EMILIANE FERNANDA SILVA FREITAS

TAXONOMIA E FILOGENIA MOLECULAR DE FUNGOS MICORRÍZICOS E ENDOFÍTICOS ASSOCIADOS AO SISTEMA RADICULAR DE ORQUÍDEAS DA MATA ATLÂNTICA

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

Orientadora: Maria Catarina Megumi Kasuya

Coorientadoras: Meiriele da Silva
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RESUMO

FREITAS, Emiliane Fernanda Silva, D.Sc., Universidade Federal de Viçosa, março de 2021. **Taxonomia e filogenia molecular de fungos micorrízicos e endofíticos associados ao sistema radicular de orquídeas da Mata Atlântica.** Orientadora: Maria Catarina Megumi Kasuya. Coorientadoras: Meiriele da Silva e Marisa Vieira de Queiroz.

Orchidaceae é a maior família entre as Angiospermas, com representantes distribuídos em todos os continentes. No Brasil, a Mata Atlântica é considerada um berço de diversidade de orquídeas, muitas das quais estão em risco de extinção. Uma característica comum entre as espécies de orquídeas é a produção de sementes sem tecido de reserva, que carecem de uma fonte externa de carbono para a germinação. Em ambiente natural essa necessidade é suprida por meio da associação com fungos micorrízicos, o que as tornam dependentes desses simbiontes. Os fungos micorrízicos de orquídeas são conhecidos como fungos rizoctonioides, um grupo polifilético que inclui taxa pertencentes às famílias Ceratobasidiaceae, Sebacinaceae, Serendipitaceae e Tulasnellaceae, e que representam um grupo de taxonomia complexa devido à escassez de caracteres morfológicos relevantes. As orquídeas abrigam também uma grande diversidade de fungos endofíticos radiculares pouco explorados, e que têm mostrado potencial para produção de metabólitos secundários com potencial para promoção de crescimento e controle de doenças. Diante disso, esta tese teve como objetivo estudar a diversidade taxonômica e a filogenia de fungos micorrízicos e endofíticos associados à *Cattleya jongheana*, uma espécie de orquídea endêmica da Mata Atlântica, e em risco de extinção. Nossos resultados mostram que *C. jongheana* se associa frequentemente com fungos micorrízicos do gênero *Tulasnella,* e com menor frequência com o gênero *Serendipita*, e que muitos dos isolados obtidos são espécies ainda desconhecidas. Dessa forma, análises de filogenia associadas a caracteres morfológicos nos permitiram caracterizar seis novas espécies de *Tulasnella* e uma nova espécie de *Serendipita,* como definido no Código Internacional de Nomenclatura para Algas, Fungos e Plantas. Entre os fungos endofíticos associados às raízes de *C. jongheana*, observamos que *Colletotrichum*, *Microdiplodia*, *Trichoderma* e *Xylaria* são os gêneros mais recorrentes, e uma nova espécie de *Neopestalotiopsis* foi descrita: *Neopestalotiopsis hadrolaeliae*. Nosso estudo contribui para o conhecimento da diversidade de fungos micorrízicos e endofíticos associados à *C. jongheana*, bem como para a descrição de novas espécies fúngicas. Informações a respeito de parceiros micorrízicos e endofíticos são essenciais

para a conservação de populações de orquídeas. Dessa forma, nossos resultados têm implicações na preservação da espécie estudada.

Palavras-chave: Orchidaceae. Micorrizas. *Tulasnella*. *Serendipita*. Conservação.

ABSTRACT

FREITAS, Emiliane Fernanda Silva, D.Sc., Universidade Federal de Viçosa, March, 2021. **Taxonomy and molecular phylogeny of mycorrhizal and endophytic fungi associated with the root system of orchids from Atlantic Forest.** Advisor: Maria Catarina Megumi Kasuya. Co-advisors: Meiriele da Silva and Marisa Vieira de Queiroz.

Orchidaceae is the largest family among Angiosperms, with representatives distributed on all continents. In Brazil, the Atlantic Forest is rich in orchid diversity, many of which are at risk of extinction. A common feature among orchid species is the production of seeds without reserve tissue, which require an external source of carbon for germination. In a natural environment, this demand is supplied by the association with mycorrhizal fungi, which make orchids dependent on these symbionts in the earliest stages of development. Mycorrhizal fungi of orchids are known as *Rhizoctonia*-like, a polyphyletic group that includes taxa belonging to the families Ceratobasidiaceae, Sebacinaceae, Serendipitaceae and Tulasnellaceae, and which represent a complex taxonomy group due to the scarcity of relevant morphological characters. Orchids also host a wide variety of under-explored root endophytic fungi, which have shown an ability for the production of secondary metabolites with the potential to promote growth and control diseases. This study aimed to investigate the taxonomic diversity and phylogeny of mycorrhizal and endophytic fungi associated with *Cattleya jongheana*, a species of orchid endemic to the Brazilian Atlantic Forest, and at risk of extinction. Our results show that *C. jongheana* is frequently associated with mycorrhizal fungi of the genus *Tulasnella*, and less frequently with the genus *Serendipita*, and that most of the isolates obtained are still unknown species. Thus, phylogeny analyses associated with morphological characters allowed us to characterize six new species of *Tulasnella* and a new species of *Serendipita*, which will be proposed as defined in the International Nomenclature Code for Algae, Fungi and Plants. Among the endophytic fungi associated with *C. jongheana* roots, we observed that *Colletotrichum*, *Microdiplodia*, *Trichoderma* and *Xylaria* are the most recurrent genera, and a new species of *Neopestalotiopsis* was described: *Neopestalotiopsis hadrolaeliae*. Our study contributes to the knowledge of the diversity of mycorrhizal and endophytic fungi associated with *C. jongheana*, in addition to the description of new fungal species. Information about mycorrhizal and endophytic partners is essential for the conservation of orchid populations. So, our results have direct implications for the preservation of the species studied.

Keywords: Orchidaceae. Micorrizas. *Tulasnella*. *Serendipita*. Conservação.

SUMÁRIO

INTRODUÇÃO GERAL

A família Orchidaceae é a mais diversa entre todas as Angiospermas com aproximadamente 27 mil espécies descritas (Govaerts 2019). As orquídeas estão distribuídas em todos os continentes e em diferentes tipos de habitat, com espécies epífitas, terrestres ou rupícolas. O Brasil abriga cerca de 2650 espécies (Giulietti et al. 2005) e, entre os biomas brasileiros, a Mata Atlântica destaca-se em diversidade de orquídeas. Apesar da grande diversidade, muitas espécies se encontram em algum grau de risco de extinção, devido à destruição de hábitats, eliminação de polinizadores e coleta predatória (Martinele & Moraes 2013).

As sementes da maioria das espécies de orquídeas são muito pequenas e não apresentam endosperma suficiente para garantir a germinação do embrião, o que as tornam dependentes de uma fonte externa de carbono (Rasmussen 1995). Em ambiente natural, o carbono é fornecido para as sementes através de associações com fungos micorrízicos (Rasmussen & Rasmussen 2009). Os simbiontes fúngicos colonizam as células do embrião da orquídea e formam enovelados de hifas, chamados *pelotons*, que são degradados liberando carbono e outros nutrientes, o que torna possível a germinação das sementes e o estabelecimento das plantas (Dearnaley et al. 2012). A associação micorrízica pode se manter até a fase adulta da planta e, dessa forma, parte do carbono é obtido a partir da fotossíntese e outra parte através dos simbiontes fúngicos (orquídeas mixotróficas), ou no caso das orquídeas aclorofiladas todo o carbono fornecido à planta é obtido através do parceiro fúngico (orquídeas micoheterotróficas) (Dearnaley et al. 2012).

Além de carbono, os simbiontes fúngicos também podem auxiliar a planta na absorção de água e outros nutrientes, como fósforo e nitrogênio (Cameron et al. 2006;

2007; Dearnaley & Cameron 2017). Os fungos micorrízicos isolados a partir de raízes de orquídeas também têm demonstrado outras funções ecológicas importantes quando inoculados em outras plantas, como controle biológico de fitopatógenos (Mosquera-Espinosa et al. 2013; Jiang et al. 2016), promoção de crescimento (Fritsche et al. 2021) e resistência a metais pesados (Herrera et al. 2018).

Entre os principais fungos micorrízicos com os quais as orquídeas se associam estão os fungos rizoctonioides do Filo Basidiomycota, um grupo polifilético que inclui taxa pertencentes às famílias Ceratobasidiaceae, Sebacinaceae, Serendipitaceae e Tulasnellaceae (Rasmussen 1995; Weiß et al. 2016). Devido à escassez de características morfológicas e culturais relevantes e a dificuldade em induzir a fase sexuada desse grupo, a identificação dos gêneros e espécies se baseava principalmente na condição nuclear e ultraestrutura dos septos, o que tornava a taxonomia dos fungos rizoctonióides muito complexa (Currah & Zelmer 1992). Atualmente, estudos taxonômicos têm utilizado uma abordagem molecular, associada a características morfológicas, para identificar e descrever novas espécies de fungos micorrízicos de orquídeas (Suárez et al. 2006; Linde et al. 2017; Solís et al. 2017; Freitas et al. 2020). Para o gênero *Tulasnella*, que tem sido o principal simbionte de orquídeas brasileiras, sabe-se que a região ITS (*Internal Transcribed Spacer*) do DNA ribossomal fornece um bom suporte para estudos filogenéticos e identificação de novas espécies (Linde et al. 2014; 2017).

O tecido cortical das raízes de orquídeas abriga também uma grande diversidade de fungos endofíticos não micorrízicos. O termo endofítico é utilizado para se referir aos "organismos que em algum momento da vida podem colonizar os tecidos internos das plantas sem causar dano aparente ao seu hospedeiro" (Petrini 1991). De modo geral, fungos endofíticos são conhecidos por produzirem metabólitos secundários com potencial para promoção de crescimento de plantas, controle de insetos e patógenos de plantas

(Porras-Alfaro & Bayman 2011). Assim, tem crescido o interesse em conhecer a diversidade de fungos endofitícos em orquídeas (Ma et al. 2015). Esses fungos pertencem predominantemente ao filo Ascomycota, e os gêneros *Colletotrichum*, *Fusarium, Trichoderma* e *Verticillium* têm sido frequentemente isolados (Ma et al 2015). Os fungos endofíticos em raízes de orquídeas têm mostrado papel importante no desenvolvimento das plantas, como promoção do crescimento e defesa contra estresse biótico e abiótico (Pant et al. 2017; Shah et al. 2018).

Cattleya jongheana (Rchb.f.) Van den Berg (sinonímia *Hadrolaeliae jongheana*) é uma orquídea brasileira, epífita e ameaçada de extinção, que ocorre em regiões da Mata Atlântica brasileira, um hotspot de biodiversidade (Neto et al. 2013). Estudos sobre fungos endofíticos e micorrízicos de *C. jongheana* são escassos. Um levantamento da biodiversidade de fungos associados às raízes desta orquídea, baseado em sequências de DNA obtidas a partir de amostras de raízes, revelou uma grande diversidade de táxa, porém apenas algumas OTUs foram identificadas em nível de gênero (Oliveira et al. 2014).

O isolamento e correta identificação de fungos micorrízicos e endofíticos de orquídeas é de grande importância para o desenvolvimento de estratégias de conservação dessas plantas. A fim de preencher a lacuna do conhecimento da diversidade de fungos associados a *C. jongheana*, o presente trabalho teve como objetivo isolar, investigar a diversidade e estudar a filogenia e taxonomia destes isolados.

REFERÊNCIAS

Cameron DD, Leake JR, Read DJ (2006) Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytologist 171:405-16. https://doi.org/10.1111/j.1469-8137.2006.01767.x

Cameron DD, Johnson I, Leake JR, Read DJ (2007) Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. Annals of botany 99:831–834. https://doi.org/10.1093/aob/mcm018

Dearnaley JD, Cameron DD (2017) Nitrogen transport in the orchid mycorrhizal symbiosis - further evidence for a mutualistic association. New Phytologist 213:10-12. https://doi.org/10.1111/nph.14357

Currah RS, Zelmer C (1992) A key and notes for the genera of fungi mycorrhizal with orchids and a new species in the genus *Epulorhiza*. Reports of the Tottori Mycological Institute 0: 43–59.

Dearnaley JDW, Martos F, Selosse, MA (2012) Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: Hock B (ed) The Mycota IX: Fungal Associations, 2nd edn. Springer, Berlin Heidelberg.

Freitas EFS, Silva M, Cruz ES, Mangaravite E, Bocayuva MF, Veloso TGR, Selosse M-A, Kasuya MCM (2020) Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species. Scientific Reports 10:7069

Fritsche Y, Lopes ME, Selosse, MA et al. (2021) *Serendipita restingae* sp. nov. (Sebacinales): an orchid mycorrhizal agaricomycete with wide host range. Mycorrhiza 31: 1–15. https://doi.org/10.1007/s00572-020-01000-7

Giulietti AM, Harley RM, De Queiroz LP, Wanderley MGL, Berg CVD (2005) Biodiversity and conservation of plants in Brazil. Conservation Biology 19:632–639. https://doi.org/10.1111/j.1523-1739.2005.00704.x

Govaerts R (2016) WCSP: World Checklist of Selected Plant Families. In: Species 2000 & ITIS catalogue of life, annual checklist. Leiden, the Netherlands.

Herrera H, Valadares R, Oliveira G et al. (2018) Adaptation and tolerance mechanisms developed by mycorrhizal *Bipinnula fimbriata* plantlets (Orchidaceae) in a heavy metal-polluted ecosystem. Mycorrhiza 28:651–663. https://doi.org/10.1007/s00572-018-0858-4

Hou XQ, Guo SX (2009) Interaction between a dark septate endophytic isolate from *Dendrobium* sp. and roots of *D. nobile* seedlings. J. Integr. Plant Biol. 51:374–381.

Jiang J-H, Tam S-L, Toda T, Chen L-C (2016) Controlling Rhizoctonia damping-off of Chinese mustard by using endomycorrhizal *Rhizoctonia* spp. isolated from orchid mycorrhizae. Plant Disease 100: 85–91.

Jiang J, Zhang K, Cheng S et al. (2019) *Fusarium oxysporum* KB-3 from *Bletilla striata*: an orchid mycorrhizal fungus. Mycorrhiza 29:531–540. https://doi.org/10.1007/s00572-019-00904-3

Johnson TR, Stewart SL, Dutra D et al. (2007) Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae) - preliminary evidence for the symbiotic culture advantage. Plant Cell Tiss Organ Cult 90:313. https://doi.org/10.1007/s11240-007-9270-z

Linde CC, Phillips RD, Crisp MD, Peakall R (2014) Congruent species delineation of *Tulasnella* using multiple loci and methods. New Phytologist 201:6–12. https://doi.org/10.1111/nph.12492

Linde CC, May TW, Phillips RD et al. (2017) New species of *Tulasnella* associated with terrestrial orchids in Australia. IMA Fungus 8:28–47. https://doi.org/10.5598/imafungus.2017.08.01.03

Ma X, Kang J, Nontachaiyapoom S, Wen T, Hyde KD (2015) Non-mycorrhizal endophytic fungi from orchids. Current Science 108: 16.

Martinelli G, Moraes MA (2013) Livro Vermelho da Flora do Brasil, 1st edn. Rio de Janeiro.

Mosquera-Espinosa AT, Bayman P, Prado GA, Gómez-Carabalí A, Otero JT (2013) The double life of *Ceratobasidium*: orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia solani* sheath blight of rice. Mycologia 105: 141–150.

Neto LM, Barros F, Vinhos F, Furtado SG, et al (2013) Orchidaceae. In: Martinelli G, Moraes MA (eds) Livro Vermelho da Flora do Brasil, 1st edn. Rio de Janeiro, pp749-818

Oliveira SF, Bocayuva MF, Veloso TGR et al. (2014) Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic Forest, Brazil. Mycorrhiza 24:55–64. https://doi.org/10.1007/s00572-013-0512-0

Pant B, Shah S, Shrestha R, Pandey S, Joshi PR (2017) An Overview on Orchid Endophytes. In: Varma A, Prasad R, Tuteja N (eds) Mycorrhiza - Nutrient Uptake, Biocontrol, Ecorestoration. Springer, Cham, pp 503-524. https://doi.org/10.1007/978-3-319-68867-1_26

Petrini O (1991) Fungal Endophytes of Tree Leaves. In: Andrews JH, Hirano SS, eds. Microbial Ecology of Leaves. New York: Springer - Verlag New York Inc., 179–197.

Porras-Alfaro A, Bayman P (2011) Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. Annual Review of Phytopathology 49: 291–315.

Rasmussen HN (1995) Terrestrial orchids: From Seed to Mycotrophic Plant. Cambridge University Press, p. 444

Rasmussen HN and Rasmussen FN (2009) Orchid mycorrhiza: implications of a mycophagous life style. *Oikos* 118: 334-345.

Shah S, Shrestha R, Maharjan S, Selosse M-A, Pant B (2019) Isolation and characterization of plant growth-promoting endophytic fungi from the roots of *Dendrobium moniliforme*. Plants 8:5. https://doi.org/10.3390/plants8010005

Solís K, Barriuso JJ, Garcés-claver A, González V (2017) *Tulasnella tubericola* (Tulasnellaceae, Cantharellales, Basidiomycota): a new Rhizoctonia-like fungus associated with mycorrhizal evergreen oak plants artificially inoculated with black truffle (*Tuber melanosporum*) in Spain. Phytotaxa 317:175–187. https://doi.org/10.11646/phytotaxa.317.3.2

Suárez JP, Weiss M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycological Research 110:1257–1270. https://doi.org/10.1016/j.mycres.2006.08.004

Weiß M, Waller F, Zuccano A, Selosse MA (2016) Sebacinales – one thousand and one interactions with land plants. New Phytologist 211:20–40. https://doi.org/10.111

CAPÍTULO 1

Neopestalotiopsis hadrolaeliae sp. nov., a new endophytic species from the roots of the endangered orchid *Hadrolaelia jongheana* in Brazil

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Neopestalotiopsis hadrolaeliae sp. nov., a new endophytic species from the roots of the endangered orchid *Hadrolaelia jongheana* in Brazil

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ABSTRACT

A new endophytic fungal species, *Neopestalotiopsis hadrolaeliae*, is described from the roots of *Hadrolaelia jongheana*, an endangered orchid species growing in Brazil. Multigene phylogenetic analyses coupled with morphological observations facilitated the identification of new species. Phylogenetic analyses based on ITS, *TUB* and *TEF* DNA sequences showed that the new taxon is different from reported *Neopestalotiopsis* species. Descriptions and illustrations and notes are provided for the new species, which we name herein as *N. hadrolaeliae*.

KEYWORDS: Ascomycota, Pestalotiopsidaceae, Phylogeny, Sordariomycetes

INTRODUCTION

The genus *Neopestalotiopsis* Maharachch., K.D. Hyde & Crous (2014:135) (Pestalotiopsidaceae) was established in 2014 with *Neopestalotiopsis protearum* (Crous & L. Swart) Maharachch., K.D. Hyde & Crous as type species, to accommodate species that previously belonged to the genus *Pestalotiopsis* (type species *Pestalotiopsis maculans*) (Maharachchikumbura *et al.* 2014). *Neopestalotiopsis* differs from *Pestalotiopsis* and *Pseudopestalotiopsis* by conidial morphology, mainly by its versicolourous median cells, and sequence data of the internal transcribed spacer regions (ITS), partial β-tubulin (*TUB*) and translation elongation factor 1-alpha (*TEF*) gene (Jeewon *et al.* 2002, 2003, Maharachchikumbura *et al.* 2014). *Neopestalotiopsis* species are commonly reported as plant pathogens, saprobe or endophytic (Jeewon *et al.* 2004, Liu *et al.* 2010, Hyde *et al.* 2016, Reddy *et al.* 2016, Shetty *et al.* 2016, Ran *et al.* 2017, Bezerra *et al.* 2018), and have been reported as epiphytic and gibberellin producers in orchids (Salazar-Cerezo *et al.* 2018).

Brazil has a great diversity of orchids with around 2,650 species and 205 genera, out of which more than 60% correspond to endemic species (Giulietti *et al.* 2005). *Hadrolaelia jongheana* (Rchb.f.) Van den Berg is an endangered Brazilian epiphytic/ lithophytic orchid that occurs in regions affected by human activity, such as Zona da Mata Mineira and Quadrilátero Ferrífero (Martinelli & Moraes 2013). Studies using DNA extracted from roots of *H. jongheana* in Brazil showed a great diversity of fungi associated with the roots of this orchid (Oliveira *et al.* 2014). Endophytes isolated from orchids can contribute to plant growth and development as well as the production of valuable secondary metabolites (Hiruma *et al.* 2016, Pant *et al.* 2017, Shah *et al*. 2018).

During a survey of cultivable mycorrhizal and endophytic fungi associated with the roots of *H. jongheana* at Parque Estadual Serra do Brigadeiro, two isolates of

Neopestalotiopsis were isolated. Later, based on morphological and molecular analysis, it was found that the two isolates belonged to the same species, here described as new. In this study, we introduce a new species of *Neopestalotiopsis hadrolaeliae* collected from Brazil based on both morphology and molecular data.

MATERIAL AND METHODS

Sample collection and isolates

All samples were collected at Parque Estadual Serra do Brigadeiro (Minas Gerais, Brazil)**.** Asymptomatic roots of *H. jongheana* were sampled and brought to the Laboratório de Associações Micorrízicas, Departamento de Microbiologia/Universidade Federal de Viçosa, in an ice box and refrigerated at 4 °C. The root samples were washed under running tap water, cut across into 2–3 mm thick fragments and surface-sterilized using 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, followed by two successive rinses in autoclaved distilled water. The root fragments were placed on potato dextrose agar medium (PDA) plates and incubated at 25 ºC. Axenic cultures were preserved on silica gel and deposited in the culture collection of the Universidade Federal de Viçosa, Coleção Octávio Almeida Drummond (COAD). The specimens were deposited at the Herbarium of the Universidade Federal de Viçosa (VIC). Mycobank numbers were obtained as outlined in Mycobank (www.mycobank.org).

Morphological studies

The morphological features were recorded following the method of Hu *et al.* (2007) for members of Pestalotiopsidaceae. Microscopic preparations were made in distilled water and 30 measurements were obtained per structure. Observations, measurements and photographic images of microscopic fungal structures were taken with an Olympus BX 53 light microscope equipped with an Olympus Q-Color5^{TM} digital high

definition color camera, using differential interference contrast (DIC) illumination. Adobe Photoshop CS5 was used for the final editing of the images acquired and photographic preparations. The fungus and colony characters were described from cultures growth on PDA and incubated for 1–2 weeks at 25 ºC. Colour terminology followed Rayner (1970).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fungal mycelia grown on PDA at 25 ºC, using the Nucleospin® Soil (MACHEREY-NAGEL GmbH & Co. KG). Three nuclear gene regions were targeted for Polymerase Chain Reaction (PCR) amplification and subsequent sequencing. The Internal Transcribed Spacer (ITS) regions of rDNA molecule were amplified using primer pairs ITS1 and ITS4 (White *et al.* 1990), the partial β-tubulin (*TUB*) gene region was amplified with primer pairs T1 and bt2b (Glass & Donaldson 1995, O'Donnel & Cigelnik 1997) and the partial translation elongation factor 1-alpha (*TEF*) was amplified using the primer pairs EF1-526F and EF1-1567R (Rehner & Buckley 2005).

Polymerase chain reactions (PCR) was performed with 50 µL reaction system consisting of 28.75 µL of double distilled water, 10 µL of 5× Taq buffer, 4 µL of $MgCl_2$ (25 Mm), 2 µL of dNTP (10 mM each), 2 µL of each primer (5 µM), 0.25 µL Taq DNA polymerase (5 U/µl), and 1.0 µL of DNA template. The thermal cycling program was carried out as follows: For ITS, an initial denaturing step was conducted at 95 °C for 2 min, followed by 39 amplification cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. For *TUB* PCR conditions, an initial denaturing step of 2 min was carried out at 94 °C, followed by 30 cycles of 50s at 94 °C, 1 min at 55 °C, 1 min at 72 °C and 10 min at 72 °C. For *TEF*, an initial step of 2

min was carried out at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 52 °C, 1 min at 72 °C and 7 min at 72 °C.

Amplified PCR fragments were checked on 1.5% agarose electrophoresis gels stained with ethidium bromide. PCR products were purified and sequenced by Macrogen, Inc. (Seoul, South Korea) using the primers mentioned above. The sequences from forward and reverse primers were used to obtain consensus sequences that were generated using the MEGA v. 7.0.26 software tool (Molecular Evolutionary Genetics Analysis) (Kumar *et al.* 2016). All sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. The new DNA sequences of ITS, *TUB* and *TEF* regions generated in this study were deposited in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (Table 1).

TABLE 1. GenBank accession numbers of *Neopestalotiopsis* isolates included in this study. The samples obtained in this study are highlighted in bold.

		GenBank accession		
Species	Culture accession No.[1]	ITS	TUB	TEF
N. alpapicalis	MFLUCC 17-2544*	MK357772	MK463545	MK463547
N. aotearoa	CBS 367.54	KM199369	KM199454	KM199526
N. asiatica	MFLUCC 12-0286	JX398983	JX399018	JX399049
N. australis	CBS 114159	KM199348	KM199432	KM199537
N. brasiliensis	COAD 2166*	MG686469	MG692400	MG692402
N. chiangmaiensis	MFLUCC 18-0113	-	MH412725	MH388404
N. chrysea	MFLUCC 12-0261	JX398985	JX399020	JX399051
N. chrysea	MFLUCC 12-0262	JX398986	JX399021	JX399052
N. clavispora	MFLUCC 12-0281*	JX398979	JX399014	JX399045
N. clavispora	MFLUCC 12-0280	JX398978	JX399013	JX399044
N. cubana	CBS 600.96	KM199347	KM199438	KM199521
N. cocoes	MFLUCC 15-0152	KX789687	-	KX789689
N. egyptiaca	CBS 140162	KP943747	KP943746	KP943748
N. egyptiaca	COAD 2167	MG686470	MG692401	MG692403
N. ellipsospora	MFLUCC 12-0283*	JX398980	JX399016	JX399047
N. ellipsospora	MFLUCC 12-0284	JX398981	JX399015	JX399046

N. eucalypticola	CBS 264.37	KM199376	KM199431	KM199551
N. foedans	CGMCC 3.9123*	JX398987	JX399022	JX399053
N. foedans	CGMCC 3.9178	JX398989	JX399024	JX399055
N. formicarum	CBS 362.72*	KM199358	KM199455	KM199517
N. formicarum	CBS 115.83	KM199344	KM199444	KM199519
N. hadrolaeliae	VIC 47180*	MK454709	MK465120	MK465122
N. hadrolaeliae	VIC 47181	MK454710	MK465121	MK465123
N. honoluluana	CBS 114495	KM199364	KM199457	KM199548
N. honoluluana	CBS 111535	KM199363	KM199461	KM199546
N. iranensis	CBS 137768	KM074048	KM074057	KM074051
N. javaensis	CBS 257.31*	KM199357	KM199437	KM199543
N. macadamiae	BRIP63737c	KX186604	KX186654	KX186627
N. magna	MFLUCC 12-652	KF582795	KF582793	KF582791
N. mesopotamica	CBS 336.86*	KM199362	KM199441	KM199555
N. mesopotamica	CBS 299.74	KM199361	KM199435	KM199541
N. musae	MFLUCC 15-0776	KX789683	KX789686	KX789685
N. natalensis	CBS 138.41*	KM199377	KM199466	KM199552
N. pandanicola	KUMCC 17-0175	-	MH412720	MH388389
N. pernambucana	RV01	KJ792466	-	KU306739
N. pernambucana	RV02	KJ792467	-	KU306740
N. phangngaensis	MFLUCC 18-0119	MH388354	MH412721	MH388390
N. piceana	CBS 394.48*	KM199368	KM199453	KM199527
N. piceana	CBS 254.32	KM199372	KM199452	KM199529
N. protearum	CBS 114178	JN712498	KM199463	KM199542
N. rosae	CBS 101057*	KM199359	KM199429	KM199523
N. rosae	CBS 124745	KM199360	KM199430	KM199524
N. rosicola	CFCC 51992*	KY885239	KY885245	KY885243
N. samarangensis	MFLUCC 12-0233*	JQ968609	JQ968610	JQ968611
N. samarangensis	CBS 115451	KM199365	KM199447	KM199556
N. saprophytica	MFLUCC 12-0282	JX398982	JX399017	JX399048
N. saprophytica	CBS 115452	KM199345	KM199433	KM199538
Neopestalotiopsis sp. Clade 4	CBS 233.79	KM199373	KM199464	KM199528
Neopestalotiopsis sp. Clade 10	CBS 110.20	KM199342	KM199442	KM199540
Neopestalotiopsis sp. Clade 15	CBS 177.25	KM199370	KM199445	KM199533
Neopestalotiopsis sp. Clade 15	CBS 274.29	KM199375	KM199448	KM199534
Neopestalotiopsis sp. Clade 15	CBS 322.76	KM199366	KM199446	KM199536
Neopestalotiopsis sp. Clade 15	CBS 664.94	KM199354	KM199449	KM199525
Neopestalotiopsis sp. Clade 20	CBS 164.42	KM199367	KM199434	KM199520
Neopestalotiopsis sp. Clade 20	CBS 360.61	KM199346	KM199440	KM199522
Neopestalotiopsis sp. Clade 22	CBS 119.75	KM199356	KM199439	KM199531

Neopestalotiopsis sp. Clade 22	CBS 266.80	KM199352	-	KM199532
Neopestalotiopsis sp. Clade 26	CBS 266.37	KM199349	KM199459	KM199547
Neopestalotiopsis sp. Clade 26	CBS 323.76	KM199350	KM199458	KM199550
Neopestalotiopsis sp. Clade 26	CBS 361.61	KM199355	KM199460	KM199549
N. steyaertii	IMI 192475*	KF582796	KF582794	KF582792
N. surinamensis	CBS 450.74*	KM199351	KM199465	KM199518
N. umbrinospora	MFLUCC 12-0285	JX398984	JX399019	JX399050
N. vitis	MFLUCC 15-1265	KU140694	KU140685	KU140676
N. zimbabwana	CBS 111495	JX556231	KM199456	KM199545
Pseudopestalotiopsis theae	MFLUCC 12-0055*	JQ683727	JQ683711	JQ683743

[1]CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; COAD: Coleção de Cultura Octávio Almeida Drummond, Universidade Ferderal de Viçosa, Viçosa, Brazil; IMI: Culture Collection of CABI Europe UK Centre, Egham, UK; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.
*Ex-type strains.

Phylogenetic analyses

Consensus sequences were compared against NCBI's GenBank nucleotide database using their mega BLAST algorithm. The most similar sequences were downloaded in FASTA format and the sequence datasets for the three genomic loci were individually aligned using the MAFFT v. 7 online portals (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh & Standley 2013). The resulting sequence alignments were manually checked and adjusted in MEGA v. 7.0.26 and concatenated with Mesquite v. 3.51 (Maddison & Maddison 2018). A phylogenetic tree was constructed using a combined ITS, *TUB* and *TEF* dataset.

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and then with the concatenated sequences (ITS, TUB and TEF). Nucleotide substitution was determined using the MrModeltest 2.3 program (Nylander 2004) for each gene region and included in the analyses. Once the likelihood scores had been calculated, the models were selected according to the Akaike Information Criterion (AIC). The results of MrModeltest recommended a GTR+I+G model for ITS, and an GTR+G model for TEF

and TUB. The phylogenetic analysis of the concatenated alignment was performed using the CIPRES web portal (Miller *et al.* 2010) and the MrBayes program v.3.1.1 (Ronquist & Heulsenbeck 2003). In MrBayes, the data were partitioned per locus, and the parameters of the nucleotide substitution models for each partition were set as described above. Two sets of four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations and sampled every 1,000th generation. The first 25% of the trees were discarded as the burn-in phase for each analysis and posterior probabilities (Rannala & Yang 1996) were determined from the remaining trees and are presented on the left of each node. Maximum likelihood (ML) analysis was implemented using the RAxML-HPC v.8 on XSEDE (8.2.12) available on the CIPRES web portal. The alignments and trees were deposited in TreeBASE (http://treebase.org/treebase-web/) (ID. 24043). The trees were visualized in FigTree (Rambaut 2009) and exported to graphics programs.

