Short communication

On *Geastrum argentinum*, a forgotten species

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ABSTRACT

Geastrum argentinum, a long forgotten species described by Spegazzini and previously regarded by Soto and Wright (2000) as a synonym of *G. fimbriatum*, has been found in several locations in Argentina. A careful analysis revealed relevant differences between those taxa, proving the validity of Spegazzini's species. Here we re-describe Spegazzini's species from the damaged type material and from new fresh collections, proposing one of them as an epitype.

The genus *Geastrum* Pers. is the most diverse one within the order Geastrales (Hosaka et al. 2006), including nearly 300 described taxa (Robert et al. 2005). Some partial revisions about the genus *Geastrum* have been performed (Ponce de León 1968; Sunhede 1989) but a comprehensive worldwide study is still needed. In Argentina, few works have been published, highlighting the new species described by Spegazzini (1899, 1902, 1912, 1927; Dios et al. 2011), and the revision of the species from Buenos Aires made by Soto and Wright (2000) which includes the synonymization of *G. argentinum* Speg. with *G. fimbriatum*. Fr. We studied the type specimens of *G. argentinum* deposited in LPS herbarium, finding one very characteristic species. Our aim is to re-describe that taxon, clarifying its taxonomic and nomenclatural status and to provide an epitype in view of the poor preservation state of Spegazzini's type material.

General methodology and terminology about morphological traits follows Sunhede (1989), with several minor exceptions. Macromorphological characteristics are referred to dried basidiomata unless otherwise stated. Color codes refer to Pantone (Anonymous 1995). Routinary micromorphological study was carried out on a light microscope. Basidiospore measurements were all made under an 100× immersion oil objective, with 10× oculars, in 3% KOH solution. For scanning electron microscopy a small piece of mature glebal mass was mounted on a sample holder and observed in a Zeiss DSM 982 Gemini. Cultures were made on common malt agar from pieces of fresh pseudoparenchymatous layer and immature glebal mass, incubated at 25 °C.

The description is based on about 250 basidiomata in all states of development. Habit photographs were taken with a Canon EOS 450D digital camera. Drawings were performed

with the aid of a camera lucida. Public herbarium acronyms follow Thiers (2012).

Protocols for DNA isolation, PCR amplification, purification of PCR products, and sequencing are described in Rubio-Casas et al. (2011), and briefly summarized here. DNA was extracted from about 20 mg of the mature glebal mass of the proposed epitype collection, with the DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions, eluting the DNA with milli-Q water in a final volume of 100 μL. The nrDNA ITS molecular marker, which comprises the internal transcribed spacer 1 (ITS1), the 5.8S nrRNA gene, and the internal transcribed spacer 2 (ITS2) regions, was amplified using the primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The cycling parameters were an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were purified using the QIAquick PCR Cleanup Kit (QIAGEN). Sequencing was carried out in an ABI 3730 sequencer (Applied Biosystems, Foster City, USA), with the same primers used for the PCR.

Fifteen GenBank additional sequences were used for the molecular analysis. Those include the four most similar sequences compared with our *G. argentinum* sequence in the BLAST search, plus all sequences included in GenBank under the name *Geastrum fimbriatum* or its heterotypic synonym *Geastrum sessile* (Sowerby) Pouzar, plus one sequence as *Geastrum* sp. that is almost identical to three of the previously selected sequences under the names *G. fimbriatum* or *G. sessile*. Two sequences of *Myriostoma coliforme* (Dicks.) Corda were used as outgroup based on the results of Hosaka et al. (2006). GenBank accession numbers and herbarium voucher information extracted from this database are provided in Table 1.

Sequences were automatically aligned using the Q-INS-i strategy of MAFFT (Katoh et al. 2002), and manually adjusted with BioEdit (Hall 1999). The alignments were deposited at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S14472). Ambiguously aligned regions were removed with Gblocks (Castresana 2000), allowing all gaps but only a maximum of 2 contiguous non-conserved positions. Phylogenetic analyses were made using both the Maximum Parsimony (MP) approach and Bayesian inference.

MP analysis was conducted with PAUP* v4.0b10 (Swofford 2002) using PaupUp v1.0.3.1 graphical interface (Calendini and Martin 2005), performing heuristic searches with the following options: "MulTrees" in effect, TBR as branch-swapping algorithm, and branches collapsing when maximum branch length is zero. All characters were equally weighted and gaps were treated as missing data. To calculate branch supports the bootstrap approach was used, performing 5000 replicates with the full-heuristic option.

