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EPULORHIZA AMONILIOIDES SP. NOV.: A NEW ANAMORPHIC SPECIES OF ORCHID MYCORRHIZA FROM BRAZIL

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ABSTRACT

In this study, a new anamorphic species of orchid mycorrhizal fungus is described and illustrated based on both morphological and molecular characteristics. Mycorrhizal fungal strains were isolated and selected from root samples of three species of rupicolous, epiphytic and terrestrial tropical orchids that are native to Brazil: *Encyclia dichroma*, *E. ghillanyi* and *Brassavola tuberculata*. *Epulorhiza amonilioides* sp. nov. is distinguished from the other species of *Epulorhiza* by the absence of monilioid cells in pure culture. Phylogenetic analyses based on 5.8S ITS rDNA and mtLSU indicated that *E. amonilioides* is phylogenetically different from other fungal lineages of *Tulasnella* previously identified on tropical and temperate orchid and Sebacinaceae species. *E. amonilioides* forms a different clade that groups with some anamorphic mycorrhizal fungi corresponding to *Tulasnella irregularis*. *E. amonilioides* is strictly related to *T. irregularis*, its putative teleomorph. *E. amonilioides* is the seventh species described for the genus and is the second from Brazil.

The genera *Epulorhiza*, *Ceratorhiza* and *Moniliopsis* (Basidiomycota) were proposed by Moore as anamorphic fungi that form a mycorrhizal association with orchids (Moore 1987). Their segregation from *Rhizoctonia* was based on the morphology of the dolipore septum and on anamorph-teleomorph connections. *Epulorhiza* was defined as bearing a dolipore septum with a non-perforated parenthosome, with *Tulasnella* J.Schröt. as its teleomorph (Moore 1987, 1996).

The circumscription of *Epulorhiza* was also marked by morphological features of isolates in pure culture, which distinguish it from the other genera of rhizoctonioid fungi (Currah and Zelmer 1992). These features are (i) slow growth, (ii) the margin or practically all the colony being submerged, (iii) monilioid cells being oval or completely spherical, and (iv) the absence of polyphenol oxidase production (Currah and Zelmer 1992; Currah et al. 1987; Zelmer and Currah 1995; Currah et al. 1997; Pereira et al. 2003). Although there are features that can be easily detected for the identification of the genus, infraspecific delimitation remains difficult (Rasmussen 1995, 2002). This is due mainly to the difficulty of defining diagnostic features and the absence of stable morphological features in pure cultures (Andersen 1990).

To combine speed and efficiency in the identification of mycorrhizal fungi at genus level, most current studies have focused on the direct identification, by PCR and sequencing, of the ITS (Internal Transcribed Spacer) region of the rDNA in root samples (Shefferson et al. 2005, 2007; Bougoure et al. 2005; Kristiansen et al. 2004; Mc Cormick et al. 2004; Suárez et al. 2006; Shimura et al. 2009; Nontachaiyapoom et

al. 2010; Roche et al. 2010). This method has enabled great progress in the study of natural populations, though it does not allow the examination of isolate morphology for a comparative analysis of morphological and molecular data (Almeida et al. 2007). This may be reflected by the current situation of *Epulorhiza*, which has only six described species (Currah and Zelmer 1992; Currah et al. 1987; Zelmer and Currah 1995; Currah et al. 1997; Pereira et al. 2003); one of them, *E. epiphytica*, was described from Brazil by Pereira, Rollemberg & Kasuya (Pereira et al. 2003).

While analyzing isolates of orchid species from the northeastern region of Brazil, especially from the Bahia state, we detected some anamorphs that not only had different morphological features in pure culture but also showed ITS rDNA and mtLSU sequences that were distinct from established sequences in GenBank. These isolates were obtained from three species of orchids: (i) *Brassavola tuberculata* Hook., epiphytic or rupicoulous plants in semi-arid areas in the interior of Bahia; (ii) *Encyclia ghillanyi* Pabst, rupicolous plants that grow on rock surfaces in semi-arid areas in the interior of Bahia; and (iii) *Encyclia dichroma* (Lindl.) Schltr., which grows in humid sandy areas along the Bahia coast and are always in patches of vegetation, together with species of Myrtaceae, Euphorbiaceae and Cactaceae, in Restinga (strand vegetation amidst Atlantic Forest). Here, we describe and illustrate these isolates as a new species of *Epulorhiza*, based on both morphological and molecular features.

