TOMÁS GOMES REIS VELOSO

FUNGAL COMMUNITY PROFILE OF *Zygopetalum maxillare* AND *Zygopetalum mackayi* (ORCHIDACEAE)

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

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APROVADA: 22 de julho de 2016.

_____
Denise Mara Soares Bazzolli
(Coorientadora)

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Melissa Faust Bocayuva Cunha
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Mateus Ferreira Santana

_____
Maria Catarina Megumi Kasuya
(Orientadora)

A Deus,

Aos meus pais,

Ao meu irmão,

Dedico.

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BIOGRAFIA

Tomás é filho de Maria das Graças Gomes Veloso e Antônio Inácio Reis Veloso. Nasceu em Viçosa, Minas Gerais. Estudou na escola Estadual Raul de Leoni durante 11 anos, formando-se no ensino médio em 2008.

De agosto de 2006 a julho 2008, ainda como estudante secundário, foi Bolsista de Iniciação Científica Júnior sob a orientação da professora Maria Catarina Megumi Kasuya. Neste período teve seu primeiro contato com a pesquisa científica, onde trabalhou com micorrizas em espécies de orquídeas ameaçadas de extinção.

Em março de 2009 iniciou seus estudos na Universidade Federal de Viçosa no curso de Engenharia Agronômica. Neste mesmo ano retornou ao Laboratório de Associações Micorrízicas, onde continuou a realizar pesquisas em micorrizas de orquídeas.

Formou-se em julho de 2014 e em agosto deste mesmo ano iniciou o mestrado no Programa de Pós-Graduação em Microbiologia Agrícola.

SUMÁRIO

Abstract

VELOSO, Tomás Gomes Reis, MS, Universidade Federal de Viçosa, july, 2016. **Fungal Community profile of *Zygopetalum maxillare* and *Zygopetalum mackayi* (ORCHIDACEAE).** Advisor: Maria Catarina Megumi Kasuya. Co-advisors: Melissa Faust Bocayuva and Denise Mara Soares Bazzolli.

The orchids seeds are minute and easily dispersed by the wind. However, due to this reduced size there is no enough reserve tissue to allow seed germination, so that the nutrients uptake in the in the first steps of life cycle is done by the association to a mycorrhizal fungus. Once distinct orchids species associate can associate with distinct fungi is important to know for each orchid which are the fungi associate, because the conservation of threatened orchids requires the conservation of the fungi. Here, culture-depedent and independent on approaches were used for studying the fungal profile community of two orchid species, *Zygopetalum maxillare*, an epiphyte, and *Zygopetalum mackayi*, a terrestrial. The results of culture-independent methods showed fungi of Ascomycota as the majority present. Both *Zygopetalum* species associate with Sebacinaceae and Ceratobasidiaceae, however, while species of Ceratobasidiaceae are shared by both orchids, Sebacinaceae are not. These results were corroborated by the results of culture-dependent approaches, although no fungi of Ceratobasidiaceae could be isolated. The isolates obtained by this study are potential to be used in seed germination and seedling development of *Z. maxillare* and *Z. mackayi* and for future use in reintroduction program.

Resumo

VELOSO, Tomás Gomes Reis, M.Sc., Universidade Federal de Viçosa, julho de 2016. **Perfil de comunidades fúngicas de *Zygopetalum maxillare* e *Zygopetalum mackayi* (Orchidaceae).** Orientadora: Maria Catarina Megumi Kasuya. Coorientadoras: Melissa Faust Bocayuva e Denise Mara Soares Bazzolli.

As orquídeas produzem sementes minúsculas que são facilmente dispersas pelo vento. Entretanto, devido ao tamanho reduzido, não apresentam endosperma suficiente para que a germinação ocorra, de modo que o suprimento de carbono e energia nos estágios iniciais de desenvolvimento é proporcinado pela associação a fungos micorrízicos. Uma vez que diferentes espécies de orquídeas podem apresentar diferentes espécies de fungos micorrízicos associados é importante conhecer a comunidade fúngica para cada orquídea uma vez que a conservação de muitas espécies de orquídeas ameaçadas de extinção provavelmente requer a presença destes fungos. Este trabalho teve como objetivos (1) avaliar o perfil de fungos associados a duas espécies de orquídeas, *Zygopetalum maxillare*, uma epífita, e *Zygopetalum mackayi*, uma terrestre, por meio de métodos dependentes e independentes de cultivo e (2) otimizar os processos de conservação e de extração de DNA de amostras radiculares colonizadas por fungos micorrízicos. O melhor método para conservar o DNA de amostras radiculares colonizadas foi o armazenamento em tampão de lise contendo Dodecil sulfato de sódio (SDS) e a extração de DNA foi otimizado através utilização de uma frequência angular de 6500 rpm durante 48 s no homogeneizador Precellys® 24. Os resultados dos métodos independentes de cultivo mostram que há uma predominância de fungos do filo Ascomycota e que ambas espécies de orquídeas se associam com espécies fúngicas das famílias Sebacinaceae e Ceratobasidiaceae. Enquanto que algumas espécies de fungos da família Ceratobasidiaceae são compartilhados por ambas as espécies de orquídeas, as espécies de Sebacinaceae não o são. Estes resultados foram corroborados com os obtidos pelas técnicas dependentes de cultivo, onde a maioria dos fungos isolados foi de Ascomycota. Entretanto, não foi possível isolar nenhum fungo da família Ceratobasidiaceae. Os isolados fúngicos obtidos neste trabalho apresentam grande potencial para serem utilizados na germinação de sementes de *Z. mackayi* e *Z. maxillare* e sua utilização em futuros programas de reintrodução.

INTRODUÇÃO GERAL

As orquídeas fazem parte da família Orchidaceae, a mais derivada e diversificada entre as monocotiledôneas. Estima-se que existam mais de 25.000 espécies distribuídas em 8 subfamílias e 880 gêneros (Dressler 2005). A distribuição das espécies é cosmopolita, ocorrendo em todos continentes, exceto Antártica (Cribb et al. 2003). O Brasil abriga cerca de 2553 espécies distribuídas em 238 gêneros, sendo que mais da metade (64%) são espécies endêmicas (Barros et al. 2016). Ocorrem em todos os 5 biomas, estando muitas em risco de extinção (Martinelli and Moraes 2013).

Orquidáceas são plantas fascinantes devido a beleza exuberante de suas flores. Desta forma, muitas espécies têm alto valor econômico, sendo exploradas comercialmente num mercado que movimenta dezenas de bilhões de dólares anualmente, que inclui não apenas a produção de mudas, mas também de derivados, como perfumes e baunilha (Medina et al. 2009). Infelizmente, justamente devido à beleza de suas flores, muitas espécies vêm sofrendo coleta indiscriminada que, somada à destruição do habitat natural por atividades antrópicas, têm aumentado o risco de extinção de muitas espécies (Martinelli and Moraes 2013).

As orquídeas produzem milhares de sementes leves e diminutas em frutos capsulares. Estas duas características fazem com que a dispersão ocorra eficientemente pelo vento. Entretanto, estas mesmas características impedem que a semente armazene reservas de carbono suficientes para o processo de germinativo. Desta forma, o suprimento de carbono é feito através da associação com um fungo micorrízico compatível. Nesta associação a obtenção dos nutrientes é feita pela digestão das hifas fúngicas através de enzimas da semente (Kuga et al. 2014).

Os fungos comumente relatados fazendo esta associação com orquídeas são basidiomicetos compreendendo espécies dos gêneros *Tulasnella*, *Sebacina*, *Ceratobasidium* e *Thanatephorus* (García et al. 2006). O número de fungos micorrízicos associados com uma orquídea varia dependendo da espécie de orquídea e do estágio do ciclo de vida na qual ela se encontra (Bidartondo and Read 2008; Phillips et al. 2011). Algumas espécies se encontram associadas a apenas um único fungo durante todo seu ciclo de vida (Phillips et al. 2011), numa associação altamente específica, enquanto outras podem se associar a vários fungos (Bidartondo and Read

2008), numa associação inespecífica. Desta forma, o estudo da especificidade é importante para que sejam utilizados fungos compatíveis no processo de germinação.

Uma associação orquídea-fungo do tipo específica pode limitar a distribuição de orquídeas quando ocorrência do fungo é restrita a poucas regiões (Swarts et al. 2010) e em casos extremos pode, inclusive, levar a eventos de especiação, como relatado para *Gastrodia nipponica* (Kinoshita et al. 2016). Desta forma, para orquídeas que apresentam distribuição geográfica limitada é importante verificar se isto é consequência de uma associação específica com um fungo, pois a conservação das populações desta espécie dependerá também da conservação das populações de seu parceiro fúngico e do habitat no qual estão inseridos. Por outro lado, podem existir casos de associações específicas em que o fungo apresenta uma ampla distribuição geográfica, fazendo com que a orquídea possa ter uma larga ocorrência (Davis et al. 2015).

De acordo com o hábito de crescimento, as orquídeas podem ser divididas basicamente em três classes: as que crescem diretamente no solo (terrestres), sobre rochas (rupícolas) e sobre árvores (epífitas). O nível de especificidade para cada hábito é bem variável (Jacquemyn et al. 2010; Swarts et al. 2010), entretanto a interação orquídea-fungo em espécies epífitas tende a ser mais conservada, tendendo a co-evoluir mais do que as terrestres (Martos et al. 2012).

Muito se tem discutido se as associações altamente específicas podem fazer com que algumas espécies se tornem raras (Swarts et al. 2010; Phillips et al. 2011; McCormick and Jacquemyn 2014). Nesse sentido, foi demonstrado que a orquídea terrestre *Caladenia huegelli*, ameaçada e de rara ocorrência no sudoeste australiano, apresenta alta especificidade de associação, de modo que os autores concluíram que sua limitada distribuição era devido a limitada distribuição de seu simbionte fúngico (Dearnaley et al. 2012). Entretanto, há os que defendem que a distribuição limitada de algumas espécies não é definida apenas pelo nível de especificidade, mas sim pela interação de vários fatores bióticos e abióticos de várias ordens (McCormick and Jacquemyn 2014).

No caso de orquídeas epífitas, tem sido observado que mesmo havendo um elevado número de espécies arbóreas numa floresta, com potencial de serem utilizadas como suporte para seu crescimento (forófito), pode ocorrer uma seleção de forófitos

por parte das orquídeas crescendo somente em algumas poucas espécies que são de fato utilizadas (Gowland et al. 2011; Adhikari et al. 2012). Fatores como a disponibilidade de luz, pH da casca, cobertura da casca por musgos, influenciam significativamente esta preferência (Gowland et al. 2011; Adhikari et al. 2016). Também foi verificado se há influência da interação orquídea-fungo na escolha das orquídeas pelo forófito, entretanto não foi possível obter resposta clara (Gowland et al. 2013).

