria*, the photosynthesis activity provides 1.47x more C to orchid than OrMF ($\%x_{dF =}$ 1.47).

No direct correlation was observed between height and ^{13}C signatures in orchid, non-orchid species and OrMF, the range of R^2 were 0.04, 0.17 and 0.03, respectively (figure 2). The values of ^{13}C signature of OrMF are closer among the samples in higher position, like 11m, 15m, 16m and 19m.

The values of $\delta^{13}C$ signature of OrMF are more correlated among highest samples, while for orchid no clear the correlation was observed and non-orchid signature are more correlated among plants occurring in lowest height (figure 3).

Table 1: δ13C signatures in the leaves of orchids, non-orchids plants and pelotons of orchid's mycorrhizal fungi (OrMF) and their metabolisms (Met) according to Silvera (2010), growing in different height (m) of the phorophyte.

Orchids				Non-orchids plants				OrMF			
Species	Height	δ^{13}C	Met	Species	Height	δ^{13}C	Met	Species	Height	δ^{13}C	Met
Orchid 1	2	-29,167	C3	Bromeliaceae	2	-31,043	C3	Orchid 1	2	-29,103	C3
Orchid 2	2	-15,133	CAM	Bromeliaceae 1	2	-28,812	C3	Orchid 2	2	-12,907	CAM
Orchid 3	2	-31,573	C3	Fern 1	2	-29,608	C3	Orchid 3	2	-31,285	C3
Octomeria	3.5	-27,635	C3	Fern 2	2	-27,446	C3	*Octomeria*	3.5	-26,119	C3
Pleurothallis	6	-29,879	C3	Bambou	2	-32,892	C3	*Pleurothallis*	6	-30,110	C3
Pleurothallis	7	-27,061	C3	Clusiaceae	2	-29,612	C3	*Pleurothallis*	7	-24,558	C3
Pleurothallis 1	8	-27,587	C3	Araceae	6	-27,618	C3	*Pleurothallis 2*	8	-27,476	C3
Pleurothallis 2	8	-27,831	C3	Pteridofita	6	-28,902	C3	*Oncidium* 2	8	-14,297	CAM
Oncidium 2	8	-17,017	CAM	Bromeliaceae	7	-25,648	C3	*Maxillaria 1*	9	-27,213	C3
Pleurothallis 3	9	-25,461	C3	Begoniaceae	7	-25,963	C3	Orchid 4	10	-26,804	C3
Maxillaria 1	9	-26,611	C3	Dicot 1	8	-31,123	C3	*Pleurothallis*	*10*	-27,276	C3
Tilandsia	10	-26,510	C3	Myrtaceae 1	8	-31,901	C3	Orchid 5	11	-25,028	C3
Orchid 4	10	-26,674	C3	Dicot 2	9	-31,416	C3	Orchid 6	11	-26,058	C3
Pleurothallis	10	-27,319	C3	Bromeliaceae 2	9	-26,805	C3	Orchid 7	11	-27,251	C3
Orchid 5	11	-26,478	C3	Fern 3	10	-26,256	C3	*Pleurothallis*	11	-26,492	C3
Orchid 6	11	-27,307	C3	Dicot 3	10	-27,456	C3	*Pleurothallis*	11	-28,389	C3
Orchid 7	11	-29,723	C3	Bromeliaceae 3	10	-26,495	C3	*Octomeria*	11	-27,383	C3
Pleurothallis	11	-28,376	C3	Bromeliaceae	10	-26,430	C3	*Pleurothallis*	12	-26,393	C3
Pleurothallis	11	-27,948	C3	Bromeliaceae	10	-16,150	CAM	*Octomeria*	12	-26,754	C3
Octomeria	11	-27,921	C3	Bromeliaceae 4	11	-25,816	C3	Orchid 8	15	-23,994	C3
Pleurothallis	12	-27,759	C3	Bromeliaceae	11	-17,103	CAM	*Octomeria*	15	-24,479	C3
Octomeria	12	-28,200	C3	Bromeliaceae	11	-26,487	C3	*Pleurothallis*	15	-26,847	C3
Orchid 8	15	-26,208	C3	Bromeliaceae	11	-25,993	C3	Maxillaria cf acicularis	15	-27,306	C3
Octomeria	15	-26,818	C3	Bromeliaceae	11	-15,514	CAM	*Pleurothallis*	15	-27,873	C3
Pleurothallis	15	-26,996	C3	Pteridofita	12	-27,551	C3	*Pleurothallis*	*15*	-27,299	C3

Table 1 cont.

