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Fungal diversity in soils across a gradient of preserved Brazilian Cerrado

Ademir Sergio Ferreira de Araujo[1*], Walderly Melgaço Bezerra[4], Vilma Maria dos Santos[1], Luis Alfredo Pinheiro Leal Nunes[1], Maria do Carmo Catanho Pereira de Lyra[2], Marcia do Vale Barreto Figueiredo[3], and Vania Maria Maciel Melo[4]

[1]*Soil Quality Lab., Agricultural Science Center, Federal University of Piauí, Teresina, PI, Brazil*
[2]*Genome Lab., Agronomic Institute of Pernambuco, Recife, PE, Brazil*
[3]*Genetic and Molecular Biology Lab., Agricultural Science Center, Federal University of Piauí, Teresina, PI, Brazil*
[4]*Microbial Ecology and Biotechnology Lab., Federal University of Ceara, Fortaleza, CE, Brazil*

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The preserved Cerrado from Northeastern Brazil presents different physicochemical properties and plant diversity, which can influence the fungal communities. Therefore, we evaluated the fungal diversity in preserved sites, at Sete Cidades National Park, across a gradient of vegetation that included Campo graminoide, Cerrado stricto sensu, Cerradao, and Floresta decidual. Of all of the operational taxonomic units (OTUs) obtained, the Floresta decidual presented the highest richness. Ascomycota were the most abundant phylum (45%), followed by Basidiomycota (32%). Basal fungi and other phyla accounted for 23% of the total dataset. Agaricomycetes, Eurotiomycetes, Lecanoromycetes, Basidiobolus, Dothideomycetes, and Taphrinomycetes were the most abundant classes of fungi found across the gradient of Cerrado vegetation. In conclusion, our study suggests that the Brazilian Cerrado from Sete Cidades National Park presents a high fungal diversity and includes sources of new fungal species for biotechnological purposes.

Keywords: soil microbiology, eukaryotic microorganisms, fungi

Introduction

The Brazilian Cerrado is an ecosystem with high biodiversity and covers approximately 25% of the Brazilian territory (Forzza *et al.*, 2010). This ecosystem usually presents vegetation distributed across a gradient that includes 'Campo graminoide', 'Cerrado stricto sensu', 'Cerradao', and 'Floresta decidual' (Coutinho, 1978; Castro *et al.*, 1999). These different types of vegetation influence the soil physicochemical properties (Ruggiero *et al.*, 2002; Lucena *et al.*, 2014) and plant diversity (Oliveira *et al.*, 2007) across this gradient.

In the Cerrado in Northeastern Brazil, the Sete Cidades National Park, which is a protected area with 6,221 ha, was established with the aim of studying and conserving its biological diversity. Specifically, the 'Programa Ecológico de Longa Duração' (PELD) has studied the plant diversity within the Sete Cidades National Park, and previous studies have confirmed the hypothesis that different levels of plant diversity exist across this gradient of the Cerrado (Castro *et al.*, 1998, 1999). However, it is unclear whether the fungal community follows this plant diversity gradient. In Brazilian Cerrado, previous studies have focused on the effect of land-use on the microbial diversity, specifically the fungal communities (Castro *et al.*, 2008; Oliveira *et al.*, 2016).

Tropical ecosystems present the highest soil biodiversity worldwide, and the fungal communities have high diversity in these ecosystems. Fungi are eukaryotic organisms in the soil that perform important functions in the C, N, and P turnover (Rachid *et al.*, 2015) and have ecological roles as decomposers, pathogens, and symbionts (Newsham *et al.*, 1995). Therefore, an evaluation of the fungal diversity is important because these microorganisms influence nutrient cycling, organic matter decomposition, and the promotion of plant growth (Crowther *et al.*, 2012; Babu *et al.*, 2015; Larsen *et al.*, 2015). Unfortunately, there is little information about the fungal communities' diversity and distribution compared with those of their plant counterparts (Karst *et al.*, 2013). Moreover, according to Karst *et al.* (2013), the diversity and distribution of fungal species are often affected by the vegetation gradient because there is a significant shift in plant diversity across the gradient. Further, these organisms are influenced by several abiotic factors, such as physicochemical properties (Posada *et al.*, 2008).