Um elevado número de ascomicetos endofíticos associados ao sistema radicular de orquídeas vem sendo realatado. Mais de quinze ordens de ascomicetos já foram relatadas como endofíticos (Herrera et al. 2010; Oliveira et al. 2014). Inclusive, já foi demonstrado que o gênero *Tuber* é capaz de formar associação micorrízica com a orquídea *Epipactis*, com formação de *pelotons*, estrutura típica da associação entre orquídeas e fungos rizoctonióides (Weiss et al. 2004). Entretanto, na maioria dos estudos os autores têm focado apenas nos fungos rizoctonióides, deixando de lado os demais endofíticos associados as raízes. Dessa forma o papel desempenhado por estes fungos permanece desconhecido. Embora existam vários trabalhos mostrando que estes são produtores de metabólitos secundários que atuam no controle de bactérias e fungos patogênicos (Cheng et al. 2012; Ratnaweera et al. 2015), poucos trabalhos investigaram o papel destes na germinação das sementes (Matsubara 2012) ou na produção de mudas (Zhao et al. 2014a).

Nos últimos anos o estudo da diversidade de fungos associados a orquídeas tem crescido consideravelmente devido ao avanço das técnicas de sequenciamento de nova geração (método independente de cultivo), que possibilitaram o estudo da associação em larga escala (Huang et al. 2014; Oja et al. 2014), tendo inclusive permitido o desenvolvimento de novos marcadores moleculares para fungos micorrízicos (Ruibal et al. 2014). Entretanto, tem-se observado uma escassez de estudos que agreguem as sequências de DNA obtidas a um dado morfológico, seja através do sequenciamento de isolados em cultura pura ou de esporos coletados do campo. E uma vez que a análise dos dados de sequenciamento depende de um banco de dados com informações curadas que são possíveis apenas quando são feitos estudos morfológicos dos fungos, muitos estudos de diversidade de fungos ficam com boa parte das sequências geradas sem identificação taxonômica ou com uma identificação apenas a nível de filo ou ordem (Oja et al. 2014). Desta forma, é necessário que estudos

realizem o isolamento de fungos para uma identificação curada dos fungos de modo que a biologia da associação possa ser melhor entendida (Khamchatra et al. 2016).

Muitas orquídeas brasileiras estão relatadas em listas de espécies ameaçadas de extinção (Martinelli and Moraes 2013). Para qualquer espécie da flora ameaçada de extinção, são necessários esforços para que a preservação da espécie seja garantida. Entretanto, o plano de conservação para orquídeas é complexo uma vez que depende da presença de polinizadores e de fungos compatíveis para que a germinação das sementes ocorra no habitat natural. Neste sentido, esforços de conservação e restauração de orquídeas ameaçadas devem considerar a coleta e o estudo de ambos os simbiontes desta associação (Krupnick et al. 2013)

Nesse contexto, o gênero *Zygopetalum* tem atualmente duas espécies incluídas no Livro Vermelho da Flora do Brasil (Martinelli and Moraes 2013). Este gênero pertence à subtribo Zygopetalinae, da subfamília Epidendroidae contém cerca de quinze espécies que ocorrem em florestas tropicais (Pridgeon et al. 2009). *Zygopetalum* spp. podem ter hábitos rupícolas, terrestres ou epifíticos, ocorrendo na América Central e América do Sul todas florestas tropicais (Pridgeon et al. 2009).

Dentre as espécies ameaçadas, *Zygopetalum maxillare* Lodd. destaca-se diante das demais espécies devido a sua ocorrência quase que exclusiva em espécies de samambaias arbóreas, a *Cyathea delgadii* e *Dicksonia sellowiana*, em ambiente úmido e sombreado (Pridgeon et al. 2009). Além disso, apresenta um hábito de crescimento dimórfico, ou seja, tem inicialmente um crescimento rizomatoso através da emissão de brotações espaçadas ao longo do rizoma, mas quando alcançam o ápice do caule do forófito passam a apresentar crescimento cespitoso. Por outro, lado a espécie *Zygopetalum mackayi* Hook., que não está em risco de extinção, é terrestre e ocorre geralmente em declives montanhosos e beiras de estradas (Pridgeon et al. 2009).

Diante disso, este trabalho consiste em investigar se as diferentes características apresentadas pelas duas espécies congêneres de *Zygopetalum* estão correlacionadas com o perfil de fungos endofíticos e micorrízicos associados e seu hábito, além de propor um método com melhor rendimento e qualidade DNA total para ser usado nas análises independentes de cultivo e que possibilita a obtenção de análises fidedignas de diversidade microbiana.

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SHORT COMMUNICATION

Written according to Mycology Progress

1. OPTIMIZATION OF DNA CONSERVATION AND EXTRACTION FROM ORCHID ROOTS COLONIZED

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Abstract

The conservation of threatened orchid species relies a suitable mycorrhizal fungus partner to enable the seeds germination, once they do not have enough energy and carbon source to germinate. Methodologies applying molecular biology are often used to study this mycorrhizal association. The great challenge to use molecular tools is too ensure a suitable DNA quality. All these methodologies share the DNA extraction as the first step of the process. Therefore, this is a key-step to ensure access to the genetic information present in the samples. So, to keep DNA integrity from the samples and optimize the DNA extraction we performed two experiments. Tp improve DNA conservation, we tested to storage orchid root samples in RNAlater™ (23 °C), Buffer Lysis (SL1, 23 °C) or freezing (-20 °C), for three to 24 days. For optimizing the DNA extraction, we evaluated the angular frequency (4000, 4400, 5300, 6100 and 6500 rpm), and time (6, 18, 48, 78 e 90) using Precellys®24 machine. The analysis of experiments showed the maximum yield of DNA is achieved using the combination of 6500 rpm with 48 s and the root samples conservation in Buffer Lysis (SL1), at room temperature (23 °C), until 17 days, allows getting a suitable integrity and concentration.

Keywords

DNA samples, Orchidaceae, Diversity, Mycorrhiza

Introduction

The most orchid mycorrhizal fungi do not display enough features to be identified using morphological methods because of the absence of sexual spores (Roberts 1999; García et al. 2006). For this reason, the studies of this association requires the employment of molecular approaches (Oja et al. 2014; Oliveira et al. 2014; Waud et al. 2014).

All the molecular approaches share a common step, the DNA obtaining, which is crucial to be optimized to ensure that the whole genetic information present in the samples be accessed (Diédhiou et al. 2014). Furthermore, is important that this method be less time consuming to allow the quickly processing of many samples, once the studies on orchid mycorrhiza diversity often use hundreds of samples (Martos et al. 2012; Oja et al. 2014).

The majority of the studies on orchid mycorrhiza using molecular tools requires storage after sampling prior to molecular analysis (Oliveira et al. 2014), thereby, a suitable method to conserve the samples prior to molecular analysis is required to avoid loss of genetic information and concentration increase of contaminants (Bainard et al. 2010).

Additionally, the choice of DNA extraction method have a significant implication on the DNA recovery and PCR success (Henderson et al. 2013; Diédhiou et al. 2014), biasing the representativeness of diversity analysis.

Here, we establish an efficient method to conserve the DNA of colonized root samples and optimize the DNA extraction process for orchid roots colonized by mycorrhizal fungus.

Material and methods

Optimization of total DNA extraction

To improve the method for conservation of samples for DNA extraction and optimized the DNA extraction itself of colonized roots by using the kit NucleoSpin® Soil from Macherey-Nagel. Aiming a suitable experimental representativeness, we used one root from three different orchid species: *Hadrolaelia jongheanna* (= *Cattleya jongheana*), *Hoffmannseggella caulescens* e *Hoffmannseggella cinnabarina*, totaling tree roots.

The first step of the DNA extraction kit used is the sample lysis, which was performed using a high-throughput tissue homogenizer Precellys®24. This machine displays a wide range of angular frequency and time, which can be adjusted for several types of samples. The right time and angular frequency used in this machine is crucial point because the non-suitable adjustment of these factors do not ensure the best DNA yield and integrity.

Experimental design

We tested five levels of angular frequency (4000, 4400, 5300, 6100 e 6500 rpm) and time (6, 18, 48, 78 e 90 s) in the high-throughput tissue homogenizer Precellys®24, in a rotatable central composite design ($\alpha = \sqrt{2}$), with four essays in the axial points ($+\sqrt{2}$ e $-\sqrt{2}$), four in the cubic points (+1 e -1) and three in the central point (0, 0), totaling eleven essays in an economic experiment design (Figure 1).

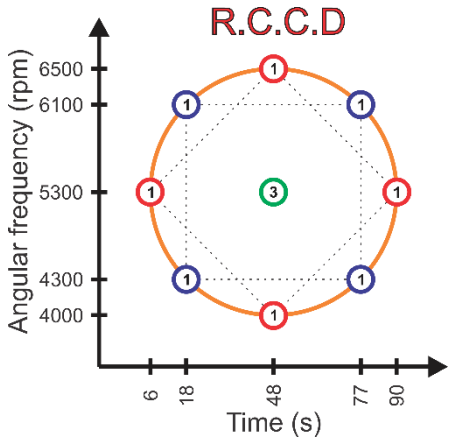

Figure 1: Rotable central composite designed to test angular frequency versus time in the step of lysis of DNA extraction. The number inside the circles indicate the number of essays per treatment.

Homogenization of samples

Orchids roots can display different features like: hardness, diameter, water content, lignification level, etc. which difficult the cell lysis and obtaining of nucleic acids integer (Moreira and Dos Santos Isaias 2008). These features can affect the yield and quantity of extracted DNA by a treatment. To avoid it, we firstly homogenized the samples such that each experimental unit showed the maximum of homogeneity. Each root was divided in segments of 1 cm long (Figure 2). The segments were sliced in 22 transversal fragments. Each experimental unit, a two mL microtube, received two transversal fragments of each segment.

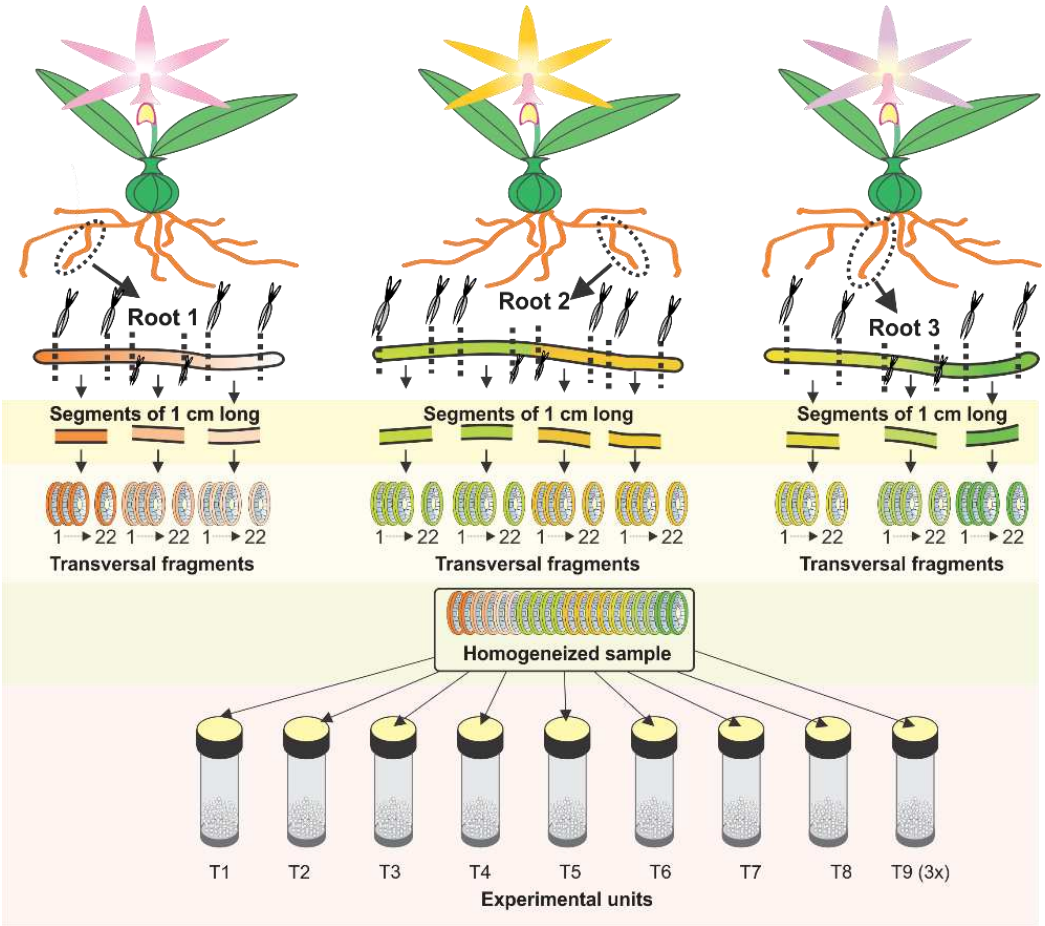

Figure 2: Strategy for homogenization of orchid roots. Each experimental unit receive two transversal fragments from each root segment. Eight treatments, T1 until T8 were performed in a unique essay and one treatment (T9) were performed in tree essays.

