



## Two new species of *Geastrum* (Geastraceae, Basidiomycota) found in Brazil

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With 4 figures

**Abstract:** We have studied molecular and morphological data of *Geastrum aculeatum* sp. nov. and *G. echinulatum* sp. nov. These two species were found in Brazil's semi-arid region and in central Amazon. It is characterized by the nature of the mycelial layer with aculeate tufts. *Geastrum echinulatum* differs from *G. aculeatum* in the size of the spores, presence of subiculum and structural details in the mycelial tufts. The phylogenetic analyses were performed through parsimony and Bayesian methods, using the *atp6* and *LSU* regions. These analyses confirm that both species are distinctly segregated from the other *Geastrum* species analyzed here.

**Key words:** Geastraceae, molecular phylogenetics, taxonomy, Neotropics.

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## Introduction

The current generic concept of *Geastrum*, described by Persoon in 1801, was based on the taxonomic treatment of Micheli (1729) as *Geaster*, which was considered an orthographic variant of *Geastrum* (Demoulin 1984). *Geastrum* is the largest genus of the family Geastraceae Corda, which is currently placed in the order Geastrales Hosaka & Castellano (Hosaka et al. 2006). This genus is well distributed geographically, with approximately 50 known species around the world (Kirk et al. 2008) and 40 records in Brazil (Trierveiler-Pereira & Baseia 2009). The systematics of this remarkable group has not yet been resolved, and molecular studies about tropical species are important for understanding the phylogeny and evolution of this gasteroid genus.

The northeast region of Brazil comprises nine states (areas), which covers 18% of the country total land area, with phytoecological domains of utmost importance for the conservation of biodiversity. Caatinga is the largest domain, with characteristic vegetation of semiarid regions, accounting for approximately 70% of the northeast region and 13% of Brazil's total territory (Lewinsohn & Prado 2003). Few species of fungi have been recorded in Brazilian semiarid areas (Fig. 1). Only four species of *Geastrum* have been described: *G. hieronymi* Henn., *G. saccatum* Fr., *G. setiferum* Baseia, *G. triplex* Jungh. and *G. xerophilum* Long (Leite & Baseia 2007, Leite et al. 2007, Drechsler-Santos et al. 2008, Silva et al. 2011).

The Amazon rainforest covers nearly 5.4 million km<sup>2</sup> of area (Malhi et al. 2008), sheltering approximately a quarter of the world terrestrial species (Dirzo & Raven 2003). 62% of the total territory is located in Brazil (MMA 2002), and despite the high biodiversity of the region, the mycobiota is still insufficiently studied (Souza and Aguiar 2004). In the Brazilian Central Amazon nine species of *Geastrum* have been recorded: *G. englerianum* Henn., *G. juruense* Henn., *G. saccatum*, *G. scleroderma* Mont., *G. fimbriatum* Fr., *G. entomophilum* Fazolino, Calonge & Baseia, *G. javanicum* (Lev.) P.Ponce, *G. lageniforme* Vittad. and *G. lilloi* L.S.Domínguez (Hennings 1904, Trierveiler-Pereira et al. 2009, Leite et al. 2011).

In this work we analyze morphological and molecular data, aiming to improve the knowledge about the diversity of this genus in the Brazilian Central Amazon and in semiarid regions, describing two new species.

## Materials and methods

**STUDIED AREA:** The studied material was collected in the following localities: 1) Parque Nacional Serra das Confusões (8°26'50"S, 42°19'47"W), 2) Serra da Jibóia (12°51'S, 39°28'W), both located in semiarid regions, and 3) Estação Experimental de Silvicultura Tropical – INPA (02°37'S, 60°09' W), part of the Amazon rainforest domain. Parque Nacional Serra das Confusões is covered with vegetation characteristic of caatinga regions and of caatinga-savannah transition zones (Ab'Sáber 1981). In Serra da Jibóia one can find sections of caatinga at the base, and typical vegetation of rocky environments at the top of the ridge (Velooso & Góes Filho 1982). Estação Experimental de Silvicultura Tropical (EEST) is covered with dense Tropical Forest (RADAM/Brazil 1978).

