

# Rediscovery of *Sclerogaster luteocarneus* (Geastrales, Agaricomycetes): A Forgotten Species

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**Abstract:** *Sclerogaster luteocarneus* described from São Leopoldo (Rio Grande do Sul, Brazil) in 1920 as *Octaviania luteocarnea*, and later cited in 1936 from Guadeloupe (French Antilles), is here newly reported from three localities in the Atlantic Forest from North- and Southeastern regions of Brazil. *Sclerogaster luteocarneus* is recognized by its lignicolous habit, different from the rest of *Sclerogaster* species known to date, which are hypogeous or semihypogeous. Based on morphological features, *S. luteocarneus* is separated from other *Sclerogaster* species mainly by its slightly verrucose and pedicellate basidiospores. Phylogenetic analyses of large subunit (LSU) nrDNA confirmed that this species is closely related to *Sclerogaster compactus*. A complete description and illustrations of *S. luteocarneus* is provided, and the affinities with other species of the genus are discussed.

**Keywords:** Taxonomy, Phallomycetidae, Gasteromycetes, Phylogeny, LSU nrDNA sequences.

## 1. INTRODUCTION

Genus *Sclerogaster* Hesse was described by Hesse in 1891 [1] to recognize gasteroid fungi with hypogeous habitat, small basidioma, gelatinous and pale yellow to green gleba in fresh specimens, becoming very hard when dried with small cavities filled by powdery spore mass of minutely warty or spiny basidiospores [2].

Hosaka *et al.* [3], based on 5-gene sequences, placed *Sclerogaster* in the class Phallomycetidae K. Hosaka, Castellano & Spatafora, and in the order Geastrales Hosaka & Castellano; position that was confirmed by Cléménçon *et al.* [4] based on morphological similarities of rhizomorph anatomy of *Sclerogaster* and other truffle-like basidiomycetes. Hosaka & Castellano [5] showed the monophyly of the genus and close relationship with Geastraceae in Geastrales, in which *Sclerogaster* formed a family of its own, Sclerogastraceae Locq. ex P.M. Kirk [6].

Twelve species are recognized in this genus [7]: *Sclerogaster compactus* (Tulasne & C. Tulasne) Saccardo [8] (syn. *S. lanatus* R. Hesse [1]); *S. candidus* (Tulasne & C. Tulasne) Zeller & Dodge [9]; *S. columellatus* (Zeller) Fogel [10]; *S. gastrosporioides* Pilát & Svrcek [11]; *S. hysteroangiooides* (Tulasne & C. Tulasne) Zeller & Dodge [9]; *S. liospermus* (Tul. & C. Tul.)

Soehner [12]; *S. luteocarneus* (Bresadola) Zeller & C.W. Dodge [9]; *S. minor* Coker & Couch [13]; *S. pacificus* Zeller & C.W. Dodge [9], *S. salisburyensis* Verwoerd [14], *S. siculus* Zeller & Dodge [9] (syn. *S. lanatus* Mattiolo [15]), and *S. xerophilus* Fogel [16]. Almost all species have been described from the Northern Hemisphere, except *S. luteocarneus* (basionym: *Octaviania luteocarnea* Bres.), that was collected from São Leopoldo (Brazil) and *S. salisburyensis* from Southern Rhodesia (South Africa). According to the protologues, all species grow hypogaeously or semihypogaeously on soil, among leaves or roots; except, *S. luteocarneus* that was described as growing on wood (Bresadola 1920: "Hab. ab ligna" [17]).

Recently, in fragments of the Atlantic Forest in Brazil, we have found gasteroid specimens growing on decaying wood that could belong to the species *S. luteocarneus*. Bresadola [7] described as *Octaviania luteocarnea* from Brazil based on a collection of J. Rick (nº 80) from São Leopoldo (Rio Grande do Sul); later Zeller & Dodge [9] made a new combination, *S. luteocarneus*, without giving more information. However, in Dodge & Zeller [2], the description of *S. luteocarneus* is based on two collections: a) J. Rick nº 51, also from Rio Grande do Sul, considered by these authors as a type (located in Lloyd Mus. 06025 and Dodge herbarium); and b) specimens collected by Duss in 1895, in Bois de Bains Jaunes (Guadeloupe, French Antilles). Even though we have done an exhaustive literature search, we have not found more literatures on *S. luteo-*

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*carneus*, nor was this species included in the research conducted by Hosaka & Castellano [5].

