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ORIGINAL ARTICLE

Diversity of endophytic fungi in *Eucalyptus microcorys* assessed by complementary isolation methods

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Abstract

Brazil is the world's largest producer country of eucalyptus. Although widely applied in the charcoal industry, no studies have focused on the microorganisms associated with *Eucalyptus microcorys*. Here, we evaluated the composition and structure of endophytic fungal communities in leaves of *E. microcorys* through two isolation techniques. A total of 120 fresh leaves were collected in a year-long survey at an eucalyptus plantation in the State of São Paulo (Brazil). Endophytic fungi were isolated by particle filtration (PF) and direct leaf fragment plating (LP) in two media: modified dicloran and synthetic nutrient agar, both supplemented with rose bengal and chloramphenicol. The isolates were grouped into morphospecies and identified by morphology and DNA sequencing. We recovered a total of 709 isolates, representing 59 taxa. All taxa found are reported as endophytic for the first time for *E. microcorys*. *Castanediella eucalypticola* and *Neophaeomoniella eucalypti* are new occurrences reported for Brazil. The LP technique recovered a higher number of taxa and isolates than the PF. However, the PF technique retrieved a higher species/isolate ratio than the LP method, 0.12 and 0.09, respectively. Fungal diversity assessed by diversity metrics did not significantly differ between isolation methods. Both techniques recovered a high number of unique taxa, demonstrating that neither method would individually represent the species richness from *E. microcorys*. The use of LP and PF provided a greater number of observed taxa and consequently new occurrence of species for Brazil.

Keywords Culture-dependent methods · Leaf plating · Particle filtration · Endophytes

Introduction

The genus *Eucalyptus* is native to Australia and adjacent islands of Oceania, but Brazil has become the world's largest producer country (Sociedade Brasileira de Silvicultura 2015). Eucalyptus is used as raw material for the production of paper, cellulose, and other derivatives that represent 4% of Brazil's GDP and 8% of all Brazilian exports (Jonker et al. 2015). Although *Eucalyptus grandis* Hill ex Maiden is the most planted species in Brazil, *E. microcorys* F. Muell is also widely cultivated in the country, due to its feasible chemical and physical properties for the production of charcoal (Oliveira et al. 2014).

There are about 700 species and sub-species of eucalyptus and at least 150 of them are associated with more than 1400 fungal species (Hyde et al. 2007). Between the years 1995 and 2003 alone, 34 new fungal species were described from eucalyptus trees (Hyde et al. 2007). In addition, Cheewangkoon et al. (2009) described 15 new fungal species associated with *Eucalyptus* spp. Although several studies continue to focus on the microbial diversity in eucalyptus (Lima et al. 2013; Massenssini et al. 2016; Souza et al. 2017; Pavlic-Zupanc et al. 2017), there are no reports in the fungal community associated with *E. microcorys* in Brazil.

Many fungi have been considered recurrent and exclusive in eucalyptus. For example, the order Capnodiales has more than 150 species associated with eucalyptus leaves (Crous and Wingfield 1997; Crous 1998; Hunter et al. 2006; Crous et al. 2006; Cheewangkoon et al. 2008; Crous et al. 2009; Aguín

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et al. 2013). Many of these fungi are endophytic or latent pathogens such as *Cladosporium*, *Mycosphaerella*, *Pseudocercospora*, *Readeriella*, and *Teratosphaeria* (Kharwar et al. 2010; Marsberg et al. 2014; Bernreiter et al. 2016). *Eucalyptus* spp. are considered a cache of fungal diversity and studies that explore the microbiota associated with these plants are essential for the discovery of new species (Cheewangkoon et al. 2009).