RESULTS

Phylogeny

The alignment consisted of 66 strains (including the outgroup sequence) (Table 1). The DNA sequence data from the ITS, *TUB* and *TEF* regions were combined for the Bayesian and Maximum likelihood analyses. The final alignment of ITS (501 characters), *TUB* (440 characters), and *TEF* (499 characters) gene regions had a total length of 1450 characters (including alignment gaps), out of which 1193 were parsimony-informative, 1323 were variable and 103 were conserved. The concatenated alignment contained a total of 65 ingroup strains with *Pseudopestalotiopsis theae* (Sawada) Maharachch., K.D. Hyde & Crous (MFLUCC12-0055) as outgroup (Table 1). Based on phylogenetic analyses and morphological comparisons, it is possible to identify the new species of

Neopestalotiopsis, *N. hadrolaeliae*. The topologies of trees generated from ML and BI analyses were essentially similar, therefore only BI is shown below (Fig.1).

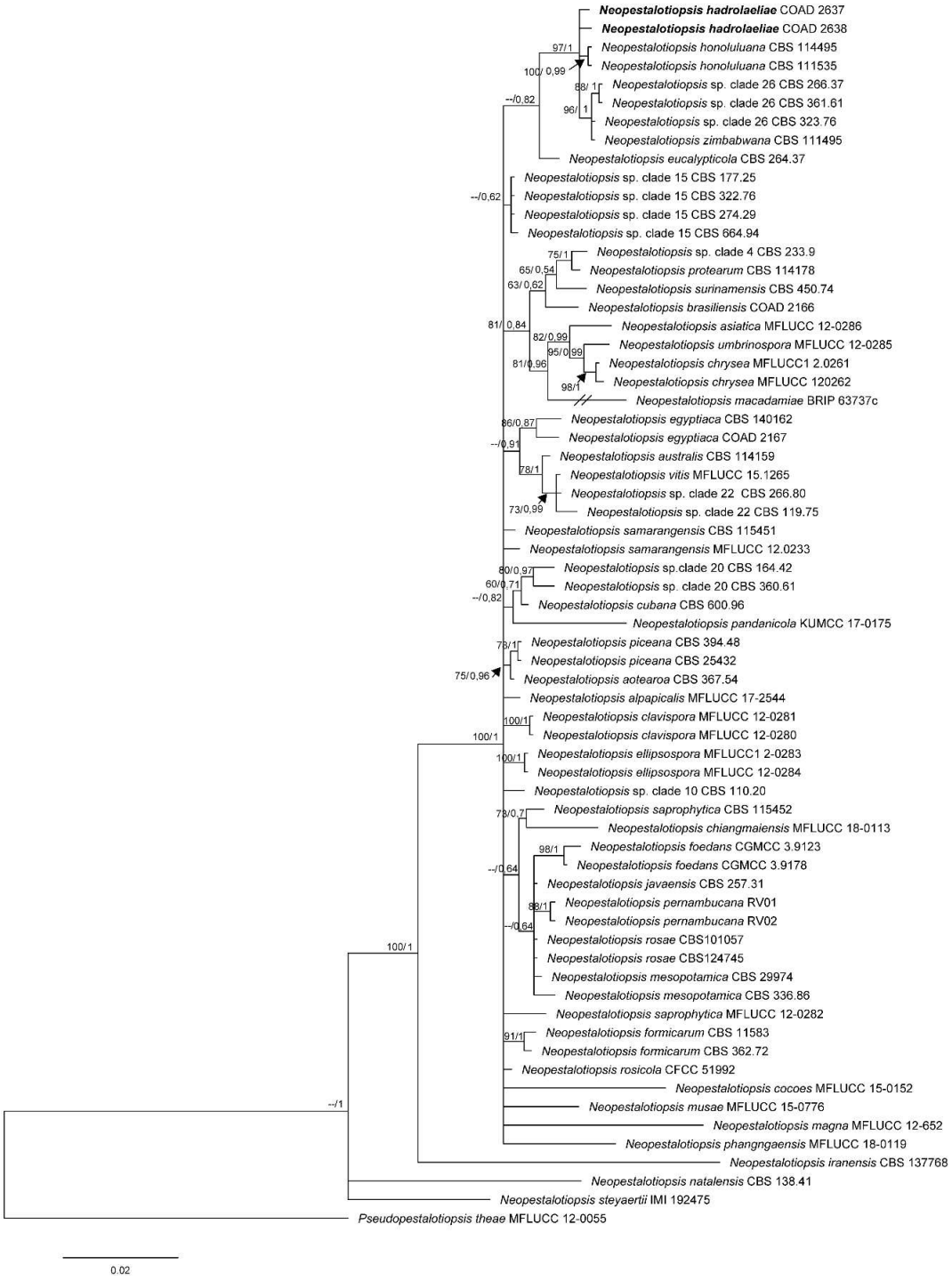

FIGURE 1. Bayesian phylogenetic tree inferred from DNA sequence data from multigene alignment (ITS, *TUB* and *TEF*). Maximum likelihood bootstrap support (MLBS > 60) and Bayesian posterior probabilities (BPP) values are indicated next to the nodes (MLBS/BPP). Specimens representing the new species are in bold face. The tree was rooted with *Pseudopestalotiopsis theae* MFLUCC 12-0055.

Taxonomy

Neopestalotiopsis hadrolaeliae E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya, *sp. nov.* (Fig. 2)

Mycobank: MB830023

Systematic position: Ascomycota, Pezizomycotina, Sordariomycetes, Xylariomycetidae, Amphispheriales, Pestalotiopsidaceae.

Type:—BRAZIL. Minas Gerais: Parque Estadual Serra do Brigadeiro, roots of *Hadrolaelia jongheana* (Rchb.f.) Chiron & V.P.Castro (Orchidaceae), February 2018, Freitas, E.F.S. (VIC47180 **holotype**, ex-type living culture COAD2637).

Etymology:— Name derived from the plant host genus *Hadrolaelia*.

Colonies on PDA attaining 54 mm diam after 7 d at 25 °C, with undulate edge, whitish, with dense aerial mycelium on the surface. Reverse of the colony white to pale brown. Sexual morph: not observed. Asexual morph: Conidiomata pycnidial in culture on PDA, globose to clavate, solitary or aggregated in clusters, semi-immersed, black, exuding globose, dark brown to black conidial masses. Conidiophores reduced to conidiogenous cells. Conidiogenous cells discrete, subcylindrical to ampulliform, hyaline, 15.5–17 × 1–4 µm. Conidia ellipsoid, straight and sometimes slightly curved, 4-septate, 19–26.5 × 5–7.5 µm ($\bar{X}$ ± SD = 22.3 ± 2.2 × 6 ± 0.5 µm); basal cell obconic with truncate base, rugose and thin-walled, 4–7 µm long; three median cells doliiform, 12–16.5 µm long ($\bar{X}$ ± SD = 14 ± 1.1 µm), rugose, versicolourous, brown to olivaceous, septa darker than the rest of the cell (second cell from base pale brown, 4–5.5 µm long; third cell darker brown, 3.5–6 µm long; fourth cell brown, 4–6 µm long); apical cell subcylindrical, thin- and smooth-walled, 3–6 µm long, hyaline; with 1–3 tubular apical appendages, arising from the apical crest, unbranched, flexuous 10–33 µm long ($\bar{X}$ ± SD = 19.6 ± 5.7 µm); basal appendage single, unbranched, centric, 2–11 µm long.

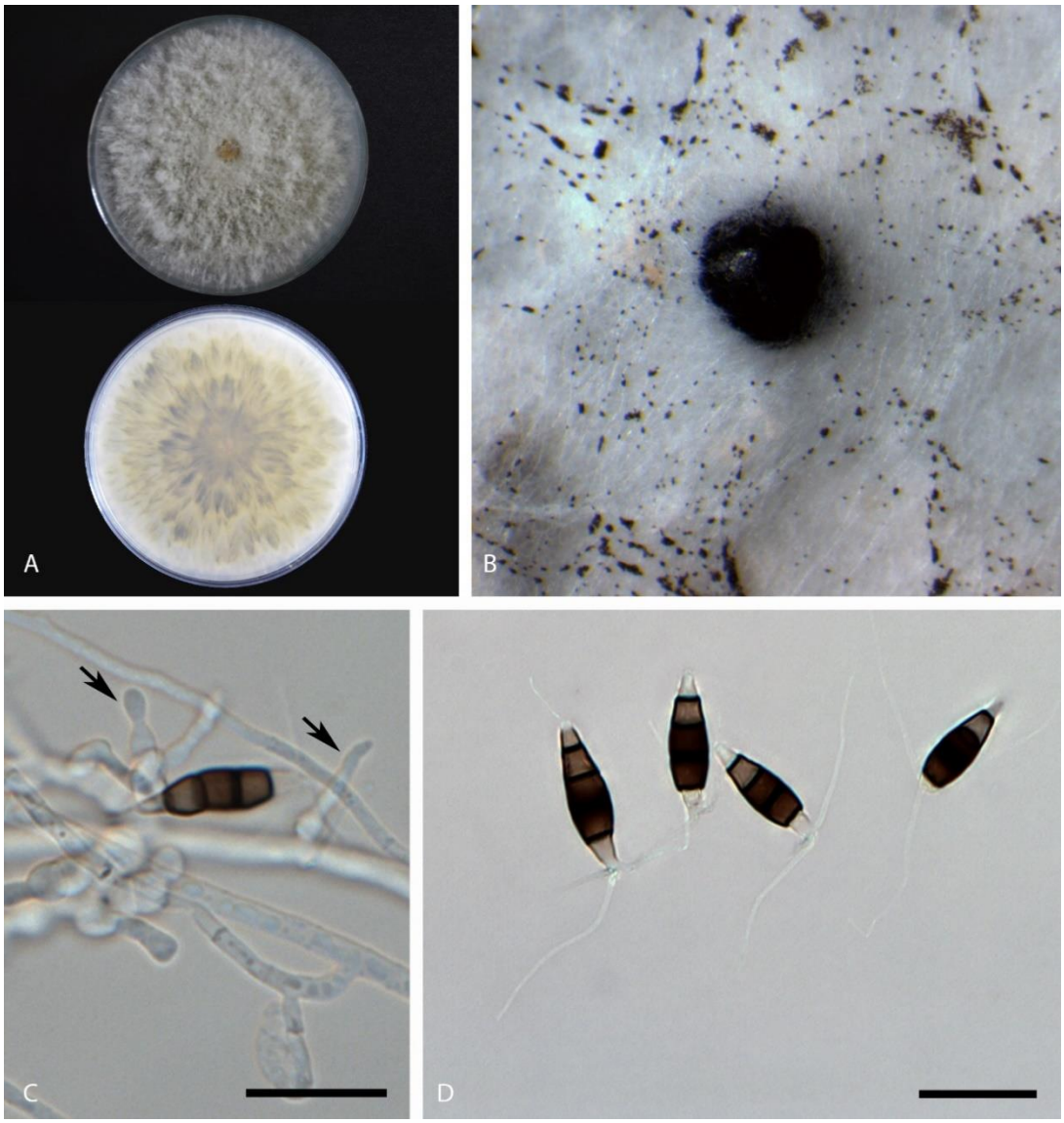

FIGURE 2. *Neopestalotiopsis hadrolaeliae* (COAD 2637, **ex-type**). **A,** Morphological aspect in PDA culture (front and reverse); **B,** Conidiomata on PDA; **C,** Conidiogenous cell; **D,** Conidia with apical appendages. Scale bars = 20 μm.

Additional specimens examined.—BRAZIL. Minas Gerais: Parque Estadual Serra do Brigadeiro, roots of *Hadrolaelia jongheana* (Rchb.f.) Chiron & V.P.Castro (Orchidaceae), COAD 2638, February 2018, Freitas, E.F.S.: *N. hadrolaeliae*

Notes:— Based on multi-gene phylogenetic analysis, *Neopestalotiopsis hadrolaeliae* is a sister taxon to *N. honoluluana* Maharachch., K.D. Hyde & Crous (2014: 141) and *N. zimbabwana* Maharachch., K.D. Hyde & Crous (2014: 149). The sister taxa are

morphologically different from *N. hadrolaeliae* by number and in length of apical appendages, and conidial size (Table2). *Neopestalotiopsis honoluluana* has 3 apical appendages with 23−40 µm long ($\bar{X}$ ± SD = 32 ± 6.0 µm) and conidia with 24−34 × 7.5−9.5 µm ($\bar{X}$± SD = 28 ± 2.3 × 8.3 ± 0.6 µm), *N. zimbabwana* has 2−3 apical appendages with 23−35 µm long ($\bar{X}$ ± SD = 28.6 ± 4 µm) and conidia with 23−29 × 7−8.5 µm (X ± SD = 25.3 ± 1.2 × 7.7 ± 0.3 µm), while *N. hadrolaeliae* has 1−3 apical appendages with 10−33 µm long ($\bar{X}$ ± SD = 19.6 ± 5.7 µm) and conidia with 19−26.5 × 5−7.5 µm ($\bar{X}$ ± SD = 22.3 ± 2.2 × 6 ± 0.5 µm). When comparing the 476 TEF1 nucleotides of *N. hadrolaeliae* and *N. honoluluana*, we note that strains differed by 4 bp. Across the 440 TUB2 nucleotides, there were 2 bp differences, while *N. hadrolaeliae* differs from *N. zimbabwana* by 4 bp in the TEF1 and 2 bp in the TUB2 gene. In a BLASTn search on NCBI GenBank, the closest matches of TUB2 sequence of *N. hadrolaeliae* is *N. honoluluana* with 98.97% identity to the strain CBS 114495 (KM199457), while the closest matches with the TEF1 sequence were with 99.79% *N. eucalypticola* strain CBS 26437 (KM199551).

TABLE 2: Comparison of morphological characters of *N. hadrolaeliae* and closely species.

	N. hadrolaeliae	***N. honoluluana***	***N. zimbabwana***
Conidiogenous cell	15.5–17 × 1–4 µm	5–20 × 2–6 µm	5–15 × 3–8 µm
Conidia	19−26.5 × 5−7.5 µm $\bar{X}$ ± SD = 22.3 ± 2.2 × 6 ± 0.5 µm	24−34 × 7.5−9.5 µm $\bar{X}$ ± SD = 28 ± 2.3 × 8.3 ± 0.6 µm	23−29 × 7−8.5 µm $\bar{X}$ ± SD = 25.3 ± 1.2 × 7.7 ± 0.3 µm
Basal cell	2.5−5.5 µm long	4.5−7 µm long	3.5−5.5 µm long
Median cells	12−16.5 µm long $\bar{X}$ ± SD = 14 ± 1.1 µm	15−20 µm long $\bar{X}$ ± SD = 17.3 ± 1.6 µm	15.5–17.5 µm long $\bar{X}$ ± SD = 16.5 ± 0.6 µm
Apical cell	3−6 µm long	4−7.5 µm long	4−6.5 µm long
Nº apical appendages	1−3	3	2−3
Apical appendages	10−33 µm long $\bar{X}$ ± SD = 19.6 ± 5.7 µm	23−40 µm long $\bar{X}$ ± SD = 32 ± 6.0 µm	23−35 µm long $\bar{X}$ ± SD = 28.6 ± 4 µm
Basal appendages	2−11 µm long	2.5−10 µm long	3−9.5 µm long

DISCUSSION

Studies on endophytic fungi in Orchidaceae have unraveled a high diversity of fungi, with over 110 genera (Ma *et al.* 2015). Genera within Pestalotiopsidaeceae, including *Neopestalotiopsis*, have been isolated as endophytic and epiphytic fungi in orchids (Chen *et al.* 2011, 2012, Tempesta *et al.* 2011, Salazar-Cerezo *et al.* 2018). However, no species of *Neopestalotiopsis* have been reported on *Hadrolaelia* (Farr & Rossman 2019).

Neopespalotiopsis hadrolaeliae is introduced as a new species associated from roots of *Hadrolaelia jongheana* in Minas Gerais, Brazil. Phylogenetically, *N. hadrolaeliae* is closely related to *N. honoluluana*, *N. zimbabwana* and *Neopestalotiopsis* sp. clade 26. When comparing *N. hadrolaeliae* with *N. honoluluana* and *N. zimbabwana* we observed 4 bp differences in the TEF1 nucleotides and 2 bp in the TUB2 nucleotides.

Similar works have shown that the evolutionary relationship among species of *Neopestalotiopsis* are not robust, with low support values (Maharachchikumbura *et al.* 2014, Jiang *et al.* 2018, Kumar *et al.* 2019). Additional studies are necessary to obtain other genes to a better separation of species (Maharachchikumbura *et al.* 2014, Kumar *et al.* 2019).

Morphologically, *N. hadrolaeliae* differs from *N. honoluluana* and *N. zimbabwana* by having smaller conidia and shorter apical appendages (Maharachchikumbura *et al.* 2014). According to Maharachcikumbura *et al.* (2014), *Neopestalotiopsis* sp. clade 26 is phylogenetically and morphologically similar to *N. zimbabwana*, and they are separated only based on geographical differences. Jeewon and Hyde (2016) have established guidelines dealing with taxonomic aspects to consider while establishing new species and these need to be considered to better understand species relationships. It appears that there could have been an overestimate of actual species in many genera, such as *Neopestalotiopsis*.

Pestalotiopsis-like taxa, including *Neopestalotiopsis* species, have been suggested as important sources of bioactive secondary metabolites with anti-tumor, anti-fungal or anti-microbial potential (Xu *et al.* 2010, 2014). Recently, an endophytic species of phytohormone-producing *Neopestalotiopsis* was isolated from the Mexican threatened orchid species *Stahopea tigrina* (Salazar-Cerezo *et al.* 2018). The production of phytohormone by *N. hadrolaeliae* will be further investigated, as well as its potential in seedling establishment, plant-growth promotion and reestablishment of young *H. jongheana* in nature.

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REFERENCES

Bezerra, J.D.P., Machado, A.R., Firmino, A.L., Rosado, A.W.C., Souza, C.A.F., Souza-Motta, C.M., Freire, K.T.L.S., Paiva, L.M., Magalhães, O.M.C., Pereira, O.L (2018) Mycological Diversity Description I. *Acta Botanica Brasilica* 32: 1–11. https://doi.org/10.1590/0102-33062018abb0154

Chen, J., Hu, K.-X., Hou, X.-Q., Guo, S.-X. (2011) Endophytic fungi assemblages from 10 *Dendrobium* medicinal plants (Orchidaceae). *World Journal of Microbiology and Biotechnology* 27: 1009–1016. https://doi.org/10.1007/s11274-010-0544-y

Chen, J., Wang, H., Guo, S.-X. (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). *Mycorrhiza* 22: 297–307. https://doi.org/10.1007/s00572-011-0404-0

Farr, D.F. & Rossman, A.Y. (2019) *Fungal Databases.* U.S. National Fungus Collections, ARS, USDA. Available from: http://nt.ars-grin.gov/fungaldatabases (acessed: 23 January 2019).

Giulietti, A.M., Harley, R.M., De Queiroz, L.P., Wanderley, M.D.G.L., Van Den Berg, C. (2005) Biodiversity and conservation of plants in Brazil. *Conservation Biology* 19: 632–639.
https://doi.org/10.1111/j.1523-1739.2005.00704.x

Glass, N.L. & Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* 61: 1323–1330.

Hiruma, K., Gerlach, N., Sacristán S., Nakano, R.T., Hacquard, S., Kracher, B., Neumann, U., Ramírez, D., Bucher, M., O'Connell, R.J., Schulze-Lefert, P. (2016) Root endophyte *Colletotrichum tofieldiae* confers plant fitness benefits that are phosphate status dependent. *Cell* 165: 464–474.
http://dx.doi.org/10.1016/j.cell.2016.02.028

Hu, H., Jeewon, R., Zhou, D., Zhou, T., Hyde, K.D. (2007) Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β-tubulin gene phylogenies. *Fungal Diversity* 24: 1–22.

Hyde, K.D., Hongsanan, S., Jeewon, R., Bhat, J.D., McKenzie, E.H., Jones, E.B.G., Phookamsak, R., Ariyawansa, H.A., Boonmee, S., Zhao, Q. (2016) Fungal diversity notes 367–490: taxonomic and phylogenetic contributions to fungal taxa. *Fungal Diversity* 80: 1–270.
https://doi.org/10.1007/s13225-016-0373-x

Jeewon, R., Liew, E.C.Y., Hyde, K.D. (2002) Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. *Molecular Phylogenetics and Evolution* 25: 378–392.

Jeewon, R., Liew, E.C.Y., Simpson, J.A., Hodgkiss, I.J., Hyde, K.D. (2003) Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. *Molecular Phylogenetics and Evolution* 27: 372–383. https://doi:10.1016/S1055-7903(03)00010-1

Jeewon R., Liew, E.C.Y., Hyde K.D. (2004) Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. *Fungal Diversity* 17: 39–55.

Jeewon, R. & Hyde, K.D. (2016) Establishing species boundaries and new taxa among fungi: recommendations to resolve taxonomic ambiguities. *Mycosphere* 7 (11): 1669–1677.

https://doi:10.5943/mycosphere/7/11/4

Jiang, N., Bonthond, G., Fan, X.L., Tian, C.M. (2018) *Neopestalotiopsis rosicola* sp. nov. causing stem canker of *Rosa chinensis* in China. *Mycotaxon* 133 (2): 271–283. https://doi.org/10.5248/133.271

Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30 (4): 772–780. https://doi.org/10.1093/molbev/mst010

Kumar, V., Cheewangkoon, R., Gentekaki, E., Maharachchikumbura, S.S.N., Brahmanage, R.S., Hyde, K.D. (2019) *Neopestalotiopsis alpapicalis* sp. nov. a new endophyte from tropical mangrove trees in Krabi Province (Thailand). *Phytotaxa* 393: 251–262.

https://doi.org/10.11646/phytotaxa.393.3.2

Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 33: 1870–1874.

https://doi.org/10.1093/molbev/msw054

Liu, A.R., Chen, S.C., Wu, S.Y., Xu, T., Guo, L.D., Jeewon, R., Wei, J.G. (2010) Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy. *Molecular Phylogenetics and Evolution* 57: 528–535.

https://doi.org/10.1016/j.ympev.2010.07.017

Ma, X., Kang, J., Nontachaiyapoom, S., Wen, T., Hyde, K.D. (2015) Non-mycorrhizal endophytic fungi from orchids. *Current Science* 109: 72–87.

Maddison, W. P. & Maddison, D.R. (2018) *Mesquite: a modular system for evolutionary analysis*, version 3.51. Available from: http://www.mesquiteproject.org/ (accessed: 23 January 2019).

Maharachchikumbura, S.S.N., Hyde, K.D., Groenewald, J.Z., Xu, J., Crous, P.W. (2014) Pestalotiopsis revisited. *Studies in Mycology* 79: 121–186.

http://dx.doi.org/10.1016/j.simyco.2014.09.005

Martinelli, G. & Moraes, M.A. (2013) *Livro Vermelho da Flora do Brasil.* Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rio de Janeiro, 1100 pp.

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. *In: Proceedings of the Gateway Computing Environments Workshop (GCE).* pp. 1−8.

https://doi.org/10.1109/GCE.2010.5676129

Nylander, J.A.A. (2004) *MrModeltest v2*. Evolutionary Biology Centre, Uppsala University, Sweden. Available from: https://github.com/nylander/MrModeltest2 (accessed: 23 January 2019).

O'Donnel, K. & Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103–116.

Oliveira, S.F., Bocayuva, M.F., Veloso, T.G.R., Bazzolli, D.M.S., da Silva, C.C., Pereira, O.L., Kasuya, M.C.M. (2014) Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic Forest, Brazil. *Mycorrhiza* 24: 55–64. https://doi.org/10.1007/s00572-013-0512-0

Pant, B., Shah, S., Shrestha, R., Pandey, P., Joshi, P.R. (2017) An Overview on Orchid Endophytes. *In:* Varma, A., Prasad, R., Tuteja, N. (Eds.*) Mycorrhiza – Nutrient Uptake, Biocontrol, Ecorestoration*. Springer, Berlin, pp. 503–524.

Rambaut, A. (2009) *FigTree version 1.4.4*. Available from: http://tree.bio.ed.ac.uk/software/figtree/ (accessed: 23 January 2019).

Ran, S.F., Maharachchikumbura, S.S.N., Ren, Y.L., Liu, H., Chen, K.R., Wang, Y.X., Wang, Y. (2017) Two new records in *Pestalotiopsidaceae* associated with Orchidaceae disease in Guangxi Province, China. *Mycosphere* 8: 121–130. https://doi.org/10.5943/mycosphere/8/1/11

Rannala, B. & Yang, Z. (1996) Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *Journal of Molecular Evolution* 43: 304–311.

Rayner, R.W. (1970) *A Mycological Color Chart*. Commonwealth Mycological Institute, Kew, 34pp.

Reddy, M.S., Murali, T.S., Suryanarayanan, T.S., Rajulu, M.B.G., Thirunavukkarasu, N. (2016) *Pestalotiopsis* species occur as generalist endophytes in trees of Western Ghats forests of southern India. *Fungal Ecology* 24: 70–75. http://dx.doi.org/10.1016/j.funeco.2016.09.002

Rehner, S.A. & Buckley, E. (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97: 84–98.

Ronquist, F. & Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574. https://10.1093/bioinformatics/btg180

Salazar-Cerezo, S., Martinez-Montiel, N., Cruz-Lopez, M.C., Martinez-Contreras, R.D. (2018) Fungal diversity and community composition of culturable fungi in *Stanhopea trigrina* cast gibberellin producers. *Frontiers in Microbiology* 9: 612. https://doi.org/10.3389/fmicb.2018.00612

Shah, S., Shrestha, R., Maharjan, S., Selosse, M-A., Pant, B. (2019) Isolation and characterization of plant growth-promoting endophytic fungi from the roots of *Dendrobium moniliforme*. *Plants* 8: 5. https://doi.org/10.3390/plants8010005

Shetty, K.G., Rivadeneira, D.V., Jayachandran, K., Walker, D.M. (2016) Isolation and molecular characterization of the fungal endophytic microbiome from conventionally and organically grown avocado trees in South Florida. *Mycological Progress* 15: 977–986. https://doi.org/10.1007/s11557-016-1219-3

Tempesta, S., Rubini, A., Pupulin, F., Rambelli, A. (2011) *Pestalotiopsis* endophytes from leaves of two orchid species collected in Costa Rica. *Cryptogamie, Mycologie* 32: 315–321. https://doi.org/10.7872/crym.v32.iss3.2011.315

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Eds.) *PCR Protocols: a guide to methods and applications.* Academic Press, San Diego, pp. 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Xu, J., Ebada, S.S., Proksch, P. (2010) *Pestalotiopsis* a highly creative genus: chemistry and bioactivity of secondary metabolites. *Fungal Diversity* 44: 15–31. https://doi.org/10.1007/s13225-010-0055-z

Xu, J., Yang, X., Lin, Q. (2014) Chemistry and biology of *Pestalotiopsis*-derived natural products. *Fungal Diversity* 66: 37–68. https://doi.org/10.1007/s13225-014-0288-3

CAPÍTULO 2

Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species

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Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species

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ABSTRACT

The genus *Tulasnella* often forms mycorrhizas with orchids and has worldwide distribution. Species of this genus are associated with a wide range of orchids, including endangered hosts. Initially, species identification relied mostly on morphological features and few cultures were preserved for later phylogenetic comparisons. In this study, a total of 50 *Tulasnella* isolates were collected from their natural sites in Minas Gerais, Brazil, cultured, and subjected to a phylogenetic analysis based on alignments of sequences of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA. Our results, based on phylogeny, integrated with nucleotide divergence and morphology, revealed the diversity of isolated *Tulasnella* species, which included four new species, namely, *Tulasnella*

brigadeiroensis, *Tulasnella hadrolaeliae*, *Tulasnella orchidis* and *Tulasnella zygopetali.* The conservation of these species is important due to their association with endangered orchid hosts and endemic features in the Brazilian Atlantic Forest.

KEYWORDS: Basidiomycota, Endangered orchids, New taxa, Orchidaceae, Phylogeny, rhizoctonia-like, Tulasnellaceae.

INTRODUCTION

Orchidaceae (or orchids) is the largest family of flowering plants, with approximately 27,000 species described[1]. The Neotropics is the region of greatest orchid diversity[2] and approximately 205 genera and 2,650 species occur in Brazil, of which about 1,800 are endemic[3]. Many orchid species are endangered, mainly due to anthropogenic pressure and dependency between orchids and other organisms, i.e. pollinators or mycorrhizal fungi[4,5].

Several endangered orchid species are listed in the Livro Vermelho da Flora do Brasil[6]. Among them, *Hadrolaelia jongheana* is an epiphytic orchid found in the Zona da Mata and Quadrilátero Ferrífero, two areas severely affected by anthropogenic activity. *Zygopetalum maxillare* is an epiphytic species which, although not officially endangered, grows almost exclusively in tree ferns[7], which limits its distribution. *Cattleya cinnabarina* and *Cattleya caulescens* are rupicolous (i.e. grow on bare rocks) and endemic to the Southeastern Brazil[8]. These species belong to Brazilian Atlantic Forest, a highly diverse but endangered hotspot of biodiversity[9]. Like all orchids, they need mycorrhizal fungi for germination due to the limited reserves in seeds[10]. The symbiotic fungus supplies the embryo with carbon and other nutrients, which enable the germination and establishment

of the orchid[11]. Orchids associate mainly with Basidiomycota often called rhizoctonia, a polyphyletic that includes taxa belonging to the families Ceratobasidiaceae, Sebacinaceae, Serendipitaceae and Tulasnellaceae[12,13].

The specificity of orchid–mycorrhizal fungi varies among species[14,12] and the distribution of mycorrhizal fungi can affect the patterns of distribution of orchids[15]. Species with low specificity for their fungal partner may be more successful in conservation strategies, such as assisted migration[8]. Despite this, specialist orchids might be widely distributed if their fungal partners are broadly distributed[16,14]. Indeed, the ecology of *Tulasnella* species from the roots of orchids remains poorly known and even though they are often considered saprotrophic[11] they may also colonize the roots of non-orchid plants[17]. The availability of compatible symbionts may directly impact the conservation of species[4].

The genus *Tulasnella* is often observed as orchid mycorrhizal fungi in temperate and tropical regions[12,18,19], and several isolates have been reported to increase seed germination and seedling growth[20–25]. Identification of mycorrhizal fungi in South American orchids, mostly conducted in Brazil, has often revealed *Tulasnella* symbionts: *Tulasnella* species were isolated from *Epidendrum secundum*[26,27], *Epidendrum dendrobioides* and *Sophronits milleri*[28], *Oeceoclades maculata*, *Epidendrum rigidum* and *Polystachya concreta*[29], *E. rigidum* and *P. concreta*[30]. Yet little is known about *Tulasnella* in the hotspot of biodiversity of the Brazilian Atlantic Forest.

Tulasnella species have complex morphological characteristics, but rarely form fruitbodies *in situ* or sexual structures *in vitro*[29–33]. As morphological characteristics are not sufficient to describe *Tulasnella* species[34], molecular approaches have been used too[32,33,35–38]. Species identification is mostly based on phylogenetic concordance of multiple unrelated genes/regions, but for this complex genus, the internal transcribed

spacer (ITS) of the nuclear ribosomal DNA was shown to be highly suitable for species delimitation in *Tulasnella*[31,38].

In a survey of cultivable mycorrhizal fungi associated with the roots of the rare-to-endangered Brazilian orchids *H. jongheana*, *C. cinnabarina*, *C. caulescens* and *Z. maxillare,* we obtained 50 isolates of *Tulasnella*. Herein, based on morphological and molecular analyses, we have evaluated the diversity of *Tulasnella* associated with these four orchids and describe potentially new *Tulasnella* species.

RESULTS

Tulasnella isolates from Brazilian Atlantic Forest

Fifty isolates of the genus *Tulasnella* were obtained in this study (Table 1), namely, twenty isolates from *C. cinnabarina* roots, fourteen from *C. caulescens* roots, nine from *H. jongheana* (eight from Parque Estadual da Serra do Brigadeiro (PESB) and one from Parque Estadual da Serra Negra (PESN)) and seven isolates from *Z. maxillare*. As they were isolated from pelotons dissected from roots, they all are likely orchid mycorrhizal fungi. All isolates from *C. cinnabarina* and *C. caulescens* were identified as *Tulasnella calospora,* whereas isolates obtained from *H. jongheana* and *Z. maxillare* are described below as four new *Tulasnella* species.

Table 1: *Tulasnella* isolates obtained in this study. Ex-type strains are indicated in bold face.

