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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF SPECIES OF *TULASNELLA* (HOMOBASIDIOMYCETES) ASSOCIATED WITH NEOTROPICAL PLANTS OF LAELIINAE (ORCHIDACEAE) OCCURING IN BRAZIL

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Introduction

Tullasnella spp. have been found forming mycorhizal associations with plants of all Orchidaceae subfamilies, and they are one of the main symbionts in partially micoheterotrophic plants (Taylor *et al.* 2002). Little is known about mycorhizal fungi of Neotropical Orchidaceae, especially in Laeliinae that occur in distinct environments such as "Restingas", Seasonal Forests and "Campos Rupestres" (Cruz *et al.* 2003, Britto *et al.* 1993, França *et al.* 1997, Withner 2000).

Some few studies in completely mycoheterotrophic Epidendroideae have been shown that these plants form mycorrhizal associations mainly with fungi of the genera *Russula*, *Thelephora*, *Sebacina*, as well as other ectomycorrhizal Basidiomycetes in trees (Taylor and Bruns, 1999, 1997, Taylor *et al.* 2003, Selosse *et al.* 2002, Girlanda *et al.* 2006). There are other studies indicating a preferential association between basidiomycetous fungi and Orchidaceae plants as in Oncidiinae with *Ceratobasidium* and *Cypripedium* with *Tulasnella* (Otero *et al.* 2002, 2004, Shefferson *et al.* 2005). These works suggest a putative specificity and recruiting of these plants in the environment where they occur.

Laeliinae plants have been intensively and indiscriminately collected in Brazil, leading to a significant reduction in their natural populations. In order to establish conservation strategies to these threatened plants as there is an indication in literature showing a preferential association between some specific fungi and Orchidaceae, the identity of symbiont fungi forming mycorrhizal associations in Brazilian Laeliineae was studied, aiming to an efficient *in situ* and *ex situ* conservation.

Methodology

COLLECTION SITES AND ISOLATION OF FUNGI

Orchidaceae plants were collected from natural populations that occur in two distinct Brazilian States. A total of 20 natural populations, including plants of Laeliinae and Pleurothallidinae were sampled. From each population, one or two individual plants were collected and their roots were sampled in a period of one to two weeks since collection date. The individuals were selected from distinct environments (Tropical Rain Forest, "Restinga", and "Campo Rupestre") and the isolation of associated fungi was carried out according to Warcup and Talbot (1967).

MORPHOLOGICAL CHARACTERIZATION OF FUNGAL COLONIES

Fungal colonies were incubated for 30 days in PDA (potato-dextrose agar) and OA (3% oat meal agar) to induce the formation of monilioid cells, and they

were further analysed to determine the form, number and array of the cells. Macroscopic and microscopic somatic features of the colonies were also described. In order to analyse the nuclear condition, hyphal nuclei were stained according to Sneh *et al.* (1991).

MOLECULAR CHARACTERIZATION OF FUNGAL ISOLATES

All the isolates were first cultivated in BDA for 15 days at 28 °C , including an *Epulorhiza epiphytica* Pereira, Rollemberg et Kasuya isolate, gently sent by Mycorrhizal Association Lab of the Federal University of Viçosa, Brazil. DNA extraction was carried out according to CTAB protocol (Doyle & Doyle, 1987). Double-stranded symmetric PCR reactions were carried out in 0.2-mL tubes in 50 µL reaction volume, using the primers ITS5 and ITS4 that amplify the Internal Transcribed Spacer (ITS region) of nuclear ribosomal DNA (White *et al*., 1990). PCR products were purified using EXOSAP and were sequenced in an automatic DNA sequencer (SCE 2410, Spectrumedix LLC). Chromatograms were edited using GAP4 software in Staden (Staden, 1996). Resulting sequences were submitted to a similarity search using BLASTn software of NCBI and aligned with Clustal X (Thompson *et al.* 1997). Phylogenetic parsimony analyses (heuristic search, TBR algorithm) were conducted in PAUP 4.0 (Swofford, 1998). Clade robustness was assessed using bootstrap proportions (1000 replications) (Felsenstein, 1985).

