



## Original Paper

# ***Geastrum tupiense*: a new earthstar fungi species from the Brazilian Atlantic Forest and a new insertion in the *Velutina* subsection (Geastrales, Basidiomycota)**

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### Abstract

*Geastrum tupiense* is a new species belonging to subsect. *Velutina* (Sect. *Mycelostroma*). The fundamental diagnostic characteristics are reddish immature basidiomata and rhizomorphs with bipyramidal crystals. It was found in two areas of the Atlantic Forest biome, considered one of the main hotspots in the world for its high level of endemism and biodiversity. Delimitation of this new species was based on morphological characters and phylogenetic analyses of ITS/LSU nrDNA. Descriptions, phylogenetic tree, photographs, and taxonomic discussion are provided.

**Key words:** biodiversity, Geastraceae, neotropics, phylogeny, taxonomy.

### Resumo

*Geastrum tupiense* é uma nova espécie pertencente a subseção *Velutina* (Sessão *Mycelostroma*). As características diagnósticas fundamentais são basidioma imaturo avermelhado e rizomorfa com cristais bipiramidais. Foi encontrado em duas áreas do bioma Mata Atlântica, considerado um dos principais hotspots do mundo pelo seu elevado nível de endemismo e biodiversidade. A delimitação desta nova espécie foi baseada em caracteres morfológicos e análises filogenéticas de ITS/LSU nrDNA. São fornecidas descrições, árvores filogenéticas, fotografias, e discussões taxonômicas.

**Palavras-chave:** biodiversidade, Geastraceae, neotrópicos, filogenia, taxonomia.

### Introduction

The genus *Geastrum* Pers. presents great diversity in tropical regions. There were 67 species records from Brazil until the publication of Accioly *et al.* (2019). In recent years, five new species have been described: *Geastrum caatingense* J.O. Sousa, M.P. Martín & Baseia, *G. parvistellum* J.O. Sousa, M.P. Martín & Baseia (Sousa *et al.* 2019); *G. hyalinum* Freitas-Neto, N.M. Assis, J.O. Sousa, Baseia (Assis *et al.* 2019);

*G. lanuginosum* R.V.B. Araújo, J.O. Sousa, M.P. Martín, Baseia & B.D.B Silva (Crous *et al.* 2019); and *G. calycicoriaceum* Freitas-Neto, J.O. Sousa, Ovrebo, M.P. Martín & Baseia (Crous *et al.* 2020); *G. squameoramulosum* T.S. Cabral, J.O. Sousa & Baseia (Cabral *et al.* 2022). The total count of 73 species in Brazilian territory is a significant data, since the total number of *Geastrum* species known scientifically reaches about 100–120 worldwide (Zamora *et al.* 2014).

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In the genus *Gastrum* some species complexes have already been proven, such as: *Gastrum hirsutum* Baseia & Calonge, *G. schweinitzii* Zeller and *G. triplex* Jungh. based on molecular techniques, mainly with data from the ITS and LSU region that provide support on identification in this fungal group (Kasuya *et al.* 2012; Accioly *et al.* 2019), besides showing that several other species await formal names and descriptions (Zamora *et al.* 2015).

The Atlantic Forest is an important Brazilian Biome that runs along the coast of the Country from northeast to south, composed mainly of two types of vegetation: coastal forest and Atlantic semideciduous forest (Morellato & Haddad 2000). Despite the efforts of researchers and conservation programs created by non-governmental organization (such as “SOS Mata Atlântica”) in the last few decades to alert and halt the deforestation of this biome, less than 8% of its original vegetation cover remains intact (Faria *et al.* 2006). This fact is alarming, since the Atlantic Forest was ranked 4<sup>th</sup> in the level of importance of global hotspots by Myers *et al.* (2000). Due to the various ecosystems associated with this vegetation domain, the diversity of species may be even greater than what has been observed so far in the Amazon rainforest (Morellato & Haddad 2000).

