

## Endophytic fungi in roots of native orchids of rupestrian grasslands (campos rupestres) in Serra do Cipó, Brazil

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Recebido em 19.IX.2019

Aceito em 19.VIII.2021

DOI 10.21826/2446-82312021v76e2021021

**ABSTRACT** – Root endophytic fungi are essential to the life cycle of orchids. Many of them act as growth promoters of the host plant and mycorrhizal endophytes are indispensable to seed germination. This study aimed to evaluate the cultivable endophytic fungal richness of five native orchids of rupestrian grasslands (campos rupestres) in Serra do Cipó, Brazil. Healthy roots were sampled and the endophytes isolated, from which total DNA was extracted and the *Internal Transcribed Spacer* (ITS) region amplified and sequenced. Analysis of 32 fungal sequences permitted the identification of 25 species of the phyla Ascomycota and Basidiomycota. Nine isolates belonged to the Basidiomycota genera *Sebacina* Tul. & C. Tul. and *Tulasnella* J. Schröt., which contain important mycorrhizal endophytes of orchids. Same fungal species were obtained from different orchids growing on distinct substrates. Fungal molecular identification indicates high endophyte richness supporting the life cycle of orchids in rupestrian grasslands.

**Keywords:** Endemic orchids, Endophytes, Mycorrhizal fungi.

**RESUMO** – Fungos endofíticos radiculares são essenciais para o ciclo de vida das orquídeas. Muitos deles atuam como promotores de crescimento da planta hospedeira e os endófitos micorrízicos são indispensáveis a germinação de sementes. Este estudo objetivou avaliar a riqueza de fungos endofíticos cultiváveis de cinco orquídeas nativas de Campos Rupestres na Serra do Cipó, Brasil. Raízes saudáveis foram amostradas e os endófitos isolados. Cada isolado teve seu DNA total extraído e a região ITS (*Internal Transcribed Spacer*) amplificada e sequenciada. A análise de 32 sequências fúngicas permitiram a identificação de 25 espécies pertencentes aos filos Ascomycota e Basidiomycota. Nove isolados pertenciam aos gêneros *Sebacina* Tul. & C. Tul. and *Tulasnella* J. Schröt. do filo Basidiomycota, que são importantes gêneros de fungos micorrízicos de orquídeas. A mesma espécie fúngica foi obtida de diferentes orquídeas crescendo em substratos distintos. A identificação molecular dos fungos indicou alta riqueza de endófitos sustentando o ciclo de vida das orquídeas em campos rupestres.

**Palavras chave:** Endófitos, Fungo micorrízico, Orquídea endêmica.

### INTRODUCTION

Orchidaceae is one of the largest botanical families with an estimated number of more than 27,000 species (Jones 2006, The Plant List 2020). The greatest importance of these plants is the ornamental potential of their flowers, which are highly diverse in size and color combinations in colorful inflorescences (Cardoso & Israel 2005), making the family of great economic value. As a result, several native species are highly threatened by predatory collection and illegal trade, in addition to the destruction of their natural environment and climate change (Fay 2018).

In their natural environment, orchids associate with endophytic microorganisms, mainly fungi and bacteria that live inside plants and grow inside their tissues, without causing symptoms of disease (Azevedo 1998, Bayman & Otero 2006). Most endophytes bring benefits to plant development and the association can, in many cases, be

considered mutualistic (Bayman & Otero 2006, Dearnaley *et al.* 2012). Even though they are observed in different parts of the plant, fungal root endophytes have gained prominence due their role in promoting orchid growth and the dependence of seed germination on the interaction with mycorrhizal endophytes (Bayman & Otero 2006, Ma *et al.* 2015, Dearnaley *et al.* 2017).

Some endophytic fungi establish mutualistic symbioses with orchids, referred to as orchid mycorrhizal associations (OMAs), and are found in roots of adult plants, thus the term “mycorrhiza” meaning “fungus-root” (from the Greek mykes = fungus and rhiza = root) (Antonioli & Kaminski 1991, Smith & Read 2008, Yeh *et al.* 2019). OMAs are characterized by the presence of pelotons, which are mycorrhizal fungal hyphae colonizing inside compatible cells (Peterson *et al.* 2004, Dearnaley *et al.* 2017). Interaction with mycorrhizal fungi is essential for orchid seed germination (Peterson *et al.* 2004, Yeh *et al.*

2019). Since orchid species produce seeds that are extremely small and devoid of reserve tissue, seeds depend on embryo basal cell infection and colonization by mycorrhizal fungi to obtain the simple sugars and nutrients required for development through peloton digestion (Dearnaley *et al.* 2012, Kuga *et al.* 2014, McCormick *et al.* 2016). This metabolic strategy, called mycoheterotrophy, continues until the formation of the first leaf and the beginning of the seedling phase (Dearnaley *et al.* 2012).

The colonization of the first roots of seedlings is confirmed by the presence of pelotons in cortical cells (Peterson *et al.* 2004). During this phase, the seedling can interact with the same species of mycorrhizal fungus or other species of mycorrhizal fungi, as well as recruit some non-mycorrhizal endophytes (Smith & Read 2008, Rasmussen *et al.* 2015). Seedlings and adult individuals of chlorophyllous orchids can have a mixed biomass carbon source (mixoheterotrophic metabolism), part coming from atmospheric carbon and part from fungal carbon (Selosse *et al.* 2006). At this stage, the digestion of pelotons in the roots has the important function of providing or enhancing orchid uptake of organic and inorganic nutrients (Smith & Read 2008, Yeh *et al.* 2019). In achlorophyllous orchids, however, digestion by fungal hyphae is the main route of obtaining carbon as well as nutrients (Dearnaley *et al.* 2012, 2017).

A great diversity of endophytic fungi has been estimated by obtaining pure cultures from healthy roots of orchids sampled in their natural habitats and the identification of such endophytes has been an important source of information (Boldrini *et al.* 2010, Shah *et al.* 2019). The cultivation of isolates allows the interaction between plant and endophyte to be studied by *in vitro* fungal inoculation of seeds and seedlings, such as in experiments of seed germination (Pereira *et al.* 2011, 2015, Valadares *et al.* 2012) and seedling growth promotion (Shah *et al.* 2019), for example.

With the increase in predatory collection of orchids and the destruction of their natural habitat, the need for conservational strategies for them in their natural environment has become evident, especially for those that are endemic and/or at risk of extinction (Fay 2018). For this reason, it is extremely important to identify the richness of fungi that naturally interact with orchids, particularly for the reintroduction of these orchids in their natural environment. Therefore, the present work aimed to evaluate the richness of cultivable endophytic fungi associated with the roots of five orchid species that occur in rupestrian grasslands (*campos rupestres*) rock outcrop areas of Serra do Cipó, Brazil. The identification of endophytic fungal isolates will not only make it possible to assess the fungal richness on the roots of these orchid species, but will also document part of the fungal community interacting with them.