Therefore, because the PELD identified different patterns in plant diversity (Castro *et al.*, 1998, 1999) and physicochemical properties (Ruggiero *et al.*, 2002) across a gradient of Cerrado within the Sete Cidades National Park, we hypothesized that there would be distinct soil fungal communities across this gradient. To test this hypothesis, we assessed the fungal taxonomic diversity by using metagenomic next-generation sequencing (NGS) and soil physicochemical properties to explore how the environment shapes the fungi alpha and beta diversity. We used the amplicon sequencing method to assess the fungal diversity as this method serves as a robust approach for reliably sequencing amplicons of large scale samples from various communities (Wu *et al.*, 2015).

*For correspondence. E-mail: asfaruaj@yahoo.com.br; Tel.: +55-86-3215-5740; Fax: +55-86-3215-5743

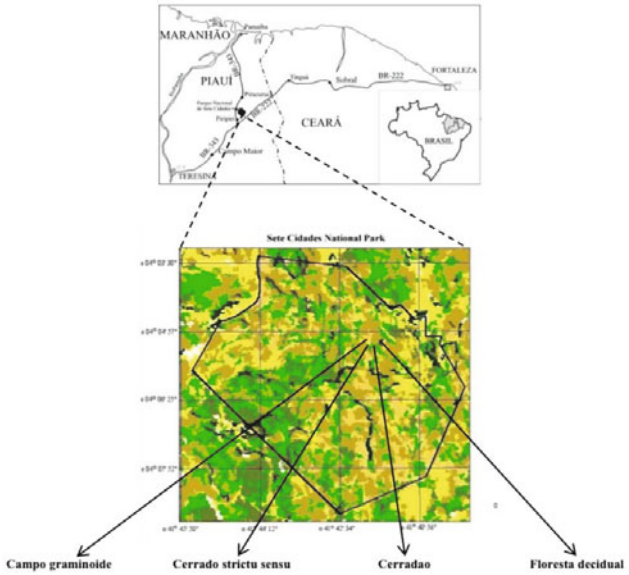

Fig. 1. **Map presenting the gradient of cerrado at Sete Cidades National Park, Brazil.**

Materials and Methods

Study area

The study was conducted within the Sete Cidades National Park (PNSC; 04º02′-08′S and 41°40′-45′W), located in the northeastern state of Piauí, Brazil. The park covers an area of 6,221 ha. The climate is sub-humid moist, with a great deficiency of water and a small annual thermal amplitude. There are two distinct seasons (wet and dry) during the year, and the annual average temperature is 25°C. The area has an annual average rainfall of 1,558 mm, which primarily occurs in February, March and April.

We evaluated preserved sites (with 1,000 m^2 each) in the Cerrado, inserted in the 'Programa Ecológico de Longa Duração' (PELD) of the Brazilian government, across a gradient of different Cerrado formations that included Campo graminoide, Cerrado stricto sensu, Cerradao, and Floresta decidual (Fig. 1 and Table 1). Basically, Campo graminoide is covered with a continuous grass stratum; Cerrado stricto sensu is covered by grass, shrubs, low trees and a woody stratum; Cerradao is covered by a woody stratum with varying density of shrubs and trees; and Floresta decidual is covered by trees (Coutinho, 1978).

Soil sampling and chemical analysis

Each preserved site (Campo graminoide, Cerrado stricto sensu, Cerradao, and Floresta decidual) was divided in three transects (replication), where soil samples (three points per transect; nine samples per site) were collected, at a depth of 0–20 cm in March (wet season) 2014. All of the soil samples were immediately stored in sealed plastic bags and transported in an ice box to the laboratory. A portion of the soil samples was stored in bags and kept at -20°C for DNA analysis (36 samples), and another portion was air-dried, sieved through a 2-mm screen and homogenized for chemical analyses (36 samples).