**MORPHOLOGICAL STUDIES:** The macroscopic studies were carried out on fresh and dry basidiomes. Color standards used were Kornerup & Wanscher (1978). Hand cut sections of dried specimens were

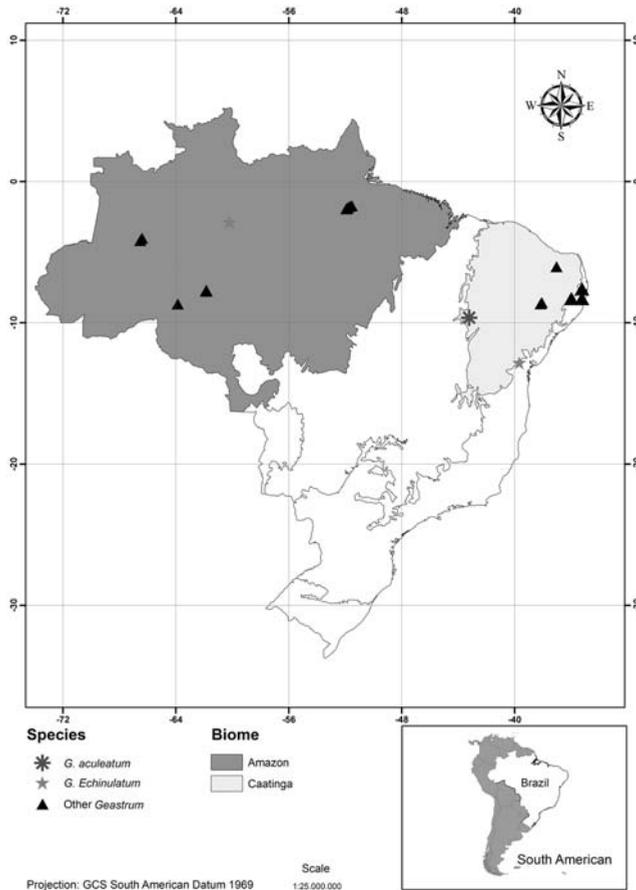


Fig. 1. Map showing *Geastrum* distribution in Brazilian semi-arid region and in central Amazon.

mounted in 5% KOH for light-microscope observations, and spore surface details were additionally analyzed under scanning electron microscope (SEM) according to Cortez et al. (2008). Basidiospore measurements including at least 20 additional randomly selected basidiospores from each specimen. The materials studied were deposited in the fungal collection from the Herbaria UFRN, URM and INPA.

### Phylogenetic Analysis

**DNA EXTRACTION:** The genomic DNA was extracted from herborized species including the holotypes of the new species, following the protocol of Gardes & Bruns (1993), with modifications. About 200 mg of peridium tissue was triturated in 500 µl of pre-heated CTAB solution (2% CTAB; 1,4 M NaCl; 100 mM Tris-HCl, pH 8,0; 20 mM EDTA; 0,2% b-mercaptoetanol) with a micropistile using a pellet pestle motor, and then incubated at 65°C for 30 min. Afterwards, 500 µl of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 10000 g for 10 min. For precipitation of DNA, 300 µl of 65% isopropanol was added and centrifuged at 12000 g for 20 min. The DNA was washed with 70% ethanol centrifuged at 12000 g for 3 min. The ethanol was then discarded and the DNA was left to dry overnight. The amount of DNA was estimated by using a NanoDrop® (Thermo Scientific) spectrophotometer, and diluted at 25 ng/µl for amplifications by PCR.

The large ribosomal subunit region of the nuclear DNA and the codant DNA region of ATPase subunit 6 were amplified using primer combinations LR0R/LR5 and ATP6-1/ATP6-2, as described by Vilgalys (1990) and Kretzer and Bruns (1999) respectively. The sequencing of fragments of PCR was done with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 or DYEnamic ET Terminator Matrix Standard, at Centro de Estudos do Genoma Humano – USP, Brazil.