Maxillaria cf acicularis	15	-27,401	C3	Bromeliaceae	12	-26,019	C3	Orchid 9	16	-24,789	C3
Pleurothallis	15	-26,122	C3	Bromeliaceae	12	-27,673	C3	*Cattleya jongheana*	*16*	-13,068	CAM
Pleurothallis	15	-27,323	C3	Dicot 4	15	-27,671	C3	*Bulbophyllum 1*	*16*	-25,337	C3
Orchid 9	16	-25,694	C3	Dicot 5	15	-29,357	C3	*Oncidium 3*	16	-25,871	C3
Cattleya jongheana	16	-15,755	CAM	Pteridofita	15	-28,513	C3	*Pleurothallis 4*	16	-23,421	C3
Bulbophyllum 1	16	-26,387	C3	Bromeliaceae	15	-25,707	C3	*Isochilus linearis*	*16*	-26,590	C3
Oncidium 3	16	-28,229	C3	Not identified	15	-28,384	C3	*Bulbophyllum*	*16*	-27,776	C3
Isochilus linearis	16	-26,732	C3	Bromeliaceae	15	-14,412	CAM	Orchid 10	18	-16,857	CAM
Polystachya concreta	16	-26,894	C3	Fern 4	16	-27,339	C3	*Oncidium cf hookeri*	*18*	-25,767	C3
Bulbophyllum	16	-27,885	C3	Dicot 6	16	-28,177	C3	Orchidaceae	18	-26,015	C3
Oncidium sp1	17	-27,983	C3	Dicot 7	16	-27,765	C3	*Bifrenaria*	*18*	*-26,925*	C3
Orchid 10	18	-18,841	CAM	Dicot 8	16	-28,447	C3	*Bifrenaria*	*19*	-27,398	C3
Oncidium cf. *hookeri*	18	-27,290	C3	Bromeliaceae	16	-27,161	C3	*Epidendrum*	*19*	-24,975	C3
Pleurothallis	18	-26,180	C3	Not identified	16	-28,012	C3	Orchidaceae	19	-27,125	C3
Orchidaceae	18	-26,963	C3	Pteridofita	16	-27,346	C3	*Maxillaria*	19	-26,433	C3
Bifrenaria	18	-28,986	C3	Bromeliaceae	17	-26,669	C3				
Bifrenaria	19	-29,287	C3	Pteridofita	17	-26,440	C3				
Epidendrum	19	-25,140	C3	Not identified	17	-28,321	C3				
Orchidaceae	19	-26,960	C3	Fern 5	18	-24,903	C3				
Maxillaria	19	-28,305	C3	Dicot 9	18	-28,007	C3				
				Fern 6	18	-29,171	C3				
				Pteridofita	18	-28,456	C3				
				Not identified	18	-27,381	C3				
				Not identified	19	-25,710	C3				
				Bromeliaceae	19	-23,817	C3				
				Suculenta	19	-29,267	C3				
				Pteridofita	19	-29,242	C3				
				Bromeliaceae	19	-14,457	CAM				
				Bromeliaceae	3.5	-27,571	C3				

Table 2: Groups of orchids and non-orchids species and orchid mycorrhizal fungi (OrMF) related to their metabolism, mycoheterotrophic (MH), autotrophic (AT), mixotrophic (MT) based on the strategy of carbon acquisition.

Group	Sample	Specie	Height (m)	δ13C/ 12C
MH	Orchid	Orchid sp. 2	2	-15,133
	OrMF	Orchid sp. 2	2	-12,907
	Orchid	*Pleurothallis*	6	-29,879
	OrMF	*Pleurothallis*	6	-30,110
	Orchid	*Oncidium*	8	-17,017
	OrMF	*Oncidium*	8	-14,297
	OrMF	*Pleurothallis*	8	-27,476
	Orchid	*Pleurothallis*	8	-27,831
	Orchid	Orchid sp. 4	10	-26,674
	OrMF	Orchid sp. 4	10	-26,804
	Orchid	*Pleurothallis*	10	-27,319
	OrMF	*Pleurothallis*	10	-27,276
	Orchid	*Pleurothallis*	11	-28,376
	OrMF	*Pleurothallis*	11	-28,389
	Orchid	*Pleurothallis*	15	-27,323
	OrMF	*Pleurothallis*	15	-27,299
	OrMF	*Pleurothallis*	15	-26,847
	Orchid	*Pleurothallis*	15	-26,996
	OrMF	*Bulbophyllum*	16	-27,776
	Orchid	*Bulbophyllum*	16	-27,885
	Orchid	*Isochilus*	16	-26,732
	OrMF	*Isochilus*	16	-26,590
	Orchid	Orchid sp. 10	18	-18,841
	OrMF	Orchid sp. 10	18	-16,857
	Orchid	Orchidaceae	18	-26,963
	OrMF	*Bifrenaria*	18	-26,925
	Orchid	Orchidaceae	19	-26,960
	OrMF	Orchidaceae	19	-27,125

Group	Sample	Specie	Height (m)	δ13C/12C
AT	Orchid	Orchid	11	-26,478
	Non-orchid	Bromeliaceae	11	-26,487
	Non-orchid	Pteridofita	12	-27,551
	Orchid	*Pleurothallis*	12	-27,759
	Non-orchid	Bromeliaceae	12	-27,673
	Orchid	*Pleurothallis*	15	-26,122
	Non-orchid	Bromeliaceae	15	-25,707
	Non-orchid	Dicot	16	-28,177
	Orchid	*Oncidium*	16	-28,229
	Orchid	*Bifrenaria*	19	-29,287
	Non-orchid	suculenta	19	-29,267
	Non-orchid	Pteridofita	19	-29,242
MT	Orchid	*Orchid* sp. 3	2	-31,573
	Non-orchid	bromeliaceae	2	-31,043
	OMF	Orchid sp. 3	2	-31,285
	Orchid	*Octomeria*	3.5	-27,635
	Non-orchid	Bromeliaceae	3.5	-27,571
	OrMF	Octomeria	3.5	-26,119
	Non-orchid	Bromeliaceae	9	-26,805
	Orchid	*Maxillaria*	9	-26,611
	OrMF	*Maxillaria*	9	-27,213

