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Morphological and molecular analyses in *Scleroderma* (Basidiomycota) associated with exotic forests in Pampa biome, southern Brazil

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Abstract

Basidiomes of the mycorrhizal genus *Scleroderma* were collected on exotic forest soils (associated with *Eucalyptus* and *Pinus*) from Pampa biome, southern part of Rio Grande do Sul State, Brazil. Three species were identified, using both morphological and molecular approaches as follows: *S. albidum*, *S. citrinum*, and *S. verrucosum*. New ITS rDNA sequences generated in this study, together with others retrieved from GenBank, showed the species nesting in two main clades headed by *S. albidum* and *S. citrinum*. Morphological descriptions are provided for these two species. Infrageneric dichotomy represents a separation into combinations of reticulate spores plus clamped hyphae and echinulate spores plus simple-septate hyphae.

Key words – gasteroid fungi – phylogenetic analysis – soil symbionts

Introduction

The Pampa bioma is one of six Brazilian biomes, being restricted to the southern part of Rio Grande do Sul State, Brazil. It comprises 176,469 km² in area, 64% of the States territory and 2.07% of Brazilian territory. This biome shares physiognomic characters with Pampean region of Uruguay and Northern Argentina (Boldrini et al. 2010, IBGE 2013). The predominant vegetation of this biome includes grasses, shrubs and riparian forests. Recent data point to nearly 3000 plant species, some being endemic to Pampean region (Boldrini et al. 2010, Iganci et al. 2011).

Large areas are cultivated with exotic forest species, mainly *Eucalyptus* spp. and *Pinus* spp. little is known of the ectomycorrhizal diversity associated with these exotic forests. Recently specimens of the genus *Scleroderma* Pers. were found as a mycorrhizal component within the Pampa biome. *Scleroderma* is found in tropical, temperate and subtropical ecosystems worldwide. About 25 species are recognized morphologically, with 12 being reported from Brazil (Guzmán 1970, Sims et al. 1995, Baseia & Milanez 2000, Giachini et al. 2000, Sobestiansky 2005, Meijer 2006, Gurgel et al. 2008, Sanon et al. 2009, Cortez et al. 2011). The diagnostic characters for the genus are gasteroid basidiome of epigeous habit, rarely hypogeous, and globose to subglobose

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basidiospores with ornamentation reticulate to echinulate (Guzmán 1970, Giachini et al. 2000, Cortez et al. 2011, Yousaf et al. 2012, Nouhra et al. 2012).

Knowledge about the genus *Scleroderma* in Pampa biome is limited, the main work is of Cortez et al. 2011, who identified seven species: *S. albidum* Pat. & Trab. emend. Guzmán, *S. bovista* Fr., *S. citrinum* Pers., *S. dictyosporum* Pat., *S. fuscum* (Corda) E. Fisch., *S. leave* Lloyd emend. Guzmán, and *S. verrucosum* (Bull.) Pers. However, a molecular approach to studying *Scleroderma* in Brazil is lacking, although it has been used elsewhere on a restricted number of species and specimens (Phosri et al. 2009, Sanon et al. 2009, Nouhra et al. 2012, Kumla et al. 2013, Zhang et al. 2013, Rusevska et al. 2014).

The purpose of this work is to contribute to the identification of *Scleroderma* species from Pampa Biome through morphological and molecular analysis in order to improve mycorrhizal fungi studies. It will also contribute to phylogenetic studies in *Scleroderma*, still incipient in worldwide scientific literature, with new molecular data available in public databases.

Materials & Methods

Isolates and morphology

Collection of species – Fresh basidiomes were obtained during mycological trips in the region of Pampa biome in Rio Grande do Sul State, Brazil. The basidiomes were collected in the top soil, near to *Eucalyptus* spp. and *Pinus* spp. trees. They were collected from September 2010 to October 2013 in municipalities of Barra do Quaraí, Bororé, Jaguarí, Pinhal Grande, Santa Maria, Santana do Livramento and São Francisco de Assis.

