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Full Length Research Paper

Diversity and antimicrobial activity of fungal endophyte communities associated with plants of Brazilian savanna ecosystems

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Fungal endophyte communities associated with leaves of *Myrciaria floribunda*, *Alchornea castaneifolia*, and *Eugenia* aff. *bimarginata* were examined, collected from Brazilian Cerrado ecosystems, and studied for their ability to produce antimicrobial activity. A total of 93 isolates of endophytic fungi were obtained and identified by sequencing of internal transcribed spacer (ITS) regions of the rRNA gene, which revealed the presence of 20 *Ascomycota* and three *Basidiomycota* taxa. The genus *Colletotrichum* is the most frequent endophyte associated with *M. floribunda* and *A. castaneifolia*. *Mycosphaerella* is the most frequent genus associated with *E.* aff. *bimarginata*. All fungal endophytic isolates were cultured and the crude extracts were screened to examine the antimicrobial activities against pathogenic microorganisms. Thirty-eight fungal extracts presented antimicrobial activity against at least one of the different target microorganisms tested. *Emericellopsis donezkii* and *Colletotrichum gloesporioides* showed the best minimum inhibitory concentration (MIC) values, which were lower or similar to MICs of known antibacterial and antifungal drugs. Our results suggest that the plants of Brazilian Cerrado shelter a diverse endophytic fungal community, which includes bioactive taxa capable of producing promising antimicrobial metabolites.

Key words: Antifungal and antibacterial activities, Cerrado ecosystem, endophytic fungi, fungal ecology.

INTRODUCTION

Endophytes are microorganisms that inhabit healthy plant tissues during at least one stage of their life cycle without causing any apparent symptom of disease or negative effects on the hosts (Petrini, 1992). Endophytes have been isolated from all plants previously studied, including bryophytes (U'Ren et al., 2010), pteridophytes (Petrini et al., 1992), gymnosperms (Soca-Chafre et al., 2011), and both monocotyledonous (Pinruan et al., 2010) and dicotyledonous angiosperms (Vaz et al., 2009, Rosa et al., 2010). These fungi colonise on all available tissues, such as roots, stems, leaves, bark, fruits, seeds, and floral organs (Petrini et al., 1992; Rodriguez et al., 2009; Vaz et al., 2009). Plants from temperate environments may harbour dozens of endophytic fungi. Many studies on tropical plants have also documented remarkable

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endophyte richness (Arnold and Lutzoni, 2007). Most endophytic fungi isolated are ascomycetous, and *Sordariomycetes* and *Dothideomycetes* classes contain the majority of foliar fungal endophyte species (Arnold and Lutzoni, 2007). Different plant hosts have been studied as targets of fungal endophytes, which can represent a rich source of bioactive metabolites. The secondary metabolites produced by fungal endophytes play an important role in metabolic interactions between microorganisms and their plant hosts, such as signalling, natural defence, and regulation of the symbiosis (Schulz and Boyle, 2005).

According to Strobel (2003), plants living in a unique ecosystem may shelter a specific fungal community, which can be a source for a variety of bioactive metabolites with potential for pharmacological and agrochemical applications. The Brazilian Cerrado is a biome composed of savanna and forest ecosystems that cover approximately 2 million km^2, representing *ca*. 22% of the land surface of Brazil, plus small areas in eastern Bolivia and north western Paraguay. Some research has shown that the Cerrado ecosystems can be harbouring a large and diverse population of plant-associated fungi with many new fungal taxa (Furlanetto and Dianese, 1997). Although interest has increased in the microbial biodiversity in Brazilian Cerrado, the diversity, taxonomic composition, host affinity, and the biotechnological potential of fungi from this ecosystem remain unclear. In this context, the aims of this work were to characterise the endophytic fungal community associated with three plants from Cerrado ecosystems and to determine the ability of these fungi to produce antimicrobial metabolites.

MATERIALS AND METHODS

Strategy of host species selection

In South America, the *Eugenia* genus occurs in Argentina, Uruguay, Paraguay, and Brazil, and some species have been used as food or medicine. The antioxidant, anti-inflamatory and cytotoxic and antimicrobial activities of *Eugenia* species have been reported (Lago et al., 2011; Stefanello et al., 2011). *Eugenia* aff. *bimarginata* Berg. (*Myrtaceae*) was selected to recover fungal endophytes due to its high abundance in the collection area. *Myrciaria floribunda* O. Berg (H. West ex Willd) (*Myrtaceae*) is native of Central and South America. Also known as rum berry or guava berry, *M. floribunda* has been grown for its edible fruits; moreover, Apel et al. (2006) showed that its essential oils have antimicrobial, anti-inflammatory, and antitumor activities. *Alchornea castaneifolia* (Willd) Juss. (*Euphorbiaceae*) is a native medicinal plant of the Amazon and Brazilian Cerrado forests that has been used for centuries by indigenous peoples of the Amazon against skin diseases, ulcers, malaria, inflammatory reactions, and pathogenic microorganisms (Hiruma-Lima et al., 2006; Costa et al., 2008).

Plant collection and identification

The plant specimens were collected in two ecological reserves of Tocantins state, North Brazil. The first area was Jalapão State Park (10°36'043"S, 46°35'836"W, elevation of approximately 740 m), which is the most extensive protected area in the Tocantins state, consisting of a mosaic of Cerrado physiognomies in transition to xerophilic "Caatinga" ecosystems. The second area was Cantão State Park (09°20'526"S, 49°58'347"W, elevation of approximately 170 m), a protected area located in the West of the Tocantins state, which represents an ecotone area among Cerrado, Amazon forest, and Pantanal ecosystems (Santos and Lolis, 2007). *E.* aff. *bimarginata* was collected at Jalapão State Park and *M. floribunda* and *A. castaneifolia* were collected at Cantão State Park in July 2007. All plant vouchers were deposited at the herbarium of the Institute of the Ciências Biológicas (BHCB) of the Universidade Federal of Minas Gerais, Brazil (http://sciweb.nybg.org/science2/IndexHerbariorum.asp).