Group	Origin of orchid carbon
MH	From OrMF
AT	From photosynthesis
MT	From photosynthesis and/or OrMF

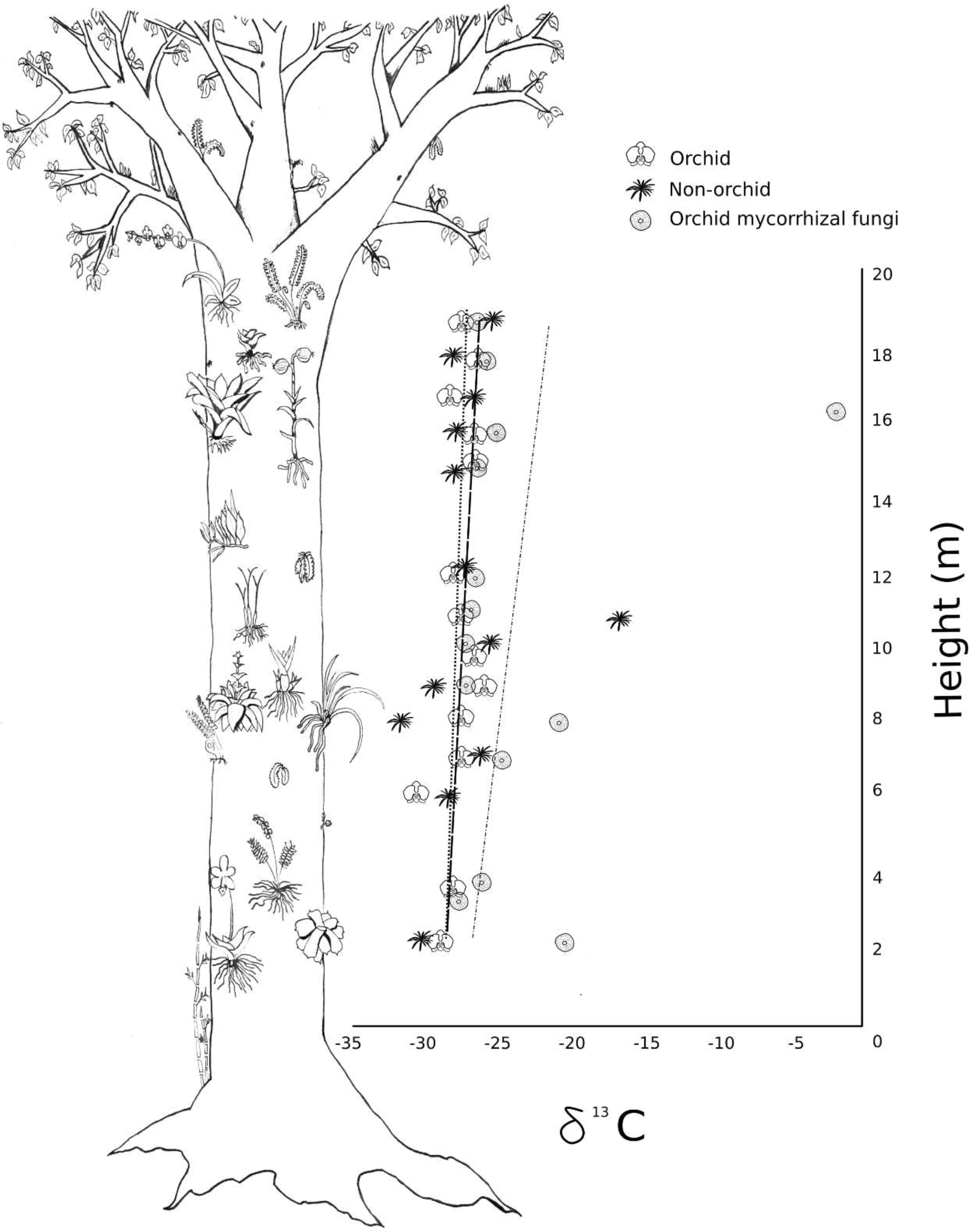

Figure 2: Draw of collected tree designer, regression analysis and ^{13}C signature in orchids, non-orchids plants and for orchid's mycorrhizal fungi (pelotons). Equation of: Orchid, $y = 0.2876x - 27.095$, R^2 0.04; Non-orchid, $y = 0.0792x - 28.418$, R^2 0.17 and Orchid mycorrhizal fungi, $y = 0.1128x - 28.317$, R^2 0.03.