**MOLECULAR PHYLOGENETIC ANALYSES:** The present study has involved two groups of molecular data. To test heterogeneity between these two groups, the incongruence length difference (ILD) test was performed on PAUP\* (Phylogenetic Analysis Using Parsimony) version 4b10 (Swofford 1998) to measure the significance of incongruence. The new sequences generated and retrieved from GenBank, originated in Hosaka and Castellano (2008) and Hosaka et al (2006), were aligned on ClustalW v.2.0.9 (Larkin et al. 2007) using default settings, and manually edited on BioEdit (Hall 1999). *Sclerogaster minor* and *S. compactus* were used as an external group based on previous analyses (Hosaka et al. 2006). The alignments were submitted to TreeBASE and may be accessed on <http://purl.org/phylo/treebase/phyloids/study/TB2:S12636?x-access-code=ba173c13b4bd2ff033d23b1cbc099f16&format=html> (Reviewer access URL).

The phylogenetic analyses were performed with concatenated data using maximum parsimony and Bayesian analysis. Both alignments were concatenated on Phyutility, designed by Smith & Dunn (2008). For maximum parsimony PAUP\* was used. Trees were calculated with heuristic search with TBR algorithm for branch-swapping and MULTREES on; the initial tree was obtained by stepwise addition with random addition sequences repeated 100 times. Gaps were treated as missing data. The support values were calculated with bootstrap of 1000 replicates, with same heuristic options above. Bayesian analysis was conducted with MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001) and the models that give the best fit for both alignments were chosen by MrModelTest (Nylander 2004). Bayesian analysis consisted in two different runs with four incrementally heated simultaneous Monte Carlo Markov chains over 2 million generations. Trees were sampled every 100 generations and, to estimate posterior probabilities, the burn-in stage was set observing when average standard deviation of split frequency (ASDSF) values dropped below 0.01. A separately Bayesian analyses with nuc-LSU and *atp6* was also performed (data not shown), and a parsimony based comparisons with Templeton test (Templeton 1983) and a likelihood based comparisons with Shimodaira-Hasegawa test (Rell optimization with 1000 bootstrap replicates) (Shimodaira & Hasegawa 1999) were performed to compare the tree topologies resulted from separately and concatenated data.

## Results

### Taxonomy

#### ***Geastrum aculeatum* B.D.B.Silva & Baseia, sp. nov.**

Figs 2a–c, 3a–c

MYCOBANK: MB 564409

Basidiomata juvenc epigaeum, depresso globosum, 10–13 mm altum, 11–16 mm latum. Exoperidium non hygroscopicum apertum 11–18 mm latum, 8–18 mm altum, fissum in 4–6 radios acutos, recurvos; stratum myceliale violaceo fusca, superficies cum caespes hypharum aculeatus; stratum medium albido vel cremeus, tenuis; stratum pseudoparenchymaticum canus caeruleus, persistens. Endoperidium 7–15 mm latum, 7–11 mm altum, sessile, globosum, laeve, cremeus vel brunneus; peristomium fibrillosum, non-delimitatum. Gleba brunnea; basidiosporae globosae vel subglobosae, 5.0–7.5 × 5.7–7.5 µm latae, brunneae, verrucosae. Hyphae capillitii longae, 3.8–5 µm latum, verruculosae, non ramificatae.

**ETYMOLOGY:** *aculeatum*, in reference to the presence of aculeate tufts covering the mycelial layer.

Unexpanded basidiomes depressed-globose, slightly umbonate, 10–13 mm tall × 11–16 mm diam., hyphal tufts aculeate, brown violet (10E5, 10E6). Expanded basidiomes 11–18 mm wide × 8–18 mm tall. EXOPERIDIUM splitting into 4–6 rays, saccate, some

