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RESEARCH ARTICLE

RICHNESS OF MACROSCOPIC FUNGI (BASIDIOMYCOTA): A SPACE TO CONSERVE

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ABSTRACT

Knowledge of the diversity and ecology of fungi in forest stands is important for understanding the interactions between these microorganisms and plant communities. The objectives of this study were to collect, identify and classify macroscopic fungi (Agaricomycetes) found in forest fragments and adjacent areas in the Forest Research Center of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) and surroundings, in the state of Rio Grande do Sul, Brazil. This search was conducted from April to June 2013, in fragments of pine, black wattle, eucalyptus, native species, mixed stands, as well as in grasslands near forest fragments. We found 40 species of fungi, 29 considered ectomycorrhizal, eights aprophytic and three lignocellulolytic species. Among the ectomycorrhizal fungi were species belonging to nine different genera: *Amanita, Descomyces, Lactarius, Pisolithus, Ramaria, Rhizopogon, Russula, Scleroderma* and *Suillus*. Seven saprophytic fungi genus were collected: *Calvatia, Gymnopilus, Lepiota, Lepista*, *Leucoagaricus, Macrolepiota* and *Stropharia*. Among lignocellulolytic fungi were three species of the *Ganoderma* genus. Samples of specimens of fungi collected in this study were stored to allow molecular studies. This scientific survey confirmed the existence of a large variety of macroscopic fungi in the forest fragments of the Forest Research Center (Rio Grande do Sul State, Brazil), demonstrating the potential of these sites as a source of mycorrhizal, saprophytic and lignocellulolytic fungi besides the importance of these habitats for the preservation of genetic heritage.

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INTRODUCTION

Despite the richness of the Brazilian flora, the country has a lack of research to provide knowledge of both the native forest species as well as the associations between forest species and symbiotic microorganisms, which would serve as a reference for programs for recovery and management of natural areas (Hobbie, 2006). The huge microbial diversity of Brazilian biomes represents an untapped reservoir of new genes and metabolisms that play pivotal roles in environmental health as well for biotechnological discovery and applications (Bruce *et al.,* 2012). In forest environments, it is important to observe the presence of several fungi classes in the development of plants and in the maintenance of ecosystem dynamics, where they contribute to nutrient cycling and decomposition of organic matter present in the litter (Rossman *et al.,* 1998; Vellinga *et al.,* 2009). Among fungi, the mycorrhizal, saprophytic and lignocellulolytic groups are frequently found in places where there are woody species (Sulzbacher, 2010). However, their occurrence in fragments of native forest and exotic forest stands in Rio Grande do Sul (RS) State has not been studied. Tedersoo (2010) described the need for field studies for the purpose of better understanding the biogeography of these microorganisms in South America. According to Sulzbacher (2010), understanding the distribution, diversity and evolutionary lines of symbiotic fungi is essential to understanding the diversity of symbiotic fungi in Brazilian ecosystems, which will allow the establishment of relationships between biotic and abiotic factors that influence the occurrence of these fungi and their symbiosis with host plants and, thus, link the occurrence of certain fungal species with specific ecosystems. Fungi are called saprophytic when the source of organic matter is decomposing, or parasites, when they grow in living material (Esposito and Azevedo, 2010). When they form associations with roots of terrestrial

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plants, they are called mycorrhizae (Stamets, 2005), which are among the key organisms in soil ecology (Copley, 2000) and reforestation programs (Marx, 1980; Brundrett *et al.,* 1996). Lignocellulolytic fungi are capable of producing lignin-degrading enzymes such as lactase, cellulase and peroxidase, which possess several industrial applications (Eriksson *et al.,* 1990; Esposito and Azevedo, 2010). Recent research involving these fungi in native and exotic forests have been carried out in different regions of Brazil (Sulzbacher *et al.,* 2013), with new species and citations reported in the northeastern (Gurgel *et al.,* 2008; Menolli *et al.,* 2009; Wartchow and Maia, 2007; Wartchow *et al.,* 2009; Wartchow and Cavalcanti, 2010) and southeastern regions (Pegler, 1997; Baseia and Milanez, 2000, 2002). In southern Brazil, the first studies were carried out during the first half of the twentieth century. Studies of the biodiversity of symbiotic fungi in this region are incipient and less expressive in terms of number and territorial extent (Giachini *et al.,* 2000, 2004; Sobestiansky, 2005; De Meijer, 2001, 2006; Sulzbacher *et al.,* 2013).