## MATERIAL AND METHODS

### Collection

The study site was in Serra do Cipó National Park, an area that belongs to the Cerrado Biome (Brazilian savanna). Serra do Cipó is located in the southern portion of Cadeia do Espinhaço in the state of Minas Gerais, Brazil (Abreu & Garcia 2005). The studied orchids were *Cattleya brevipedunculata* (Cogn.) Fowl, *Epidendrum saxatile* Lindl., *Epidendrum secundum* Jacq., *Grobya cipoensis* F. Barros & Lourenço, and *Pleurothallis teres* Lindl. All of which are native to Serra do Cipó, with *G. cipoensis* being endemic to this mountain range and *C. brevipedunculata* endemic to the state of Minas Gerais. The studied orchids were located on two rock outcrop areas with different habitats, but both with a predominance of species of *Vellozia* (Tab. 1). *Grobya cipoensis* and *C. brevipedunculata* are epiphytes and grow on *Vellozia gigantea* N.L. Menezes & Mello-Silva, while *Epidendrum saxatile* is also epiphyte but grows on *Vellozia piresiana* L.B.S. *Epidendrum* spp. and *P. teres* are saxicolous and grow in organic matter accumulated in rock fractures or on the edges of the outcrop (Tab. 1).

Three to five individuals were selected for each species. Samples consisted of entire individuals when the plants were of small size or when roots were well adhered to the substrate. In other cases, 10 to 15 root fragments were sampled per individual. The samples were packed in plastic bags and transported in a thermal bag to the Microbial Ecology Laboratory (Federal University of Viçosa, Rio Paranaíba Campus), where they were stored at 4 °C until isolation.

### Isolation of endophytic fungi

The roots were washed in running water and superficially disinfected in a series of immersions in 70 % ethanol for 1 min and 0.5 % sodium hypochlorite for 5 min, followed by three washes in sterile deionized water (Pereira *et al.* 2005). The roots were then aseptically sectioned transversely into fragments under a magnifying glass. Mycorrhizal colonization was confirmed by the presence of pelotons in cortical tissue. Fragments containing pelotons had their velamen removed and the colonized cortex portion was transferred to Petri dishes containing PDA medium (Potato Dextrose Agar, HIMEDIA). On average, five portions of cortex were transferred to each plate and five plates per plant were inoculated. The plates were then incubated at 25 °C and monitored for 15 days. When mycelium was observed growing from the root fragments, they were transferred to another plate containing PDA medium. Successive subcultures were performed to obtain pure culture. The cultural characteristics of fungal isolates were evaluated in PDA medium considering color (front and back of the colony), aspect and aerial mycelium (presence or absence).

**Table 1.** Species, habitat, collection site and sample codes of native orchids whose roots were sampled in rupestrian grasslands rock outcrop areas of Serra do Cipó, Brazil

Species	Habit	Collection site	Sample code
<i>Cattleya brevipedunculata</i>	Epiphyte	On <i>Vellozia gigantea</i>	SOA3, SOC2, SOC3 & SOC4
<i>Epidendrum saxatile</i>	Epiphyte	On <i>Vellozia piresiana</i>	EE2
<i>Epidendrum secundum</i>	Saxicolous	Rock fracture	EP2, ES2, ES3 & ES4
<i>Grobya cipoensis</i>	Epiphyte	On <i>Vellozia gigantea</i>	GC2, GC3 & GC4
<i>Pleurothallis teres</i>	Rupicolous	On rocks	PT2 & PT3

### Total DNA Extraction of endophytic fungi and ITS region amplification and sequencing

For molecular identification of the obtained endophytic fungi, total DNA was extracted from a portion of the mycelium grown in a Erlenmeyer flask containing Martin liquid medium (glucose 10 g L<sup>-1</sup>; peptone 5 g L<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub> 1 g L<sup>-1</sup>; MgSO<sub>4</sub> 0.2 g L<sup>-1</sup>; rose bengal 0.06 g L<sup>-1</sup>; and streptomycin 0.03 g L<sup>-1</sup>) or from a scraping of mycelium obtained in Petri dishes with PDA medium. The mycelium was transferred to 1.5 ml microtubes and the DNA was extracted using the NucleoSpin® Soil extraction kit (Macherey Nagel), according to the manufacturer's instructions. The DNA extracts were suspended in 20 µL of ultrapure water and quantified by electrophoresis in 0.8 % agarose gel with ethidium bromide 0.7 mg mL<sup>-1</sup>.

The ITS region of nuclear rDNA was amplified from total DNA of the fungal endophytes. The primer pair ITS1/ITS4 (White *et al.* 1990) and the polymerase chain reaction conditions described by Gardes & Bruns (1993) were used. The two strands of the amplified fragments were sequenced by Macrogen Inc. (Geumchun-gu, South Korea) using BigDye TM and a 3730xl Automatic Sequencer (Applied Biosystems).

### Sequence analysis

Contigs were constructed from the two sequence strands of endophytic ITS using the Sequencher program version 4.5 (Gene Codes). Species richness was determined based on the number of Operational Taxonomic Units (OTUs), where each OTU represented sequences with at least 97 % similarity. For this, the sequences were initially aligned by the ClustalW method (Katoh *et al.* 2005) using the MEGA program (Stecher *et al.* 2020). The alignment was then analyzed by the *dist.seqs* command to generate a matrix of calculated uncorrected pairwise distances, which was used to obtain the OTU list by the *cluster* command, both using the program Mothur (Schloss *et al.* 2009). Subsequently, the isolates were compared to sequences deposited in the NCBI database (GenBank, <http://www.ncbi.nlm.nih.gov>) and the UNITE Community database ([https://mothur.org/wiki/unite\\_its\\_database/](https://mothur.org/wiki/unite_its_database/)). Comparisons with the NCBI sequences were performed using the BLASTn algorithm (Altschul *et al.* 1997) and using the sequence with the

lowest e-value and the highest identity percentage as a reference. Comparisons with UNITE Community reference sequences were performed using the command *classify.seqs* of the Mothur program. These data were plotted using the program RStudio v1.1.463.