MATERIALS AND METHODS

Study area, isolation and morphological characterization of the fungi — Mycorrhizal fungi were isolated from plants of nine populations (four populations of *Encyclia dichroma*, four of *E. ghillanyi* and one of *Brassavola tuberculata*) distributed from south to northeast Bahia (Table 1).

Mycorrhizal fungi were directly isolated

Table 1. Locations of the 10 plant populations and the number of *E. amonilioides* isolates analyzed.

Species	Code	Number of plants sampled	Number of *E. amonilioides* strains analyzed	Location
Encyclia dichroma	CAM	22	6	Between the municipality of Camaçarí and Salvador/ Restinga - Northeast of Bahia 12°43'S, 38°9'W
	AS	13	8	Municipality of Salvador/ sandy dunes next to the Airport/Restinga - Northeast of Bahia 12°55'17,53"S, 38°19'27,47"W
	STA	14	5	Municipality of Santa Luzia, Conjunto Piatam Mirim, Betânia/Restinga - South of Bahia 15°25'44"S, 39°20'2,02"W
	BEL	16	7	Municipality of Belmonte in the direction to Barrolândia/Restinga - South of Bahia 15°52'22"S, 38°52'22"W
Encyclia ghillanyi	MA	17	7	Municipality of Maracás/Caatinga, on rocky surfaces - South of Bahia 13°26'39,27"S, 40°26'6,78"W
	MTO	10	4	Municipality of Santa Terezinha, Morro do Cruzeiro/ Caatinga, onrocky surfaces – Center-West of Bahia 12°46'30,35"S, 39°31'19,90"W
	ITIR	17	4	Municipality of Itiruçú/ Caatinga, on rocky surfaces - South of Bahia 13°32'1,78"S, 40°9'4,22"W
	SER	16	4	Municipality of Serrinha/ Caatinga, on rocky surfaces - Northeast of Bahia 11°37'29"S, 38°58'25"W
	ORO	10	3	Serra do Orobó/ Seasonal Forest, on rocky surfaces – Center-West of Bahia 12°29'48,98"S, 40°32'59,03"W
Brassavola tuberculata	BRA	1	1	Districto of Ibuaçú / Governador João Durval - Feira de Santana – Caatinga Northeast of Bahia 12°16'17,49"S, 38°57'18,47"W

from pelotons using a modified version of the protocol of Warcup and Talbot (1967). The isolates were cultured in six different culture media and incubated for 60 days at 28°C in darkness to evaluate morphology in pure culture and to allow the formation of monilioid cells and sclerotia. The media were potato dextrose agar (PDA; Difco), cornmeal agar (CMA; Difco), malt extract agar, tap water agar, coconut milk agar (Zelmer and Currah 1995), and oat meal agar (30 g oat meal, 15 g agar, 1000 ml water).

The growth rate was determined by averaging three independent radial measurements obtained every 48 hours, in PDA at 28°C in darkness, over two weeks. The number of nuclei per cell was observed in young hyphae stained with HCl-Giemsa (Sakena 1961). Observations of micromorphological features were carried out with both light microscopy (Zeiss) and scanning electron microscopy (SEM; LEO 1430VP). Enzymatic assays to evaluate cellulase and polyphenol oxidase were performed according to Smith (1977) and Davidson et al. (1938), respectively. The pure cultures were deposited in the CCMB (Culture Collection of Microorganisms of Bahia): CCMB 513-519.