The soil chemical properties were determined and measured using standard laboratory protocols. The soil pH was determined in a 1:2.5 soil/water extract. The available P and exchangeable K^+ were extracted using the Mehlich-1 extraction method and determined by colorimetry and photometry, respectively (Table 2). The total organic C (TOC) was determined by the wet combustion method using a mixture of potassium dichromate and sulfuric acid under heating.

DNA extraction and library preparation

The soil DNA was extracted from 0.5 g (total humid weight) of soil using the PowerLyzer PowerSoil DNA Isolation Kit (MoBIO Laboratories), according to the manufacturer's instructions. DNA extraction was performed in triplicate for each soil sample. The quality and relative quantity of the extracted DNA were determined using a Thermo Scientific NanoDrop 2000.

The V9 region of the 18S rRNA gene was amplified with region-specific primers (1391F/1510R; Amaral-Zettler *et al.*, 2009). Each 25 µl PCR reaction contained 14.8 µl of PCR water (Certified Nuclease-free, Promega), 2.5 µl of 10× high-fidelity PCR buffer (Invitrogen), 1.0 µl of 50 mM $MgSO_4$, 0.5 µl of dNTP (10 mM each), 0.5 µl of each primer (10 µM concentration, 200 pM final), 1.0 unit of platinum Taq polymerase high-fidelity (Invitrogen), and 4.0 µl of template DNA. The conditions for this second-round PCR were as follows: 95°C for 3 min to denature the DNA, with 8 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 min, with a final extension of 5 min at 72°C. After the first step of amplification, the amplicons were subjected to a second round of PCR

Table 1. **Vegetation indices of the evaluated sites (Oliveira *et al.*, 2007)**

	Campo graminoide	Cerrado stricto sensu	Cerradao	Floresta decidual
Plant richness*	4.7	11	17	18
Plant diversity**	0.2	0.85	1.10	1.11
Plant density***	4.7	27.1	35.0	51.8
Vegetation****	a	b	c	d

* species/100 m^2; ** H/100 m^2; *** individual/100 m^2; **** species of plants present
[a] *Andropogon fastigiatus; Aristida longifólia; Eragrostis maypurensis*
[b] *Andropogon fastigiatus; Aristida longifolia; Terminalia fagifolia; Magonia pubescens; Hymenaea courbaril; Plathymenia reticulata; Qualea grandiflora; Combretum mellifluum; Lippia origanoides; Anacardium occidentale; Simarouba versicolor; Vatairea macrocarpa*
[c] *Aspidosperma discolor; Parkia platycephala; Terminalia fagifolia; Piptadenia moniliformis; Plathymenia reticulata; Qualea parviflora; Anacardium occidentale; Copaifera coriácea; Thiloa glaucocarpa; Casearia grandiflora.*
[d] *Aspidosperma multiflorum; Aspidosperma subincanum; Campomanesia aromática; Casearia lasiophylla; Casearia ulmifolia; Copaifera coriácea; Ephedranthus pisocarpus; Piptadenia moniliformis; Pterocarpus violaceus; Thiloa galucocarpa.*

Table 2. Average of soil physicochemical properties at different sites across the gradient of Cerrado