Sequences of *Tulasnella* and *Serendipita* were aligned separately with reference sequences available from the NCBI database and the UNITE Community database (Abarenkov *et al.* 2020). *Tulasnella* sequences were analyzed with other sequences of the same genus. *Serendipita* sequences were analyzed initially with sequences of the order Sebaciales and later with sequences of the family Serendipitaceae. The sets of sequences were aligned with the MAFFT v7.453 program using the L-INS-i strategy. The Hasegawa-Kishino-Yano DNA replacement model was obtained after analyzing the alignment of the sequences in the MrModeltest program (Nylander 2004), based on the Akaike information criterion. Phylogenetic reconstruction was performed by Bayesian inference using MrBayes 3.2.6 software (Ronquist *et al.* 2012) and the MCMC method (100,000,000 generations, 2 runs and 4 chains) at CIPRES Science Gateway (Miller *et al.* 2010). The results of the Bayesian analysis were evaluated in the program Tracer v1.7.1 (Rambaut *et al.* 2018) and the trees edited in FigTree v1.4.4. The SH DOI codes of each sequence used in the phylogenetic analyses are presented on the trees according to the identification provided by the UNITE platform. The access codes for NCBI or other platforms were also reported.

## RESULTS

Sixty-three isolates of endophytic fungi were obtained, of which 32 were identified molecularly (Tab. 2). The other isolates did not maintain viability until DNA extraction or presented problems during amplification or sequencing phases, preventing the acquisition of sequence data for molecular identification. The 32 isolates were clustered among 25 OTUs (Fig. 1), suggesting a richness of 25 species of endophytic fungi associated with the studied orchids. The orchid *C. brevipedunculata* had the most isolates (10) and OTUs (7), while *G. cipoensis* had the fewest, with three isolates and three OTUs (Fig. 1). *Epidendrum saxatile*, *E. secundum* and *P. teres* had, respectively four, eight and seven isolates and four, seven and five OTUs.

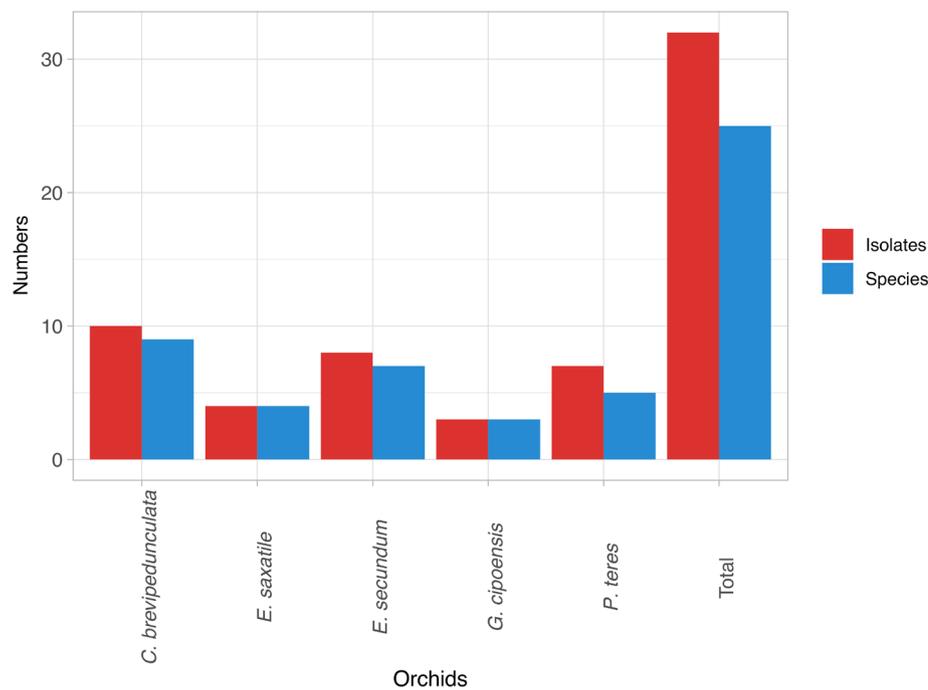
**Table 2.** Classification of endophytic fungal isolated from roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in an area of rupestrian field at Serra do Cipó/ Brazil. The classification was based on ITS sequence analysis of the fungal isolates. Phylum, order, genus and identification were chosen considering the lower “e-value” and higher identity percentage (Id%), to NCBI platform, and by “bootstrap” value (Bs), to UNITE platform

Phylum	Order	OTU	Isolates	Access	Identification	NCBI				UNITE		
						Access	Identification	E-value	Id%	Level*	Identification	Bs
Ascomycota	Boloniales	Otu10	GC3A1	KX185547	Endoxyla sp.	NR_111790	Endoxyla mallochii	3.00E-127	93.18	c	Sordariomycetes	98
	Botryosphaerales	Otu22	SOC3C2	KX185536	Endomelanconiopsis sp.	NR_158434	Endomelanconiopsis freycinetiae	0	98.41	s	Endomelanconiopsis microspora	96
		Otu18	EP2C5	KX185561	Neofusicoccum sp.	NR_152945	Neofusicoccum mangiferae	1.00E-131	94.97	f	Botryosphaeriaceae	94
Capnodiales	Otu11	ES2C1	KX185543	Toxicocladosporium sp.	NR_111765	Toxicocladosporium strelitziae	1.00E-155	97.49	g	Toxicocladosporium sp.	100	
Coniochaetales	Otu07	SOC2A3	KX185532	Coniochaeta sp.	NR_144912	Coniochaeta decumbens	0	99.77	f	Coniochaetaeaceae	85	
	Otu07	SOC2C3	KX185537	Coniochaeta sp.	NR_157478	Coniochaeta cipronana	0	98.37	f	Coniochaetaeaceae	97	
	Otu16	PT3B5	KX185556	Coniochaeta fasciculata	NR_154770	Coniochaeta fasciculata	0	98.40	s	Lecytophora fasciculata	95	
Eurotiales	Otu23	SOC4C2	KX185534	Aspergillus sp.	NR_135361	Aspergillus tabacinus	0	100.00	s	Aspergillus subversicolor	100	
	Otu01	PT2B5	KX185560	Penicillium citrinum	NR_121224	Penicillium citrinum	2.00E-114	100.00	s	Penicillium citrinum	92	
	Otu01	EE2B1	KX185562	Penicillium citrinum	NR_121224	Penicillium citrinum	0	100.00	s	Penicillium citrinum	92	
	Otu06	SOC3A3	KX185535	Talaromyces sp.	NR_120179	Talaromyces amestolkiae	4.00E-166	100.00	g	Talaromyces sp.	100	
	Otu06	GC4D2	KX185548	Talaromyces stollii	NR_111781	Talaromyces stollii	0	99.75	s	Talaromyces stollii	100	
Helotiales	Otu05	ES4C5	KX185540	Pezicula sp.	NR_155611	Pezicula melanigena	0	97.55	g	Cryptosporiopsis sp.	100	
	Otu05	ES4C3	KX185545	Pezicula sp.	NR_155611	Pezicula melanigena	3.00E-177	97.51	g	Cryptosporiopsis sp.	100	
	Otu02	PT2C2	KX185557	Pezicula sp.	NR_155611	Pezicula melanigena	0	97.39	f	Dermateaceae	100	
	Otu02	PT2C5	KX185558	Pezicula sp.	NR_155611	Pezicula melanigena	0	97.18	f	Dermateaceae	100	
	Otu19	ES2C2	KX185543	Scytalidium sp.	NR_160179	Scytalidium vaccinii	1.00E-171	93.38	c	Leotiomyces	100	
Hypocreales	Otu09	GC3B5	KX185546	Fusarium sp.	NR_130690	Fusarium keratoplasticum	5.00E-175	99.70	g	Fusarium sp.	84	
Trichosphaerales	Otu08	ES3B5	KX185544	Nigrospora sp.	NR_153480	Nigrospora hainanensis	6.00E-164	97.34	o	Trichosphaerales	100	
Xylariales	Otu13	SOC3A4	KX185531	Xylaria sp.	NR_153200	Xylaria bambusicola	0	94.61	g	Xylaria sp.	98	
	Otu12	EE2A	KX185550	Daldinia sp.	NR_147520	Daldinia hausknechtii	0	98.85	s	Daldinia loculata	95	
	Otu24	SOC2C4	KX185533	Hypoxyylon sp.	NR_145212	Hypoxyylon hinnuleum	4.00E-141	88.89	g	Hypoxyylon sp.	80	
	Otu14	EE2B5	KX185552	Nodulisporium sp.	NR_166005	Nodulisporium indicum	0	93.13	o	Xylariales	100	

