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ORIGINAL ARTICLE

Obtaining monokaryotic and dikaryotic mycelial cultures of two Amazonian strains of *Geastrum* (Geastraceae, Basidiomycota)

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ABSTRACT

The high diversity of the genus *Geastrum* and the difficulty of obtaining mycelial cultures impairs the study of the ecophysiology and the exploration of the biotechnological potential of the taxon. In this study, different culture media were tested to obtain mycelial cultures for *G. lloydianum* and *G. subiculosum* collected in the Brazilian Amazon. Data on spore germination, and isolation of monokaryotic cultures and *in vitro* sexual reproduction are presented, as well as a brief morphological description of the cultures obtained. For both species, Potato Dextrose Agar (PDA) was the most promising of the tested culture media. The highest growth in agar culture ever recorded for this genus is reported (4.9 mm per week for *G. lloydianum* and 7.5 mm for *G. subiculosum*). In the PDA culture medium, spores germinated after 35-40 days of incubation and the isolation of monokaryotic cultures of the two species, as well as *in vitro* sexual crosses, were successfully performed.

KEYWORDS: earthstars; gasteroid fungus; *Geastrum lloydianum*; *Geastrum subiculosum*; mycelial growth

Obtenção de culturas miceliais monocariótica e dicariótica de duas linhagens amazônicas de *Geastrum* (Geastraceae, Basidiomycota)

RESUMO

A alta diversidade do gênero *Geastrum* e a dificuldade de obtenção de culturas miceliais prejudicam o estudo ecofisiológico e a exploração do potencial biotecnológico do táxon. Nesse estudo, foram testados diferentes meios de cultivo, visando a obtenção de culturas miceliais para *G. lloydianum* e *G. subiculosum* coletadas na Amazônia brasileira. A germinação dos esporos, o isolamento das culturas monocarióticas e o cruzamento sexual *in vitro* são apresentados, além de uma breve descrição morfológica das culturas obtidas. O meio de cultura Batata Dextrose Ágar (BDA) foi o mais promissor dentre os meios de cultura testados no cultivo das duas espécies. Reportamos o maior crescimento em cultura de ágar já registrado para esse gênero (4,9 mm por semana para *G. lloydianum* e 7,5 mm por semana para *G. subiculosum*). Nesse meio de cultivo, os esporos germinaram após 35-40 dias de incubação e o isolamento de culturas monocarióticas das duas espécies, assim com os cruzamentos sexuais *in vitro*, foram realizados com sucesso.

PALAVRAS-CHAVE: estrela-da-terra; crescimento micelial; fungo gasteroide; *Geastrum lloydianum*; *Geastrum subiculosum*

INTRODUCTION

Geastrum Pers. is one of the genera of Basidiomycetes popularly known as earth stars, due to the aspect of the exoperium, that forms rays on dehiscence, conferring a stellariform appearance to mature basidioma (Hemmes and Desjardin 2011; Jeppson *et al.* 2013). The genus is polyphyletic (Wilson *et al.* 2011), with approximately 120 species (Zamora *et al.* 2014), making it the most diversified in the family Geastraceae Corda (Hosaka *et al.* 2006). Collectively, members of the genus have the ability to colonize a wide variety of environments (Zamora *et al.* 2013), including sandy soils, and those rich in organic material, as well as acting as a decomposer of wood (Cortez *et al.* 2011) and having ectomicorrizal associations (Karun and Sridhar 2014).

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Despite the many studies on *Geastrum* and the contining discovery of new species (Hemmes and Desjardin 2011; Leite *et al.* 2011; Trierveiler-Pereira *et al.* 2011; Silva *et al.* 2011, 2013; Zamora *et al.* 2013, 2014, 2015; Cabral *et al.* 2014; Crous *et al.* 2015; Caffot *et al.* 2016), the monograph by Sunhede (1989) is, by far, the most complete study on this genus, as it not only considers the morphological aspects of the basidiomas, but also the isolation and the characteristics of the mycelial cultures of diverse European species.

Sunhede (1989) reported the slow *in vitro* growth of *Geastrum* cultures, describing a range of 0.5 to 3.7 mm per week for some species. Stoytchev *et al.* (2001), studying *G. pouzarii* V.J. Staněk, recorded growth of 3 to 4 mm in six weeks. Zamora *et al.* (2014) in a study of *G. argentinum* Speg., observed up to 4 mm of growth per week. All these authors considered, it is necessary to improve mycelial growth in this group, mainly due to its potential for antimicrobial, anti-inflammatory, astringent and anti-hemorrhagic activity (Guerra-Dore *et al.* 2007) in the biotechnological production of bio-active compounds (Liu and Zhang 2004) and in bioremediation (Chittaragi *et al.* 2013; Sevindik *et al.* 2017; Kuhar *et al.* 2016; Santana *et al.* 2016).