Genomic DNA extraction, ITS rDNA amplification and amplicon sequencing — Isolates were cultivated in broth malt extract for 15 days at 28°C in darkness, and DNA was extracted according to the CTAB protocol of Doyle and Doyle (1987) with some modifications. ITS and mtLSU regions of the isolates were amplified using primers ITS5-ITS4 (White et al. 1990) and ML5-ML6 (Bruns et al. 1998). PCR was performed in a final volume of 25 µL and cycled as follows. For the ITS rDNA region, the thermal profile was 94°C for 4 min, 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min, and a final extension at 72°C for 7 min. For the mtLSU region, the thermal profile was 95°C for 3 min, 35 cycles of 95°C for 30 sec, 53°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplicons were purified by digestion with exonuclease and alkaline phosphatase (ExoSAP-IT, GE Healthcare), and sequenced in an ABI 3130XL automated sequencer (Applied Biosystems). The new sequences were deposited in GenBank under the numbers JF907598 - JF907605 and treebase ID 11445.

Phylogenetic analysis — Chromatograms were edited with PREGAP4 and GAP4 (Staden et al. 1998). Sequence similarity was verified using BLASTn (Zhang et al. 2000) with available sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and was subsequently aligned with ClustalX (Thompson et al. 1997). Ambiguous aligned regions with high proportion of gaps were excluded.

To enable wider phylogenetic analysis, only the 5.8S nuclear rDNA region was used, which allowed the inclusion of most *Epulorhiza/Tulasnella* and *Sebacina* sequences in GenBank. This approach allowed us to evaluate the degree of divergence between our sequences and all other sequences obtained from mycorrhizal fungi of both tropical and temperate orchids. These other sequences were also included in the sequence analysis of both genes of *E. epiphytica*, to evaluate their positioning relative to GenBank sequences of different lineages.

Maximum Likelihood (ML) phylogenetic analysis was carried out online using RAxML version 7.0.3 (Stamatakis et al. 2006), in the CIPRES web interfaceportal (http://www.phylo.org/sub_sections/portal) using the GTRMIX DNA substitution model with 1000 bootstrap iterations (Felsenstein 1985). We selected *Thanatephorus cucumeris* AF354097 and *Waitea circinata* AD001658 as external sequences for the analyses of 5.8S ITS rDNA and mtLSU, respectively, because they are taxa of Ceratobasidiaceae, which is phylogenetically

closer to Tulasnellaceae than other Basidiomycota (Moncalvo et al. 2006).

RESULTS

Epulorhiza amonilioides P.R.M.Almeida, Van den Berg & A.Góes-Neto, **sp. nov.** (Figure 1). Mycobank: MB 561179

Coloniae in agaro PDA et agaro CMA lentissime crescentes, eburneae vel pallido-eburneae, grumosae vel laeves cum mycelio aerio parvo margine submersa integra vel diffusa. In agaro PDA odor dulcis. Hyphae hyalinae ordinatim septatae, 2.5 µm diametro, cellulis hyphalibus binucleatis. Cellulae monilioides absentes. Sclerotium 0.5-1 mm diametro, sine cellulis monilioidibus, indifferentiatum ubi submersum et cum cortice pallido et medula obscura super agaro. Auctus 0.004-0.1mm in horas prope 28C. Oxidasis polyphenolis negativa, tenuiter cellulolytica.

In PDA and CMA, colonies with slow growth in culture, cream to pale cream in color, with clotted to flat aspect and scarce aerial mycelia with submerged margin entire or diffuse. Hyaline hyphae regularly septate, 2.5 µm in diameter and possessing binucleate cells. Absence of monilioid cells. In PDA, sweet scent. Sclerotia 0.5-1 mm in diameter, without formation of monilioid cells, undifferentiated when submerged and with cortex and dark medulla on agar.

ETYMOLOGY: *amonilioides* (Latin) refers to the abscence of monilioid cells.

HOLOTYPE: Colonies in PDA, isolated from *Encyclia dichroma* (Lindl.) Schltr., collected in sandy dunes (Restinga, Coastal Atlantic Forest) next to the airport in the municipality of Salvador, State of Bahia, Brazil, January, 20th 2007, 12° 55' 17.53" S, 38° 19' 27.47" W; deposited as CCMB 513-514 .

GROWTH RATE: In PDA at 28°C, 0.004-0.1 mm/h.

ENZYMATIC ASSAY: Absence of activity for polyphenol oxidase and weak cellulolytic activity.