Nucleotide substitution models were selected with jModelTest (Posada 2008), using the Bayesian Information Criterion (BIC) as optimality criterion for the Bayesian analysis (substitution model HKY + Γ). Bayesian analysis was done using the Metropolis Coupled Markov Chain Monte Carlo (MC^3) approach implemented in MrBayes 3.2 (Huelsenbeck and Ronquist 2003). Four parallel runs were executed, each one starting with a random tree, with 6 chains, 5 of which were incrementally heated with a temperature of 0.2, and length preset to 10,000,000 generations, sampling every 100th tree. Runs were automatically stopped when the average standard deviation across runs dropped below 0.005. The first 50% of the analysis was discarded as burn-in. The 50% majority-rule tree with branch lengths and posterior probabilities (pp), indicating branch supports, was calculated from the post-burn-in trees.

We consider 75% bootstrap as the minimum value for well-supported clades for the MP analysis, and 0.95 posterior probability for the Bayesian analysis. Phylogenetic trees were drawn with FigTree (Rambaut 2007).

Geastrum argentinum Speg., Fungi Argentini novi vel critici, Anal. Mus. Nac. Bs. As. 6: 193. 1899 [1898], as 'Geaster argentinus'.   Figs. 1, 2.
MycoBank no.: MB 536291.

Type: BUENOS AIRES: partido de La Plata, La Plata, Apr 1888, Spegazzini (LPS-13349) (Holotype). Partido Lomas de Zamora, Llavallol, on and among fallen *Ligustrum lucidum* stumps, 12 May 2012, F. Kuhar and J.C. Zamora (LPS-48446, Epitype, selected here).

Additional specimens examined: BUENOS AIRES: partido Lomas de Zamora, Llavallol, sobre tocón cubierto de tierra, A. Martínez, (BAFC-25603). 19 Jun 1993, M.M. Dios (BAFC-33165). CATAMARCA: La Viña, sobre mantillo entre musgos, 20 Mar 1995, V. Suárez and A. Gottlieb (BAFC-33671). ENTRE RÍOS: Colón, 15 May 1986, P. de Giorgio (BAFC-30753). SALTA: Salta, El Ucumar, 10 Apr 2009, G. Rolón and L. Papinutti (herb. F. Kuhar-10068). 13 Apr 2012, L. Papinutti, G. Rolón and J.C. Zamora (herb. J.C. Zamora-491). TUCUMÁN: 14 Nov 1906, Spegazzini (LPS-13356).

Basidiomata mostly medium-sized, with a conspicuous basal mycelial tuft, arising from a rather dense, whitish (Pantone 1225) subiculum. Unexpanded basidiomata epigeous, globose to subglobose, normally perfectly rounded, 15−32 mm diam. Exoperidium splitting to about the middle into (4−)5−9 subequal to unequal rays, non-hygrometric, saccate, ray tips recurving under the exoperidial disc, (25−)36−75(−80) mm. Mycelial layer easily peeling off, very

Table 1 − Sequences used in the phylogenetic analysis, with information extracted from the GenBank database.