Endophytic fungal isolation

Leaves from 30 individuals of each plant species were sampled. Three leaves of each individual were collected, placed in sterile plastic bags and stored less than 24 h at 10°C before the isolation of the endophytic fungi. From the leaves, three fragments (approximately 0.5 cm in length and 0.5 cm wide) of each leaf were plated. The fragments were surface sterilised by immersion in ethanol 70% (1 min) and 2% sodium hypochlorite (3 min), followed by washing with sterile distilled water (2 min) (Collado et al., 1996). The fragments were plated on Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with Chloramphenicol at 100 µg/ml (Collado et al., 1996) and were incubated at 25°C. In order to test the effectiveness of surface sterilisation, 100 µl of the final rinsing water was plated on PDA and incubated at 28°C. Hyphal growth was monitored over an eight-week period. Using aseptic technique, endophytes were transferred to axenic culture on PDA in 60 mm Petri plates and photographed after complete growth. Long-term preservation of the filamentous fungal colonies was carried out in cryotubes with 30% sterile glycerol at -80°C and in sterile distilled water at room temperature. All pure cultures of the endophytic fungal isolates were deposited into the Culture Collection of Microorganisms and Cells of Universidade Federal of Minas Gerais. For calculation purposes and statistical analysis, each individual tree was considered a sample unit. When two or more fungal isolates from the same sample were identified as belonging to the same species, they were considered to be a single isolate. The total occurrence of filamentous fungi and yeasts corresponded to the total number of isolates in each sample.

Fungal identification

Pure cultures of the fungal isolates were grouped based on morphological characters including aerial mycelium form, colony colours, surface texture and margin characters. Because most endophytes did not produce sexual or asexual reproduction structures in regular culture media, at least fifty percent of each morphospecies was selected for identification by sequencing of the ITS region of the rRNA gene. The protocol for DNA extraction of the endophytic fungi was performed according to Rosa et al. (2009). The ITS domains of the large subunit of the rRNA gene were amplified using the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), as described by White et al. (1990). Amplification of ITS and sequencing protocols were

performed as described to Vaz et al. (2009). Sequencing was carried out using an ET dynamic terminator kit in a MegaBACE 1000/automated 96 capillary DNA sequencer (GE Healthcare, Buckinghamshire, UK). Consensus sequence data of the endophytic fungi were deposited in GenBank under the accession numbers FJ466701 to FJ466730 Phylogenetic relationships were estimated using MEGA program Version 4.0 (Tamura et al., 2007). The Maximum Composite Likelihood model was used to estimate evolutionary distance with bootstrap values calculated from 1,000 replicate runs. In the presented study, a 3% cut-off value was used to define the species level based on the sequencing of the ITS domains (Nilsson et al., 2008).

Endophyte richness, diversity, and similarity among plant hosts

Species accumulation curves were generated for each plant species using EstimateS, version 8.0 (Colwell, 2005). Normality testing was performed using the Shapiro-Wilk test using a software package R (R Development Core Team, 2005). To examine similarity of the fungal communities among tree hosts we used similarity Sorenson's and Jaccard indexes based on presence/absence data only. In this study the individual plant was considered the sample unit, being each of the three sampled leaves considered a replicate for calculation purposes. The Sorenson's similarity coefficient (C_S) was calculated according to the following formula:

Cs=2c/(a+b),

And the Jaccard similarity coefficient (C*j*) is defined by the formula:

C*j*=*c*/*a*+*b*+*c*,

Where *c*; the number of fungal species coexisting in both plants, *a*; the total number of fungal species in one plant, and *b* is the total number of fungal species on the other plant.

All results were obtained with 95% confidence, and the boostrap values were calculated from 1.000 iterations. The antimicrobial results were submitted to a preliminary multiple correspondence analysis (MCA) as described in Vaz et al. (2009). Twelve variables were analyzed: plant (*M*. *floribunda*, *E.*aff. *bimarginata* and *A. castaneifolia*), antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella typhimurium*, *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Cryptococcus neoformans* (yes or no for each antimicrobial activity). All samples are illustrated in the graphic, but only the representative samples are considered for the results analysis. The MCA and classification analysis were conducted using SPAD 5.5 software (SPAD, Paris, France).

Antimicrobial assays

In vitro antimicrobial susceptibility tests were performed using the protocols described by Vaz et al. (2009) with a panel of the following eleven microorganisms: *E. coli* ATCC 25922, *S. aureus* ATCC 12600, *P. aeruginosa* ATCC 27853, *B. cereus* ATCC 11778, *S. typhimurium* ATCC 14028, *C. albicans* ATCC 18804, *C. krusei* ATCC 2159, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750 and *C. neoformans* ATCC 32608. Inoculum of the target microorganisms were adjusted to a McFarland no. 1 standard in optical density for yeasts, corresponding to 3×10^8 cfu/ml. A McFarland 0.5 standard in optical density for bacteria corresponded to 1 to 2×10^8 cfu/ml. The concentrations were confirmed via spectrophotometer readings at 580 and 626 nm for yeasts and bacteria, respectively. Yeast samples were inoculated using a swab on Sabouraud agar (1% peptone, 4% dextrose and 2% agar), and the bacteria were plated on Brain Heart Infusion agar (Difco, USA) also using a swab.

Crude extract stock solutions at 10 mg/ml were prepared in dimethyl sulphoxide (DMSO, Merck, USA) and stored at -40°C. These solutions were diluted with water and assayed at a final concentration of 100 µg/ml (DMSO concentrations remained below 1%). The extract solutions at 10 mg/ml in DMSO were applied (10 µl) on disk blanks on the Petri dishes containing the target microorganisms and incubated for 24 to 48 h at 37°C. After incubation, the inhibition zones around the application points were measured. Amphotericin B (Sigma, USA) and chloramphenicol (Sigma, USA), both at 100 µg/ml, were used as positive controls for yeasts and bacteria, respectively. Solvent (DMSO) and extracts of culture media were used as the negative controls.

Minimum inhibitory concentration

All methanolic extracts that presented antimicrobial activity in the screening above were evaluated for the minimum inhibitory concentration (MIC) against the same target microorganisms used in the screening test. The MIC values of the methanolic extracts were determined using the agar microdilution method according to the National Committee for Clinical Laboratory Standards protocols against bacteria and yeasts (NCCLS, 2004). The MICs values were defined as the lowest concentration of test samples that inhibited the visible growth of microorganisms.

RESULTS

Fungal endophyte communities

A total of 93 isolates of endophytic fungi were obtained (21 recovered from *M. floribunda*, 37 from *A. castaneifolia*, and 35 from *E.* aff. *bimarginata*), which were grouped, and 41 representative fungal isolates were identified by sequencing the ITS region of the large subunit of the rRNA gene. Molecular identification revealed 23 different endophytic taxa, from which 20 represented *Ascomycota* and three *Basidiomycota* species (Table 1). The most frequent endophytic fungus isolated from *M. floribunda* and *A. castaneifolia* was *Colletotrichum gloesporioides* (Penz.) Penz. and Sacc. (anamorph of *Glomerella cingulata*).

Ten fungal endophytes showed high divergence in the ITS region when compared with fungal sequences of other fungi deposited in the GenBank database and were identified at the genus level. These endophytes were identified as belonging to two genera of Sordariomycetes, four genera of Dothideomycetes and one genus of Leotiomycetes. The phylogenetic position of the Sordariomycetes endophytic fungi isolated in the present

Table 1. Frequency of isolation for the endophytic fungi obtained from *Eugenia* aff. *bimarginata*, *Myrciaria floribunda*, and *Alchornea castaneifolia* and closest related species according to % similarity of the ITS region of the rRNA gene by alignment with sequences of related species retrieved from the GenBank database.