Table 2. Cont.

Phylum	Order	OTU	Isolates	Access	Identification	NCBI				UNITE		Bs
						Access	Identification	E-value	Id%	Level*	Identification	
Basidiomycota	Cantharellales	Otu21	SOC2A5	KX185539	Tulasnella sp.	NR_160570	Tulasnella cumulopuntiooides	1.00E-146	87.97	f	Tulasnellaceae	92
		Otu25	EE2B3	KX185551	Tulasnella sp.	NR_160569	Tulasnella dendritica	3.00E-48	86.63	p	Basidiomycota	100
		Otu03	PT2A5	KX185555	Tulasnella sp.	NR_160570	Tulasnella cumulopuntiooides	2.00E-154	88.41	g	Epulorhiza sp.	100
		Otu03	PT3B3	KX185553	Tulasnella sp.	NR_160570	Tulasnella cumulopuntiooides	2.00E-164	88.20	g	Epulorhiza sp.	100
		Otu15	PT3C2	KX185554	Tulasnella sp.	NR_160570	Tulasnella cumulopuntiooides	4.00E-176	90.55	g	Tulasnella sp.	100
		Otu17	SOA3	KX185559	Tulasnella sp.	NR_160569	Tulasnella dendritica	9.00E-48	86.55	g	Tulasnella sp.	86
	Sebacinales	Otu04	SOC2B3	KX185538	Serendipita sp.	NR_144842	Serendipita herbamans	2.00E-93	88.50	o	Serendipitaceae	93
		Otu04	ES3A1	KX185541	Serendipita sp.	NR_144842	Serendipita herbamans	1.00E-105	85.95	o	Serendipitaceae	82
		Otu20	ES2A4	KX185542	Serendipita sp.	NR_144842	Serendipita herbamans	7.00E-104	86.42	o	Serendipitaceae	98

\* p: phylum; o: order; c: class; f: family; g: genera; s: species.



**Figure 1.** Number of isolates and species of endophytic fungi obtained from roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in a rupestrian grasslands of Serra do Cipó, Brazil. Species definition was based on ITS sequence analysis, considering isolates with greater than 97% similarity as belonging to the same fungal species.

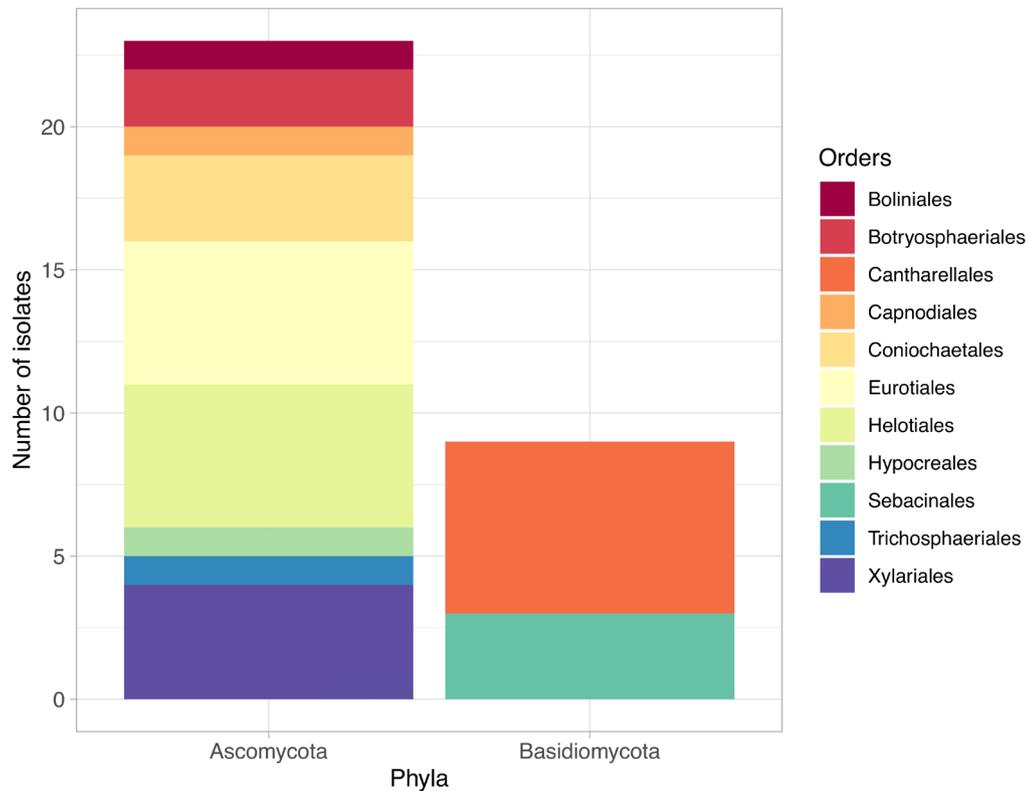
Comparisons of the endophytic ITS sequences to those from NCBI and UNITE failed to identify all of the isolates at species level. However, the association between the results obtained from classifications using the different databases allowed identifying the endophytes to the genus level (Tab. 2).