Results and discussion

IDENTIFICATION OF ISOLATES FROM LAELIINAE

According to morphological characterization, the isolates belong to the genus *Tulasnella* (Basidiomycetes) (Rasmussen 1995, Currah and Zelmer 1992, Currah *et al.* 1997b), but the somatic characters were not stable enough to differentiate the groups. All the colonies presented an entire submersed margin and binucleate hyphae (Fig.1). In all the isolates monilioid cells showed a very high morphological plasticity with cell chains ranging from 3 to 15 cells with or without ramification. Andersen (1990) pointed out that somatic features were not reliable, since there is not even one character that could be taken as a parameter in intraspecific level. The three isolates showed a growing pattern typical of rhizoctonoid fungi, but they did not produce monilioid cells even when they were submitted to distinct culture media.

All the sequences were compared to NCBI database, revealing that the isolates belonged to different lineages of *Tulasnella* including *T. violea* and *T. calospora*. Some sequences were considerably difficult to align and they were initially excluded from the phylogeny. In the phylogenetic tree (Fig. 2) some of the isolates represented lineages of *Tulasnella calospora* and others were lineages of *Epulorhiza epiphytica*, both of them significantly supported by bootstrap analysis. *E. epiphytica* is the only species described for Brazil and it was isolated from host plants that naturally occur in the State of Minas Gerais (Pereira *et al.* 2003). These results suggest that all the isolates are distinct lineages of *Tulasnella*, and that this possibly reflects the different environments where host plants occur.

RELATIONSHIPS BETWEEN LAELIINAE AND TULASNELACEAE

In accordance to the results, although host plants live in completely different environments where the research availability is distinct, one can observe the strong trend of studied plants to form mycorrhizal associations with fungi of the genus *Tulasnella* (Almeida 2006). Studies on Australian orchids revealed that Diurideae plants has a strict specificity relationship with the fungi *Sebacina vermifera* and some lineages of *Tulasnella*, including *Tulasnella calospora*, which has been considered as a universal species (Rasmussen, 1995, Warcup, 1981, 1988, 1971). Inside Diurideae, all the studied species that belong to Drakaeinae and Diuridinae associate to *Tulasnella*, and all the studied species (except for those from genera *Lyperanthus* and *Bumettia*) that belong to Caladeniinae present a strict relationship with *Sebacina* (Warcup, 1981, Dressler, 1993). As all the isolates were obtained from *pelotons*, they are mycorrizal fungi.

Despite of the great advances obtained with the direct identification of fungi by molecular techniques such as PCR and sequencing, the morphological study of the isolates is still very important, mainly for the establishing of true biological entities or species.