More than 80 years have passed since this species has been described. The aim of this paper was to confirm if our collections from the Atlantic Forest belong to *S. luteocarneus*, as well as to provide an update and exhaustive description of this species using morphological and molecular data (ITS and LSU nrDNA), and to demonstrate its phylogenetic relationship with other *Sclerogaster* species.

## 2. MATERIAL AND METHODS

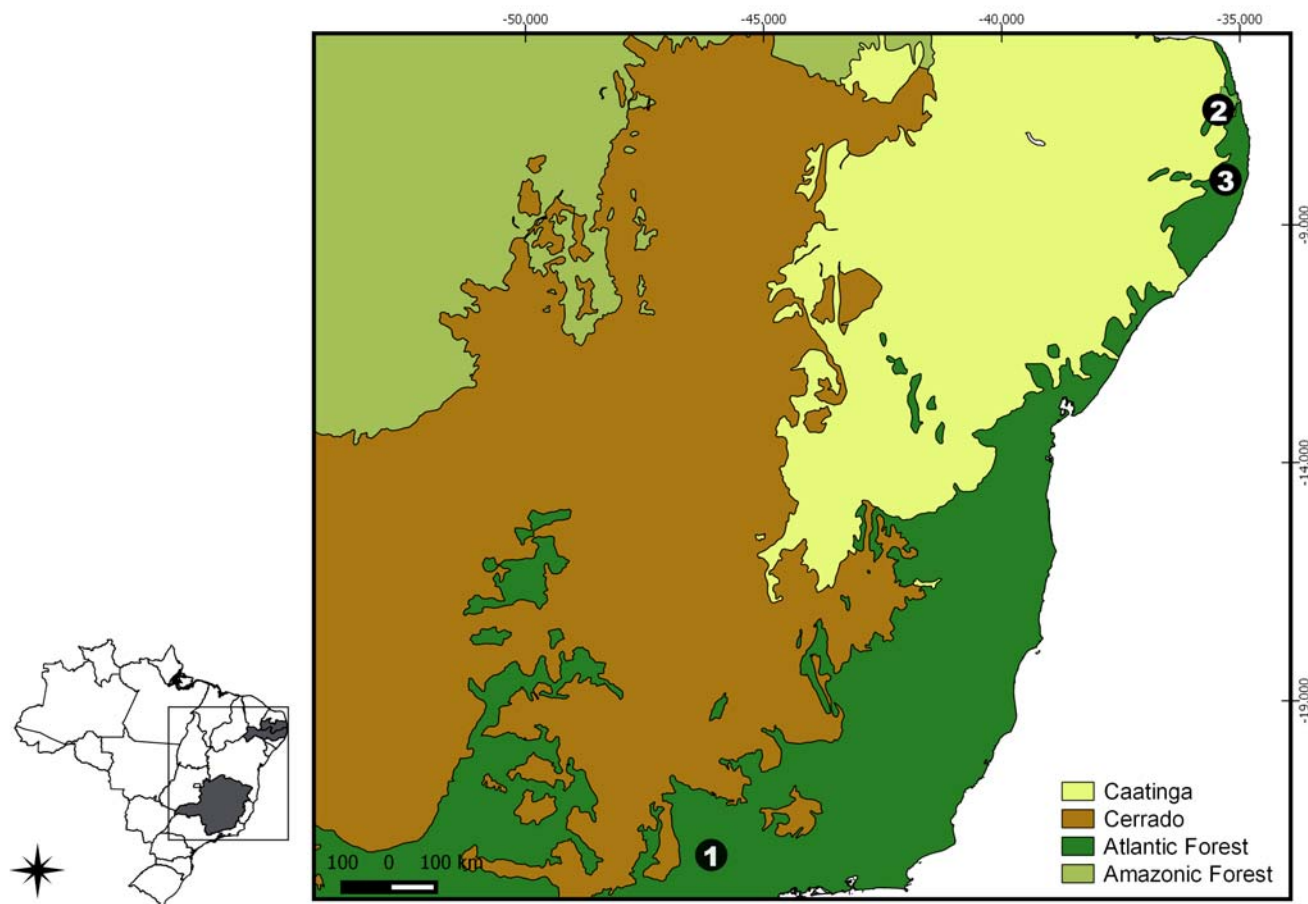
### 2.1. Sampling and Morphological studies