Diversity studies of fungi in eucalyptus have been based on culture-dependent techniques, usually involving fungal isolation from disinfected plant surfaces, fragmented and inoculated in artificial media (Schulz et al. 1993). However, the direct plating of plant tissues may have limitations on fungal diversity analysis due to the potential bias favoring fast-growing species that sporulate profusely (Paulus et al. 2003). To circumvent these drawbacks, alternative methods that better represent the endophytic community of a plant host have been applied. The particle filtration technique is used to isolate fungi from leaf litter (Bills and Polishook 1994). This method is based on substrate fragmentation and removal of conidia from the plant surface. As a result, the reduced particle size favors isolation from somatic mycelia embedded in the plant matrix, which increases the chance of recovering isolates that actually colonize the plant matrix, including slow-growing fungi otherwise missed by other methods (Bills and Polishook 1994). This technique better represents the community in the substrate, since the presence of species with a high sporulation rate is less. Some studies have recommended a particle filtration protocol to isolate fungi of litter due to its efficiency in evaluating the diversity of these microorganisms (Bills and Polishook 1994; Paulus et al. 2003; Collado et al. 2007). This technique also has been used for isolation of fungi associated with marine environments such as coral reefs (Barathikannan et al. 2017) and deep-sea sediments (Damare et al. 2006; Singh et al. 2010; Zhang et al. 2014). De Abreu and collaborators (2010) recovered over a thousand endophytic fungal isolates using this technique from the plant species *Phoradendron perrottettii* (DC.) Eichler and *Tapirira guianensis* Aubl. Aside from the latter study, little is known about the efficiency of this technique for endophytic fungi.

Considering the lack of knowledge about endophytic fungi of *E. microcorys*, the aim of this study was to evaluate the diversity of these fungi in a plant species not yet investigated for its endophytes, using two isolation techniques: direct leaf plating and particle filtration.

Material and methods

Study site and sampling

We sampled *E. microcorys* at "Floresta Estadual Edmundo Navarro de Andrade" (FEENA), in the municipality of Rio Claro, State of São Paulo, Brazil. FEENA has more than 2000 ha of eucalyptus planted, and it is known as the "cradle of eucalyptus" in Brazil. Currently, the site is considered to have the highest concentration of eucalyptus species (more than 100) outside their native range in Australia. The climate of this area is characterized by hot and humid summers and cold and dry winters.

Three plants of *E. microcorys* were selected: specimen #1 (S22° 24.876' W47° 32.616′), specimen #2 (S22° 24.891' W47° 32.622′), and specimen #3 (S22° 24.891' W47° 32.648′), approximately 7 m apart. During a year-long survey (April 2015 to April 2016), we sampled each plant specimen twice (in wet and dry seasons). At each sampling, 20 fresh and apparently healthy leaves were collected, thus a total of 120 leaves were sampled at the end of the experiment (20 leaves × 2 sampling periods × 3 plant specimens).

Endophytic fungi isolation

After collection, leaves were processed according to the method described in Crous et al. (1997) with modifications. First, the leaves were washed in running tap water and gently rubbed by hand for 5 min to remove debris. Then, the leaves were surface disinfected, which consisted of immersion for 1 min in 2% NaOCl, 30 s in 70% ethanol, and 1 min in sterile distilled water (SDW). To check for the absence of epiphytic fungi (control), 100 μL of the washing water was surface-spread on the culture media. To compare media with different levels of carbon sources, we selected dicloran rose bengal (DRB, without dicloran, nutrient-rich medium, Paulus et al. 2003) and synthetic nutrient agar (SNA, nutrient-poor medium, Nirenberg 1976), both supplemented with 150 μg mL^{-1} of chloramphenicol and bengal rose. After surface disinfection, two isolation techniques were employed: leaf plating (LP) and particle filtration (PF).

We carried out a modified version of the LP technique (Petrini 1991). Briefly, leaf fragments of approximately 1 × 1 cm^2 were cut and three fragments were inoculated on DRB and SNA media. An adaptation of the PF technique by Bills and Polishook (1994) was used. Two grams of leaves were triturated in a blender for 1 min with 50 mL of SDW. Then, fragments were washed with SDW jets and filtered through a set of four sieves with descending mesh openings (1.0, 0.7, 0.25, and 0.18 mm). The particles retained in the smallest mesh were transferred to disposable tubes and re-suspended in SDW (up to 50 mL). This suspension was shaken for 1 min and allowed to rest for 10 min. The supernatant was discarded and the tube was again filled with SDW (up to 50 mL). We repeated this procedure four times and, finally, the fragments were re-suspended in 20 mL of SDW. Aliquots of 100 μL of this suspension were surface-spread on DRB and SNA media.

All plates were incubated at 25 °C in the dark for 14 days and observed daily. Once a fungus colony appeared this was

transferred onto PCA (potato carrot agar) medium to check for axenic cultures and for further identification.

