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Abstract: Geastrum minutisporum is characterized by a basidioma up to 25 mm wide, exoperidium saccate to planar, non-hygroscopic; a globose to subglobose, sessile and greyish brown endoperidium; a silky fibrillose peristoma, lighter or darker than the endoperidium and with globose, slightly asperulate basidiospores, 2-2.5-3 µm in diam., with a short apiculus up to 0.5 µm long. Specimens were collected at the Reserva Hídrica Provincial Pampa de Achala located in the upper portion of Sierras Grandes from Córdoba, central Argentina. Morphology and phylogenetic analysis based on ITS sequences support Geastrum minutisporum as a new species.

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Editorial Office
Mycoscience

We appreciate the attention in our manuscript and the opportunity to correct it and learn with you and from you.

All reviewers and Editorial Office comments and suggestions have improved our manuscript and all of them wer accepted and the MS is according your suggestions and they have been addressed point by point and are listed below:

Editorial Office suggestions:

There are many careless mistakes in the manuscript. See the uploaded file (MYC-D-14-00149R1_okadaREV.pdf), Instructions, Sample pages, and recent issues of the journal.

The manuscript was carefully revised in order to correct languages mistakes. The uploaded file in the Mycoscience page was checked too.

Change Figures F and G from Plate n° 3

Figures were replaced, also B-D.

1- Please once again check the "guide for authors" found in http://www.elsevier.com/journals... This page also includes the sample pages in Word format. Please pay an attention to the format of references (no issue numbers, only volume is necessary, for example).

Checked and corrected.

2- Page 3, "worldwide distributed" > distributed worldwide

It was changed.

3- Page 3, "*Geastrum* species have always been identified... suggested to separate species too." > Revise the sentence in plain, grammatically correct English. For example, "*Geastrum* species have always been identified by differences in spore size and ornamentation as well as chemical reactions and rhizomorph structure when macroscopic features were ..."

It was revised.

4- Page 4, "N. Z." > Please spell it out as "New Zealand" (?)

It was changed.

5- Page 4, "For phylogenetic tree and discussion section..." > Revise it since no "Discussion" section is present as it is a short communication..

It was done.

6- Page 4, "GenBank instead the current names..." > GenBank instead of the current names...

It was done.

7- Page 5, "with 699 characters total" > with a total of 699 characters

It was done.

8- Page 5, "Gene sequences: holotype..." > It is my personal suggestions, but because only the ITS sequences were obtained (which can be easily justified because the ITS is considered an official DNA barcode marker for Fungi) and "Gene" can be ambiguous (could mean any sequences from the genome), why don't you more specifically say "ITS sequences"?

It was specified.

9- Page 6, "There are many *Geastrum* with... and we will separate them." > Revise the sentence in plain, grammatically correct English.
For example, "There are several species of *Geastrum* with spore size less than 4 micro meters or similar to *G. minutisporum*, but they are morphologically distinguishable.

The sentence was revised and corrected.

10- Page 6, "The mean spore size of *G. minutisporum* is... the largest ones." > Revise the sentence in plain, grammatically correct English. For example, "The mean spore size of G. *minutisporum* is 2.5+0.2 micro meters, with the minimum of 2 micrometers and the maximum of 3 micrometers in diam."

The sentence was modified.

11- Page 7, "In second place G. lloydianum... and arched exoperidium." > revise as "In second place, spore range of *G. lloydianum* is..."

The sentence was modified.

12- Page 7, "Finally, *G. velutinum*... not branched." > The sentence is too long. Make two separate sentences or revise it it in plain, grammatically correct English and avoid making a partial sentence.

The sentence was modified in to complete sentences.

13- Page 7, "Thus, spore size measured with light microscopy..." > I disagree with its statement. It is true that spore size can sometimes separate two closely related species, but not always so. As the authors also mentioned in the sentences above in the same page, the main diagnostic characters are the COMBINATION of spore and macroscopic characters. There are so many species of *Geastrum* with very similar spore morphology and thus cannot be

distinguished by spore size alone. Similarly, *G. minutisporum* does not seem to be distinguished by spore features alone. Revise the sentence accordingly.

Please see our clarified version

14- Page 7: It may change again following the editorial policy, but I suggest you make a heading before the key. It may look like "Key to the species of *Geastrum* in *Polylepis* woodlands in Argentina"?

We accept and incorporate the suggestion indicated because the identification key belongs to species with similar morphologies and not to species inhabiting *Polylepis* forests. It could be confusing without a heading.

Highlights

1- We described a new *Geastrum* species from Argentina.

2- It was collected in the endangered ecosystem: *Polylepis australis* woodlands.

3-We conducted molecular and morphological analysis.

4-We discuss about position of our species within *Geastrum* species

5-We provide full plate designs to illustrate our findings.

Short communication

Geastrum minutisporum sp. nov. from *Polylepis australis* woodlands in central Argentina

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Text: 10 pages; figures: 3

Abstract

Geastrum minutisporum is characterized by a basidioma up to 25 mm wide, exoperidium saccate to planar, non-hygroscopic; a globose to subglobose, sessile and greyish brown endoperidium; a silky fibrillose peristoma, lighter or darker than the endoperidium and with globose, slightly asperulate basidiospores, 2–2.5–3 µm in diam., with a short apiculus up to 0.5 µm long. Specimens were collected at the Reserva Hídrica Provincial Pampa de Achala located in the upper portion of Sierras Grandes from Córdoba, central Argentina. Morphology and phylogenetic analysis based on ITS sequences support *Geastrum minutisporum* as a new species.