Identification and morphological description – Fresh basidiomata were collected and analyzed macro- and microscopically following Brundrett et al. (1996). Basidiomes were photographed *in situ* and their colour names were compared and noted according to Munsell Soil Color Charts (2009). Microscopical characters were analyzed in optical microscope (Olympus CX40), from handmade sections with razor blades, and rehydrated in 3% KOH (v/v). At least 35 measurements of each microstructure were obtained, usually including ornamentation of the spores (or not for comparison with literature). For scanning electronic microscopy (SEM) of spores, herbarium specimens were mounted directly on aluminum stubs with a carbon band and subsequently covered with a layer of gold with 15 nm in thickness, using a Balzers SCD 050 Sputter. Spores were examined in microscope model JEOL – JSM 6060. Basidiomes were dried in Marconi dryer model MA033 at 45–50°C. Specimens were deposited at SMDB herbarium (Department of Biology, Federal University of Santa Maria**)**.

Molecular analysis

For DNA sample, a fraction of the fresh basidiome was removed for storage in CTAB (Gardes & Bruns 1993) at -20° C until the time of analysis. DNA was extracted with the DNeasy® Plant Mini Kit (Qiagen, São Paulo, Brazil) kit. For the extraction of DNA from herbarium specimens, EZNA® Forensic DNA Extraction Kit (Omega Bio-tek, product Nº. D3591-01) was used. The complete region in nrDNA (ITS1-5.8S-ITS2) was amplified with primers ITS1 and ITS4 (White et al. 1990). The amplification reaction of the rDNA fragments was performed according Baldoni et al. (2012). After the PCR amplification, electrophoresis was performed in 1.5% agarose gel and 1X TBE buffer. The DNA samples were stained with BlueGreen Loading Dye I ® (LGC Biotechnology, Cotia, Brazil) and observed under UV light. The PCR products were purified with Gen Elute PCR Clean-up Kit® (Sigma, St. Louis, USA) kit, following the manufacturer's instructions.

Sequencing of the samples was performed into the sequencer, ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequenced fragments were analyzed using the Staden Package 2.0.0b program (Staden et al. 2003) for obtaining consensus sequences, and then deposited in GenBank. The sequences were aligned in Bioedit program. The phylogenetic relationship of the specimens was reconstructed based on analyses of the ITS region in MEGA 5.0 software (Tamura

et al. 2011), with the analysis of Maximum Likelihood (ML) in a total of 1000 replications for all reconstructions. The model of nucleotide substitution General Time Reversible model was estimated using JModelTest as the best model to solve the data (Posada et al. 2006), performed with uniform rates and parameters for partial exemption (95%). Selected closely related sequences for phylogenetic analysis of the genus *Scleroderma* were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Forty-four sequences (Table 1) including the outgroup taxa *Pisolithus tinctorius* (AF374632) and *Pisolithus albus* (AF440868) were used for analyses.

Table 1 – List of species included in phylogenetic analysis, herbarium number, place of origin, and GenBank accession numbers.