In this study, we tested different mycelial growth media for the Amazon strains of *G. lloydianum* and *G. subiculosum,* report spore germination and the succesful rearing of monokaryotic and dikaryotic cultures, *in vitro* sexual reproduction for both species, and describe the characteristics of the different cultures obtained.

MATERIAL AND METHODS

Basidioma collection

Mature basidiomas of *G. lloydianum* and *G. subiculosum* were collected by hand at Campus III of Instituto Nacional de Pesquisas da Amazônia (INPA), in the city of Manaus, Amazonas state, Brazil (3°5'33.0"S, 59°59135.0"W).

Basidioma preservation followed the methodology proposed by Lodge *et al.* (2004). The material was identified following the descriptions of Sunhede (1989), Calonge *et al.* (2005), Cabral *et al.* (2014) and Sousa *et al.* (2014). Part of the collected material was used for evidence and was deposited in the INPA Herbarium (*G. lloydianum* INPA-Fungos 259923 and *G. subiculosum* INPA-Fungos 259933).

Obtaining dikaryotic mycelia

Dikaryotic mycelia of *G. lloydianum* and *G. subiculosum* were individually isolated from sections removed from the pseudoparenchymatous layer of a fresh basidial exoperidium and fragments of approximately 1 × 1 mm were inoculated onto a 90 mm diameter Petri dish containing 15 mL of medium (PDA, Difco®), and then incubated at 25 °C, in the absence of light in a Biological Oxygen Demand (BOD) chamber. After one week, 2 × 2 mm agar blocks containing mycelium were harvested and placed on new plates of the same volume and content to give pure cultures.

Evaluation of the mycelial culture medium

Mycelial growth of *G. lloydianum* and *G. subiculosum* was tested in three solid culture media: Potato Agar Dextrose (PDA, Difco®), Malt Extract Peptone Agar (MEPA) [3% Malt Extract (Becton Dickinson); 0.3% soy peptone (Acumedia); 1.5% agar (Becton Dickinson)], and Sabouraud Dextrose Agar (SDA) Becton Dickinson®. Culture media were autoclaved at 121 °C for 15 minutes and 15 mL poured into 90 mm diameter Petri dishes. A 2 × 2 mm fragment from each culture was transferred to the center of a Petri dish and maintained at 25 °C in the absence of light in the BOD chamber.

The experiment was conducted with a completely randomized design, using five replicates per treatment for each species. On experimental day 42, diameter of colonies and mycelial mass dry weight were measured, following Vargas-Isla and Ishikawa (2008). Mean colony masses and diameters were submitted to analysis of variance (two-way ANOVA) followed by a Tukey test, if significant, with level of significance set at $p < 0.05$. Statistical analyzes were performed with the ASSISTAT program (7.7 beta).

Obtaining a monokaryotic mycelium

Basidiospores of *G. lloydianum* and *G. subiculosum* were obtained from suspensions of spores in sterile distilled water, plus 20 µL of Tween 80 (Sigma-Aldrich®) stirred lightly on a Vortex® agitator. A 50-µL aliquot was then spread onto a Petri-dish surface, to test which culture provided the best dikaryotic growth medium for each species, and maintained at 25 °C, in the absence of light in a BOD chamber.

Germinated basidiospores were selected under an optical microscope and transferred to new Petri dishes of volume and content equal to those described above, then maintained under the same conditions and analyzed for growth and absence of connections over a five week period. For each species, the ten monokaryotic cultures showing the highest mycelial growth rates were selected, multiplied with transfer to new Petri dishes and used for crossing tests.

Mycelial crossing

Crosses were determined by pairing the ten monokaryotic cultures of each species. Fragments measuring 2 × 2 mm of the various monokaryotic cultures were inoculated in pairs, seperated by distances of 2 mm from each other in Petri dishes with 15 mL of the culture medium that provided the best growth rates in the dikaryotic cultures. These were maintained at five pairs per plate with three replicates and kept at 25 °C in a BOD chamber in the absence of light and analyzed over a five-week period for the formation of clamp connections.