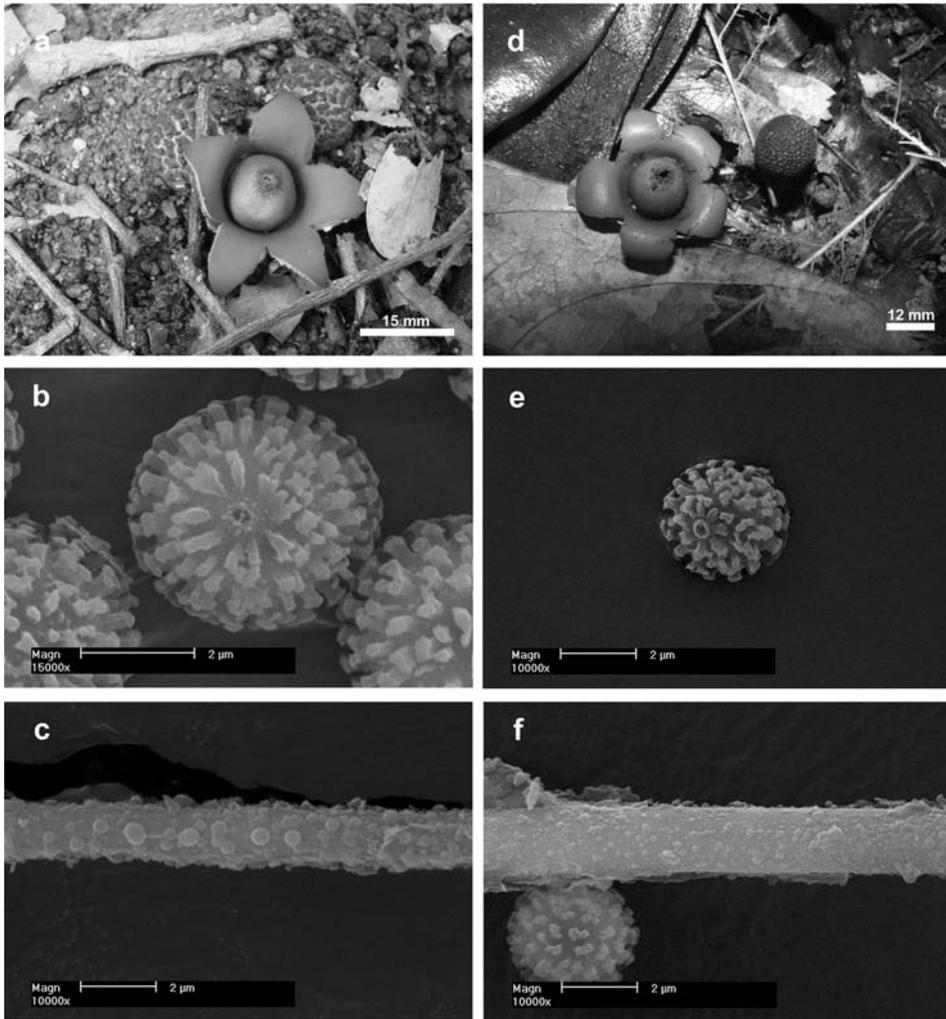


Fig. 2. *G. aculeatum*. a–c: a. Fresh basidiomata, b. Basidiospores under SEM, c. Eucapillitium under SEM. *G. echinulatum*. d–f: d. Fresh basidiomata, e. Basidiospores under SEM, c. Eucapillitium under SEM.

revolute, nonhygroscopic. Mycelial layer brown violet (10E5, 10E6), persistent, felted, forming units aculeate hyphal tufts, slightly encrusted with sand, falling off with time and leaving the fibrous layer exposed. Fibrous layer yellowish white (4A2), papery. Pseudoparenchymatous layer grayish violet (19D3) to bluish gray (19D2), thick, persistent. ENDOPERIDIAL body 7–15 mm wide × 7–11 mm tall (including peristome), sessile, globose to subglobose, glabrous, grayish brown (9F3, 10F3). Apophysis absent. PERISTOME fibrillose, not delimited, concolorous with endoperidium, appanate

to slightly conical. Columella white to beige, rounded to flat in cross section. GLEBA gray (10F1) to brownish gray (10F2).