Classical techniques such as morphological studies based on stereoscopic and optical microscopy and molecular analyses are essential to accurately provide information needed to catalogue and understand the biodiversity of ectomycorrhizal fungi in the southern state of RS. They can also identify and characterize species that are under strong environmental pressure and in danger of extinction. In addition, they make it possible to access information of great relevance related to the association of these organisms with forest species, exotic and/or native, occurring naturally or cultivated, in RS. This characterization will also make it possible to draw comparisons between the diversity of ectomycorrhizae in forest stands of monocultures (*Eucalyptus* spp., *Pinus* spp. and *Acacia mearnsii*) in relation to the diversity of ectomycorrhizae in native forest fragments. The objective of this study was to collect, identify and classify fungi found in exotic, native and mixed forest fragments in the Forest Research Center of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Santa Maria, Rio Grande do Sul State, Brazil.

MATERIALS AND METHODS

The survey of macroscopic fungi was carried out in stands of exotic (*Eucalyptus* spp., *Pinus* spp. and *Acacia mearnsii* de Wild.) and native (*Araucaria angustifolia* (Bert.) O. Kuntze) forest species, as well as in mixed forest stands (native and exotic species) and natural fields close to forest fragments located in the Forest Research Center of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), and surroundings, in Santa Maria, District of Boca do Monte (RS State, Brazil). In this region, there are predominately deep, imperfectly drained soils with low natural fertility (Abrão *et al.,* 1988). The climate of the region is classified as humid subtropical (Cfa), according to Köppen's system, with mean temperature values for the hottest month exceeding 22° C (Menegat, 1998). The average annual rainfall is 1.769 mm, with well-distributed rainfall throughout the year (Schumacher *et al.,* 2008). Fungi were collected from April to June 2013, through weekly observations of the evaluated environments. Basidioma were removed from their place of origin, photographed in loco and later in the laboratory, recording data for each specimen, such as: collection date and site, characteristics of the reproductive structures (color, consistency, insertion in the soil, shape and measurements), according to Largent (1977), while the microscopic analysis was performed according to Largent et al. (1977). The fungi were carefully removed from the soil, numbered and packed in paper bags for transportation to the laboratory. Whole specimens or parts of the collected fungi were dehydrated in a drying oven at 37°C until reaching a constant mass. Subsequently, they were kept in properly identified paper containers and stored in plastic boxes to keep the Herbarium collection.

Basidioma portions were arranged in microcentrifuge tubes containing 1mL of 2% CTAB cationic detergent (cetyltrimethylammonium chloride). The material was stored in at least two replicates, and maintained at a low temperature until its DNA extraction. For extraction of genomic DNA, the DNeasy® Plant Mini Kit (Qiagen) extraction kit was used, following the manufacturer's instructions for use. The fungal tissue was removed from the CTAB and sectioned in portions of approximately 20 mg. The polymerase chain reaction (PCR) followed the protocol described by Lupatini et al. (2008). The reaction was carried out in a total volume of 25 µl, having as components: 10 ng template DNA, 25 pmol of each primer oligonucleotide (ITS 1 and ITS 4), 10 mMTr is-HCl (pH 8.3), 50 mM of the reaction buffer, 2 mM MgCl 2, 2.5 µM each dNTP (dGTP, dCTP, dATP, dTTP) and 1 unit of Taq DNA polymerase (Invitrogen®). The primer oligonucleotides ITS 1 (5' TTC CGT AGG TGA ACC TGC GG 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') described by White et al. (1990) were used for the amplification of the rDNA transcript internal space (ITS). The amplification reactions were carried out using an initial denaturation at 94° C for 2 minutes, followed by 35 cycles consisting of: denaturation at 94° C for 1 minute, annealing at 55° C for 1 minute, and extension at 72° C for 1 minute and 30 seconds. A final extension was performed at 72° C for 10 minutes.