UFMGCB code	Closest related species	Similarity	No. of bp analyzed	Identification and GenBank accession number	Number of isolates/abundance (%)		
					EB	MF	AC
2013	*Aspergillus sydowii* [AY373868]	99	528	*Aspergillus sydowii*	1/2.86	-	-
1989, 1990	*Botryosphaeria dothidea* [AY259092]	96	513	*Botryosphaeria* sp. [HQ184171]	-	3/14.29	-
2029	*Cladosporium cladosporioides* [GQ221853]	98	510	*Cladosporium cladosporioides*	3/8.57	-	-
1937, 1997, 2003, 2004	*Colletotrichum gloeosporioides* [EU552111]	98	534	*Colletotrichum gloeosporioides*	3/8.57	8/38.10	14/37.84
1952	*Diaporthe phaseolorum* [AY577815]	99	531	*Diaporthe phaseolorum*	-	-	1/2.70
1941, 1967, 1971	*Didymella bryoniae* [EU167573]	97	526	*Didymella bryoniae*	-	-	7/18.92
1984	*Trametes* sp. [AF519892]	95	604	*Trametes* sp. [HQ184178]	-	1/4.76	-
1966, 2001	*Emericellopsis donezkii* [AY632658]	99	502	*Emericellopsis donezkii*	-	-	2/5.41
1967	*Eutypella scoparia* [EU436688]	96	526	*Eutypella* sp. [HQ184171]	-	-	1/2.70
2014	*Filobasidium capsuligenum* [AF444382]	84	570	*Filobasidium* sp.[HQ184180]	2/5.71	-	-
1974, 1978	*Fusarium decemcellulare* [FJ545369]	97	521	*Fusarium decemcellulare*	-	4/19.05	-
1936, 1953, 1955, 2043	*Gibberella moniliformis* [GQ168842]	99	501	*Gibberella moniliformis*	3/8.57	-	9/24.32
2045	*Glomerella acutata* [DQ286133]	99	8536	*Glomerella acutata*	-	4/19.05	-
2024, 2026, 2038	*Mycosphaerella pseudoellipsoidea* [EU167585]	97	442	*Mycosphaerella* sp. [HQ184178]	11/31.43	-	-
1976	*Neofusicoccum batangarum* [FJ900608]	99	512	*Neofusicoccum batangarum*	-	1/4.76	-
1932	*Nigrospora oryzae* [DQ219433]	98	483	*Nigrospora oryzae*	-	-	1/2.70
2058	*Letendraea helminthicola* [EU715680]	94	612	*Letendraea* sp. [HQ184176]	3/8.57	-	-
1960	*Penicillium sclerotiorum* [AF033404]	98	467	*Penicillium sclerotium*	2/5.71	-	2/5.41
2037	*Phaeosphaeriopsis musae* [DQ885894]	92	516	*Phaeosphaeriopsis* sp. [HQ184175]	1/2.86	-	-
2033	*Pseudeurotium desertorum* [AY129288]	94	519	*Pseudeurotion* sp. [HQ184177]	2/2.86	-	-
2012, 2060	*Pseudozyma* sp. [AJ876488]	91	545	*Pseudozyma* sp. [HQ184179]	2/5.71	-	-
2036	*Rhytidhysteron rufulum* [AM711974]	98	415	*Rhytidhysteron rufulum*	3/5.71	-	-
2020	*Xylaria* sp. [FJ175168]	99	552	*Xylaria* sp. [HQ184172]	1/2.86	-	-

UFMGCB: Culture collection of the Universidade Federal of Minas Gerais. EB: *Eugenia* aff. *bimarginata*, MF: *Myrciaria floribunda*, AC: *Alchornea castaneifolia.*

work is shown in Figure 1. The isolate UFMGCB 2020 presented 99% similarity with Xylaria sp. (GenBank access number FJ175168) and 94% sequence similarity with isolates of endophytic fungi associated with the bryophyte Herbertus alpines and the plant Plagiochila ramosissima from New Zealand (Table 1). In addition, the UFMGCB 2020 isolate showed 93% similarity with 18 nucleotide differences and 12 gaps with Xylaria castorea (GenBank access number AF163030). These fungi formed a branch in the phylogenetic tree that was strongly supported (Figure 1).

The UFMGCB 1967 isolate showed 15 nucleotide differences with sequence of *Eutypella scoparia* (GenBank access number EU436688), which was obtained as an endophytic fungus associated with the tropical fruit tree *Garcinia dulcis* in southern Thailand. Figure 2 shows the phylogenetic position of the fungal species of *Dothideomycetes* isolated in our study. The UFMGCB 1990 isolate showed 99% similarity with *Botryosphaeria* sp. (GenBank access number DQ480360), an endophytic fungus associated with *Garcinia* sp. plant hosts in southern Thailand, and 96% similarity with *Botryosphaeria dothidea* isolated from *Vitis vinifera* (Vitaceae) in Spain (GenBank access number EU650670); our isolate was identified as *Botryosphaeria* sp. The UFMGCB 2026 isolate showed 97% similarity with *Mycosphaerella pseudoellipsoidea* (EU167585) and *M. gregaria* (EU167580), which were obtained from leaves of *Eucalyptus nitens* and *Eucalyptus globule*s in South Africa and Western Australia, respectively. In our work, this isolate was identified as *Mycosphaerella* sp.

The UFMGCB 2058 isolate was identified as *Letendraea* sp. because it showed 94% query coverage and 94% similarity with an endophytic fungus *Letendraea helminthicola* strain SGLMf23 (EU715680) isolated from *Taxus globosa* in Mexico. The UFMGCB 2037 isolate showed a 6% nucleotide difference when compared with *Ascochyta* sp. CBS 117477 (GU230751) and an 8% nucleotide difference in relation to the ITS sequence of *Phaeosphaeriopsis musae* (DQ885894). This isolate was putatively identified as *Ascochyta* sp. (Figure 2).

Figure 3 shows the phylogenetic position of the fungal species of *Leotiomycetes* isolated in our study. UFMGCB 2033 isolate showed a 6% difference with an uncultured fungus (FN397281) obtained from *Tuber melanosporum* truffle-ground soil and a 6% difference in relation to *Pseudeurotium desertorum* CBS 986.72 (AY129288). Based on these data, this isolate was identified as *Pseudeurotiom* sp. The yeast isolate UFMGCB 2012 presented 91% similarity with *Pseudozyma* sp. (GenBank access number AJ876488). The taxon UFMGCB 1984 showed 95% similarity with *Trametes* sp. (GenBank access number AF519892), *Coriolopsis gallica* (GenBank access number AY684172) and *Trametes troggi* strain 4222 (GenBank access number GU199348), and it was identified as *Trametes* sp. The isolate UFMGCB 2014 showed 84% similarity with the type strain of the yeast *Filobasidium capsuligenum* (GenBank access number AF444382), and it was identified putatively as *Filobasidium* sp.