All measurements of the concentration and the integrity of the extracted DNA was carried out on NanoDrop 2000c and 0.8% agarose gel electrophoresis. The data were submitted to ANOVA and the means compared by Student test ($p<0.05$), using R software (Team 2012).

Improvement of conservation of colonized roots for DNA extraction

Three conservation methods for the roots samples: (1) storage in RNAlater (2) storage in the Buffer Lysis of NucleoSpin® Soil kit (SL1 solution, which contains Sodium dodecyl sulfate - SDS) at room temperature and (3) storage at -20 °C without any solution. The samples were homogenized according the latest item and distributed in the three treatments with two experimental replicates. After three days the DNA extractions and the measurement of concentration and integrity DNA were performed. The treatment that showed the best DNA integrity and concentration (Tuckey test, $p<0.05$) was chosen to continue evaluating the conservation along one month.

All statistical analysis were performed using R software (Team 2012) and the graphics using SigmaPlot 12.2.

Results and discussion

Optimization of DNA extraction

For the time factor both linear (p-value = 0.041) and quadratic (p-value = 0.019) terms of regression were significant by Student's t test ($p<0.05$, Figure 3), whereas for angular frequency factor only the linear was significant (Figure 3A). Once that optimization of any process requires a regression with significant negative quadratic components for all factors, new points would necessary to reach it. However, for the homogenizer Precellys®24 the maximum angular frequency is 6500 rpm.

The results of DNA integrity (Figure 4) validated the results found in the surface response (Figure 5), being the level of factors that showed the maximum DNA concentration and integrity were obtained by the combination of 6500 rpm and 48 s. These results corroborate with a previous study (Miller et al. 1999), which showed that time and angular frequency have a strongly effect on the DNA yield and integrity.

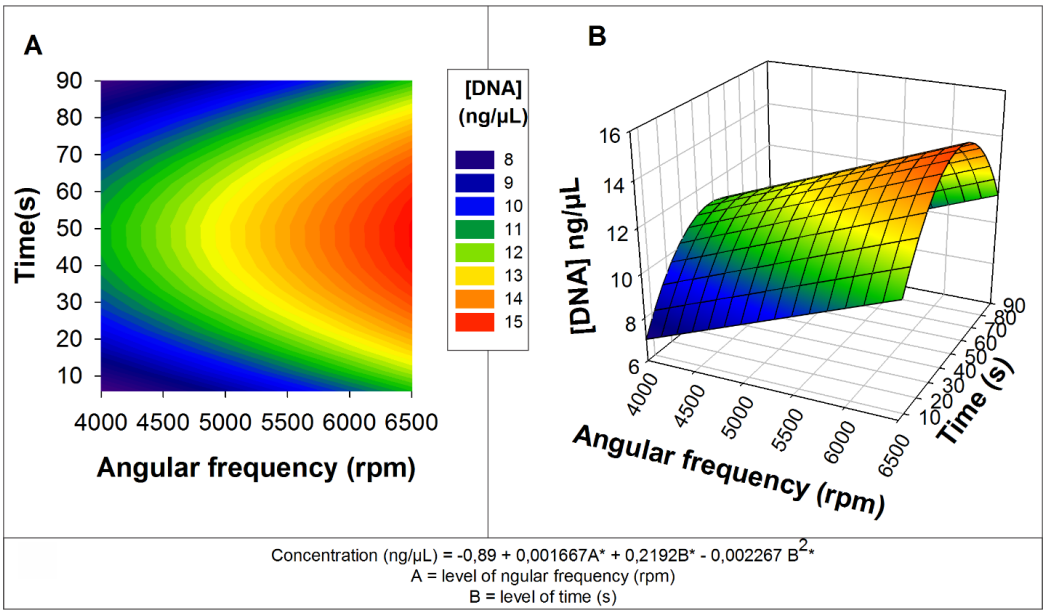

Figure 3: DNA concentration in response to different combinations of time and angular frequency on Precellys®24. Terms followed by "*" are significantly different of zero by Student's t test at significance level of 5%.

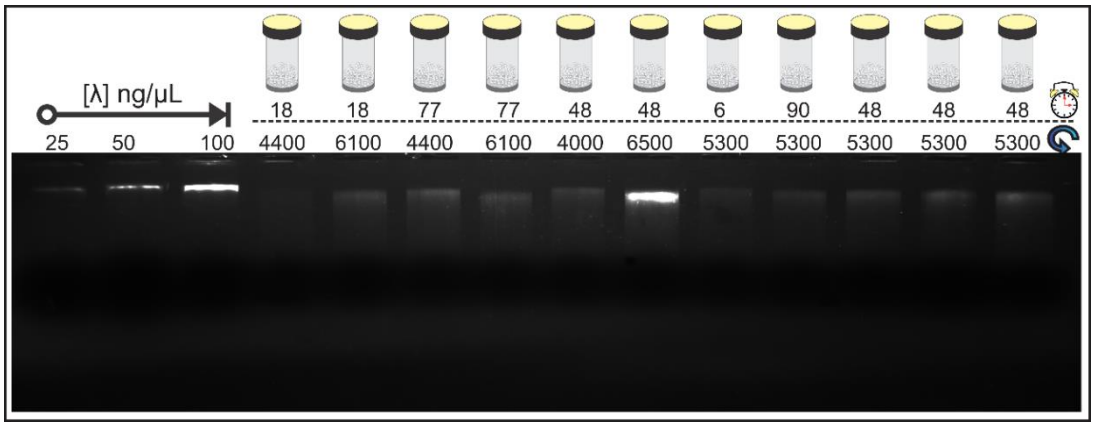

Figure 4: DNA concentration in response to different combinations of time and angular frequency on Precellys®24. The λ phage were used as a marker of integrity and concentration.

Improvement of conservation of colonized roots for DNA extraction

After three days of conservation, the three treatments did not display any difference in the genomic DNA concentration (Figure 5A). However, when we analyzed the integrity of DNA, samples stored in freezer (-20 °C) almost totally were degraded (Figure 5B). The samples stored on Lysis Buffer (SL1) and RNAlater did not show difference ($p>0.05$) in concentration (Figure 5A), however, the integrity was better in Lysis Buffer (Figure 5B).

Evaluations at 10, 17 and 24th days were done only using Lysis Buffer (SL1) (Figure 5). This decision was done considering that although the three treatments did not show differences (p-value = 0.504) in DNA concentration at 3 days (Figure 5A), the freezer treatment have not ensured integrity and RNAlater procedure requires additional steps which increase the chance of contamination.

Until 17 days of storage at room temperature, the integrity (Figure 6A) and concentration (Figure 6B) of extracted DNA were maintained (*p-value* = 0.73) and decrease about 47% after 24 days (Figure 6B). In fact, the storage for about two week at root temperature has been shown the DNA quality (Lauber et al. 2010).

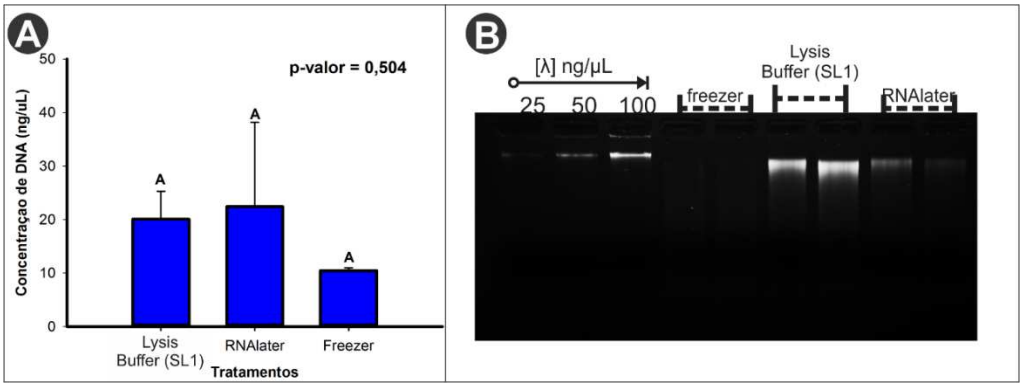

Figure 5: Determination of (A) DNA concentration, measured in NanoDrop, and (B) integrity in agarose gel 0,8 % electrophoresis extracted from colonized root samples after 3 days of preservation in freezer, RNAlater and Lysis Buffer (SL1). The means were compared by Tukey test at the 0.05 significance level.