After amplification, electrophoresis of the 1.5% (m/v) agarose gel PCR products was carried out in a horizontal vessel containing the submerged gel in TBE 1X buffer (90 mMTris-borate, 2 mM EDTA, pH 8.0). Purification of the PCR products was performed according to a protocol based on the use of 13% (m/v) PEG 8000 described by Dunn and Blattner (1987). The cleaning procedure was repeated three times. Finally, the pellets were dried at room temperature with subsequent elution in 8 µL of ultrapure water, according to Green and Sambrook (2012). The material was sent for sequencing. The primer oligonucleotides used where ITS 1 and ITS 4. Sequencing was performed in the Mega BACE 5000 (Amersham Bioscience) sequencer, following the protocol provided by the manufacturer. With the sequences analyzed, the consensus sequence was obtained using the Staden program (Staden *et al.,* 2001), while sequence alignment was obtained using the ClustalW algorithm. GenBank sequences were chosen using the Megablast Algorithm, available on the NCBI BLAST platform (http://www.ncbi.nlm.nih.gov), and phylogenetic analyses were performed using the MEGA program (Tamura *et al.,* 2006).

RESULTS AND DISCUSSION

A total of 40 macroscopic fungi species were collected and identified, of which 29 were ectomycorrhizal, seven were saprophytic and three lignocellulolytic species. Among the ectomycorrhizal fungi, 29 species belonging to seven families and to nine different genera were found: *Amanita* (3 specimens), *Descomyces, Lactarius, Pisolithus* (2 specimens), *Ramaria, Rhizopogon* (2 specimens), *Russula* (5 specimens),

Table 1. Macroscopic fungi diversity in exotic, native and mixed forest stands at the Experimental Center of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) of Rio Grande do Sul State, Brazil

Family	Fungus (number of species)	Ecossystem	Collected in
		Ectomycorrhizal fungi	
Amanitaceae	*Amanita muscaria* (Fr.) S. F. Gray	*Pinus echinata*, *pinus* sp., mixed forest	June
	Amanita sp. (2)	*Pinus echinata*, mixed forest	June
Boletaceae	*Suillus* sp. (4)	*Araucaria angustifolia*, native forest fragment, *Pinus* sp.	May
	Suillus granulatus (L.) Snell	Natural field near *Pinus taeda*	May
	Suillus luteus[b] (L.) Gray	*Pinus taeda*	May
Hymenogasteraceae	*Descomyces* sp.	*Pinus taeda*	June
Ramariaceae	*Ramaria toxica* Toledo & Petersen	Roots of *Centrolobium* sp. in mixed natural forest with eucalyptus species	June
Rhizopogonaceae	*Rhizopogon* sp. (2)	*Pinus* sp.	May
Russulaceae	*Russula* sp. (4)	*Pinus taeda*, *Pinus echinata*, mixed forest	May
	Russula amethistina Quélet	*Pinus* sp.	June
	Lactarius deliciosus (L.) Gray	native forest fragment; *Pinus taeda*	April
Sclerodermataceae	*Scleroderma* sp. (5)	*Eucalyptus dunnii*, *Pinus echinata*	May
	Scleroderma citrinum Persoon	*Eucalyptus dunnii*, *Pinus echinata*	May
	Scleroderma albidum Patouillard & Trabut	*Eucalyptus dunnii*	June
	Scleroderma bovista[b] Fr.	*Eucalyptus grandis*	May
	Pisolithus sp.	*Eucalyptus dunnii*, roadside next to mixed settlement with predominance of native species	June
	Pisolithus tinctorius[b] (Persoon) Coker & Couch	*Eucalyptus dunnii*	June
		Saprophytic fungi	
Agaricaceae	*Macrolepiota* sp. (2)	*Pinus taeda*, impacted natural field[a]	May
	Lepiota sp.	*Acacia mearnsii*	April
	Leucoagaricus sp.	*Araucaria angustifolia*	May
Lycoperdaceae	*Calvatia* sp.	*Eucalyptus dunnii*	June
Strophariaceae	*Gymnopilus spectabilis* (Fr.) Sing. var. *pampeanus* (Speg.) Sing[c]	*Acacia mearnsii*, *Eucalyptus camaldulensis*; *Eucalyptus* grandis, *Eucalyptus* sp.	May and June
	Stropharia rugosoannulata Farlowex Murrill	Impacted natural field[a]	May
Tricholomataceae	*Lepista*sp.	*Allophylus edulis*	June
		Lignocellulolytic fungi	
Ganodermataceae	*Ganoderma tornatum* (Pers.)	*Acacia mearnsii*	April
	Ganoderma sessile Murrill	*Acacia mearnsii*	April
	Ganoderma sp.	*Acacia mearnsii*	April

[a]Naturalfield next to a native forest species fragment, but receiving regular mowing. [b]Identified through molecular techniques.
[c]Fungal specie with the capacity to degrade cellulose.