Fieldwork was carried out during the rainy season from 2012 to 2014 in remains of the Atlantic Forest areas in southeast and northeast Brazil (Figure 1): (1) Reserva Biológica Municipal de Santa Rita Mitzi Brandão, Santa Rita do Sapucaí City, Minas Gerais State; (2) Reserva Ecológica Estadual Mata do Pau-Ferro in Areia City, Paraíba State; and (3) Parque Dois Irmãos, Recife City, Pernambuco State. Fresh and dry

specimens were photographed. Macro- and micro morphological analyses were based on dried basidiomata. Microscopic examinations were made from cross sections of the peridium and gleba mounted in 5% KOH, in Cotton Blue stain and in Melzer's reagent [18-19]. Thirty randomly selected basidiospores were measured using oil immersion (including ornamentation) after brief boiling, and the statistical calculation followed that of Souza *et al.* [20]. The microscopic measurements and photographs were obtained under light microscope (LM) Nikon Eclipse Ni with NIS-Elements support software. The scanning electron microscope (SEM) studies of the basidiospores were done following Cortez *et al.* [21], under a Shimadzu SSX-550 microscope. Air-dried vouchers are deposited in the UFRN Herbarium.

### 2.2. DNA Isolation, PCR Amplification and Sequencing

Genomic DNA was extracted from gleba of three dried specimens using DNeasy™ Plant Mini Kit (Qiagen, Valencia, California, USA), following the instructions of the manufacturers; lysis buffer incubation was done overnight at 55 °C.



**Figure 1:** Map of Brazil showing the localities where *Sclerogaster luteocarneus* was collected.

Total DNA was used for PCR amplification of the ca. 1450 bp region (domains D1-D2) of the large subunit (LSU nrDNA) and the internal transcribed spacer region (ITS nrDNA) of nuclear ribosomal gene. The primers LR0R [22] and LR7 [23] were used to amplify the region of the LSU nrDNA and the primers ITS1F [24] and ITS4 [25] were used to amplify the whole ITS region, including the 5.8S ribosomal RNA gene cluster and flanking parts of the small subunit (SSU) and large subunit (LSU) nuclear ribosomal genes. Individual reactions of a final volume of 25  $\mu$ L were carried out using illustra™ PuReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare, Buckinghamshire, UK) with a 10 pmol/ $\mu$ L primer concentration using the thermal cycling conditions of Martín & Winka [26].

Negative controls lacking fungal DNA were run for each experiment to check for contamination. The reactions were run with the following parameters for the LSU nrDNA: initial denaturation at 94 °C for 5 min, then 36 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min; for the ITS nrDNA: initial denaturation at 95 °C for 5 min, then 5 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, followed by 33 cycles of denaturation at 72 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C, with a final extension at 72 °C for 10 min.

The PCR products were purified using the QIAquick Gel PCR Purification (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced using the same amplification primers. Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA) was used to edit the resulting electropherograms and to assemble contiguous sequences. BLAST searches with megablast option were used to compare the sequences obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases [27].

### 2.3. Sequence Alignment and Phylogenetic Analyses

The LSU nrDNA sequences obtained were aligned using Se-Al v2.0a11 Carbon [28] with other Geastrales sequences. The sequences were compared with homologous sequences retrieved from the EMBL/GenBank/DBJ databases [29]; all FJ sequences included in Figure 2 were generated by our research group within the framework of other studies. In order to root the trees generated by the LSU analyses, four *Geastrum* sequences were included as outgroup. Alignment gaps

were marked as "-", and unresolved nucleotides and unknown sequences were indicated with "N".