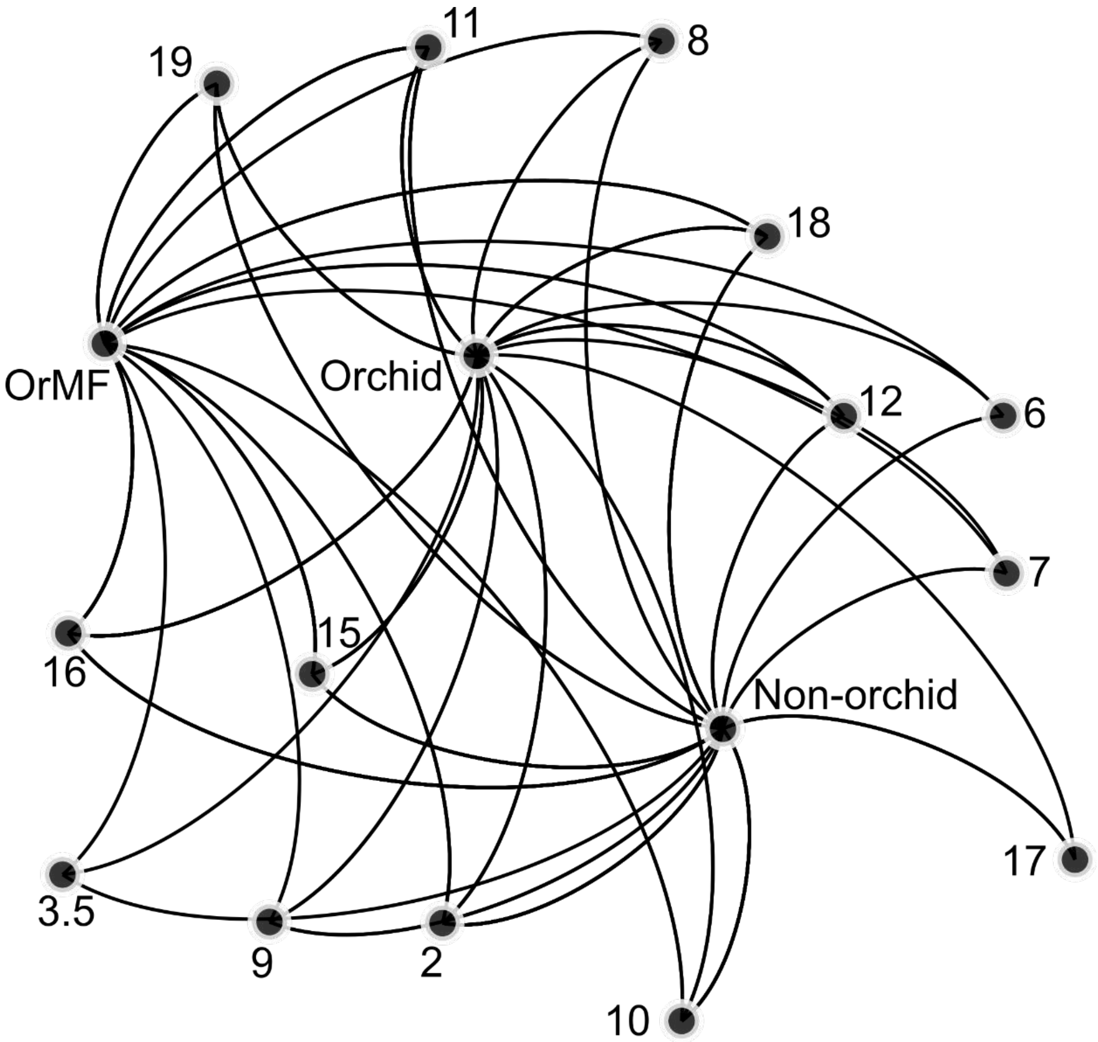

Figure 3: Artificial neural network for δ^{13}C signature for orchids and non-orchids species and orchid's mycorrhizal fungi (OrMF) growing in different height (represented by the number, in meter) of phorophyte (dots). The values of δ^{13}C signature of OrMF are more correlated among highest samples.

Discussion

This is the first study in carbon flux using ^{13}C signature in epiphytic tropical orchids and the results of this study can contribute for better understanding about the ecological mycorrhizal dynamics in epiphytic tropical orchids, as the first study of isotopes measures provided greater capacity for elucidation of different ecological mechanisms (Craig, 1957). Studies that began with plants are now applied to their interaction with the environment, including other organisms (O'Leary, 1988). The use of isotopes approach can provide the understand of many fungi ecological questions, and the interaction with the plants (Hobbie *et al.*, 2001).

Even adult individuals of chlorophyllic orchids are photosynthetically active, we verified the importance of mycorrhization by the presence of pelotons in all orchids analyzed. It is confirm the importance of interaction between OrMF and orchid (Martos *et al.*, 2012). Here we focus in the C flux, however another nutrients can also be changed between OrMF and orchid, like vitamins (Selosse, 2014). So the association OrMF - orchid is more complex than only C flow, involving many others nutritional aspects that have to be investigated.