| Taxon (GenBank name)	GB accession number	Herbarium specimen
Myriostoma coliforme	EU784376	K(M)37233
Myriostoma coliforme	JN845203	TNS TKG-GE-50801
Geastrum argentinum	KC018077	LPS-48446
Geastrum fimbriatum	EU784228	K(M)124394
Geastrum fimbriatum	EU784230	K(M)64513
Geastrum fimbriatum	JN845093	L 837179
Geastrum fimbriatum	JN845094	TENN 61511
Geastrum fimbriatum	AB509807	Unknown
Geastrum cf. *fimbriatum*	EU784229	K(M)135404
Geastrum sessile	JN845123	TENN 39858
Geastrum sp.	JN845180	TNS KH-JPN09-202
Geastrum sp.	JN845181	TNS KH-JPN09-203
Geastrum sp.	JN845182	TNS KH-JPN09-223
Geastrum sp.	JN845183	TNS KH-JPN09-268
Geastrum sp.	JN845195	TNS Sakamoto186
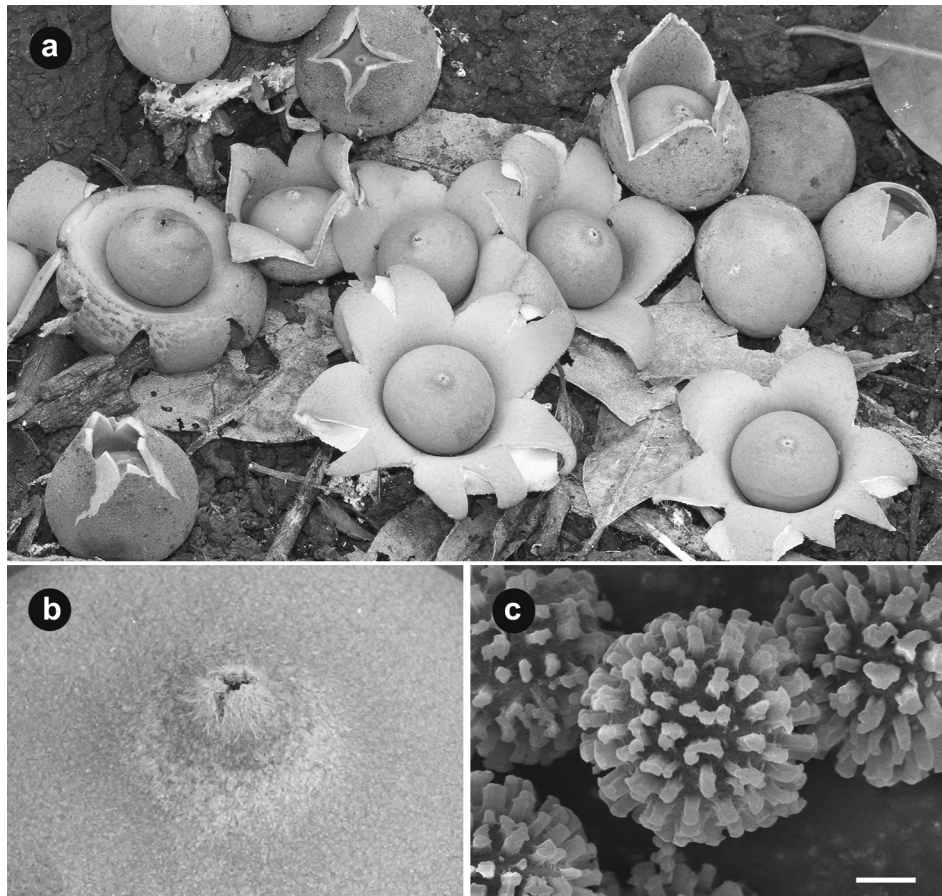
Fig. 1 — *Geastrum argentinum* (LPS-48446). a: Basidiomata from part of the epitype collection (Humid Pampas ecoregion). b: Detail of the peristome. c: Basidiospore (SEM). *Bar*: 1 μm.

coriaceous and usually splitting irregularly or substellate; outer surface not encrusting debris, reddish to dark reddish brown (Pantone 490) when fresh, yellowish (Pantone 1385) to orangish or brownish-ochraceous (Pantone 1535) when dry, velvety and with minute (<0.3 mm high) conical or irregular tufts of hyphae. Fibrous layer papyraceous, whitish. Pseudo-parenchymatous layer up to 2.0 mm thick when fresh, surface at first pinkish beige to orangish, with reddish tints (Pantone 170), dark brown (Pantone 497) when dry. Endoperidial body sessile, globose, (9—)13—26 mm diam., beige to dark greyish-brown (Pantone 156), smooth. Apophysis absent. Stalk lacking. Peristome finely fibrillose, indistinctly delimited, flat to broadly conical, concolorous or slightly lighter or darker than the surrounding endoperidium. Mature gleba dark gray-brown (Pantone 497). Columella dirty white (Pantone 1225), rather weak, somewhat columnar to club-shaped.