Table 2. Species of ectomycorrhizal fungi with identification based on the ITS sequence of rDNA

Placeofcollection	Closestspecies	AcessGenBank*	Similarity
Litter of *Eucalyptus grandis* Hill ex. Maiden	*Scleroderma bovista*	HM237175	99%
Litter of *Eucalyptus dunnii* Maiden	*Pisolithus tinctorius*	AF374700	99%

*Accession number in GenBank of the species with the highest similarity to the sample.

Scleroderma (8 specimens) and *Suillus* (6 specimens). Saprophytic fungi belonged to four families and to seven genera: *Macrolepiota* (2 specimens), *Calvatia, Gymnopilus, Lepiota*, *Lepista*, *Leucoagaricus* and *Stropharia*. Among the lignocellulolytic fungi, three species of the genus *Ganoderma* were found (Table 1). Some representatives of the fungi diversity found in the different ecosystems are represented in Figure 1.Molecular analyses enabled the identification of two ectomycorrhizal species, based on the sequence of the rDNA ITS region (Table 2). Most of the material was found in exotic forest stands of *Eucalyptus* sp., *Pinus* sp. and *Acacia mearnsii* (Tab 1). The low occurrence of ectomycorrhizal fungi in fragments of native forests in the south of Brazil has been observed by other authors (Andrade *et al.,* 2000, Sulzbacher *et al.,* 2013). Thus, a greater effort to increase the sample is necessary in this region for more accurate conclusions. These organisms may produce their fruiting bodies at other times of the year, or every two, three, seven years, for example (Alexopoulos *et al.,* 1995). Although some research has reported the absence of ectomycorrhizal associations in native forest species of RS, both in natural and *in vitro* conditions (Silva *et al.,* 2009; Andreazza *et al.,* 2011), this does not indicate that the establish of this type of symbiosis is impossible.

It is possible that the absence of ectomycorrhizae in the root system of certain plants is related to the reduced availability of inocula of the fungi at the site, reducing the occurrence of ectomycorrhizal species with roots (Silva *et al.,* 2009). The biotic factor should be considered both phytocentric and mycocentric, that is, emphasizing the diversity of plant species with which a fungal species can form mycorrhizae (the plant as a compatible symbiont) or the associations of fungi that develop symbiosis with certain specific plant taxa (Rinaldi *et al.,* 2008). However, very little is known about this in Brazil. Considering the ecological importance of mycorrhizal fungal selectivity of symbionts, some studies havereported such associations as beneficial and have contributed to a better understanding of the environmental factors that affect the diversity of fungal species along the evolutionary and ecological scale (Rinaldi *et al.,* 2008). Regarding the ectomycorrhizal collection sites, the same association pattern described by Oliveira and Giachini (1999) and Molina et al. (2005) was observed,in that although ectomycorrhizal fungi are associated with a restricted group of plants (approximately 5% of known species), 90% of symbioses formed are with temperate tree species, mainly those belonging to families Pinaceae (95%), Fagaceae (94%), Betulaceae (70%) and Salicaceae (83%) (Table 1 and Fig 2).