The Moraceae and Myrtaceae are tropical families of tree, with several representatives in subtropical and northern temperate regions. Some species of Moracae can product latex, and more important is one woody family plant (Denardi *et al.*, 2005; Ribeiro, 2011), as well as the Myrtaceae, the most common family in Atlantic Forest, both can able support Bromeliaceae, Orchidaceae and others epiphytic species (Guilherme *et al.*, 2004). Moraceae and Myrtaceae has woody trunk, and their bark are substrate for fungi saprophytic activity like OrMF, indeed it is a carbon source for OrMF as well as indirectly for the orchid. All orchids samples were mycorrhizal, including the samples which we did not get pelotons, it is confirm the importance of OrMF for orchids, seedling or at adulthood (Voyron *et al.*, 2017).

Some orchids showed very small root, with few cortex cell, therefore for the low fungi quantity were impossibility the pickup peloton to ^{13}C analysis with tools used. Methods to extract pelotons from root can be development to help OrMF studies in the future, as by centrifugation process.

We found the percentage (11.11%) of CAM in orchid's species, similar value observed in Costa Rica (9.5%) (Silvera *et al.*, 2010). However for Bromeliaceae, the percentage was four fold lower than observed in Costa Rica (Silvera *et al.*, 2010). The low prevalence of orchid and bromeliaceae with CAM compared with C3 metabolism showed the impact of environment features in PESB, like the frequent fog, and the presence of a small river of sampling area, not causing water stress. Although CAM is more common in epiphytic orchids (Roberts *et al.*, 2008), we found no predominance of this metabolism in tropical epiphytic orchids from PESB. Furthermore the morphological adaptations presents in same species, like pseudobulbs and succulent leaves (Zhang *et al.*, 2016) and lower stomatal density (Eburneo *et al.*, 2017) can promote survival under stress conditions.

The average of ^{13}C signature from OrMF was closer to the values already observed in fruit body of ectomycorrhizal fungi (Hobbie *et al.*, 2001), that can indicate a standard of carbon ratio for mycorrhizal fungi strategy. The measurement of carbon isotopes in OrMF in the initial stages of the life cycle of orchids may present different values of those sampled in adult individuals, depending on the ecology of the interaction, where the fungus supplies the carbon to the plant, since saprophytic fungi present average values of -22.77, lower than mycorrhizal ones (Hobbie *et al.*, 2001).

A study with *Quercus kelloggii* in California/USA revels that in high elevation, the stomatic density is higher than in low altimetry, with the variation in 3.5% per 100 m altitude gain (Kouwenberg, 2007). Although the abundance and stomatic activity are able to change the ^{13}C signature, we were not able to detect significant differences in the orchids analyzed because the difference among the samples were only 17 m. Studies with more samples, can be able to detect ^{13}C signature with difference between the orchid and non-orchid leaves, and OrMF. Other factors as luminosity measurement, morphological, a higher gradient of height and temporal sampling can help to can provide the greater understanding the ecological approach, once the fungi profile change along to cycling of life (Hynson *et al.*, 2013).

Strong evidence of epiphytic orchids as MH was observed, with more prevalence of MH than autotrophic and mixotrophic ones (table 2-A), showing the dependence of organic carbon in epiphytic tropical orchids, as observed for other orchids ((Bahram *et al.*, 2015). The MH are common, but the real mechanisms of carbon flux, and what molecules are exchanged is unclean (Jacquemyn *et al.*, 2017).

Since organic matter have more ^{13}C than in the atmosphere, the MH orchids (table 2-A) were more enriched with ^{13}C than orchids classified as fully autotrophic and mixotrophic ones (Gebauer *et al.*, 2016). The orchid can present ^{13}C signature similar to non-orchid plant, since it can form "fungus bridge" promoting link between these plants (Roberts *et al.*, 2008).

Although the presence of mixotrophic orchid was low, they has been showed very import to C cycle, since the mixotrophic orchid are able to use C from OrMF and atmosphere (Jacquemyn *et al.*, 2017).

ANN confirm that OrMF receive carbon from photosynthesis activity of adult epiphytic tropical orchids (figure 3). Considering that ^{13}C signature of bark is not variable by the height, this difference on carbon may be correlated by the interaction between OrMF and orchid. We trust which more sampling, with environment data, like radiation, humidity, photosynthesis ratio and identification of OrMF can provides more information and help for the better understanding of OrMF dynamics with the orchid and environment.

Conclusion

- The secondary Atlantic Forest provide condition for establishment of epiphytic orchid without temperature and hydric stress, with the prevalence of C3 orchids.
- Tropical epiphytes orchids maintain the association with mycorrhizal fungi, and mycorrhizal fungi receive more carbon from the orchids growing in the top of phorophyte than those in the bottom.

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Author contributions

CA Vieira developed the research and wrote the paper; CA Vieira, MF Bocayuva, TRG Veloso, ES Cruz, EF Freitas, IR Silva, JJ Milagres and MA Selosse collected and laboratory analyses; MCM Kasuya and MA Selosse collaborated to the research design and proposal and reviewed the entire manuscript.

