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

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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.

Orchidaceae (or orchids) is the largest family of flowering plants, with approximately 27,000 species described. The Neotropics is the region of greatest orchid diversity and approximately 205 genera and 2,650 species occur in Brazil, of which about 1,800 are endemic. Many orchid species are endangered, mainly due to anthropogenic pressure and dependency between orchids and other organisms, i.e. pollinators or mycorrhizal fungi. Several endangered orchid species are listed in the *Livro Vermelho da Flora do Brasil*. 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, which limits its distribution. *Cattleya cinnabarina* and *Cattleya caulescens* are rupicolous (i.e. grow on bare rocks) and endemic to the Southeastern Brazil. These species belong to Brazilian Atlantic Forest, a highly diverse but endangered hotspot of biodiversity. Like all orchids, they need mycorrhizal fungi for germination due to the limited reserves in seeds. The symbiotic fungus supplies the embryo with carbon and other nutrients, which enable the germination and establishment of the orchid. Orchids associate mainly with Basidiomycota often called rhizoctonia, a polyphyletic that includes taxa belonging to the families Sebacinaeae, Serendipitaceae, Ceratobasidiaceae and Tulasnellaceae.

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

The genus *Tulasnella* is often observed as orchid mycorrhizal fungi in temperate and tropical regions, and several isolates have been reported to increase seed germination and seedling growth. Identification of mycorrhizal fungi in South American orchids, mostly conducted in Brazil, has often revealed *Tulasnella* symbionts. *Tulasnella* species were isolated from *Epipendrum* species, *Epipendrum* dendrobioidei, and *Sophronits* milleri, *Oeceoclades maculata*, *Epidendrum rigidum* and *Polystachya concreta*, *E. rigidum* and *P. concreta*. 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. As morphological characteristics are not sufficient to describe *Tulasnella* species, molecular approaches and phylogenetic analyses, pairwise sequence divergence and morphological features (see below). The nuclear ribosomal DNA was shown to be highly suitable for species delimitation in *Tulasnella*.

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 described 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* roots (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 pellets 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.

**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.

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 hadroelaetica*, *Tulasnella brigadeiroensis*, *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 hadroelaetica* formed a well-supported clade (Maximum likelihood (ML)/Posterior probabilities (PP) = 100/1), which is a sister group of *T. albida* and *T. pruina*. *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 analyzes 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.

**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. 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 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 47 bp (8.1%).
- **Type:** BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, isolated from roots of the orchid *Hadroelaetia 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°C, 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 (X ± SD = 2 ± 0.3 μm), hyaline, with binucleate cells. Molinoid cells not observed. Sexual morph not observed.
- **Substrate or host:** Roots of *Hadroelaetia jongheana*.
- **Additional material examined:** BRAZIL: Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of *Hadroelaetia 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.

**Tulasnella calospora** Juél, Bh. K. svenska Vet-Akad. Handl. 23: 23 (1897). (Fig. 3)
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 (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.

| 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    | MK191993              |
|                        | COAD 2853              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191994              |
|                        | COAD 2854              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192007              |
|                        | COAD 2855              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191995              |
|                        | COAD 2856              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191996              |
|                        | COAD 2857              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191997              |
|                        | COAD 2858              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191998              |
|                        | COAD 2859              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191999              |
|                        | COAD 2860              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192000              |
|                        | COAD 2861              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192002              |
|                        | COAD 2862              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192005              |
|                        | COAD 2863              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192003              |
|                        | COAD 2864              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191974              |
|                        | COAD 2865              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191975              |
|                        | COAD 2866              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192006              |
|                        | COAD 2867              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191976              |
|                        | COAD 2868              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191977              |
|                        | COAD 2869              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191978              |
|                        | COAD 2870              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191979              |
|                        | COAD 2871              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191980              |
|                        | COAD 2873              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191981              |
|                        | COAD 2874              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191982              |
|                        | COAD 2875              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191983              |
|                        | COAD 2876              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191984              |
|                        | COAD 2877              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191985              |
|                        | COAD 2878              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191986              |
|                        | COAD 2879              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192004              |
|                        | COAD 2880              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191987              |
|                        | COAD 2881              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191988              |
|                        | COAD 2882              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK192008              |
|                        | COAD 2883              | Cattleya caulescens      | Mariana - MG    | Rupicolous    | MK191989              |
| Tulasnella brigadetroensis 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              |

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

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 (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.

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. Freitas (holotype VIC47304, ex-type culture COAD2889).