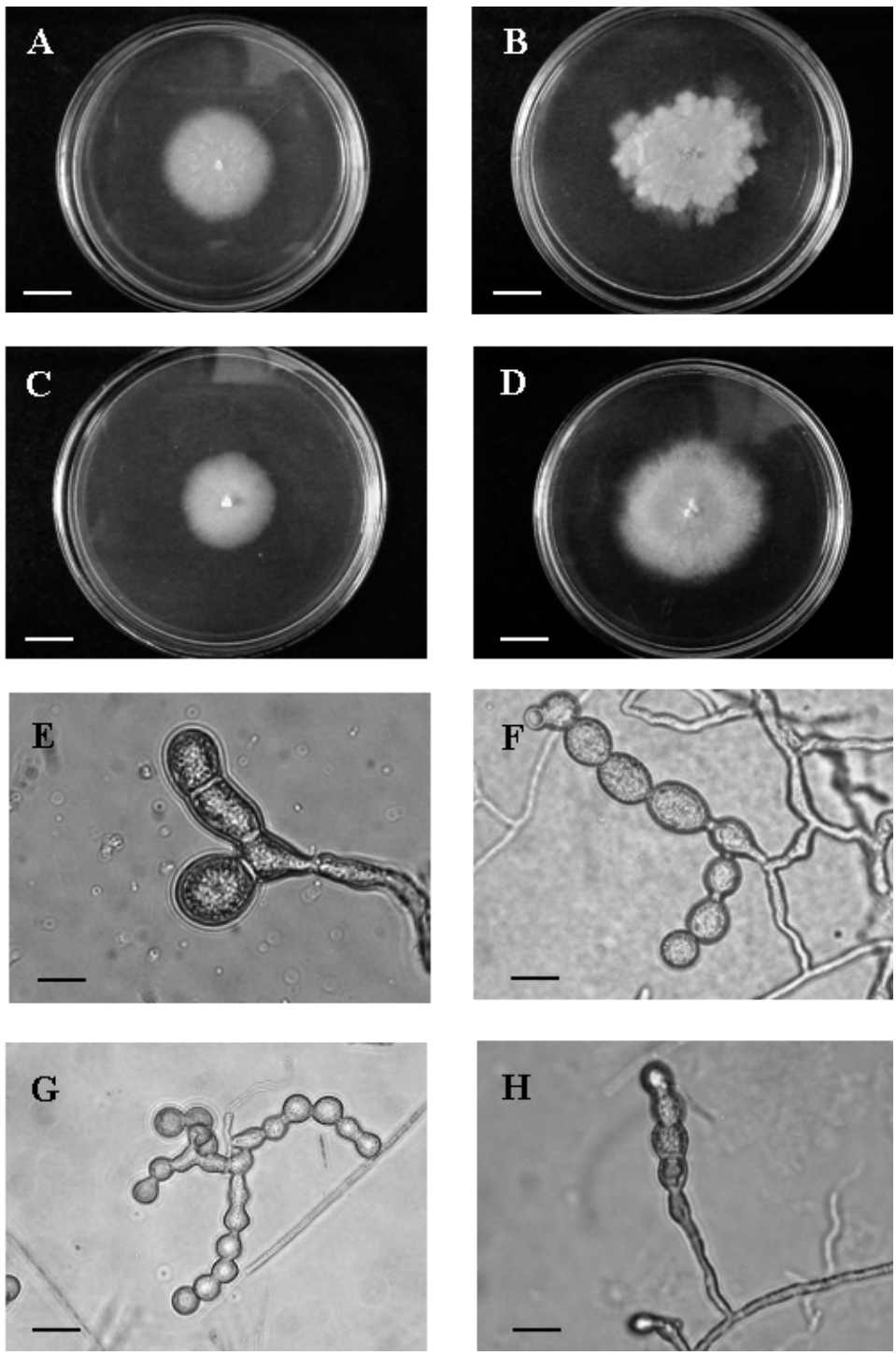

FIGURE 1. Any isolates of plants of Laeliinae. **A.** Isolate of *Acianthera hamosa*. **B.** *Cattleya elongata*. **C.** *Brassavola tuberculata*. **D.** *Dimerandra emarginata*. Scale bar is 1 cm. Any monilioid cells of other isolates. **E.** Isolate of *Sophronitis flavasulina*. **F.** *Sophronitis pabstii*. Scale bar is 3 ?m, **G.** *Epidendrum orchidiflorum* and **H**. *Cattleya tenuis.* Scale bar 5 ?m.