Site	Moisture (%)	TOC (g/kg)	pH	P (mg/kg)	K ($cmol_c$/kg)	CEC ($cmol_c$/kg)
Campo graminoide	7.3 ± 1.3[c]	4.3 ± 0.9[c]	4.5 ± 0.1[c]	3.9 ± 0.1[c]	1.4 ± 0.1[b]	2.28 ± 0.2[b]
Cerrado stricto sensu	10.5 ± 2.1[b]	8.3 ± 4 [b]	4.3 ± 0.1[b]	3.9 ± 0.3[b]	1.8 ± 0.1[b]	2.31 ± 0.1[b]
Cerradao	11.9 ± 3.7[b]	9.1 ± 2.9[b]	4.6 ± 0.2[b]	4.5 ± 0.2[b]	1.6 ± 0.2[b]	2.35 ± 0.3[b]
Floresta decidual	31.8 ± 5.8[a]	15.2 ± 4.8[a]	4.9 ± 0.3[a]	5.3 ± 0.3[a]	3.8 ± 0.3[a]	4.91 ± 0.5[a]

TOC, total organic C; CEC, cation exchange capacity
Different letters in each column indicate statistically significant differences ($P < 0.01$).

to insert the Illumina NexteraXT indexes (Illumina), according to the manufacturer's instructions. After this second step, the PCR products were cleaned by using Agencourt AMPure XP–PCR purification beads (Beckman Coulter), according to the manufacturer's manual, and were then quantified using a dsDNA BR assay Kit (Invitrogen) on a Qubit 2.0 fluorometer. Once quantified, different volumes of each of the products were pooled into a single tube such that each amplicon was represented equally. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 8.0 pM with a 20% PhiX spike for loading into the Illumina MiSeq sequencer.

Processing and analysis of sequencing data

Sequence data were processed using QIIME (Caporaso *et al*., 2010a) following the UPARSE standard pipeline (Edgar, 2013) according to Brazilian Microbiome Project (Pylro *et al*., 2014, 2016) to produce an OTU table and a set of representative sequences. Briefly, reads were truncated at 150 bp and quality-filtered using a maximum expected error value of 0.5. Pre-filtered reads were dereplicated and singletons were removed using USEARCH 7.0 (Edgar, 2010). These sequences were clustered into OTUs at a 97% similarity cutoff following the UPARSE pipeline. After clustering, sequences were aligned and taxonomically classified against the Silva database (version 111) (Quast *et al*., 2013) using the PyNAST algorithm (Caporaso *et al*., 2010b). The sequences were submitted to the NCBI Sequence Read Archive under the number SRP091586.

The strategy of rarefaction (random sub-sampling) was used to normalize the number of sequences per sample and evaluate the sequencing effort. Good's index was calculated to estimate the coverage reached using the rarefaction level chosen. The microbial diversity was evaluated using observed operational taxonomic units (OTUs), ACE, Chao1, Shannon, and Simpson as alpha diversity metrics (calculated from nine soil samples per site). Beta diversity matrices were generated using unweighted UniFrac phylogenetic distances (Lozupone and Knight, 2005) and evaluated by bi-dimensional Principal Coordinates Analysis (PCoA).

Results

The soil physicochemical properties were similar in Cerrado stricto sensu and Cerradao and contrasted with those of Campo graminoide and Floresta decidual (Table 2). The Campo graminoide and Floresta decidual presented the lowest and highest soil moisture and organic matter content, respectively. The soil pH, P, K, and CEC did not vary among the Campo Graminoide, Cerrado stricto sensu and Cerradao, but they were highest in the Floresta decidual.

A total of 439,255 reads were clustered in 1,971 OTUs using Brazilian Microbiome Project pipeline for 18S amplicons. Alpha- and beta-diversity analyses were performed with a subsample of 1,081 reads per sample. 'Floresta decidual' presented highest richness, as revealed by Chao1 and Faith's PD estimators, followed by 'Cerradao', 'Cerrado *stricto sensu*' and 'Campo graminoide' (Table 3). Rarefaction curves (calculated using a cutoff of 0.03 similarity of 18S rRNA genes) yielded the same pattern of richness as Chao1 and Faith's PD and revealed a similar depth for all sites.