Identity	Culture accession no.	Orchid Host	Origin	Habitat	GenBank accession no.
Tulasnella calospora	COAD 2850	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192009
	COAD 2851	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192010
	COAD 2852	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191991

	COAD 2853	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191993
	COAD 2854	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191994
	COAD 2855	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192007
	COAD 2856	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191995
	COAD 2857	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191996
	COAD 2858	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191997
	COAD 2859	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191998
	COAD 2860	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK191999
	COAD 2861	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192000
	COAD 2862	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192005
	COAD 2863	*Cattleya caulescens*	Mariana - MG	Rupicolous	MK192003
	COAD 2864	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191974
	COAD 2865	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191975
	COAD 2866	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK192006
	COAD 2867	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191976
	COAD 2868	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191977
	COAD 2869	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191978
	COAD 2870	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191979
	COAD 2871	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191980
	COAD 2873	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191981
	COAD 2874	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191982
	COAD 2875	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191983
	COAD 2876	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191984
	COAD 2877	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191985
	COAD 2878	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191986
	COAD 2879	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK192004
	COAD 2880	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191987

	COAD 2881	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191988
	COAD 2882	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK192008
	COAD 2883	*Cattleya cinnabarina*	Mariana - MG	Rupicolous	MK191989
Tulasnella brigadeiroesis sp. nov.	**COAD 2884**	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MK192001
	COAD 3007	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MT090025
	COAD 3008	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MT090026
Tulasnella hadrolaeliae sp. nov.	COAD 2887	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MN385724
	COAD 2888	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MN385725
	COAD 2889	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MN385726
	COAD 2890	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MN385727
	COAD 2891	*Hadrolaelia jongheana*	Araponga - MG	Epiphytic	MN385728
Tulasnella orchidis sp. nov.	**COAD 2893**	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385729
	COAD 2894	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385731
	COAD 2895	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385730
Tulasnella zygopetali sp. nov.	**COAD 2896**	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385732
	COAD 2897	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385733

	COAD 2898	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385734
	COAD 2899	*Zygopetalum maxillare*	Araponga - MG	Epiphytic	MN385735
Tulasnella sp.	COAD 2885	*Hadrolaelia jongheana*	Itamarandiba - MG	Epiphytic	MK192002

Phylogeny

The ITS alignment consisted of 93 strains (including the outgroup sequence), of which 43 are from NCBI or UNITE and 50 from this study (Tables 1 and 2) and had a total length of 583 characters (including alignment gaps). Among these, 371 characters were parsimony-informative, 419 were variable and 147 were conserved.

Table 2: GenBank and UNITE accession numbers of additional *Tulasnella* isolates included in the phylogenetic analysis. Ex-type strains are indicated in bold face.

Species	**Strain No.**	**Origin**	**GenBank accession No.**	**UNITE accession No.**
Epulorhiza amonilioides	3S	Brazil	JF907600	
Epulorhiza amonilioides	aero8	Brazil	KC928335	
Epulorhiza anaticula	UAMH 5428	Canada	EU218891	
Epulorhiza anaticula	13O004	South Korea	KT164598	SH1174351.08FU
Tulasnella albida	KC110	Unknown	AY373294	
Tulasnella asymmetrica	MAFF 305808	Australia	KC152356	

Tulasnella asymmetrica	AL.LM4.4.1	Australia	MH134544	SH1541682.08FU
Tulasnella bifrons	BPI 724849	Canada	AY373290	
Tulasnella calospora	MAFF P305801	Ecuador	DQ388041	
Tulasnella calospora	MAFF P305802	Ecuador	DQ388042	
Tulasnella calospora	MAFF P305803	Ecuador	DQ388043	
Tulasnella calospora	MAFF P305804	Ecuador	DQ388044	
Tulasnella calospora	MAFF P305805	Ecuador	DQ388045	
Tulasnella calospora	FCb4	China	KC796458	SH1554832.08FU
Tulasnella danica	KC388	USA	AY373297	
Tulasnella eichleriana	KC852	Unknown	AY373292	
Tulasnella eichleriana	K(M)143600	United Kingdom	KC152381	
Tulasnella irregularis	JHW 0632	Australia	EU218889	
Tulasnella irregularis	D1-KT-TC-1	Thailand	GU166413	
Tulasnella irregularis	C3-DT-TC-2	Thailand	GU166423	SH1561236.08FU
Tulasnella prima	CLM159	Australia	KF476556	
Tulasnella prima	07033-45	Australia	HM196800	
Tulasnella pruinosa	DAOM 17641	Unknown	AY373295	
Tulasnella pruinosa	AFTOL ID610	Unknown	DQ457642	SH1549691.08FU
Tulasnella secunda	CLM009	Australia	KF476575	
Tulasnella secunda	CLM222	Australia	KF476568	
Tulasnella sp.	141	USA	AY373264	
Tulasnella sp.	10 MM-2016	USA	KU664580	
Tulasnella sphagneti	CLM541	Australia	KY095117	
Tulasnella sphagneti	CLM583	Australia	KY445922	
Tulasnella tomaculum	KC429	Unknown	AY373296	
Tulasnella tubericola	EP-15	Spain	KX929166	

Tulasnella tubericola	EP-1	Spain	KX774345	
Tulasnella violea	FO24380a	Germany	KC152439	SH1555437.08FU
Tulasnella violea	DC292	Germany	KC152432	
Tulasnella warcupii	CLM027	Australia	KF476596	
Tulasnella warcupii	CLM007	Australia	KF476600	
Uncultured *Tulasnella*	Clone 33tu-12	China	HM230652	
Botryobasidium botryosum	AFTOL ID604	Germany	DQ267124	

Our phylogenetic analyses confirmed that mycorrhizal fungi isolated from the studied orchid species were *Tulasnella* (Fig. 1). Among these, four species are new in this genus and are described below, namely, *Tulasnella brigadeiroensis*, *Tulasnella hadrolaeliae*, *Tulasnella orchidis* and *Tulasnella zygopetali*. The newly proposed species are based on phylogenetic analyses, pairwise sequence divergence and morphological features (see below). The clades containing the Brazilian *Tulasnella* isolates are highlighted in the phylogenetic tree (Fig. 1).

Phylogenetically, all isolates of *Tulasnella* from *C. caulescens* and *C. cinnabarina* are grouped in a clade including *T. calospora* isolates, close to another group composed of *T. tubericola* and *T. bifrons* (Fig. 1). The new species *Tulasnella hadrolaeliae* formed a clade (Maximum likelihood (ML)/Posterior probabilities (PP) = 100/1), which is a sister group of *T. albida* and *T. pruinosa*. *Tulasnella brigadeiroensis* isolates were grouped in a monophyletic clade. *Tulasnella orchidis,* isolated from *Z. maxillare,* clustered in a sister clade to *T. brigadeiroensis* and *Tulasnella* sp. COAD 2885. Finally, isolates of *Tulasnella zygopetali* obtained from *Z. maxillare* formed a strongly supported clade (ML/PP = 100/1), distinct from other *Tulasnella* species. Although the phylogenetic analyses indicate that *Tulasnella* sp. COAD 2885 may represent a new species, it will not be formally described here since only one isolate was obtained during our study.

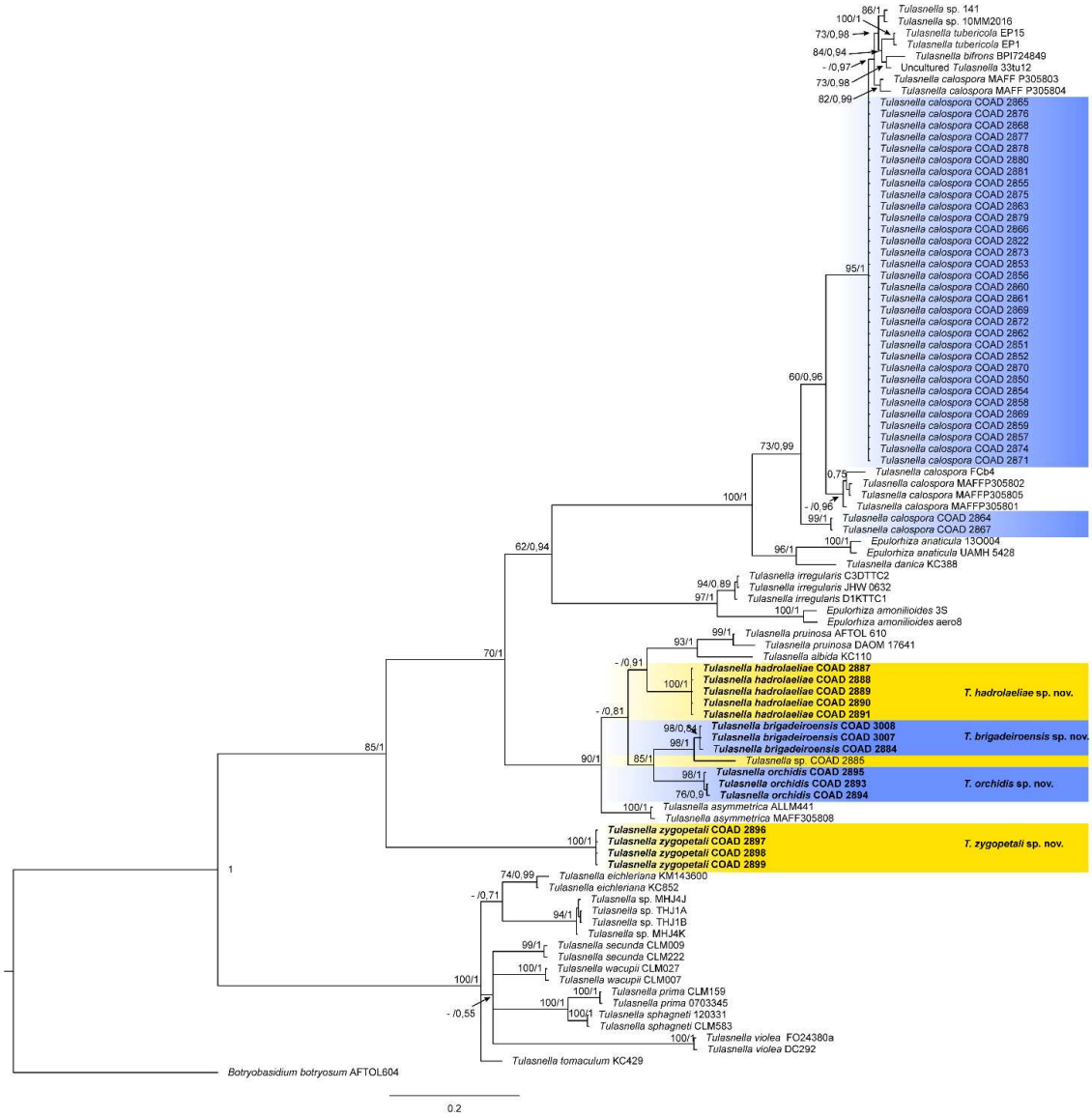

Fig. 1. Bayesian phylogenetic tree for *Tulasnella* based on ITS alignment. Maximum likelihood bootstrap support (ML > 60) and Bayesian posterior probabilities (PP) values are indicated next to the nodes (ML/PP). Species from Brazil are in the colored block and the new species described in this paper are indicated in bold face. *Botryobasidium botryosum* (AFTOL604) was used as the outgroup.

Divergence within and between clades

The Kimura-2-parameter distances between *Tulasnella* species ranged from 1.9 to 65.2% (Table 3). The divergence within *Tulasnella* species described here was lower than 0.6%. The nucleotide divergence between *Tulasnella* sp. COAD 2885 and *T. brigadeiroensis* was 7.5%, far above the 3% threshold suggested by Linde *et al.*[31] in *Tulasnella*, and supposedly belong to two different species. For some species, it was not possible to calculate the divergence within the clade, because only one isolate was used in the analysis.

Taxonomy

Tulasnella brigadeiroensis E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya, **sp. nov.** (Fig. 2)

Mycobank: MB832785

Etymology:— Referring to Parque Estadual Serra do Brigadeiro, where the type species was isolated.

Diagnosis: Tulasnella brigadeiroensis is phylogenetically closely related to *T. orchidis.* In a comparison of the 583 ITS nucleotides, *T. brigadeiroensis* differs from *T. orchidis* by 47bp (8.1%).

Type:—**BRAZIL:** Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of the orchid *Hadrolaelia jongheana*, February 2018, E.F.S. Freitas (holotype VIC47299, ex-type culture COAD2884).

Description: Colonies on PDA attaining 31 mm diam after 8 d at 25 ºC, white to cream, with undulate and submersed edge, aerial mycelium present. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 1.5–2.5 µm diam

Table 3. Estimates of percentage nucleotide divergence by the Kimura-2P distances for *Tulasnella* within and between species. There was a total of 272 positions in the final dataset. All positions containing gaps and missing data were eliminated.

	Within taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	2.2																					
2	–	8.2																				
3	1.2	16.8	14.4																			
4	0.4	16.4	14.9	2.5																		
5	–	18.1	15.1	4.3	4.4																	
6	0.4	32.9	32.7	33.7	33.6	33.5																
7	0.0	33.4	33.2	38.8	38.4	37.4	10.9															
8	–	32.9	33.9	37.3	38.2	38.7	12.6	9.0														
9	0.2	33.0	30.8	33.2	34.4	34.4	5.9	11.0	12.1													
10	–	38.2	36.6	38.8	40.4	40.1	11.7	15.9	15.9	7.5												
11	0.0	31.5	30.2	32.8	32.6	32.2	8.8	11.2	12.0	8.8	14.6											
12	0.5	34.4	32.6	34.4	35.1	33.0	8.2	10.4	13.6	5.4	10.5	8.1										
13	0.0	33.9	33.3	32.9	32.4	35.0	27.9	28.7	31.3	26.2	28.9	22.8	26.9									
14	3.4	34.4	35.0	35.9	35.1	38.1	33.6	33.1	36.1	30.1	34.8	29.1	31.0	8.6								
15	0.0	47.1	44.0	43.5	42.6	42.7	37.0	36.6	44.6	37.3	41.6	40.0	36.2	41.0	48.0							
16	0.7	58.8	57.7	60.4	61.6	63.6	49.4	48.0	52.4	46.3	51.4	54.0	49.1	51.3	52.5	50.2						
17	0.0	57.1	55.2	59.4	60.7	60.9	48.7	46.6	50.8	45.7	48.6	54.2	47.7	51.4	56.0	51.7	7.0					
18	–	57.3	56.2	59.5	60.9	61.1	48.0	46.7	49.4	45.1	50.1	51.1	47.2	50.7	53.7	48.8	3.8	5.0				
19	0.4	60.9	59.8	63.0	64.0	65.2	49.9	51.4	49.6	47.4	50.4	52.4	49.0	53.7	61.8	54.7	8.6	9.7	8.0			
20	0.0	61.3	60.2	62.6	64.1	64.3	54.6	52.4	55.3	50.6	56.1	55.7	54.3	54.4	56.7	51.0	8.8	10.7	8.2	11.8		
21	0.0	59.2	57.7	61.6	63.1	63.3	52.3	50.1	53.0	48.4	53.7	53.3	52.0	55.1	55.8	48.7	9.2	11.2	8.6	12.2	1.9	
22	0.4	62.9	62.6	59.4	60.4	59.8	53.9	49.3	51.3	52.1	54.5	51.4	52.2	52.5	56.5	54.8	18.0	17.6	16.1	19.0	21.1	20.5

1 = *Tulasnella anaticula*, 2 = *T. danica*, 3 = *T. calospora*, 4 = *T. tubericola*, 5 = *T. bifrons*, 6 = *T. asymmetrica*, 7 = *T. pruinosa*, 8 = *T. albida*, 9 = *T. brigadeiroesis*, 10 = *Tulasnella* sp. COAD 2885, 11 = *T. hadrolaelia*, 12 = *T. orchidis*, 13 = *T. irregulares*, 14 = *T. amonilioides*, 15 = *T. zygopetali*, 16 = *T. eichleriana*, 17 = *T. secunda*, 18 = *T. tomaculum*, 19 = *T. wacupii*, 20 = *T. prima*, 21 = *T. sphagneti*, 22 = *T. violea*

($\overline{X} \pm SD = 2 \pm 0.3$ µm), hyaline, with binucleate cells. Molinioid cells not observed. Sexual morph not observed.

Substrate or host: Roots of *Hadrolaelia jongheana.*

Additional material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Hadrolaelia jongheana*, October 2019, E.F.S. Freitas (COAD3007, COAD3008). This species was isolated three times from two roots. There was no difference between the morphology of the isolates.

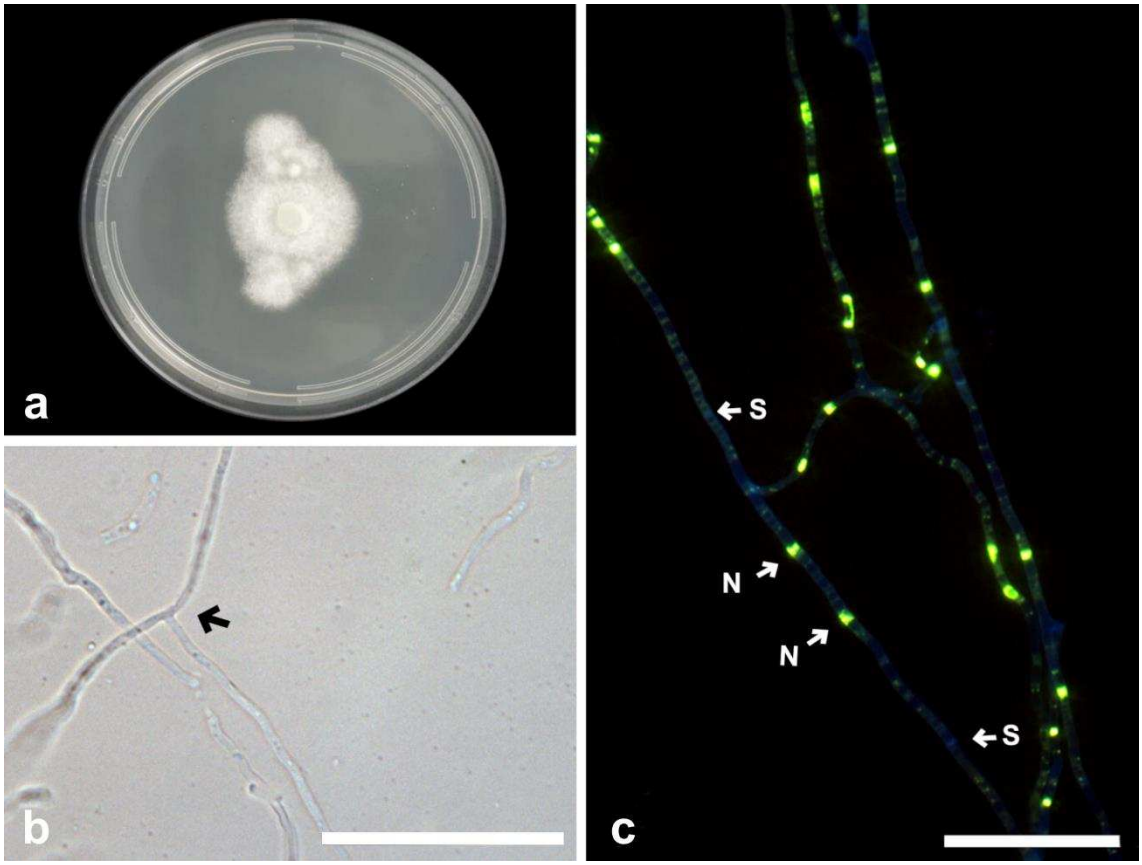

Fig. 2. *Tulasnella brigadeiroensis* (COAD2884). **a.** Eight-day-old PDA culture. **b.** Hyphae with branching at right angles. **c.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). Bars = 50 µm

Tulasnella calospora Juel, Bih. K. svenska Vet-Akad. Handl. 23: 23 (1897). (Fig. 3)

Description: Colonies on PDA attaining 45–67 mm diam after 8 d, at 25 °C, white to cream, with undulate and submersed edge, some cultures showing aerial mycelium. Hyphae from cultures are regularly septate, with branching at right angles, 3–4 µm diam ($\overline{X}$ ± SD = 3.5 ± 0.3 µm), hyaline, with binucleate cells. Molinioid hyaline, barrel to elongated barrel-shaped, in branched chains with more than five cells. Sexual morph not observed.

Substrate or host: Roots of *Cattleya caulescens* and *Cattleya cinnabarina*

Additional material examined—BRAZIL. Minas Gerais, Mariana, Mina da Alegria, Vale S.A., isolated from roots of *Cattleya caulescens*, COAD 2850–COAD2863; and from roots of *Cattleya cinnabarina*, COAD2864–2883, 2010, Bocayuva, M.F. There was no difference between the morphology of the isolates.

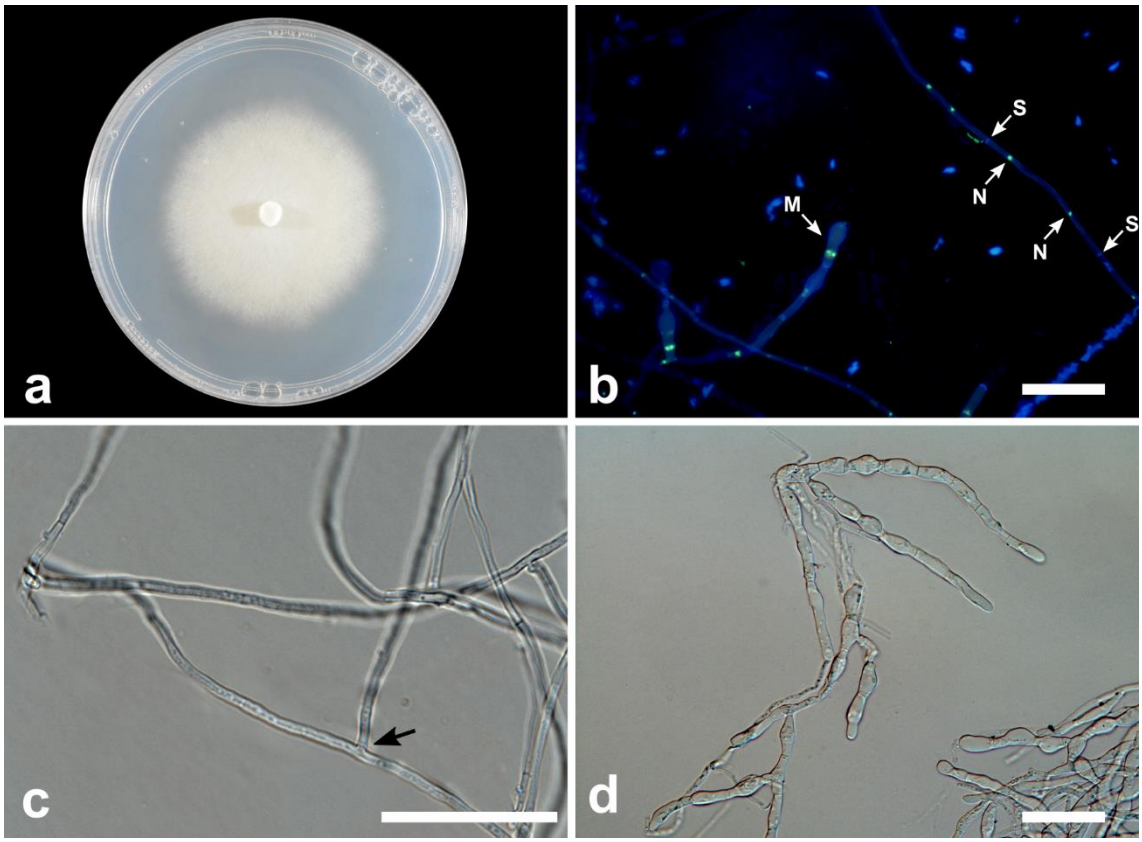

Fig. 3. *Tulasnella calospora* (COAD2869). **a.** Eight-day-old PDA culture. **b.** Hyphae stained with SYBR Green I showing binucleate cells (MC = monilioid cell; N = nuclei; S = septa). **c.** Hyphae with branching at right angles. **d.** Monilioid cell chains in CMA. Bars = 50 µm

Tulasnella hadrolaeliae E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya, **sp. nov.** (Fig. 4)

Mycobank: MB832786

Etymology: — Name derived from the plant host genus *Hadrolaelia*.

Diagnosis: Tulasnella hadrolaeliae is phylogenetically closely related to *T. albida* and *T. pruinosa.* In a comparison of the ITS nucleotides, *T. hadrolaeliae* differed from *T. albida* by 64 bp (11%) and from *T. pruinosa* by 73 bp (12.5%).

Type:—**BRAZIL:** Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of orchid *Hadrolaelia jongheana*, February 2018, E.F.S. Freitas (holotype VIC47304, ex-type culture COAD2889).

Description: Colonies on PDA showed very slow-growing (56–59 mm diam after 30 d at 25 ºC), white to cream, showing concentric rings, with undulate and submersed edge, aerial mycelium present. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 2–3.5 µm diam ($\overline{X}$ ± SD = 2.5 ± 0.3 µm), hyaline, with binucleate cells and thin-walled. Molinioid cells not observed. Sexual morph not observed.

Substrate or host: Roots of *Hadrolaelia jongheana.*

Additional material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Hadrolaelia jongheana*, February 2018, E.F.S. Freitas (COAD2887, COAD2888, COAD2890, COAD2891). This species was isolated five times from three roots. There was no difference between the morphology of the isolates.

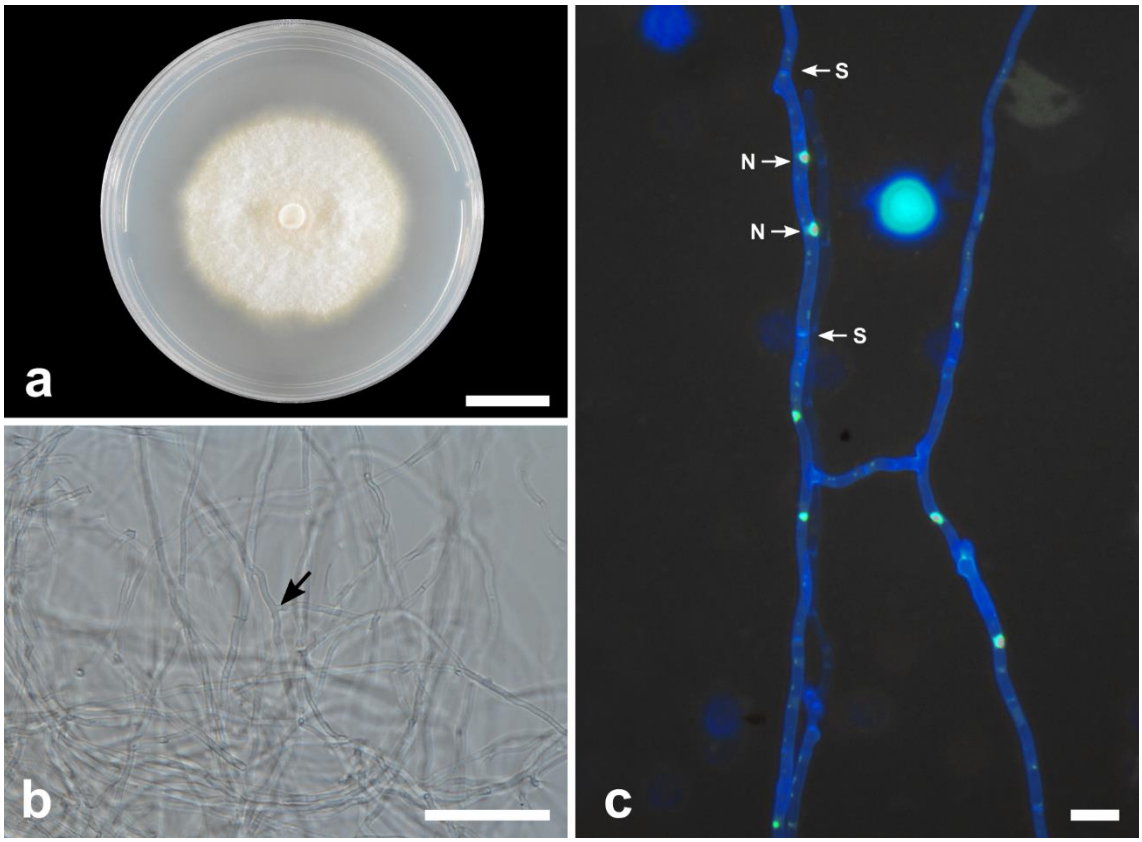

Fig. 4. *Tulasnella hadrolaeliae* (COAD2889). **a.** Thirty-day-old PDA culture. **b.** Hyphae with branching at right angles. **c.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). Bars: B = 50 µm; C = 10 µm

Tulasnella orchidis E.S. Cruz, E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya, **sp. nov.** (Fig. 5)

Mycobank: MB832787

Etymology:— Name derived from the nature of host, an orchid, from which it was isolated.

Diagnosis: Tulasnella orchidis differs from *T. brigadeiroensis* by the culture characteristics on PDA, colonies forming concentric rings with undulate edge, whereas *T. brigadeiroensis* show uniform colonies with regular edge. In a comparison of the 583 ITS nucleotides, *T. orchidis* differed from *T. brigadeiroensis* by 47 bp (8%).

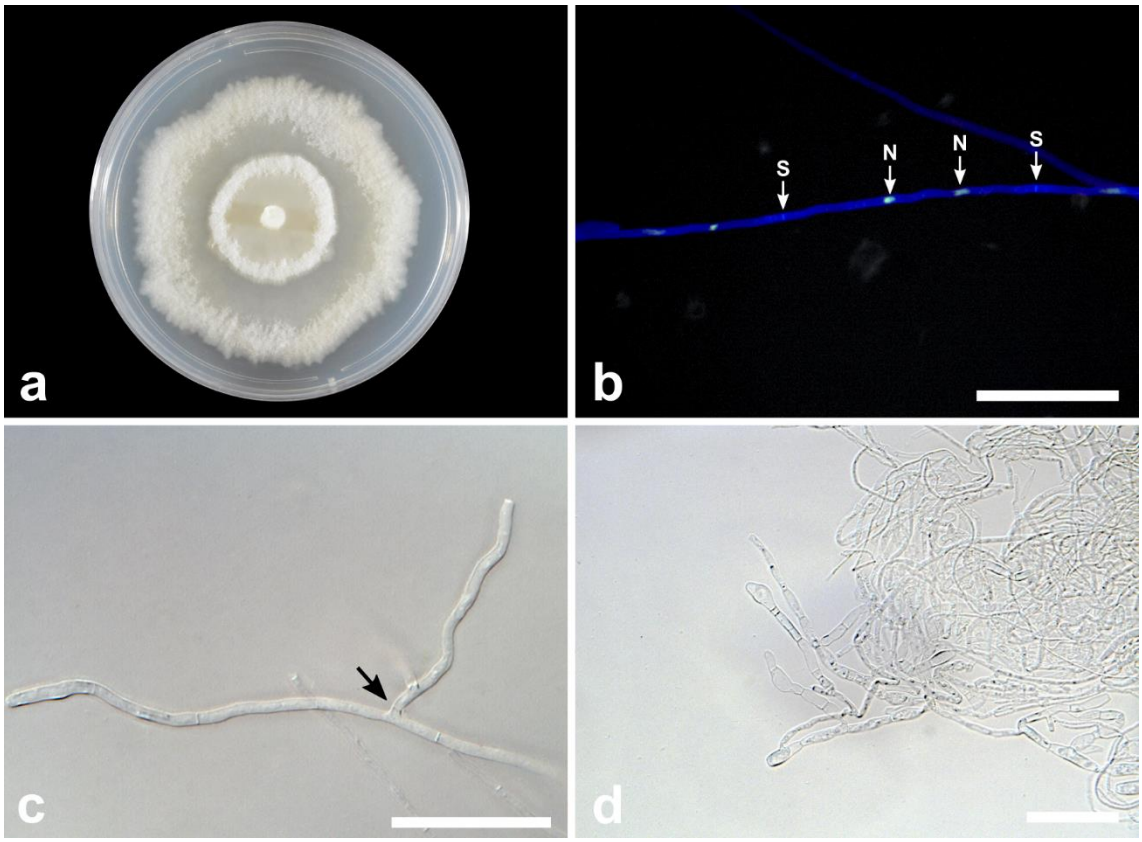

Fig. 5. *Tulasnella orchidis* (COAD2893). **a.** Fourteen-day-old PDA culture. **b.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c.** Hyphae with branching at right angles. **d.** Monilioid cell chains in CMA. Bars = 50 µm

Type:—**BRAZIL:** Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of *Zygopetalum maxillare*, February 2019, E.S. Cruz (holotype VIC47308, ex-type culture COAD2893).

Description: Colonies on PDA attaining 62–71 mm diam after 14 d, at 25 °C, white to cream, with undulate and submersed edge, showing concentric rings, no formation of aerial mycelium. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 2.5–4.5 µm diam ($\overline{X}$ ± SD = 3.5 ± 0.5 µm), hyaline, with binucleate cells and thin-walled. Molinioid cells hyaline, barrel to elliptical-shaped, 5–11.5 µm diam ($\overline{X}$ ± SD = 8 ± 2 µm) and in branched chains. Sexual morph not observed.