Description: Colonies on PDA showed very slow-growing (56–59 mm diam after 30 d at 25 °C), white to cream, showing concentric rings, with undulate and submersed edge, aerial mycelium present.

| Species                | Strain No. | Origin | GenBank accession No. | UNITE accession No. |
|------------------------|------------|--------|------------------------|---------------------|
| Epulorhiza amonilioides| 35         | Brazil | JF907600               |                     |
| Epulorhiza amonilioides| aer08      | Brazil | KC928335               |                     |
| Epulorhiza anaticula   | UAMH 5428  | Canada | EU218891               |                     |
| Epulorhiza anaticula   | 130004     | South Korea | KT164598 | SH1174351.08FU |
| Tulasnella albida      | KC110      | Unknown | AY373294               |                     |
| Tulasnella asymmetrica | MAFF 305808 clone C001 | Australia | KC152356               |                     |
| Tulasnella asymmetrica | ALLM4.4.1  | Australia | MH134544 | SH1541682.08FU |
| Tulasnella bifrons     | BPI 724849 | Canada | AY373290               |                     |
| Tulasnella calopora    | MAFF P305801 | Ecuador | DQ388041               |                     |
| Tulasnella calopora    | MAFF P305802 | Ecuador | DQ388042               |                     |
| Tulasnella calopora    | MAFF P305803 | Ecuador | DQ388045               |                     |
| Tulasnella calopora    | MAFF P305804 | Ecuador | DQ388044               |                     |
| Tulasnella calopora    | MAFF P305805 | Ecuador | DQ388045               |                     |
| Tulasnella calopora    | Fcb4       | China | KC796458 | SH1554832.08FU |
| Tulasnella danica      | KC388      | USA    | AY373297               |                     |
| Tulasnella eichleriana | KC852      | Unknown | AY373292               |                     |
| Tulasnella eichleriiana| K(M)143600 | United Kingdom | KC152381     |                     |
| Tulasnella irregularis | JHW 0632  | Australia | EU21889 |                     |
| Tulasnella irregularis | D1-K7-T/-C-1 | Thailand | GU166413 | SH1561236.08FU |
| Tulasnella irregularis | C3-DY-T/-C-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 | KY995117 |                     |
| Tulasnella sphagneti   | CLM583     | Australia | KY445922 |                     |
| Tulasnella tomaculam   | KC429      | Unknown | AY373296               |                     |
| Tulasnella tubericola  | EP-15      | Spain   | KX929166               |                     |
| Tulasnella tubericola  | EP-1       | Spain   | KX774345               |                     |
| Tulasnella violea      | FO24380a   | Germany | KCI52439 | SH1555437.08FU |
| Tulasnella violea      | DC292      | Germany | KCI52432 |                     |
| Tulasnella warcupii    | CLM027     | Australia |KF476596  |                     |
| Tulasnella warcupii    | CLM007     | Australia |KF476600  |                     |
| Uncultured Tulasnella  | Clone 33tu-12 | China | HM230652 |                     |
| Botryobasidium botryosum | AFTOL ID604 | Germany | DQ267124 |                     |

Table 2. GenBank and UNITE accession numbers of additional Tulasnella isolates included in the phylogenetic analysis. Ex-type strains are indicated in bold face.
colony white to cream. Hyphae are regularly septate with branching at right angles, 2–3.5 μm diam (± SD = 2.5 ± 0.3 μm), hyaline, with binucleate cells and thin-walled. Molinoid 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.

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

*Mycobank*: MB832787

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**Figure 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 is indicated in bold face. *Botryobasidium botryosum* (AFTOL604) was used as the outgroup.
| 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          |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 6          | 0.4  | 32.9 | 32.7 | 33.7 | 33.6 | 33.5 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 7          | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 8          |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 9          | 0.2  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 10         |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 11         | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 12         | 0.5  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 13         | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 14         | 1.7  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 15         | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 16         | 0.7  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 17         | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 18         | 0.8  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 19         | 0.4  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 20         | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 21         | 2.2  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

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. 1 = *Tulasnella anaticula*, 2 = *T. danica*, 3 = *T. calospora*, 4 = *T. tubercula*, 5 = *T. bifrons*, 6 = *T. asymmertica*, 7 = *T. pruinosa*, 8 = *T. albida*, 9 = *T. brigadeiroensis*, 10 = *Tulasnella* sp. COAD 2885, 11 = *T. hadrolelaiae*, 12 = *T. orchidis*, 13 = *T. irregularues*, 14 = *T. amonilioides*, 15 = *T. zygopectali*, 16 = *T. eichleriana*, 17 = *T. secunda*, 18 = *T. tomaculum*, 19 = *T. wacupii*, 20 = *T. prima*, 21 = *T. sphagneti*, 22 = *T. violae.*

Etyymology:—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%).