The Atlantic Forest is also representatively important for the Brazilian mycobiota. Even with numerous non-inventoried areas (most of them occurring only in areas of environmental protection), only the genus *Gastrum* has 50 species catalogued for this biome, including the recently described new species: *G. pusillipilosum* J.O. Sousa, Alfredo, R.J. Ferreira, M.P. Martín & Baseia (Crous *et al.* 2016); *G. magnosporum* J.O. Sousa, B.D.B. Silva, P. Marinho, M.P. Martín & Baseia (Crous *et al.* 2018); *G. baculicrystallum* J.O. Sousa, Accioly, Baseia & M.P. Martín, *G. brunneocapillatum* J.O. Sousa, Accioly, M.P. Martín & Baseia and *G. neoamericanum* J.O. Sousa, Accioly, M.P. Martín & Baseia (Accioly *et al.* 2019); and two of the five species already mentioned as new to Brazil after Accioly *et al.* (2019): *G. lanuginosum* (Crous *et al.* 2019); and *G. calycicoriaceum* (Crous *et al.* 2020). Thus, this study contributes to the growth of knowledge about *Gastrum* in Brazil with the description of a new species endemic to the Atlantic Forest. Besides that, it provides new data for the phylogeny of the group, which is essential for modern taxonomic studies that are not based only on the specimen's morphology.

## Materials and Methods

### Collection site

The specimens were collected in two Atlantic Rainforest regions of Brazil (Fig. 1): the Mata do Pau Ferro State Park is located in the municipality of Areia, microregion of Paraíba Marsh (Northeastern Brazil) with an area of about 607 ha. Due to the orographic effect on precipitation and temperature reduction, this forest fragment is considered “Brejo de Altitude” and is part of the Atlantic Forest biome (Freitas *et al.* 2020). The second area, Serra de Santa Rita Mitzi Brandão Biological Reserve, is an important region of water supply in the state of Minas Gerais (Southeastern Brazil), located in the municipality of Santa Rita do Sapucaí. It belongs to an area classified as a transition between the Semideciduous Seasonal Forest and the Mixed Ombrophylous Forest and has an average annual rainfall of 1,500 mm (Alfredo *et al.* 2016).

### Morphological analyses

The materials were analyzed in the Laboratório de Biologia de Fungos (LBF), Departamento de Botânica e Zoologia (DBZ) - Universidade Federal do Rio Grande do Norte (UFRN) using as reference Sunhede (1989), Baseia *et al.* (2014), Sousa *et al.* (2014) and Zamora *et al.* (2014) for the methodological procedures and morphological analyses of the specimens. Analyses of macroscopic characters were done based on detailed observations with the naked eye and with the aid of a stereo microscope. The color chart of Kornerup & Wanscher (1978) was used to determine and describe the colors. For observation of the microstructures of the specimens, small portions of the basidiomata were removed by hand, under a stereo microscope, and the layers were separated with the aid of two histological tweezers and placed on separate microscopic slides containing 5% potassium hydroxide (KOH). The basidia were analyzed using Congo red dye for better visualization. All the microstructures were separated and observed under a Nikon Eclipse Ni (LM) optical microscope with Nikon DS-Ri1 camera attached, usually using 40x or 100x magnification objectives (Baseia *et al.* 2014). At least 30 basidiospores (including ornamentations) and 20 measurements of the other microstructures were randomly chosen and measured on the microscopic slide. All measurements and image captures were performed using the software NIS - Elements AR v.4.51.00. Statistical measurements

such as the mean of the basidiospore diameter and height, standard deviation ( $x \pm SD$ , respectively), and the ratio between mean width and height ( $Q_m$ ) followed the methodology proposed by Sousa *et al.* (2017).

### Molecular analyses

The molecular procedures of DNA extraction, amplification, and purification were performed at the Laboratório de Genética Molecular de Plantas (LGMP), Departamento de Biologia Celular e Genética - UFRN. Fragments of the gleba were used for DNA extraction. The extraction protocol used was an adaptation of the QIAGEN DNeasy Plant Mini Kit protocol (Qiagen, Valencia, California, U.S.A.), based on Martin & Winka (2000) for fungal studies. The amplification was performed by polymerase chain reaction (PCR), having as target regions: the Internal Transcribed Spacer (ITS) and Large Subunit region (LSU) of nuclear ribosomal DNA. Amplification, purification and sequencing protocols, and the phylogenetic analyses of

ITS/LSU nrDNA were based on Accioly *et al.* 2019, deposited in protocols.io (<dx.doi.org/10.17504/protocols.io.wpdfdi6>). The ITS and LSU sequences of the new species were compared with homologous sequences from GenBank (Tab. 1), from collections already published with both sequences (ITS and LSU).