After confirming clamp connection formation in cultures of both species, the three cultures with the highest growth for each species were selected and the original crossing-derived dikaryotic mycelium grown-on in new Petri dishes to multiply the stock. After growth, five 2 × 2 mm fragments were inoculated into 125 mL capacity Erlenmeyer flasks, with 50 mL of the liquid of the culture medium (agar absent) which suported the best dikaryotic culture growth and, in triplicate, maintained in a BOD at 25 °C in the dark.

After five weeks growth, mycelia were separated from the medium by vacuum-pump filtration, washed three times in sterile distilled water and subjected to DNA extraction following Raeder and Broda (1985).

Amplification of the rDNA internal transcriber-spacer region (ITS) was performed with previously described primers and protocols (Gardes and Bruns 1993). PCR product purification was undertaken with ExoSAP-IT (Affymetrix Inc.), and sequencing was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit GFX (Amersham Pharmacia®), using the same primers.

Sequences of *G. hariotii* Lloyd, *G. pectinatum* Pers., *G. triplex* Jungh. and *G. parvistriatum* J.C. Zamora & Calonge were added in order to test the effectiveness of crosses for species separation. *Myriostoma coliforme* (Dicks.) Corda was used as an outgroup. Sequences were first aligned with Clustal X 2.1 (Larkin *et al.* 2007), and then BioEdit (Hall 1999). An ITS-based distance matrix was calculated using the Kimura-2 parameter replacement model with MEGA (Tamura *et al.* 2013) to assess between species divergence.

Maximum parsimony (MP) phylogenetic analyses with ITS concatenation were performed. For this, PAUP (Swofford 1998) was used, and the trees assembled using a heuristic search for branch exchange using the TBR algorithm. The initial tree was obtained by stepwise addition of 100 repeated random sequencess and 1000 bootstrap repititions. The resulting tree was edited with FigTree (http://tree.bio.Ed.ac.uk/software/figtree/).

RESULTS

Taxonomy

Geastrum lloydianum Rick, Brotéria 5: 26 (1906). Figure 1a-b.

≡*Geaster lloydianum* Rick (1906) [MB#528074]

≡*Geastrum saccatum* var. *lloydianum* (Rick) Rick (1961) [MB#349502]

Basidioma curved expanded, from 14 to 27 mm in height, including the peristomium, 14 to 27 mm in diameter, non-hygroscopic, fibrous papyraceous, cotonous, persistent fleshy mycelial layer, detaching itself in irregular brown sections. Endoprosthesis dark grayish brown to dark brown, globose deprescent, with apophysis present, sessile and short stipulate,

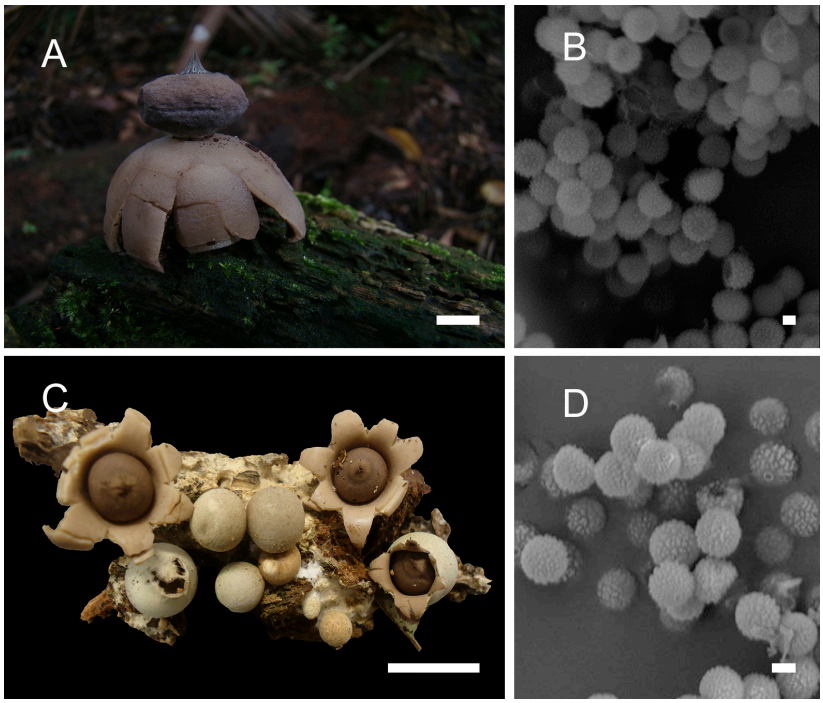

Figure 1. *Geastrum lloydianum*: A) Immature and expanded basidioma; B) Basidiospores. *Geastrum subiculosum*: C) Immature and expanded basidioma; D) Basidiospores. Scale bar: A; C = 1 cm; B; D = 3 µm. This figure is in color in the electronic version.

peristomium conical grooved, not delimited and concolorate with endoperidium. Basidiospores globose to subglobose, measuring 3.6 to 5.4 µm in diameter, slightly warty, apicule vermilion in presence of KOH.