Twenty-three isolates were classified as belonging to the phylum Ascomycota and nine to the phylum Basidiomycota (Tab. 2, Fig. 2). Among the isolates of Ascomycota were 16 different genera and nine orders: Boliniales, Botryosphaeriales, Capnodiales, Coniochaetales, Eurotiales, Helotiales, Hypocreales, Trichosphaeriales and Xylariales (Tab. 2, Fig. 2 and 3). The isolates of Basidiomycota belonged to two genera, *Tulasnella* and *Serendipita*, of two different orders, Cantharellales and Sebaciniales, respectively (Tab. 2, Figs. 2, 3).

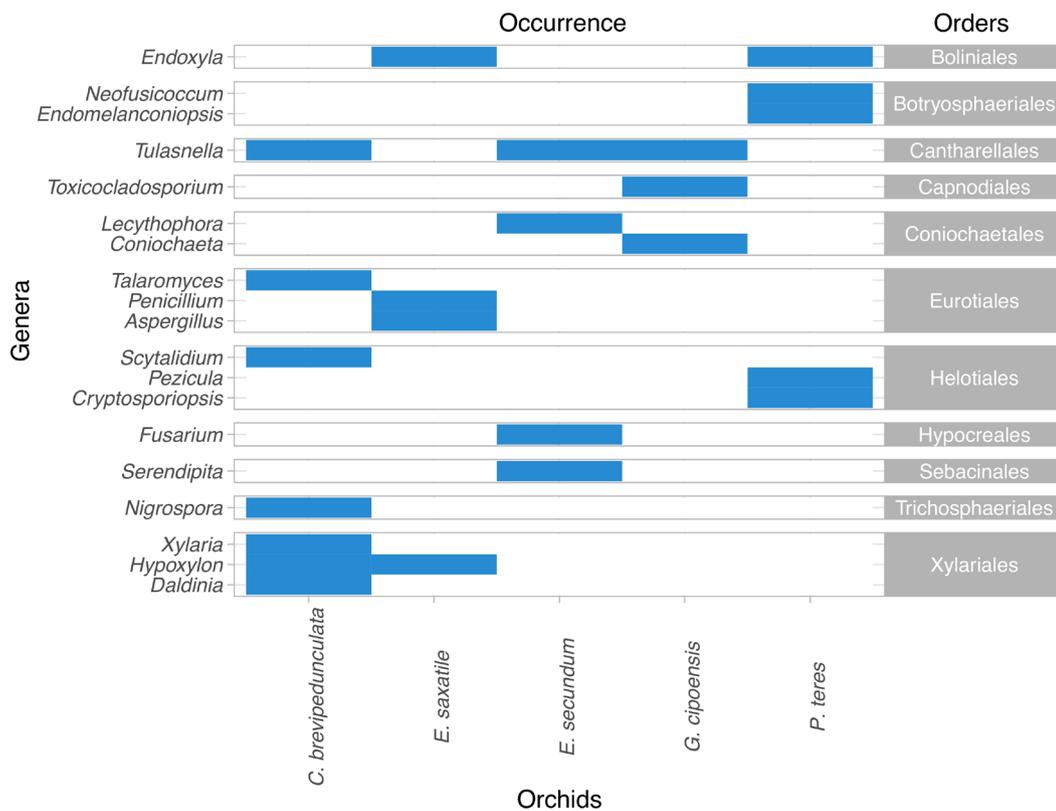
Phylogenetic analysis considering 198 Sebaciniales reference sequences confirmed that isolates SOC2B3, ES3A1 and ES2A4 belonged to the genus *Seperendita* of the family Serendipitaceae (data not shown). A phylogenetic reconstruction was performed with 34 sequences from this family (Fig. 4), disregarding the sequences of *Serendipita indica* (Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken) M. Weiß *et al.* and *S. williamsii* (A. Zuccaro & M. Weiß) M. Weiß *et al.*, which differed greatly from the studied endophytic sequences. Two sequences of *Paulisebacina allantoidea* (R. Kirschner & Oberw.) Oberw., family Sebacinaceae, were used as an outgroup. The isolates SOC2B3, ES3A1 and ES2A4 did not group with sequences of any of the previously identified species, and only with sequences of fungi classified as Serendipitaceae; however, they presented proximity to the clade of

*Serendipita vermifera* (Oberw.) P. Roberts. The ES2A4 isolate grouped with a mycorrhizal fungus of the Brazilian orchid *Hoffmannseggella caulescens* (Lindl.) HGJones (SH197470.07FU). The isolates SOC2B3 and ES3A1 grouped with mycorrhizal fungi of *Hoffmannseggella cinnabarina* (Batem. Ex Lindl.; SH197471.07FU, SH197472.07FU and SH197474.07FU), also from Brazil, and of orchids from Reunion Island (SH197483.07FU and SH105390.07FU) (Fig. 4B).