A maximum parsimony analysis (MP) was carried out; minimum length Fitch trees were constructed using heuristic searches tree-bisection-reconnection (TBR) branch swapping (100 maximum trees were saved), collapsing branches if maximum length was zero and with the MulTrees option on in PAUP\*4.0b10 [30]. Gaps were treated as missing data. Nonparametric bootstrap (MPbs) support [31] for each clade, based on 10,000 replicates using the fast-step option, was tested. The consistency index, CI [32], retention index, RI [33], and rescaled consistency index, RC [33] were obtained. A second analysis was done by maximum likelihood approach (ML) assuming the HKY + I + G model selected by PAUP\*4.0b10. Nonparametric bootstrap (MLbs) based on 10,000 replicates using the fast-step option was tested.

Analyses of ITS sequences were not performed since only one sequence under *Sclerogaster* sp. (Acc. Number KP191950, from New Zealand, voucher PDD100944) is available at the EMBL/GenBank/DBJ databases. The ITS sequences obtained in this study were deposited in GenBank for the purpose of DNA barcoding.

## 3. RESULTS AND DISCUSSION

Six sequences were generated in this study, three to each marker. Blast search of the ITS and LSU sequences confirmed that the three collections belong to the genus *Sclerogaster*. Sequences have been deposited at the EMBL/GenBank/DBJ databases with the numbers KT923663-KT923668.

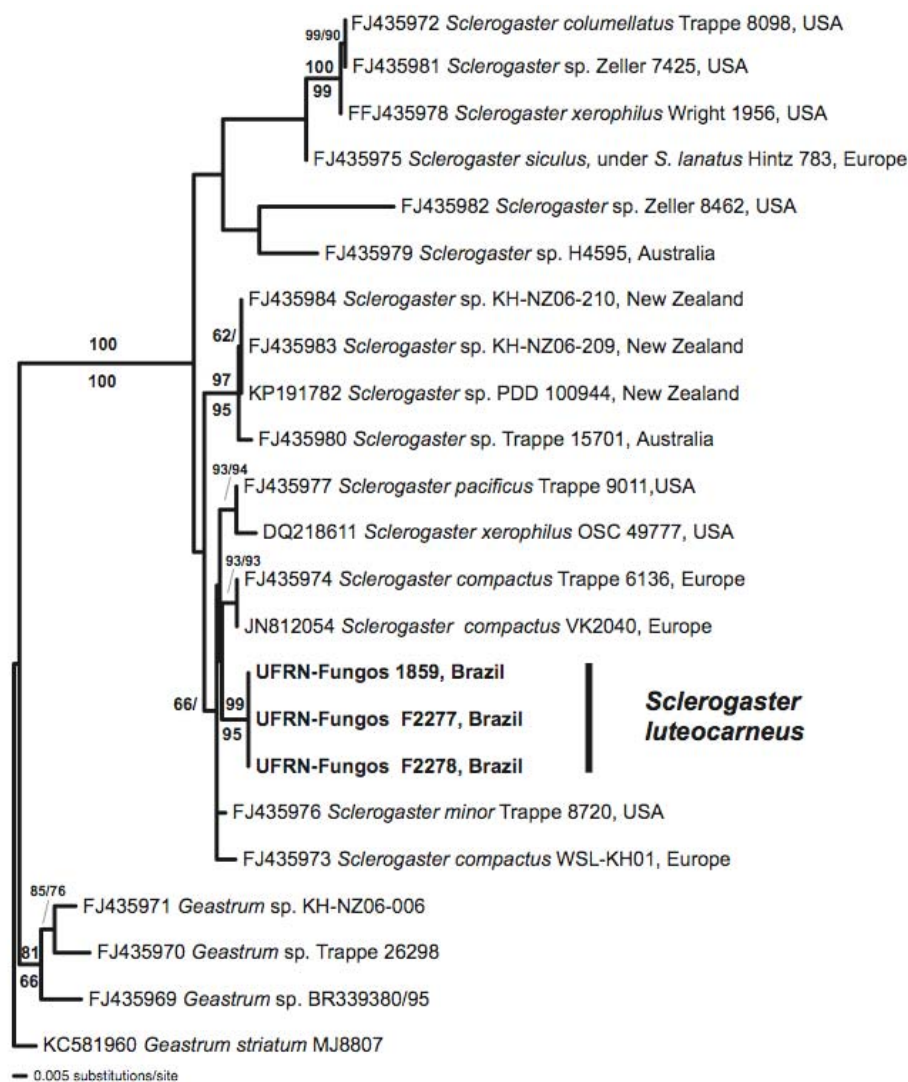
The three LSU sequences were aligned with 16 sequences downloaded from GenBank under *Sclerogaster* and four *Geastrum* sequences to produce a matrix of 683 unambiguously aligned nucleotide positions. Among them, 556 positions were constant, 41 were parsimony-uninformative and 86 were parsimony-informative. In the maximum parsimony analysis under heuristic search, all the 100 most parsimonious trees (MPTs) obtained with tree length=206, consistency index CI = 0.7330, retention index RI = 0.8485, and rescaled consistency index RC = 0.6219. Two trees were obtained from the maximum likelihood analyses (one of them shown in Figure 2). The strict consensus obtained from the MP and ML analysis (data not shown) showed similar topologies to Figure 2. In both analyses, the ingroup (*Sclerogaster*) formed a highly