Material examined: Brazil. Amazonas, Manaus. INPA Campus III, 18 II 2014. Santana, MDF. INPA 259923.

Geastrum subiculosum Cooke & Massee, Grevillea 15: 97 (1887). Figure 1c-d.

≡*Geaster subiculosum* Cooke & Massee (1887) [MB#528132]

=*Geaster subiculosum* Cooke & Massee (1887) [MB#528132]

Immature basidioma subglobous to obovoid, with surface smooth to slightly wrinkled on the subicule, white yellowish to yellowish gray fading to beige. Basidioma expanded with persistent fleshy mycelial layer of yellowish brown to light yellow, with revolte rays cream. Endoperidium dark grayish brown, peristome mamiform, fibrillous, non-delimited, concolor with endoperidium saculiform expansion. Basidiospores small, globose to subglobous, measuring 2.4 to 3.3 µm in diameter, slightly warty, hyaline.

Material examined: Brazil. Amazonas, Manaus. INPA Campus III, 22 III 2014. Santana, MDF. INPA 259933.

Evaluation of culture medium in dikaryotic growth

Despite slow growth, cultures of the two species developed in all tested media (Figure 2). PDA and MEPA produced the best colony diameter values for *G. lloydianum*, with grow rates of some 4.5 and 4.9 mm per week, respectively. For *G. subiculosum*, PDA was the best medium, with growth of around 7.5 mm per week. For *G. lloydianum*, no significant statistical difference was observed in colony biomass between the culture media. However, for *G. subiculosum*, PDA and DAS produced the best results (Table 1).

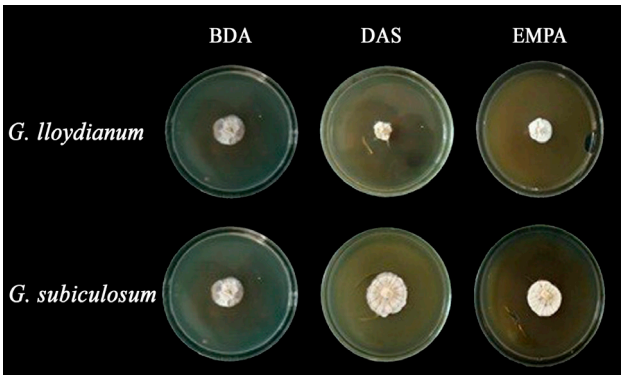

Figure 2. Mycelial cultures of *Geastrum lloydianum* and *G. subiculosum* after 40 days of incubation in different culture media at 25 °C, in the dark. PDA = Potato Dextrose Agar; DAS = Dextrose Agar Sabouraud; MEPA = Malt Extract Peptone Agar. This figure is in color in the electronic version.

Table 1. Evaluation of growth in different culture media by diameter (mm) and biomass (mg) for *Geastrum lloydianum* and *G. subiculosum* over 42 days of incubation at 25 °C in the dark.

Species	Mycelial diameter (mm)			Colony biomass (mg)		
	PDA	DAS	MEPA	PDA	DAS	MEPA
Geastrum lloydianum	22.3bA	11.7bB	24.7aA	0.07bA	0.05bA	0.04aA
G. subiculosum	37.7aA	30.7aB	26.3aC	0.25aA	0.11aB	0.07aC
CV (%)		10.27			20.02	

BDA = Potato Dextrose Agar; DAS = Dextrose Agar Sabouraud; MEPA = Malt Extract Peptone Agar; VC = Coefficient of Variation. Letters with different sufixes indicate significantly different values among groups (ANOVA, $p < 0.05$). Small letters refer to comparison between isolates and capital letters, to comparison among treatments.

During the first week of cultivation, *G. lloydianum* and *G. subiculosusm* hyphae showed little or no clamp branching of more distant connections. At 42 days, the cultures showed uneven margins, larger hyphae, numerous clamp connections, and the presence of chlamydospores in terminal and interim positions on the hyphae.