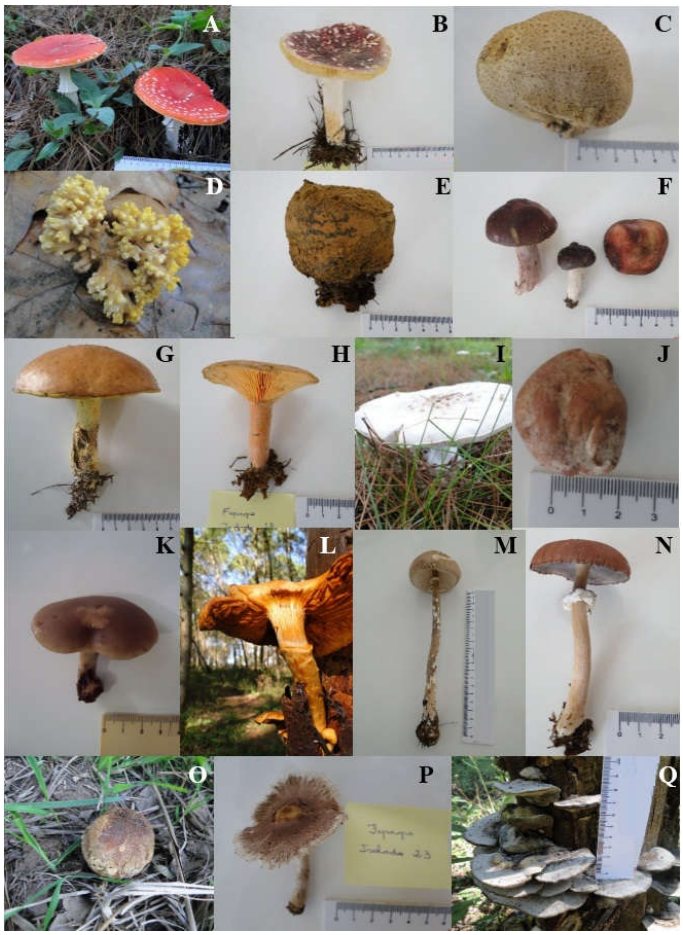

Figure 1. Some macroscopic fungi specimens collected in Experimental Center of Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) of Rio Grande do Sul State, Brazil. *Amanita muscaria* (A), *Russula* sp. (B), *Scleroderma citrinum* (C), *Ramaria toxica* (D), *Pisolithus* sp. (E), *Russula amethystina* (F), *Suillus* sp. (G), *Lactarius deliciosus* (H), *Amanita* sp. (I), *Descomyces* sp. (J), *Lepista* sp. (K), *Gymnopilus spectabilis* (L), *Macrolepiota* sp. (M), *Stropharia rugosoannulata* (N), *Calvatia* sp. (O), *Leucoagaricus* sp. (P) and *Ganoderma* sp.(Q).

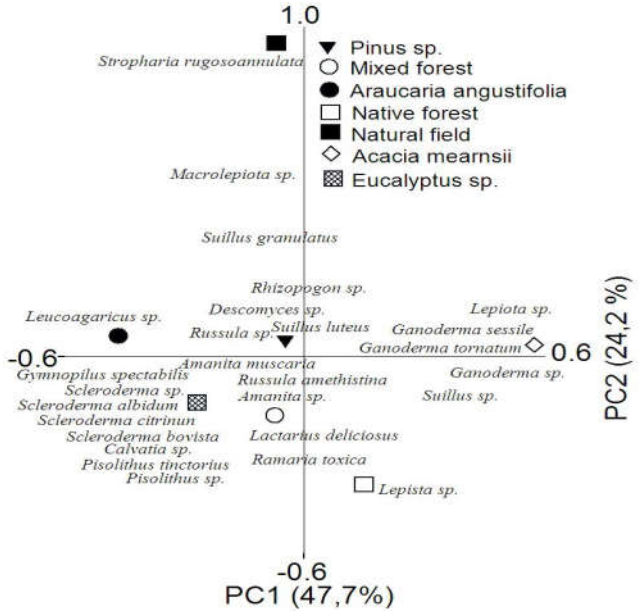

Figure 2. Graphic representation of canonical correlation analysis (CCA) between collection sites and ectomycorrhizal, saprophytic and lignocellulolytic genera and species collected in different ecosystems in the central region of Rio Grande do Sul State, Brazil.