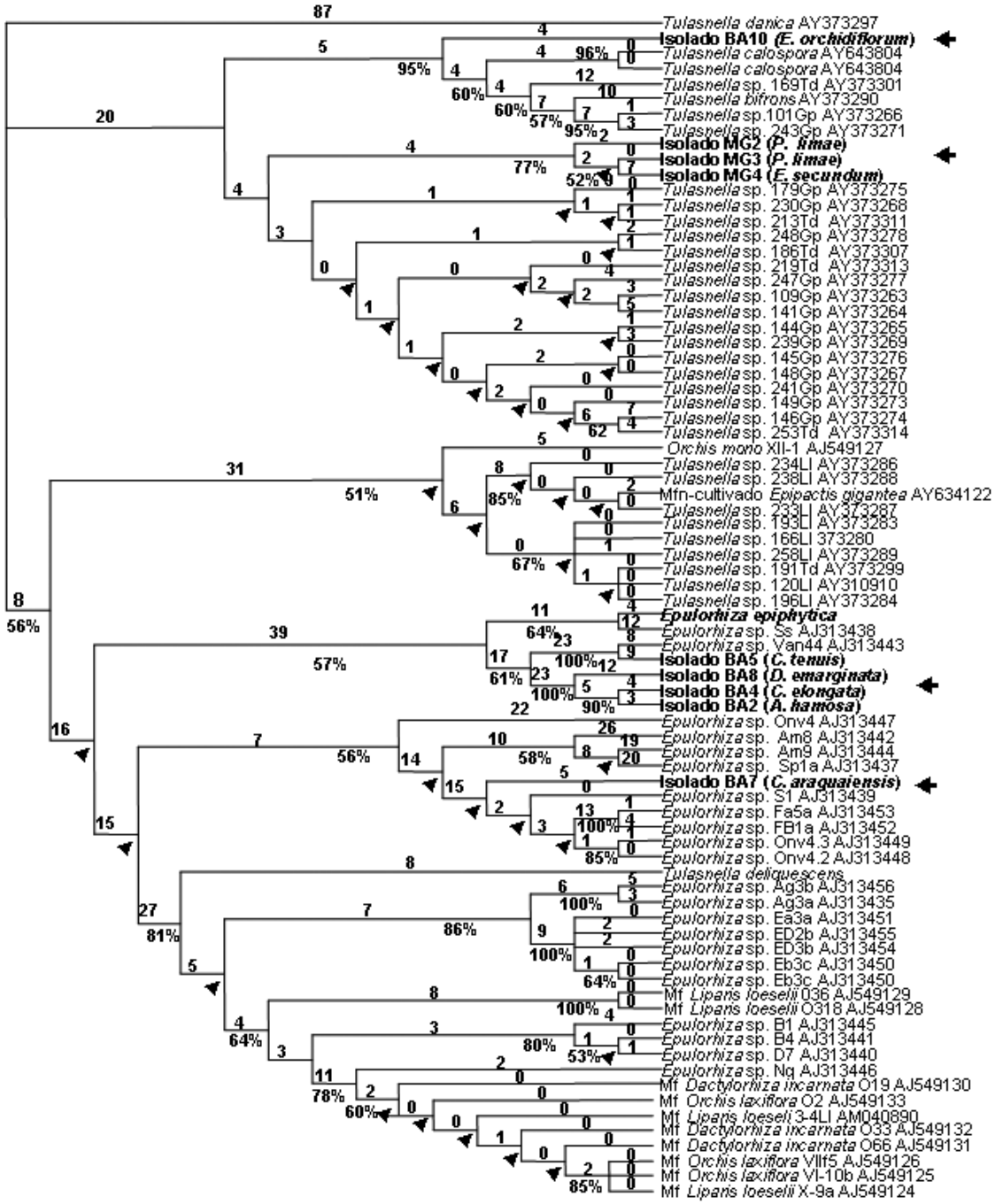

FIGURE 2**.** Fungal internal transcribed spacer phylogeny suggesting that the isolates of Laeliinae form mycorrhizal associations with fungi of the genus *Tulasnella*. The arrows show where the isolates of Laeliinae are.

Currently these studies have been decreasing, which reflects, for instance, the insignificant number of anamorphic fungi of described *Epulorhiza* species (Currah and Zelmer, 1992, Zelmer and Currah, 1995, Currah *et al.* 1997a, Pereira *et al.* 2003), as well as the high number of sequences deposited in GenBank without any definition in the specific level (McCormick *et al.* 2004, Shefferson *et al.* 2005).

It is not known if this putative preference could be extended to all genera inside Laeliinae. Some studies has already pointed out this possible preferential relationship in the mycorrhizal association in some few species of Laeliinae (Curtis, 1939, Nogueira *et al.* 2005, Pereira *et al.* 2001, 2003, Zettler *et al.* 1999). Future investigations will be carried out in order to verify the pattern of mycorrhizal association in Laeliinae genera for the development of a future program of symbiotic propagation of threatened Brazilian species.

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