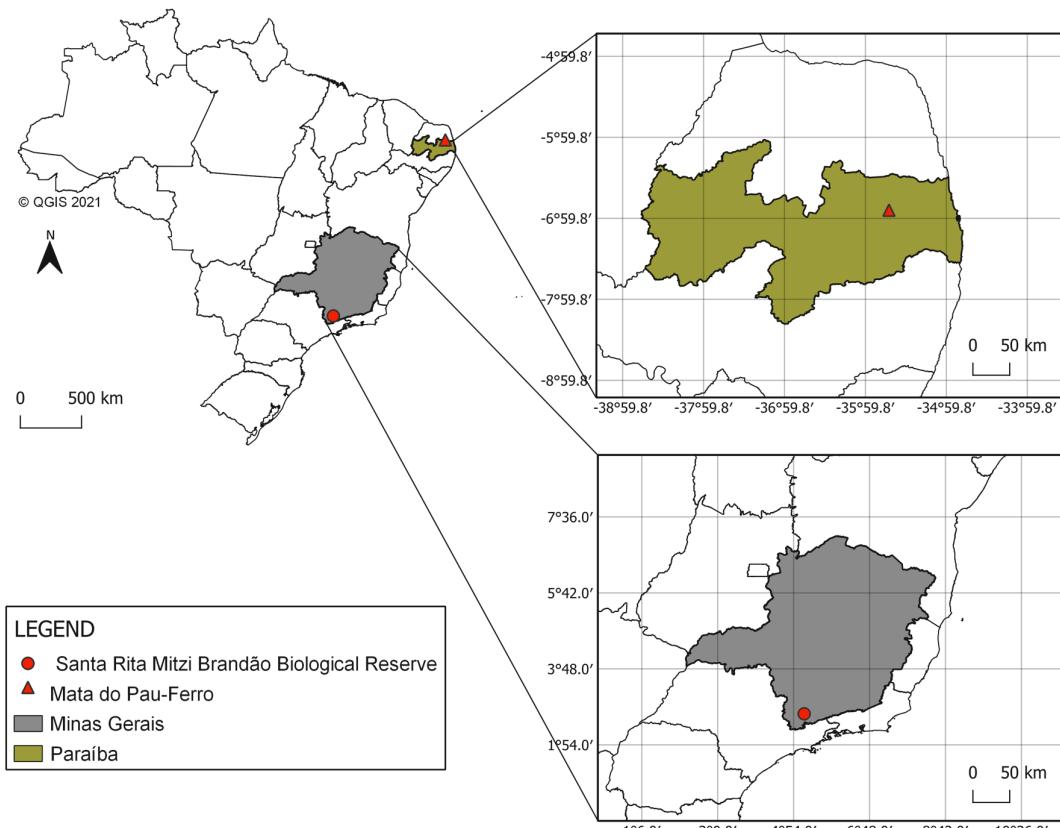
## Results

### Taxonomy

*Gastrum tupiense* J.O. Sousa, Freitas-Neto & Baseia, sp. nov. Mycobank: 839424.

*Gastrum tupiense* differs from other *Gastrum* species mainly by reddish unexpanded basidiomata with coriaceous surface and wrinkled regions; mycelial layer surface coriaceous with hyphae inflated at the apex (until 11.2  $\mu\text{m}$  diam.) and rhizomorph hyphae covered by bipyramidal crystal aggregates.

Unexpanded basidiomata epigaeous, reddish when fresh (7C8), dark blond (5D4) to bronze (5E5) when dried, subglobose with umbo when



**Figure 1** – Map showing the collection sites, Mata do Pau Ferro State Park (PB, Brazil) and Serra de Santa Rita Mitzi Brandão Biological Reserve (MG, Brazil).

**Table 1** – Specimens and sequences used to recover the phylogenetic trees. The access numbers of the sequences of the new species *Gastrum tupiense* are indicated with asterisks.