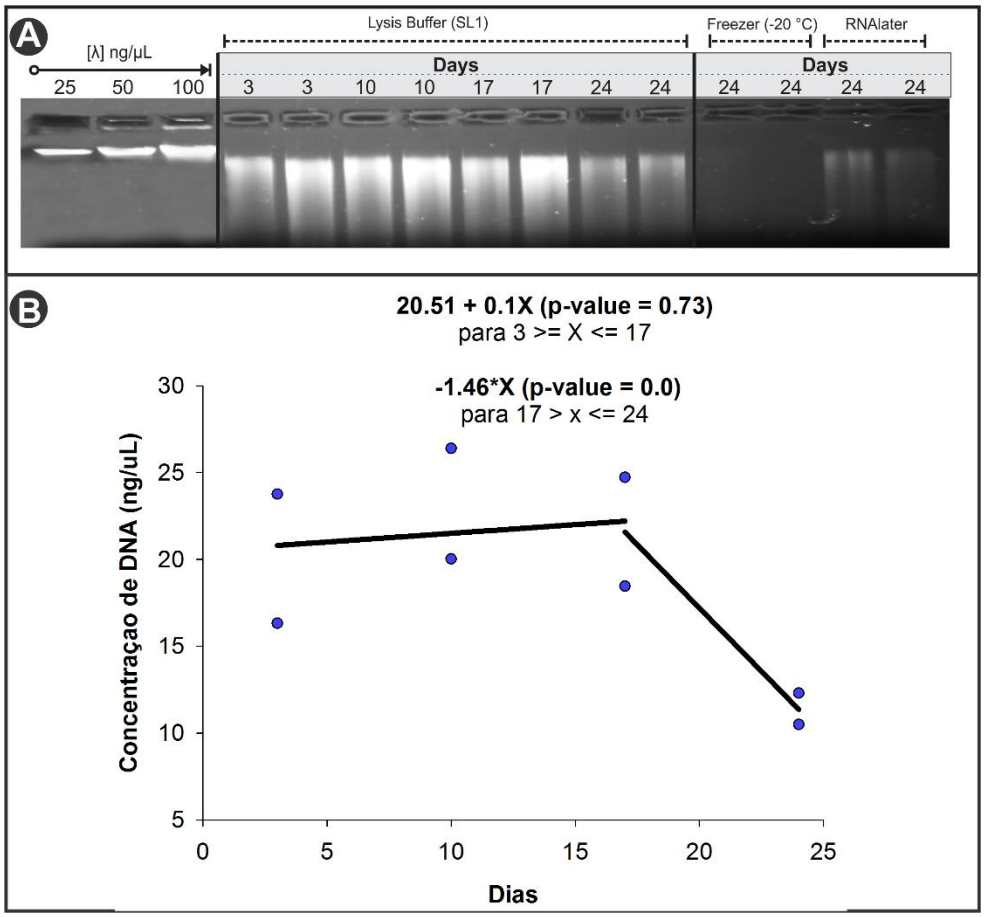

Figure 6: (A) Integrity in 0.8% agarose gel electrophoresis and (B) concentration of extracted DNA of colonized orchid roots, storage in Lysis Buffer (SL1) along 24 days.

CONCLUSIONS

The maximum DNA extraction is optimized by the combination of 6500 rpm and 48 s using the Precellys Machine and the best way to conserve samples before DNA extraction is the storage on Lysis Buffer SL1.

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2. FUNGAL COMMUNITY PROFILE OF *Zygopetalum maxillare* AND *Zygopetalum mackayi* in BRAZILIAN ATLANTIC FOREST (ORCHIDACEAE)

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Abstract

Orchids relies a fungal partner to germinate their the seeds. In some cases, this process can be performed by a specific fungus species. Consequently, if this fungal species is on restrict spatial occurrence, the orchid occurrence is also limited. Two orchid species of the same genus, *Zygopetalum mackayi* (a terrestrial) and *Zygopetalum maxillare* (an epiphytic), show a distinct spectrum of geographic occurrence. While *Z. mackayi* shows a broad distribution, *Z. maxillare* is usually found only on two types of tree ferns, *Cyathea delgadii* and *Dicksonia sellowiana*. So, our question is if this restrict occurrence is related to an association with specific mycorrhizal fungal species. From each species, 3 root samples of 5 plants were sampled in four localities of Atlantic Forest, in Brazil. The community profile of each species was evaluated after extraction of total DNA from colonized roots and the ITS1 region was sequenced. We verified that the overall fungal community of both orchids are defined by the host species with no significant influence of the locality. Fungal community structure of *Z. maxillare* is more sensitive to change due to enviromental factors than *Z. mackayi*. Both species are associate with Ceratobasidiaceae and Sebacinaceae. However, only species of Ceratobasidiaceae are shared by both *Zygopetalum.* The exclusively occurrence of *Z. maxillare* in tree ferns is probably realted to the specific association with specific *Sebacina* species.

Keywords

Host specificity, mycorrhiza, endophytes, tropical orchid, Atlantic forest

Introduction

Species of Orchidaceae depend on association with mycorrhizal fungi to complete their life cycle. It occurs because the dust seed doesn't have endosperm to afford germination (Rasmussen et al. 2015), requiring a symbiotic fungal partner to provide nutrients.

The most common fungal partners of orchids is *Rhizoctonia*, belonging to Basidiomycetes, which present the anamorphic similar stage (García et al. 2006). So, they are usually called rhizoctonias, an polyphyletic group which contains distant related fungi taxa, like Sebacinaceae, Ceratobasidiaceae and Tulasnellaceae, all Agaricomycetes (García et al. 2006). However, studies have shown that non-rhizoctonias fungi can also be associated with orchids (Selosse et al. 2004; Kottke et al. 2010; Matsubara 2012).

The association of orchid-fungus may limit the distribution of some orchids whether the fungus is restrict to few regions (Swarts et al. 2010). Furthermore, in some cases symbiosis specialization triggers the speciation of orchids (Kinoshita et al. 2016). If the fungus is widely distributed, the orchids can have a broad occurrence (Davis et al. 2015). Therefore, for orchids displaying restrict occurrence it is important to verify if this feature is defined by association with one or few symbiont fungi, aiming to create a suitable management for endangered populations. Besides, mycorrhizal fungi associate with one orchid can varies in number and in time (Phillips et al. 2011; De Long et al. 2013; Kartzinel et al. 2013; Rasmussen and Rasmussen 2014)

Other studies have been shown that mycorrhizal in epiphytic orchids is more conserved than terrestrial ones (Martos et al. 2012). Furthermore, it has been reported that high mycorrhizal specialization can be the cause of rarity of some orchid species (Swarts et al. 2010). Sometimes the rarity is not explained just by mycorrhizal specialization (Phillips et al. 2011; Ding et al. 2014). Otherwise, little is known about how the distribution of epiphytic orchids can be affected by fungi distribution, especially in the tropical environment (Oliveira et al. 2014).

Some epiphytic orchids occur mainly in few phorophytes species of the total potential tree hosts of a forest. Attempts trying to answer whether this pattern occurs due to mycorrhizal specialization have been done, however there is not a clearly explanation (Gowland et al. 2011; Gowland et al. 2013; Riofrio et al. 2013).

Many biotic and abiotic factors provided by phorophyte can act on preference of orchid for it, like: humidity, light/shade, chemical composition of bark, presence of suitable mycorrhizal fungi (Adhikari et al. 2016). Therefore, research efforts must regard the main factors of the distribution of these species. The orchid genus *Zygopetalum*, contains fifteen species, epiphyte or terrestrial, all in tropical forests (Pridgeon et al., 2009) and until now little research have been done about this genus, and focusing mainly on assymbiotic germination (Nagaraju and Mani 2005; Hong et al. 2010).

Zygopetalum maxillare Lodd. is an epiphyte that grows almost exclusively in tree ferns, such as *Cyathea* sp. and *Dicksonia sellowiana* Hook., in wet and shady conditions (Pridgeon et al. 2009). Due to the phorophyte specificity, the cultivation of this species is laborious. On the other side, *Zygopetalum mackayi* Hook. is a terrestrial widespread species. Both *Zygopetalum* species occur in Brazilian Atlantic Forest, one of the most important hotspot. Many factors,including illegal logging and anthropogenic forest destruction (Pereira et al. 2015), has resulted the inclusion of these species in the The Red Book of Brazilian Flora, under “least concern” (LC) IUCN category (Martinelli and Moraes 2013). So, considering that both species have a conservation appeal, it is important to investigate the fungi profile community, and also to investigate if the restrict occurrence of *Z. maxillare* in tree ferns is defined by an specific mycorrhizal fungi association, while the broad occurrence of *Z. mackayi* is related to a generalist symbiose.

Materials and Methods

Study site

The study was conducted in four fragment of Atlantic Forest, southeast Brazil (Figure 1), where the both species (*Z. mackayi* and *Z. maxillare*) coexist. Two areas are localized in Rio de Janeiro State: [1] APA Palmares, Paty do Alferes (S 22° 25' 10", W 43° 25' 21") and [2] RPPN Bacchus, Macaé de Cima, Nova Friburgo, (S 22° 17' 14" W 42° 32' 1"), and two in Minas Gerais State: [3] Serra do Funil, Rio Preto (S

22º 05' 21" W 43º 49' 40") and [4] Parque Estadual da Serra do Brigadeiro, Araponga (S 20° 42' 55" W 42° 26' 51").

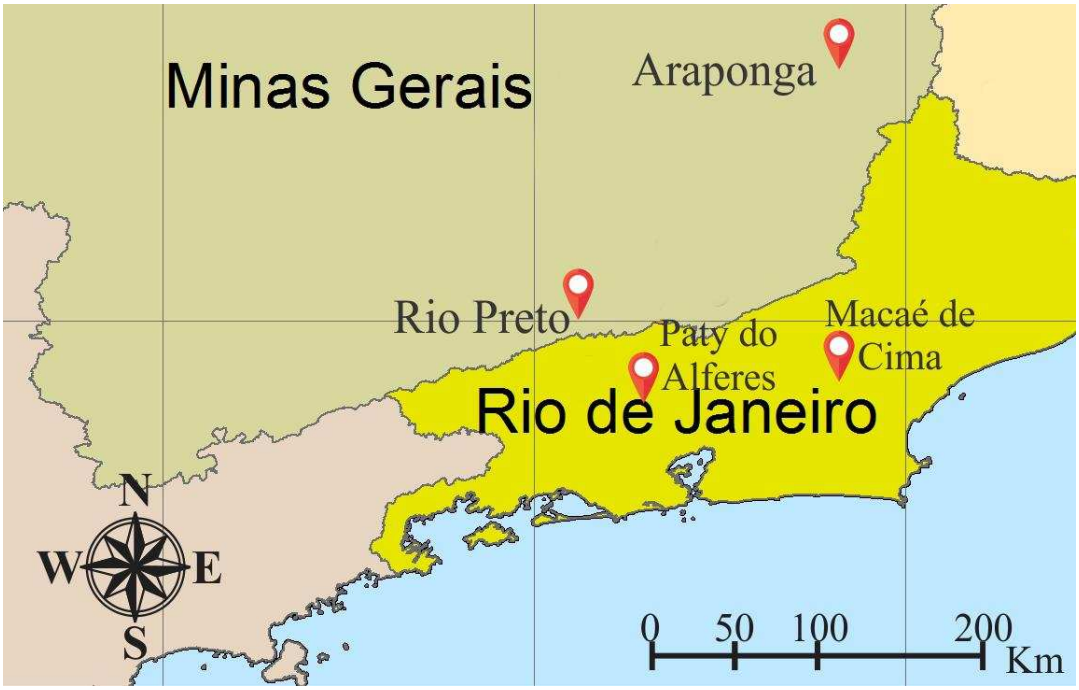

Figure 1: Samples of *Zygopetalum maxillare* and *Zygopetallum mackayi* were collected in four sites of Atlantic Forest, where these both species coexist, in Rio de Janeiro and Minas Gerais States.

In each locality we collected 3 root fragments from 5 plants of each orchid species, totaling 15 samples for each specie for each locality. Prior of DNA extraction, the roots were sliced in 22 thin transversal fragments and kept in Lysis buffer SL1 of kit NucleoSpin® Soil (Macherey-Nagel) for conservation.