supported monophyletic group (MPbs = 100%, MLbs = 100%).

The three new LSU sequences of the samples from Brazil were clustered as a well-supported clade (MPbs=99%, MLbs=95%), which was shown as a sister group to the two sequences labeled as *S. compactus* (FJ435974 and JN812054), however, this relationship was not well supported (MPbs and MLbs <50%). *Sclerogaster compactus* is morphologically similar to the Brazilian specimens with similar basidiospore sizes (4–6 µm and 4.5–6.5 µm) and patterns of basidiospore ornamentation (slightly verrucose), but *S. compactus* differs by the basidioma size (6 mm in *S. compactus* vs. 15 mm in Brazilian specimens; dried specimens), the hypogeous habit, and non-pedicellate basidiospores.

According to our results, three sequences under *S. minor*, *S. pacificus*, and *S. xerophilus* (all hypogeous) were grouped in the same clade with *S. compactus* (Figure 2) and the Brazilian specimens. However, those three species have bigger basidiospores of 7.4 µm or larger in diameter [9,13,16]. Moreover, *S. xerophilus* differs from Brazilian specimens because the peridium of the former species is easily separable from the gleba [16].

Figure 2 also shows that sequences from Brazilian specimens are very different from those of *S. collumelatus* and *S. siculus* (under *S. lanatus* in GenBank). Morphologically, *S. collumelatus* differs from the Brazilian specimens by its basidiomata with columella. *Sclerogaster siculus* has smaller basidiomata (0.6 × 0.4



**Figure 2:** Phylogenetic position of *Sclerogaster luteocarneus* specimens from Brazil, based on LSU sequences; one of two maximum likelihood trees generated assuming the HKY + I + G model selected by PAUP\*4.0b10 is shown. All isolates are labeled with GenBank accession numbers, name, isolate and geographical origin as indicated in GenBank (except to *S. lanatus*, that the current name *S. siculus* is also indicated). Maximum parsimony bootstrap support (above branches), and maximum likelihood bootstrap support (below branches) greater than 50 % are indicated.

mm), the outer peridium layer that is not pseudoparenchymatous, and the basidiospores minutely echinate (4.1–5.6  $\mu\text{m}$ ).

Sequences of other *Sclerogaster* species were not available at the EMBL/GenBank/DDBJ databases. However, morphological characters are different enough to distinguish these species from our specimens: in *S. candidus* the peridium is thinner (80–90  $\mu\text{m}$  thick), and the basidiospores are bigger (6.5–8.5  $\mu\text{m}$ ) [2]; in *S. hysteroangioides* the basidiomata are smaller (0.6  $\times$  0.4 mm), the outer peridium layer is not pseudoparenchymatous, and the basidiospores are minutely echinate (4.5–6.5  $\mu\text{m}$ ); in *S. liospermus* the basidiomata are smaller ("size of a pea", [2]) and the basidiospores are smooth (6–7  $\mu\text{m}$ ); and, finally, in *S. salisburyensis* the basidiospores are bigger (10.8–14.4  $\mu\text{m}$ ) and reticulate [34].