Substrate or host: Roots of *Zygopetalum maxillare.*

Additional material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Zygopetalum maxillare*, February 2019, E.S. Cruz (COAD2894, COAD289). This species was isolated three times from the same root. There was no difference between the morphology of the isolates.

Tulasnella zygopetali E.S. Cruz, E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya, **sp. nov.** (Fig. 6)

Mycobank: MB832789

Etymology: — Name derived from the plant host genus *Zygopetalum*, from which it was first collected.

Diagnosis: Tulasnella zygopetali is phylogenetically different from other *Tulasnella* species. Morphologically, *T. zygopetali* differs from other *Tulasnella* species described here as it has wider hyphae (3–6 µm diam) and monilioid cells (6.5–12.5 µm diam). In a comparison of the 583 ITS nucleotides, *T. zygopetali* differed from *T. brigadeiroensis* by 134 bp (23%), from *T. hadrolaeliae* by 148 bp (25.4%) and from *T. orchidis* by 135 bp (23%).

Type:—**BRAZIL:** Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of *Zygopetalum maxillare*, February 2019, E.S. Cruz (holotype VIC47311, ex-type culture COAD2896).

Description: Colonies on PDA attaining 86 mm diam after 8 d, at 25 °C, white to cream, with regular and submersed edge, dense aerial mycelium. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 3–6 µm diam ($\overline{X} \pm SD = 4 \pm 0.9$ µm), hyaline, with binucleate cells and thin-walled. Molinioid cells hyaline, elongated barrel-shaped, 6.5–12.5 µm diam ($\overline{X} \pm SD = 10 \pm 1.5$ µm), in branched chains with more than five cells. Sexual morph not observed.

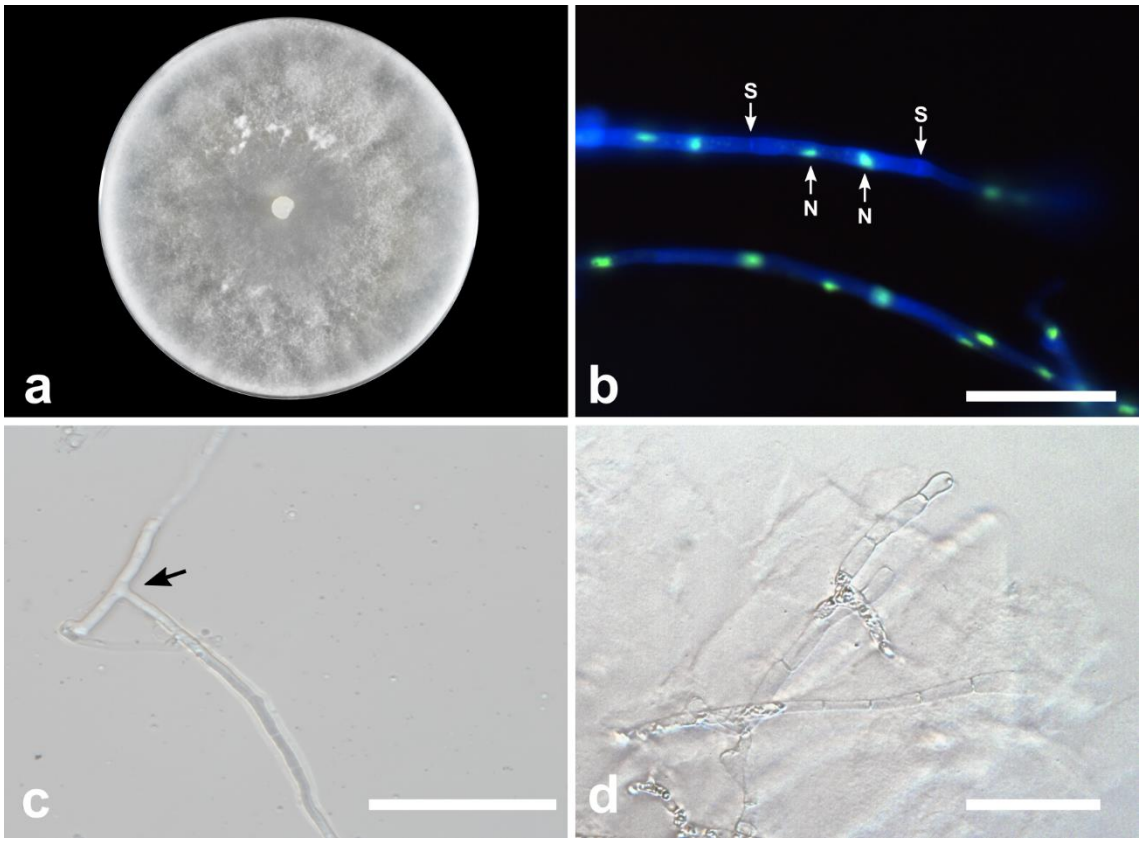

Fig. 6. *Tulasnella zygopetali* (COAD2896). **a.** Eight-day-old PDA culture. **b.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c.** Hyphae with branching at right angles. **d.** Monilioid cell chains in CMA. Bars = 50 µm

Substrate or host: Roots of *Zygopetalum maxillare.*

Additional material examined—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Zygopetalum maxillare*, February 2019, E.S. Cruz (COAD2897, COAD2898, COAD2899). This species was isolated four times from the same root. There was no difference between the morphology of the isolates.

DISCUSSION

We investigated *Tulasnella* species associated with the roots of four Brazilian orchids from different vegetations of the Atlantic Forest, where this fungal genus is little

known. A previous study of the same area, based only on the molecular approach, observed high fungal community diversity in roots of *H. jongheana*, *C. caulescens* and *C. cinnabarina* orchids, but no *Tulasnella* was identified[8]. The authors suggested that *Tulasnella* sequences were not detected due to the primers used. Indeed, universal fungal primers such as ITS1F/ITS4 often do not detect *Tulasnella* species due to a high rate of molecular evolution of nuclear rDNA genes in this genus[35,39].

The genus *Tulasnella* (Tulasnellaceae) was described in 1888 by Schröter, with *Tulasnella lilacina* J. Schröt. as the type species, and nowadays there are 73 accepted species in Index Fungorum[40]. Due to the lack of molecular data from the type specimen, many *Tulasnella* species are described only by morphological-based approaches[38]. Morphological characters, such as size and shape of hyphae, basidia, sterigmata and basidiospore, when used alone, may lead to incorrect species identification[34], e.g. because they are affected by cultural conditions. For species delimitation, we have combined both molecular and morphological data as recommended by Cruz *et al.*[34,36], using ITS as suggested by Linde *et al.*[38].

Among the species of the genus *Tulasnella, T. calospora* is considered as a nearly universal orchid symbiont[41]. It has been isolated from orchids in Asia[42,43], Australia[44,45], Europe[46] and South America[47,48]. However, the definition of *T. calospora* species is still unclear, since phylogenies have shown taxonomic problems concerning this species[35]. In Brazil, *T. calospora* was obtained from the roots of the orchids *Oeceoclades maculata*[29], *Epidendrum secundum*, *Acianthera limae* and *Polystachya concreta*[48] in the Zona da Mata and Quadrilátero Ferrífero regions of the state of Minas Gerais. Herein, *T. calospora* was isolated from *C. caulescens* and *C. cinnabarina* roots also sampled in the Quadrilátero Ferrífero region. These results suggest that *T. calospora* is a nonspecific orchid symbiont broadly distributed in the studied region.

The present study also yielded information for four species, which likely are only a small fraction of the unknown *Tulasnella* species diversity. *Tulasnella hadrolaeliae* and *T. brigadeiroensis* are mycorrhizal fungi isolated from pelotons in the roots of *H. jongheana*, an endangered epiphytic orchid. *Tulasnella brigadeiroensis* was collected at two different times: first (February 2018) just one isolate was obtained, and second (October 2019) two additional isolates of the new species *T. brigadeiroensis* were collected. *Tulasnella zygopetali* and *T. orchidis* were isolated from pelotons from the same individual of *Zygopetallum maxillare*. *Zygopetalum maxillare* is an epiphytic orchid with high specificity in a host tree relationship[7]. In PESB, *Z. maxillare* grows exclusively on the stems of tree ferns.

The new *Tulasnella* species studied here were described using a polyphasic approach. Phylogenetically, *T. hadrolaeliae* formed a sister clade with *T. albida* and *T. pruinosa.* However, the definition of the phylogenetic species of *T. albida* cannot be confirmed due to the absence of molecular data from the type specimen[49]. Additionally, morphological characters cannot distinguish *T. albida* and *T. pruinosa*[34]. Therefore, as for *T. calospora*, molecular data from the type specimen are required to confirm the delimitation of the species *T. albida* and *T. pruinosa*[49].

Tulasnella brigadeiroensis and *T. orchidis* formed well-supported sisters clades. *Tulasnella brigadeiroensis* and *Tulasnella* sp. COAD 2885 showed high percentage sequence divergence between clades (7.5%). This value is higher than the 3% sequence divergence cut-off value proposed for species delimitation[50] or 3–5% divergence used for *Tulasnella* species[38]. Regarding the other new species described here, the interspecific nucleotide divergence ranged from 5.4 to 41.6%. These values are comparable to or even higher than those found in previous studies on *Tulasnella*[33,34,38].

Knowledge of the diversity of orchid mycorrhizal fungi is important for successful conservation strategies[4], together with their maintenance in culture collection. Our study contributes to the description of diversity of *Tulasnella* associated with orchids of the Brazilian Atlantic Forest, which is relevant for conservation of these orchids and for knowledge of fungal richness in this hotspot of biodiversity. Further studies are required to verify the potential of new species to support seed germination, seedling development and, consequently, orchid conservation programs.

CONCLUSIONS

Phylogenetic analyses, integrated with nucleotide divergence and morphological characteristics, showed the diversity of *Tulasnella* species associated with orchids of the Brazilian Atlantic Forest, including the description of four novel *Tulasnella* species. This is the first study using a polyphasic approach to the description of *Tulasnella* in Brazil, and it suggests that further studies will uncover more diversity. The cultivation of these species may help the strategies of conservation of endangered Brazilian orchids.

METHODS

Sample collection and isolates

Root samples of the epiphytic orchid *H. jongheana* were collected from the PESB (Araponga – MG, Brazil) and PESN (Itamarandiba – MG, Brazil) (Fig. 7). *Zygopetalum maxillare* samples were also obtained from PESB, while *C. cinnabarina* and *C. caulescens* were sampled from iron mining areas in the Quadrilátero Ferrífero region (Mariana – MG, Brazil) (Fig. 7). Apparently healthy roots were analyzed at the Laboratório de Associações Micorrízicas (DMB/UFV). The root samples were gently

washed under running tap water, cut into pieces of transversal root fragments, 2–3 mm thick, surface-sterilized in 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, followed by two successive rinses of sterile distilled water. These fragments were then examined under a stereomicroscope, after slicing into several thin transversal slices. Cells containing pelotons were placed on potato dextrose agar (PDA) medium without antibiotics and then incubated at 25 ºC in the dark. Axenic cultures were preserved on rice grains in an ultrafreezer at -72 ºC or silica gel and were deposited in the Coleção Octávio Almeida Drummond collection (COAD) at the Universidade Federal de Viçosa. Representative specimens were deposited at the herbarium of the Universidade Federal de Viçosa (VIC).

Fig. 7. Investigated orchids: **a**, flower of *Hadrolaelia jongheana*; **b**, *Zygopetalum maxillare*; **c**, flower of *Cattleya cinnabarina*; **d**, flower of *Cattleya caulescens*.

Morphology

The fungus and colony characteristics were described from cultures grown on PDA at 25 ºC in the dark for 1–4 weeks depending on their growth rate. Measurements of colony diameters were taken using digital calipers. Color terminology followed Rayner[51]. The nuclear condition was observed from young hyphae after staining with SYBR Green I according to Meinhardt *et al.*[52]. The isolates were transferred to Corn Meal Agar (CMA) medium and incubated at 25 ºC in the dark, for 4–6 weeks, to induce monilioid cell formation[29]. Observations, measurements and photographic images of microscopic fungal structures were recorded using an Olympus BX53 light microscope, with an Olympus Q-Color5TM digital high-definition color camera and differential interference contrast (DIC) illumination. Adobe Photoshop CS5 was used for the final editing of the acquired images and photographic preparations.

DNA extraction, PCR amplification and sequencing

The genomic DNA was extracted from fungal mycelia grown on PDA at 25 ºC for 4 weeks, using the Nucleospin® Soil (MACHEREY-NAGEL GmbH & Co. KG), in accordance with the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using primer pairs ITS1 and ITS4[53]. Each polymerase chain reaction (PCR) was performed in 50 µL containing 10–20 ng of DNA template, 1× Taq buffer, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.4 mM of each dNTP, and 1.0 U Taq DNA polymerase (Cellco Biotec do Brasil Ltda, São Paulo, Brazil). PCR was carried out using a MyCyclerTM Thermal Cycler (Bio-Rad Laboratories B.V., Veenendal, The Netherlands) with an initial denaturation at 95 °C, for 2 min, followed by 39 PCR cycles (denaturation at 95 °C for 1 min; annealing at 50 °C for 1 min; extension at 72 °C for 1 min) before a final extension at 72 °C for 10 min.

The PCR products were visualized on 1% agarose gels stained with ethidium bromide to assess product size and quality, purified and then sequenced from the two strands using the primers ITS1 and ITS4[53]. Consensus sequences were generated using the MEGA v.7.0.26 software tool[54]. All sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. The sequences were deposited in GenBank (see accession numbers in Table 1).

Phylogenetic analyses

Consensus sequences were compared against NCBI's GenBank nucleotide databases by using the BLASTn algorithm. The most similar sequences were downloaded in FASTA format and aligned with our sequences by using the MAFFT v. 7 online portals[55]. The resulting sequence alignments were manually checked and adjusted in MEGA v.7.0.26 software tool[54].

Bayesian inference (BI) analyses employing a Markov Chain Monte Carlo method were performed on all sequences. Nucleotide substitution models were determined using the MrModeltest 2.3 program[56] and, once the likelihood scores had been calculated, the models were selected according to the Akaike information criterion (AIC). The results of MrModeltest recommended a GTR+G model for ITS, and a dirichlet (1,1,1,1) state frequency distribution and a gamma distributed rate variation were set. The phylogenetic analysis was performed using the CIPRES web portal[57] and the MrBayes program v.3.1.1[58]. Two sets of four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations and sampling every 1,000th generation. The first 25% of the trees were discarded as the burn-in phase for each analysis. Posterior probabilities[59] were determined from the remaining trees and are presented on the left of each node.

Maximum likelihood (ML) analysis was implemented using the RAxML-HPC v.8 on XSEDE (8.2.12) available on the CIPRES web portal. Parameters for maximum likelihood were set to rapid bootstrapping and the analysis was carried out using 1000 replicates. Alignments and trees were deposited in TreeBASE (http://treebase.org/treebase-web/) (25158). The trees were visualized in FigTree V1.4.4[60] and the layout of the tree for publication was done using Adobe Illustrator v. CC.

Divergence between clades

In order to assess the sequence divergence between and within the clades obtained in the phylogeny tree, the Kimura-2-parameter distances were calculated as implemented in MEGA v.7.0.26[61]. The analysis involved 85 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 272 positions in the final dataset.

Data availability

All materials examined were deposited in the public culture collection of the Coleção Octávio Almeida Drummond (COAD), of the Universidade Federal de Viçosa. Alignments and tree files generated during the current study are available at TreeBASE (accession https://www.treebase.org/treebase-web/home.html; study 25158). All sequence files are available from the GenBank database. The complete list of accession numbers is included in Table 1. They will be made available to the public after the publication of the paper.

REFERENCES

1. Govaerts, R. WCSP: World Checklist of Selected Plant Families. In: Species 2000 & ITIS catalogue of life, 2016 annual checklist. Leiden, the Netherlands (2016).

2. Pridgeon, A. The illustrated encyclopaedia of orchids. Timber, Portland (1995).

3. Giulietti, A. M., Harley, R. M, De Queiroz L. P., Wanderley, M. G. L., Berg, C. V. D. Biodiversity and conservation of plants in Brazil. *Conservation Biology* **19**, 632−639, https://doi.org/10.1111/j.1523-1739.2005.00704.x (2005).

4. Swarts, N. D., Dixon, K. W. Terrestrial orchid conservation in the age of extinction. *Annals of Botany* **104**, 543–556, https://doi.org/10.1093/aob/mcp025 (2009).

5. Selosse, M.-A. The latest news from biological interactions in orchids: in love, head to toe. *New Phytologist* **202**, 337–340, https://doi.org/10.1111/nph.12769 (2014).

6. Martinelli, G., Moraes, M. A. *Livro Vermelho da Flora do Brasil*, 1st edn. Rio de Janeiro (2013).

7. Pridgeon, A. M., Cribb, P. J., Chase, M. W., Rasmussen, F. N. *Genera Orchidacearum: Epidendroideae* (Part Two), 5th vol. Oxford, New York (2009).

8. Oliveira, S. F., Bocayuva, M. F., Veloso, T. G. R., Bazzolli, D. M. S., Silva, C. C., Pereira, O. L., Kasuya, M. C. M. Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic Forest, Brazil. *Mycorrhiza* **24**, 55−64, https://doi.org/10.1007/s00572-013-0512-0 (2014).

9. Myers, N., Mittermeier, R. A, Mittermeier, C. G., Fonseca, G. A. B., Kent, J. Biodiversity hotspots for conservation priorities. *Nature* **403**, 853–858 (2000).

10. Dearnaley, J. W. D., Perotto, S., Selosse, M. A. Structure and development of orchid mycorrhizas. In: Martin, F. (ed) *Molecular Mycorrhizal Symbiosis*. Wiley-Blackwell, Hoboken, New Jersey, 63-86 (2016).

11. Smith, S. E., Read, D. J. *Mycorrhizal Symbiosis*, 3rd edn. Adelaide, Australia. (2008)

12. Dearnaley, J. D. W., Martos, F., Selosse, M. A. Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: Hock B (ed) *The Mycota IX: Fungal Associations*, 2nd edn. Springer, Berlin Heidelberg (2012).

13. Weiß, M., Waller, F., Zuccano, A., Selosse, M. A. Sebacinales – one thousand and one interactions with land plants. *New Phytologist* **211**, 20–40, https://doi.org/10.1111/nph.13977 (2016).

14. Jacquemyn, H., Honnay, O., Cammue, B. P. A., Brys, R., Lievens, B. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus Orchis. *Molecular Ecology* **19**, 4086–4095, https://doi.org/10.1111/j.1365-294X.2010.04785.x (2010).

15. McCormick, M. K., Whigham, D. F., Canchani-Viruet, A. Mycorrhizal fungi affect orchid distribution and population dynamics. *New Phytologist* **219**, 1207–1215, https://doi.org/10.1111/nph.15223 (2018).

16. Otero, J. T., Flanagan, N. S., Herre, E. A., Ackerman, J. D., Bayman, P. Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). *American Journal of Botany* **94**, 1944–1950, https://doi.org/10.3732/ajb.94.12.1944 (2007).

17. Girlanda, M., Segreto, R., Cafasso, D., Liebel, H. T., Rodda, M., Ercole, E., Cozzolino, S., Gebauer, G., Perotto, S. Photosynthetic Mediterranean meadow orchids

feature partial mycoheterotrophy and specific mycorrhizal associations. *American Journal of Botany* **98**,1148–1163, https://doi.org/10.3732/ajb.1000486 (2011).

18. Martos, F., Munoz, F., Pailler, T., Kottke, I., Gonneau, C., Selosse, M. A. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology* **21**, 5098–5109, https://doi.org/10.1111/j.1365-294X.2012.05692.x (2012).

19. Xing, X. et al. The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest. *Oikos* **128**, 1254-1264, https://doi.org/10.1111/oik.06363 (2019).

20. McCormick, M. K., Whigham, D. F., O'Neill, J. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* **163**, 425–438. https://doi.org/10.1111/j.1469-8137.2004.01114.x (2004)

21. Pereira, M. C., Pereira, O. L., Costa, M. D., Rocha, R. B., Kasuya, M. C. M. Diversidade de fungos micorrízicos Epulorhiza spp. isolados de Epidendrum secundum (Orchidaceae). *Revista Brasileira de Ciência do Solo* **33**, 1187–1197, https://doi.org/10.1590/S0100-06832009000500012 (2009).

22. Zi, X. M., Sheng, C. L., Goodale, U. M., Shao, S. C., Gao, J. Y. In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, Dendrobium aphyllum (Orchidaceae). *Mycorrhiza* **24**, 487–499, https://doi.org/10.1007/s00572-014-0565-8 (2014).

23. Khamchatra, N., Dixon, K. W., Tantiwiwat, S., Piapukiew, J. Symbiotic seed germination of an endangered epiphytic slipper orchid, *Paphiopedilum villosum* (Lindl.) Stein. from Thailand. *South African Journal Botany* **104**, 76–81, https://doi.org/10.1016/j.sajb.2015.11.012 (2016).

24. Meng, Y. -Y., Fan, X, -L., Zhou, L. -R., Shao, S. -C., Liu, Q., Selosse, M. -A., Gao, J. -Y. Symbiotic fungi undergo a taxonomic and functional bottleneck during orchid seed germination: a case study on *Dendrobium moniliforme*. *Symbiosis* **79**, 205-212, https://doi.org/10.1007/s13199-019-00647-x (2019).

25. Meng, Y. Y., Zhang, W. L., Selosse, M. A., Gao, J. Y. Are fungi from adult orchid roots the best symbionts at germination? A case study. *Mycorrhiza* **29**, 541–547, https://doi.org/10.1007/s00572-019-00907-0 (2019).

26. Pereira, M. C. et al. Morphological and molecular characterization of Tulasnella spp. fungi isolated from the roots of *Epidendrum secundum*, a widespread Brazilian orchid. *Symbiosis* **62**, 111–121, https://doi.org/10.1007/s13199-014-0276-0 (2014).

27. Pereira, M. C., Pereira, O. L., Costa, M. D., Rocha, R. B., Kasuya, M. C. M. Diversidade de fungos micorrízicos *Epulorhiza* spp. isolados de *Epidendrum secundum* (Orchidaceae). *Revista Brasileira de Ciência do Solo* **33**, 1187–1197 (2009).

28. Nogueira, R. E., Pereira, O. L., Kasuya, M. C. M., Lanna, M. C. S., Mendonça M. P. Fungos micorrízicos associados a orquídeas em campos rupestres na região do Quadrilátero Ferrífero, MG, Brasil. *Acta Botanica Brasilica* **19**(3), 417–424. https://doi.org/10.1590/S0102-33062005000300001 (2005).

29. Pereira, O. L., Kasuya, M. C. M., Borges, A. C., Araújo, E. F. Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. *Canadian Journal Botany* **83**, 54–65, https://doi.org/10.1139/b04-151 (2005).

30. Pereira, O. L., Rollemberg, C. L., Borges, A. C., Matsuoka, K., Kasuya, M. C. M. *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. *Mycoscience* **44**, 153-155, https://doi.org/10.1007/s10267-002-0087-7 (2003).

31. Linde, C. C., Phillips, R. D., Crisp, M. D., Peakall, R. Congruent species delineation of *Tulasnella* using multiple loci and methods. *New Phytologist* **201**, 6–12, https://doi.org/10.1111/nph.12492 (2014).

32. Solís, K., Barriuso, J. J., Garcés-claver, A., González, V. *Tulasnella tubericola* (Tulasnellaceae, Cantharellales, Basidiomycota): a new Rhizoctonia-like fungus associated with mycorrhizal evergreen oak plants artificially inoculated with black truffle (*Tuber melanosporum*) in Spain. *Phytotaxa* **317**, 175–187, https://doi.org/10.11646/phytotaxa.317.3.2 (2017).

33. Rachanarin, C., Suwannarach, N., Kumla, J., Srimuang, K.O., Mckenzie, E. H. C., Lumyong, S. A new endophytic fungus, *Tulasnella phuhinrongklaensis* (Cantharellales, Basidiomycota) isolated from roots of the terrestrial orchid, *Phalaenopsis pulcherrima*. *Phytotaxa* **374**, 99–109, https://doi.org/10.11646/phytotaxa.374.2.1 (2018).

34. Cruz, D., Suárez, J. P., Kottke, I., Piepenbring, M. Cryptic species revealed by molecular phylogenetic analysis of sequences obtained from basidiomata of *Tulasnella*. *Mycologia* **106**, 708–722, https://doi.org/10.3852/12-386 (2014).

35. Suárez, J. P., Weiß, M., Abele, A., Garnica, S., Oberwinkler, F., Kottke, I. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research* **110**, 1257–1270, https://doi.org/10.1016/j.mycres.2006.08.004 (2006).

36. Cruz, D., Suárez, J. P., Kottke, I., Piepenbring, M., Oberwinkler, F. Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8S sequence data of basidiomata from a tropical Andean forest. *Mycological Progress* **10**, 229–238, https://doi.org/10.1007/s11557-010-0692-3 (2011).

37. Fujimori, S., Abe, J. P., Okane, I., Yamaoka, Y. Three new species in the genus *Tulasnella* isolated from orchid mycorrhiza of *Spiranthes sinensis var. amoena* (Orchidaceae). *Mycoscience* **60**, 71–81, https://doi.org/10.1016/j.myc.2018.09.003 (2019).

38. Linde, C. C., May, T. W., Phillips, R. D., Ruibal, M., Smith, L. M., Peakall, R. New species of *Tulasnella* associated with terrestrial orchids in Australia. *IMA Fungus* **8**, 27–47, https://doi.org/10.5598/imafungus.2017.08.01.03 (2017).

39. Moncalvo, J. M. et al. The cantharelloid clade: dealing with incongruent gene trees and phylogenetic reconstruction methods. *Mycologia* **98**, 937–948, https://doi.org/10.1080/15572536.2006.11832623 (2006).

40. Kirk, P., Bridge, P. Index Fungorum. http://www.indexfungorum.org/ (2019).

41. Hadley, G. Non-Specificity of symbiotic infection in orchid mycorrhiza. *New Phytologist* **69**, 1015–1023 (1970).

42. Tan, X. M. et al. Isolation and identification of endophytic fungi in roots of nine *Holcoglossum* plants (Orchidaceae) collected from Yunnan, Guangxi, and Hainan Provinces of China. *Current Microbiology* **64**, 140-147, https://doi.org/10.1007/s00284-011-0045-8 (2012).

43. Ding, R. et al. Identity and specificity of rhizoctonia-like fungi from different populations of *Liparis japonica* (Orchidaceae) in northeast China. *PLOS ONE* **9**, e105573, https://doi.org/10.1371/journal.pone.0105573 (2014).

44. Warcupii, J. H. The mycorrhizal relathionships of Australian orchids. *New Phytologist* **87**, 371–381, https://doi.org/10.1111/j.1469-8137.1981.tb03208.x (1981).

45. De Long, J. R., Swarts, N. D., Dixon, K. W., Egerton-Warburton, L. M. Mycorrhizal preference promotes habitat invasion by a native Australian orchid: *Microtis media*. *Annals of Botany* **111**, 409–418, https://doi.org/10.1093/aob/mcs294 (2013).

46. Warcupii, J. H., Talbot, P. H. B. Perfect states of rhizoctonias associated with orchids. *New Phytologist* **66**, 631–64, https://doi.org/10.1111/j.1469-8137.1967.tb05434.x (1967).

47. Steinfort, U., Verdugo, G., Besoain, X., Cisternas, M. A. Mycorrhizal association and symbiotic germination of the terrestrial orchid *Bipinnula fimbriata* (Poepp.) Johnst (Orchidaceae). *Flora* **205**, 811–817, https://doi.org/10.1016/j.flora.2010.01.005 (2010).

48. Nogueira, R. E., Berg, C. van den, Pereira, O. L., Kasuya, M. C. M. Isolation and molecular characterization of Rhizoctonia-like fungi associated with orchid roots in the Quadrilátero Ferrífero and Zona da Mata regions of the state of Minas Gerais, Brazil. *Acta Botanica Brasilica* **28,** 298–300, https://doi.org/10.1590/s0102-33062014000200017 (2014).

49. Cruz, D., Suárez, J. P., Piepenbring, M. Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. *Nova Hedwigia* **102**, 279–338, https://doi.org/10.1127/nova_hedwigia/2015/0304 (2016).

50. Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N., Larsson, K. H. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics* **4**, 193-201, (2008).

51. Rayner, R.W. A Mycological Color Chart. Commonwealth Mycological Institute, Kew, (1970).

52. Meinhardt, L. W., Bellato, C. D. M., Tsai, S. M. SYBR® Green I used to evaluate the nuclei number of fungal mycelia. *Biotechniques* **31**, 42–46 (2001).

53. White, T. J., Bruns, T., Lee, S., Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: A Guide of Methods and Applications*. Academic Press, San Diego, 315–322 (1990).

54. Kumar, S., Stecher, G., Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets brief communication. *Molecular Evolutionary Genetics* **33**, 1870–1874, https://doi.org/10.1093/molbev/msw054 (2016).

55. Katoh, K., Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–778, https://doi.org/10.1093/molbev/mst010 (2013).

56. Nylander, J. A. A. MrModeltest version 2. Evolutionary Biology Centre, Uppsala, Sweden. https://github.com/nylander/MrModeltest2 (2004).

57. Miller, M. A., Pfeiffer, W., Schwartz, T. Creating the CIPRES science gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop. https://doi.org/10.1109/GCE.2010.5676129 (2010).

58. Ronquist, F., Huelsenbeck, J.P. MrBayes 3: Beyesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574, https://doi.org/10.1093/bioinformatics/btg180 (2003).

59. Rannala, B., Yang, Z. Proballity distribution of molecular evolutionary trees: A new method of phylogenetic inference. *Journal of Molecular Evolution* **43**, 304-311 (1996).

60. Rambaut, A. FigTree version 1.4.4. In: *Molecular evolution, phylogenetics and epidemiology*. http://tree.bio.ed.ac.uk/software/figtree/ (2009).

61. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. MEGA5: Molecular evolutionary genetics analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739, https://doi.org/10.1093/molbev/msr121 (2011).

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AUTHORS' CONTRIBUTIONS

E.F.S.F and M.S designed the study. Material preparation and data collection were performed by E.F.S.F., E.S.C., M.F.B. and T.G.R.V. Analyses were performed by E.F.S.F, M.S. and E.M. The first draft of the manuscript was written by E.F.S.F and was revised by M.S. All authors commented on previous versions of the manuscript. The work was substantially revised by M.-A.S. and supervised by M.C.M.K. All authors read and approved the final manuscript.

ADDITIONAL INFORMATION

Competing interests The authors declare that they have no competing interests.

CAPÍTULO 3

Two news *Tulasnella* species, mycorrhizal fungi from *Cattleya jongheana* (Orchidaceae).

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Cantharellales Gäum

Notes: The order Cantharellales shows a morphological complexity, which becomes difficult the use of these characters to define taxa within the order (Gónzalez et al. 2016). Cantharellales is divided into four families (Botryobasidiaceae, Ceratobasidiaceae, Hydinaceae and Tulasnellaceae) according to septal pore structure and secondary spore production (Hibbet et al. 2014). Fungi from Cantharellales have different nutritional modes such as saprotrophy, pathogenic and mycorrhizal symbiotic (Moncalvo et al. 2006).

Tulasnellaceae Juel

Notes: The family Tulasnellaceae was included in Cantharellales based on the phylogeny of mt-rDNA (Hibbet and Thorn, 2001) and accommodate the genera *Pseudotulasnella* Lowy, *Stilbotulasnella* Oberw. & Bandoni, and *Tulasnella* J. Schröt. (Cruz et al. 2016). Tulasnellaceae exhibit septa with imperforate parenthesomes and can produce secondary spores (Hibbet et al. 2014). The family is also characterized by hyaline hyphae, with or without clamp connections, bi- or multinucleate. Basidiomata are resupinate or effused. Holobasidia are globose, sphaeropendiculate to clavate, and with four sterigmata per basidium. Basidiospores are hyaline, thin-walled, smooth and show a range of shapes (Cruz et al. 2016).

Tulasnella J. Schröt.