Species	Strain /Specimen	Origin	GenBank
Scleroderma albidum	ICN 154608	Santa Maria, Brazil	**KJ676532**
Scleroderma albidum	SMDB 14.507	Barra do Quaraí, Brazil	**KJ676521**
Scleroderma albidum	SMDB 14.503	Bororé, Brazil	**KJ676522**
Scleroderma albidum	SMDB 14.517	Bororé, Brazil	**KJ676523**
Scleroderma albidum	SMDB 14.516	Jaguarí, Brazil	**KJ676524**
Scleroderma albidum	SMDB 14.508	Jaguarí, Brazil	**KJ676525**
Scleroderma albidum	SMDB 14.512	Santa Maria, Brazil	**KJ676526**
Scleroderma albidum	SMDB 14.513	Pinhal Grande, Brazil	**KJ676527**
Scleroderma albidum	SMDB 14.510	Sant. do Livramento, Brazil	**KJ676528**
Scleroderma albidum	SMDB 14.509	Sant. do Livramento, Brazil	**KJ676529**
Scleroderma albidum	SMDB 14.511	Sant. do Livramento, Brazil	**KJ676530**
Scleroderma albidum	SMDB 14.514	São Franc. de Assis, Brazil	**KJ676531**
Scleroderma areolatum	PBM2205	USA	EU718116
Scleroderma areolatum	JMP00080	USA	EU819438
Scleroderma areolatum	RBG/Kew K(M)125392	England	EU784407
Scleroderma areolatum	E00278286	Dane County, WI, USA	FM213353
Scleroderma areolatum	F:PGK193	USA	GQ166910
Scleroderma aurantium	8-5	Sichuan, China	HM237174
Scleroderma bovista	K(M)105588	England	EU784409
Scleroderma bovista	RT00034	USA	EU819517
Scleroderma bovista	BCN-MPM1989	Catalonia, Spain	FM213340
Scleroderma bovista	5-1	Sichuan, China	HM237175
Scleroderma bovista	O1A_1	USA	JX030276
Scleroderma bovista	O1Q_1	USA	JX030277
Scleroderma bovista	Scl_1-1-2LC	USA	JX030217
Scleroderma bovista	Scl_1-2-2LC2	USA	JX030218
Scleroderma bovista	-	Japan	AB099901
Scleroderma bovista	K80S09	New Zealand	GQ267487
Scleroderma bovista	CM9	Pakistan	KF881875
Scleroderma citrinum	Scl_1-3-1H	USA	JX030202
Scleroderma citrinum	Scl_2-2-1H	USA	JX030205
Scleroderma citrinum	Scl_2-2-1HC	USA	JX030207
Scleroderma citrinum	CH1-127	USA	JX079368
Scleroderma citrinum	(DNA 778)	Germany	HM189957
Scleroderma citrinum	JMP0082	USA	EU819440
Scleroderma citrinum	SMDB: 14.500	Santa Maria, Brazil	**KJ679575**
Scleroderma citrinum	SMDB: 14.499	Santa Maria, Brazil	**KJ679576**
Scleroderma verrucosum	ICN: 154625	Santa Maria, Brazil	**KJ676520**
Scleroderma verrucosum	06MCF7265_E10/45-09	Macedonia	HF933241
Scleroderma verrucosum	K(M)30670	-	EU784415
Scleroderma verrucosum	07MCF7984_E10/45-11	Macedonia	HF933232
Scleroderma sp.	P091	Estônia	FN669245
Scleroderma sp.	5-2	China, Sichuan	HM237172
Scleroderma sp.	ScT-X-08	"Montenegro: Tivat"	JQ685726
Pisolithus albus	T25070	Australia	AF440868
Pisolithus tinctorius	MARX270	Georgia, USA	AF374632

Bold *Scleroderma* species obtained from Pampa biome / RS

Results

Taxonomy descriptions

***Scleroderma albidum* Pat. & Trab. emend. Guzmán,** Darwiniana 16: 295 (1970) Figs. 1–2

Basidiomata epigeous, 6–39 mm high, 6.7–37 mm in diam., globose to subglobose; surface smooth, cracked, to squamulous, background yellow (8/6–7/6 2.5Y) to brownish yellow (6/6 0YR), cracks small (±0.5 mm), irregular, olive yellow (6/8 2.5Y) at the top; squamules small, very pale brown (8/3–8/4 10YR) light olive brown (5/6 2.5Y), dark yellow brown (4/4 10YR), yellow (8/8–7/8 2.5Y) in the base. Rhizomorphs more aggregated at the base, pale brown (8/4 2.5Y), branched, narrowing towards the end, 0.3 mm in diam. Peridium 0.5–1.65 mm when fresh, rubbery in consistence, very pale brown (8/4 10YR). Gleba compact when young, becoming pulverulent at maturity, light brownish gray (6/2 10YR), grayish black (2.5 10YR), reddish brown (4/3 10YR) to dark brown (3/2 10YR).

Basidiospores globose, echinulate, dark brown in KOH, 8–17 × 9–17 µm in diam., including ornamentation, n=120/2, coated by crowded curved spines. Basidia not observed. Peridium consisting of two distinct layers. Exoperidium a layer formed by hyphae simple-septate, with slightly thickened walls (ca. 1 µm), interwoven, often ramified and superimposed, hyaline to yellowish brown, 1.5–8.5 µm in diam. n=120/2. Endoperidium consisting of hyphae pseudoparenquimatous, hyaline, thick-walled (ca. 1 µm), rounded at the end, 2–21 µm in diam. n=120/2. Trama 1.5–9 µm in diam., hyphae hyaline, thin-walled (ca. 1µm), branched, simple-septate, clamp connections not observed. Conductive hyphae present, somewhat irregular in profile, filled with an amorphous yellowish brown content, up to 3–16 µm in diam. Clamp connections absent.