The samples do not fit the assumptions of a normal distribution, which could be confirmed by the low *P* value in the Shapiro-Wilk test (*M. floribunda*, P=2.978 10^{-5}; *A. castaneifolia*, P=4.261 10^{-5}; and *E. bimarginata*, P=9.463 10^{-4}). The accumulation curves of *E.* aff. *bimarginata* and *A. castaneifolia* did not reach an asymptote, and the accumulation curve of *M. floribunda* approximated an asymptote (Figure 4). The similarity index values showed that the taxa composition was variable among the different hosts and the highest similarity was between *A. castaneifolia* and *E.* aff. *bimarginata* (Table 3). This result is likely related to the occurrence of 10 endophytic taxa only in *E.* aff. *bimarginata* samples, whereas six taxa occurred only in *A. castaneifolia* samples and five only in *M. floribunda*.

Antimicrobial activity

Thirty isolates (33.3%) displayed antimicrobial activity against at least one target microorganism with inhibition zones ranging from 7 to 35 mm in diameter. Table 2 shows that the MIC values of methanolic extracts from endophytic fungi ranged from 7.8 to 500 µg/ml against *C. krusei*, 7.8 to >1.000 µg/ml against *C. parapsilosis*, 62.5 to 500 µg/ml against *Cr. neoformans*, 250 µg/ml against *C. albicans* and >1.000 µg/ml against *C. glabrata*. In addition, the MIC values ranged from 62.5 to >1.000 µg/ml against *S. aureus* and 7.8 to >1.000 µg/ml against *B. cereus*. Differing from these, the MIC values for *S. thyphymurium* were higher than 1.000 µg/ml. The extracts of *C. gloesporioides* UFMGCB 2002 and *Emericellopsis donezkii* UFMGCB 2001 showed the strongest antifungal activities, and the best MIC values. Two isolates of *E. donezkii* (UFMGCB 1966 and UFMGCB 2001) showed the strongest antibacterial activity. The data were clustered into five classes using multivariate statistical analyses (Figure 5). The first group was characterized by extracts of endophytic fungi from the *M. floribunda* presenting activity against *S. typhimurium*, The second group was comprised of endophytic fungi from *A. castaneifolia*, and presented activity against *C. glabrata,* with no activity against *C. parapsilosis*. The fifth group was characterized by endophytic fungi collected from *E.* aff. *bimarginata* with activity against *C. parapsilosis* and *C. albicans.* No conclusion about the antimicrobial activity and the species host from which the fungi were collected from could be concluded for the third and fourth groups. The relative proportion of each group is showed in the Figure 5.

DISCUSSION

The most frequent endophytic fungus isolated from the two plant species, *M. floribunda* and *A. castaneifolia,* was *C. gloesporioides*. *C. gloesporioides* has been commonly isolated as an endophyte from several plant species (Joshee et al., 2009; Higgins et al., 2011; Vega et al., 2010), and it is considered a generalist species.

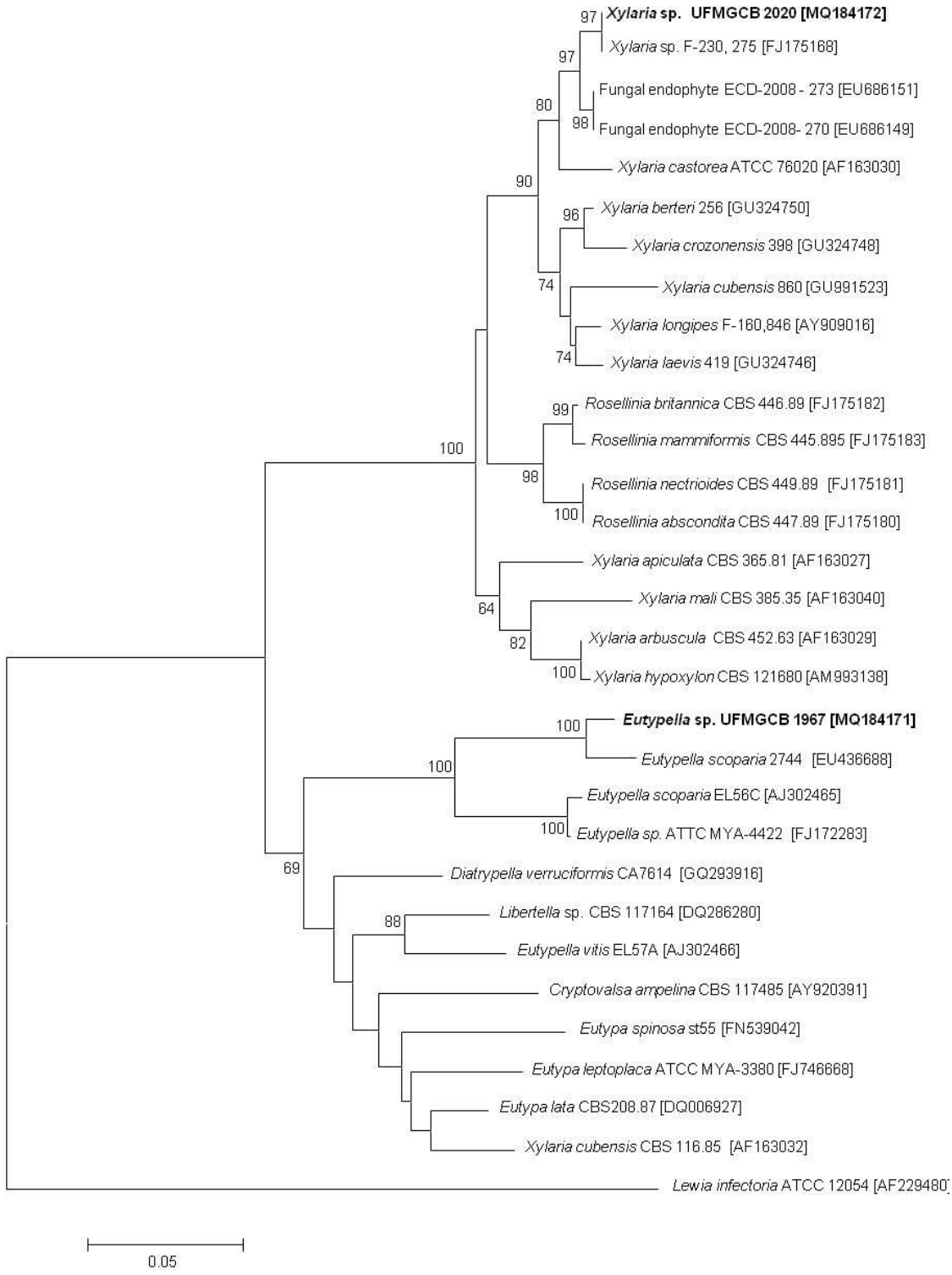

Figure 1. Phylogenetic tree based on the rRNA gene sequence (ITS1-5.8S-ITS2) showing the closest relatives of *Sordariomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Lewia infectoria* ATCC12054 [AF229480] as the out-group.