Species	Country	Collection number	GenBank accession number	
			ITS	LSU
<i>G. verrucoramulosum</i>	Brazil	INPA 264956	KX670831	NG_060680
<i>G. cf. stipitatum</i>	Brazil	Zamora 528	KF988345	KF988576
<i>G. inpaense</i>	Brazil	INPA 233330	KJ127023	KJ127017
<i>G. inpaense</i>	Brazil	INPA 255834	KJ127024	KJ127018
<i>G. albonigrum</i>	Brazil	UFRN-Fungos 1989	KJ127026	KJ127019
<i>G. albonigrum</i>	Panama	MAF 36140	KF988349	KF988468
<i>G. rufescens</i>	Slovakia	Adamcik 060628	KC582009	KC582009
<i>G. rufescens</i>	Spain	HerbZamora 253	KF988424	KF988552
<i>G. rufescens</i>	Sweden	VW 072006	KC582011	KC582011
<i>G. rufescens</i>	Sweden	MJ 6268	KC582010	KC582010
<i>G. rufescens</i>	Spain	HerbZamora 274	KF988425	KF988553
<i>G. aculeum</i>	Brazil	UFRN-Fungos 168	MK940899	MK940899
<i>G. caririense</i>	Brazil	UFRN-Fungos 2266	MF158626	MF158627
<i>G. echinulatum</i>	Brazil	UFRN-Fungos 1682	MK940900	MK940900
<i>G. echinulatum</i>	Brazil	ALCB 133026	MK94090	MK94090
<i>G. argentinum</i>	Argentina	MAF 82605	KF988353	KF988473
<i>G. argentinum</i>	Argentina	LPS 48446	KF988352	KF988472
<i>G. ishikawai</i>	Brazil	UFRN-Fungos 2785	KX753671	KX765817
<i>G. lanuginosum</i>	Brazil	ALCB 046912	MK940901	MK936167
<i>G. velutinum</i>	China	HFJAU 0291	MN258637	MN258637
<i>G. velutinum</i>	China	HFJAU 0467	MN258638	MN258638
<i>Gastrum</i> sp.	Japan	TNS Sakamoto 82	JN845197	JN845321
<i>Gastrum</i> sp.	USA	TENN 068889	KY744188	KY744188
<i>Gastrum</i> sp.	Argentina	AM_AR15-003	KY462273	KY462273
<i>G. velutinum</i>	New Zealand	PDD 2886	JN845173	JN845297
<i>G. javanicum</i>	Japan	TNS90902	JN845100	JN845218
<i>G. velutinum</i>	Spain	HerbRibes 31120762	KF988448	KF988583
<i>G. taylorii</i>	Argentina	CORDEN9	JN845204	JN845329
<i>G. velutinum</i>	Argentina	MAF 83785	KF988446	KF988581
<i>G. velutinum</i>	Argentina	MAF 83786	KF988447	KF988582
<i>G. calycicoriaceum</i>	Peru	MAF 83787	KF988449	KF988584
<i>G. calycicoriaceum</i>	Panama	UFRN-Fungos 3002	MT183521	MT183522
<i>G. calycicoriaceum</i>	Brazil	UFRN-Fungos 1215	KJ127031	JQ683663
<i>G. tupiense</i> sp. nov.	Brazil	UFRN-Fungos 2290	MW981354*	MW981349*
<i>G. tupiense</i> sp. nov.	Brazil	UFRN-Fungos 2291	MW981353*	MW981359*
<i>G. fornicatum</i>	Spain	MAF 30749	KF988375	KF988497