Material examined – Brazil, Rio Grande do Sul State, Bororé, 24.VIII.2011, leg. M.A. Sulzbacher, A. Silveira & R.B. Steffen (SMDB 14.503; 14.505; 14.517); Barra do Quaraí, 23.VIII.2011, leg. M.A. Sulzbacher, A. Silveira & R.B. Steffen (SMDB 14.507); Jaguarí, Serro do Chapadão, 19.I.2011, leg. D.P. Golle, M.A. Sulzbacher & R.B. Steffen (SMDB 14. 508); 23.X.2010, leg. D.P. Golle, M.A. Sulzbacher, D.B. Baldoni & M. Lupatini (SMDB 14.516); Pinhal Grande, 31.III.2013, leg. D.F. Montagner (SMDB 14.513); Santana do Livramento, 5.IV.2013, leg. D.F. Montagner, A. Silveira, G. Coelho & D.B. Baldoni (SMDB 14.509; 14.510; 14.511); Santa Maria, 18.III.2013, leg. D.F. Montagner & A. Moro (SMDB 14.512); São Francisco de Assis, 23.V.2013, leg. D.F. Montagner & L. Morandini (SMDB 14.514).

Discussion – Basidiome morphological features of *S. albidum* are somewhat variable, mainly in peridium thickness and spore size, likely reflecting the lack of knowledge on onthogenic basidiome development. Some specimens present spore size and ornamentation, thin peridium, and small squamulae similar those of *S. verrucosum*. It also resembles *S. cepa* by the spore size, peridium hyphal structure and thickness (Guzmán 1970). *S. albidum* is also macroscopically similar to *S. bovista*, but the former presents a thin peridium and echinulate basidiospores (Nouhra et al. 2012).

Known distribution – Common in Southern Hemisphere, Asia, North America, and Europe (Guzmán 1970, Sulzbacher et al. 2013). Brazil: including the states of Pernambuco (Gurgel et al. 2008); Minas Gerais, Rio de Janeiro, and São Paulo (Guzmán 1970); Santa Catarina (Giachini et al. 2000); and Rio Grande do Sul (Cortez et al. 2011). Associated with *Eucalyptus* spp.; probably ectomycorrhizal in *Eucalyptus* plantations.

***Scleroderma citrinum* Pers.,** Syn. Meth. Fung. 1:153 (1801) Figs. 3–4

Basidiomata epigeous, 17.4–38 mm high, 25–67 mm in diam.; smooth depressed on top, globose to subglobose, yellowish brown (5/4 10YR) to light yellowish brown (6/4 10YR) when young, and to pale brown (8/4 10YR), yellow (7/8–8/8 5YR) and brownish yellow (6/6–6/8 10YR) at maturity. Surface smooth when young, cracked at the sides and top when mature, cracks yellow

(5/6 10YR) to dark yellowish brown (4/4 10YR), covered by scales irregular in shape (±1 mm) yellowish brown (5/8 10YR) concolorous to the cracks to brownish yellow (6/6 10YR). Rhizomorphs numerous, small, 0.20–0.50 mm, concolorous aggregated the base, white (8/2 2.5Y) to pale yellow (8/4 10YR). Peridium 4 mm thick, rubbery when fresh, very pale yellow (9/2 2.5Y), bruising reddish yellow (7/6–6/6 7.5YR), dehiscence irregular, occurring by rupture of the apical portion. Gleba compact when young, white (8/1 5YR) to black (2.5/1 5YR), becoming powdery at maturity, olive brown (4/4 2.5Y) to olive yellow (6/8 5Y).

Basidiospores globose reticulate, yellowish brown in KOH, 9–12 × 14–16 µm in diam., n=120/2, including ornamentation; Basidia not observed; Peridium consisting of three layers. Exoperidium formed by hyphae interwoven, thin-walled (ca. 1 µm), hyaline, brownish yellow to rust brown in KOH, thick, 4–12 µm in diam. n=120/2. Mesoperidium formed by hyphae interwoven, septate, of hyaline hyphae, narrower than those of exoperidium, 2–8 µm in diam. n=120/2, slightly thickened walls (ca. 2 µm). Endoperidium formed by hyphae fibulate 2–4 µm in diam., thin-walled, hyaline. Clamp connections present.