Analyses of unweighted UniFrac phylogenetic distance showed the behavior of fungal communities across the gradient (Fig. 2). When taken in account unweighted Unifrac metrics (OTUs presence/absence), fungal communities from 'Campo graminoide' and 'Floresta decidual' appeared more distinct from each other, while 'Cerrado *stricto sensu*' and 'Cerradao' were quite similar (Fig. 2A). In the other hand, weighted Unifrac metrics explained 52.08% of variation suggesting that the ordination of the fungal communities in all sites are more related to the OTUs relative abundance (Fig. 2B).

Ascomycota, Basidiomycota, and basal fungi were the most abundant phyla (47%, 30%, and 18%, respectively) and other phyla accounted for 5% of total dataset. Dominance of the most abundant phyla varied according to different sites (Fig. 3). Ascomycota was the most abundant in 'Cerrado *stricto sensu*' and 'Cerradao' (Fig. 3B and D), whereas Basidiomycota was the most abundant phyla in 'Floresta decidual' (Fig. 3C). 'Campo graminoide' presented a codominance of Ascomycota and Basal fungi (Fig. 3A).

Agaricomycetes (33.6%), Eurotiomycetes (31%), Lecanoromycetes (19.6%), Basidiobolus (4.6%), Dothideomycetes

Table 3. Fungal richness and diversity estimation (calculated from nine soil samples for each site) derived from 18S rRNA across a gradient of Cerrado

Sites	Chao 1	Faith's PD	Shannon	Observed OTUs	Good's Coverage
Campo graminoide	421[a]	25.30	6.33	363[a]	0.9276
Cerrado stricto sensu	445[ab]	25.97	6.03	368[a]	0.9153
Cerradao	519[bc]	28.69	6.13	439[ab]	0.9181
Floresta decidual	540[c]	28.86	5.68	468[b]	0.9053

Different letters in each column indicate statistically significant differences ($P < 0.01$).

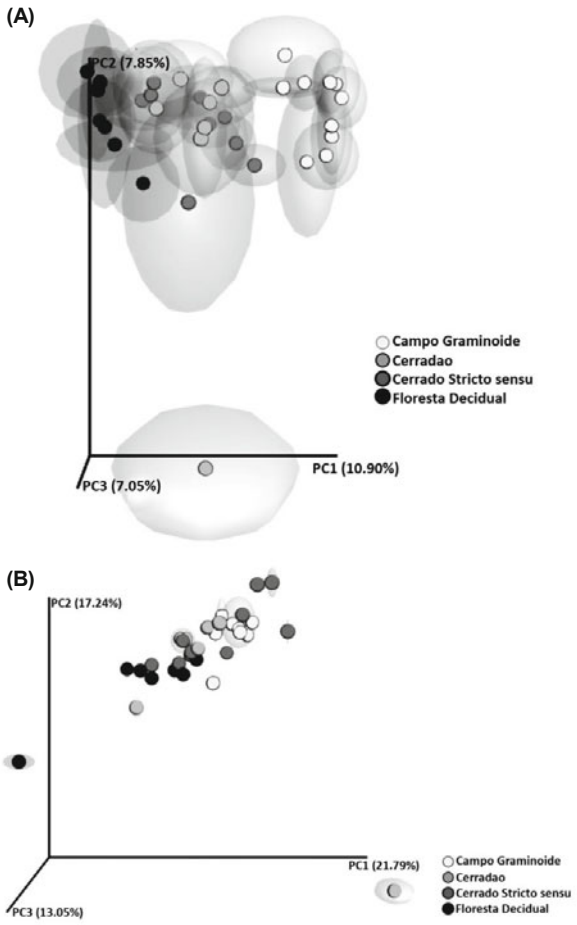

Fig. 2. Principal coordinate analysis of fungal communities in soils across a gradient of tropical Cerrado from Northeast Brazil. Sequences were rarified at the same sequencing depth (1,080 reads) and the abundances matrices were generated using unweighted (A) and weighted (B) UniFrac phylogenetic distances.