Notes: Tulasnella was described in 1888 by Schröter, based on *Tulasnella lilacina* J. Schröt. as the type species (Schröter, 1888). This genus hardly forms fruitbodies *in situ* or sexual structures *in vitro* (Pereira et al. 2003; 2005; Cruz et al. 2011) and shows a high

rate of molecular evolution in nuclear rDNA (Moncalvo et al. 2006). Nowadays, molecular analyses based on ITS rDNA have been used as the main tool to delimit *Tulasnella* species (Linde et al. 2017; Sólis et al. 2017; Freitas et al. 2020), and 73 accepted species are listed in Index Fungorum (http://www.indexfungorum.org). Species of this genus have been studied as important orchid mycorrhizal fungi in different countries (Suárez et al. 2006; Pereira et al. 2014, Fujimori et al. 2018; Meng et al 2019; Freitas et al. 2020). *Tulasnella* is reported also as saprotrophs on decayed wood (Cruz et al. 2011) as well as endophytic in roots of non-orchids plants (Girlanda et al. 2011). In this study, we introduce two new *Tulasnella* species, isolated from the roots of *Cattleya jongheana* (Orchidaceae).

***Tulasnella* sp. 1** E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya

MycoBank number: MB836541; Fig. 1

Holotype: VIC47407.

Description: Colonies on PDA attaining 31 mm diam after 8 d, at 25 °C, white to cream, with regular and submersed edge, scarce aerial mycelium. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angle, 2–4 µm diam ($\overline{X}$ ± SD = 4 ± 0.4 µm), hyaline, with binucleate cells and thin-walled. Molinioid cells, hyaline, barrel to globose-shaped, thick-walled, 9–13 µm diam ($\overline{X}$ ± SD = 10.5 ± 1.2 µm), in branched chains. Sexual morph not observed.

Material examined: Brazil, Minas Gerais, Parque Estadual da Serra do Brigadeiro, isolated from roots of *Cattleya jongheana* (Orchidaceae), 2019, E.F.S. Freitas (holotype VIC47407, ex-type culture COAD3099). Additional specimen examined: Brazil, Minas Gerais, Parque Estadual da Serra do Brigadeiro, isolated from roots of *Cattleya jongheana* (Orchidaceae), 2019, E.F.S. Freitas (COAD3100).

GenBank number: ITS = MT808332 (Holotype), MT808333.

Notes: Tulasnella sp. 1 is phylogenetically closely related to *T. eichleriana* (Fig. 2). The Kimura-2-parameter distances between *T. mycorrhiza* and *T. eichleriana* showed high percentage sequence divergence between clades (8.3%) (data not shown).

Ecology: Tulasnella sp. 1 was isolated from pelotons dissected from *C. jongheana* roots, which suggests that it is orchid mycorrhizal fungi.

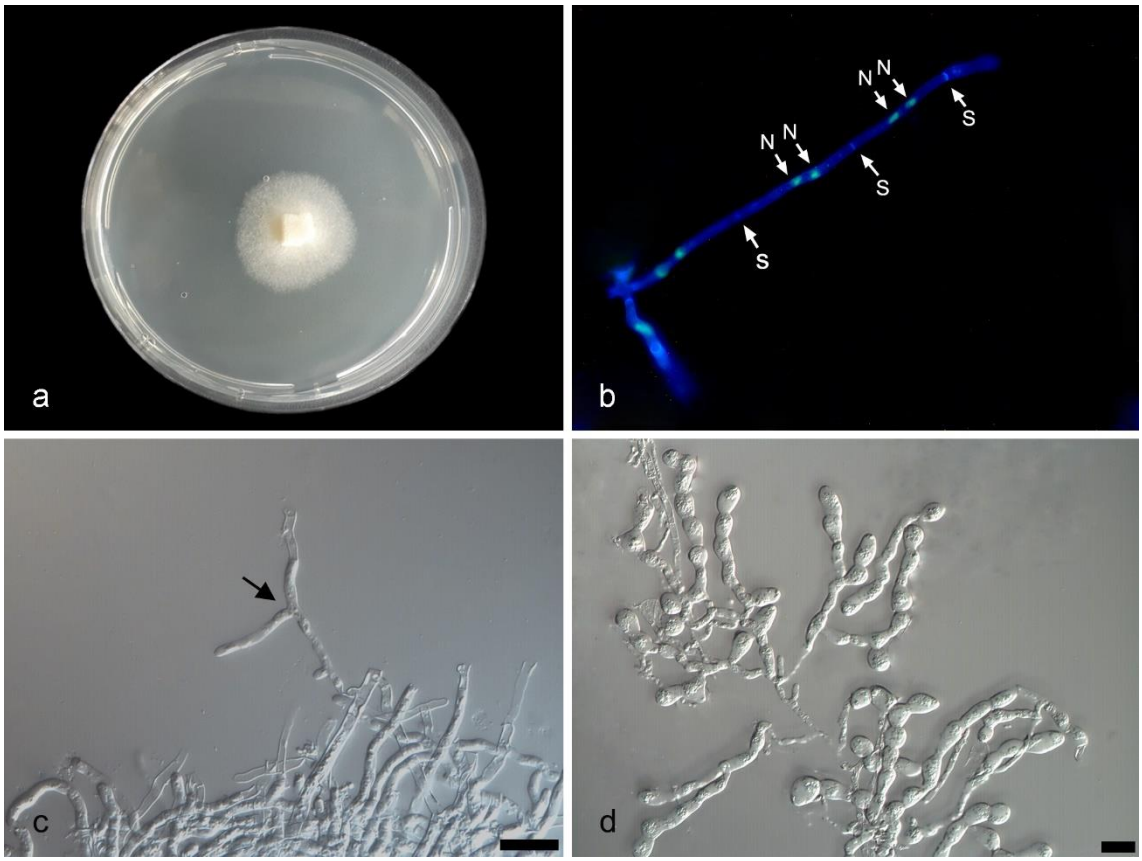

Figure 1: *Tulasnella* sp. 1 (COAD3099). **a.** Eight-day-old PDA culture. **b.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c.** Hyphae with branching at right angle. **d.** Monilioid cell chains in CMA. Bars: C and D= 20 µm

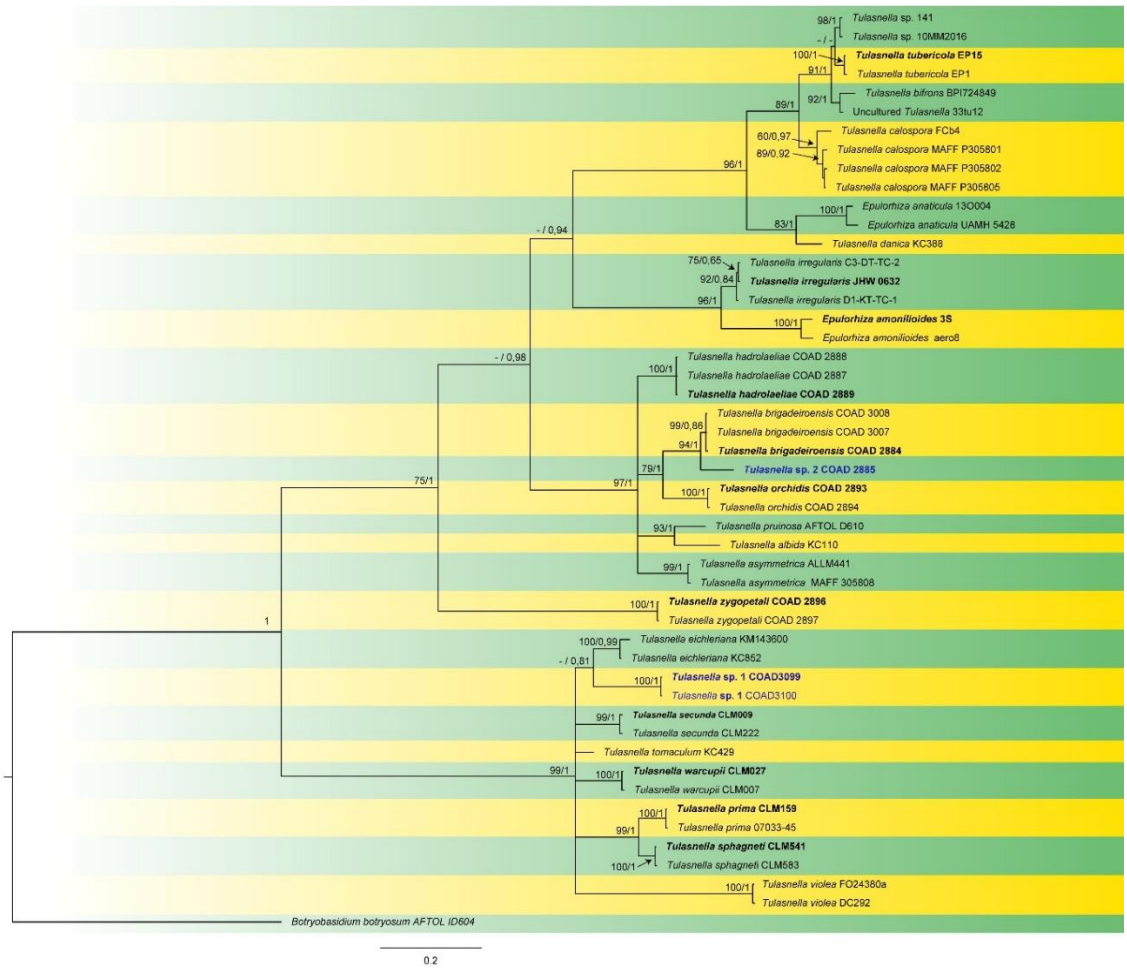

Figure 2: Bayesian phylogenetic tree for *Tulasnella* species based on ITS region of ribosomal DNA. Fifty-six strains are included in the gene sequence analyses with a total of 580 characters after alignment. The tree is rooted with *Botryobasidium botryosum* (AFTOL ID604). Tree topology of the maximum likelihood analysis was similar to the Bayesian analysis. Estimated base frequencies were as follows: A = 0.1926, C = 0.2860, G = 0.2754, T = 0.2460; substitution rates AC = 1.3236, AG = 3.2693, AT = 1.6134, CG = 1.1187, CT = 4.8406, GT = 1.00000; gamma distribution shape parameter α = 0.5218. The ex-type strains are indicated in bold. The news species are indicated in blue. Maximum likelihood bootstrap support (ML > 60) and Bayesian posterior probabilities (PP) values are indicated next to the nodes (ML/PP).

***Tulasnella* sp. 2** E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya

MycoBank number: MB832788; Fig. 3

Holotype: VIC47300

Description: Colonies on PDA attaining 41 mm diam after 8 d, at 25 °C, white to cream, with regular and submersed edge, scarce aerial mycelium. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angle, 2–3 µm diam ($\overline{X} \pm SD = 2.5 \pm 0.3$ µm), hyaline, with binucleate cells and thin-walled. Molinioid cells, hyaline, barrel to elongated barrel-shaped, 5–8 µm diam ($\overline{X} \pm SD = 7 \pm 1$ µm), in branched chains. Sexual morph not observed.

Material examined: Brazil, Minas Gerais, Parque Estadual da Serra Negra, isolated from roots of *Cattleya jongheana* (Orchidaceae), 2010, M.F. Bocayuva (holotype VIC47300, ex-type culture COAD2885).

GenBank number: ITS = MK192002

Notes: Tulasnella sp. 2 is phylogenetically closely related to *T. brigadeiroensis* (Fig. 2), a species also isolated from roots of *C. jongheana* in Brazil (Freitas et al. 2020). The Kimura-2-parameter distances between *T. brigadeiroensis* and *Tulasnella* sp. 2 showed high percentage sequence divergence between clades (6.2%) (data not shown).

Ecology: *Tulasnella* sp. 2 was isolated from pelotons dissected from *C. jongheana* roots, which suggests that it is orchid mycorrhizal fungi.

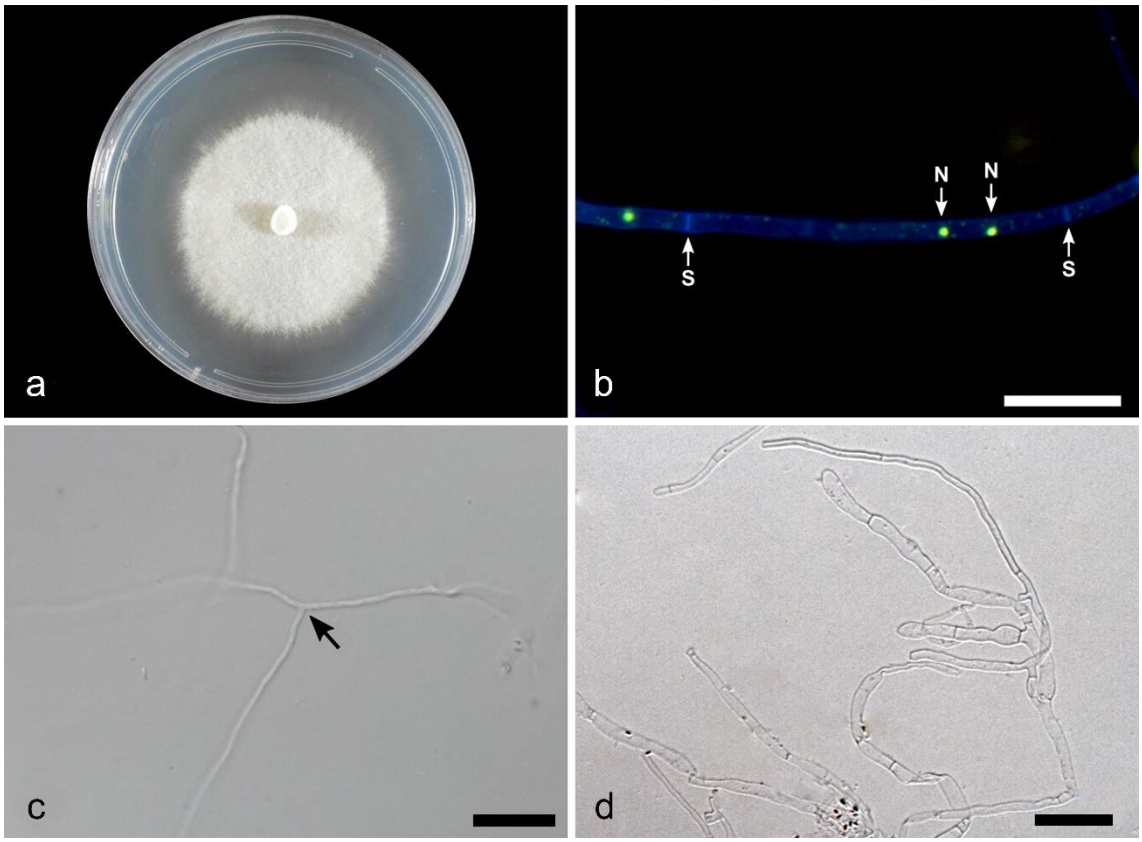

Figure 3: *Tulasnella* sp. 2 (COAD2885). **a.** Eight-day-old PDA culture. **b.** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c.** Hyphae with branching at right angle. **d.** Monilioid cell chains in CMA. Bars: B = 20 µm; C = 40 µm; D = 50 µm

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REFERENCES

Cruz D, Suárez JP, Kottke I, Piepenbring M, Oberwinkler F (2011) Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8S sequence data of basidiomata from a tropical Andean forest. Mycological Progress 10:229–238

Cruz D, Suárez JP, Piepenbring M (2016) Morphological revision of Tulasnellaceae, with two new species of *Tulasnella* and new records of *Tulasnella* spp. for Ecuador. Nova Hedwigia 102:279–338

Freitas EFS, Silva M, Cruz ES, Mangaravite E, Bocayuva MF, Veloso TGR, Selosse M-A, Kasuya MCM (2020) Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species. Scientific Reports 10:7069

Fujimori S, Abe JP, Okane I, Yamaoka Y (2019) Three new species in the genus *Tulasnella* isolated from orchid mycorrhiza of *Spiranthes sinensis* var. amoena (Orchidaceae). Mycoscience 60:71–81

Girlanda M, Segreto R, Cafasso D, Liebel HT, Rodda M, Ercole E, Cozzolino S, Gebauer G, Perotto S (2011) Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. American Journal of Botany 98:1148–1163

Gónzalez D, Rodriguez-Carres M, Boekhout T, Stalpers J, Kuramae EE, Nakatani AK, Vilgalys R, Cubeta MA (2016) Phylogenetic relationships of *Rhizoctonia* fungi within the Cantharellales. Fungal Biology 120:603–619

Hibbett DS, Bauer R, Binder M, Giachini AJ, Hosaka K, Justo A, Larsson E, Larsson KH, Lawrey JD, Miettinen O, Nagy LG (2014) Agaricomycetes. In: McLaughlin

DJ, Spatafora JW, eds. The Mycota VIIA Systematics and Evolution. Springer, Berlin, pp 373–429

Hibbett DS, Thorn RG (2001) Basidiomycota: Homobasidiomycetes. In: McLaughlin DJ, McLaughlin EG, Lemke PA, eds. The Mycota VIIB Systematics and Evolution. Berlin: Springer-Verlag. p 121–168

Index Fungorum (2020). http://www.indexfungorum.org/

Linde CC, May TW, Phillips RD, Ruibal M, Smith LM, Peakall R (2017) New species of *Tulasnella* associated with terrestrial orchids in Australia. IMA Fungus 8:27–47

Meng,Y-Y, Fan X-L, Zhou L-R, Shao S-C, Liu Q, Selosse M-A, Gao J-Y (2019) Symbiotic fungi undergo a taxonomic and functional bottleneck during orchid seed germination: a case study on *Dendrobium moniliforme*. Symbiosis 79:205–212

Moncalvo JM, Nilsson RH, Koster B, Dunham SM, Bernauer T et al. (2006) The cantharelloid clade: Dealing with incongruent gene trees and phylogenetic reconstruction methods. Mycologia 98: 937–948

Pereira MC, Coelho IS, Valadares RBS, Oliveira SF, Bocayuva M, Pereira OL, Araújo EF, Kasuya MCM (2014) Morphological and molecular characterization of *Tulasnella* spp. fungi isolated from the roots of *Epidendrum secundum*, a widespread Brazilian orchid. Symbiosis 62:111–121

Pereira OL, Kasuya MCM, Borges AC, Araújo EF (2005) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Canadian Journal Botany 83:54–65

Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, Kasuya MCM (2003) *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. Mycoscience 44:153–155

Schröter J (1888) Kryptogamen-Flora von Schlesien 3-1(4):385–512

Solís K, Barriuso JJ, Garcés-claver A, González V (2017) *Tulasnella tubericola* (Tulasnellaceae, Cantharellales, Basidiomycota): a new *Rhizoctonia*-like fungus associated with mycorrhizal evergreen oak plants artificially inoculated with black truffle (*Tuber melanosporum*) in Spain. Phytotaxa 317:175–187

Suárez, JP, Weiß M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycological Research 110:1257–1270

CAPÍTULO 4

Diversity of cultivable endophytic and mycorrhizal fungi isolated from *Cattleya jongheana* (Orchidaceae), and description of a new *Serendipita* species

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Diversity of cultivable endophytic and mycorrhizal fungi isolated from *Cattleya jongheana* (Orchidaceae) in Brazil, with a description of new *Serendipita* species

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Abstract

Brazilian Atlantic Forest is an important biodiversity hotspot in the world, which is strongest endangered due to anthropogenic activities. The biome stands out also by richness in orchids species, several of them at risk of extinction. Orchids associate with mycorrhizal and endophytic fungi, developing a mutualism relation. In this study, we investigated the diversity and composition of mycobionts in the roots of *Cattleya jongheana*, an endangered orchid from the Brazilian Atlantic Forest. Isolates were

obtained, cultured and molecularly identified through sequencing of internal transcribed spacer region (ITS) of the nuclear ribosomal DNA. Additionally, phylogenetic analyses were performed with mycorrhizal isolates. We identified 212 fungal isolates, corresponding to 32 genera. Endophytic fungi from Ascomycota were dominant (66,45%) and included *Colletotrichum*, *Microdiplodia, Trichoderma* and *Xylaria.* Mycorrhizal fungal were all members of Basidiomycota, and *Tulasnella* was the most frequent genera obtained (27,36%). *Serendipita* species were isolated less frequently (3,77%), and based on phylogeny and morphological data one new species is described.

Keywords: Endangered orchids, Hadrolaelia jongheana, Tulasnellaceae, Serendipitaceae, Phylogeny

Declarations

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Competing interests: The authors declare that they have no competing interests.

Availability of data and material: Materials examined from new species were deposited in the public culture collection of the Coleção Octávio Almeida Drummond (COAD), and materials from other isolates were deposited in the collection of Laboratório de Associações Micorrízicas, of the Universidade Federal de Viçosa. Alignments and tree files generated during the current study are available at TreeBASE (accession https://www.treebase.org/treebase-web/home.html). All sequence files are available from the GenBank database and the complete list of accession numbers is included in

Supplementary material 1. They will be made available to the public after the publication of the paper.

Authors' contributions: E.F.S.F and M.S designed the study. All authors contributed to the material preparation. Data collection were performed by E.F.S.F. Analyses were performed by E.F.S.F, M.S. and C.A.V. The first draft of the manuscript was written by E.F.S.F and was revised by M.S. All authors commented on previous versions of the manuscript. The work was substantially revised and supervised by M.C.M.K. All authors read and approved the final manuscript.

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INTRODUCTION

Brazilian Atlantic Forest is one important biodiversity hotspot of the world, which hosts about 8000 species of endemic plants (Myers 2000), and with the highest number of threatened species in Brazil (Martinelli et al 2013). Currently, the biome has only 28% of its original cover (Rezende et al 2018) and the degradation is resulting mainly from anthropic activities, as agriculture and livestock (Guerra et al 2020). Brazilian Atlantic Forest also stands out by the diversity of orchids with more than 1400 described species, several of which are threatened of extinction (Neto et al. 2013). The diversity of species and high level of threat in the Orchidaceae family is also associated to the complexity of ecological interactions which occur in these plants, such as interactions with pollinators, non-mycorrhizal endophytic fungi and mycorrhizal fungi (Mehra 2020).

Although 70% of orchids are epiphytic and most of them grow in tropical environments (Gentry and Dodson 1987), studies on mycorrhizal and endophytic fungi from orchids around the world have been focused on temperate terrestrial species (Ma et al 2015; Rasmussen et al 2015; McCormick et al 2018), and little is known about epiphytic orchids in tropical environments. Regardless of the region or lifestyle, the mycorrhizal association in orchids involves fungi from Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae, and *Tulasnella* has been the genus most frequently observed (Dearnaley et al 2012).

Since 2002, our research group has been dedicated to studies of orchids mycorrhizal and endophytic fungi from Brazilian Atlantic Forest and Campos Rupestres in Minas Gerais, focusing mainly on fungal diversity and symbiotic seed germination (Pereira et al. 2002, 2003, 2005a, b, c; Nogueira et al. 2005, 2014; Pereira et al. 2009, 2011a, b, 2014, 2015; Oliveira et al. 2014; da Silva et al. 2018; Freitas et al. 2019, 2020; Vieira et al. 2020). Among the orchids studied, in recent years more attention has been

given to *Cattleya jongheana*, a species epiphytic or rupicolous, with slow grown, and have been over-colleted due to its ornamental and commercial potential, which leads it to be cited as endangered species in the Livro Vermelho da Flora do Brasil (Neto et al. 2013).

Continuing studies on fungi diversity in orchids tropical species, 210 isolates were obtained in this work during an investigation of cultivable endophytic and mycorrhizal fungi associated with *C. jongheana* roots. The isolates were identified based on molecular phylogeny and morphological characterization. A new species of *Serendipita* will be described, illustrated and discussed in this paper.

MATERIAL AND METHODS

Sample collection and isolates

Samples of *C. jongheana* were collected from the Parque Estadual da Serra do Brigadeiro (PESB), a fragment of Brazilian Atlantic Forest (Araponga – MG, Brazil). Three, apparently healthy, roots per plant were collected from nine adult individuals of *C. jongheana*. The samples were placed into plastic bags and transported, under refrigeration, to the Laboratório de Associações Micorrízicas (Departamento de Microbiologia/Universidade Federal de Viçosa). The root samples were gently washed under running tap water, cut into pieces of transversal root fragments, 2–3 cm thick, surface-sterilized in 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, followed by two successive rinses of sterile distilled water.

To isolating of mycorrhizal fungi, fragments were examined under a stereomicroscope, after slicing into several thin transversal slices, and the velamen was removed. Cells containing pelotons were placed on potato dextrose agar (PDA) medium without antibiotics and then incubated at 25 °C in the dark. To isolating of endophytes

fungi, 3–5 mm diameter fragments were transferred to Petri plate containing PDA medium and incubated at 25 ºC in the dark. Axenic cultures were preserved on rice grains in an ultrafreezer at -72 °C or silica gel and deposited in Fungi Collection from the Laboratório de Associações Micorrízicas/Bioagro/UFV. Axenic cultures were preserved on silica gel and deposited in the culture collection of the Universidade Federal de Viçosa, Coleção Octávio Almeida Drummond (COAD).

Molecular Analyses

The genomic DNA was extracted from fungal mycelia grown on PDA at 25 ºC for 1–3 weeks, using the Nucleospin® Soil (MACHEREY-NAGEL GmbH & Co. KG), following with the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer (ITS rDNA) region was amplified using primer pairs ITS1 and ITS4 (White et al.1990). Each polymerase chain reaction (PCR) was performed in 50 µL containing 10–20 ng of DNA template, 1× Taq buffer, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.4 mM of each dNTP, and 1.0 U Taq DNA polymerase (Cellco Biotec do Brasil Ltda, São Paulo, Brazil). PCR was carried out with an initial denaturation at 95 °C, for 2 min, followed by 39 PCR cycles (denaturation at 95 °C for 1 min; annealing at 50 °C for 1 min; extension at 72 °C for 1 min) before a final extension at 72 °C for 10 min. The primers ITS3Seb (specific for Sebacinales) and TW13, following Selosse et al. (2007), were used to isolates previously identified as *Serendita* spp.

The PCR products were visualized by electrophoresis on 1,5% agarose gels stained with ethidium bromide to assess product size and quality, then they are purified and sequenced from the two strands using the primers mentioned above. All sequences were checked manually, and nucleotides with ambiguous positions were clarified using

both primer direction sequences. The sequences were deposited in GenBank and accession numbers are available in the supplementary material.

Sequences obtained were compared against sequences available on GenBank database (National Center for Biotechnology Information) using BLAST. Genera delimitation of all isolates was defined using a similarity threshold of 97% for ITS sequences. For species delimitation of *Serendipta* and *Tulasnella* species, phylogenetic analysis based on Bayesian inference (BI) and/or Maximum likelihood (ML) combined with morphological analysis were performed, as follows below.

Diversity analyses

Diversity statistics, including Simpson and Shannon diversity index were calculated from the absolute values of the number of occurrences of each genus observed with 9,999 bootstrap, using software Past v 4.02 (Hammer et al. 2001). The sampling effort was determined in a rarefaction curve also using the software Past.

Phylogenetic analyses of mycorrhizal fungi

A phylogenetic relationship of the *Tulasnella* and *Serendipita* isolates was generated individually. Sequences available on NCBI and UNITE were downloaded in FASTA format and aligned with our sequences using the MAFFT v. 7 online portals (Katoh and Standley 2013). The resulting sequence alignments were manually checked and adjusted in MEGA v.7.0.26 software tool (Kumar et al. 2016).

Bayesian inference analyses employing a Markov Chain Monte Carlo method were performed on all sequences. Nucleotide substitution models were determined using the MrModeltest 2.3 program (Nylander 2004) and, once the likelihood scores had been calculated, the models were selected according to the Akaike information criterion (AIC).

The results of MrModeltest for Tulasnellaceae recommended a GTR+G model for ITS. For Serendipitaceae and Sebacinaceae analyses the model recommended was GTR+I+G.

The phylogenetic analysis was performed using the CIPRES web portal (Miller et al. 2010) and the MrBayes program v.3.2.7a (Ronquist and Huelsebeck 2003). Two sets of four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations and sampling every 1,000th generation. The first 25% of the trees were discarded as the burn-in phase for each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from the remaining trees and are presented on the left of each node. Maximum likelihood analysis was implemented using the RAxML-HPC v.8 on XSEDE (8.2.12) available on the CIPRES web portal. Parameters for maximum likelihood were set to rapid bootstrapping and the analysis was carried out using 1000 replicates. Alignments and trees were deposited in TreeBASE (http://treebase.org/treebase-web/). The trees were visualized in FigTree V1.4.4 (Rambaut 2009) and the layout of the tree for publication was done using Adobe Illustrator v. CC.

Morphology of *Tulasnella* and *Serendipita* isolates

The colony characteristics were described from cultures grown on PDA at 25 ºC in the dark for 8 days. The nuclear condition was observed from hyphae grown on PDA at 25 ºC in the dark for 8 days, after staining with SYBR Green I according to Meinhardt et al. (2001). To induce monilioid cell formation the isolates were transferred to Corn Meal Agar (CMA) medium and incubated at 25 ºC in the dark, for 4–6 weeks (Pereira et al. 2005). Observations, measurements, and photographic images of microscopic fungal structures were recorded using an Olympus BX53 light microscope, with an Olympus Q-Color5TM digital high-definition color camera and differential interference contrast

(DIC) illumination. Adobe Photoshop CS5 was used for the final editing of the acquired images and photographic preparations.

RESULTS

Composition of fungal communities from *C. jongheana* roots

A total of 212 isolates of endophytic and mycorrhizal fungi were obtained from roots of *C. jongheana*, and the sequences were deposited in Genbank database (Supplementary material 1). The isolates belong to the phyla Ascomycota (143 isolates, 67.45%) and Basidiomycota (66 isolates, 31.13%), and they are distributed in 32 genera (Fig.1; Supplementary material 2). Endophytes root-associated fungi were all belonging to Ascomycota. Among them *Xylaria*, *Colletotrichum*, *Trichoderma* and *Microdiplodia* were the genera most recurrent, with frequences 13.68%, 10.38%, 6.60% and 5.19%, respectively. *Fusarium*, *Neopestalotiopsis* and *Pestalotiopsis* were also, among other genera, obtained from *C. jongheana* roots (Fig.1).

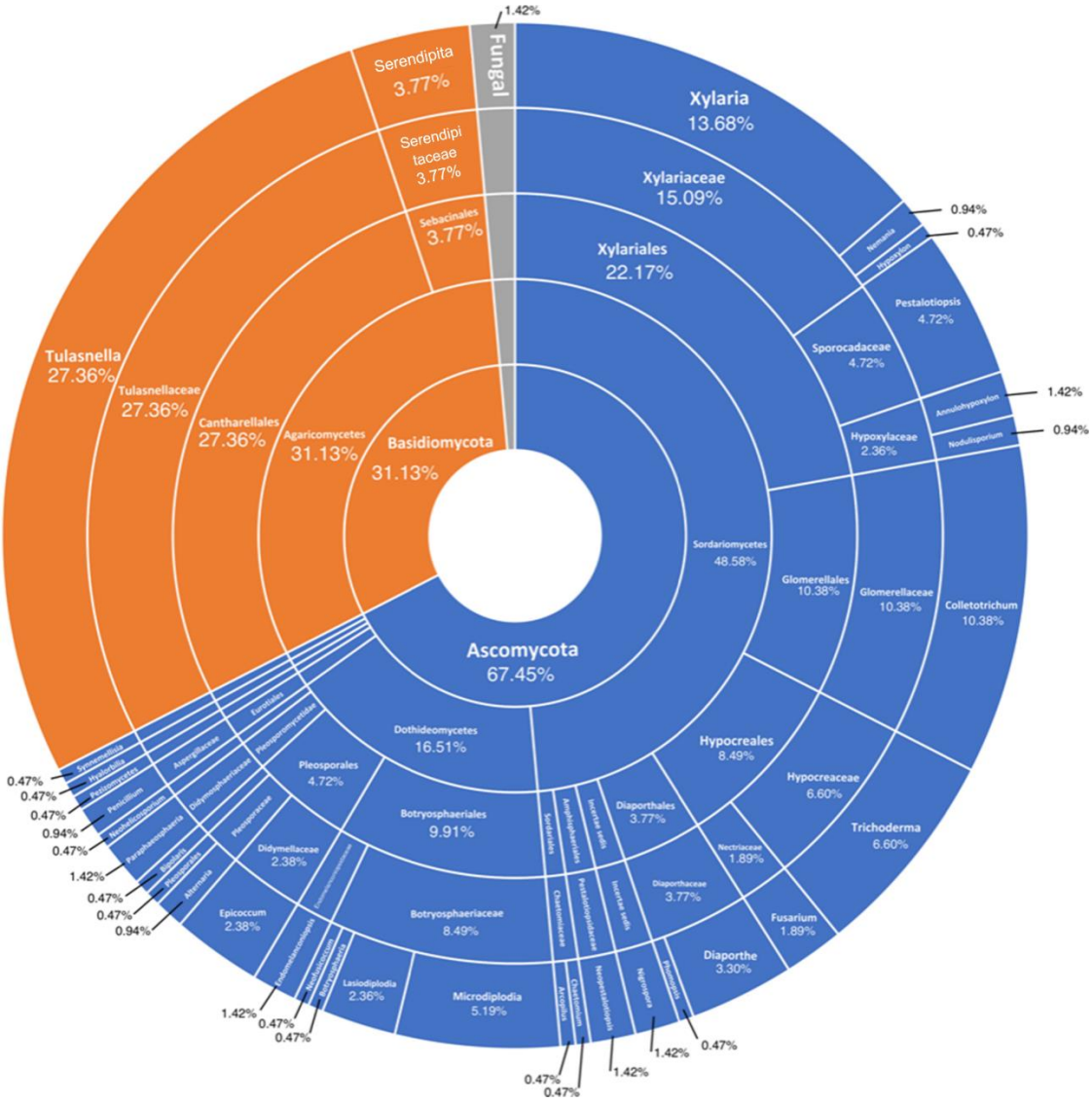

Fig. 1 Percentage of endophytic and mycorrhizal taxa after isolation in potato dextrose agar (PDA) and identified by molecular methods.