BASIDIOSPORES globose to subglobose, 5.0–7.5 × 5.7–7.5 µm, verrucose under MO, under SEM the ornamentation is columnar, with rounded ends to almost flat, dark brown in 5% KOH. EUCAPILLITIUM 3.8–5 µm diam., rough, encrusted with amorphous substance, hyphal thin walls (up 0.8 µm), without pores, without septa, yellowish in 5% KOH. Mycelial layer composed of thin sinuous-walled hyphae, lumen absent, 2.5–5.0 µm diam., yellow to hyaline in 5% KOH. Fibrous layer composed of thin straight walled hyphae, 1.6–3.8 µm diam., yellow to hyaline in 5% KOH. Pseudoparenchymatous layer composed of subglobose, citriform to elongated hyphae, 16.5–31.7 µm diam. × 12.7–33.0 µm in length, walls >1 µm thick, hyaline to pale yellowish in 5% KOH.

MATERIAL EXAMINED: Brazil, Piauí, Parque Nacional Serra das Confusões, growing on stony soil, 31 March 2011, leg. B.D.B.Silva (UFRN-Fungos 1681 holotype; URM 81000 isotype).

**Geastrum echinulatum** T.S.Cabral, B.D.B.Silva & Baseia, **sp. nov.** Figs 2d–f, 3d–f

MYCOBANK: MB 564414

Basidiomata juvene epigaeum, subglobosae vel ovalis, 16–17 mm altum, 11–13 mm latum, in subiculo album. Exoperidium non hygroscopicum apertum 18–22 mm latum, 14–29 mm altum, fissum in 5 radios acutos; stratum myceliale brunneolum, superficies cum caespes hypharum; stratum medium albo-flavescens, papyraceus; stratum pseudoparenchymaticum rubro brunneolum, rigidus, persistens. Endoperidium 10–11 mm latum, 8–10 mm altum, subglobosae, laeve, sessile, brunneo cinereo; peristomium fibrillosum, non-delimitatum. Gleba brunnea; basidiosporae globosae, 3.8–5 µm latae, brunneae, dense verrucosae. Hyphae capillitii longae, 2.5–4.4 µm latum, verruculosae.

ETYMOLOGY: *echinulatum*, in reference to the presence of peaky tufts in mycelial layer.

Unexpanded basidiomes subglobose to oval, 16–17 mm tall × 11–13 mm diam., in wood, growing on a white subiculum, attached to the substrate by several white rhizomorphs up 1 mm thick, surface covered with hyphal tuft gregarious, light brown violet (6D5) to dark brown (6F5). Expanded basidiomes 18–22 mm wide × 14–29 mm tall. EXOPERIDIUM splitting into 5 rays, saccate, nonhygroscopic. Mycelial layer light brown (6D4, 6E5), persistent, forming hyphal tuft separate in the base, becoming gregarious at the tips of the rays, not encrusted with sand. Fibrous layer yellowish white (4A2), papery. Pseudoparenchymatous layer brownish red (8D3) when fresh, becoming dark brown (7F4), rigid, persistent. ENDOPERIDIAL body 10–11 mm wide × 8–10 mm tall (including peristome), sessile, subglobose, glabrous, brownish gray (6D3). Apophysis absent. PERISTOME fibrillose, not delimited, concolorous with endoperidium, appanate. Columella white to yellow, rounded to columnar in section. GLEBA dark brown (7D4).

BASIDIOSPORES globose, 3.8–5 µm, coarsely verrucose under MO, under SEM the ornamentation is ± columnar, with rounded ends to almost flat, brown in 5% KOH. EUCAPILLITIUM 2.5–4.4 µm diam., slightly rough, encrusted with amorphous substance, hyphal thin walls (up 1.3 µm), without pores, without septa, yellowish in 5% KOH. Mycelial layer composed of sinuous-walled hyphae, 3.8–8.9 µm diam., hyaline to yellowish in 5% KOH. Fibrous layer composed of straight and thin walled hyphae, 2.4–3.8 µm diam., hyaline to yellowish in 5% KOH. Pseudoparenchymatous layer composed of subglobose to elliptic hyphae, 19–36.8 µm diam. × 26.7–53.3 µm in length, hyaline to yellowish in 5% KOH.