ADDITIONAL ISOLATES: Colonies in PDA, isolated from *Encyclia ghillanyi* Pabst, collected on rocky surfaces in the municipalities of Itiruçu, Santa Terezinha and Ruy Barbosa, State of Bahia, Brazil, July, 17th 2007; deposited as CCMB 515-519.

DISCUSSION

Taxonomic conclusions based on molecular data — *Epulorhiza amonilioides* differs from all other *Epulorhiza* species in that it does not produce monilioid cells in culture. This is particularly rare in *Epulorhiza*, because all species and even most strains produce monilioid cells, an important feature that is common among *Rhizoctonia* s. l. (Saksena and Vaartaja 1960, 1961; Currah and Zelmer 1992; Currah et al. 1997; Roberts 1999; Rasmussen 1995, 2002). Although *E. amonilioides* does not produce monilioid cells, all other morphological features in culture are shared with the species and strains of this genus. These features include very slow growth rate, production of sclerotia and regularly septated hyphae with right-angle branches (Currah and Zelmer 1992; Currah et al. 1987; Zelmer and Currah 1995; Currah et al. 1997; Pereira et al. 2003), which are considered to be of low taxonomic value for the group (Andersen 1990). However, the absence of monilioid cells has persisted during four years of observation in same conditions of pure culture (PDA), and can be considered a diagnostic feature for this new species. Furthermore, in vitro symbiotic germination experiments were performed with *E. amonilioides*, and we verified its ability to germinate seeds of *Cattleya sincorana* (Schltr.) Van den Berg (data not shown). This species of epiphytic orchid grows on rocky surfaces and as an epiphyte on *Vellozia* (Veloziaceae) in Bahia, Brazil.

We observed great variation in the

macromorphological features of cultured strains of *E. amonilioides* isolated from populations of *Encyclia dichroma* and *E. ghillanyi* in different geographic areas of Bahia (data not shown). *E. amonilioides* strains with contrasting morphological features were found in the plants from the municipalities of Salvador and Maracás, which are close to the extreme limits of the geographical distribution of these plants in Bahia. The morphology of these strains on PDA ranged from pale cream-colored colonies of flat aspect that did not produce sclerotia to cream-colored colonies with