Since the three collections from the Atlantic Forest were found on decaying wood, and have unique morphological features that fit with the protologue of *Octavinia luteocarnea* [17], and the description of *S. luteocarneus* from Dodge & Zeller [2], and differ from the rest of the species already described under this genus, we confirm the identification of Atlantic Forest collections as belonging to the forgotten species *Sclerogaster luteocarneus*, and an updated description is provided below; moreover, an epitype is proposed.

### 3.1. Taxonomy

***Sclerogaster luteocarneus*** (Bres.) Zeller & Dodge, Ann. Mo. Bot. Gard 22: 370, 1935 (Figures 3-5).

Type:

J. Rick n° 51 (Lloyd Mus. 06025 and Dodge), from Rio Grande do Sul, is considered in Dodge & Zeller 1936 [2], as a type specimen (Not seen).

Epitype (Designated Here):

BRASIL, Minas Gerais, Santa Rita do Sapucaí, Reserva Biológica Municipal Cyro de Luna Dias, 22°25'00.0"S, 46°10'00.0"W, 03.01.2014, D.S. Alfredo & P. Lavor, DSA 208 (UFRN-Fungos 2278!; ITS sequence GenBank KT923665, LSU sequence GenBank KT923668).

#### 3.1.1. Macroscopic Characters

Basidiomata immature, 6–11 mm wide, globose to subglobose, lignicolous, growing on white subiculum in decaying wood. Basidiomata mature, 8–15 mm wide,

subglobose, lignicolous, sessile, attached to white subiculum. Peridium with two layers, not separable, 150  $\mu\text{m}$  thick; outer layer smooth in fresh specimens, later wrinkled, buff yellow becoming brown at maturity; inner layer wrinkled, cream. Gleba yellowish and gelatinous in fresh basidioma, becoming brown to dark brown and hard in dried basidioma, composed of small chambers (up to 0.5 mm wide). Columella absent.

#### 3.1.2. Microscopic Characters

Peridium outer layer pseudoparenchymatous with elements globose, subglobose to irregular shape (10–29  $\mu\text{m} \times 9$ –18  $\mu\text{m}$ , with walls of 0.5–1  $\mu\text{m}$  thickness), hyaline to yellowish in 5% KOH and negative reaction in Melzer. Peridium inner layer formed by hyphae of 3–12  $\mu\text{m}$  wide, septate, with occasional clamp connections, weakly dextrinoid, and hyaline in 5% KOH. Basidia not observed. Capillitium and paracapillitium absent. Basidiospores 4.5–6.5  $\mu\text{m} \times 4$ –6  $\mu\text{m}$  ( $x = 5.3 \pm 0.5 \times 5.2 \pm 0.5$ ,  $Q_m = 1.04$ ,  $n = 30$ ), globose, subglobose to ovoid, slightly verrucose, pedicellate (0.5–1.5  $\mu\text{m}$  length), pale yellow in 5% KOH, weakly dextrinoid.