Material examined – Brazil, Rio Grande do Sul State, Santa Maria, 28.III.2013, leg. D.F. Montagner (SMDB 14.499; 14.500; 14.501; 14.502).

Discussion –*S. citrinum* is the easiest recognized species in the area, usually being found in *Pinus* Plantations. It grows somewhat variable in size, but usually is found as bigger basidiomes with an appearance of orange fruits on soil. They are characterized by thicker squamulose peridium with more vivid brownish colours, dehiscence irregular, and distinctly reticulate basidiospores (Nouhra et al. 2012).

Known distribution – North America, Central Europe, Asia, Africa, South America (Guzmán 1970, Sulzbacher et al. 2013). Brazil: States of Paraíba (Gurgel et al. 2008); São Paulo (Bononi et al. 1981); Paraná (Meijer 2006); Santa Catarina (Giachini et al. 2000); and Rio Grande do Sul (Sobestiansky 2005, Cortez et al. 2011). Associated with *Pinus elliottii* Engelm. and *P. taeda* L. plantations; ectomycorrhizal.

Fig. 1 – Basidiomes and spores of *Scleroderma* species (SEM). 1–2 *S. albidum*. 3–4 *S. citrinum*.

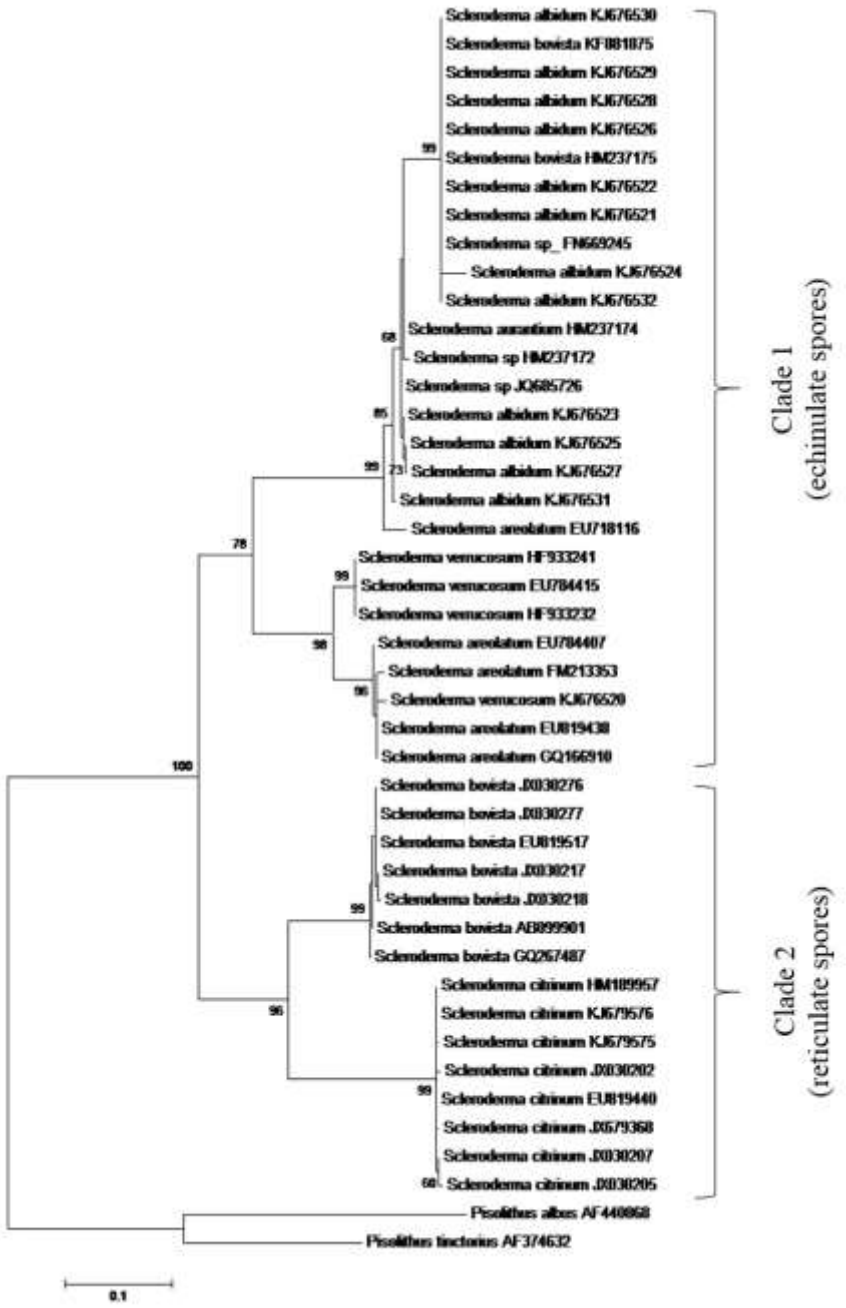

Fig. 2 – Phylogenetic reconstruction of the genus *Scleroderma*, obtained from ITS1-5.8S-ITS2 sequences. Bootstrap values (in %) are from maximum likelihood (ML) analyses (1000 bootstraps). The 15 *Scleroderma* new sequences generated in this study are labeled with the isolation DNA code in Table 1. Sequences from *Pisolithus tinctorius* and *P. albus* were used as outgroup.

Phylogenetic analyses

After sequencing, 15 fragments were obtained from 610 bp to 831 bp of nrDNA ITS region. Phylogenetic analysis based on ITS sequences supported the genus segregation into two major clades; clade 1 for species with echinulate-subreticulate spores plus simple-septate hyphae and clade 2 for species with reticulate spores plus clamped hyphae. This data supports that obtained by other authors (Phosri et al. 2009, Sanon et al. 2009, Nouhra et al. 2012, Kumla et al. 2013, Zhang et al. 2013, Rusevska et al. 2014). An infrageneric separation was initially proposed by Guzmán (1970), but instead, splitting *Scleroderma* in three sections based on basidiospore ornamentation, as follows: Section *Scleroderma* for reticulate spores; *Aculeatispora* for spiny spores; and additionally *Sclerangium* for subreticulate spores.