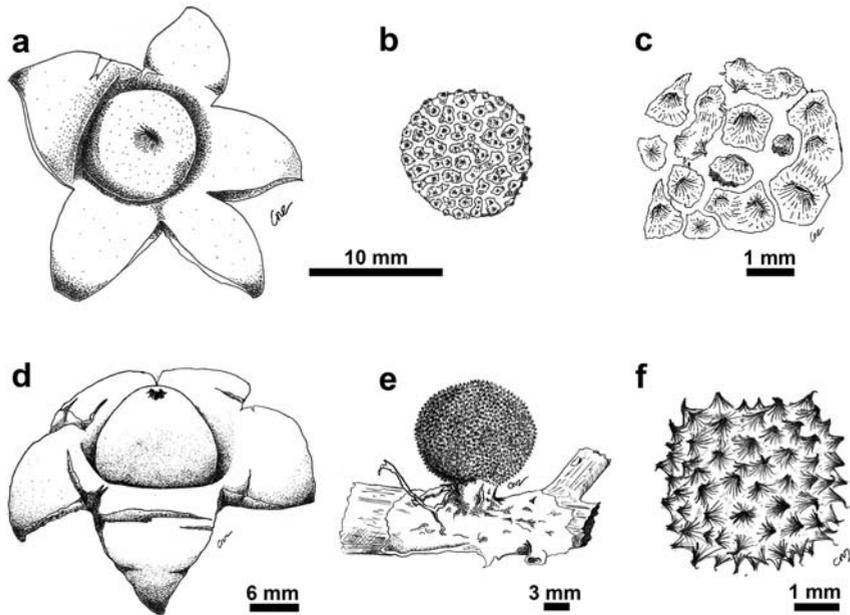


Fig. 3. Schematic drawing. a–c. *Geastrum aculeatum*; d–f. *Geastrum echinulatum*. a,d. expanded basidiome; b,e. immature basidiome; c,f. mycelial layer showing the hyphal tufts organization pattern.

MATERIAL EXAMINED: Brazil, Amazonas, Estação Experimental de Silvicultura Tropical (BR-174, km 45), growing over decayed wood, 10 May 2011, leg. T.S.Cabral (INPA 240002 holotype; UFRN-Fungos 1683 isotype); Brazil, Bahia, Serra da Jibóia, on wood, 22 September 2010, leg. B.D.B.Silva & Baseia (UFRN-Fungos 1682 paratype).

### Molecular Phylogenetic Analyses

Sequences from nuc-LSU and atp6 regions were successfully obtained from the species analyzed, with GenBank accession numbers: JQ683661, JQ683668 (*G. aculeatum* UFRN-Fungos 1681); JQ683659, JQ683665 (*G. echinulatum* INPA240001); JQ683667 (*G. echinulatum* UFRN-Fungos 1682, from which nuc-LSU region was not possible to amplify); JQ683660, JQ683666 (*G. sp.* INPA240005); JQ683662, JQ683670 (*G. hirsutum* UFRN-Fungos 1214); JQ683663, JQ683669 (*G. javanicum* UFRN-Fungos 1215); JQ683664, JQ683671 (*G. schweinitzii* UFRN-Fungos 1741). After the BLAST search, a total of 46 sequences were retrieved from GenBank.

The ILD test did not show significant heterogeneity ( $P=0.17$ ) between the datasets. The Templeton and Shimodaira-Hasegawa tests performed to compare tree topologies resulted from separately and concatenated datasets showed that the tree from concatenated dataset is significantly better than the others, suggesting that the use of concatenated data improve phylogenetic analysis in this group.

With concatenated data, in maximum parsimony analysis, from 1146 total characters, 405 were variable, from which 296 were parsimony informative (205 from atp6 and 91

from nuc-LSU). The analysis using heuristic search has led to one most parsimonious tree with 1129 steps, with CI=0.490, RI=0.516 and RC=0.253. MrModelTest chose GTR+I+G as the best model for both datasets. In Bayesian analysis, the average standard deviation of split frequencies dropped below 0.01 after generation 535000. Based on that, the first 5400 trees were discarded as burn-in period, and the remaining 14600 were used to calculate the consensus tree. After discarding the burn-in phase, the trees had a likelihood score of -6482.79 (total from the two runs) with the potential scale reduction factor (PSRF) of 1.000–1.001, suggesting a sufficient number of generations in analyses.