Morphological and molecular identification

We preliminary grouped fungal isolates in morphospecies based on the colony morphology and microscopic characteristics of reproductive structures following taxonomic keys (Carmichael et al. 1980; Barber et al. 2005; Cheewangkoon et al. 2008; Lombard et al. 2010). Representative isolates of each morphospecies were submitted to molecular identification using the internal transcribed spacer region (ITS), as the universal barcode marker. Genomic DNA was extracted from isolates grown on PCA medium for 7 days at 25 °C. Mycelia were harvested and mechanically broken by agitation in lysis buffer supplemented with glass beads (425–600 μm in diameter, SIGMA), following a modified protocol by Möller et al. (1992). Briefly, we added 5 μL of proteinase K and incubated it at 65 °C for 30 min. Then, 140 μL of 5 M NaCl and 64 μL of 10% CTAB were added and incubated at 65 °C for 60 min. The tubes were centrifuged at 10,000 rpm (Eppendorf microcentrifuge, 5424) for 30 s and 600 μL of chloroform with isoamyl alcohol (24:1) were added. Then, the tubes were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and transferred to 1.5 mL tubes. After this procedure, 300 μL of 100% ice-cold isopropanol and 50 μL of 3M sodium acetate pH 5.2 were added. The suspension was centrifuged at 10,000 rpm for 10 min and removed by single inversion of the tubes, followed by washing with 600 μL of 70% ethanol and centrifuged at 10,000 rpm for 10 min. The ethanol was removed by single inversion. After ethanol drying, 30 μL of Tris EDTA buffer (10 mM Tris; 1 mM EDTA) were added.

The ITS region was amplified with primers ITS4 and ITS5 (White et al. 1990). Amplification reactions consisted of 0.2 mM of each dNTP, ×1 KCl buffer, 2 mM $MgCl_2$, 0.4 μM of each primer, and 0.04 U of Taq polymerase to a final volume of 25 μL. The reactions followed these conditions: 94 °C/3 min, 35 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/2 min. For the isolates that were not identified with ITS, we also amplified the partial ribosomal large subunit (LSU) and small subunit (SSU) regions, using primers LR0/LR5 and NS1/NS4, respectively, according to Thambugala et al. (2014). Amplicon purification was performed with Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The sequencing reaction was performed using BigDye® Terminator v. 3.1 Cycle Sequencing Kit (ThermoFisher), according to the manufacturer's protocol, and then applied in a ABI 3500 sequencer.

Forward and reverse sequences were assembled in BioEdit v. 7.0.5.3 (Hall 1999). Then, contigs were compared with homologous sequences deposited in the databases of the National Center for Biotechnology Information (NCBI)—GenBank (http://www.ncbi.nlm.nih.gov/genbank) by BLASTn (Altschul et al. 1997) and MycoBank (http://www.mycobank.org/). In addition, for the majority of fungal isolates, we carried out phylogenetic analysis to help in the barcode identification. The phylogenetic trees (not shown) were generated in MEGA v.5.2 (Tamura et al. 2011), using the neighbor-joining algorithm, Kimura 2-parameter as nucleotide substitution model and 1000 bootstrap pseudoreplicates. Representative sequences generated in this study were deposited at the NCBI—GenBank under accessions: MF663538–MF663596 (for ITS) and MF664751 (for LSU).

Comparison between isolation techniques

We used a combination of diverse metrics with multivariate analysis to compare both isolation techniques in recovering the fungal community. Simpson (1-*D*) and Shannon (*H'*) diversity indices were calculated and compared by the nonparametric Mann-Whitney test to verify significant differences. Rarefaction curves with 95% confidence intervals were generated using the analytical solution of "Mao tau" to compare the fungi richness obtained by the different isolation techniques. The similarity index (SIMPER) was calculated to verify the contribution of each species to the general differences in fungal composition. In addition, to explore hidden patterns in the structure of fungal communities, we carried out an ordination nonmetric multidimensional scaling analysis (NMDS). For this analysis, we built a matrix with taxa and samples displayed into columns and rows, respectively. The Bray-Curtis index was applied as the distance measure, whose principle is the use of the abundance of the taxa to quantify the dissimilarity between samples. To measure differences observed in NMDS, we carried out a similarity analysis (ANOSIM). The diversity analyses were performed in Past v. 2.17c. (Hammer et al. 2001) and statistical analyses in R v. 3.0.1 (R Core Team 2016). For a better comparison between the techniques, we normalized the data (using the species/isolate ratio).