dried, 12.4–21.1 × 14.9–19.6 mm, surface coriaceous, with wrinkled regions, non-encrusted with debris. Subiculum white (2A2), rhizomorphs present, 22.2–27.1 mm, encrusted with debris. Expanded basidiomata saccate, 13.2–16.3 mm high (including peristome) × 17.4–37.8 mm wide. Exoperidium splitting into 6–9 triangular rays, revolute, rolling up under basidiomata, non-hygroscopic. Mycelial layer brownish orange (5C3) persistent, some basidiomata non-persistent and ephemeral, surface coriaceous, slightly encrusted. Fibrous layer yellowish white (2A2), coriaceous. Pseudoparenchymatous layer light orange (6A4) when fresh, soot brown (5F5) when dried, some rimose, persistent, with an inconspicuous collar in some basidiomata. Endoperidial body greyish brown (8F3), subglobose, 9.2–12.7 × 10.4–14.3 mm, sessile, surface glabrous, non-pruinose. Apophysis absent. Peristome fimbriate, greyish brown (7E3), distinctly delimited by a line lighter than the endoperidium, mammiform < 2 mm high. Gleba greyish brown (7F3). Rhizomorphs composed of hyaline, thin hyphae, surface covered by bipyramidal crystals, 3.5–9.6 × 1.2–2.2 µm, in an irregular arrangement. Mycelial layer composed of yellowish to hyaline hyphae, some sinuous, inflated at the apex, 4.3–11.2 µm diam, surface non-encrusted, some branched, thick-walled (0.6–1.6 µm) and evident lumen. Fibrous layer composed of yellowish to hyaline hyphae, 2.3–4.7 µm diam, surface non-encrusted, non-branched, thick-walled (0.6–1.5 µm) and lumen evident. Pseudoparenchymatous layer composed of yellowish cells, subglobose, oval to elongated in shape, 26.5–52.1 × 21.3–32.1 µm, thick-walled (0.8–2.4 µm). Eucapillitium with pale brown hyphae, 3–5.6 µm diam, surface non-encrusted, covered by warts, thin walls (0.3–0.8 µm) and evident lumen. Basidia yellowish, clavate, 20 × 6.5–7, with 6 sterigmata. Basidiospore brownish, globose to subglobose, 4.3–5 × 4.2–4.7 µm ( $x = 4.5 \pm 0.2 \times 4.41 \pm 0.2 \mu\text{m}$ ,  $Q_m = 1.02$ ,  $n = 30$ ), conspicuous ornamentation under LM. Warts between 0.4–0.9 µm high.

**Material examined:** Typus: PARAIBA: Areia, Mata do Pau Ferro State Park, 06°58'12"S, 35°42'15"W, 606 m a.s.l, on leaf litter, 21.VII.2014, J.O. Sousa *et al.* JM123, holotype UFRN-Fungos 2290, GenBank ITS= MW981353, LSU= MW981354; 22.VII.2014, J.O. Sousa *et al.* JM143 isotype UFRN-Fungos 2291, GenBank ITS= MW981349, LSU= MW981350.

**Additional materials examined:** BRAZIL. MINAS GERAIS: Santa Rita de Sapucaí, Serra de Santa

Rita Mitzi Brandão Biological Reserve, 22°11'44"S, 45°44'32"W, on wood, 23.II.2012, D.S. Alfredo & P.R. Lavor DSA 41, paratype UFRN-Fungos 2038; DSA46, paratype UFRN-Fungos 2039.