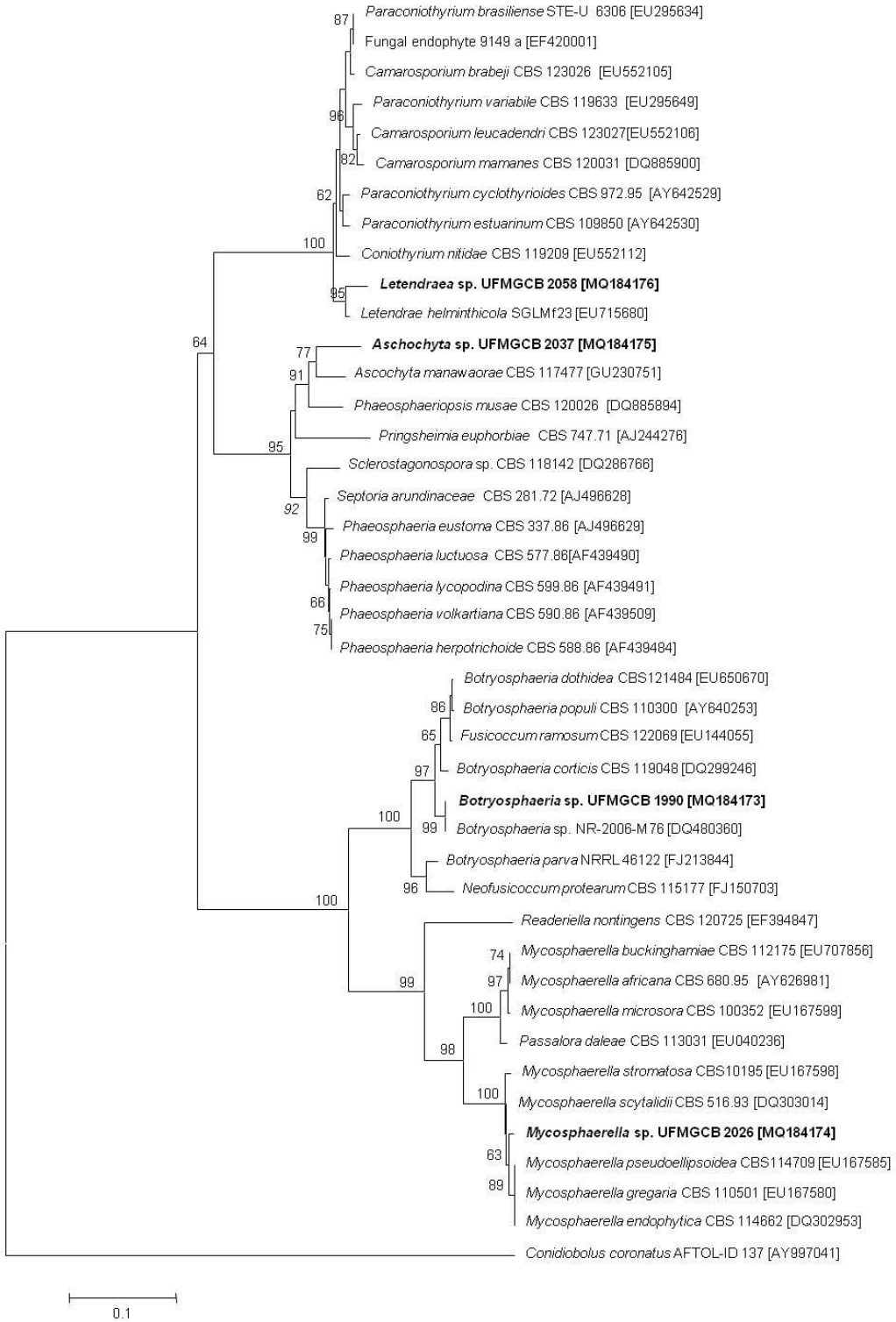

Figure 2. Phylogenetic tree based on the 5 rRNA gene sequence (ITS1-5.8S-ITS2) showing closest relatives of *Dothydeomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Conidiobolus coronatus* AFTOL-ID137 [AY997041] as the out-group.