All fungal cultures obtained from pelotons belonged to Basidiomycota, and are potential mycorrhizae. These Basidiomycota cultures showed relatively slow growth rates compared to Ascomycota cultures obtained. *Tulasnella* was the dominant genus, with frequency of 27.36% (58 isolates) (Fig 1) and isolated from all of the nine plants sampled. *Serendipita* genus also related as mycorrhizae in orchid, was also isolated, but less frequently than *Tulasnella* (8 isolates, 3.77%).

Diversity of fungal communities

Rarefaction curves showed a tendency to saturation for genus obtained from isolation of orchids roots (Fig 2), indicating that the sampling effort was sufficient to cover the diversity of fungal communities associated with *C. jongheana*. Shannon–Wiener and Simpson diversity indices based on genera frequencies were 2.68 and 0.88, respectively. The value of the Simpson diversity index suggests a low diversity, which can be explained by the high frequency of *Tulasnella* isolates.

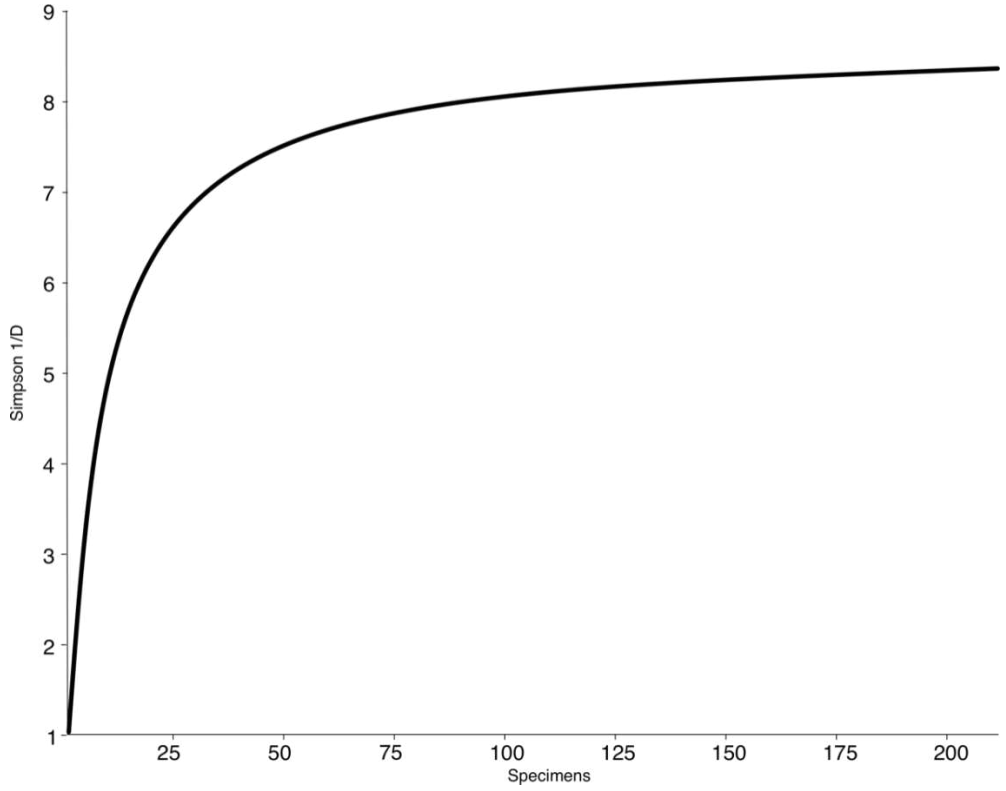

Fig. 2 Rarefaction curve of diversity (Simpson) of genera of fungi obtained from *C. jongheana* roots.

Phylogenetic analyses

Phylogenetic analyses of *Tulasnella* consisted of 104 strains - including the outgroup sequence - (Supplementary material 3), out of which 48 are from nucleotide databases (NCBI or UNITE) and 56 from this research. The alignment had a total length

of 597 nucleotides bases (including alignment gaps), of which 396 were parsimony-informative, 454 were variable and 139 were conserved. Bayesian Inference tree is shown below (Fig. 2). *Tulasnella* isolates obtained from *C. jongheana* roots in this study could be assigned to five different species, and they are highlighted on blue box in the phylogenetic tree (Fig. 2). All isolates were grouped in clades representative of species already described in the literature, and 55 isolates showed similarity to species recently described from *C. jongheana* roots. Most isolates (44) were clustered in a clade included sequences recorded as *Tulasnella* sp.1 (submitted). Three isolates formed a well-supported clade (PP = 1) together sequences from *Tulasnella hadrolaeliae*, and eight isolates were arranged in a clade representing *Tulasnella brigadeiroensis* (PP = 0.92). One isolate (EM190) was disposed between two clades between sequences from *Tulasnella calospora*.

Phylogenetic analyses of Serendipitaceae and Sebacinaceae comprised 50 strains - including the outgroup sequence - (Supplementary material 4), out of which 43 are from nucleotide databases (NCBI or UNITE) and seven were obtained in *C. jongheana* roots. The alignment yielded a total length of 652 nucleotide bases (including alignment gaps), of which 167 were parsimony-informative, 216 were variable and 427 were conserved. The ML and Bayesian analyses generated trees with the same topology, and just Bayesian Inference is shown below (Fig. 3). Isolates obtained in this study cluster within Serendipitaceae family and could be assigned to two different taxa. The first taxon consisting of three isolates and is close to *Serendipita vermifera* species complex. Even though the phylogenetic analyses suggest that these isolates may represent a new species, we will be more conservative and we will keep these isolates with the *S. vermifera* species complex. The second taxon is composed by four isolates and formed a strongly supported clade (ML/PP = 100/1), which is a sister group of *Serendipita indica*, *Serendipita*

williamsii and *Serendipita sacchari*. This taxon is here proposed as a new specie, named *Serendipita* sp. 1. A comparison of the ITS sequence dataset indicates that *Serendipita* sp.1 differs from *S. indica* in 17/652 bp (2.6%), from *S. williamsii* in 19/652 bp (2.9%) and from *S. sacchari* in 21/652 bp (3.2%).

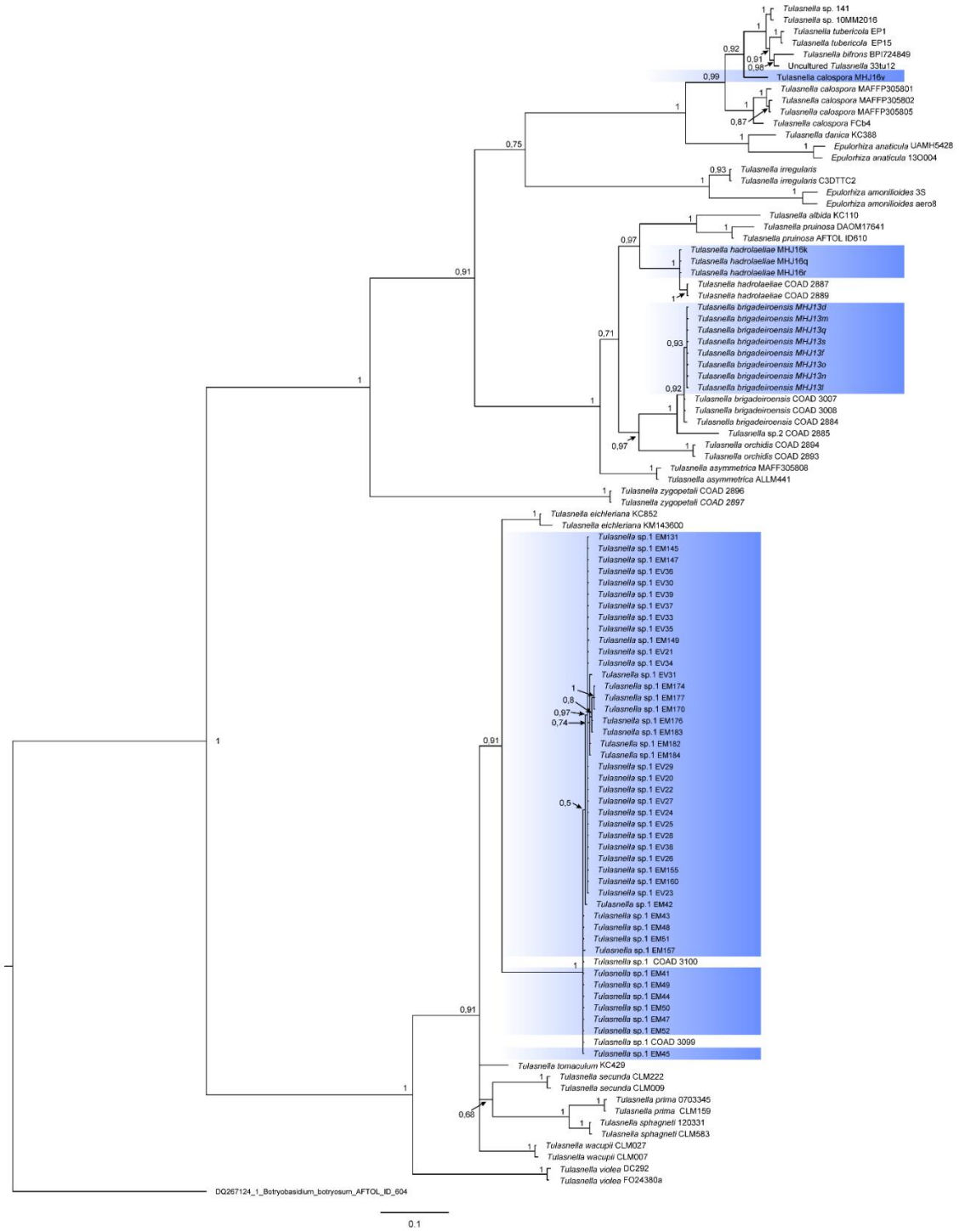

Fig. 2 Bayesian phylogenetic tree for *Tulasnella* based on ITS alignment. Bayesian posterior

probabilities (PP) values are indicated next to the nodes. Species from this study are in the blue block. *Botryobasidium botryosum* (AFTOL604) was used as the outgroup.

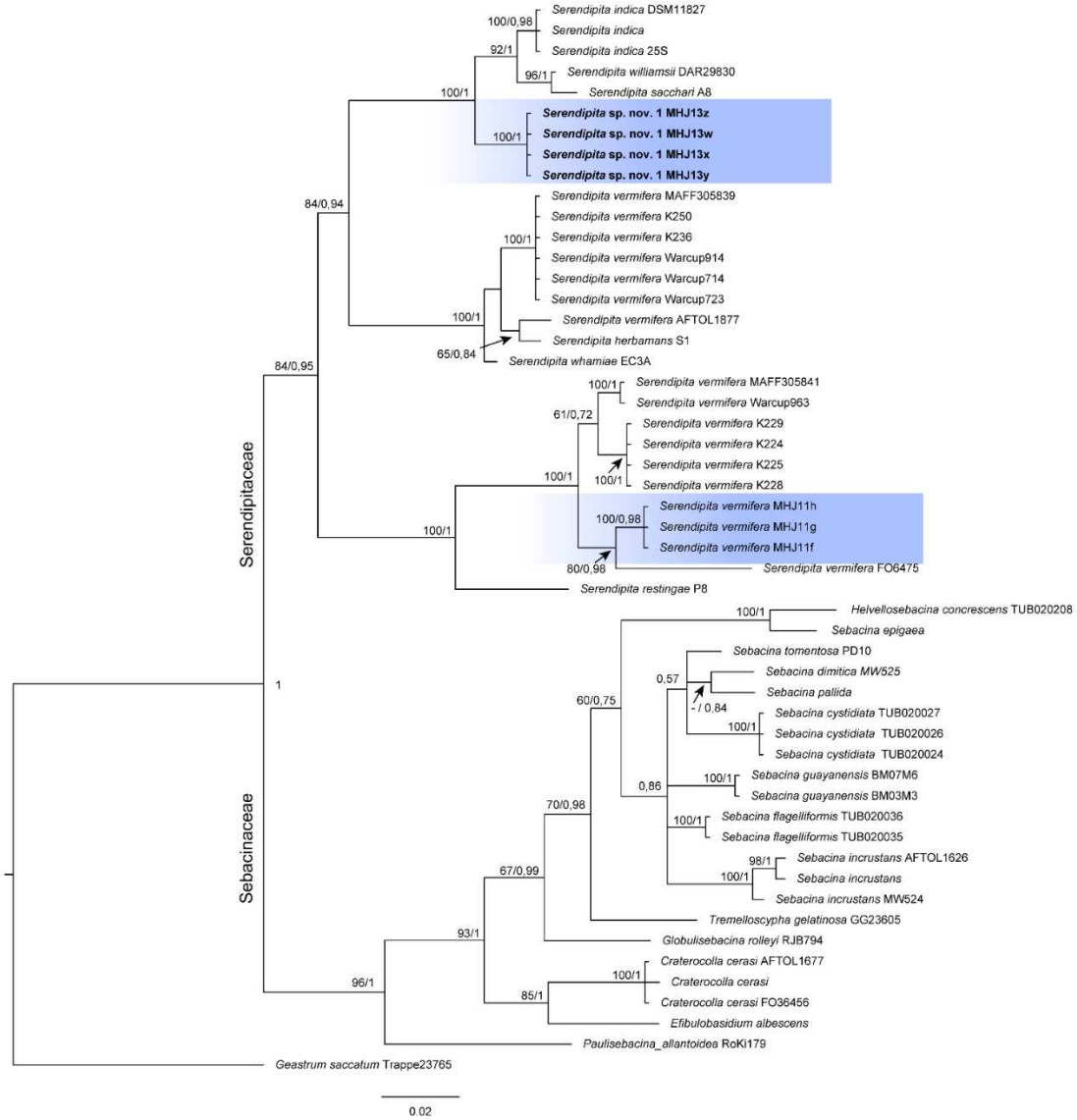

Fig. 3 Bayesian phylogenetic tree for Serendipitaceae and Sebacinaceae based on ITS alignment. Maximum likelihood bootstrap support (ML > 60) and Bayesian posterior probabilities (PP) values are indicated next to the nodes (ML/PP). Species from this study are in the blue block and the new species described in this paper are indicated in bold face. *Geastrum saccatum* (Trappe23765) was used as the outgroup.

Taxonomy

***Serendipita* sp. 1** E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya (Fig. 4)

Diagnosis: Serendipita sp.1 differs from *S. indica* and *S. sacchari* by its smaller chlamydospores, and from *S. williamsii* by a greater number of nuclei in the chlamydospores.

Type:—**BRAZIL:** Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of the orchid *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ13Y).

Description: Colonies on PDA attaining 24 mm diam after 8 d at 25 ºC in the dark, white to cream, with submersed edge, aerial mycelium present. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, with 3–4.8 µm diam ($\overline{X}$ ± SD = 4.0 ± 0.6 µm), hyaline, lacking clamp connections. Hyphae are binucleate. Molinioid cells hyaline were formed in CMA and are globose to sub-globose in branched chains, with diameter of 2.5–4.0 µm ($\overline{X}$ ± SD = 3.4 ± 0.6 µm). Chlamydospores were globose to sub-globose, multinucleate, with 9.5–15.5 µm ($\bar{X}$ ± SD = 12.0 ± 1.5) in length and 8.0–13.0 µm ($\bar{X}$ ± SD = 10.3 ± 1.8 µm) in width, formed at the tips of the hyphae. Neither clamp connections nor sexual morph were observed.

Substrate or host: Roots of *Cattleya jongheana.*

Additional material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ13W, MHJ13X, MHJ13Z).

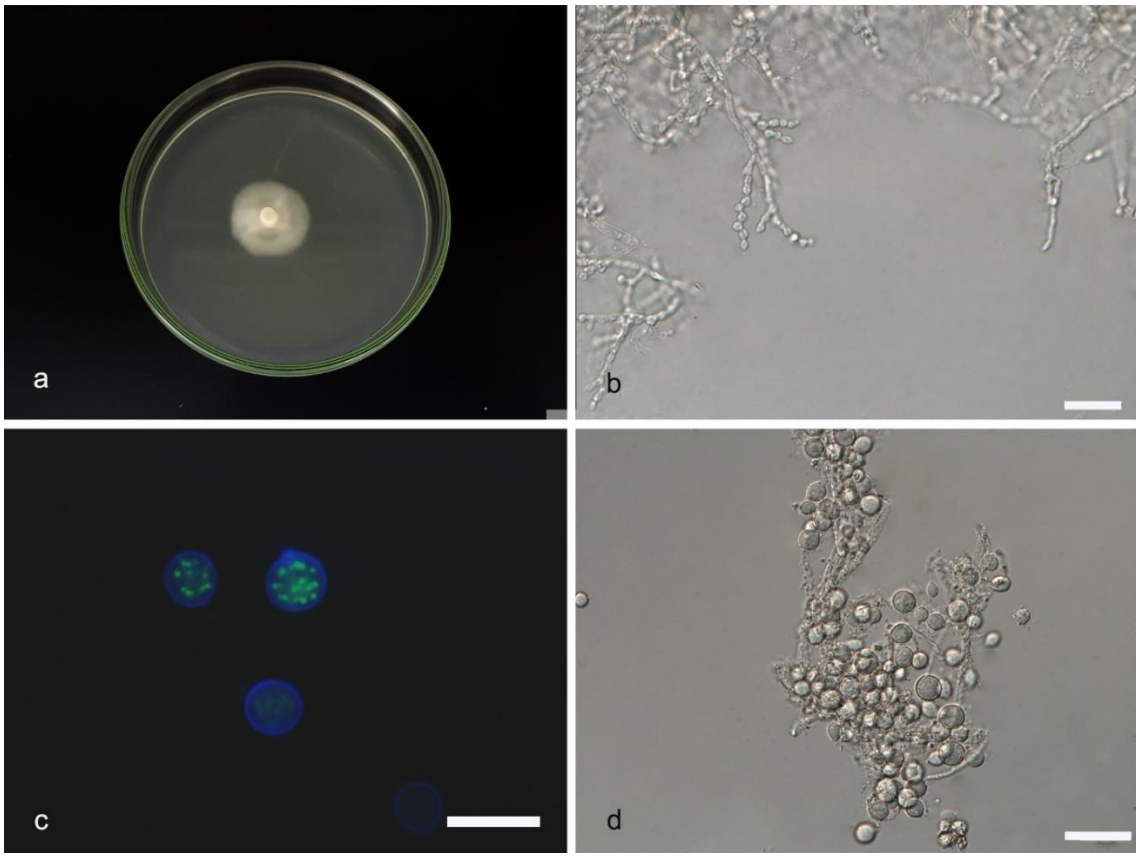

Fig. 4 *Serendipita* sp.1 **a** Eight-day-old PDA culture. **b** Monilioid cell chains in CMA. **c** Chlamydospores stained with SYBR Green. **d** Chlamydospores. Bars: b, d = 20 µm; c = 25 µm

Serendipita vermifera (Oberw.) P. Roberts (1993) (Fig. 5)

Description: Colonies on PDA achieving 19 mm diam after 8 d at 25 ºC, white to cream, with submersed edge, aerial mycelium scarce. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 1.5–2.4 µm diam ($\overline{X}$ ± SD = 1.8 ± 0.2 µm), hyaline, with binucleate cells, lacking clamp connections. The fungus produces molinioid cells hyaline in branched chains, globose, with diameter of 2.5–3.8 µm ($\overline{X}$ ± SD = 3.2 ± 0.4 µm). Neither chlamydospores nor sexual morph were observed.

Substrate or host: Roots of *Cattleya jongheana.*

Material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ11G).

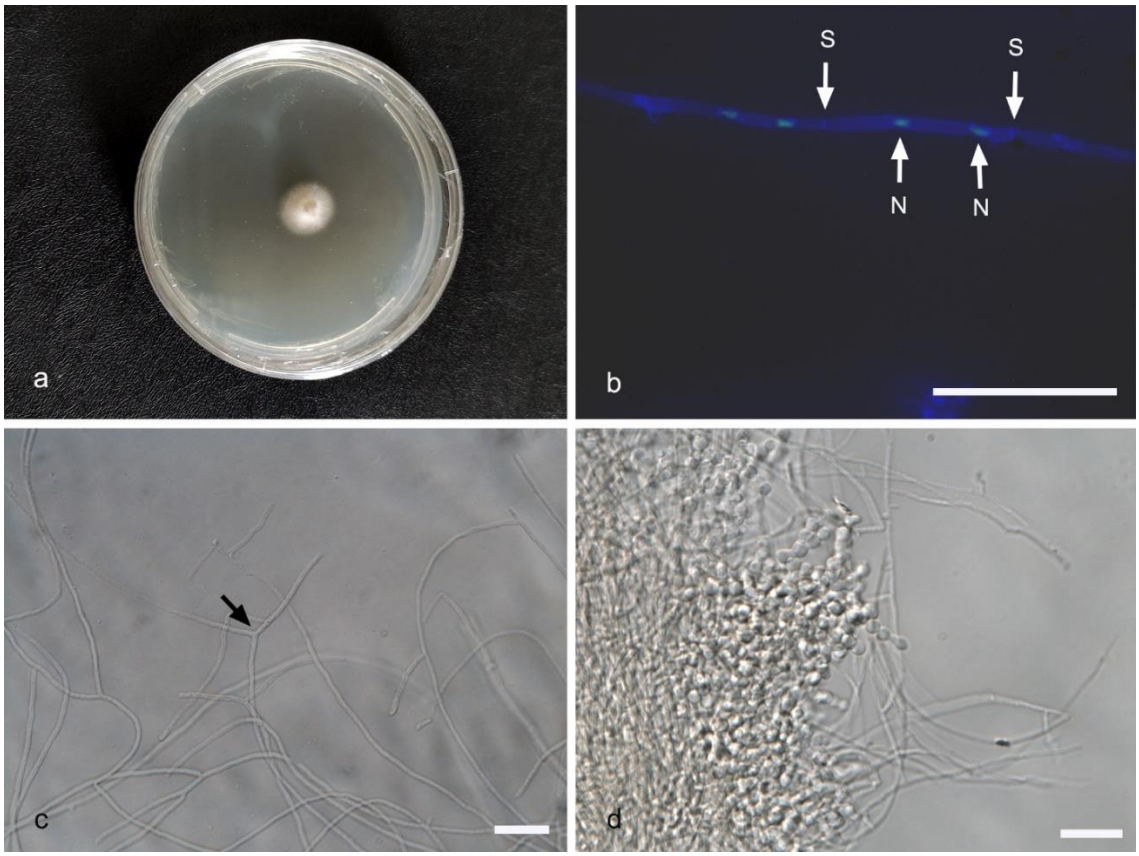

Fig. 5 *Serendipita vermifera.* **a** Eight-day-old PDA culture. **b** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c** Hyphae with branching at right angles. **d** Monilioid cell chains in CMA. Bars: b = 25 µm; c, d = 20 µm

Tulasnella brigadeiroensis E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya (2020). (Fig. 6)

Description: Colonies on PDA achieving 30 mm diam after 8 d at 25 ºC, white to cream, with submersed edge, aerial mycelium scarce. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angles, 2.0–3.0 µm diam ($\overline{X} \pm$ SD = 2.6 ± 0.25 µm), hyaline, with binucleate cells. Molinioid cells not observed. Sexual morph not observed.

Substrate or host: Roots of *Cattleya jongheana.*

Material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ13D, MHJ13F, MHJ13L, MHJ13M, MHJ13N, MHJ13O, MHJ13Q, MHJ13S). There was no difference between the morphology of the isolates.

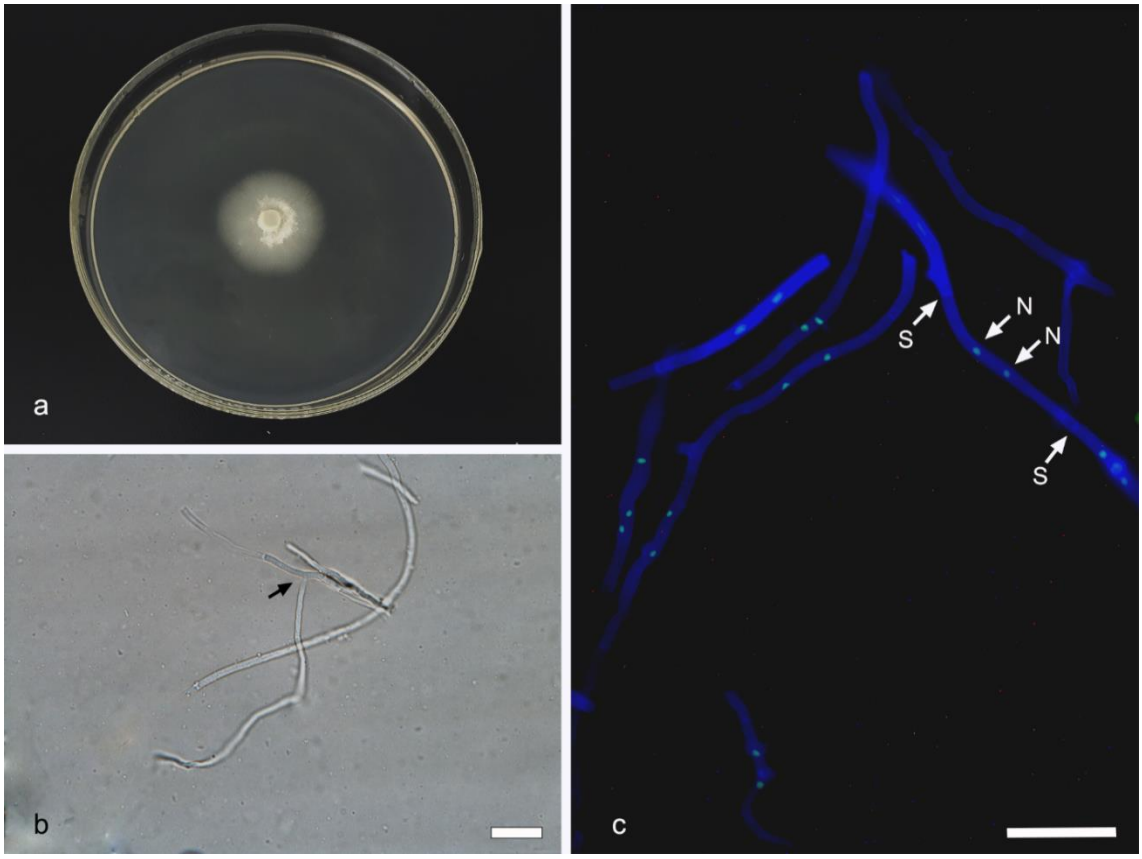

Fig. 6 *Tulasnella brigadeiroensis*. **a** Eight-day-old PDA culture. **b** Hyphae with branching at right angles. **c** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). Bars: b = 20 µm; c = 25 µm

Tulasnella calospora Juel, Bih. K. svenska Vet-Akad. Handl. 23: 23 (1897). (Fig. 7)

Description: Colonies on PDA attaining 15 mm diam after 8 d, at 25 °C, white to cream, with undulate and submersed edge, some cultures showing aerial mycelium. Hyphae from cultures are regularly septate, with branching at right angles, 1.8–2.7 µm diam ($\overline{X}$ ± SD =

2.3 ± 0.2 µm), hyaline, with binucleate cells. Molinioid cells not observed. Sexual morph not observed.

Substrate or host: Roots of *Cattleya jongheana.*

Material examined— BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ16v).

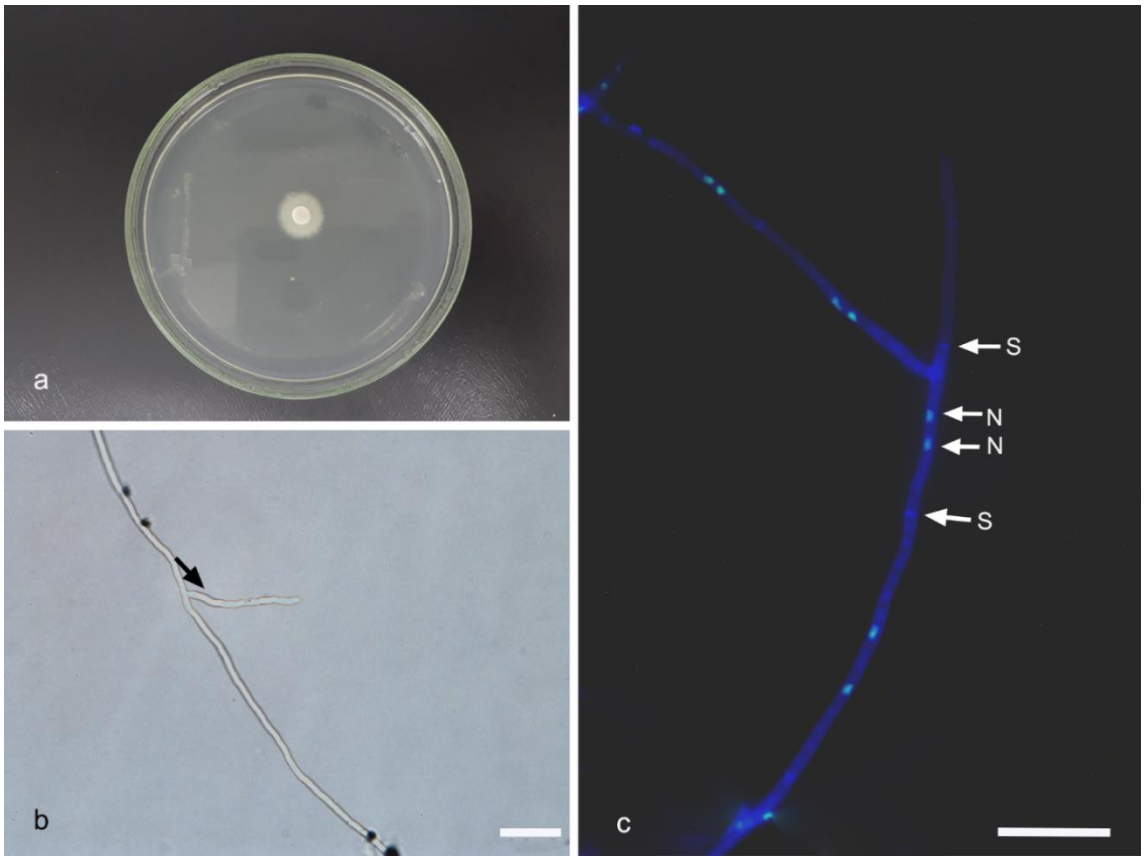

Fig. 7 *Tulasnella calospora.* **a** Eight-day-old PDA culture. **b** Hyphae with branching at right angles. **c** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). Bars: b = 20 µm; c = 25 µm

Tulasnella hadrolaeliae E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya (2020). (Fig. 8)

Description: Colonies on PDA exhibited slow-growing, attaining 17 mm diam after 8 d at 25 ºC, mycelium white to cream, with undulate and submersed edge, aerial mycelium absent to scarce. Reverse of the colony white to cream. Hyphae are regularly septate with

branching at right angles, 2.5–4.5 µm diam ($\overline{X}$ ± SD = 3.5 ± 0.5 µm), hyaline, with binucleate cells and thin-walled. Molinioid cell hyaline, barrel to elongated barrel-shaped, in branched chains, 4.5–6.0 µm diam ($\overline{X}$ ± SD = 5.5 ± 0.3 µm). Sexual morph not observed.

Substrate or host: Roots of *Cattleya jongheana.*

Material examined.—BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Cattleya jongheana*, October 2019, E.F.S. Freitas (MHJ16k, MHJ16q, MHJ16r). There was no difference between the morphology of the isolates.