Basidia mostly subglobose to lecythiform, thin- to slightly thick-walled, 12.0—16.5 × 7.5—11.5(—13.0) μm. Basidiospores brown, globose, (4.4—)4.8—5.6 μm, with cylindrical warts, 0.5—1.0 μm high. Mycelial layer double-layered; inner layer of thin-walled, branched, clamped hyphae, strongly glued together; outer layer consisting of thick-walled, aseptate, radially arranged hyphae, progressively widened to the apical part, 1.5—7.0(—9.0) μm diam., strongly and usually abruptly dilated at the end to form an up to 30.0 μm diam. vesicle, which is only slightly thick-walled and therefore fragile.

Cultures in common malt agar growing very slowly (up to 4 mm/week), sparse, hyaline, with uneven margins. Hyphae in young colonies straight, narrow, almost unbranched, with scarce clamp connections. Older cultures show broader hyphae, with guttulated repetitive globose swellings, consecutive proliferative clamp connections and chlamydospore-like bodies in terminal and intercalar position. Oxidases reactions strongly positive for laccase and negative for lignin-peroxidase and Mn-peroxidase.

Molecular results

The final alignment after excluding ambiguous regions has 542 total characters, 382 of which are constant, 111 are variable and parsimony-informative, and 49 are variable and parsimony-uninformative. Search for trees under Maximum Parsimony criterion retained 5 best trees, all of them with a length of 235 steps and a similar topology. The Consistency Index (CI) was 0.838, the Retention Index (RI) was 0.863, and the Rescaled Consistency Index (RC) was 0.723.

The Bayesian analysis was automatically stopped after 820,000 generations, when the deviation of split frequencies across runs dropped below 0.005. Then, a total of 32,800 trees had been sampled. The 50% majority-rule consensus tree was obtained from the 50% post-burn-in trees. This consensus tree is shown in Fig. 3, with bootstrap values from the MP analysis
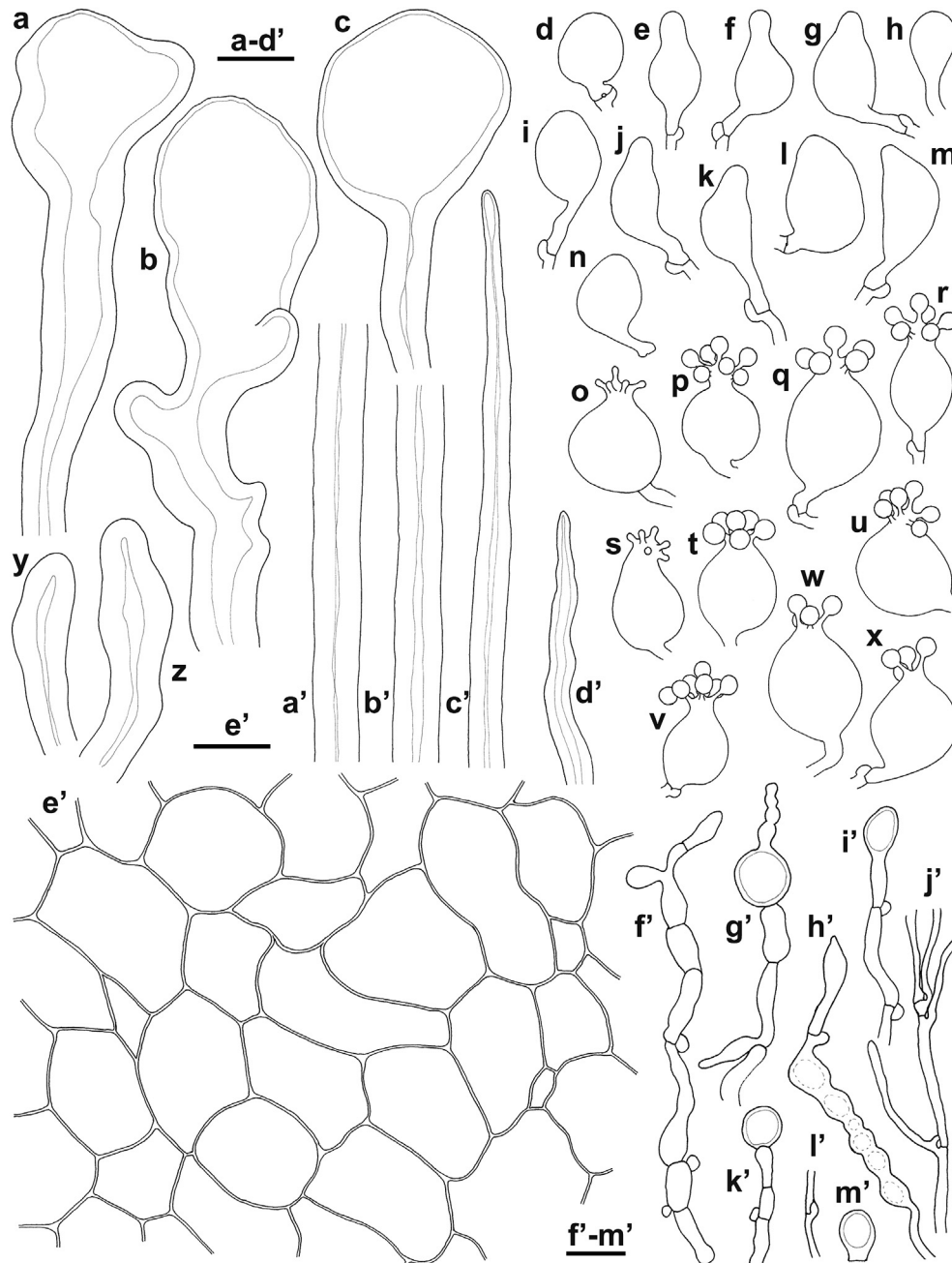
Fig. 2 – Light microscope micrographs of *Geastrum argentinum*. a–c: Inflated terminal cells of the mycelial layer external surface. d–x: Basidia. y–z: Protruding hyphae of the endoperidial surface. a′–c′: Capillitium. d′: Hypha from the peristome. e′: Pseudoparenchymatous layer hyphal cells. f′–m′: Hyphae and chlamydospores from malt agar cultures. *Bars*: a–z, a′–d′ 10 μm; e′ 25 μm; f′–m′ 12.5 μm.