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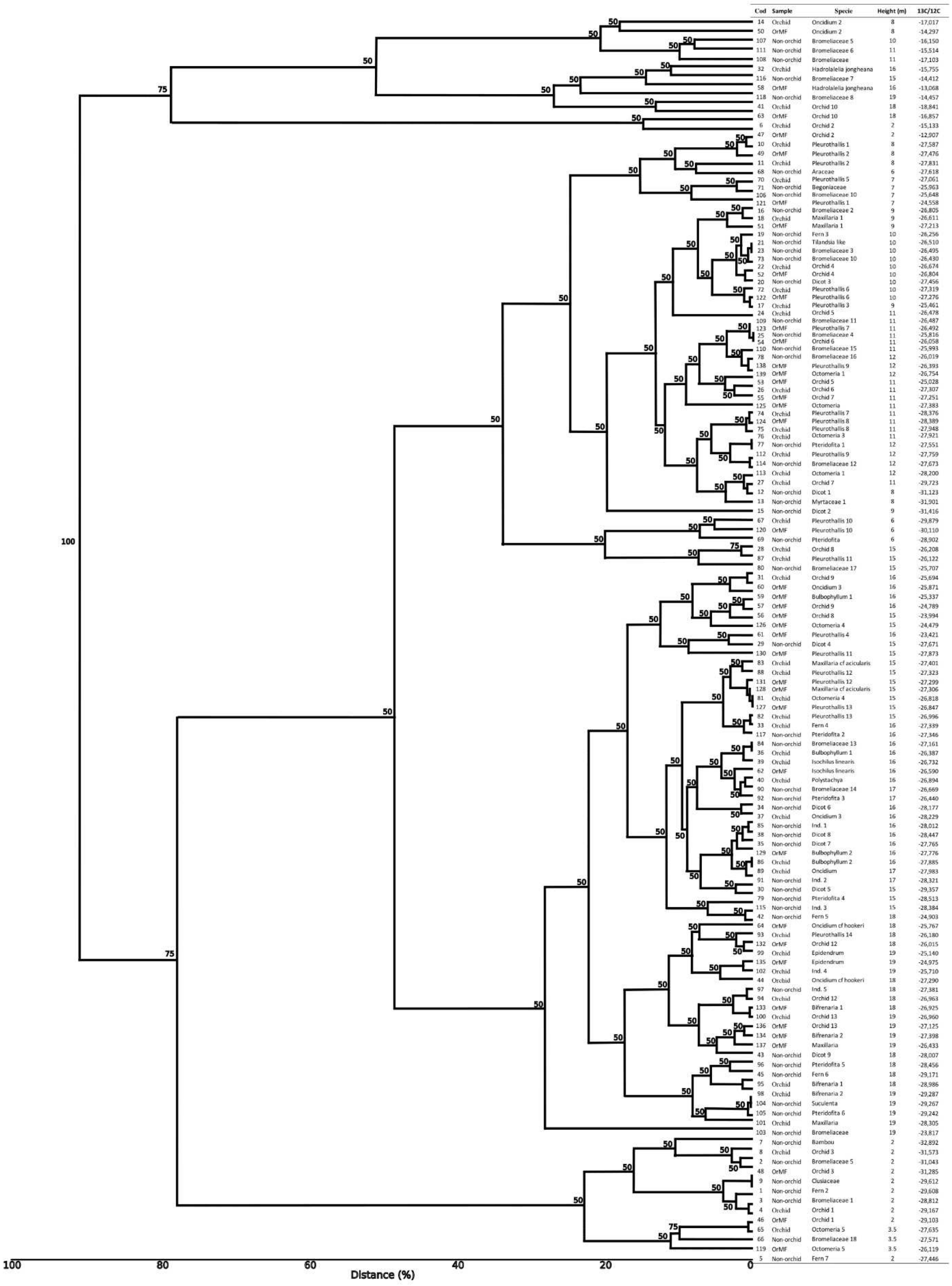
Supplementary material