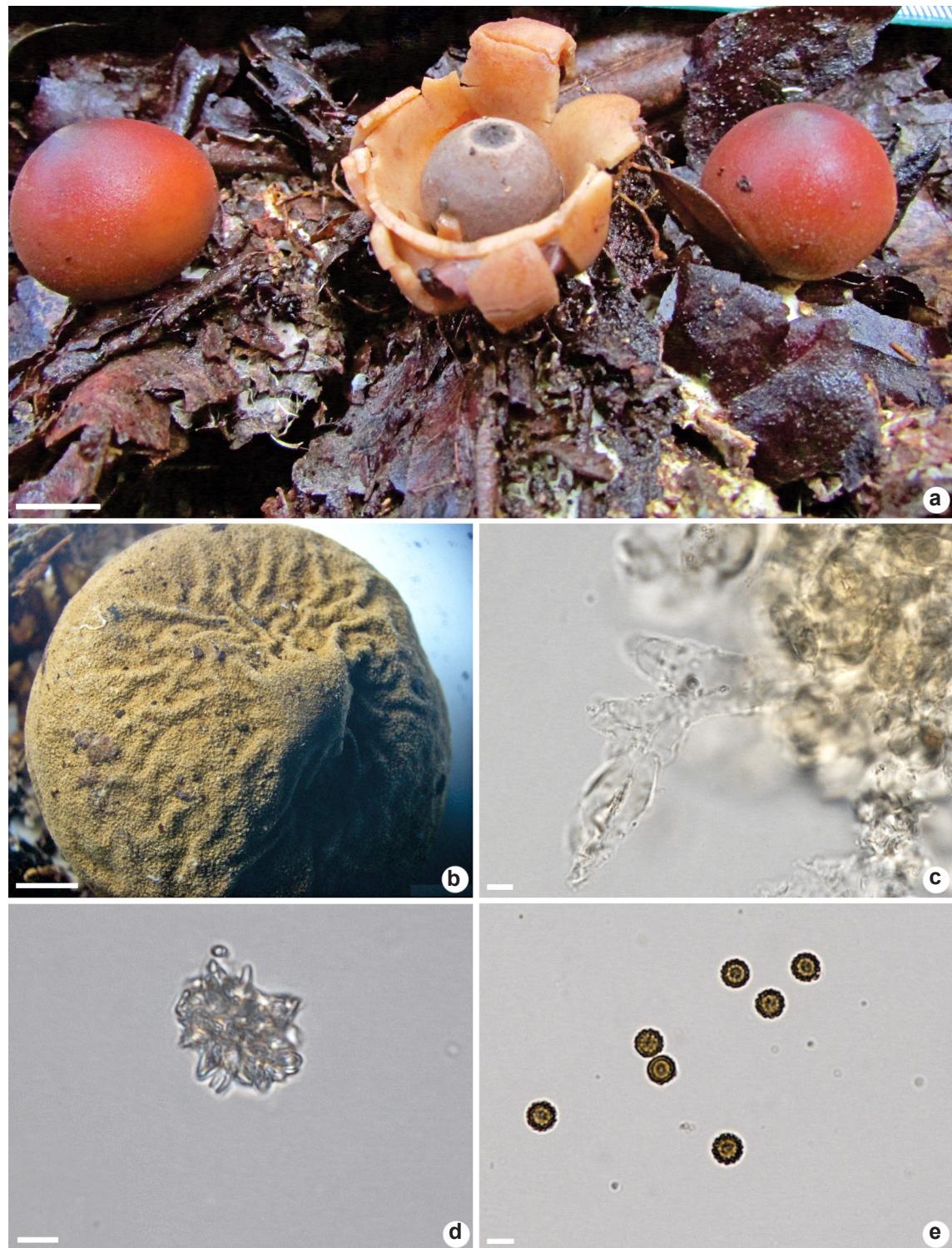
In honor of the Tupinambá indigenous people who inhabited the Brazilian coast, where the new species was collected.

The specimens were found in the Atlantic Rainforest of the states of Paraíba and Minas Gerais, Brazil. Growing on wood and leaf litter, with gregarious habit. Until now, the distribution of *G. tupiense* is restricted to Brazilian Atlantic Rainforest.

*Geastrum tupiense* is mainly characterized by reddish unexpanded basidiomata with coriaceous surface and wrinkled regions, mycelial layer surface coriaceous with hyphae inflated at the apex (up to 11.2 µm diam.), fimbriate and delimited peristome, basidiospores 4.2–5 µm and rhizomorph hyphae with bipyramidal crystals (Fig. 2).

As shown by our phylogenetic analyses that section *Exareolata* De Toni and sect. *Mycelostroma* (Henn.) P.Ponce de León, subsect. *Velutina* J.C. Zamora form well delimited phylogenetic clusters with a high level of confidence (100% posterior probabilities). *Geastrum forniciatum* (Huds.) Hook. was used as an outgroup because it presented molecular and morphological characters distinct from the species included in the analyses. (ITS and LSU concatenated, Fig. 3). *Geastrum tupiense* is inserted in sect. *Mycelostroma*, subsect. *Velutina*. Inside this subsection, the species *G. velutinum* Morgan and *G. calycicoriaceum* (Crous *et al.* 2020) has some features in common with *G. tupiense* (mycelial layer with coriaceous surface, delimited peristome, presence of subiculum and rhizomorph). *Geastrum velutinum* differs from *G. tupiense* by the fibrillose peristome, velutinous tufts in the mycelial layer, brownish to violet endoperidium, and basidiospores less than 4 µm (Dissing & Lange 1962). *Geastrum calycicoriaceum* is distinguished by the dark color in the unexpanded basidiomata, mycelial layer predominantly ephemeral, peeling-off forming a cup under the fruit body and composed of thin hyphae (up to 5 µm), smaller basidiospores (up to 4.1 µm) with smaller warts (up to 0.5 µm), as well as rhizomorph with acicular crystals (Crous *et al.* 2020).