DNA extraction, PCR amplification and sequencing

The segment of each root destined for direct DNA extraction was processed using Nucleospin Soil® (Macherey-Nagel), following manufacturer's instructions using 48 s and 6500 rpm in the Lysis step in Precellys® 24. The extracted DNA was quantified by spectrophotometer in a NanoDrop 2000c spectrophotometer.

Prior to sequencing, PCR were performed using the pair of primers ITS1/ITS4Tul, to verify the association with Tulasnellaceae family. Because many primers designed to amplify fungal DNA from orchids are not able to amplify *Tulasnella*, a well-known orchid symbiont. As we did not have detect *Tulasnella* in the samples, the sequencing was carried out ITS1F/ITS2 (Table 1).

Table 1: Primers used in this study for amplification of ITS1 region

Primer	Sequence (5' → 3')	Target region	Reference
ITS1F	CTTGGTCATTTAGAGGAAGTAA	ITS1	(Gardes and Bruns 1993)
ITS2	GCTGCGTTCTTCATCGATGC	ITS1	(White et al. 1990)

The ITS1 region was amplified with the pair of primers ITS1F and ITS2 (Table 1) overhanging a compatible Illumina tail. Each PCR was performed in a final volume of 25 µL, containing 12 µL of MoBio PCR Water, 1 µL of DNA (1 ng/µL), 1 µLf of each primer (5 µM) and 10 µL of 5 Prime HotMasterMix (Mobio Laboratories Inc., Carlsbad, CA, USA). The cycling condition was carried out with an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 60 s and 72 °C for 90 s; and a final extension at 72 °C for 10 min.

The amplifcons were purified using the UltraClean® PCR Clean-Up Kit (MObio Laboratories Inc., Carlsbad, CA, USA) and quantified by Qubit® (Invitrogen by Life Technologies). Each amplicon was diluted town to 2 nM in a library pool, then they were denatured and diluted to a final concentrion of 6.75 pM for sequencing in Illumina MiSeq 2 x 250 bp.

Analysis

The analysis were performed using the Quantitative Insights Into Microbial Ecology Sofware (QIIME 1.8.0 software) (Caporaso et al. 2010; Bokulich et al. 2013), according to Brazilian Microbiome Project protocol for ITS sequences (Pylro et al. 2014). In the cleaning step, all reads which present at least one of the following parameters: length short than 140 pb, quality below 25 at Phred scale, one or more base ambiguity were discarded. The ITS1 region was extracted using ITSx (Bengtsson-Palme et al. 2013). All remained reads were clustered in OTUs at 97% of identity, using Uparse method (Edgar 2013), and each OTU was assigned in a taxon by blast tool available on Qiime using the UNITE database (Koljalg et al. 2014). Only samples that shows coverage above 0.9 were used to further analysis. The diversty indexes, component principal Analysis (PCoA), rarefaction curves and fungi co-ocurrence were done by QIIME scripts (Caporaso et al. 2010; Caporaso et al. 2011).

Results

Sequencing

Analyses of ITS1 sequences

After taking off the low quality sequences, 640.870 sequences containing only ITS1 region (using the primers ITS1F) were retained. For *Z. maxillare*, the number of sequences were 320.973 (16.049 sequences per sample) and for *Z. mackayi* 319.897 (18.817 reads per sample). The coverage values showed that the sampling could access the most of apparent diversity (Table 2).

The fungal diversity in *Z. mackayi* and *Z. maxillare* did not differ for all evaluated index of diversity and equitability (p-value > 0,05; Table 2). The Good's coverage indexes were higher than 97% for all datasets, showing that the sampling efforts was enough to cover fungal diversity.

Table 2: Summary of sequences of ITS1 region after filter off low quality reads

Orchid species	ITS region	N° of reads	n° OUTs[1]	Goods' Coverage index (%)	Chao1[2]	Shannon[2]	Equitability
Z. maxillare	ITS1	320973	1636	99,12 ± 0,80	268,25 **A**	4,07 **A**	0,53 **A**
Z. *makcayi*		319897	1644	97,43 ± 0,32	267,54 **A**	3,5 **A**	0,55 **A**

[1]OTUs presenting reads clustered at 97 % identity threshold.
[2]Means in the same collum with the same letter are not significantly different from each other at the 0.05 level F-test.

When we tested the slope of the last three points, the fitted equations showed a linear coefficient statistically equal to zero, which is an indicative that the diversity achieved a plateau and the sampling effort was enough to cover the apparent fungal diversity (Figure 2).

Influence of host and environment on the fungal community structure

A principal component analysis (PCoA) based on Bray-Curtis dissimilarity was done aiming to verify if the fungal profile community of each orchid species are different (Figure 3a). The samples of each species did not overlap with other, showing a different composition of fungi defined by the host (p-value = 0.008). The number of fungi species shared by both orchid species was below than 30% (Figure 3b). More than 2/3 of the fungi species occurred only in *Z. maxillare* or *Z. mackayi*, elucidating the differences found in PCoA (Figure 3a).

Samples from different towns (PERMANOVA p-value = 0.08) and states (PERMANOVA p-value = 0.21) show no influence of locality on the fungal community structure.

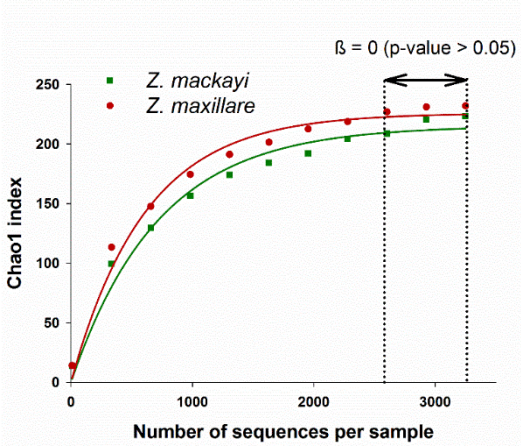

Figure 2: Rarefaction curve for ITS1 region by Chao1 diversity index to verify the sequencing depth. A regression slope test (at 0.05 significance level) was done to verify whether further sampling would yield the Chao1 diversity index.

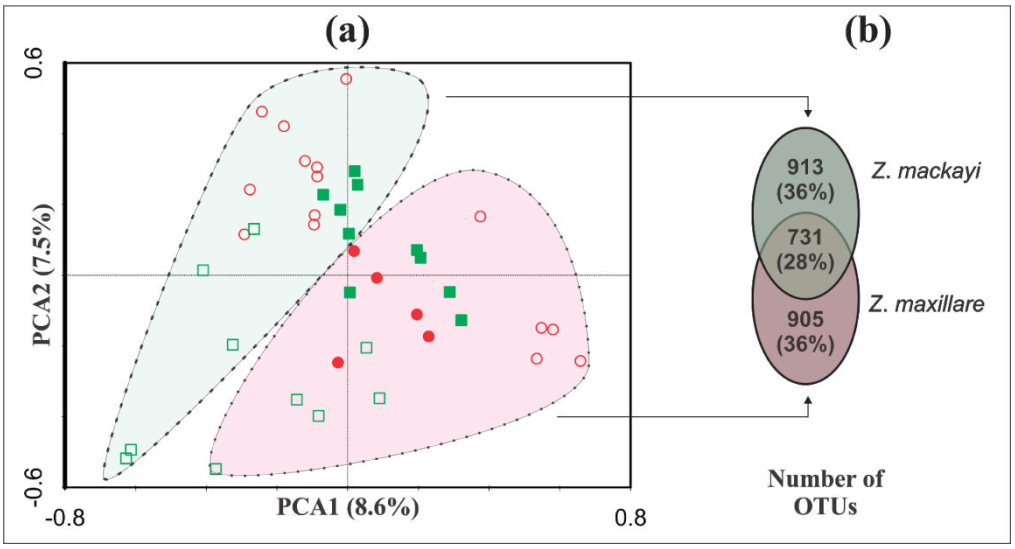

Figure 3: (a) Principal component analysis based on Bray-Curtis dissimilarity for fungal community habiting roots of *Zygopetalum maxillare* (pink shade) *and Zygopetalum mackayi* (green shade) in four localities (☐ Paty do Alferes; ■ Macaé de Cima; ● Serra do Funil; ○Araponga). The numbers in parenthesis indicate the percentage level of explanation by each principal component (PCA1 or PCA2). (b) Number of OTUs, shared or non-shared, between the two orchid species. Each OTU was built using a cutoff threshold of 97%.

The fungal community of *Z. maxillare* was more influence by environmental factors than *Z. mackayi* (Table 3). So, we can see that for the epiphytic orchid, according to the elevation of the altitude, the Shanon index, equitability and dominance of fungal community increases by relative humidity increasing, but when the relative humidity or

temperature increases the equitability decreases (Table 3). Otherwise, fungal community composition of *Z. mackayi* did not undergo influence of these factors.

Table 3: Pearson' coefficient matrix between the environmental factors and the fungal community indexes

Index	***Zygopetalum maxillare***			***Zygopetalum mackayi***		
	Relative humidity	Average Temperature	Altitude	Relative humidity	Average Temperature	Altitude
Shannon	-381^{ns}	-0.403^{ns}	-0.494*	0.261^{ns}	0.229 ^{ns}	0.071 ^{ns}
Equitability	-0.450*	-469*	-0.465*	0.362 ^{ns}	0.293 ^{ns}	0.129 ^{ns}
Dominance	0.240^{ns}	0.381 ^{ns}	0.453*	0.293 ^{ns}	0.166 ^{ns}	0.264 ^{ns}

^{ns} No significant and *significant at 0.05 confidence level by Student t-test.

Fungal community composition

For both orchid species, the number of Ascomycota were greater than Basidiomycota (Figure 4). However, the number of Ascomycota was much higher for *Z. mackayi* than *Z. maxillare*. Sequences assigned as belonging to Zygomycota phylum were more abundant in *Z. maxillare* than *Z. mackayi*. In the dataset we found high percentage of unidentified reads and reads without similar sequence on database (no blast hit).

The majority of reads could not be identified in family or order levels. Further, many reads did not get any hit by blast during the annotation step (Figure 4). The most abundant well-known mycorrhizal fungi found in *Z. maxillare* roots were Sebacinaceae (2.16%). However, fungi from Ceratobasiciaceae were also found in low proportion (1.76%). Although in low proportion, in *Z. mackayi*, Ceratobasiaceae was higher (0.64%) than Sebacinaceae (0.54%).