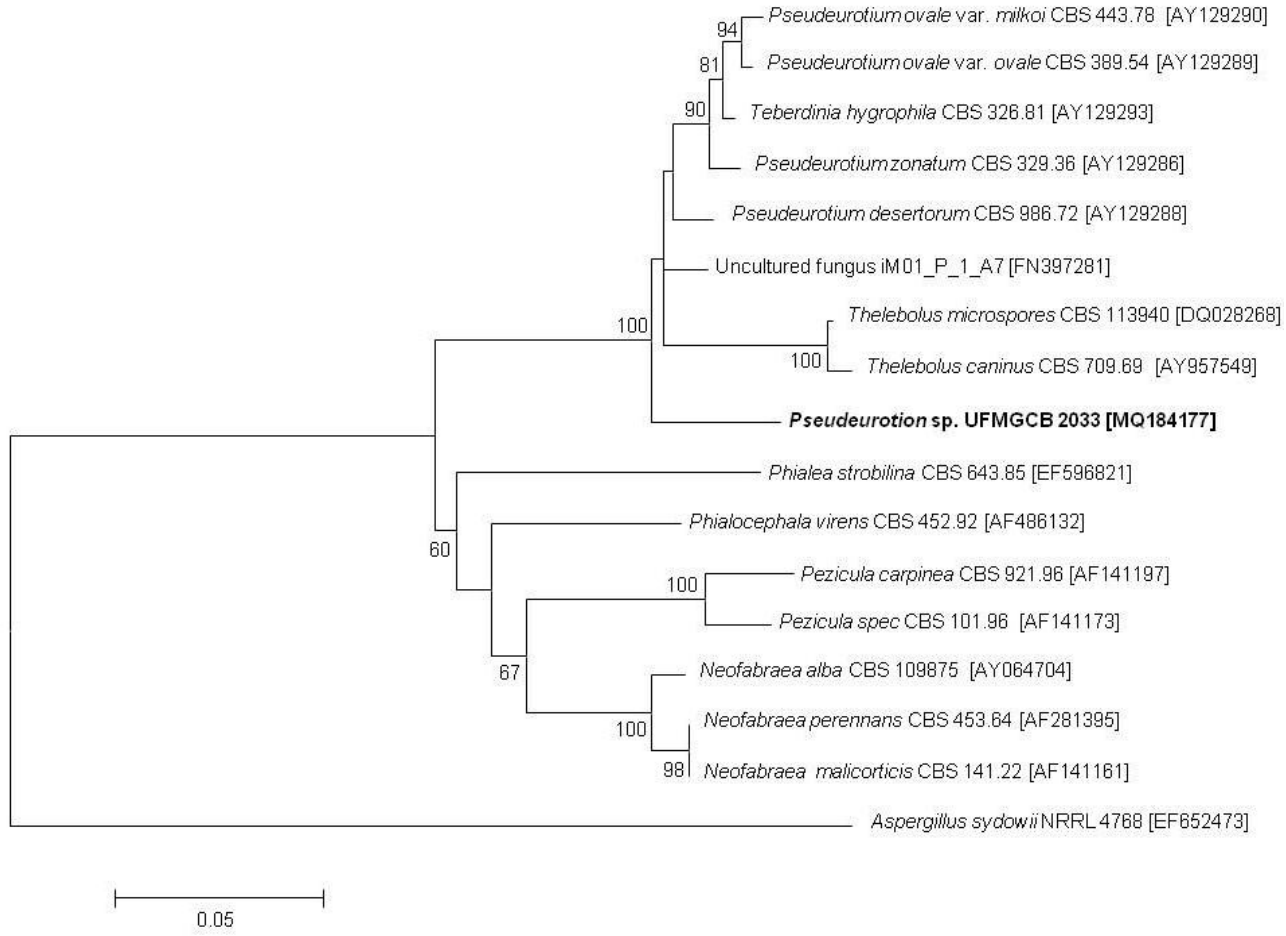

Figure 3. Phylogenetic tree based on the 5 rRNA gene sequence (ITS1-5.8S-ITS2) showing closest relatives of *Leotiomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Aspergillus sydowii* [EF652473] as the out-group.

Mycosphaerella sp. was the most frequent fungus among the 13 species associated with *E.* aff. *bimarginata* in Jalapão State Park and may be considered prevalent among the fungal species associated with this host plant. *Mycosphaerella* species have been recovered as endophytes (Johsee et al., 2009; Márquez et al., 2010), although some species of *Mycosphaerella* are phytopathogenic fungi able to cause serious diseases in different plant species (Crous et al., 2009). *Giberella moniliformis* and *Didymella bryoniae* were also frequent in *A. castaneifolia* samples. *G. moniliformis* and *Penicillium sclerotiorum* were common to *A. castaneifolia* and *E.* aff. *bimarginata,* which were collected in different geographic areas presenting different phytophysiognomies. This result indicated that these two fungal species may be ubiquitous in Cerrado ecosystems. The species of *Colletotrichum*, *Gibberella*, and *Penicillium* are frequently found as endophytes in many tropical, temperate, and polar plants (Rosa et al., 2009; U'Ren et al., 2009; González et al., 2011) and have adapted to a wide range of geographical sites, climatic conditions, ecological habitats, and host plants. Ten isolates of endophytic fungi isolated in our work could represent new species, since their ITS sequences showed high divergence when compared with fungal sequences of other species deposited in GenBank. This result shows that the three plant species can be a reservoir of new fungal species.

The samples do not fit the assumptions of a normal distribution, and the accumulation curves of *Eugenia* aff. *bimarginata* and *A. castaneifolia* did not reach an asymptote, and this is a pattern frequently found

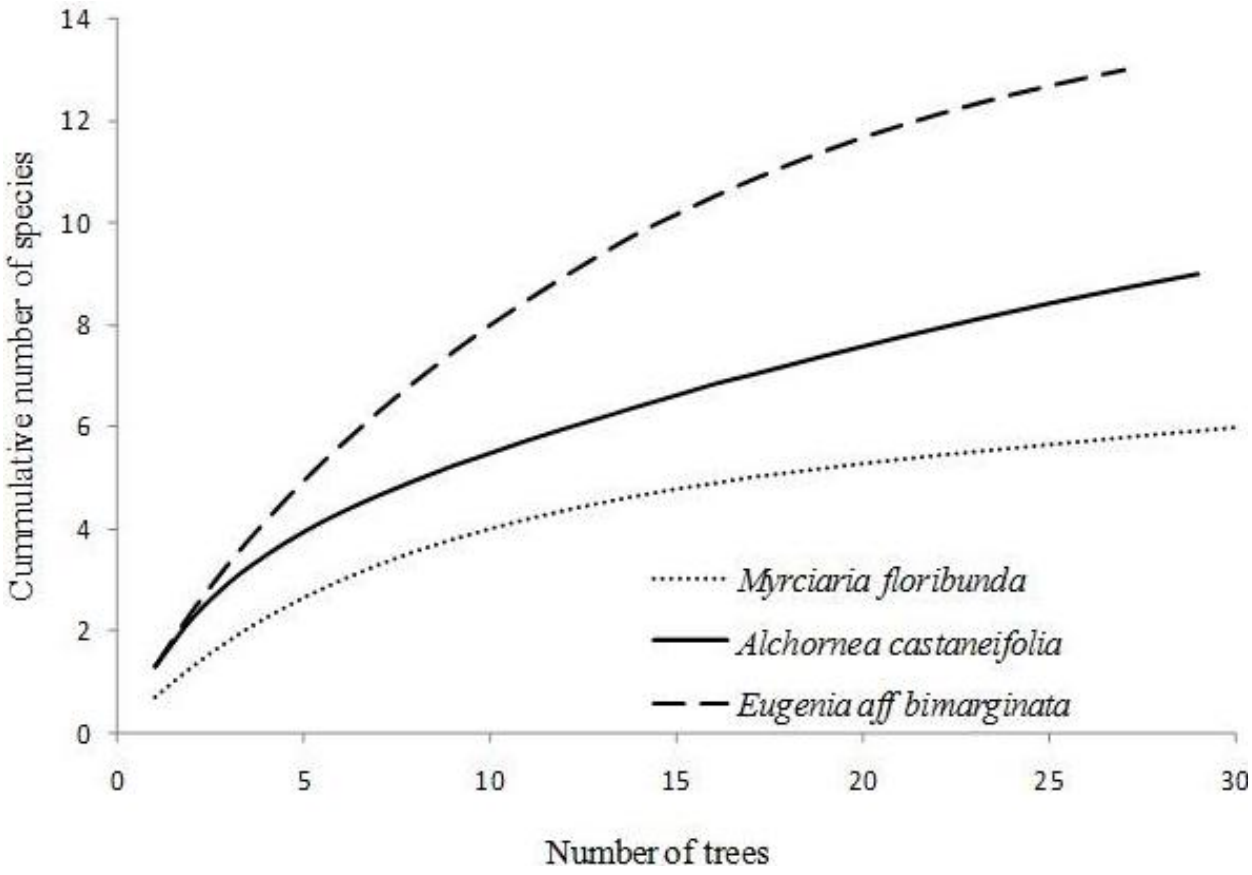

Figure 4. Species accumulation curves for fungal endophytes from healthy leaves of three host species (*Eugenia* aff. *bimarginata*, *Alchornea castaneifolia* and *Myrciaria floribunda*) based on the Mao Tao estimator calculated using EstimateS v.8.0 (6).