Another similar species to *G. tupiense* is *Geastrum javanicum* Lév. due to the presence of basidiomata saccate, endoperidial body sessile, greyish, mycelial layer with coriaceous surface, subiculum and persistent rhizomorph (Ponce de



**Figure 2 – a-e.** *Geastrum tupiense* sp. nov. – a. unexpanded and expanded basidiomata *in situ* (UFRN-Fungos 2290, holotype); b. unexpanded basidiomata with coriaceous surface and wrinkled regions; c-e. microstructures under LM – c. mycelial layer with hyphae inflated at the apex; d. bypyramidal crystals of the rhizomorph; e. basidiospores. Scale bars = 10 mm (basidiomata *in situ*), 5 mm (unexpanded basidiomata) and all microstructures 5 µm.

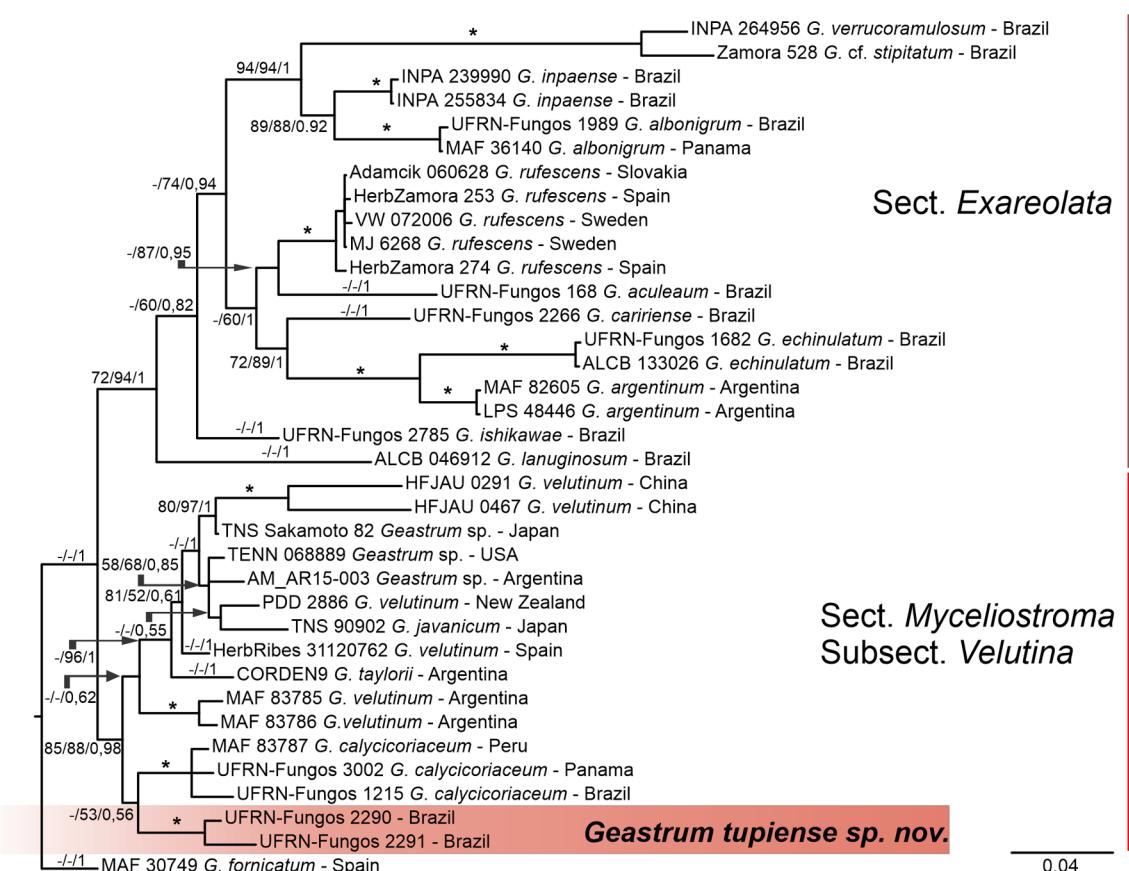
León 1968), but *G. tupiense* differs in having larger basidiospores (up to 5 µm), immature basidiomata with wrinkled regions, and fimbriate and delimited peristome.