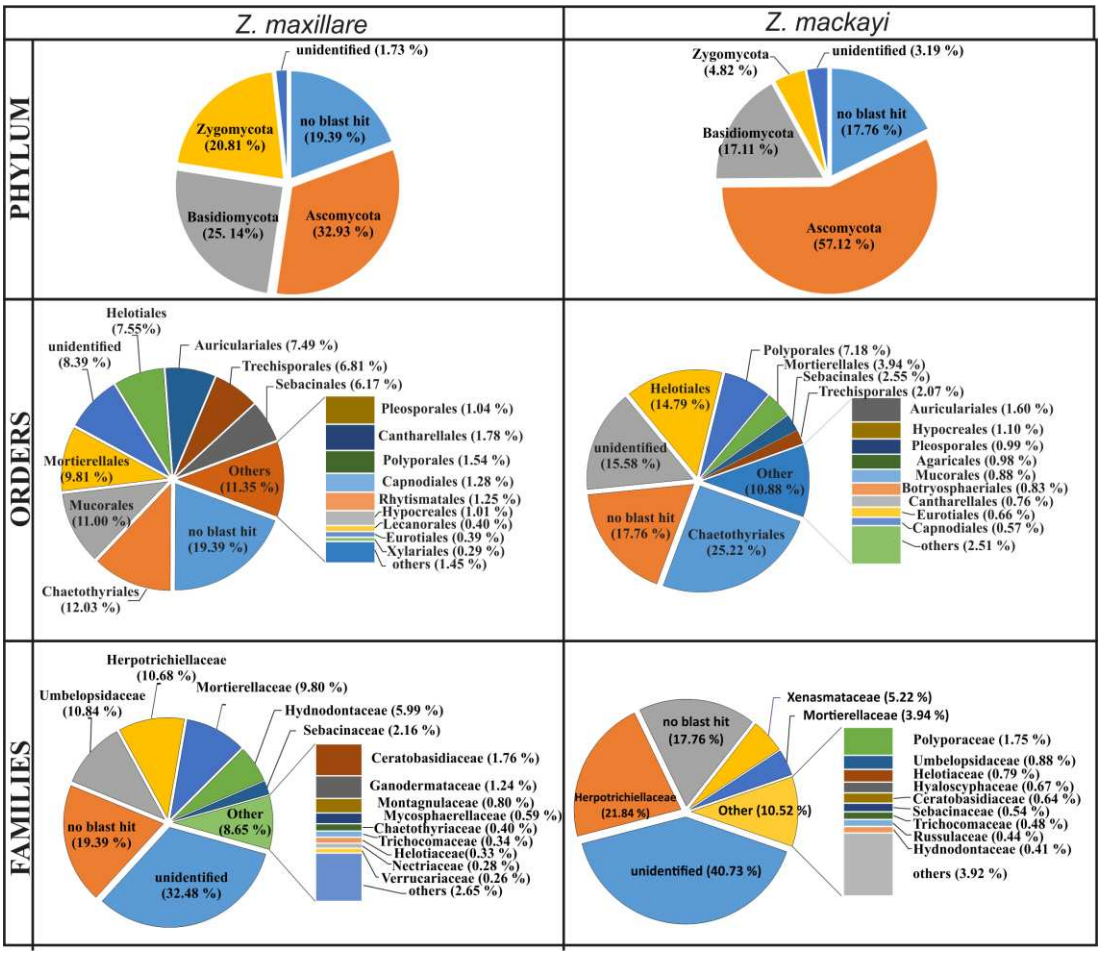

Figure 4: Fungi composition by Phylum, Order and Family levels for *Zygopetalum maxillare* and *Zygopetalum mackayi*. The frequency of each taxon are shown in parentheses.

Mycorrhizal fungi community

A total of five and six species of Sebacinaceae were recovered, respectively, from *Z. maxillare* and *Z. mackayi* (Figure 5). In both orchids it was not observed sharing of Sebacinaceae fungi, showing that both orchid species associates with different species of this family (Figure 5). Both *Zygopetalum* species have associated with Sebacinales Clade B. For *Z. maxillare* we recovered a total of 10 species of Ceratobasidiaceae and for *Z. mackayi*, 5 species. However, both orchids shared three fungi species of Sebacinaceae (Figure 5).

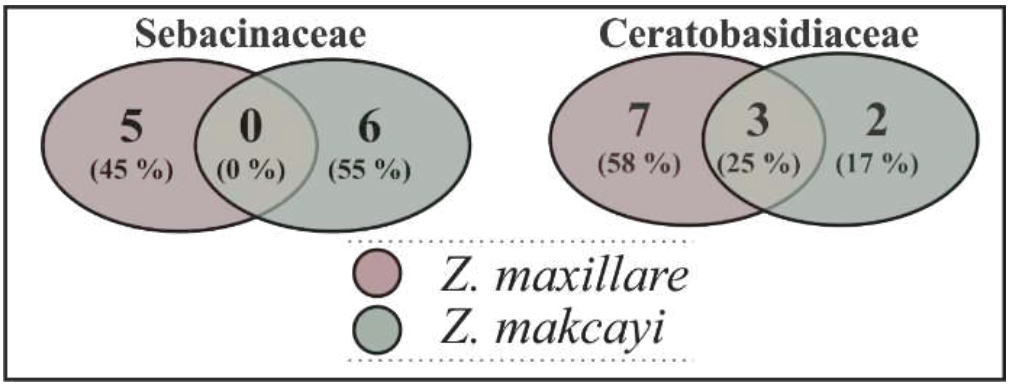

Figure 5: Venn-diagrams showing the number of OTUs (threshold at 97 % similarity cut-off) found in *Z. maxillare* (pink) and *Z. mackayi* (green). The number in the overlapped area indicates the number of OTUs shared by both species.

Discussion

This work is the first next generation sequencing study to investigate the fungal community of Brazilian orchids roots. Although there was no difference in the fungal diversity index of *Zygopetalum* species, both communities present different fungal composition.

The difference in the fungal community of *Zygopetalum* species were defined by the host tree (p-value = 0.189) and not by the locality where these species occur (p-value = 0.008), corroborating with previous studies of Martos et al. (2012), which shows that host tree influences the fungal community on orchids roots. The influence on the fungal community by the host tree were observed in other types of mycorrhizas (Tedersoo et al. 2010; Torrecillas et al. 2012). Indeed, it was confirmed that the endophytic fungal community structure is strongly shaped by the host, instead of the locality (Persoh 2013).

The epiphytic species, *Z. maxillare*, were more influenced by environmental factors than the terrestrial species, *Z. mackayi* (Table 2). This is in according with previously studies which have shown that epiphytic communities are more sensitive to environmental factors (Song et al. 2011; Ding et al. 2016). The influence of humidity on equitability (Table 2) only on fungal community of *Z. maxillare* can be related to where the orchid grow, since this species grows on phorophytes, generally, in forest area with close vegetation. Then, the humidity is higher and are maintained for longer time than in open vegetation, where *Z. mackayi* occurs. In fact, a study showed that the air humidity was the most significant factors affecting the epiphytic

vascular communities (Song et al. 2011). Therefore, here we find that fungal community undergoes the same influence.

The low equitability in high humidity level is probably because of the dominance of few opportunistic fungi that grow due to higher availability of water. In contrast to reported on literature (Geml et al. 2014), we observed a decreasing in fungal diversity when the altitude increases.

Studies applying direct DNA sequencing from the roots have shown a dominance of ascomycetes endophytes (Selosse et al. 2004; Oliveira et al. 2014; Waud et al. 2016). The similar pattern has been achieved when culture-based methods are used (Herrera et al. 2010; Huang and Zhang 2015). It can be due to in the velamen of orchids many ascomycetes have been found (Herrera et al. 2010), most of them with unknown function. However, recent studies have shown these fungi can exercise antimicrobial activity against fungi and bacteria (Ma et al. 2015; Barnes et al. 2016). However, in a rare case, an ascomycete was reported forming a true orchid mycorrhizae (Selosse et al. 2004).

The two families from the Zygomycota, Umbelopsidaceae and Mortierellaceae, found in both *Zygopetalum* species roots, were also found in another study (Huang and Zhang 2015). Furtheremore, *Umbelopsis nana* (Umbelopsidaceae) has been improved the macro and micronutrients uptake of *Cymbidium* seedlings (Zhao et al. 2014b), complementing the nutrients provided by mycorrhizal fungi.

When we focus the analysis only on the well-known mycorrhizal fungi, we can observe that species of Ceratobasidiaceae are shared by both *Zygopetalum* species, while Sebacinaceae species are not (Figure 5). In this way, both orchid plants display different specificity for some Sebacinaceae species.

Indeed, Sebacinaceae is huge family and present many types of mycorrhizas besides orchid mycorrhizas with many nutritional modes (Hibbett et al. 2014). Additionally, it has been proposed that the genus *Sebacina*, which forms true orchid mycorrhizas, is probably a polyphyletic group harboring many cryptic species (Weiss et al. 2004). The fact of some Sebacinaceae species has been not shared by *Zygopetalum* species could be the reason for the occurrence of *Z. maxillare* only in tree-ferns.

A low percentage of Sebacinaceae species was observed in the samples of both *Zygopetalum* species (Figure 4). As our collect was done only a once in the adult orchid, we suggest that Sebacinaceae and Ceratobasidiaceae species were partially replaced by others during the plant development, but they were not totally excluded, because they maybe develop a crucial role in the future seed germination. In fact some orchids have a different partner in each step of development (Bidartondo and Read 2008), changing the fungal partner during the life cycle (Rasmussen et al. 2015). Although, some orchid species have kept the same fungi from the germination to the mature phase (Phillips et al. 2011).

This specificity is set up during the seed germination step of the orchid life cycle, displaying a bottleneck that could be related to the restrict occurrence of *Z. maxillare* on the tree ferns. For this reason, studies could try to isolate the species of Sebacinaceae from both *Zygopetalum* species and do assays of symbiotic germination to verify if these Sebacinaceae species found in adults plants are responsible for the restrict occurrence of *Z. maxillare* on the tree fern.

Conclusions

The congeners species *Z. mackayi* and *Z. maxillare* display different fungal community structures and this difference is also observed in the mycorrhizal taxon. Although both species present association with species of Sebacinaceae and Ceratobasidiaceae, they do not share the same species of Sebacinaceae. Future studies with isolation and germination assays are necessary to investigate the specificity of symbiosis, which can be a bottleneck for the restrict occurrence of *Z. maxillare* on tree ferns.

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3. ROOT MYCORRHIZAL FUNGI ON TERRESTRIAL AND EPYPHITIC *Zygopetalum* (ORCHIDACEAE) SPECIES

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Abstract

All orchids rely on mycorrhizal fungi to provide nutrients for seed germination. So, it is interesting to isolate and identify them for future studies, since it is important to conserve the germoplasm bank for use them in the reintroduction and conservation program. Here we isolated fungi from an epiphyte (*Zygopetalum maxillar*e) and a terrestrial (*Zygopetalum mackayi*). It was obtained 12 isolates from *Z. maxillar*e and 14 from the *Z. mackayi*. There were a dominance of Ascomycota (77%) compared to Basidiomycota (23%). Among the well-known orchid mycorrhizal fungi, four isolates were identified as *Sebacina* (15%). A Bayesian approach showed a distinct phylogenetic position of the mycorrhizal fungi of both *Zygopetalum*. These finds suggest that although these species associates with fungi of *Sebacina*, they belong to distinct species.

Keywords

*Zygopetalum maxillar*e, *Zygopetalum mackayi,* mycorrhiza, pure culture, Atlantic Forest

Introduction

Orchids are fascinating plants due their exuberant beautiful flowers. However, inside their roots is hidden another riveting feature: a symbiotic relationship with different fungi. All orchids associate with mycorrhizal fungi in at least one stage of their life cycle, since the minute size of the seeds, which do not contain endosperm, as nutritive tissue, so that the fungal partner performs the nutrients uptake in the germination (Rasmussen et al. 2015).