Results

We recovered a total of 709 isolates from leaves of *Eucalytpus microcorys*. After identification, the isolates were grouped in 59 taxa (Table S1). All of them are reported for the first time as endophytic for *E. microcorys*. The phylum Ascomycota was the most dominant (99% of the isolates), regardless of the technique used, and was represented by the orders: Xylariales (334 isolates), Botryosphaeriales (113), Diaporthales (110), Capnodiales (28), Pleosporales (8), Sordariales (7), Hypocreales (4), Mytilinidiales (1), and Phaeomoniellales (1). Cantharellales (1) and Polyporales (3) were the only orders representing the phylum Basidiomycota (1% of the isolates). A total of 99 out of 709 isolates could not

Table 1 Diversity metrics describing the fungal communities from leaves of *Eucalyptus microcorys* obtained by two isolation techniques: leaf plating (LP) and particle filtration (PF)

Diversity metrics	LP	PF
Richness	48	27
Species/isolates ratio	0.09	0.12
Number of isolates	489	220
Shannon index (*H'*)	2.69	2.54
Simpson index (*1-D*)	0.86	0.88
Chao-1	69.38	49

be identified or assigned to an order. The ascomycetous species *Castanediella eucalypticola* and *Neophaeomoniella eucalypti* were reported for the first time in Brazil.

Regarding the isolation techniques, the LP and PF methods recovered 489 and 220 isolates, respectively. The LP method obtained higher species richness than the PF technique (48 and 27, respectively, Table 1). However, the species/isolates ratio was higher in PF (0.12) when compared to LP (0.09, Table 1). The LP and PF methods recovered similar diversity of fungi, as noted by the absence of significant differences in the Simpson and Shannon indices (Mann-Whitney, $P > 0.05$). Although the techniques presented similar diversity indices, only 16 out of 59 taxa (27.1%) were shared.

Rarefaction curves did not reach an asymptote, even considering both techniques (Fig. 1). This suggests that the richness would continue to increase with a greater sampling effort, regardless of the isolation technique. The LP technique recovered a larger number of rare species (singletons and doubletons), including *Annulohypoxylon moriforme*, *Cytospora austromontana*, *Marssonina californica*, *Nemania abortiva*, *Neophaeomoniella eucalypti*, *Pantospora guazumae*, and *Preussia minimoides* (Table 2).

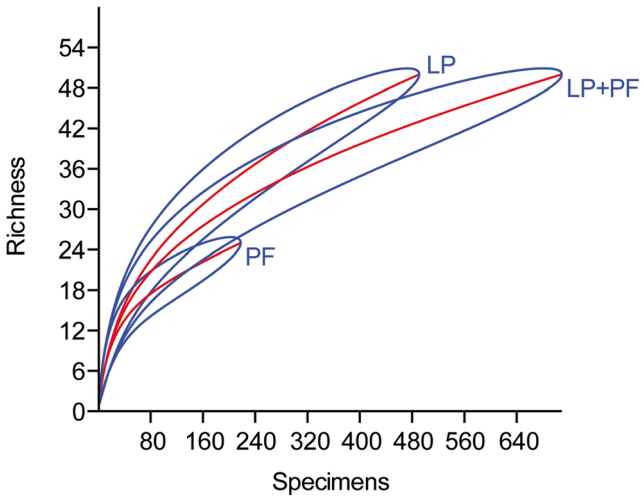

Fig. 1 Rarefaction curves of endophytic fungi richness associated with *Eucalyptus microcorys* leaves recovered by the different isolation techniques. LP, leaf plating technique; PF, particle filtration technique. LP + PF: combination of the two techniques. Blue lines: confidence interval at 95%

The ANOSIM showed significant differences between community compositions obtained by the two techniques ($R = 0.56$, $P = 0.004$). The SIMPER test showed 87.3% dissimilarity among the communities recovered in both techniques, with *Xylaria globosa* (19%), *Xylaria apiculata* (8.3%), *Pseudoplagiostoma eucalypti* (5.7%), *Phyllosticta capitalensis* (5.5%), *Neofusicoccum* sp.2 (4.7%), and *Diaporthe* sp. (4.3%) as the fungi that accounted for the majority of the dissimilarity. These were also the most abundant species (Table 2).

The NMDS analysis showed a clear pattern in the distribution of fungal communities with stress coefficient equal 0. Coordinate 1 (representing 98% of the distribution) suggests structural differences in fungal communities as samples separates by isolation technique: LP (left) and PF (right, Fig. 2). On the other hand, coordinate 2 suggests no structuring of fungal communities by sampling periods (Fig. 2).