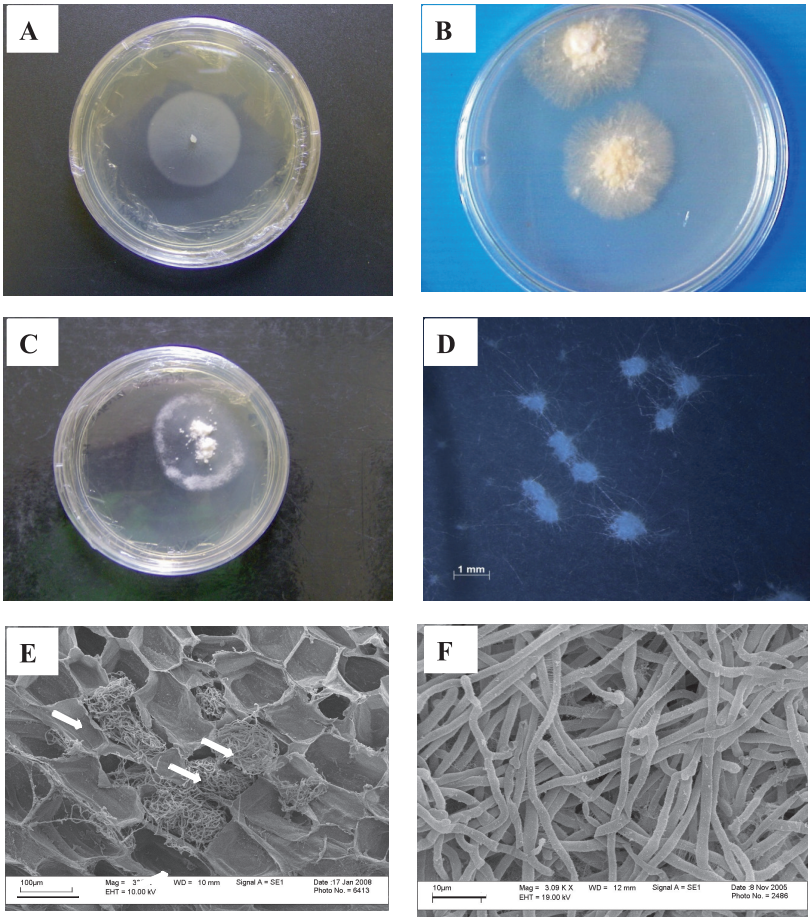

Figure 1. *Epulorhiza amonilioides*. A – C show colonies with distinct macromorphological features. A, a strain isolated from *E. dichroma* in the population of SA (on sandy dunes in the municipality of Salvador). B, a strain isolated from *B. tuberculata*. C, a strain isolated from *E. ghillanyi* in the population of MA (on inselbergs in the municipality of Maracás). D, sclerotia formed on agar, observed in colonies isolated from *E. ghillanyi* in the population of MA. E, SEM image of root cortex tissue of *E. dichroma*, showing colonization through the formation of pelotons (indicated by arrows). F, SEM image of *E. amonilioides* mycelia, without the formation of monilioid cells.

clotted aspect that produced sclerotia (Figure 1). Therefore, as previously pointed out by Andersen (1990), there are considerable morphological variations within a single species of *Epulorhiza*, even when the analyses are performed on a geographical scale (as in our study).

In the topologies of Figures 2 and 3, *E. amonilioides* forms a monophyletic group distinct from all previously identified *Tulasnella* strains isolated from tropical and temperate orchids. Moreover, none of the *Epulorhiza/Tulasnella* taxa groups with any Sebacinaceae (Selosse et al. 2002; Ma et al. 2003; Shefferson et al. 2005, 2007; Bougoure et al. 2005; Kristiansen et al. 2004; McCormick et al. 2004; Porras-Alfaro & Bayman 2007; Suárez et al. 2006; Shimura et al. 2009; Illyes et al. 2009; Nontachaiyapoom et al. 2010; Roche et al. 2010). The 5.8S rDNA and mtLSU data demonstrate the phylogenetic relationships among the groups of *Tulasnella*, and place the different isolates into distinct groups. From the 5.8S rDNA data, four groups were retrieved (Figure 2). The first, clade A, with significant statistical support from bootstrapping, includes *E. amonilioides*, orchid mycorrhizal fungi from Thailand (Nontachaiyapoom et al. 2010), and other mycorrhizal fungi isolated from orchids in temperate areas (Illyes et al. 2009). These mycorrhizal fungi are more strictly related to *Tulasnella irregularis* Warcup & P.H.B. Talbot (Warcup and Talbot 1980). Second, clade B groups a vast number of mycorrhizal fungi identified in studies with orchids from both temperate and tropical areas (Ma et al. 2003; McCormick et al. 2004; Porras-Alfaro and Bayman 2007). In our study, this clade belongs to the group of *E. epiphytica/Tulasnella danica* Hauerslev. Clade C, the third group, also includes mycorrhizal fungi isolated and identified in temperate and tropical orchids; however, all the mycorrhizal fungi that represent *T. calospora* (Boud.) Juel strains are part of this group (Warcup and Talbot, 1967; McCormick et al. 2004; Suárez et al. 2006). Finally, clade D is formed by *T. violea* (Quél.) Bourdot & Galzin and *T. pruinosa* Bourdot & Galzin, which have already been established in previous phylogenetic studies as distinct species from all other *Tulasnella* strains (McCormick et al. 2004; Suárez et al. 2006; Roche et al. 2010).

The mtLSU data also agreed with the 5.8S rDNA data, as *E. amonilioides* forms a monophyletic group with *T. irregularis* (Figure 3). Furthermore, by analyzing mtLSU data separately, some species of *Tulasnella*, specifically *T. violea* and *T. calospora*, seem to be polyphyletic. These findings have already been pointed out by Roche et al. (2010), who suggested the possibility of sequence misidentification in GenBank.