in samples from tropical studies (Gazis and Chaverri, 2010, Joshee et al., 2009). This result suggests that further sampling could recover other endophytic fungi associated with these plants. However, the accumulation curve of *M. floribunda* approximated an asymptote, which indicated that increasing sampling will not significantly increase the number of species found, although species richness in *M. floribunda* is lower than in the other host trees sampled.

The similarity index indicated a low similarity among the fungal communities of the host plants, and this result is likely related to the occurrence of 10 endophytic taxa only in *E.* aff. *bimarginata* samples, whereas six taxa occurred only in *A. castaneifolia* samples and five only in *M. floribunda*. It is also related to the common occurrence of accidental species, defined here as those with one or two isolates per host.

Brazil has a long tradition in medicinal plant use and many of them are used in the form of crude extracts, infusions or plasters to treat common infections. Several works have showed the capacity of endophytic fungi to produce bioactive natural products with potential therapeutic interest (Strobel et al., 2003; Rodriguez et al., 2009; Liu et al., 2010; Vaz et al., 2009). Therefore, considering that the plant species studied has a popular use to treat infectious disease we decided test the endophytic fungi associated with these plants against pathogenic microorganisms. Thirty fungal isolates displayed antimicrobial activity against at least one target microorganism. Isolates of *E. donezkii* and *C. gloesporioides* showed the best minimum inhibitory concentration (MIC) values, which were lower or similar to MICs of known antibacterial and antifungal drugs. One isolate each of *C. gloesporioides* (UFMGCB 2002) and *E. donezkii* (UFMGCB 2001) showed the strongest antifungal activities. The *Colletotrichum* genus includes phytopathogenic, saprophytic, and endophytic species (Joshee et al., 2009; Gazis and Chaverri, 2010). Several works have reported that *Colletotrichum* species are a potential source of bioactive metabolites, such as

Table 2. Minimal inhibitory concentration (MIC) in µg/ml against yeasts and bacteria of crude extracts obtained from endophytic fungi isolated from *Eugenia* aff. *bimarginata*, *Myrciaria floribunda*, and *Alchornea castaneifolia*.

Host plant	UFMGCB Code	Endophytic species	Individual tree	Yeasts					Bacteria		
				CA	CK	CP	CG	CN	SA	ST	BC
E. bimarginata	2027	*Gibberella moniliformis*	30	>1.000	-	-	-	250	-	-	1.000
	2015	*G. moniliformis*	11	-	-	1.000	-	-	-	-	-
	2025	*Mycosphaerella pseudoellipsoidea*	28	-	-	>1.000	-	-	-	-	-
	2031	*Mycosphaerella* sp.	5	-	500	-	-	-	-	-	250
	2032	*Mycosphaerella* sp.	6	-	-	-	-	-	1.000	-	-
	2038	*Mycosphaerella* sp.	23	250	-	-	-	-	-	-	-
	2040	*Mycosphaerella* sp.	29	-	-	>1.000	-	250	-	-	-
	2059	*Mycosphaerella* sp.	11	-	-	-	-	-	250	-	-
M. floribunda	1990	*Botryosphaeria* sp.	11	-	-	-	-	-	-	>1.000	250
	1991	*Botryosphaeria* sp.	11	-	-	-	>1.000	-	-	-	-
	1987	*Coletotrichum gloeosporioides*	1	250	-	>1.000	-	-	-	-	-
	1974	*Fusarium decemcellulare*	13	-	-	-	-	500	-	-	-
	1978	*F. decemcellulare*	13	-	-	>1.000	-	-	-	-	-
	1985	*F. decemcellulare*	13	-	-	-	-	500	-	>1.000	>1.000
	1988	*F. decemcellulare*	13	-	250	-	-	500	-	>1.000	-
	2044	*Glomerella acutata*	14	-	500	-	-	-	-	-	-
	1986	*Glomerella acutata*	14	-	-	500	-	-	-	>1.000	500
A. castaneifolia	2002	*C. gloeosporioides*	1	-	**7.8**	**7.8**	-	500	>1.000	-	250
	1935	*C. gloeosporioides*	1	-	-	-	1.000	500	-	-	-
	1938	*C. gloeosporioides*	1	-	-	-	1.000	-	-	-	-
	1959	*C. gloeosporioides*	1	-	-	-	-	500	-	-	-
	1961	*C. gloesporioides*	2	-	-	-	>1.000	-	-	-	-
	2007	*C. gloeosporioides*	1	-	-	125	-	-	-	-	-
	1941	*Didymella bryoniae*	4	-	-	-	-	500	-	-	-
	1946	*Didymella bryoniae*	4	-	-	-	1	-	-	-	-
	1966	*Emericellopsis donezkii*	5	-	-	-	-	-	**62.5**	-	**7.8**
	2001	*Emericellopsis donezkii*	5	-	250	-	-	**62.5**	**62.5**	-	**15**
	1994	*G. moniliformis*	3	-	-	-	-	-	-	>1.000	125

Table 2. Contd

	1996	*G. moniliformis*	1	-	-	-	-	1.000	-	-	-
	1963	*G. moniliformis*	3	-	-	-	-	500	-	-	250

UFMGCB: Culture collection of the Universidade Federal of Minas Gerais. Ca: *Candida albicans*; Ck: *Candida krusei*; Cp: *C. parapsilosis*; Cg: *Candida glabrata*; Cn: *Cryptococcus neoformans*; Sa: *Staphylococcus aureus*; St: *Salmonella thyphimurium*; Bc: *Bacillus cereus*; - no inhibition. Only results for fungal isolates with at least one antimicrobial activity are shown.

Table 3. Sorenson's and Jaccard similarity coefficients of endophytic fungi from the three plants.