Another interesting result was in relation to culture media. The DRB medium was more efficient in the recovery of isolates compared to the SNA, regardless of the technique, 56 and 29 taxa, respectively, because 30 taxa were only observed on the DRB and 3 only by SNA (Table 2). Regarding the isolation time, there was no growth after 10 days of cultivation, regardless of the technique used. Both techniques had the highest number of taxa recovered between the second and fifth day of inoculation.

Discussion

This is the first study on the diversity of endophytic fungi in *Eucalyptus microcorys*. Our evaluation of endophytes from this previously unexplored eucalyptus species suggests a high fungal diversity, in addition to new species records for Brazil. We also showed the impact of the isolation method on the recovery of endophytic fungi. Either LP or PF alone would not recover the breadth of fungal taxa found when considering both methods together. The low number of shared taxa between isolation techniques indicates that culture-dependent studies focused on the endophytic fungal diversity would benefit when different isolation methods are considered.

The use of two or more methods to assess the microbial diversity is expected to increase the number of species recovered. Although LP is widely used for endophytic fungi isolation, PF was useful in the isolation of these microorganisms. Despite the fact that LP recovered a greater number of isolates, PF recovered a more diverse community, according to the species/isolates ratio. This may have occurred due to the diversity of cultivable species increases as the density of the inoculum (leaf fragments) decreases (Collado et al. 2007). This technique also favored sporulating fungi found in leaves

Table 2 Fungi (in number of isolates) recovered from *Eucalyptus microcorys* using two isolation techniques: leaf plating (LP) and particle filtration (PF) and two culture media

Fungi	LP		PF		Total
	DRB	SNA	DRB	SNA	
Annulohypoxylon moriforme (Henn.) Ju, Rogers & Hsieh 2005	1	0	0	0	1
Apoharknessia insueta (B. Sutton) Crous & Lee 2004	3	0	0	0	3
Calonectria sp. De Not. 1867	1	0	1	0	2
Castanediella eucalypticola Crous & Wingf. 2016	0	1	4	5	10
Ceratobasidiaceae G.W. Martin 1948	1	0	0	0	1
Cladosporium cladosporioides (Fresen.) Vries 1952 species complex	3	0	0	1	4
Colletotrichum sp.1 Corda 1831	9	8	0	1	18
Colletotrichum sp.2	6	1	0	0	7
Colletotrichum sp.3	20	1	0	0	21
Cytospora austromontana Adams & Wingf. 2005	1	0	0	0	1
C. eucalypticola Van der Westh. 1965	0	0	2	3	5
C. variostromatica Adams & Wingf. 2005	5	0	0	0	5
Daldinia sp.1 Ces. & De Not. 1863	19	2	4	0	25
Daldinia sp.2	3	0	0	0	3
Diaporthe phaseolorum (Cooke & Ellis) Sacc. 1882	25	7	0	0	32
Dothistroma sp. Hulbary 1941	0	0	2	0	2
Epicoccum sp. Link 1816	4	2	0	0	6
Fusicladium amoenum (Castañeda & Dugan) Crous, Schub & Braun 2007	0	0	1	0	1
Geomyces sp.1 Traaen 1914	0	1	0	0	1
Geomyces sp.2	0	1	0	0	1
Gliocephalotrichum bacillisporum Decock & Huret 2006	0	0	0	2	2
Hypoxylon sp.1 Bull. 1791	2	0	0	0	2
Hypoxylon sp.2	1	0	0	0	1
Marssonina californica (Ellis & Everh.) Magnus 1906	1	0	0	0	1
Nemania sp. Gray 1821	12	6	1	0	19
N. abortiva Rogers, Ju & Hemmes 2006	1	0	0	0	1
N. difusa (Sowerby) Gray 1821	8	3	12	0	23
Neofusicoccum sp. Crous, Slippers & Phillips 2006	10	0	0	0	10
N. parvum (Pennycook & Samuels) Crous, Slippers & Phillips 2006	24	0	6	19	49
Neophaeomoniella eucalypti Rooney-Lath. & Crous 2015	1	0	0	0	1
Neurospora discreta Perkins & Raju 1986	1	0	4	2	7
Nigrospora oryzae (Berk. & Broome) Petch 1924	12	0	1	0	13
Pantospora guazumae Cif. 1938	2	0	0	0	2
Phlebia floridensis Nakasone & Burds. 1995	0	0	1	0	1
Phyllosticta capitalensis Henn. 1908	8	0	30	16	54
Preussia minimoides (S.I. Ahmed & Cain) Valldos. & Guarro 1990	2	0	0	0	2
Pseudocercospora norchiensis Crous 2007	0	0	2	5	7
Pseudoplagiostoma eucalypti Cheew., Wingf. & Crous 2010	18	1	41	3	63
Pseudoplagiostoma sp. Cheew., Wingf. & Crous 2010	0	0	1	0	1
Readeriella eucalypti (Gonz. Frag.) Crous 2006	0	0	4	9	13
Satchmopsis brasiliensis Sutton & Hodges 1975	0	0	3	9	12
Skeletocutis diluta (Rajchenb.) David & Rajchenb. 1992	0	1	1	0	2
Teratosphaeria ohnowa (Crous & Wingf.) Crous & Braun 2007	0	0	1	0	1
Xylaria sp.1 Hill ex Schrank 1789	2	1	0	0	3
Xylaria sp.2	1	0	0	0	1
Xylaria sp.3	6	2	1	0	9