As shown in topology of *Scleroderma* phylogenetics tree (Fig. 2), Clade 1 included only species with echinulate to subreticulate spores, such as *S. albidum, S. verrucosum,* and *S. areolatum*. Most sequences (13 among 15) generated for this study nested together in a conspecific group we called *S. albidum* clade. This fact is remarkable, because they seemed to represent a group of different species by preliminary morphology observation, showing some variation in basidiome appearance, spore ornamentation and spore size. All specimens were shown to be genetically closely related, without dependence on their diverse collection localities. In this clade *S. albidum*, sequences named as *Scleroderma bovista* (KF881875; HM237175) and *S. aurantium* (HM237174), two taxa known by their reticulate spores and clamped hyphae (Coker & Couch 1928), must represent erroneous identifications by presenting highest similarity with other *S. albidum* sequences – obtained from specimens with echinulate spores and simple-septate hyphae. Sequences of *Scleroderma* sp. (FN669245; JQ685728) from Estonia and Montenegro also claded along with *S. albidum* specimens and are herein conceived as conspecific. The collection of *Scleroderma verrucosum* ICN 154625 clustering in *S. areolatum* clade may represent a misidentification.

Clade 2 (Fig.2) nested only species with reticulate spores plus fibulate hyphae coinciding with sect. *Scleroderma* sensu Guzmán (1970); it formed two groups (*S. bovista* and *S. citrinum*). A pair of Brazilian sequences (KJ679576; KJ679575) formed a well supported *S. citrinum* clade (BT value 96%) and high BLAST similarity (98%) with other sequences from China, USA and Germany.

A restricted set of morphological characters and their states have been a recognized problem in *Scleroderma* taxonomy providing insufficient data for discriminating species. Another question comes from variable basidiome structure, which can be influenced by soil and environment conditions (Kazuya et al. 2008). Molecular data on the genus must be increasingly generated and used for establishing the limits and phylogenetic relationships of the species (Sanon et al. 2009).

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