Although the species *Gastrum aculeatum* B.D.B. Silva & Baseia, *G. caririense*, *G. echinulatum* T.S. Cabral, B.D.B. Silva & Baseia and *G. lanuginosum* belong to a distinct phylogenetic section (sect. *Exareolata*) from *G. tupiense*, they can also be compared morphologically because they are similar in color and size. However, *G. aculeatum* and *G. echinulatum* differs from *G. tupiense* by having mycelial layer covering (respectively in each species) by aculeos and peaky tufts, peristome non-delimited and dark unexpanded basidiomata (Silva *et al.* 2013). *G. caririense* differs in the non-delimited peristome, presence of pseudoparenchymatous collar and

larger basidiospores (4.5–7 µm) (Crous *et al.* 2017), while the *G. lanuginosum* is distinguished by the lighter endoperidium and pseudoparenchymatous layer, smaller basidiospores (3–4 µm), and mycelial layer with strangulated-acuminate hyphae forming wooly appearance (Crous *et al.* 2019).

## Discussion

The identification of fungi through classical taxonomy using only morphological aspects can aggravate taxonomic problems, especially when the objects of study are organisms with identical morphology or that present inconspicuous differences in their phenotype, known as cryptic or semi-cryptic species (Vizzini *et al.* 2013). This is mainly because taxonomists assign different weights to morphological characteristics, in addition to the subjectivity relating to some



**Figure 3** – Phylogenetic tree based on combined dataset (ITS/LSU). Section clades are colored and named on the right. Tree topology based on Bayesian analyses. *Geastrum forniciatum* was used as out-group. Numbers on branches are Parsimony and Maximum likelihood bootstrap, as well as Posterior Probabilities from Bayesian analysis. Asterisk (\*) represents the fully supported branches in Parsimony, Maximum likelihood and Bayesian analysis. The new *Geastrum* species is indicated in **bold**.

characters, generating a barrier to the establishment of widely accepted classifications (Stielow *et al.* 2011). Integrative taxonomy using at least two identification perspectives (such as morphological and molecular analysis) is on its way to becoming an excellent alternative in solving problems like these.

The problem generated by identification based only on morphological characters can create a range of hidden species known as species complexes: these organisms are named and attributed to existing species, masking their real diversity (Accioly *et al.* 2019). *Gastrum calycicoriaceum* and *G. tupiense* are phylogenetically close and are inserted in the subsect. *Velutina*. This subsection is characterized by humicolous species with a well-developed rhizomorph system covered by narrow prism-shaped crystals, and basidiomata not caespitose (Zamora *et al.* 2014). Both species are from South American (Neotropical) and they were previously identified as *G. javanicum*, despite having as type locality Java Island (Southeast Asia), a distinct ecoregion (Indo-Malay) (Olson *et al.* 2001). Furthermore, *G. javanicum* presents uncertainties regarding its nomenclature and systematic position. According to Ponce de Léon (1968), using only morphological data, *G. javanicum* and *G. velutinum* are considered synonyms. These species (*G. calycicoriaceum*, *G. tupiense*, *G. velutinum* and *G. javanicum*) have almost similar phenotypic characteristics and may be a new species complex in the genus *Gastrum*. From the revision of the genus proposed by Zamora *et al.* (2014), using both morphology and molecular tools, the type collection of the *Gastrum javanicum* was not recovered; thus, it was considered “enigmatic”. Therefore, we hope that our work can contribute to more robust phylogenetic studies involving the subsection *Velutina* that can elucidate the incongruities related to the species in this subsection.

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