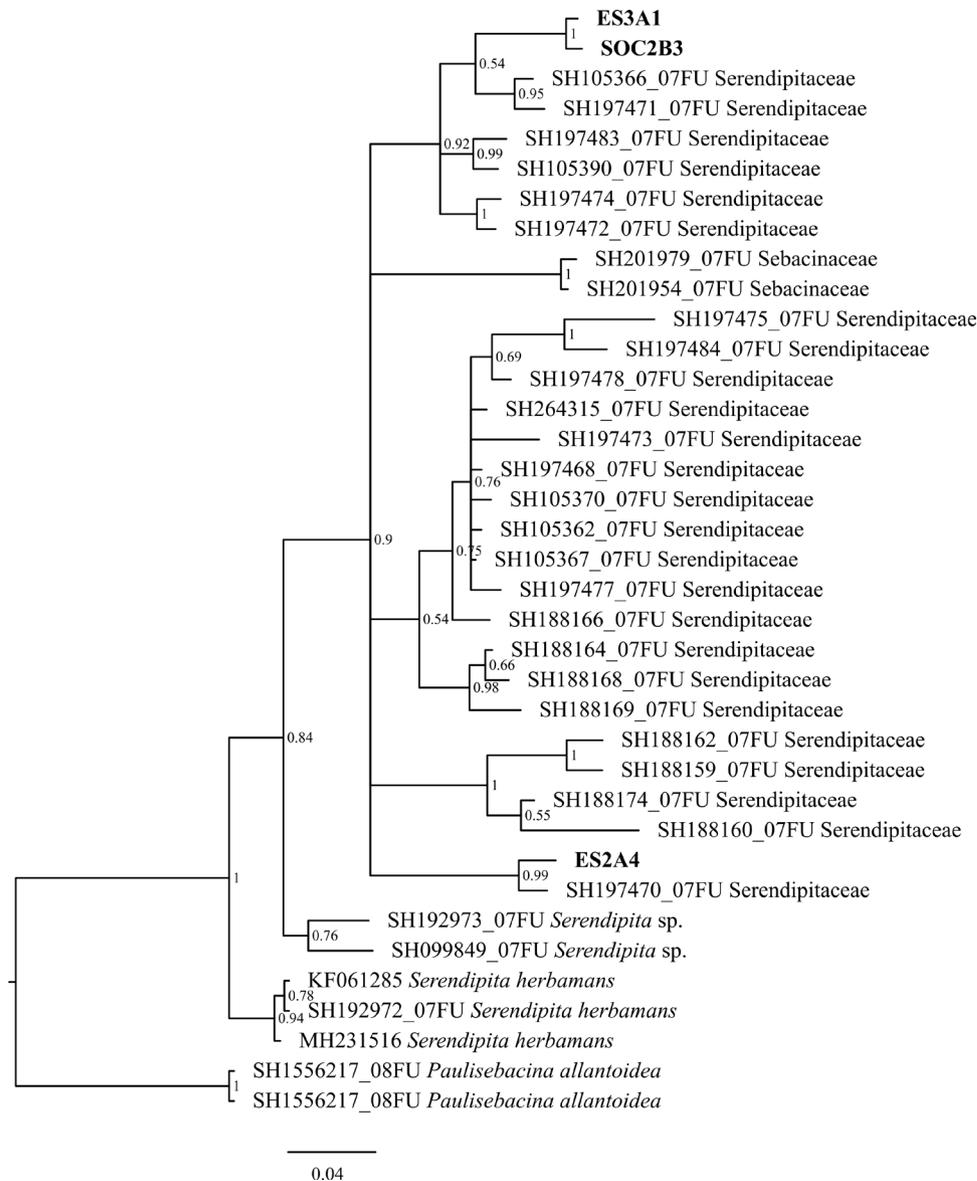
Phylogenetic analysis containing 282 Tulasnellaceae reference sequences confirmed the classification of isolates EE2B3, PT3C2, PT3B3, PT2A5, SOA3 and SOC2A5 as *Tulasnella* sp. (data not included). A phylogenetic reconstruction was performed with 70 *Tulasnella* sequences, considering the species *Tulasnella eichleriana* Bres., *T. tomaculum* P. Roberts and *T. violea* (Quél.) Bourdot & Galzin as outgroups (Fig. 5). Isolate EE2B3 formed a clade with three isolates of *Tulasnella*: two mycorrhizal orchids from Ecuador (SH193016.07FU and SH193015.07FU) and one from Costa Rica (SH193018.07FU). Isolate SOA3 grouped with symbionts of orchids from Japan (SH203337.07FU and SH203339.07FU) and Reunion Island (SH203338.07FU). Isolate SOC2A5 showed high similarity with two mycorrhizal *Tulasnella* from Ecuador (SH195570.07FU and SH192337.07FU), one from Reunion Island (SH195571.07FU) and another from Costa Rica (SH195573.07FU). Isolate PT3C2 grouped with isolates from Ecuador (SH199030.07FU and SH213190.07FU) and South Africa (SH213195.07FU). Finally, isolates PT3B3 and PT2A5 grouped with a *Tulasnella* from the United States (SH212756.07FU), in a clade close to *T. caulospora* (Boud.) Juel (SH212756.07FU).



**Figure 2.** Number of fungal isolates at the levels of phylum and order obtained from roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in an area of rupestrian grasslands at Serra do Cipó/ Brazil.



**Figure 3.** Fungal genera occurring as endophytic in roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in a rupestrian grasslands of Serra do Cipó, Brazil.



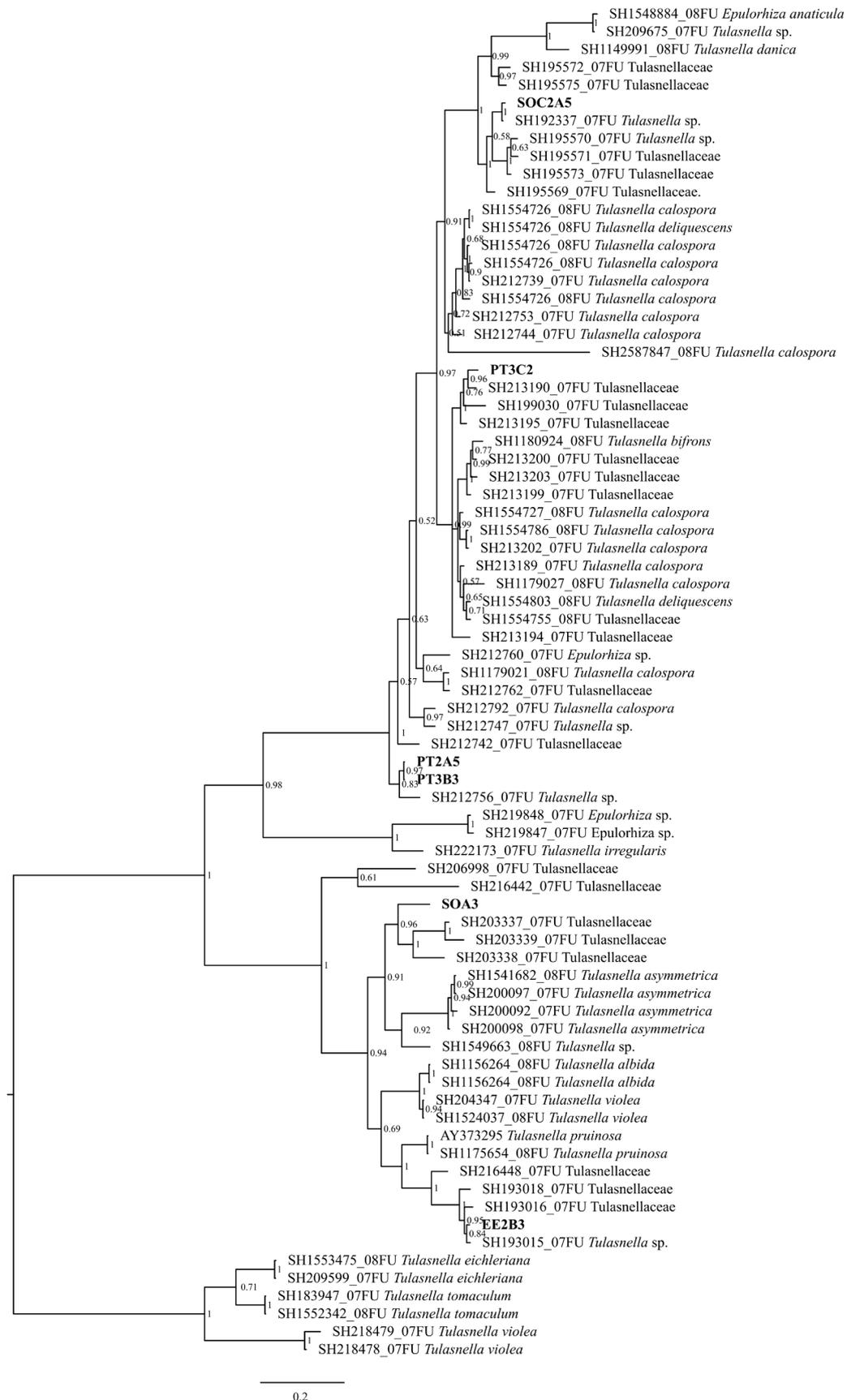
**Figure 4.** Phylogenetic inference of the endophytic fungal genus *Serendipita* generated by Bayesian analysis of ITS sequences. *A posteriori* probabilities are indicated on the nodes. Bold codes and respective NCBI access numbers for the isolates obtained from roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in a rupesrian grasslands of Serra do Cipó, Brazil. Identification and accession number for NCBI and UNITE platform are provided to reference sequences.