Some orchid seeds are able to germinate only if a specific fungal species is present (Phillips et al. 2011), while others can germinate with many (Bidartondo and Read 2008). Therefore, studies isolating and identifying the fungi associated with orchid have been done. In many of these studies, Basidiomycota fungi belonging to *Sebacinaceae*, *Tulasnellaceae* and *Ceratobasidiaceae* families are usually reported. However, a number of endophytic Ascomycota and Zygomycota fungi have also been reported associate with orchid plants (Huang and Zhang 2015). The role of these fungi is poorly understood and requires more studies. Studies addressing these fungi have shown that they can increase the nutrient uptake during germination (Zhao et al. 2014b) and even promoting seed germination (Vujanovic 2000).

Since the advancing of high-throughput sequencing, the studies of orchid mycorrhizas has been increased (Huang et al. 2014; Oja et al. 2014) and it is an excellent approach to study the fungal community. However, as it is a culture-independent approach, it depends on accurate information deposited in databases that comes from the sequencing of identified pure cultures or spores from the field. In this way, it is important to perform isolation of fungi (Khamchatra et al. 2016) for understanding the biology of the association.

In The Red Book of Brazilian Flora (Martinelli and Moraes 2013) some orchids have been reported. It is alarming because the preservation of orchid species is more complex than other plants due to the requirement of its fungal partner. In order that, conservation and restauration efforts of threatened orchid species should consider also the fungal symbionts (Krupnick et al. 2013).

Here we studied *Zygopetalum maxillare* Lodd. and *Zygopetalum mackayi* Hook. Two species of the same genus but with different growth habits. A previously study using a culture-independent method has been shown that both congeners species

are associates with different endophytes and mycorrhizal fungi (*Sebacina*) (Veloso et al., non-published data). *Zygopetalum maxilare* is listed in the Red Book of Brazilian Flora, and there is no information related to root endophytic fungi. So, the aim of this study was to isolate and identify endophytic fungi associate with adult plants of *Z. maxillare* and *Z. mackayi*, occurring in the Brazilian Atlantic Forest, as well as to identify them and to analyse the phylogenetic relationship of between the mycorrhizal fungi.

Material and Methods

Sampling sites

The sampling was performed in four different localities in the Atlantic Forest: in Serra do Brigadeiro State Park, Araponga and Serra do Funil, Rio Preto (Minas Gerais state), and Palmares Environmental Protection Area, Paty do Alferes and Bacchus Natural Heritage Private Reserve, Macaé de Cima (Rio de Janeiro state). In each locality, 15 roots of *Z. maxillare* and 15 roots of *Z. mackayi* were sampled. They were stored at 4 °C, until the isolation process.

Fungal isolation

The roots were cut in small fragments of 2 cm long for superficially disinfestation using ethanol 70% for 1 min and sodium hypochlorite 0.5 % for 5 min followed by rinsing in sterilized water. Each disinfested root was sliced in several thin transversal slices and the clusters of cells harboring pelotons were transferred to a Petri dish containing Potate Dextrose Agar (PDA). To identify which colonies are rhizoctonias, the fungi were inoculate on Corn Meal Agar (CMA) at 25 °C to verify the presence of moniliod cells, typical of this group.

DNA extraction, PCR amplification and sequencing

For fungi indentification, the total DNA extraction of the isolates was carried out using NucleoSpin Plant II® (Macherey-Nagel) according to the manufacture's instructions. The DNA integrity was evaluated in 0.8 % agarose gel electrophoresis stained with ethidium bromide, and the quantification determined by NanoDrop 2000c. One nanogram of extracted DNA of each fungal isolate was used for PCR using

the pair of primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes and Bruns 1993) and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al. 1990), to amplify the ITS1-5.8s-ITS2 region. The reactions were performed according to the manufactures recommendations with Go TaqTM (Promega, Madison, USA). The cycling condition was 95 °C for 2 min (initial denaturation), 39 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Two additional reactions, one with 1 ng of total DNA of *Sebacina* sp. and other with MiliQ water (DNA-free) were used as positive and negative control of the PCR, respectively.

The sequencing of PCR products were done by ABI 3730XL capillary sequencer. The annotation of fungi identification will be performed by BLAST tool, available on NCBI.

Sequence analyses

The sequences were analyzed using Geneious software (version 9.1.4). The regions of reads with error score above 0.01 were discarded. For taxonomic annotation, the sequences were BLASTed against nr database from GenBank. The closest match found was choose by the following parameters: (1°) lowest e-value, (2°) highest Normalized Bit Score, (3°) highest query coverage and (4°) highest pairwise identity.

Phylogenetic analyses of Sebacinales

The Sebacinaceae sequences were queried against GenBank database using Blastn (optimized for more dissimilar sequences - discontinuous megablast) to recover close related sequences of Sebacinaceae. A multiple alignment was performed by MAFFT using the L-INS-I method (Katoh et al. 2005). For phylogenetic inference we used Bayesian approach with MrBayes 3.2.6 available on CIPRES Science Gateway (version 3.3) (Miller et al. 2010). The models of substitution was estimated using Akaike Information Criterion generated by MrModeltest (Nylander 2004). Four independent runs with 4 Markovian Monte Carlo were turned by 15 million generations. The first one million of trees were discarded and the remaining were used to generate a consensus tree.

Results

26 fungi were isolated and identified. 12 from *Z. maxillare* and fourteen from *Z. mackayi* from four different localities, belonging to 10 orders and 16 genera (Table 1). The isolates were represented by 23% Basidiomycota and 77% Ascomycota. Four isolates (15%) belong to *Sebacina* genus, a typical orchid mycorrhizal fungus, was isolated only from Araponga. Only one fungus was recovered from *Z. mackayi* of Serra do Funil while 11 (42%) were isolated from the roots collected in Paty do Alferes (Table 1). All sequences showed a pairwise identity above 95% against their closest match on GenBank, what allowed the identification of isolates until the genus level. However the isolate ZMT18 were identified only to Class level as Sordariomycetes.

The isolated fungi showed very well different cultural characteristics in color and shape of colony (Figure 1), besides presenting distinct growth rate. Only *Sebacina* presented the typical rhizoctonia features, a well-known orchid mycorrhizal fungus (Figure 1B).

A Bayesian inference used to verify the phylogenetic placement of four *Sebacina* isolates showed two well-supported clades with a high posteriori probability (Figure 2). Each clade presented fungi isolates from the same orchid, *Z. maxillare* or *Z. mackayi*, without any overlapping, suggesting that they belong to distinct species.

Table 1 – Taxonomic assignment of *Zygopetalum maxillare* and *Zygopetalum makayi* isoaltes based on the closest match on GenBank.

	Closest match on GenBank									
Isolate's code	**e-value**	**Bit-score**	**Query coverage (%)**	**Pairwise Identity (%)**	**Accession number**	**Genus**	**Order**	**Locality**	**State**	**Host**
ZME01	0	1007.54	100.00%	99.80%	AB512404	*Xylaria*	Xylariales	Paty do alferes	RJ	*Z. maxillare*
ZME02	0	897.00	100.00%	99.00%	KP991624	*Fimetariella*	Sordariales	Paty do alferes	RJ	*Z. maxillare*
ZME06	0	836.00	96.00%	95.00%	HQ889710	*Camarops*	Boliniales	Paty do alferes	RJ	*Z. maxillare*
ZME11	0	933.191	100.00%	99.80%	KF313104	*Chaetomium*	Sordariales	Paty do alferes	RJ	*Z. maxillare*
ZME12	0	910.119	100.00%	98.80%	KC218448	*Plectosphaerella*	incertae sedis	Paty do alferes	RJ	*Z. maxillare*
ZME13	0	850.515	97.79%	96.00%	KJ188562	Dactylaria	Helotiales	Paty do alferes	RJ	*Z. maxillare*
ZME14	0	1090.64	100.00%	99.80%	HQ248212	*Marasmius*	Agaricales	Macaé de Cima	RJ	*Z. maxillare*
ZME15	0	888.00	99.00%	96.00%	HG937132	*Chaetomium*	Sordariales	Macaé de Cima	RJ	*Z. maxillare*
ZME19	0	1009.39	100.00%	96.10%	HQ154302	*Sebacina*	Sebacinales	Araponga	MG	*Z. maxillare*
ZME20	0	994.618	100.00%	95.80%	HQ154302	*Sebacina*	Sebacinales	Araponga	MG	*Z. maxillare*
ZME22	0	1026.01	99.46%	100.00%	KP133223	*Nemania*	Xylariales	Araponga	MG	*Z. maxillare*
ZME27	0	1035.09	100.00%	100.00%	AJ230675	*Trichoderma*	Hypocreales	Araponga	MG	*Z. mackayi*
ZMT01	0	846.886	100.00%	95.20%	KT581859	*Phoma*	Pleosporales	Paty do alferes	RJ	*Z. mackayi*
ZMT03	0	527.415	99.09%	95.70%	AY254156	*Myrothecium*	Hypocreales	Paty do alferes	RJ	*Z. mackayi*
ZMT04	$2.43E^{-157}$	565.958	97.30%	96.90%	AY254156	*Myrothecium*	Hypocreales	Paty do alferes	RJ	*Z. mackayi*
ZMT07	0	979.336	100.00%	99.20%	JF439501	*Penicillium*	Eurotiales	Paty do alferes	RJ	*Z. mackayi*
ZMT08	0	933.191	100.00%	100.00%	KU255048	*Cladosporium*	Capnodiales	Paty do alferes	RJ	*Z. mackayi*
ZMT09	0	906.00	100.00%	96.00%	HG937132	*Chaetomium*	Sordariales	Macaé de Cima	RJ	*Z. mackayi*
ZMT10	0	908.00	100.00%	96.00%	HG937132	*Chaetomium*	Sordariales	Macaé de Cima	RJ	*Z. mackayi*
ZMT17	0	789.64	100.00%	94.80%	KT224813	*Camarops*	Boliniales	Macaé de Cima	RJ	*Z. mackayi*
ZMT18	0	856.284	96.30%	98.30%	AB847034	Unknown	-	Macaé de Cima	RJ	*Z. mackayi*
ZMT26	0	1123.88	100.00%	99.20%	AF280759	*Schizophyllum*	Agaricales	Serra do Funil	MG	*Z. mackayi*
ZMT27	0	1070.33	99.66%	100.00%	AJ230675	*Trichoderma*	Hypocreales	Araponga	MG	*Z. mackayi*
ZMT28	0	962.031	100.00%	99.80%	KM458824	*Penicillium*	Eurotiales	Araponga	MG	*Z. mackayi*
ZMT29	0	953	100.00%	95.00%	JX317148	*Sebacina*	Sebacinales	Araponga	MG	*Z. mackayi*
ZMT30	0	953	100.00%	95.00%	JX317148	*Sebacina*	Sebacinales	Araponga	MG	*Z. mackayi*