#### 3.1.3. Specimens Examined

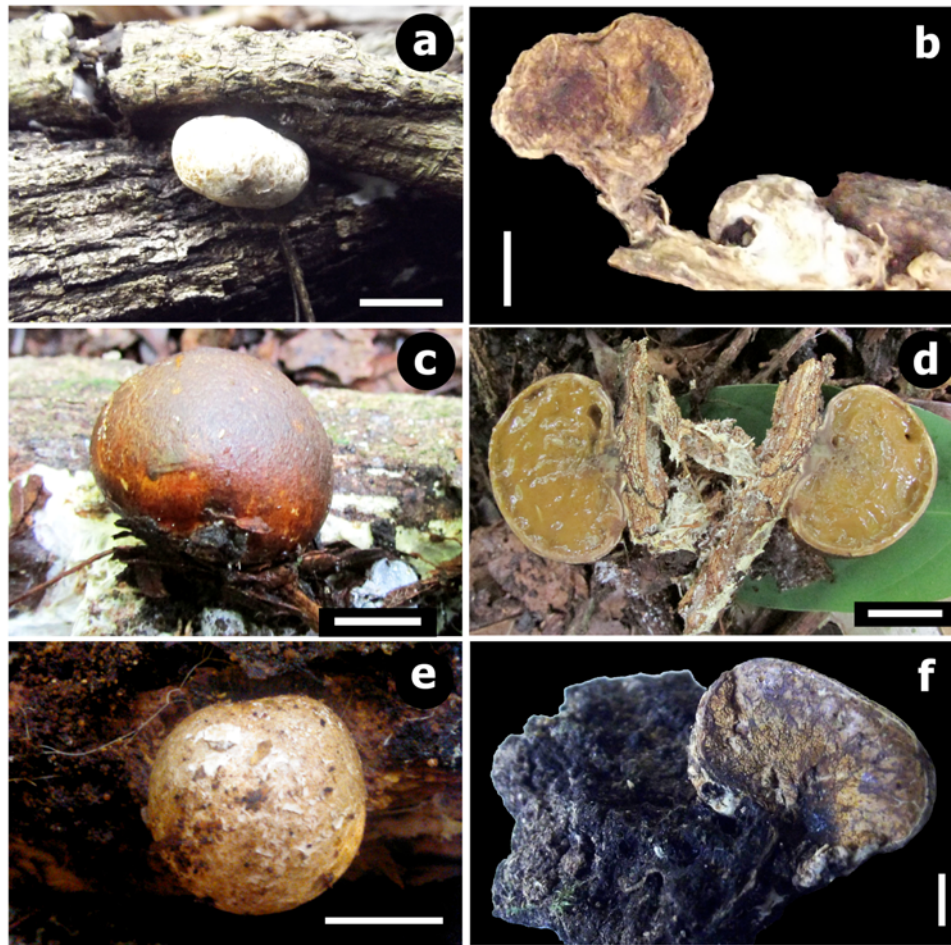
BRASIL, Minas Gerais, Santa Rita do Sapucaí, Reserva Biológica Municipal Mitzi Brandão, 22°25'00.0"S, 46°10'00.0"W, 03.01.2014, D.S. Alfredo & P. Lavor, DSA 208 (UFRN-Fungos 2278, duplex in MA-Fungi; ITS sequence GenBank KT923665, LSU sequence GenBank KT923668); Paraíba, Areia, Reserva Ecológica Estadual Mata do Pau-Ferro, 06°57'899"S, 35°44'956"W, 642 m, 18.07.2012, D.S. Alfredo, DSA 98 (UFRN-Fungos 1859, duplex in MA-Fungi; ITS sequence GenBank KT923663, LSU sequence GenBank KT923666); idem, 16.07.2013, D.S. Alfredo, DSA 172 (UFRN-Fungos 2277, duplex in MA-Fungi; ITS sequence GenBank KT923664, LSU sequence GenBank KT923667); Pernambuco, Recife, Parque Dois Irmãos, 08°05'01.07"S, 35°30'00.0"W, 21.06.2003, I.G. Baseia, s/n (UFRN-Fungos 1470).

### 4. CONCLUSION

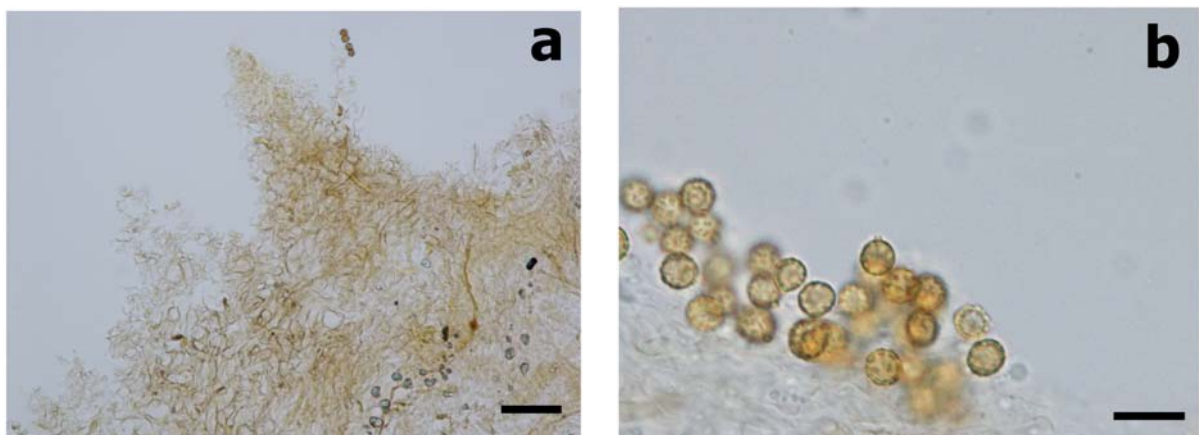
*Sclerogaster luteocarneus* has been found in the Brazilian Atlantic Forest with records from the south region (Rio Grande do Sul State), southeastern (Minas Gerais State) northeastern region (Pernambuco and Paraíba State) and in Guadeloupe, French Antilles.