## DISCUSSION

Twenty-five OTUs were identified by the ITS sequence analysis of the 32 isolates, suggesting that 25 different fungal species establish associations with the roots of the studied orchids (Fig. 1). More than 70% of the OTUs were unique and only seven were represented by more than one isolate (Table 2). These values can be considered high when compared to the number of possible species obtained by other studies of cultivable endophytes (Pereira *et al.* 2005, 2014, Valadares *et al.* 2012, 2015). The present work, however, sampled different species of orchids growing on different substrates, while the other works investigated fewer orchids and less diverse habits. Since 25 species of fungi were determined from the 32 identified isolates,

a greater effort at isolation would be expected to reveal even higher richness of endophytic fungi in association with the studied orchids.

Some of the studied orchids had associations with the same OTU, even when growing on different substrates. This was observed for the epiphytic orchid *E. saxatile*, which interacted with a fungus also isolated from the rupicolous species *P. teres* (Otu01); and for the epiphytic orchid *C. brevipedunculata* and the saxicolous *E. secundum*, which interacted with Otu04 isolates (Table 2). *Cattleya brevipedunculata* and *G. cipoensis*, growing as epiphytes, interacted with Otu06 isolates (Table 2). Adult plants of some orchids establish mycorrhizal associations to more easily obtain nutrients from the substrate (Dearlaney *et al.* 2017). Some plant species, however, may not establish



**Figure 5.** Phylogenetic inference of the endophytic fungal genus *Tulasnella* generated by Bayesian analysis of ITS sequences. *A posteriori* probabilities are indicated on the nodes. Bold codes and respective NCBI access numbers for isolates obtained from roots of the five native orchids (*Cattleya brevipedunculata*, *Epidendrum saxatile*, *E. secundum*, *Grobya cipoensis* and *Pleurothallis teres*) sampled in a rupestrian grasslands of Serra do Cipó, Brazil. Identification and accession number for NCBI and UNITE platform are provided to reference sequences.

associations with the same mycorrhizal fungus when co-occurring in the same region and/or on the same substrate, to avoid competition (Valadares *et al.* 2015). But our data corroborate the observation that different species of orchids interact with the same mycorrhizal fungal isolates when they co-occur in the same area and/or substrate (Chen *et al.* 2019, Herrera *et al.* 2019).

The number of isolates for Ascomycota was more than 2.5 times that for Basidiomycota (Fig. 2, Table 2). Only the genera *Serendipita* and *Tulasnella* were observed among Basidiomycota isolates, and both genera are reported as mycorrhizal on several orchids (Dearnaley *et al.* 2017). In addition, in contrast with several studies on tropical orchids, the genus *Ceratobasidium* was not detected in the present work (Mosquera-Espinosa *et al.* 2013, Valadares *et al.* 2015).

During the isolation protocol employed here, only root fragments containing pelotons were transferred to isolation media. Since pelotons are considered a characteristic structure of mycorrhizal association in orchids, this protocol would favor the isolation of the mycorrhizal fungi (Pereira *et al.* 2014, Valadares *et al.* 2012, 2015). However, non-mycorrhizal endophytic fungi grow in roots without forming easily visible structures (Bayman & Otero 2006) and can co-occur with the mycorrhizal fungi in root fragments (Azevedo 1998). If a non-mycorrhizal fungus is colonizing the root and presents a faster growth rate, it will be recultivated to the detriment of mycorrhizal fungi, making it possible to isolate non-mycorrhizal endophytes even by transferring fragments of the cortex containing pelotons.

Mycorrhizal and non-mycorrhizal endophytic fungi are extremely important to orchid maintenance in the field (Bayman & Otero 2006, Salazar-Cerezo *et al.* 2018). Non-mycorrhizal fungi of Ascomycota and Basidiomycota have been described in other works (Waud *et al.* 2016, Salazar-Cerezo *et al.* 2018, Shah *et al.* 2019). Studies using independent cultivation methods molecularly identified a great diversity of fungal endophytes, revealing that the diversity of symbionts associated with orchid roots is much more complex than previously thought (Dearnaley *et al.* 2017, Waud *et al.* 2016, Shah *et al.* 2019). Faced with a large amount of information on the endophytic community of plants, the challenge is to understand the role of the fungal diversity of this microbial community interacting with a plant. Methods for proposing functional roles based on fungal classification have been developed (<http://www.stbates.org/guilds/app.php>), and permit a partial understanding of the role of each fungus detected in a sample of an environment (Cevallos *et al.* 2018).

Non-mycorrhizal and mycorrhizal fungi are assigned the roles of enhancing plant access to nutrients; protecting against desiccation; providing protection against pathogens by releasing secondary metabolites and by inducing plant resistance; and producing phytohormones that assist in plant development (Dutta *et al.* 2014, Ma *et al.* 2015). Moreover, mycorrhizal fungi are also responsible for the germination of orchid seeds under natural conditions (Peterson *et al.*

2004, Dearnaley *et al.* 2017, Yeh *et al.* 2019). Thus, non-mycorrhizal fungi are a fundamental component of the microbial community of the substrate.

Some Basidiomycota of the classes Atractiellomycetes and Agaricomycetes, as well as Ascomycota of the class Pezizomycetes, were listed as possibly being mycorrhizal (Dearnaley *et al.* 2012, 2017, Waud *et al.* 2016). However, among the isolates obtained in this study, only the Basidiomycota isolates of the genera *Sebacina* and *Tulasnella*, both from the class Agaricomycetes, can be considered mycorrhizal. Since the isolates of Ascomycota obtained in this study were not listed as mycorrhizal with orchids, they were considered as non-mycorrhizal endophytes.