(3.4%), and Taphrinomycetes (1%) were the most abundant classes of fungi found across gradient of Cerrado. Other classes accounted for 6.8% of total dataset. From Basidiomycota group, Agaricomycetes were the most abundant in 'Floresta decidual'. This class, together with Eurotiomycetes and Lecanoromycetes (both Ascomycota classes), were equally represented in 'Cerrado *stricto sensu*' and 'Cerradao' (Fig. 4).

Discussion

The physicochemical properties of the soil and the status of vegetation across the gradient of Cerrado showed that both Cerrado stricto sensu and Cerradao present high similarity, whereas Campo graminoide and Floresta decidual are contrasting sites. Campo graminoide showed a high presence of grasses species and a lower soil pH, organic C content, and soil humidity. In contrast, Floresta decidual presented a high dominance of trees species and soil properties, highlighted by high organic C, TOC, pH, and moisture. Thus, these contrasting soil properties and plant communities were important drivers for separating the fungal communities across the biome.

Next-generation sequencing survey in the four Cerrado sites retrieved more than 1,900 OTUs of eukaryotes, and this estimate are higher than those of previous studies that evaluated the soil of Cerrado from the Brazilian Central Plateau (Castro *et al.*, 2008, 2016). Floresta decidual had more fungal richness, according to the rarefaction curves, which also showed small variation in the total number of OTUs from all of the evaluated sites. Conversely, Campo graminoide appears to present the highest diversity, as indicated by the alpha diversity indices. This pattern disagrees with a recent study, which highlighted the forest as the habitat that has greater fungal diversity in the Brazilian Cerrado (Castro *et al.*, 2016). The highest value of fungal diversity in Campo graminoide was

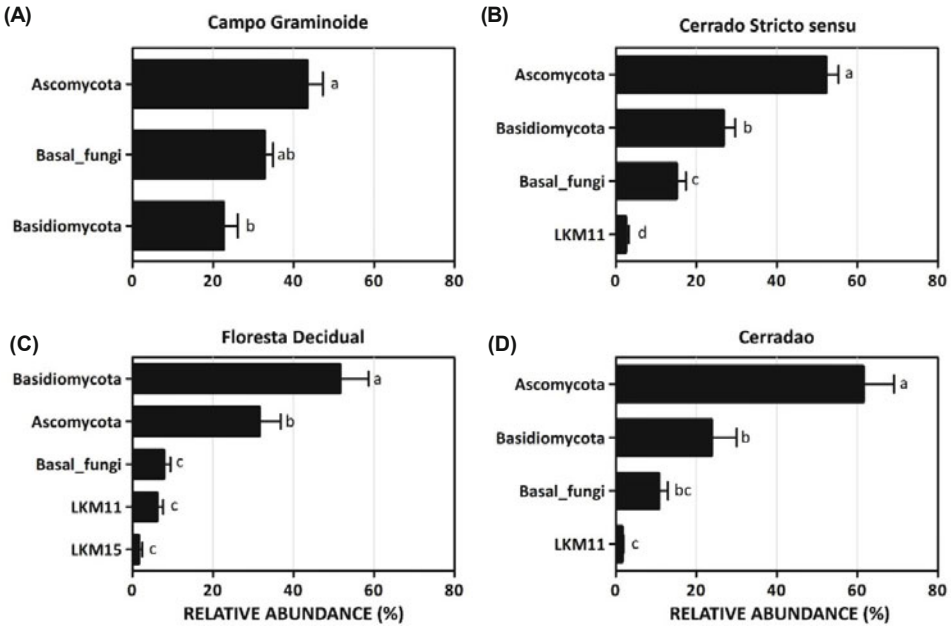

Fig. 3. Relative abundance (RA>1%) of fungal phyla from each soil library derived from Cerrado areas. (A) 'Campo Graminoide'; (B) 'Cerrado *stricto sensu*'; (C) 'Cerradao'; (D) 'Floresta Decidual'. *Bars*, standard error. Different letters indicate statistically significant differences between groups ($P < 0.01$).

explained by the dominance of basal fungi in that site. This group comprises four diverse fungal subgroups (arbuscular mycorrhizal fungi, microsporidia, Chytrids, and Zygomycetes) that form the base of the fungal phylogenetic tree (Leeder *et al*., 2011).