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

MycoBank: MB832789

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

Diagnosis: *Tulasnella zygopectali* is phylogenetically different from other *Tulasnella* species. Morphologically, *T. zygopectali* differs from other *Tulasnella* species described here as it has wider hyphae (3–6 μm) and monilioid cells (6.5–12.5 μm diam). In a comparison of the 583 ITS nucleotides, *T. zygopectali* differed from *T. brigadeiroensis* by 134 bp (23%), from *T. amonilioides* by 134 bp (23%) and from *T. orchidis* by 134 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 (**X** ± **SD** = 4 ± 0.9 μm), hyaline, with binucleate cells and thin-walled. Molinioid cells hyaline, elongated barrel-shaped, 6.5–12.5 μm diam (**X** ± **SD** = 10 ± 1.5 μm), in branched chains with more than five cells. 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 (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. 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.

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. Due to the lack of molecular data from the type specimen, many *Tulasnella* species are described only by morphological-based approaches. Morphological characters, such as size and shape of hyphae, basidia, sterigmata and basidiospore, when used alone, may lead to incorrect species identification, 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., using ITS as suggested by Linde et al. 

Among the species of the genus *Tulasnella*, *T. calospora* is considered as a nearly universal orchid symbiont. It has been isolated from orchids in Asia, Australia, Europe and South America. However, the definition of *T. calospora* species is still unclear, since phylogenies have shown taxonomic problems concerning this species. In Brazil, *T. calospora* was obtained from the roots of the orchids *Oeceoclades maculata*, *Epipendrum secundum*, *Acianthera limacae* and *Polystachya concreta* 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 *Zygopetalum maxillare*. *Zygopetalum maxillare* is an
Figure 3. *Tulasnella calospora* (COAD2869). (a) Eight-day-old PDA culture. (b) Hyphae stained with SYBR Green I showing binucleate cells (M = monilioid cell; N = nuclei; S = septa). (c) Hyphae with branching at right angles. (d) Monilioid cell chains in CMA. Bars = 50 μm.

Figure 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.
**Figure 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.

**Figure 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.
epiphytic orchid with high specificity in a host tree relationship. 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. Additionally, morphological characters cannot distinguish T. albida and T. pruinosa. 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.

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 or 3–5% divergence used for Tulasnella species. 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.

Knowledge of the diversity of orchid mycorrhizal fungi is important for successful conservation strategies, 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
Representative specimens were deposited at the Fungarium of the Universidade Federal de Viçosa (VIC). Stems were deposited in the Coleção Oswaldo Almeida Drummond collection (COAD) at the Universidade Federal de Viçosa. Axenic cultures were preserved on rice grains in an ultrafreezer at −20°C or silica gel and were deposited in the Coleção Oswaldo Almeida Drummond collection (COAD) at the Universidade Federal de Viçosa (VIC).

Morphology. The fungus and colony characteristics were described from cultures grown on PDA at 25 °C 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. The nuclear condition was observed from young hyphae after staining with SYBR Green I according to Meinhardt et al.24. The isolates were transferred to Corn Meal Agar (CMA) medium and incubated at 25 °C in the dark, for 4–6 weeks, to induce monilioid cell formation49. Observations, measurements and photographic images of microscopic fungal structures were recorded using an Olympus BX53 light microscope, with an Olympus Q-Cam TM 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 °C 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. Each polymerase chain reaction (PCR) was performed in 50 μL containing 10–20 ng of DNA template, 1X Taq buffer, 2 mM MgCl2, 0.2 μM of each primer, 0.4 mM of each dNTP, and 1.0 U Taq DNA polymerase (Cellico Biotech do Brasil Ltda., São Paulo, Brazil). PCR was carried out using a MyCycler™ 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. Consensus sequences were generated using the MEGA v.7.0.26 software tool. 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. The resulting sequence alignments were manually checked and adjusted in MEGA v.7.0.26 software tool.48

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 program50 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 portal51 and the MrBayes program v.3.1.152. 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 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 V1.4.450 and the layout of the tree for publication was done using Adobe Illustrator v. CC.

Divergence between clades and haplotype network. 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.7.0.2641. 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 Oswaldo 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.

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Author 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.

Competing interests
The authors declare no competing interests.

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