Parsimony and Bayesian analyses resulted in trees with similar topologies; however only consensus tree obtained after exclusion of the burn-in stage in MrBayes is illustrated in Fig. 4. The numbers on nodes represents posterior probabilities (PP) and bootstrap (BT) values.

## Discussion

The phylogenetic tree resulting from the molecular analyses shows *G. echinulatum* INPA 240002, *G. sp.* INPA 240005, *G. echinulatum* UFRN-Fungos 1682 and *G. aculeatum* grouping in a clade (PP:0.88, BT:54). Though not highly supported (PP<0.95; BT<70), it is clear that these species are phylogenetically distant from the others analyzed here. These species have hyphal tufts in the mycelial layer, organized in different ways. Primarily, that seems to indicate a synapomorphy in *Geastrum*. *Geastrum echinulatum* UFRN-Fungos 1682 and *G. echinulatum* INPA240002 are the same species, however they were collected in the states of Bahia and Amazonas respectively. *Geastrum sp.* INPA 240005 is probably a new species, but it was not described in this work since only one basidiome has been found. We believe it is a new species due to the morphological differences, such as delimited peristome, non-persistent pseudoparenchymatous layer, and the habitat of growing in soil.

*Geastrum aculeatum* is characterized chiefly by the presence of aculeate hyphal tufts on the mycelial layer, large basidiospores ( $5.0\text{--}7.5 \times 5.7\text{--}7.5 \mu\text{m}$ ) and violet gray to bluish gray pseudoparenchymatous layer when fresh; whereas *G. echinulatum* is characterized by the presence of subiculum,  $3.8\text{--}5 \mu\text{m}$  spores and also by aculeate hyphal tufts in the mycelial layer, yet it is not possible to find gaps between this one's tufts.

*Geastrum aculeatum* and *G. echinulatum* resemble *G. litchiforme* Desjardin & Hemmes in the surface of the immature basidiome, with tufts of hyphae arranged on the surface of the mycelial layer. However, *G. litchiforme* has smaller spores ( $3.2\text{--}3.8 \mu\text{m}$ ), collar-shaped pseudoparenchymatous layer around the endoperidium and cup-shaped mycelial layer (Hemmes & Desjardin 2011). One also notices that the organization pattern of the hyphal tufts in *G. aculeatum* resembles that of *G. litchiforme* better than the one of *G. echinulatum*.

Macroscopically, *G. aculeatum* resembles *G. saccatum*, specifically in the saccate basidiome, fibrillose peristome and sessile endoperidium. However, *G. saccatum* Fr. has smaller spores ( $4.6\text{--}6 \mu\text{m}$ ), delimited peristome and non-hygroscopic exoperidium (Sunhede 1989).