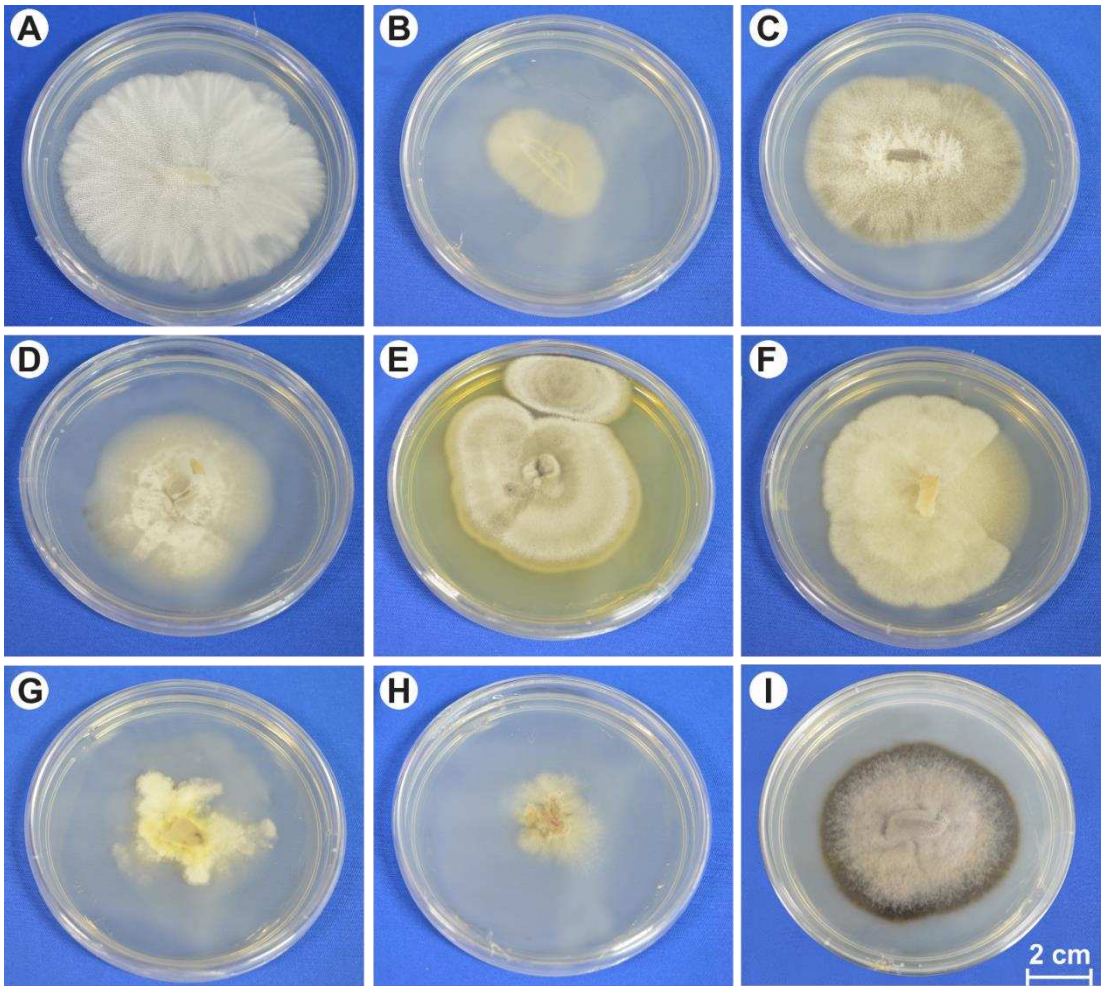

Figure 1: Cultures of some endophytic/mycorrhizal fungi isolates from *Zygopetalum maxillare*: (A) *Xylaria*; (B) *Sebacina* and *Zygopetalum mackayi*: (C) *Plectosphaerella*; (D) *Chaetomium*; (E) *Cladosporium*; (F) *Myrothecium*; (G) *Nemania*; (H) *Camarops*; (I) *Dactylaria*.

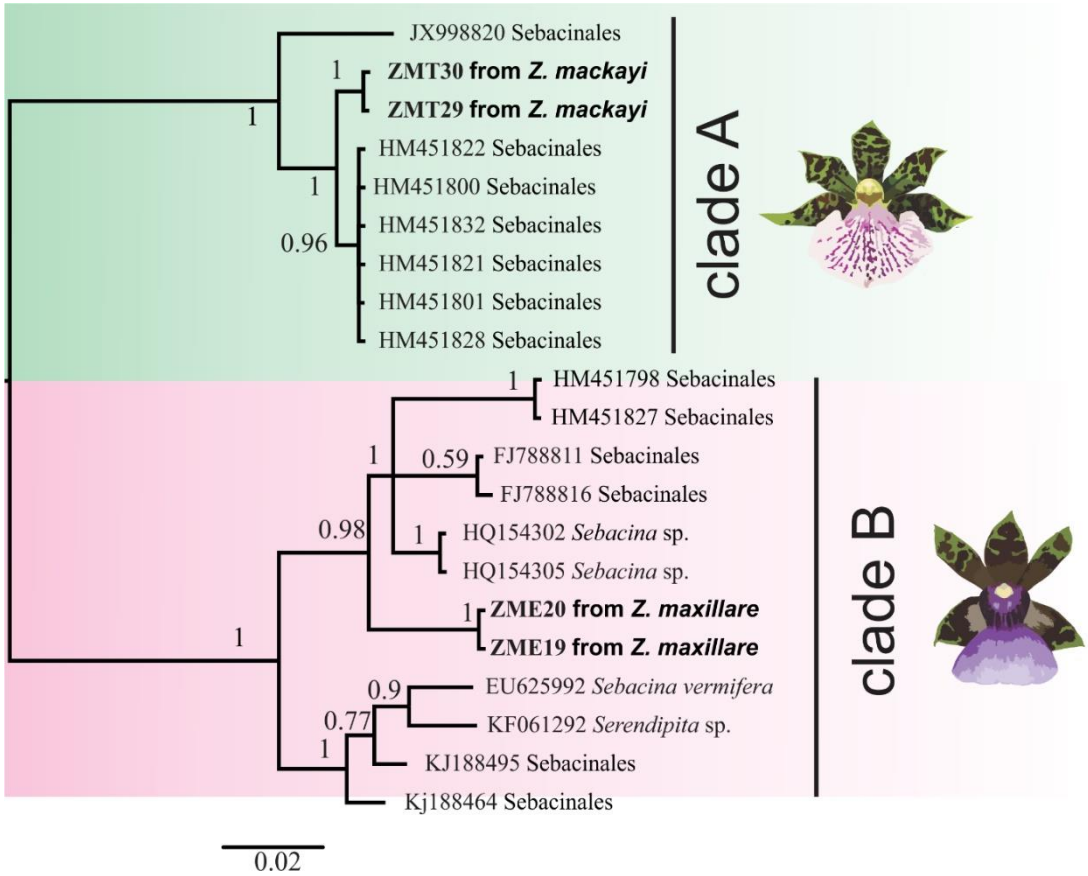

Figure 2: Phylogenetic placement of *Sebacina* isolates (in bold) from *Zygopetalum maxillare* and *Zygopetalum mackayi*. Tree inferred using the Bayesian approach. The number in the nodes represent the posterior probability.

Discussion

Studies related to fungal diversity in orchid roots, either using culture-dependent methods (Jiang et al. 2011) or culture-independent approach (Oliveira et al. 2014), have shown that Ascomycota are the majority of fungi present on orchid roots (Herrera et al. 2010). The non-mycorrhizal fungi isolated from orchids are usually reported as producers of secondary metabolites that can act controlling other micro-organisms, usually gram-positive bacteria (Ratnaweera et al. 2015; Dissanayake et al. 2016) or producing antioxidant compounds (Gubiani et al. 2014). Some studies have applied these fungi in seed germination, and have been demonstrated they are able to improve the nutrients uptake or resistance of seedlings to pathogens (Zhu et al. 2011; Cheng et al. 2012)

Many endophytic fungi have been isolated from the orchid roots as found in *Zygopetalum* species (Table 1). *Thichoderma* has also been isolated from *Dendobrium* (Zi et al. 2014) and *Hoffmannseggella caulescens* (Oliveria et al, 2014). Xylaria is

usually reported as endophytic of orchid roots and some of them have shown to produce antibacterial compounds against gram-postive bacteria (Ratnaweera et al. 2014). In fact, there is a high diversity of *Xylaria* fungi that can associate with orchids, even within of a single orchid genus (Chen et al. 2013). The presence of *Chaetomium* in orchid roots have been reported (Jiang et al. 2011; Sawmya et al. 2013; Dissanayake et al. 2016), not only in orchid, but in others species of plants and it has been shown to produce antimicrobial compounds against *Bacillus* and *Streptococcus* (Dissanayake et al. 2016).

The usual mycorrhizal fungi isolated from Brazilian orchids has been Tulasnella (Pereira et al. 2009; Nogueira et al. 2014). Surprising, only *Sebacina* was isolated as rhizoctonia mycorrhizal fungi. In a previous culture-independent study using these two *Zygopetalum* species it was observed them associated with Sebacinaceae and Ceratobasidiaceae (Veloso et al, non-published data). We verified that the *Sebacina* isolates from the same orchid species clustered together in a same clade while isolates from different species split in two different clades (Figure 3), showing a distinct phylogeny. Once the order Sebacinales harbors a lot huge range of fungi with different life habits, as saprophytes, endophytes and mycorrhizal, it is not surprising to find different species in these orchids. However, additional study need be done to verify if these differences between the isolates reflect in the germination step of the life cycle of *Z. maxillare* and *Z. mackayi*, since some orchids are able to germinate only in the presence of fungi isolated from their own root system (Pereira et al. 2005; Bonnardeaux et al. 2007).

Conclusions

Z. maxillare and *Z. mackayi* associate with different endophytic fungi, having a dominance of Ascomycota (77%) compared to Basidiomycota (23%). *Sebacina* was the only known mycorrhizal fungi common in *Zygopetalum*, but the isolates from each orchid species belong to different *Sebacina* species.

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CONCLUSÕES E CONSIDERAÇÕES FINAIS

Neste estudo foi proposta uma metodologia eficiente para conservar o DNA de amostras radiculares colonizadas por fungos micorrízicos destinadas ao processo de extração de DNA. Este método consiste no armazenamento no tampão de lise SL1, o qual contém o detergente SDS, e na utilização de uma frequência angular de 6500 rpm e tempo de 48 s no homogeneizadar Precellys®24 durante a etapa de lise celular. Essa metodologia otimiza a extração de DNA e a conservação do DNA, o que permite a acessar toda informação genética presente nas amostras.

Pela análise dos dados independentes de cultivo, foi verificado que as espécies *Zygopetalum maxillare* e *Zygopetalum mackayi*, embora do mesmo gênero, apresentam um perfil diferente de fungos associados, sendo a maioria do filo Ascomycota. Ambas se associam com fungos das famílias Sebacinaceae e Ceratobasidiaceae. Entretanto, não compartilham nenhuma espécie da família Sebacinaceae.

Os resultados do isolamento de fungos corroboram com os observados através da metodologia independente de cultivo. Ou seja, para o estudo da diversidade de fungos associados as orquídeas são importantes o uso de ambas metodologias.

Os isolados fúngicos obtidos neste trabalho tem potencial para serem utilizados na germinação ou produção de mudas das espécies de orquídeas estudadas e também para futuros programas de reintrodução dessas orquídeas, caso esses isolados sejam compatíveis.