	E.* aff. *bimarginata	***M. floribunda***	***A.castaneifolia***
Eugenia aff. *bimarginata*	-	0.133 / 0.063	0.400 / 0.167
Myrciaria floribunda	0.133 / 0.063	-	0.200 / 0.091
Alchornea castaneifolia	0.400 / 0.167	0.200 / 0.091	-

colletotric acid, which has an antimicrobial substance with activity against bacteria and fungi (Suryanarayanan et al., 2009; Liu et at., 2010), and gloesporone, a fungal germination inhibitor (Meyer et al., 1987). Two isolates of *E. donezkii* showed the strongest antibacterial activity. Berg et al. (1996) isolated bergofungin from the extract of *E. donezki*, a peptaibol-type antibiotic that showed antibacterial activity against *B. cereus*. According to Saleem et al. (2010), promising compounds useful to development of antimicrobial drugs should show MICs values ranging from 0.02 to 10 µg/ml. The extracts of endophytic isolates UFMGCB 2002 and UFMGCB 2001 showed the best MIC values, which were lower or near the MICs of known antibacterial and antifungal drugs. For example, penicillin was effective (range: 0.012 to > 32 µg/ml) against *B. cereus* (Turnbull et al., 2004), and fluconazole was effective against both *C. krusei* (16 to 64 µg/ml) and *C. parasilosis* (2 to 8 µg/ml) (NCCLS, 2004). Fungal crude extracts are generally a mixture of active and non-active compounds, and low MICs may be suggestive of good antimicrobial activity. The extracts of these endophytic fungal are potential candidates for further studies to isolate the bioactive compounds, which can serve as models for specific drugs (antibacterial or antifungal), as well as drugs with broad antibiotic effects. The majority of fungal isolates obtained in this study that belonged to the same species and obtained from the same host tree exhibited different antimicrobial activity patterns against different target microorganisms. Some works also reported differences in the biological activities among fungal isolates of the same plant species (Vaz et al., 2009). These results suggest that more than one fungal isolate of each species should be tested when searching for biological activities. The high number of methanolic extracts with antimicrobial activity may be considered as a possible indicator for the capacity of these endophytic fungi to produce active compounds against the target pathogens

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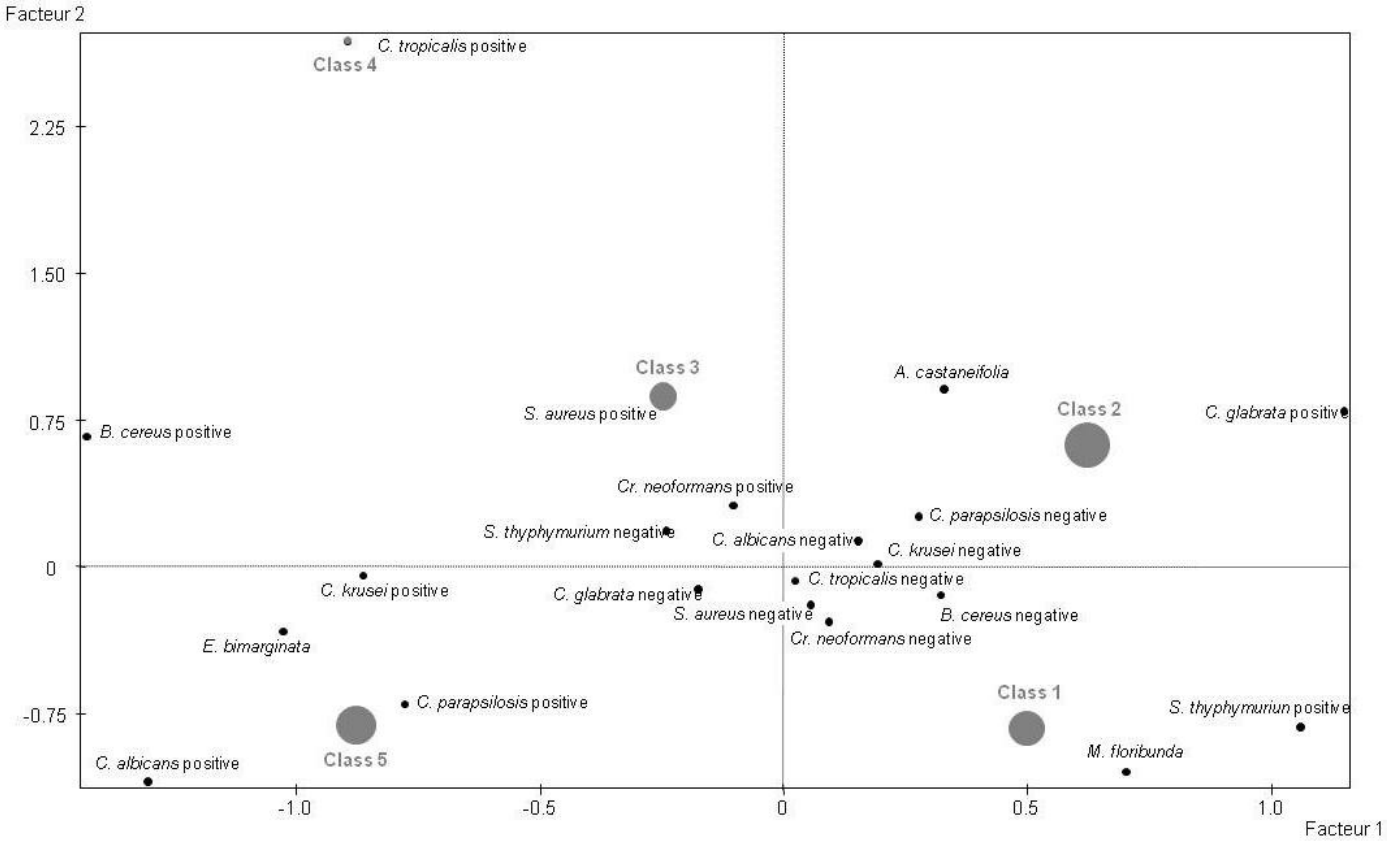

Figure 5. Multiple correspondence analyses about the association among endophytic fungi and species host (*Myrciaria floribunda*, *Alchornea castaneifolia*, and *Eugenia* aff. *bimarginata*) and antimicrobial activity (positive or negative) against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella typhimurium*, *Candida albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *Cryptococcus neoformans* (yes or no for each antimicrobial activity). Class 1: Endophytic fungi isolated from *M. floribunda* presenting activity against *S. typhimurium*. Class 2: Endophytic fungi from *A. castaneifolia* presenting activity against *C. parapsilosis* and no activity against *C. parapsilosis*. Class 5: Endophytic fungi from *Eugenia* aff. *bimarginata* with antimicrobial activity against *C. parapsilosis* and *Candida albican.* Class 3 and 4: No conclusions about these groups.

Brazilian laws.

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