Table 2 (continued)

Fungi	LP		PF		Total
	DRB	SNA	DRB	SNA	
Xylaria sp.4	1	0	0	0	1
Xylaria sp.5	2	0	0	0	2
Xylaria sp.6	1	1	0	0	2
X. globosa (Pers.) Mont. 1855	109	49	7	1	166
X. berteroi (Mont.) Cooke ex Rogers & Ju 2012	2	0	0	0	2
X. apiculata Cooke 1879	46	18	0	0	64
FNI 1	0	0	1	0	1
FNI 2	1	0	0	0	1
FNI 3	1	0	0	0	1
FNI 4	1	0	0	0	1
FNI 5	1	0	0	0	1
FNI 6	1	1	1	12	15
FNI 7	2	1	0	0	3
Total	381	108	132	88	709

FNI non-identified fungi *DRB* dicloran rose bengal *SNA* synthetic nutrient agar

of *E. microcorys*, as *Pseudoplagiostoma eucalypti*, *Readeriella eucalypti*, and *Satchmopsis brasiliensis*. Previous studies in various substrates showed the efficiency of PF in recovering an increased diversity compared to traditional isolation methods (Bills and Polishook 1994; Paulus et al. 2003; Collado et al. 2007). Because there is little

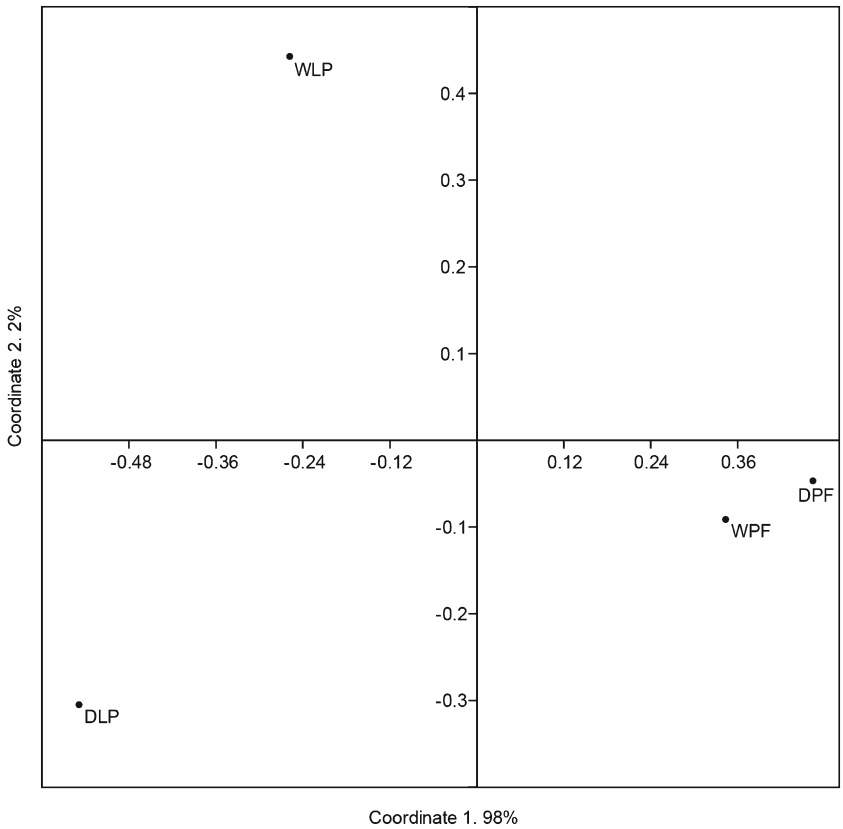

Fig. 2 Nonmetric multidimensional scaling analysis (NMDS) demonstrating the distribution of the endophytic fungal community in leaves of *Eucalyptus microcorys*. W, wet; D, dry; LP, leaf plating technique; PF, particle filtration technique. Stress: 0.0

knowledge about the applicability of this technique for endophytic fungi, our results suggest that PF may be considered when assessing the endophytic fungal diversity, supporting previous claims on this matter (Unterseher and Schnittler 2009).

The order Xylariales was the most abundant, and *Xylaria* was the most recurrent taxa. This genus is considered cosmopolitan (except for the poles) and apparently has no host specificity. *Xylaria* spp. are frequently reported as endophytes in several plant species such as: *Arundina graminifolia* (D. Don) Hochr. (Orchidaceae), *Embothrium coccineum* Forst. & Forst. (Proteaceae), *Khaya anthotheca* (Welw.) C. DC. (Meliaceae), *Piper aduncum* L. (Piperaceae), *Vanilla planifolia* Jacks. ex Andrews (Orchidaceae), including *Eucalyptus* spp. (Lupo et al. 2001; Oliveira et al. 2011; Linnakoski et al. 2012; González-Teuber 2016; Rajulu et al. 2016). The LP technique allowed the isolation of a large number of xylariaceous fungi, recovering 296 out of 334 isolates. Perhaps, these fast-growing fungi may have outnumbered others in the isolation plates due to the size of the leaf fragment. In contrast, the PF method may have reduced the load of these microorganisms from the samples used for isolation. Nevertheless, *Castanediella eucalypticola* (Xylariaceae) was only isolated by the PF method. This species was described by Crous et al. (2016) on leaf spots of *Eucalyptus robusta* Sm. in France, and it is reported here as a new occurrence for Brazil (and America).