The species grouped in the same clade as *S. luteocarneus* are distinguished by their morphological characters, such as the size of the basidiomata, the





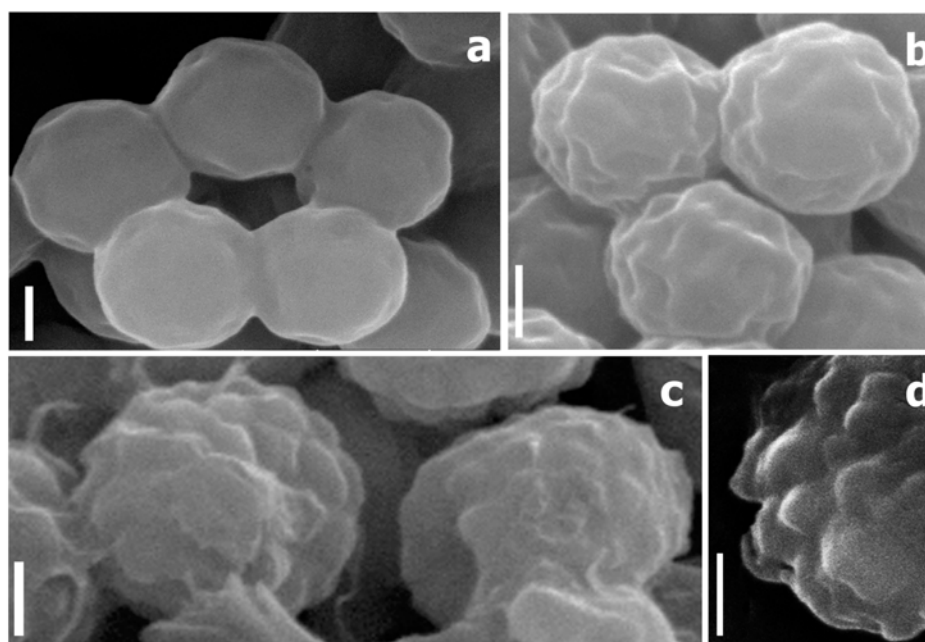
**Figure 3:** *Sclerogaster luteocarneus* basidiomata. **a-b:** UFRN-Fungos 1859; **c-d:** UFRN-Fungos 2277; **e-f:** UFRN-Fungos 2278. *In situ:* a, c-d, e; *Ex situ:* b, f. Bars: a, c-d, e = 10 mm; Bars: b, f = 2.5 mm.



**Figure 4:** *Sclerogaster luteocarneus*. **a:** Peridium, pseudoparenchymatous outer layer; **b:** Basidiospores. Bar: a = 50  $\mu$ m; Bar: b = 10  $\mu$ m.

outer peridium layer, as well as the size and ornamentation of the basidiospores. More molecular analyses are needed in this genus, since from some species no sequences are available in the EMBL/GenBank/ DDBJ databases, not even the barcoding sequence (ITS nrDNA).

We can conclude that, even after an exhaustive literature search, the epigeous growth habit seems to be unique of *S. luteocarneus*, since there is no other record of epigeous species. Moreover, *S. luteocarneus* has not been found for 80 years, so we decided to epytype this species.



**Figure 5:** *Sclerogaster luteocarneus* scanning electron micrographs of spores at different maturation stages. **a,c-d:** UFRN-Fungos 1859; **b:** UFRN-Fungos 1470. a-b: immature spores; c-d: mature spore. Bars: a-d= 1  $\mu$ m.

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