Cod	Sample	Specie	Height (m)	13C/12C
14	Orchid	Oncidium 2	8	-17,017
50	OrMF	Oncidium 2	8	-14,297
107	Non-orchid	Bromeliaceae 5	10	-16,150
111	Non-orchid	Bromeliaceae 6	11	-15,514
108	Non-orchid	Bromeliaceae	11	-17,103
32	Orchid	Hadrolalelia jongheana	16	-15,755
116	Non-orchid	Bromeliaceae 7	15	-14,412
58	OrMF	Hadrolalelia jongheana	16	-13,068
118	Non-orchid	Bromeliaceae 8	19	-14,457
41	Orchid	Orchid 10	18	-18,841
63	OrMF	Orchid 10	18	-16,857
6	Orchid	Orchid 2	2	-15,133
47	OrMF	Orchid 2	2	-12,907
10	Orchid	Pleurothallis 1	8	-27,587
49	OrMF	Pleurothallis 2	8	-27,476
11	Orchid	Pleurothallis 2	8	-27,831
68	Non-orchid	Araceae	6	-27,618
70	Orchid	Pleurothallis 5	7	-27,061
71	Non-orchid	Begoniaceae	7	-25,963
106	Non-orchid	Bromeliaceae 10	7	-25,648
121	OrMF	Pleurothallis 1	7	-24,558
16	Non-orchid	Bromeliaceae 2	9	-26,805
18	Orchid	Maxillaria 1	9	-26,611
51	OrMF	Maxillaria 1	9	-27,213
19	Non-orchid	Fern 3	10	-26,256
21	Non-orchid	Tilandsia like	10	-26,510
23	Non-orchid	Bromeliaceae 3	10	-26,495
73	Non-orchid	Bromeliaceae 10	10	-26,430
22	Orchid	Orchid 4	10	-26,674
52	OrMF	Orchid 4	10	-26,804
20	Non-orchid	Dicot 3	10	-27,456
72	Orchid	Pleurothallis 6	10	-27,319
122	OrMF	Pleurothallis 6	10	-27,276
17	Orchid	Pleurothallis 3	9	-25,461
24	Orchid	Orchid 5	11	-26,478
109	Non-orchid	Bromeliaceae 11	11	-26,487
123	OrMF	Pleurothallis 7	11	-26,492
25	Non-orchid	Bromeliaceae 4	11	-25,816
54	OrMF	Orchid 6	11	-26,058
110	Non-orchid	Bromeliaceae 15	11	-25,993
78	Non-orchid	Bromeliaceae 16	12	-26,019
138	OrMF	Pleurothallis 9	12	-26,393
139	OrMF	Octomeria 1	12	-26,754
53	OrMF	Orchid 5	11	-25,028
26	Orchid	Orchid 6	11	-27,307
55	OrMF	Orchid 7	11	-27,251
125	OrMF	Octomeria	11	-27,383
74	Orchid	Pleurothallis 7	11	-28,376
124	OrMF	Pleurothallis 8	11	-28,389
75	Orchid	Pleurothallis 8	11	-27,948
76	Orchid	Octomeria 3	11	-27,921
77	Non-orchid	Pteridofita 1	12	-27,551
112	Orchid	Pleurothallis 9	12	-27,759
114	Non-orchid	Bromeliaceae 12	12	-27,673
113	Orchid	Octomeria 1	12	-28,200
27	Orchid	Orchid 7	11	-29,723
12	Non-orchid	Dicot 1	8	-31,123
13	Non-orchid	Myrtaceae 1	8	-31,901
15	Non-orchid	Dicot 2	9	-31,416
67	Orchid	Pleurothallis 10	6	-29,879
120	OrMF	Pleurothallis 10	6	-30,110
69	Non-orchid	Pteridofita	6	-28,902
28	Orchid	Orchid 8	15	-26,208
87	Orchid	Pleurothallis 11	15	-26,122
80	Non-orchid	Bromeliaceae 17	15	-25,707
31	Orchid	Orchid 9	16	-25,694
60	OrMF	Oncidium 3	16	-25,871
59	OrMF	Bulbophyllum 1	16	-25,337
57	OrMF	Orchid 9	16	-24,789
56	OrMF	Orchid 8	15	-23,994
126	OrMF	Octomeria 4	15	-24,479
61	OrMF	Pleurothallis 4	16	-23,421
29	Non-orchid	Dicot 4	15	-27,671
130	OrMF	Pleurothallis 11	15	-27,873
83	Orchid	Maxillaria cf acicularis	15	-27,401
88	Orchid	Pleurothallis 12	15	-27,323
131	OrMF	Pleurothallis 12	15	-27,299
128	OrMF	Maxillaria cf acicularis	15	-27,306
81	Orchid	Octomeria 4	15	-26,818
127	OrMF	Pleurothallis 13	15	-26,847
82	Orchid	Pleurothallis 13	15	-26,996
33	Orchid	Fern 4	16	-27,339
117	Non-orchid	Pteridofita 2	16	-27,346
84	Non-orchid	Bromeliaceae 13	16	-27,161
36	Orchid	Bulbophyllum 1	16	-26,387
39	Orchid	Isochilus linearis	16	-26,732
62	OrMF	Isochilus linearis	16	-26,590
40	Orchid	Polystachya	16	-26,894
90	Non-orchid	Bromeliaceae 14	17	-26,669
92	Non-orchid	Pteridofita 3	17	-26,440
34	Non-orchid	Dicot 6	16	-28,177
37	Orchid	Oncidium 3	16	-28,229
85	Non-orchid	Ind. 1	16	-28,012
38	Non-orchid	Dicot 8	16	-28,447
35	Non-orchid	Dicot 7	16	-27,765
129	OrMF	Bulbophyllum 2	16	-27,776
86	Orchid	Bulbophyllum 2	16	-27,885
89	Orchid	Oncidium	17	-27,983
91	Non-orchid	Ind. 2	17	-28,321
30	Non-orchid	Dicot 5	15	-29,357
79	Non-orchid	Pteridofita 4	15	-28,513
115	Non-orchid	Ind. 3	15	-28,384
42	Non-orchid	Fern 5	18	-24,903
64	OrMF	Oncidium cf hookeri	18	-25,767
93	Orchid	Pleurothallis 14	18	-26,180
132	OrMF	Orchid 12	18	-26,015
99	Orchid	Epidendrum	19	-25,140
135	OrMF	Epidendrum	19	-24,975
102	Orchid	Ind. 4	19	-25,710
44	Orchid	Oncidium cf hookeri	18	-27,290
97	Non-orchid	Ind. 5	18	-27,381
94	Orchid	Orchid 12	18	-26,963
133	OrMF	Bifrenaria 1	18	-26,925
100	Orchid	Orchid 13	19	-26,960
136	OrMF	Orchid 13	19	-27,125
134	OrMF	Bifrenaria 2	19	-27,398
137	OrMF	Maxillaria	19	-26,433
43	Non-orchid	Dicot 9	18	-28,007
96	Non-orchid	Pteridofita 5	18	-28,456
45	Non-orchid	Fern 6	18	-29,171
95	Orchid	Bifrenaria 1	18	-28,986
98	Orchid	Bifrenaria 2	19	-29,287
104	Non-orchid	Suculenta	19	-29,267
105	Non-orchid	Pteridofita 6	19	-29,242
101	Orchid	Maxillaria	19	-28,305
103	Non-orchid	Bromeliaceae	19	-23,817
7	Non-orchid	Bambou	2	-32,892
8	Orchid	Orchid 3	2	-31,573
2	Non-orchid	Bromeliaceae 5	2	-31,043
48	OrMF	Orchid 3	2	-31,285
9	Non-orchid	Clusiaceae	2	-29,612
1	Non-orchid	Fern 2	2	-29,608
3	Non-orchid	Bromeliaceae 1	2	-28,812
4	Orchid	Orchid 1	2	-29,167
46	OrMF	Orchid 1	2	-29,103
65	Orchid	Octomeria 5	3.5	-27,635
66	Non-orchid	Bromeliaceae 18	3.5	-27,571
119	OrMF	Octomeria 5	3.5	-26,119
5	Non-orchid	Fern 7	2	-27,446