and posterior probabilities from the Bayesian analysis indicated on branches.

The sequence obtained from a specimen of *G. argentinum* is rather isolated, and the closest species seems to be an undetermined taxon, represented by four sequences (JN845180–JN845183). Those sequences have a maximum identity between 86% and 87% in BLAST searches, and are grouped as the sister group of *G. argentinum*, but well-differentiated from it. *Geastrum argentinum* is placed in a well-supported clade (bs = 100, pp = 1.0) as a sister taxon of another well-supported clade in the MP analysis (bs = 100) formed by those four sequences of that unknown species. Specimens determined as *G. fimbriatum*, *G.* cf. *fimbriatum*, and *G. sessile* from GenBank are non-monophyletic and likely represent five different evolutionary lineages, one of them including 4 sequences grouped in a well-supported clade (bs = 100, pp = 1.0), and the others isolated along the phylogenetic tree.
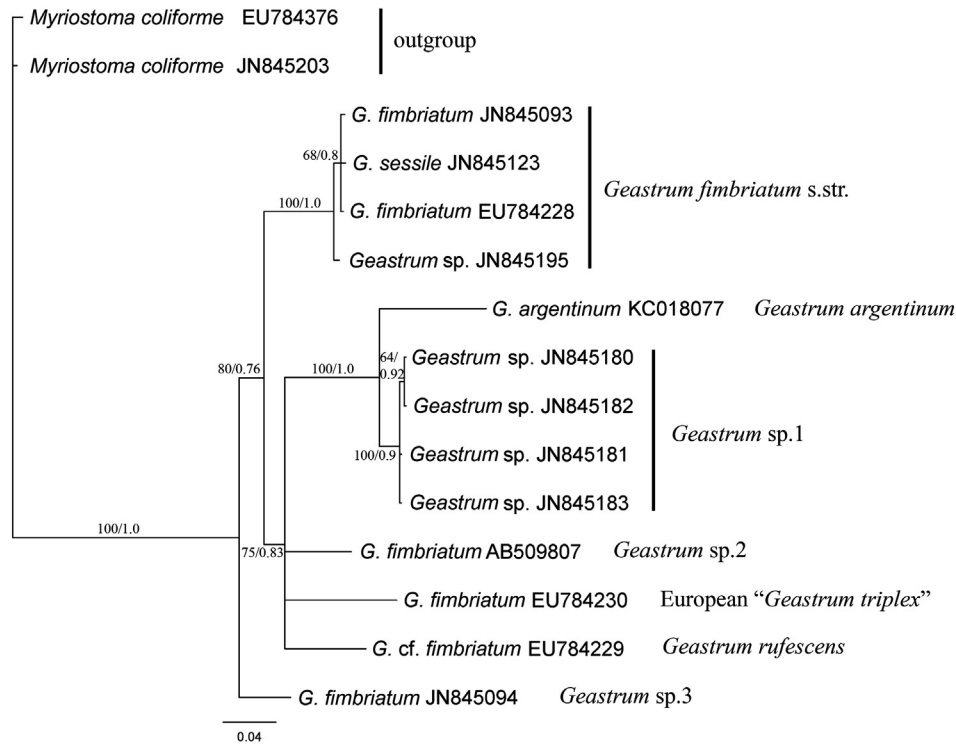
Fig. 3 — 50% Majority-rule consensus tree obtained from the Bayesian analysis. Numbers on branches indicate bootstrap values from the Maximum Parsimony analysis with PAUP*, and posterior probabilities from the Bayesian analysis respectively. Terminals in the tree are identified by the GenBank name and accession number, and our present taxonomic opinion is indicated on the right side.