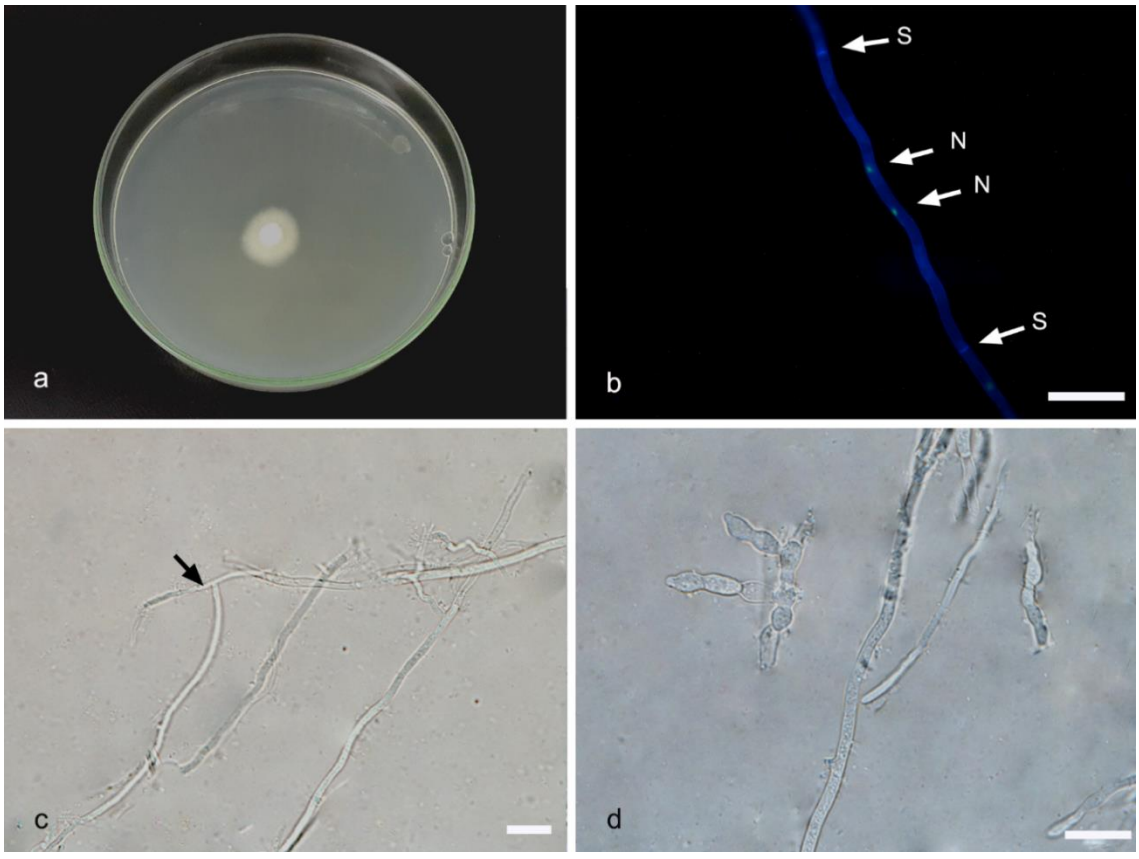

Fig. 8 *Tulasnella hadrolaeliae.* **a** Eight-day-old PDA culture. **b** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c** Hyphae with branching at right angles. **d** Monilioid cell chains in CMA. Bars: b = 25 µm; c, d = 20 µm

***Tulasnella* sp. 1** E.F.S. Freitas, Meir. Silva & M.C.M. Kasuya. (Fig. 9)

Description: Colonies on PDA attaining 18 mm diam after 8 d, at 25 °C, white to cream, with regular and submersed edge, absent aerial mycelium. Reverse of the colony white to cream. Hyphae are regularly septate with branching at right angle, 2.5–4.5 µm diam ($\overline{X}$ ± SD = 3 ± 0.5 µm), hyaline, with binucleate cells and thin-walled. Molinioid cells, hyaline, barrel to globose-shaped, thick-walled, 8.5–13.5 µm diam ($\overline{X}$ ± SD = 11.5 ± 1.2 µm), in branched chains. Sexual morph not observed.

Material examined: Brazil, Minas Gerais, Parque Estadual da Serra do Brigadeiro, isolated from roots of *Cattleya jongheana* (Orchidaceae), 2019, E.F.S. Freitas (EM41, EM42, EM43, EM44, EM45, EM47, EM48, EM49, EM50, EM51, EM52, EM131, EM145, EM147, EM149, EM155, EM157, EM160, EM170, EM174, EM176, EM177, EM182, EM183, EM184, EV20, EV21, EV22, EV23, EV24, EV25, EV26, EV27, EV28, EV29, EV30, EV31, EV33, EV34, EV35, EV36, EV37, EV38, EV39).

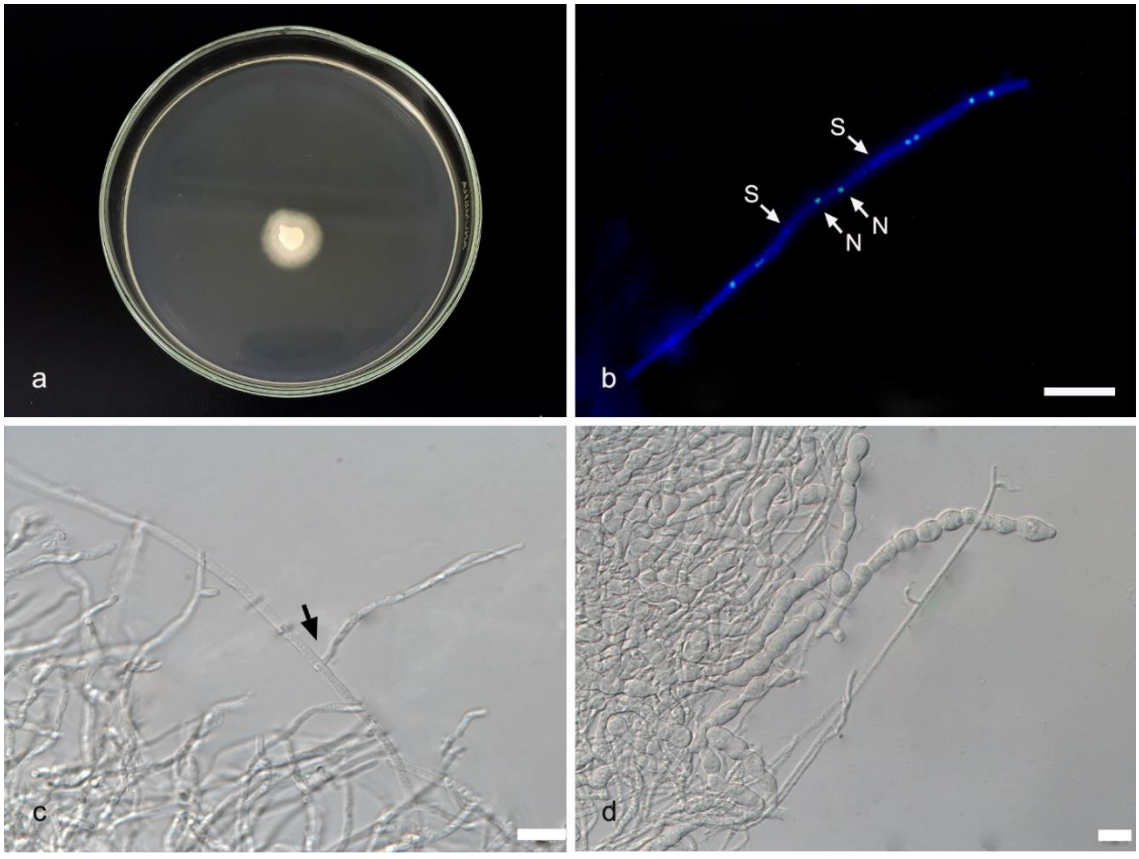

Fig. 9 *Tulasnella* sp.1. **a** Eight-day-old PDA culture. **b** Hyphae stained with SYBR Green I showing binucleate cells (N = nuclei; S = septa). **c** Hyphae with branching at right angles. **d** Monilioid cell chains in CMA. Bars: b = 25 µm; c, d = 20 µm

DISCUSSION

Studies about endophytic and mycorrhizal fungi associate with a range of orchids have revealed a great fungal diversity, however, this diversity in orchids from the *Cattleya* genus has been poorly investigated (Ma et al. 2015). The first study on the diversity and composition of the fungal community of *C. jongheana* roots in Brazil shown a high diversity of fungal communities, composed mainly of Ascomycota and Basidiomycota, but many sequences were from unidentified species, which suggests a hidden diversity of mycorrhizal and endophytic fungi associated with this orchid. (Oliveira et al. 2014). Recently molecular tools associated with culture-based methods have allowed exploring this diversity and described new species from genera *Colletotrichum* (da Silva et al. 2018), *Neopestalotiopsis* (Freitas et al. 2019) and *Tulasnella* (Freitas et al. 2020). Herein we continue to study the composition and diversity of endophytic and mycorrhizal fungi from roots of *C. jongheana*, as well as taxonomy and phylogeny of genera *Tulasnella* and *Serendipita*, known symbionts of orchids.

Our study shows that *C. jongheana* can be associated with a wide range of fungi, of which 67.45% were endophytic ascomycetous and 31.13% were mycorrhizal basidiomycetous. This information agrees with other studies, which indicated that endophytic fungi are present in greater numbers than mycorrhizal fungi in roots of orchids (Novotná et al. 2018). Among endophytic isolates belonging to Ascomycota from the present survey, the most frequent genera were *Colletotrichum*, *Microdiplodia*, *Trichoderma* and *Xylaria*, which have been described in association with other orchids

(Ma et al. 2015). *Xylaria* species has been frequently observed in association with roots in epiphytic and lithophytic orchids worldwide (Bayman and Otero 2006), and more recently it was also reported from roots of *Pogoniopsis schenckii*, an aclorophyllated orchid (Sisti et al. 2019). *Trichoderma* sp. has been identified in roots from adult individuals (Novotná et al. 2018; Shah et al. 2019), as well as in the first germination stages of orchid in the wild (Chen et al. 2019), and some isolates have been showing potential in the germination of seeds *in vitro* (Sisti et al. 2019). *Colletotrichum* sp. and *Trichoderma* sp. were observed also in leaves of *Dendobrium* species and exhibited a different level of pathogenicity in seedlings (Sarsaiya et al. 2020). The role of the endophytic fungi in orchids is not well understood, but in general they are recognized for the production of bioactive compounds, with antimicrobial activities (Vaz et al. 2009; Ratnaweera et al. 2014; Barnes et al. 2016) or growth promoting activities (Zhang et al. 2013; Shah et al. 2019).

Mycorrhizal orchids fungi isolated from the peloton in the roots of *C. jongheana* in the present study belong to *Tulasnella* and *Serendipita* genus. *Tulasnella* was the genus with the highest frequency (27.36%) among all other mycorrhizal or endophytic fungi. This genus has been frequently recorded as forming mycorrhizal association in orchids worldwide (McCormick et al. 2004; Roche et al. 2010; Linde et al. 2017; Meng et al 2019; Nguyen et al. 2020) and in Brazil, *Tulasnella* has been isolated from *Oeceoclades maculate* (Pereira et al. 2005), *Epidendrum secundum*, *Acianthera limae* and *Polystachya concreta* (Nogueira et al. 2014). Furthermore, recent studies described four new species of *Tulasnella* from *C. jongheana* roots in Brazil - *T. brigadeiroensis*, *T. hadrolaeliae*, *T. orchidis* and *T. zygopetali* – and it showed that the same host plants can associate with more than one *Tulasnella* symbiont (Freitas et al. 2020). *Tulasnella* sp.1 (COAD3099, COAD3100) and *Tulasnella* sp.2 (COAD2885) were isolated previously in *C. jongheana*

and will be proposed as new species (submitted). Here, *T. brigadeiroensis*, *T. hadrolaeliae* and *Tulasnella* sp.1 were isolated from *C. jongheana* again, and besides that *Tulasnella* sp.1 was obtained repeatedly (44 isolates) in different individuals, suggesting that these species are predominant in *C. jongheana*.

On the other hand, this is the first report of *T. calospora* in *C. jongheana* in Brazil, and only one isolate was obtained. Recently, *T. calospora* was isolated several times from roots of *Cattleya cinnabarina* and *Cattleya caulescens*, two rupicolous orchids, and the authors suggested that *T. calospora* is a common and largely distributed orchid symbiont (Freitas et al. 2020). However, mycorrhizal preferences have been demonstrated in epiphytic orchids, which may vary between closely related orchids (Otero et al. 2004; Suárez et al. 2006), and *T. calospora* does not seem to be a preferred symbiont in *C. jongheana*.

Serependita species also were isolated from *C. jongheana* roots, but less frequently than with *Tulasnella*, which is in accordance with other studies (Suárez et al. 2008; Martos et al. 2012). Serendipitaceae was introduced in 2016 to accommodate clades previously assigned to Sebacinales group B and contains a single genus, *Serendipita* P.Roberts (Weiß et al. 2016). Currently, there are 14 species *Serendipita* listed in Index Fungorum (www.indexfungorum.org) and Mycobank databases (Robert et al. 2005), however, some species need both morphological and molecular confirmation (Oberwinkler et al. 2014). *Serendipita* has been isolated from orchids roots since the 20th century, when Warcup and Talbot isolated fungi from Australian orchids and identify them as *Serendipita vermifera* (Warcup and Talbot 1967). Nowadays, we know that *S. vermifera* strains belong to different non identified species and are treated as a species complex (Weiß et al. 2016).

Recently, new *Serendipita* species were described associated with orchids roots: *Serendipta whamiae* from the Australian orchid *Eriochilus cucullatus* (Crous et al. 2020), and *Serendipita restingae* from Brazilian orchid *Epidendrum fulgens* (Fritsche et al. 2020). Here, we obtained two clades from *Serendipita* isolates: the first clade belongs to *Serendipita vermifera* species complex and the second one is the new species *Serendipita* sp. 1. *Serendipita vermifera* species complex also have been found in association highly specifics with terrestrial orchids from *Caladenia* genus in Australia, evidencing that high mycorrhizal specificity is likely to be the limiting factor in the distribution of this genera (Swarts et al. 2010; Wright. et al 2010). Furthermore, *S. vermifera* species complex also interacts with other plants forming ectomycorrhizal, ericoid mycorrhizae associations or as endophytic fungi (Oberwinkler et al. 2013; Wei ß et al. 2016), and can play an important role in plant development, such as growth promotion, nutrient uptake, resistance against biotic and abiotic stresses, as have already been reviewed (Oberwinkler et al. 2013; Ray and Craven 2016).

The new species *Serendipita* sp. 1 is phylogenetically close to *S. indica*, *S. williamsii* and *S. sacchari*, but differs from them by phylogenetic analyses of the ITS region. Morphologically, *Serendipita* sp. 1 can be distinguished from *S. indica* and *S. sacchari* by having smaller chlamydospores (Verma et al. 1998; Xie et al. 2020), and from *S. williamsii* by greater nuclear number in the chlamydospores (Basiewicz et al. 2012). *Serendipita indica* is well known for its broad host range, besides its ability to improve grown of plants (Varma et al. 1999; Rai et al. 2001; Achatz et al. 2010), to induce resistance to diseases and tolerance to abiotic stresses (Waller et al. 2005). Recent studies also have shown a positive effect of inoculation of *S. indica* and *S. williamsii* on plant growth (Venneman et al. 2017; 2020), evidencing the biotechnological potential of Serendipitaceae in agriculture.

Our study contributes to knowledge of the diversity of mycorrhizal and endophytic fungi associates with roots of *C. jongheana*, besides of description of one new species from *Serendipita* genera. Further studies will be carried out to investigate the potential of these isolates on seeds germination, grown promotion and defense of plant against pathogens, which is important for the conservation of *C. jongheana*, as well other orchids.

REFERENCES

Achatz B, von Rüden S, Andrade D et al. (2010) Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development. Plant Soil 333:59–70. https://doi.org/10.1007/s11104-010-0319-0

Barnes EC, Jumpathong J, Lumyong S, Voigt K, Hertweck C (2016) Daldionin, an Unprecedented Binaphthyl Derivative, and Diverse Polyketide Congeners from a Fungal Orchid Endophyte. Chemistry - A European Journal 22:4551–4555. https://doi.org/10.1002/chem.201504005

Basiewicz M, Weiß M, Kogel,KH, Langen G, Zorn H, Zuccaro A (2012) Molecular and phenotypic characterization of *Sebacina vermifera* strains associated with orchids, and the description of *Piriformospora williamsii* sp. nov.. Fungal Biology 116(2): 204-213. https://doi.org/10.1016/j.funbio.2011.11.003

Bayman P, Otero JT (2006) Microbial Endophytes of Orchid Roots. In: Schulz B, Boyle C, Sieber TN (eds) Microbial Root Endophytes. Soil Biology. Springer, Berlin, pp153-177. https://doi.org/10.1007/3-540-33526-9_9

Chen L, Wang YC, Qin LY, He HY, Yu XL, Yang MZ, Zhang HB (2019) Dynamics of fungal communities during *Gastrodia elata* growth. BMC Microbiol 19:158. https://doi.org/10.1186/s12866-019-1501-z

Crous PW, Cowan DA, Maggs-Kölling G, Yilmaz N et al (2020) Fungal Planet description sheets: 1112–1181. Persoonia - Molecular Phylogeny and Evolution of Fungi 45:251-409. https://doi.org/10.3767/persoonia.2020.45.10

Da Silva M, Cruz ES, Veloso TGR, Miranda L, Pereira OL, Bocayuva MF, Kasuya MCM (2018) *Colletotrichum serranegrense* sp. nov., a new endophytic species from the roots of the endangered Brazilian epiphytic orchid *Cattleya jongheana*. Phytotaxa 351(2):163–170. https://doi.org/10.11646/phytotaxa.351.2.4

Freitas EFS, Da Silva M, Barros MVP, Kasuya MCM (2019) *Neopestalotiopsis hadrolaeliae* sp. nov., a new endophytic species from the roots of the endangered orchid *Hadrolaelia jongheana* in Brazil. Phytotaxa 416(3):211–220. https://doi.org/10.11646/phytotaxa.416.3.2

Freitas EFS, Da Silva M, Cruz ES et al. (2020) Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species. Scientific Reports 10:7069. https://doi.org/10.1038/s41598-020-63885-w

Fritsche Y, Lopes ME, Selosse MA et al. (2020) *Serendipita restingae* sp. nov. (Sebacinales): an orchid mycorrhizal agaricomycete with wide host range. Mycorrhiza 31:1–15. https://doi.org/10.1007/s00572-020-01000-7

Gentry A, Dodson C (1987) Diversity and Biogeography of Neotropical Vascular Epiphytes. Annals of the Missouri Botanical Garden 74:205-233. https://doi.org/10.2307/2399395

Hou XQ, Guo SX (2009) Interaction between a dark septate endophytic isolate from *Dendrobium* sp. and roots of *D. nobile* seedlings. J. Integr. Plant Biol. 51(4):374–381.

Index Fungorum (2021) http://www.indexfungorum.org/. Acessed 28 January 2021

Jiang J, Zhang K, Cheng S et al. (2019) *Fusarium oxysporum* KB-3 from *Bletilla striata*: an orchid mycorrhizal fungus. Mycorrhiza 29:531–540. https://doi.org/10.1007/s00572-019-00904-3

Katoh K & Standley DM. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30:772–778. https://doi.org/10.1093/molbev/mst010

Kumar S, Stecher G & Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874. https://doi.org/10.1093/molbev/msw054 .

Linde CC, May TW, Phillips RD, Ruibal M, Smith LM, Peakall R (2017) New species of *Tulasnella* associated with terrestrial orchids in Australia. IMA Fungus 8:28–47. https://doi.org/10.5598/imafungus.2017.08.01.03

Ma X, Kang J, Nontachaiyapoom S, Wen T, Hyde KD (2015) Non-mycorrhizal endophytic fungi from orchids. Current Science 109:72–87.

Martos F, Munoz F, Pailler T, Kottke I, Gonneau C, Selosse MA (2012) The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. Mol Ecol. 21:5098-109. https://doi.org/10.1111/j.1365-294X.2012.05692.x

McCormick MK, Whigham DF and O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytologist 163:425-438. https://doi.org/10.1111/j.1469-8137.2004.01114.x

McCormick MK, Whigham DF, Canchani-Viruet A (2018) Mycorrhizal fungi affect orchid distribution and population dynamics. New Phytologist 219:1207-1215. https://doi.org/10.1111/nph.15223

Mehra S (2020) Fungal Endophytes: Australian Terrestrial Orchids. IntechOpen. https://doi.org/10.5772/intechopen.91976

Meinhardt LW, Bellato CDM, Tsai SM (2001) SYBR® Green I used to evaluate the nuclei number of fungal mycelia. Biotechniques 31:42–46.

Meng YY, Zhang WL, Selosse MA, Gao JY (2019) Are fungi from adult orchid roots the best symbionts at germination? A case study. Mycorrhiza 29:541-547. https://doi.org/10.1007/s00572-019-00907-0

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop. https://doi.org/10.1109/GCE.2010.5676129 .

Neto LM, Barros F, Vinhos F, Furtado SG, et al (2013) Orchidaceae. In: Martinelli G, Moraes MA (eds) Livro Vermelho da Flora do Brasil, 1st edn. Rio de Janeiro, pp749-818

Nguyen DQ, Li Hua, Tran TT, Sivasithamparam K, Jones MGK, Wylie SJ (2020) Four *Tulasnella* taxa associated with populations of the Australian evergreen terrestrial orchid *Cryptostylis ovata.* Fungal Biology 124:24-33. https://doi.org/10.1016/j.funbio.2019.10.006

Nogueira RE, Van den Berg C, Pereira OL, Kasuya MCM, Lanna MCS, Mendonça, MP (2005) Fungos micorrízicos associados a orquídeas em campos rupestres na região do Quadrilátero Ferrífero, MG, Brasil. Acta Botanica Brasílica 19:417-424.

Nogueira RE, Van den Berg C, Pereira OL, Kasuya MCM (2014) Isolation and molecular characterization of Rhizoctonia-like fungi associated with orchid roots in the Quadrilátero Ferrífero and Zona da Mata regions of the state of Minas Gerais, Brazil. Acta Botanica Brasilica 28:298–300. https://doi.org/10.1590/s0102-33062014000200017

Novotná A, Benítez Á, Herrera P, Cruz D, Filipczyková E, Suárez JP (2018) High diversity of root-associated fungi isolated from three epiphytic orchids in southern Ecuador. Mycoscience 59: 24-32. https://doi.org/10.1016/j.myc.2017.07.007

Nylander JAA (2004) MrModeltest version 2. Evolutionary Biology Centre, Sweden. https://github.com/nylander/MrModeltest2

Oberwinkler F, Riess K, Bauer R et al. (2013) Enigmatic Sebacinales. Mycol Progress 12:1–27. https://doi.org/10.1007/s11557-012-0880-4

Oberwinkler F, Riess K, Bauer R, et al. (2014) Morphology and molecules: the Sebacinales, a case study. Mycol Progress 13:445–470. https://doi.org/10.1007/s11557-014-0983-1

Oliveira SF, Bocayuva MF, Veloso TGR et al. (2014) Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic Forest, Brazil. Mycorrhiza 24:55–64. https://doi.org/10.1007/s00572-013-0512-0

Otero JT, Ackerman JD, Bayman P (2004) Differences in mycorrhizal preferences between two tropical orchids. Molecular Ecology 13:2393-2404. https://doi.org/10.1111/j.1365-294X.2004.02223.x

Pereira MC, Rocha DI, Veloso TGR, Pereira OL, Francino DMT, Meira RMSA, Kasuya MCM (2015) Characterization of seed germination and protocorm development of *Cyrtopodium glutiniferum* (Orchidaceae) promoted by mycorrhizal fungi *Epulorhiza* spp.. Acta Botanica Brasilica 29:567-574.

Pereira MC, Coelho IS, Valadares RBS, Oliveira SF, Bocayuva M, Pereira OL, Araújo EF, Kasuya MCM (2014) Morphological and molecular characterization of *Tulasnella* spp. fungi isolated from the roots of *Epidendrum secundum*, a widespread Brazilian orchid. Symbiosis.

Pereira MC, Pereira OL, Costa MD, Rocha RB, Kasuya MCM (2009) Diversidade de fungos micorrízicos *Epulorhiza* spp. isolados de *Epidendrum secundum* (Orchidaceae). Revista Brasileira de Ciência do Solo 33: 1187-1197.

Pereira MC, Torres DP, Guimaraes FAR, Pereira OL, Kasuya MCM (2011b) Germinação de sementes e desenvolvimento de protocormos de *Epidendrum secundum* Jacq. (Orchidaceae) em associação com fungos micorrízicos do gênero *Epulorhiza*. Acta Botanica Brasílica 25:534-541.

Pereira MC, Vieira MV, Tótola MR, Kasuya MCM (2011a) Total fatty acid composition in the characterization and identification of orchid mycorrhizal fungi *Epulorhiza* spp.. Revista Brasileira de Ciência do Solo 31:1159-1165.

Pereira OL, Cavallazzi JRP, Rollemberg CL, Kasuya, MCM (2002) *Sphenospora Kevorkianii*, a rust fungus (Uredinales: Raveneliaceae) on the orchid *Pleurothallis mentigera*. Brazilian Journal of Microbiology 33:155-156.

Pereira OL, Kasuya MCM, Borges AC, Araújo EF (2005a) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Canadian Journal Botany 83:54–65. https://doi.org/10.1139/b04-151

Pereira OL, Kasuya MCM, Rollemberg CL, Borges AC (2005b) Indução in vitro da germinação de sementes de *Oncidium flexuosum* (Orchidaceae) por fungos micorrízicos rizoctonióides. Revista Brasileira de Ciência do Solo 29:199-206.

Pereira OL, Kasuya MCM, Rollemberg CL, Montandon G (2005c) Isolamento e identificação de fungos micorrízicos rizoctonióides associados a três espécies de orquídeas epífitas neotropicais no Brasil. Revista Brasileira de Ciência do Solo 29:191-197.

Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, Kasuya MCM (2003) *Epulorhiza epiphytica* sp. nov. isolated from mycorrhyzal roots os epiphytic orchid species from Brazil. Mycoscience: 44:153-155.

Rai M, Acharya D, Singh A, et al. (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128. https://doi.org/10.1007/s005720100115

Rambaut A (2009) FigTree version 1.4.4. Molecular evolution, phylogenetics and epidemiology. http://tree.bio.ed.ac.uk/software/figtree/

Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T (2015) Germination and seedling establishment in orchids: a complex of requirements. Annals of Botany, 116:391–402. https://doi.org/10.1093/aob/mcv087

Ratnaweera PB, Williams DE, de Silva ED, Wijesundera RLC, Dalisay DS, Andersen RJ (2014) Helvolic acid, an antibacterial nortriterpenoid from a fungal endophyte,

Xylaria sp. of orchid *Anoectochilus setaceus* endemic to Sri Lanka. Mycology 5:23-28. https://doi.org/10.1080/21501203.2014.892905

Ray P, Craven KD (2016) *Sebacina vermifera*: a unique root symbiont with vast agronomic potential. World J Microbiol Biotechnol 32:16. https://doi.org/10.1007/s11274-015-1970-7

Robert V, Stegehuis G and Stalpers J (2005) The MycoBank engine and related databases. https://www.mycobank.org/. Accessed 28 January 2021

Roche SA, Carter RJ, Peakall R, Smith LM, Whitehead MR and Linde CC (2010) A narrow group of monophyletic *Tulasnella* (Tulasnellaceae) symbiont lineages are associated with multiple species of *Chiloglottis* (Orchidaceae): Implications for orchid diversity. American Journal of Botany 97:1313-1327. https://doi.org/10.3732/ajb.1000049

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Beyesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574, https://doi.org/10.1093/bioinformatics/btg180

Sarsaiya S, Jain A, Jia Q, Fan X, Shu F, Chen Z, Zhou Q, Shi J, Chen J. (2020) Molecular identification of endophytic fungi and their pathogenicity evaluation against *Dendrobium nobile* and *Dendrobium officinale*. International Journal of Molecular Sciences 21:316. https://doi.org/10.3390/ijms21010316

Shah S, Shrestha R, Maharjan S, Selosse M-A, Pant B (2019) Isolation and characterization of plant growth-promoting endophytic fungi from the roots of *Dendrobium moniliforme*. Plants 8:5. https://doi.org/10.3390/plants8010005

Sisti LS, Flores-Borges DNA, Andrade SALd, Koehler S, Bonatelli ML and Mayer JLS (2019) The role of non-mycorrhizal fungi in germination of the mycoheterotrophic

orchid *Pogoniopsis schenckii* Cogn. Frontiers in Plant Science 10:1589. https://doi.org/10.3389/fpls.2019.01589

Suárez JP, Weiß M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycological Research 110:1257–1270. https://doi.org/10.1016/j.mycres.2006.08.004

Suárez JP, Weiß M, Abele A, et al. (2008) Members of Sebacinales subgroup B form mycorrhizae with epiphytic orchids in a neotropical mountain rain forest. Mycological Progress 7:75. https://doi.org/10.1007/s11557-008-0554-4

Swarts ND, Sinclair EA, Francis A, Dixon KW (2010) Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Molecular Ecology 19: 3226-3242. https://doi.org/10.1111/j.1365-294X.2010.04736.x

Varma A, Verma S, Sudha, Sahay N, Bütehorn B, Franken P (1999). *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Applied and Environmental Microbiology 65:2741-2744. https://doi.org/10.1128/AEM.65.6.2741-2744.1999

Vaz AB, Mota RC, Bomfim MR, Vieira ML, Zani CL, Rosa CA, Rosa LH (2009) Antimicrobial activity of endophytic fungi associated with Orchidaceae in Brazil. Canadian Journal of Microbiology 55:1381-1391. https://doi.org/10.1139/W09-101

Venneman J, Audenaert K, Verwaeren J, Baert G, Boeckx P, Moango AM, Dhed'a BD, Vereecke D and Haesaert G (2017) Congolese rhizospheric soils as a rich source of new plant growth-promoting endophytic *Piriformospora* Isolates. Frontiers in Microbiology 8:212. https://doi.org/10.3389/fmicb.2017.00212

Venneman J, Vandermeersch L, Walgraeve C, Audenaert K, Ameye M, Verwaeren J, Steppe K, Van Langenhove H, Haesaert G and Vereecke D (2020) Respiratory CO2 combined with a blend of volatiles emitted by endophytic *Serendipita* strains strongly stimulate growth of *Arabidopsis* implicating auxin and cytokinin signaling. Frontiers in Plant Science 11:544435. https://doi.org/10.3389/fpls.2020.544435

Verma S, Varma A, Rexer K, Hassel A, Kost G, Sarbhoy A, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90: 896-903. https://doi.org/doi:10.2307/3761331

Vieira CA, Veloso TGR, Moreira BC, Freitas EFS, Bazzolli DMS, Kasuya MCM (2020) Fungus used for germination is supplanted after reintroduction of *Hadrolaelia jongheana* (Orchidaceae). Revista Agrária Acadêmica 3:148-161. https://doi.org/10.32406/v3n62020/148-161/agrariacad

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, et al. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proceedings of the National Academy of Sciences 102:13386-13391. https://doi.org/10.1073/pnas.0504423102

Warcup JH and Talbot PHB (1967) Perfects states of Rhizoctonias associated with orchids. New Phytologist 66:631-641.

Weiß M, Waller F, Zuccano A, Selosse MA (2016) Sebacinales – one thousand and one interactions with land plants. New Phytologist 211:20–40. https://doi.org/10.1111/nph.13977

White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ,

White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322

Wright MM, Cross R, Cousens RD, et al. (2010) Taxonomic and functional characterization of fungi from the *Sebacina vermifera* complex from common and rare orchids in the genus *Caladenia*. Mycorrhiza 20:375–390. https://doi.org/10.1007/s00572-009-0290-x

Xie L, Long YY, Zhang Y, Chen YL, Zhang WL (2020) *Serendipita sacchari* sp. nov. from a sugarcane rhizosphere in southern China. Mycotaxon 135: 579-587. https://doi.org/10.5248/135.579

Zhang FS, Lv YL, Zhao Y, Guo SX (2013) Promoting role of an endophyte on the growth and contents of kinsenosides and flavonoids of *Anoectochilus formosanus* Hayata, a rare and threatened medicinal Orchidaceae plant. Journal of Zhejiang University 14: 785–792.