Figure S1: Cluster analysis from $\delta^{13}C$ signatures and higher Myrtaceae and Moraceae phorophyties. OrMF, orchid mycorrhizal fungi. Numbers in the arms indicate the probability of distance

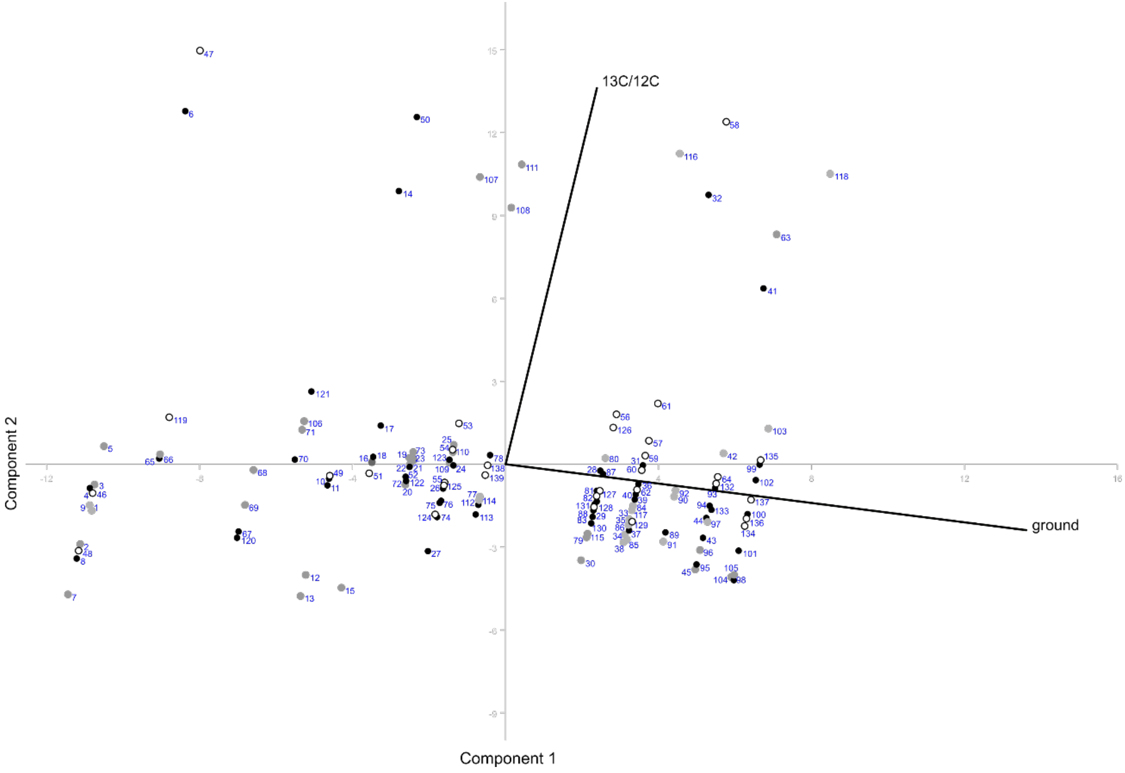


Figure S2. Principal component analysis of the δ13C signature. The samples were distributed without standard, thought the ground and δ^{13}C ratio was relevance, not able to explain the relationship between ground and 13C signatures. Grey circle: non-orchids; Black circle: orchid and open circle: OrMF – orchid mycorrhizal fungi. Component 1 explained for 14.4502% and 2 for 26.3309%, total 40.78%. For the code number see the figure S1.