Comparison with other species

Soto and Wright (2000) considered *G. argentinum* as a synonym of *G. fimbriatum* Fr., but we found many morphological differences between the Argentinian and the European species. *G. fimbriatum* has a mycelial layer strongly encrusted with debris, not coriaceous, whitish to pale yellowish. The hyphae of its outer part are not conspicuously inflated. Finally, in *G. fimbriatum* the spore print is brown to pale brown and the basidiospores are pale brown to ochraceous, much less and differently ornamented, and smaller. According to all these dissimilarities, we can conclude that the two species might be not only different, but also not even closely related.

Although the principal aim of this work is to re-describe *G. argentinum* instead of performing a phylogenetic analysis of it and its potential relatives, some general conclusions can be extracted from the phylogenetic analysis. The apparent isolation of *G. argentinum* from the most similar taxa has been mentioned in the results, but trickier is the identity of *G. fimbriatum*. All the GenBank sequences bearing the names *G. fimbriatum*, *G.* cf. *fimbriatum*, or *G. sessile* are well-differentiated from *G. argentinum*, and dispersed along the tree likely representing up to five different taxa. Problems with the identification of herbarium specimens of *G. fimbriatum* were noted by Zamora et al. (2009), where three sequences present in GenBank as *G. fimbriatum* were grouped in three different clades representing three clear morphological and phylogenetic species. Considering that the nrITS is a good molecular

marker for species-identification purposes (Schoch et al. 2012) we marked these possible determinations in the phylogenetic tree to show our taxonomic view, although a revision of the herbarium specimens is desirable to confirm our assumptions. Kasuya et al. (2012) showed an excellent example where an assumed "single morphological species" included in fact several phylogenetic species. Some morphological characters, such as the pseudoparenchymatous collar in the "*G. triplex* complex" (Kasuya et al. 2012) may have been overvalued as taxonomic traits, and this might be the case of some of the morphological features of *G. fimbriatum*, such as the non-delimited fibrillose peristome, classically used to characterize the species.

Geastrum javanicum Lev. is an enigmatic species with several records along tropical and subtropical areas. The vague description from Ponce de León (1968) has been followed by most recent authors applying the name to almost any species where the mycelial layer easily peels off, and/or to species where basidiomata arise from a subiculum. Leveillé (1846) described it as a species with a reddish subiculum, a verrucose surface of the mycelial layer, concolorous with the subiculum, a blackish inner exoperidial surface, a red endoperidium, and not conspicuously ornamented basidiospores. Most of these traits do not match with those of *G. argentinum*. Finally, *Geastrum lloydii* Bres. & Pat. seems to be another extremely close taxon but its mycelial layer is dark colored, almost black, and densely velutinate which deviates from our concept of *G. argentinum*.

Epitypification

The holotype specimen in herb. LPS is in a rather poor condition, being very fragmented, only partially showing some diagnostic characteristics. Thus, we found important to select an epitype of recent material for this species. The selected epitype is composed by 125 basidiomata in different states of development, collected from the same mycelium and agreeing in all morphological characteristics with those of the protologue. This specimen was found in a place close to La Plata, near the type locality.

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