Obtaining monokaryotic mycelia

The germination period of basidiospores of *G. lloydianum* and *G. subiculosum* was similar, ranging from 35 to 40 days after incubation. Under these conditions, it was possible to isolate 84 monokaryotic mycelia of *G. lloydianum* and 96 of *G. subiculosum*.

Cultures grew, on average, 1.2 mm per week, but differed in mycelial morphology. Monokaryotic cultures of *G. lloydianum* on PDA were thicker, with a denser mycelium and a slightly irregular border, while *G. subiculosum* hyphae were thinner, less dense, with a thin appearence and irregular growth.

The slow development of the two species made it difficult to analyze *in vitro* pairings. However, the presence of multiple clamp connctions in the resulting mycelium indicated dikaryotic mycelium establishment for all pairs (Figure 3), thus indicating sexual crossing (Taylor *et al.* 2000; Taylor *et al.* 2006). The dikaryotic mycelia resulting from *in vitro* crossings were grouped with the dikaryotic mycelia isolated from the respective basidiomas (Figure 4), showing that the method can likely be employed with other species of the group.

DISCUSSION

The slow *in vitro* growth rates of some *Geastrum* species was first reported by Sunhede (1989), who noted a growth rate variation of 0.5 to 3.7 mm per week. Stoytchev *et al.* (2001) reported growth of 3 to 4 mm after six weeks for *G. pouzarii* V.J. Staněk, and Zamora *et al.* (2014) observed growth of 4 mm per week for *G. argentinum* Speg. All these species were grown in a malt extract medium. In this same culture medium, *G. lloydianum* and *G. subiculosum* showed higher growth speeds of about 4.6 and 5.2 mm per week, respectively.

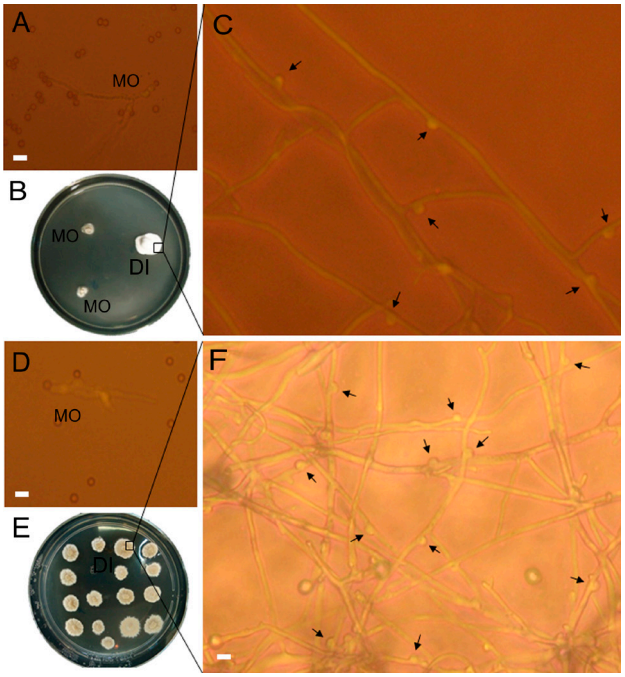

Figure 3. *In vitro* crossover for two species of *Geastrum*. A-C) *G. subiculosum*; D-F) *G. lloydianum*, where A) and D) is the monokaryotic mycelium 35 days after incubation; B) is the monokaryotic and dikaryotic mycelium resulting from crossover; E) is the dikaryotic mycelium resulting from crossover; C) and F) show connection clamps indicating success at crossover. MO = monokaryotic mycelium; DI = dikaryotic mycelium. This figure is in color in the electronic version.

Both our species showed best results with PDA. This has also been observed for other *Geastrum* species (*G. schweinitzii* (Berk. & M.A. Curtis) Zeller, *G. triplex* and *G. echinulatum* B.D.B. Silva & Baseia (M.D.F. Santana, unpublished data). Although the values are still low, means for mycelial growth in PDA were the most promising recorded so far for the genus.

In vitro germination of sexual spores is the key first step in monokaryotic culture isolation, and can even be used to determine the fungal reproduction system (Anderson *et al.* 1980; Carvalho *et al.* 1997; Capelari and Fungaro 2003), a field of research still little explored for gasteroid fungi. However, the basidiospores of only a few species of *Geastrum* have germinated under laboratory conditions, the main examples to date being the few cases described by Sunhede (1989) and the report by Stoytchev *et al.* (2001) for *G. pouzarii*.