The analysis of phylogenetic distance indicated that the fungal community presented significant differences among the sites. Cerrado stricto sensu and Cerradao showed high similarities in their fungal communities, which may be related to the similar status of vegetation and soil properties. Conversely, the contrast in the fungal communities among Floresta decidual and Campo graminoide reflected the differences in the vegetation and soil properties of these sites. Similar findings were observed by Castro *et al.* (2016), who observed a distinction in the fungal communities between Campo graminoide and Floresta decidual from Cerrado stricto sensu and Cerradao.

The results showed the dominance of Ascomycota and Basidiomycota (more than 70% of the total) across the gradient of Cerrado from Sete Cidades National Park. Ascomycota were dominant in Cerrado stricto sensu and Cerradao, probably because these areas share similar physicochemical characteristics and vegetation; i.e., they are mainly composed of grasses, shrubs, low trees, and a woody stratum. Therefore, there is a high variety of plant litter and different C compounds. Ascomycota are known for producing enzymes (Ma *et al*., 2013) that are involved in the degradation of biopolymers, such as cellulose and hemicellulose (Castro *et al*., 2016) found in plant litter, which may explain their high abundance in Cerrado stricto sensu and Cerradao.

In contrast, Basidiomycota presented the highest dominance in Floresta decidual, which confirms the characteristic of this phylum to survive better under conditions of high organic matter content (Castro *et al*., 2016). In addition, the group of Basidiomycetes is involved in C cycling in several forest environments and acts as wood decomposers, mainly with high contents of complex C compounds (Paul, 2014). Therefore, Floresta decidual, with its dominance of trees with high lignin content, contributed to the highest dominance of Basidiomycota in this site.

Agaricomycetes were the most abundant class of fungi found across the gradient of Cerrado and predominated in Floresta decidual. Representatives of this class of fungi can be found in soils from different environments (Tedersoo *et al*., 2010), where they are key players in wood and litter decomposition (Gerlach *et al*., 2013). Furthermore, some species can be pathogens (Hibbett, 2006), parasites (Ghobad-Nejhad *et al*., 2010), and symbionts, such as ectomycorrhizas of trees (Tedersoo *et al*., 2010).

Eurotiomycetes, Lecaromycetes, Pezizomycetes, and Dothideomycetes were the most abundant Ascomycetes across the gradient. These fungi are filamentous endophytes, such as pathogens, associated with tropical plants (Arnold and Lutzoni, 2007), and present a high biochemical diversity and potential to be used in bioprospecting (Arnold, 2011). Specially, the class Eurotiomycetes presents chemical diversity and antibiotic activity (Lutzoni *et al*., 2011) that are interesting for biotechnological use. The class Lecanoromycetes is the third largest known class of fungi (after Agaricomycetes and Dothideomycetes), and more than 95% participate in lichen association (Kirk *et al*., 2008). Ecologically, lichens are known as decomposers and can fix nitrogen from the atmosphere and release for plants (Seneviratne and Indrasena, 2006). In addition, lichens produce metabolites with antiseptic activities and antimicrobial compounds for medicinal use (Muller, 2001). Taphrinomycetes are early diverging ascomycetes in the phylum Ascomycota (Sugiyama *et al*., 2006). Fungi of this class depend largely on their host plants and have limited growth in soil substrate in the absence of their hosts (Jumpponen, 2003).