According to Oliveira and Giachini (1999), in Brazil, the Basidiomycetes frequently colonize and fruit pine and eucalyptus species, with the most frequently found fungal species being of the genera *Pisolithus* Alb. and Schwein., *Scleroderma* Pers., *Rhizopogon* Fr., *Amanita* Pers. and *Lactarius* Pers. Studying global patterns of ectomycorrhizal introductions, Vellinga et al. (2009) observed that some plants were moved around the world with their intact root system. These roots may have harbored ectomycorrhizal fungi and the transport of plants may have facilitated their introduction into new ecosystems. At least 200 species of ectomycorrhizal fungi from the Ascomycota and Basidiomycota phyla have been moved from native environments to new habitats. Most of these introductions are associated with plantations of *Pinus* sp. and *Eucalyptus* sp. in the Southern Hemisphere (Giachini *et al.,* 2000; Vellinga *et al.,* 2009). Three species of lignocellulolytic fungi (Tab 1 and Fig 2), belonging to the genus *Ganoderma*, were also found, besides the species *Gymnopilus spectabilis*, which although saprophytic, is capable of cellulose degradation. For the industrial applications of these organisms, the ability of these groups to degrade recalcitrant substances such as textile dyes (Kamida *et al.,* 2005), petroleum derivatives (Isikhuemhen *et al.,* 2003), phenolic compounds (Jacques *et al.,* 2007) and lignocellulosic residues (Peres *et al.,* 2002; Alonso *et al.,* 2007) has been evaluated, in addition to their use in the production of enzymes (Faria, 2010). According to Souza and Rosado (2009), these characteristics make lignocellulolytic fungi important biotechnological tools to promote the biodegradation of recalcitrant compounds. In addition, recent studies have shown that structures formed by these fungi (sclerotia) produce secondary metabolites that are capable of assisting in future biotechnological studies, as well as in genomic sequencing studies (Smith *et al.,* 2015).

The Forest Research Center (29° 38’ 41’’S 53° 56’ 3’’W and 29° 40’ 27’’S 53° 54’ 27’’W) of the Fundação Estadual dePesquisa Agropecuária (FEPAGRO)has a long tradition in forestry research. Throughout its 75 years of existence, it has accumulated a wealth of native and exotic tree species, which places it in a privileged situation due to its diversity. In its area today, there are more than 40 stands of tree species, serving as support for research projects with a variety of objectives, both from the institution itself, as well as other teaching and research institutions. Due to the low anthropization, over time, growth of implanted stand and natural regeneration has occurred leading to formation of edaphic microfauna, characterized by mycorrhizae and growth promoting fungi, associated to the forest species present in the area. Recent research on the occurrence of macroscopic fungi in forest stands of the Forest Research Center has provided a description of the new species *Gloeoporus guerreroanus* (Coelho *et al.,* 2006), *Hypochnella verrucospora* (Coelho *et al.,* 2010) and *Echinoporia inermis* (Coelho, 2008). In addition, the first records inBrazil of the species *Chondrogaster pachysporus* (Sulzbacher *et al.,* 2010), *Hysterangium affinee H. inflatum* (Cortez *et al.,* 2011), as well as the first record in South America of wood degrading species *Gelatoporia subvermispora* (Baldoni *et al.,* 2012) and *Sarcoporia polyspora* (Baldoni *et al.,* 2015) were reported. In addition to macroscopic fungi, a number of fungal strains of the genus *Trichoderma* have been isolated, a specific group widely recognized for their agricultural application in biological disease control and plant growth promotion (Steffen *et al.,* 2016). These publications demonstrate the importance of

maintaining the forests of the Forest Research Center for the preservation of the genetic patrimony of macroscopic fungi and ensuring continuity of taxonomic and diversity studies (Coelho and Silveira, 2014), as well as those about association with tree mycorrhizal species in native forest species (Andreazza *et al.,* 2008). There is a great diversity of macroscopic fungi in forest fragments of the Forest Research Center and surroundings, demonstrating the potential of these sites as a source of mycorrhizal inocula and of organisms with potential for biotechnological use in industries and plant production. Data of this nature are fundamental to increase the knowledge of the biodiversity of organisms present in certain places and periods, which greatly facilitates future studies, as well as to ensure preservation of the genetic patrimony.

Conclusion

Forty species of macroscopic fungi were identified in exotic, native and mixed forest fragments at the Forest Research Center of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), in Santa Maria, Rio Grande do Sul, Brazil, between April and June 2013. Most of the collected fungi were ectomycorrhizae (29 species, 9 genera, 7 families), but there were also species of lignocellulolytic fungi (8 species, 7 genera, 4 families) and saprophytic fungi (3 species, 1 genera, 1 family). The occurrence of ectomycorrhizal species in native forest stands demonstrates the need for further in-depth studies aiming to detect the natural establishment of this type of association in species that have been little studied until the present moment.

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