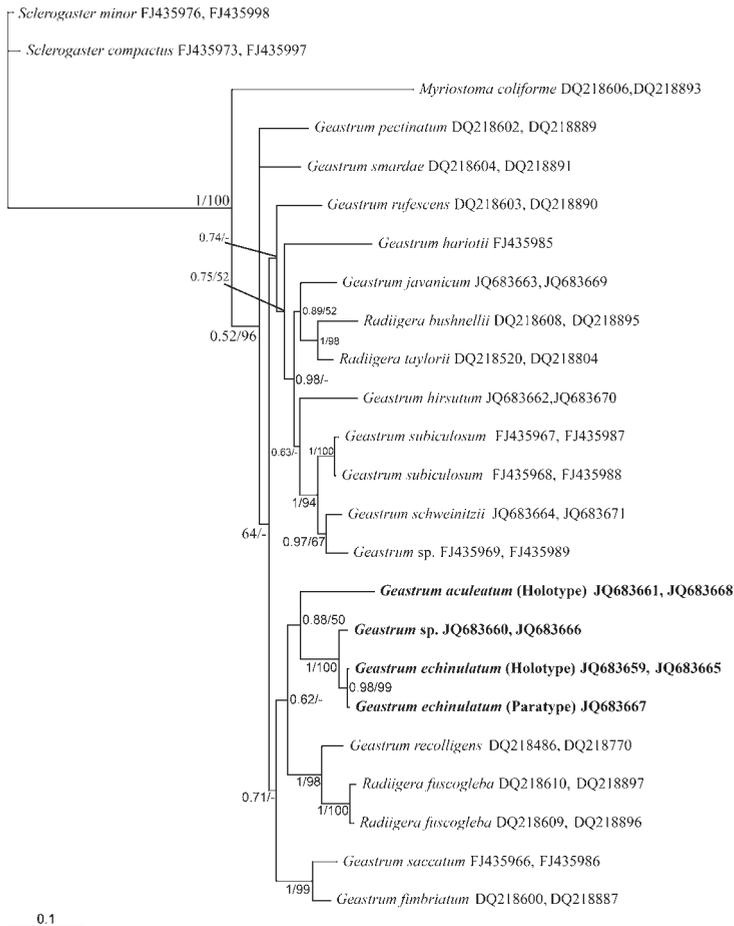


Fig. 4. A 50% majority rule consensus tree computed after exclusion of burn in period in MrBayes. Node numbers correspond to posterior probabilities (PP) and bootstrap values. The tree shows *Geastrum* species that have in common hyphal tufts on mycelial layer clustered together, which may indicate a synapomorphy in the genus.

A few species of *Geastrum* are similar in basidiospores size to *G. aculeatum*: *G. berkeleyi* Masee (5.5–7.0 μm), *G. floriforme* Vittad. (5.5–7.2 μm), *G. pectinatum* Pers. (5.5–7.0 μm), *G. pouzarii* V.J.Staněk (5.5–7.0 μm) and *G. pseudolimbatum* Hollós (5.5–7.0 μm). However, *G. berkeleyi*, *G. pectinatum* and *G. pouzarii* have delimited and sulcate peristome, and pedicellate endoperidium, whereas *G. floriforme* differs for having involute rays over the endoperidium, larger amount of rays (5–13) and furfuraceous endoperidium at the beginning of the development. *Geastrum pseudolimbatum* differs for pedicellate endoperidium, with small warts on the surface, and arched exoperidium, with tips recurved towards the endoperidium when fresh

(Cunningham 1944, Bottomley 1948, Sunhede 1989, Pegler et al. 1995, Bates 2004). On the phylogenetic tree this separation is clear, and it is possible to observe *G. aculeatum* and *G. pectinatum* grouping in different clades.

*Geastrum echinulatum* resembles *G. javanicum* and *G. velutinum* Morgan, in the reddish color of the pseudoparenchymatous layer, tomentous mycelial layer and presence of subiculum, but *G. javanicum* and *G. velutinum* have a velutinous mycelial layer that separates from the basidiome becoming semifornicate and delimited peristome.

The presence of subiculum is observed in few species of *Geastrum*: *G. hirsutum* Baseia and Calonge, *G. lilloi*, *G. schweinitzii* (Berk. & Curt.) Zeller, *G. subiculosum* (Cooke & Masee) G.H.Cunningham, *G. pleosporus* Douanla-Meli and *G. mirabile* Mont. However *G. hirsutum* differs from *G. echinulatum* in the cespituous basidiomes, hirsute mycelial layer, delimited peristome and smaller spores (2.5–3µm); *G. lilloi* has small basidiome (up to 2 cm), smooth and velvety mycelial layer, presence of microsclereids, and smaller basidiospores (2.5–3.5µm). *G. schweinitzii* and *G. mirabile* also have cespituous basidiome and smaller spores (3.2–3.8µm and 3–4 µm). *G. subiculosum* differs in the slightly warty spores; and *G. pleosporus* has a mycelial layer that easily separates from the fibrous layer, and delimited peristome. Moreover, observing the phylogenetic tree it is possible to see that *G. echinulatum* is phylogenetically distant from *G. subiculosum*, *G. schweinitzii*, *G. javanicum* and *G. hirsutum*, species that have in common the presence of subiculum.

Based on molecular and morphological data, and on intense comparison with other species, it is clear that *G. aculeatum* and *G. echinulatum* are new species of *Geastrum*, and that broadens our knowledge about this genus.

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