Based on all the morphological and molecular data in the present study, *T. irregularis* is probably the teleomorph of *E. amonilioides*. Sequence analysis shows high identity (99-100%) between *E. amonilioides* and *T. irregularis* for both the ITS rDNA and mtLSU sequences (Figures 2 and 3). Furthermore, the sequences obtained from *E. amonilioides* were identical between isolates from the same plant and even between isolates from the three different studied plant hosts.

Although Nontachaiyapoom et al. (2010) found some orchid mycorrhizal fungi in Thailand that are phylogenetically related to *E. amonilioides/T. irregularis*, these fungi have not yet been described as new anamorphs. Moreover, *E. amonilioides* has features that contrast with Thai isolates [according to the descriptions of Nontachaiyapoom et al. (2010)]. *E. amonilioides* is more strictly related to *T. irregularis* (Figures 2 and 3) than the anamorphs described in Nontachaiyapoom et al. (2010). Thus, *E. amonilioides* is the seventh species described for the genus (Currah and Zelmer 1992; Currah et al.

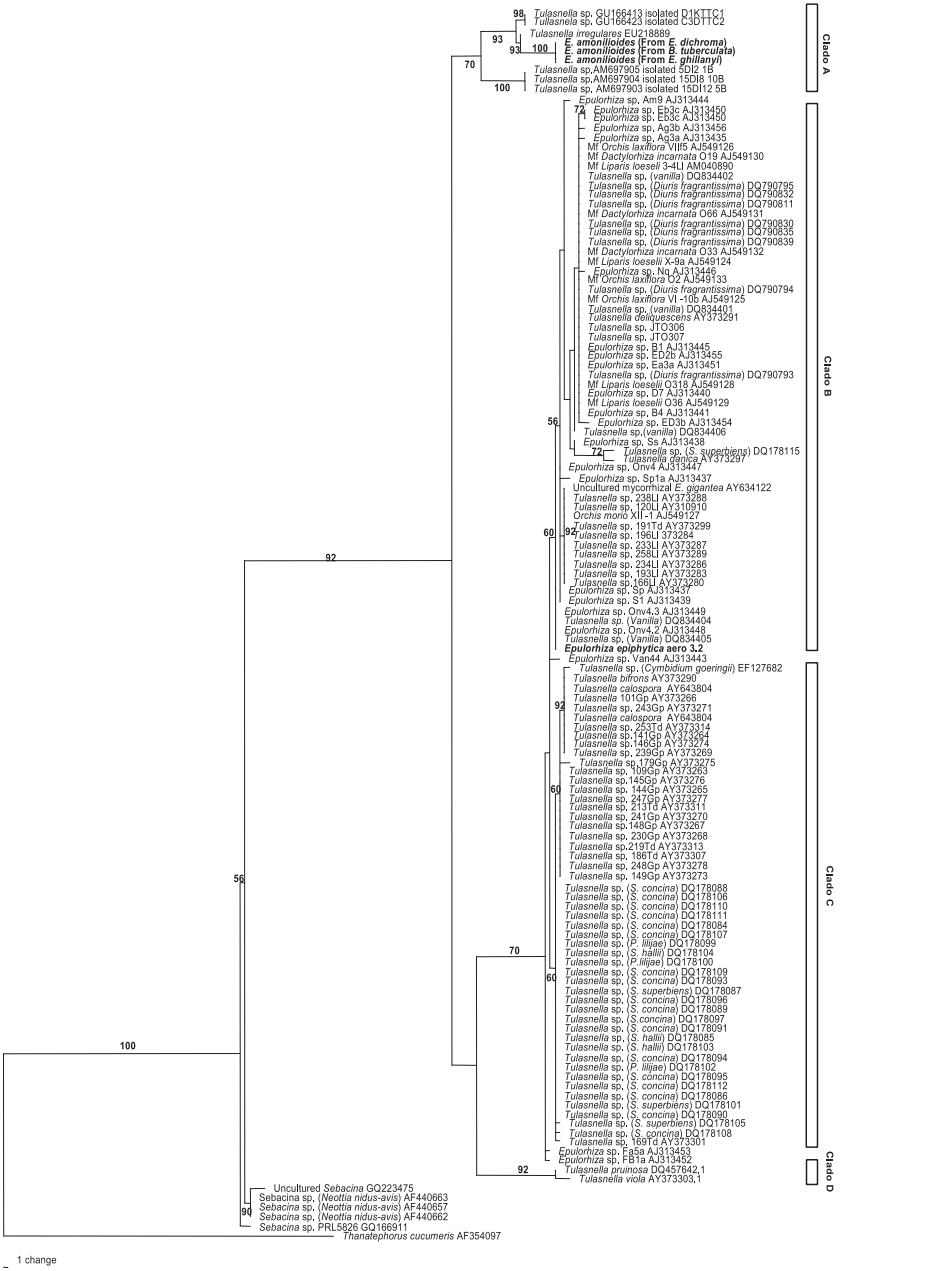

Figure 2. Maximum likelihood tree of *Tulasnella* strains using the 5.8S ITS nuclear rDNA region as the data set. Numbers next to the branches represent bootstrap values. Only bootstrap values greater than or equal to 50% are shown.

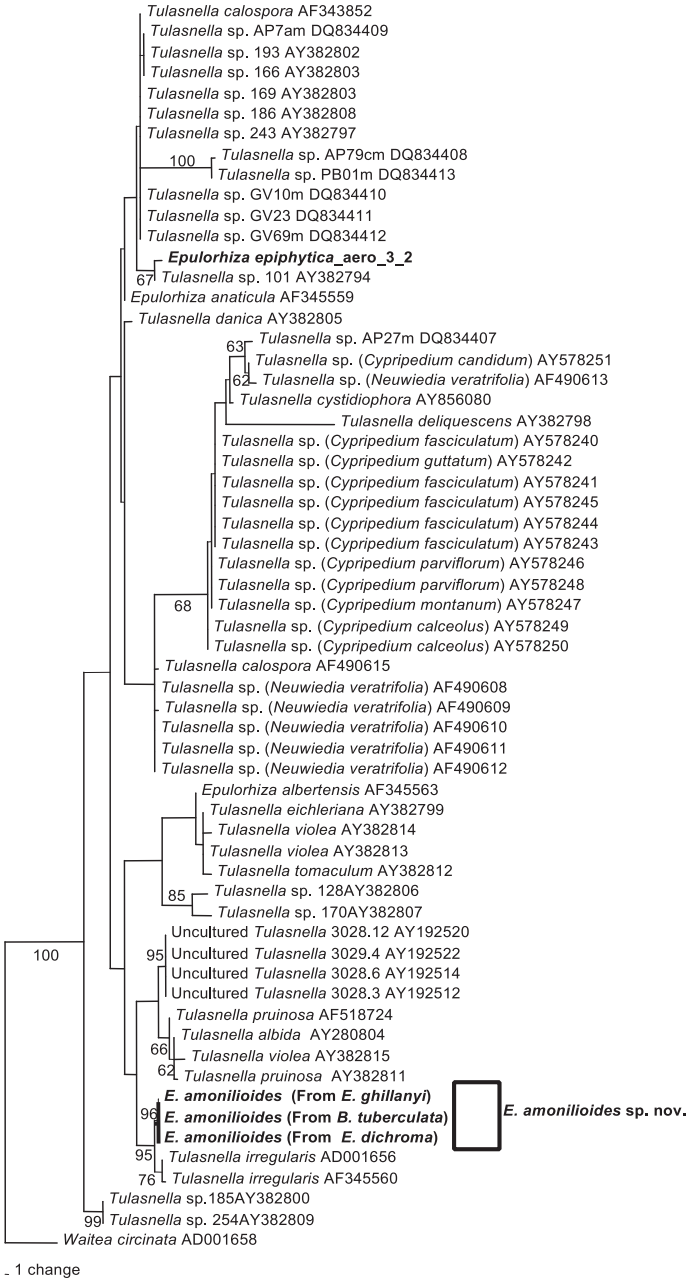

Figure 3. Maximum likelihood tree of *Tulasnella* strains using the mtLSU region as the data set. Numbers next to the branches represent bootstrap values. Only bootstrap values greater than or equal to 50% are shown.

1987; Zelmer and Currah 1995; Currah et al. 1997; Pereira et al. 2003), and the second from Brazil.

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