Zi XM, Sheng CL, Goodale UM, SHAO SC, Gao JY (2014) In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). Mycorrhiza 24:487-499. https://doi.org/10.1007/s00572-014-0565-8

Electronic supplementary material 1: Isolates obtained in this study.

Query	Closest Match BLAST	Query coverage	% Pairwise Identity	Putative Ecology
EHJ1A	*Alternaria tenuissima* (MT601957)	100.00%	100.0%	Endophyte
EHJ1B	*Epicoccum nigrum* (MT582797)	100.00%	100.0%	Endophyte
EHJ1C	*Diaporthe malorum* (NR158411)	99.19%	100.0%	Endophyte
EHJ1D	*Alternaria tenuissima* (MT601957)	100.00%	100.0%	Endophyte
EHJ1E	*Sordariomycetes* sp. (KR708970)	100.00%	100.0%	Endophyte
MHJ1A	*Fusarium decemcellulare* (MK482193)	97.76%	98.8%	Endophyte
EHJ2D	*Colletotrichum* sp. (FJ042520)	100.00%	99.8%	Endophyte
MHJ2A	*Tulasnella* sp. (KC291647)	81.75%	99.8%	Orchid Mycorrhiza
MHJ2B	*Xylaria* sp. (MG814037)	100.00%	99.6%	Endophyte
EHJ4B	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ4C	E*ndomelanconiopsis microspora* (MK371761)	100.00%	100.0%	Endophyte
EHJ4E	*Trichoderma caerulescens* (MT217122)	100.00%	99.6%	Endophyte
EHJ4F	*Paraphaeosphaeria* sp. (HQ631065)	96.69%	98.8%	Endophyte
EHJ4H	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ4I	*Pestalotiopsis* sp. (KP230825)	100.00%	100.0%	Endophyte
EHJ4J	*Diaporthales* sp. (JF773672)	99.59%	99.2%	Endophyte
EHJ4K	*Colletotrichum* sp. (FJ042520)	100.00%	99.6%	Endophyte
EHJ4L	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ4N	*Epicoccum nigrum* (MT582797)	100.00%	100.0%	Endophyte
EHJ4O	*Epicoccum nigrum* (MT582797)	100.00%	100.0%	Endophyte
EHJ4P	*Epicoccum nigrum* (MT582797)	100.00%	100.0%	Endophyte
EHJ5B	*Xylaria berteroi* (MN148282)	100.00%	100.0%	Endophyte
EHJ5C	*Colletotrichum cliviae* (JX902436)	100.00%	100.0%	Endophyte
EHJ5F	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ5G	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ5H	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ5K	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ5L	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ5M	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ6B	*Colletotrichum* sp. (FJ042520)	100.00%	100.0%	Endophyte
EHJ6C	*Arcopilus aureus* (MT626577)	100.00%	100.0%	Endophyte
EHJ6D	*Xylaria adscendens* (KP133292)	100.00%	99.8%	Endophyte
EHJ6F	*Lasiodiplodia theobromae* (MT764993)	100.00%	100.0%	Endophyte
EHJ6I	*Nodulisporium* sp. (MW020707)	100.00%	100.0%	Endophyte
EHJ6J	*Colletotrichum crassipes* (KP050648)	100.00%	99.6%	Endophyte
EHJ6L	*Xylaria* sp. (MT643822)	100.00%	99.6%	Endophyte
EHJ7A	*Lasiodiplodia theobromae* (MT764993)	100.00%	100.0%	Endophyte
EHJ7B	*Colletotrichum theobromicola* (MT470601)	100.00%	100.0%	Endophyte
EHJ7C	*Diaporthe passiflorae* (KR534744)	100.00%	99.8%	Endophyte
EHJ7D	*Lasiodiplodia theobromae* (MT764993)	100.00%	100.0%	Endophyte
EHJ7G	*Lasiodiplodia theobromae* (MT764993)	100.00%	100.0%	Endophyte
EHJ7I	*Phomopsis* sp. (MT000108)	100.00%	99.8%	Endophyte
EHJ7K	*Pestalotiopsis microspora* (MT378372)	100.00%	100.0%	Endophyte
EHJ7L	*Colletotrichum crassipes* (KP050648)	100.00%	99.6%	Endophyte
EHJ7M	*Diaporthe cissampeli* (NR147594)	100.00%	89.7%	Endophyte
EHJ8A	*Xylaria* sp. (MT643822)	100.00%	99.8%	Endophyte
EHJ8B	Xylariales sp. (KU747562)	100.00%	98.4%	Endophyte
EHJ8C	Xylariales sp. (KU747562)	100.00%	98.2%	Endophyte

EHJ8D	*Xylaria* sp. (MH046901)	100.00%	98.8%	Endophyte
EHJ8E	Xylariales sp. (KU747817)	100.00%	99.5%	Endophyte
EHJ8F	*Xylaria enteroleuca* (AM084368)	100.00%	99.8%	Endophyte
EHJ8G	*Annulohypoxylon stygium* (MH370739)	100.00%	100.0%	Endophyte
EHJ8H	*Annulohypoxylon stygium* (KY250416)	100.00%	99.9%	Endophyte
EHJ8J	*Xylaria plebeja* (KY250416)	100.00%	98.4%	Endophyte
EHJ8L	Xylariales sp. (MW045865)	100.00%	99.3%	Endophyte
EHJ8N	*Xylaria intracolorata* (MF770878)	99.81%	96.9%	Endophyte
EHJ8O	*Pestalotiopsis microspora* (MT597834)	100.00%	99.6%	Endophyte
EHJ8Q	*Endomelanconiopsis microspora* (MK371761)	100.00%	99.8%	Endophyte
EHJ9A	*Trichoderma viride* (MT939298)	100.00%	100.0%	Endophyte
EHJ9B	*Trichoderma viride* (MT939298)	100.00%	100.0%	Endophyte
EHJ9C	*Trichoderma viride* (MT939298)	100.00%	100.0%	Endophyte
EHJ9D	*Pestalotiopsis microspora* (MT378372)	100.00%	100.0%	Endophyte
EHJ9F	*Xylaria intracolorata* (MF770878)	100.00%	97.4%	Endophyte
EHJ9I	*Diaporthe paranensis* (NR111857)	99.39%	99.0%	Endophyte
EHJ9K	*Trichoderma viride* (MT755726)	100.00%	100.0%	Endophyte
EHJ9L	*Trichoderma viride* (MT939298)	100.00%	100.0%	Endophyte
EHJ9M	*Trichoderma viride* (MT939298)	100.00%	100.0%	Endophyte
EHJ9N	*Trichoderma koningiopsis* (MH347310)	100.00%	100.0%	Endophyte
EHJ10A	*Xylaria allantoidea* (KR534657)	100.00%	99.8%	Endophyte
EHJ10B	Fungal (FJ612983)	99.62%	99.4%	Endophyte
EHJ10C	*Neofusicoccum ribis* (MK557959)	100.00%	100.0%	Endophyte
EHJ10D	*Trichoderma viride* (MN594481)	100.00%	100.0%	Endophyte
EHJ10E	*Trichoderma viride* (MN594481)	100.00%	100.0%	Endophyte
EHJ11A	*Trichoderma viride* (MN594481)	100.00%	100.0%	Endophyte
EHJ11B	*Paraconiothyrium estuarinum* (KJ572126)	99.60%	100.0%	Endophyte
EHJ11C	*Trichoderma koningiopsis* (MN636272)	100.00%	100.0%	Endophyte
EHJ11D	Xylariaceae (KU747833)	100.00%	97.8%	Endophyte
EHJ11E	X*ylaria intracolorata* (MF770878)	100.00%	96.7%	Endophyte
EHJ11F	*Trichoderma* sp. (KU556538)	99.61%	100.0%	Endophyte
EHJ11G	Fungal (KM265968)	100.00%	99.6%	Endophyte
MHJ11A	Fungal (GU166405)	99.80%	89.2%	Endophyte
MHJ11F	Uncultured *Sebacina* (HQ154305)	100.00%	97.0%	Orchid Mycorrhiza
MHJ11G	Uncultured *Sebacina* (HQ154305)	99.12%	97.0%	Orchid Mycorrhiza
MHJ11H	Uncultured *Sebacina* (HQ154305)	99.64%	96.8%	Orchid Mycorrhiza
EHJ12A	*Hypoxylon anthochroum* (KY992584)	99.78%	99.8%	Endophyte
EHJ12A	*Nodulisporium* sp. (MN184851)	100.00%	99.6%	Endophyte
EHJ12B	Xylariaceae (KU747729)	99.17%	98.9%	Endophyte
EHJ12C	P*enicillium melinii* (NR077155)	100.00%	100.0%	Endophyte
EHJ12E	*Diaporthe passiflorae* (MN077411)	98.99%	99.4%	Endophyte
EHJ12F	*Xylaria* sp. (KJ404200)	99.40%	100.0%	Endophyte
EHJ12I	*Penicillium melinii* (NR077155)	100.00%	100.0%	Endophyte
EHJ12J	*Nemania* sp. (MG545055)	100.00%	100.0%	Endophyte
EM41	*Tulasnella* sp. (JX546234)	99.49%	99.7%	Orchid Mycorrhiza
EM42	*Tulasnella* sp. (JX546234)	99.49%	99.7%	Orchid Mycorrhiza
EM43	*Tulasnella* sp. (JX546234)	99.62%	99.7%	Orchid Mycorrhiza
EM44	*Tulasnella* sp. (JX546234)	99.74%	99.7%	Orchid Mycorrhiza
EM45	*Tulasnella* sp. (JX546234)	99.61%	99.5%	Orchid Mycorrhiza
EM47	*Tulasnella* sp. (JX546234)	99.87%	99.6%	Orchid Mycorrhiza
EM48	*Tulasnella* sp. (JX546234)	99.23%	99.7%	Orchid Mycorrhiza
EM49	*Tulasnella* sp. (JX546234)	99.11%	99.7%	Orchid Mycorrhiza

EM50	*Tulasnella* sp. (JX546234)	99.74%	99.7%	Orchid Mycorrhiza
EM51	*Tulasnella* sp. (JX546234)	99.74%	99.7%	Orchid Mycorrhiza
EM52	*Tulasnella* sp. (JX546234)	99.87%	99.7%	Orchid Mycorrhiza
EHJ13A	*Xylaria* sp. (GQ334444)	100.00%	100.0%	Endophyte
EHJ13B	*Fusarium babinda* (MK968883)	100.00%	100.0%	Endophyte
EHJ13D	*Fusarium babinda* (MK968883)	99.57%	100.0%	Endophyte
EHJ13E	*Fusarium verticillioides* (KY860652)	100.00%	99.8%	Endophyte
EHJ13F	*Colletotrichum* sp. (KP748193)	99.60%	99.2%	Endophyte
EHJ13G	*Colletotrichum cliviae* (JX902436)	100.00%	100.0%	Endophyte
EHJ13H	*Xylaria subtorulosa*	87.30%	97.0%	Endophyte
EHJ13I	*Xylaria venosula* (EF026149)	99.80%	99.8%	Endophyte
EHJ13J	*Xylaria sp.* (JQ862679)	99.79%	99.6%	Endophyte
MHJ13D	*Tulasnella* sp. (GU166427)	99.42%	92.8%	Orchid Mycorrhiza
MHJ13E	*Tulasnella* sp. (JX546234)	100.00%	100.0%	Orchid Mycorrhiza
MHJ13F	*Tulasnella* sp. (GU166420)	99.81%	92.9%	Orchid Mycorrhiza
MHJ13L	*Tulasnella* sp. (GU166420)	99.80%	93.0%	Orchid Mycorrhiza
MHJ13M	*Tulasnella* sp. (GU166420)	99.42%	92.9%	Orchid Mycorrhiza
MHJ13N	*Tulasnella* sp. (GU166427)	100.00%	92.8%	Orchid Mycorrhiza
MHJ13O	*Tulasnella* sp. (GU166427)	99.04%	92.8%	Orchid Mycorrhiza
MHJ13Q	*Tulasnella* sp. (GU166420)	99.42%	93.0%	Orchid Mycorrhiza
MHJ13S	*Tulasnella* sp. (GU166427)	99.21%	92.8%	Orchid Mycorrhiza
MHJ13W	Uncultured *Sebacina* (GU189681)	87.99%	96.3%	Orchid Mycorrhiza
MHJ13X	Uncultured *Sebacina* (GU189681)	100.00%	96.9%	Orchid Mycorrhiza
MHJ13Y	Uncultured *Sebacina* (GU189681)	99.78%	98.2%	Orchid Mycorrhiza
MHJ13Z	Uncultured *Sebacina* (GU189681)	100.00%	98.3%	Orchid Mycorrhiza
MHJ13A8	*Xylariaceae* sp. (AB741592)	58.02%	89.5%	Endophyte
EHJ14C	*Colletotrichum crassipes* (KP050648)	99.59%	99.6%	Endophyte
EHJ14D	*Microdiplodia* sp. (MN421894)	99.38%	98.1%	Endophyte
EHJ14E	*Paraphaeosphaeria* sp. (LC115035)	100.00%	98.4%	Endophyte
EHJ14E	Pleosporales (KP306979)	100.00%	98.4%	Endophyte
EHJ14F	*Endomelanconiopsis microspora* (MK371761)	100.00%	100.0%	Endophyte
EHJ14F	*Botryosphaeria* sp. (FJ527864)	100.00%	100.0%	Endophyte
EHJ14F	*Colletotrichum crassipes* (KP050648)	99.18%	99.6%	Endophyte
EHJ14H	Pezizomycetes (KX909062)	71.78%	99.0%	Endophyte
EHJ14J	*Annulohypoxylon stygium* (KP170485)	99.37%	98.3%	Endophyte
EHJ14K	*Epicoccum nigrum*	100.00%	98.7%	Endophyte
EHJ14L	*Chaetomium homopilatum* (LT993569)	100.00%	98.4%	Endophyte
MHJ14A	*Microdiplodia* sp. (MN421894)	98.97%	99.8%	Endophyte
MHJ14B	*Microdiplodia* sp. (MN421894)	93.00%	99.8%	Endophyte
MHJ14E	*Microdiplodia* sp. (MN421894)	99.38%	98.1%	Endophyte
MHJ14F	*Microdiplodia* sp. (MN421894)	99.17%	97.9%	Endophyte
MHJ14G	*Microdiplodia* sp. (MN421894)	99.08%	100.0%	Endophyte
MHJ14I	*Microdiplodia* sp. (MN421894)	99.59%	97.9%	Endophyte
MHJ14K	Uncultured *Sebacina* (GU189681)	99.82%	97.5%	Orchid Mycorrhiza
MHJ14L	*Microdiplodia* sp. (MN421894)	99.79%	99.8%	Endophyte
MHJ14M	*Microdiplodia* sp. (MN421894)	99.16%	99.8%	Endophyte
MHJ14N	*Microdiplodia* sp. (MN421894)	99.58%	98.1%	Endophyte
MHJ14O	*Microdiplodia* sp. (MN421894)	99.17%	99.8%	Endophyte
MHJ14Q	*Hyalorbilia* sp. (DQ656651)	84.07%	84.3%	Endophyte
EM131	*Tulasnella* sp. (JX546234)	98.06%	99.7%	Orchid Mycorrhiza
EHJ15A	*Xylaria berteroi* (MN148282)	100.00%	100.0%	Endophyte
EHJ15C	*Nigrospora oryzae* (MF380826)	99.59%	99.4%	Endophyte

EHJ15D	*Xylaria venosula* (EF026149)	100.00%	99.8%	Endophyte
EHJ15E	*Colletotrichum gloeosporioides* (MK937679)	100.00%	100.0%	Endophyte
EHJ15E	*Nigrospora* sp. (MG976425)	100.00%	100.0%	Endophyte
EHJ15F	*Colletotrichum gloeosporioides* (MK937679)	99.79%	100.0%	Endophyte
EHJ15F	*Nigrospora* sp. (MG976425)	99.79%	100.0%	Endophyte
EHJ15G	*Bipolaris* sp. (KF852586)	100.00%	100.0%	Endophyte
EM145	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EM147	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EM149	*Tulasnella* sp. (JX546234)	94.87%	96.8%	Orchid Mycorrhiza
MHJ15G	*Neohelicosporium laxisporum* (NR160381)	98.17%	88.7%	Endophyte
MHJ15J	*Nemania macrocarpa* (NR160210)	100.00%	96.8%	Endophyte
MHJ15J	*Xylaria longipes* (MN588219)	99.80%	88.6%	Endophyte
EM155	*Tulasnella* sp. (JX546234)	99.36%	99.7%	Orchid Mycorrhiza
EM157	*Tulasnella* sp. (JX546234)	99.87%	99.6%	Orchid Mycorrhiza
EM160	*Tulasnella* sp. (JX546234)	100.00%	99.7%	Orchid Mycorrhiza
EHJ16A	*Pestalotiopsis* sp. (EU047944)	99.78%	99.8%	Endophyte
EHJ16A	*Neopestalotiopsis eucalypticola* (MN483278)	100.00%	99.6%	Endophyte
EHJ16B	*Neopestalotiopsis cubana* (MN520027)	100.00%	100.0%	Endophyte
EHJ16C	*Pestalotiopsis sp.* (EU047944)	100.00%	99.8%	Endophyte
EHJ16C	*Neopestalotiopsis* sp. (MN700118)	100.00%	99.6%	Endophyte
EHJ16D	*Epicoccum nigrum*	100.00%	98.7%	Endophyte
EHJ16E	*Pestalotiopsis cocculi* (KU720062)	100.00%	100.0%	Endophyte
EHJ16F	*Diaporthe* sp. (KU523580)	99.59%	100.0%	Endophyte
EHJ16G	*Lasiodiplodia theobromae* (MN759434)	100.00%	100.0%	Endophyte
EHJ16H	*Trichoderma viride* (MH971268)	100.00%	100.0%	Endophyte
MHJ16A	*SynnEMellisia aurantia* (NR154444)	99.80%	94.7%	Endophyte
EM170	*Tulasnella* sp. (JX546234)	100.00%	99.6%	Orchid Mycorrhiza
EM174	*Tulasnella* sp. (JX546234)	99.87%	99.5%	Orchid Mycorrhiza
MHJ16G	*NEMania macrocarpa* (NR160210)	100.00%	96.6%	Endophyte
EM176	*Tulasnella* sp. (JX546234)	99.87%	99.7%	Orchid Mycorrhiza
EM177	*Tulasnella* sp. (JX546234)	99.87%	99.6%	Orchid Mycorrhiza
MHJ16K	*Tulasnella* sp. (KC928365)	100.00%	98.3%	Orchid Mycorrhiza
EM182	*Tulasnella* sp. (KC928365)	99.45%	99.9%	Orchid Mycorrhiza
EM183	*Tulasnella* sp. (KC928365)	99.30%	99.7%	Orchid Mycorrhiza
MHJ16P	*Tulasnella* sp. (KC928365)	99.41%	99.9%	Orchid Mycorrhiza
MHJ16Q	*Tulasnella* sp. (KC928365)	100.00%	98.3%	Orchid Mycorrhiza
MHJ16R	*Tulasnella* sp. (KC928365)	100.00%	98.3%	Orchid Mycorrhiza
MHJ16V	*Tulasnella* sp. (JX546234)	100.00%	99.2%	Orchid Mycorrhiza
EV20	*Tulasnella* sp. (JX546234)	100.00%	100.0%	Orchid Mycorrhiza
EV21	*Tulasnella* sp. (JX546234)	99.87%	99.9%	Orchid Mycorrhiza
EV22	*Tulasnella* sp. (JX546234)	90.05%	97.1%	Orchid Mycorrhiza
EV23	*Tulasnella* sp. (JX546234)	100.00%	99.6%	Orchid Mycorrhiza
EV24	*Tulasnella* sp. (JX546234)	98.98%	99.9%	Orchid Mycorrhiza
EV25	*Tulasnella* sp. (JX546234)	98.98%	99.9%	Orchid Mycorrhiza
EV26	*Tulasnella* sp. (JX546234)	99.43%	100.0%	Orchid Mycorrhiza
EV27	*Tulasnella* sp. (JX546234)	98.98%	100.0%	Orchid Mycorrhiza
EV28	*Tulasnella* sp. (JX546234)	98.98%	100.0%	Orchid Mycorrhiza
EV29	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EV30	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EV31	*Tulasnella* sp. (JX546234)	99.74%	99.6%	Orchid Mycorrhiza
EV32	*Xylariaceae* (AB741589)	100.00%	93.3%	Endophyte
EV33	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza

EV34	*Tulasnella* sp. (JX546234)	99.87%	99.9%	Orchid Mycorrhiza
EV35	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EV36	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza
EV37	*Tulasnella* sp. (JX546234)	99.74%	99.9%	Orchid Mycorrhiza
EV38	*Tulasnella* sp. (JX546234)	99.36%	99.9%	Orchid Mycorrhiza
EV39	*Tulasnella* sp. (JX546234)	99.62%	99.9%	Orchid Mycorrhiza

Electronic supplementary material 2: Details of composition of mycorrhizal and endophytic fungal isolated from *Cattleya jongheana* roots.

Phylum	%	Class	%	Order	%	Family	%	Genus	%	N observado
Ascomycota	67,45	Dothideomycetes	16,51	Botryosphaeriales	9,91	Botryosphaeriaceae	8,49	*Microdiplodia*	5,19	11
								Lasiodiplodia	2,36	5
								Neofusicoccum	0,47	1
								Botryosphaeria	0,47	1
						Endomelanconiopsidaceae	1,42	*Endomelanconiopsis*	1,42	3
				Pleosporales	4,72	Pleosporaceae	1,42	*Alternaria*	0,94	2
								-	0,47	1
						Massarinaceae	0,47	*Bipolaris*	0,47	1
						Didymellaceae	2,83	*Epicoccum*	2,83	6
				Pleosporomycetidae	1,42	Didymosphaeriaceae	1,42	*Paraphaeosphaeria*	1,42	3
				Tubeufiales	0,47	Tubeufiaceae	0,47	*Neohelicosporium*	0,47	1
		Eurotiomycetes	0,94	Eurotiales	0,94	Aspergillaceae	0,94	*Penicillium*	0,94	2
		Incertae sedis	0,47	Incertae sedis	0,47	Incertae sedis	0,47	*Synnemellisia*	0,47	1
		Orbiliomycetes	0,47	Orbiliales	0,47	Orbiliaceae	0,47	*Hyalorbilia*	0,47	1
		Pezizomycetes	0,47	Incertae sedis	0,47	Incertae sedis	0,47	-	0,47	1
		Sordariomycetes	48,58	Amphisphaeriales	1,42	Pestalotiopsidaceae	1,42	*Neopestalotiopsis*	1,42	3
				Diaporthales	3,77	Diaporthaceae	3,77	*Diaporthe*	3,30	7
								Phomopsis	0,47	1
				Glomerellales	10,38	Glomerellaceae	10,38	*Colletotrichum*	10,38	22
				Hypocreales	8,49	Hypocreaceae	6,60	*Trichoderma*	6,60	14
						Nectriaceae	1,89	*Fusarium*	1,89	4
				Incertae sedis	1,42	Incertae sedis	1,42	*Nigrospora*	1,42	3
				Sordariales	0,94	Chaetomiaceae	0,94	*Arcopilus*	0,47	1
								Chaetomium	0,47	1

				Xylariales	22,17	Hypoxylaceae	2,36	*Annulohypoxylon*	1,42	3
								Nodulisporium	0,94	2
						Sporocadaceae	4,72	*Pestalotiopsis*	4,72	10
						Xylariaceae	15,09	*Xylaria*	13,68	29
								Nemania	0,94	2
								Hypoxylon	0,47	1
Basidiomycota	31,13	Agaricomycetes	31,13	Cantharellales	27,36	Tulasnellaceae	27,36	*Tulasnella*	27,36	58
				Sebacinales	3,77	Sebacinaceae	3,77	*Sebacina*	3,77	8
Fungi	1,42	Fungi	1,42	Fungi	1,42	Fungi	1,42	Fungal	1,42	3

Electronic supplementary material 3: GenBank and UNITE accession numbers of additional *Tulasnella* isolates included in the phylogenetic analysis. Strains obtained from this study are indicated in bold face.

Species	Strain No.	Origin	GenBank accession No.	UNITE accession No.
Epulorhiza amonilioides	3S	Brazil	JF907600	JF907600
Epulorhiza amonilioides	aero8	Brazil	KC928335	KC928335
Epulorhiza anaticula	UAMH 5428	Canada	EU218891	
Epulorhiza anaticula	13O004	South Korea	KT164598	KT164598
Tulasnella albida	KC110	United Kingdom	AY373294	AY373294
Tulasnella asymmetrica	MAFF 305808	Australia	KC152356	KC152356
Tulasnella asymmetrica	AL.LM4.4.1	Australia	MH134544	MH134544
Tulasnella bifrons	BPI 724849	Canada	AY373290	AY373290
Tulasnella brigadeiroensis	COAD2884	Brazil	MK192001	
Tulasnella brigadeiroensis	COAD3007	Brazil	MT090025	MT090025
Tulasnella brigadeiroensis	COAD3008	Brazil	MT090026	MT090026
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella brigadeiroensis		Brazil		
Tulasnella calospora	MAFF P305801	Ecuador	DQ388041	DQ388041
Tulasnella calospora	MAFF P305802	Ecuador	DQ388042	
Tulasnella calospora	MAFF P305805	Ecuador	DQ388045	
Tulasnella calospora	FCb4	China	KC796458	KC796458
Tulasnella calospora		Brazil		
Tulasnella danica	KC388	USA	AY373297	AY373297
Tulasnella eichleriana	KC852	United Kingdom	AY373292	AY373292
Tulasnella hadrolaeliae	COAD2887	Brazil	MN385724	
Tulasnella hadrolaeliae	COAD2889	Brazil	MN385726	

Tulasnella hadrolaeliae		Brazil		
Tulasnella hadrolaeliae		Brazil		
Tulasnella hadrolaeliae		Brazil		
Tulasnella irregularis	JHW 0632	Australia	EU218889	
Tulasnella irregularis	C3-DT-TC-2	Thailand	GU166423	GU166423
Tulasnella orchidis	COAD2893	Brazil	MN385729	MN385729
Tulasnella orchidis	COAD2894	Brazil	MN385731	MN385731
Tulasnella prima	CLM159	Australia	KF476556	
Tulasnella prima	07033-45	Australia	HM196800	
Tulasnella pruinosa	DAOM 17641	Unknown	AY373295	
Tulasnella pruinosa	AFTOL ID610	Unknown	DQ457642	DQ457642
Tulasnella secunda	CLM009	Australia	KF476575	KF476575
Tulasnella secunda	CLM222	Australia	KF476568	
Tulasnella sp.	141	USA	AY373264	
Tulasnella sp.	10 MM-2016	USA	KU664580	
Tulasnella sp.1	COAD3099	Brazil	MT808332	
Tulasnella sp.1	COAD3100	Brazil	MT808333	
***Tulasnella* sp.1**		Brazil		
***Tulasnella* sp.1**		Brazil		
***Tulasnella* sp.1**		Brazil		
***Tulasnella* sp.1**		Brazil		
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***Tulasnella* sp.1**		Brazil		
***Tulasnella* sp.1**		Brazil		
***Tulasnella* sp.1**		Brazil		
Tulasnella sp.2	COAD2885	Brazil	MK192002	
Tulasnella sphagneti	CLM541	Australia	KY095117	KY095117
Tulasnella sphagneti	CLM583	Australia	KY445922	
Tulasnella tomaculum	KC429	United Kingdom	AY373296	AY373296
Tulasnella tubericola	EP-15	Spain	KX929166	
Tulasnella tubericola	EP-1	Spain	KX774345	
Tulasnella violea	FO24380a	Germany	KC152439	KC152439
Tulasnella violea	DC292	Germany	KC152432	
Tulasnella warcupii	CLM027	Australia	KF476596	KF476596

Tulasnella warcupii	CLM007	Australia	KF476600
Tulasnella zygopetali	COAD2896	Brazil	MN385732
Tulasnella zygopetali	COAD2897	Brazil	MN385733
Uncultured *Tulasnella*	Clone 33tu-12	China	HM230652
Botryobasidium botryosum	AFTOL ID604	Germany	DQ267124

Electronic supplementary material 4: GenBank and UNITE accession numbers of additional Sebacinaceae and Serendipitaceae isolates included in the phylogenetic analysis. Ex-type strains are indicated in bold face. Strains obtained from this study are indicated in bold face.

Species	**Strain No.**	**Origin**	**GenBank accession No.**	**UNITE accession No.**
Craterocolla cerasi	VKummer 02.12.2001	Germany	AY505542	-
Craterocolla cerasi	AFTOL1677	Germany	DQ520103	-
Craterocolla cerasi	FO36456	Germany	AF291308	-
Efibulobasidium albescens	-	Canada	AF384860	AF384860
Globulisebacina rolleyi	RJB794	Canada	AY509550	-
Helvellosebacina concrescens	TUB020208	USA	KF061275	-
Paulisebacina_allantoidea	Roki179	Germany	AF291367	-
Sebacina cystidiata	TUB020024	Germany	KF000452	-
Sebacina cystidiata	TUB020026	Germany	KF000454	-
Sebacina cystidiata	TUB020027	Germany	KF000455	KF000455
Sebacina dimitica	MW525	Germany	AF291364	-
Sebacina epigaea	-	Germany	AF291267	-
Sebacina flagelliformis	TUB020035	Germany	KF000463	-
Sebacina flagelliformis	TUB020036	Germany	KF000464	KF000464
Sebacina guayanensis	BM03M3	Venezuela	KF773775	KF773775
Sebacina guayanensis	BM07M6	Venezuela	KF773777	-
Sebacina incrustans	-	Germany	AY143340	AY143340
Sebacina incrustans	AFTOL1626	USA	DQ521406	-
Sebacina incrustans	MW524	Germany	AF291365	-
Sebacina pallida	-	Venezuela	AF384862	AF384862
Sebacina tomentosa	PD10	Venezuela	KF773779	KF773779
Serendipita herbamans	S1	Germany	KF061285	KF061285
Serendipita indica	-	India	AY505557	-
Serendipita indica	-	India	AY293202	-
Serendipita indica	DSM11827	India	KF061284	KF061284
Serendipita restingae	P8	Brazil	MN595219	-
Serendipita sacchari.	A8	China	KY496809	-

***Serendipita* sp. 1**		Brazil		-
***Serendipita* sp. 1**		Brazil		-
***Serendipita* sp. 1**		Brazil		-
Serendipita vermifera		Brazil		-
Serendipita vermifera		Brazil		-
Serendipita vermifera		Brazil		-
Serendipita vermifera	AFTOL1877	Germany	DQ520096	-
Serendipita vermifera	FO6475	Germany	HM030724	-
Serendipita vermifera	K225	Australia	EU625992	EU625992
Serendipita vermifera	K228	Australia	EU625993	-
Serendipita vermifera	K229	Australia	EU625994	
Serendipita vermifera	K224	Australia	EU625991	EU625991
Serendipita vermifera	K236	Ecuador	EU625995	EU625995
Serendipita vermifera	K250	Ecuador	EU626001	EU626001
Serendipita vermifera	MAFF305839	Australia	KF061290	-
Serendipita vermifera	MAFF305841	Australia	KF061292	KF061292
Serendipita vermifera	Warcup714	Australia	AY505549	-
Serendipita vermifera	Warcup723	Australia	AF291366	-
Serendipita vermifera	Warcup914	Australia	AY505552	-
Serendipita vermifera	Warcup963	Australia	AY505554	-
Serendipita whamiae	EC3A	Australia	MT422063	-
Serendipita williamsii	DAR29830	Australia	AY505556	-
Tremelloscypha gelatinosa	GG23605		AF291308	-
Geastrum saccatum	Trappe23765		AY574646	-

CONCLUSÕES GERAIS

O presente trabalho contribuiu para o conhecimento dos micobiontes com os quais *C. caulescens*, *C. cinnabarina*, *C. jongheana* and *Z. maxillare* se associam. Baseado em métodos dependentes de cultivo e posterior análises de sequencias de DNA, 212 isolados de fungos micorrízicos e endofíticos foram investigados. Este é o primeiro estudo que descreve a diversidade e composição de fungos cultiváveis associados as raízes das orquídeas estudadas.

Nosso estudo fornece também a descrição de oito novas espécies, a saber*: Neopestalotiopsis hadrolaeliae, Serendipita* sp. 1*, Tulasnella brigadeiroensis, T. hadrolaeliae, T. orchidis, T. zygopetali, Tulasnella* sp. 1 e *Tulasnella* sp. 2. Trabalhos futuros poderão investigar o potencial destas espécies na germinação de sementes e desenvolvimento de plantas de orquídeas para fins de reintrodução e, consequentemente, de conservação de espécies.