Botryosphaeriales were also largely represented in this study. More than 25 species in several genera of Botryosphaeriaceae have been reported from *Eucalyptus* leaves as endophytes and pathogens (Barradas et al. 2016). The genus *Neofusiccocum* is considered an aggressive pathogen of several plants, including eucalyptus species. *Botryosphaeria dothidea* (Moug.) Ces. & De Not., previously classified as a *Neofusiccocum* anamorph, causes severe damage to crops worldwide, and it is reported to cause latent endophytic infections (Smith et al. 1996). In addition, *Phyllosticta capitalensis* is associated with a wide range of hosts, causing symptoms such as leaf spots and fruit necrosis (Wulandari et al. 2009; Wang et al. 2012; Wikee et al. 2013).

Another new occurrence recovered for Brazil was *Neophaeomoniella eucalypti* (Phaeomoniellales), previously reported by Rooney-Lath and Crous (Crous et al. 2015) on stems of *Eucalyptus globulus* in California (USA). In this study, *N. eucalypti* was considered a rare species, because only one isolate was recovered by the LP technique.

Culture media also influence the recovering of endophytic fungal diversity. DRB (nutrient-rich) recovered a higher number of isolates and species diversity than SNA (poor in carbon sources). Fungi grow more slowly on SNA than on DRB, probably due to the low amount of nutrients present in the former. The efficiency of DRB may be attributed to the amount of simple sugars available. Other studies have demonstrated the effectiveness of the DRB for isolating saprobe fungi (Bills and Polishook 1994; Polishook et al. 1996; Costa et al. 2015). Although SNA was not efficient for recovering the endophytic fungi, Fakhrunnisa and Ghaffar (2006) had satisfactory results using this culture medium for the isolation of *Fusarium*. Apparently, the use of carbon-rich media, such as modified DRB and others commonly used (potato-dextrose agar and oatmeal agar), may be useful for the isolation of endophytic fungi.

The similar diversity indices and the high dissimilarity index of the communities recovered by the different techniques demonstrate they complement each other. For optimizing endophytic fungal isolation, we suggest that PF could be used as a complimentary method to LP to assess the communities of these fungi.

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