Even if germination rates are low (less than 1% in this study, for both species) these are the first records of basidiospore germination for both *G. subiculosum* and *G. lloydianum*. Basidiospore germination for gasteroid fungi, such as *Pisolithus* Alb. & Schwein, for example, ranges from 0.001 to 0.38% (Bulmer 1964; Silvério 2013). This may be the possible cause of the lack of success with previous reported attempts, such as by Kuhar and Papinutti (2009) for *G. episcopale* Kuhar & Papin, synonym for *G. violaceum* Rick.

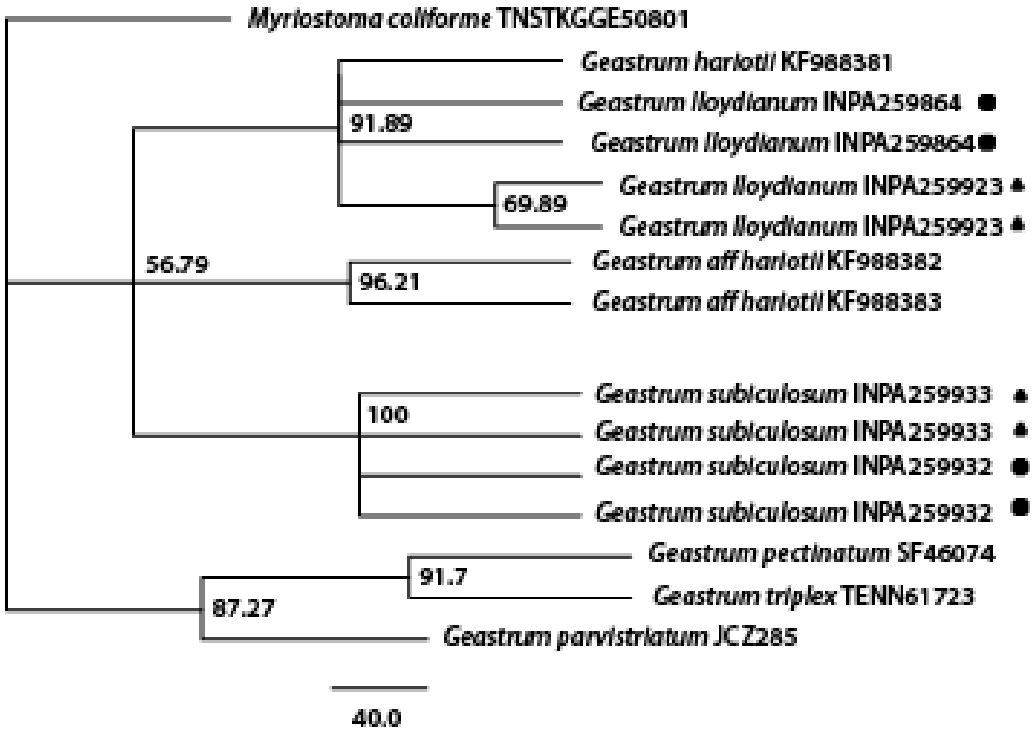

Figure 4. Molecular similarity between basidioma-derived dikaryotic mycelia (square) and dikaryotic mycelia derived from *in vitro* crossing (triangle) for Amazonian strains of *Geastrum lloydianum* and *G. subiculosum*. The tree was constructed based on the rDNA ITS region sequences (ITS1, 5.8S and ITS2).

Low germination rates under laboratory conditions were historically reported in the literature for gasteroid fungi (Bulmer 1964; Fries 1978, 1987; Carvalho *et al.* 1997; Silvério 2013). For *Rhizopogon roseolus* (Corda) Th. Fr., the first germination may take two to three weeks (Kawai *et al.* 2008) and for *Pisolithus arhizus* (Scop.) Rauschert., eight weeks after incubation (Carvalho *et al.* 1997).

CONCLUSIONS

PDA culture medium was the most promising for mycelial growth of Amazonian strains of *Geastrum lloydianum* and *G. subiculosum*. This culture medium was also effective for the germination of basidiospores and for obtaining monokaryotic cultures. It is necessary to improve the production of *Geastrum* mycelial cultures, as well as to apply the methodology used in here to other species, in oredr to broaden the evaluation of its applicability for ecophysiological and biotechnological studies of *Geastrum*.

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