Ascomycota corresponded to more than 70 % of the isolates and OTUs in this study, and were obtained from all the orchids studied (Table 2), which is in accordance with their reported abundances in the endophytic communities of other orchids (Waud *et al.* 2016, Shah *et al.* 2019). This indicates the importance of Ascomycota as orchid endophytes that promote the growth of their hosts (Azevedo 1998, Selosse *et al.* 2004, Bayman & Otero 2006, Salazar-Cerezo *et al.* 2018).

Some roles of the obtained Ascomycota groups are known. Species of the orders Botryosphaeriales and Capnodiales are found as pathogens, endophytes and saprobes degrading cellulose and other complex carbohydrates (Schoch *et al.* 2006). Eurotiales, represented here by the genera *Aspergillus* and *Penicillium*, comprises a group of morphologically and ecologically divergent fungi that are capable of growing in extreme environments and producing several secondary metabolites, such as penicillin (Geiser *et al.* 2006). Endophytic *Penicillium* isolates of orchids have shown the ability to produce gibberellins, thus suggesting potential for regulating plant growth (Salazar-Cerezo *et al.* 2018). The order Helotiales, one of the largest in the phylum Ascomycota (Wang *et al.* 2006a, 2006b), has been reported as being among the taxa with the greatest occurrence in other orchid species (Ma *et al.* 2015, Jaquemyn *et al.* 2016). These fungi have varied ecological and nutritional characteristics, and develop well in various ecosystems as pathogens, endophytes, mycorrhizae, ectomycorrhizae, parasites, terrestrial saprobes, and aquatic saprobes (Wang *et al.* 2006b). The present study obtained five isolates of this order, but only from *E. secundum* and *P. teres*, two orchid species observed to be preferentially saxicolous and rupicolous (Table 2).

The present study obtained a high number of representatives of the class Sordariomycetes, which includes the orders Xylariales, Trichosphaeriales, Hypocreales, Coniochaetales and Coniochaetales (Table 2). According to Zhang *et al.* (2006), this class includes endophytic organisms of plants, including mycoparasites and saprobes, the latter being involved in the decomposition and cycling of nutrients. Their hosts also benefit from increased resistance to infection by pathogens and insect predation through the production of bioactive compounds (Ma *et al.* 2015).

Microorganisms of the genera *Fusarium* and *Xylaria* are known to be plant pathogens, but they may have potential for use in the germination of orchid seeds (Salifah *et al.* 2011). The genera *Coniochaeta* and *Fusarium* also were reported as endophytic of terrestrial orchids (Lee & Eom 2017, Lee *et al.* 2017).

According to Hibbett *et al.* (2007), fungi of the class Agaricomycetes are common in all terrestrial environments and can be pathogens, parasites and symbionts of plants and animals, and many form fruiting bodies known as mushrooms. This class was represented here by *Serendipita* and *Tulasnella*, which belong to the orders Cantharellales and Sebaciales, respectively (Table. 2). Many fungi of these orders are referred to as rhizoctonioids (Roberts 1999, García *et al.* 2006). They are the most important mycorrhizal fungi of orchids (Dearnaley *et al.* 2017), and have been frequently reported as mycorrhizal with them (Robert 1999, García *et al.*, 2006, Dearnaley *et al.*, 2017).

Even with the large number of described species of *Sebaciales* and *Tulasnella*, and the availability of their sequences from NCBI and UNITE, it was not possible to identify the *Serendipita* and *Tulasnella* isolates of this work to species level due to the low similarity they exhibited with sequences in these databases (Fig. 4 and 5). The clades in which they were found contain only sequences of fungi without identification, and so they may represent undescribed species.

The *Serendipita* isolates of the present study grouped with endophytes from other orchids and were closest to endophytes from two orchids also from rupestrian grasslands in Minas Gerais, Brazil (Fig. 4). However, interpreting this as speciation due to geographical location is questionable, since a *Serendipita* observed in orchids from Reunion Island is in the same clade. The clade containing the *Serendipita* fungi of the present study was near the clade containing *S. vermifera*, a fungus often described as mycorrhizal of orchids and plants of other families (Weiss *et al.* 2004, Weiß *et al.* 2016). *Serendipita vermifera* represents a fungal complex, assembling isolates from different regions of the world that present similar morphological features, but high ITS variation (Weiß *et al.* 2016). These taxonomical complexes emerge from difficulties related to the association of morphological and molecular data in classification, and highlights the need to search for additional useful morphological and/or molecular characters that can enlighten fungal classification.

The same limitation has been reported for *Tulasnella* concerning the species *T. calospora*. Although mycorrhizal fungi isolated with various orchids have been reported as belonging to this species, phylogenetic analyses have suggested that this taxon encompasses different fungal species (Suarez *et al.* 2006). Four of the *Tulasnella* isolates of the present study (PT2A5, PT3B3, PT3C2 and SOC2A5) were placed in clades close to those containing *T. calospora* (Fig. 5), but the distances observed between the studied isolates and *T. calospora*, on phylogenetic tree

and in comparisons with NCBI and UNITE sequences, revealed insufficient similarity to confirm this identification. Likewise, the *Tulasnella* isolates EE2B3 and SOA3 were not sufficiently similar to any sequence of any previously identified fungal species. These six isolates were similar to *Tulasnella* fungi observed in association with orchids in different parts of the world, indicating that they are cosmopolitan species.

Works with Brazilian orchids have demonstrated that the isolates from Cantharellales and Sebaciales, especially the genera *Tulasnella* and *Ceratobasidium*, is essential for the germination of orchid seeds, the early development of embryos and the growth of seedling development (Pereira *et al.* 2011, 2015, Guimarães *et al.* 2013, Valadares *et al.* 2012), and are frequently observed in association with adult plants (Pereira *et al.* 2005, Valadares *et al.* 2015). Therefore, *Sebacia* and *Tulasnella* isolates, the only mycorrhizal fungi isolates found, may be the main fungi responsible for orchid seed germination in the studied rupestrian grasslands. The structure of an endophytic fungal community, and its variation throughout the life cycle stages of a plant, are critical factors for maintaining orchids in natural conditions and deserving of further attention (Rasmussen *et al.* 2015, Shao *et al.* 2017). The high number of Ascomycota isolates suggests they are important components of the endophytic community of orchid roots in the studied rupestrian grasslands. Additionally, further studies are needed that describe species of fungal endophytes and study their roles in the life cycle of orchids.

## ACKNOWLEDGEMENT

We thank the the Minas Gerais State Research Support Foundation (FAPEMIG) and National Council for Scientific and Technological Development (CNPq) for maintaining the scientific initiation scholarships and for financing the projects (CAG-APQ-01744-13 and APQ-429102/2016-0, respectively).

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