As representatives of basal fungi, the class Basidiobolus comprises a group of filamentous fungi that are commonly found in tropical regions of Africa, South America, and Asia (Gugnani, 1999). These fungi act on the decomposition of organic

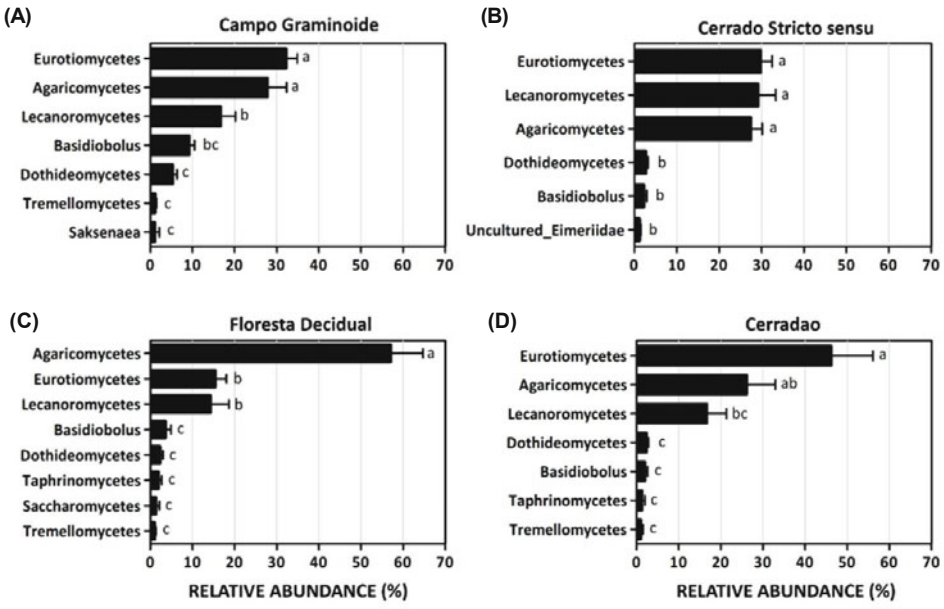

Fig. 4. Relative abundance (RA>1%) of fungal classes from each soil library derived from Cerrado areas. (A) 'Campo Graminoide'; (B) 'Cerrado *stricto sensu*'; (C) 'Cerradao'; (D) 'Floresta Decidual'. *Bars*, standard error. Different letters indicate statistically significant differences between groups ($P < 0.01$).

matter and serve as pathogens that cause diseases in humans (Kahn *et al*., 2001). The main representative species from this class is *Basidiobolus ranarum*, which is present in decaying leaf litter and acts as a commensal in the gut of frogs (Gugnani, 1999). In this study, we detected unclassified fungi that represented approximately 10% of the total OTUs, which suggests that a significant amount of fungal species are still unknown and reinforces the idea that further studies are necessary to assess this important genetic resource.

The published studies about fungal communities in soils of the Cerrado have compared the conversion of native Cerrado for croplands, and they found marked shifts in fungal diversity (Castro *et al*., 2008; Oliveira *et al*., 2016). In contrast, studies that focused on the fungal diversity in the soil of preserved native Cerrado physiognomies are scarce. Recently, Castro *et al*. (2016) have surveyed the fungal diversity in the Cerrado from the Central Plateau, Brazil, and compared four physiognomies that ranged from savanna grassland to forest formations. The results observed by Castro *et al.* (2016) showed a dominance of Ascomycota and Basidiomycota in all of the physiognomies. Similarly, our study compared a gradient of Cerrado from Northeastern Brazil and found a dominance of Ascomycota and Basidiomycota; however, the distribution of the fungal communities was different than those observed by Castro *et al*. (2016). Similarly, there was a different abundance of phyla according to physicochemical properties and plant coverage, i.e., Ascomycetes in Cerrado and Cerradao and Basidiomycetes in Floresta decidual. Therefore, our results highlight the hypothesis of the existence of a hotspot of fungal diversity in preserved Cerrado inside the Sete Cidades National Park. Our results indicate a potential source of new fungal species for biotechnological purposes and industrial applications in preserved Cerrado from protected National Parks in Northeastern Brazil.

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