Keywords

Earthstars, Gasteroid fungi, Phylogeny, Tabaquillo

Polylepis Lodge belongs to the Rosaceae family and its species are distributed along South America's Andes high mountains, and occur in deep canyons and ravines along water sheds where it grows in isolated patches (Simpson 1979; Cingolani et al. 2004; Enrico et al. 2004; Renison et al. 2006, 2013). *Polylepis australis* Bitt. forms small patches of mixed woodlands with *Maytenus boaria* Molina at elevations between 1300 and 2600 m a.s.l. above the timberline in Argentinean Puna, Yungas and Chaquean phytogeographic regions (Cabrera 1994). Studies on gasteroid mycobiota from these woodlands have shown new and endemic species (Hernández Caffot et al. 2011, 2013).

Geastrum Pers. includes 50 species distributed worldwide (Kirk et al. 2008) however many species have been described since then (i.e. Fazolino et al. 2008; da Silva et al. 2013; Sousa Cabral et al. 2014). Mycologists have recorded more than 30 *Geastrum* species for Argentina (i.e. Spegazzini 1912, 1927; Domínguez de Toledo 1986, 1989; Soto and Wright 2000; Kuhar et al. 2012; Hernández Caffot et al. 2013; Zamora et al. 2014) and all basidiomata morphologies within *Geastrum* are well represented in the country.

Geastrum species have always been identified by differences in spore size and ornamentation as well as chemical reactions and rhizomorph structure when macroscopic features were were not enough to separate them. Kasuya et al. (2010) mentioned the relevance of the episporium ornamentation and spore size as an informative character, useful to identify for example species within the *G. triplex* Jungh. complex. Zamora et al. (2013) introduces chemical analysis, rizomorph structure and calcium oxalate crystals to differentiate within species. Molecular studies have shown that long distance dispersal may not occur frequently, supporting the idea that Southern Hemisphere species are different from those in the Northern Hemisphere (Kasuya et al. 2012). Thus, molecular studies are needed to verify, identify and differentiate South American taxa from species from other continents.

The aim of the present study is to contribute to the knowledge of gasteroid mycobiota describing a new species for science, *Geastrum minutisporum*, collected in *Polylepis australis* high altitude woodlands from Córdoba, central Argentina.

Specimens were collected in the Reserva Hídrica Provincial Pampa de Achala, located in the upper portion of Sierras Grandes in Córdoba, central Argentina, at elevations between 1800 to 2300 m a.s.l. where mean temperatures of the coldest and warmest month are 5.0 and 11.4º C respectively, and there is no frost-free period (Cingolani et al. 2003). Mean annual precipitation is 840 mm, with rainfall concentrated in the warmest months between October and April (Cabido 1985). Vegetation consists of a mosaic of tussock grasslands, grazing lawns, granite outcrop communities, *P. australis* woodlands and eroded areas with exposed rock surfaces (Cingolani et al. 2004, 2008).

Specimens were dried, kept frozen at –5° C for a week and deposited in the herbarium Museo Botánico de Córdoba from Universidad Nacional de Córdoba (CORD). Morphological features were studied in laboratory under stereoscope (Wild M3Z) and light microscope (Nikon Stereoscopic Zoom Microscope SMZ745T). Scanning electron microscopy (SEM) of basidiospores was made with a Zeiss LEO 1450VP. Dried

specimens color is according to Munsell (1994).

DNA was extracted from glebal tissue with a CTAB (hexadecyltrimethyl ammonium bromide) method as described by Doyle and Doyle (1990). For amplifying the ITS region, the primer combination of ITS5 and ITS4 (White et al. 1990) was used. PCR reactions were performed in 25 mL reaction tubes with 1.13ReddyMixTM PCR Master Mix (ABgeneH, Thermo Fisher Scientific Inc., UK) according to manufacturer instructions. Cycling parameters for the ITS was 1 cycle of 95° C for 5 min, 30 cycles of 95° C for 1 min, 55°C for 30 s, and 72° C for 1 min, with a final extension at 72° C for 10 min. Amplified products were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing with the BigDyeTM terminator kit and run on ABI 3730XL. ITS sequences of three specimens of *G. minutisporum* MLHC 14 (type), 15 and 16 (isotypes), generated for this study have been deposited in GenBank and were combined into a dataset with additional GenBank sequences (Fig. 1) to evaluate new accession in a broad phylogenetic scheme. A total of 27 sequences including the outgrup taxa *Myriostoma coliforme* (Dicks.) Corda were used. Thus, of the 26 *Geastrum* species included in the phylogram from USA, England, France, Japan, Spain, Sweden, Belgium, New Zealand and Russia, 16 have been reported for Argentina, and we report three new sequences which correspond to *G. minutisporum* from *P. australis* woodlands. For phylogenetic tree we decided to use the names stated in herbaria vouchers and GenBank instead of the current names for some species: i.e. *G. mirabile* Mont. (1855) is considered a synonym of *Geastrum schweinitzii* (Berk. & Curtis) Zeller (1948) and *G. velutinum* Morgan (1895) which is considered, by some authors, as a synonym of *G. javanicum* Lèv. (1846) sensu Ponce de Leon (1968).

DNA sequences were initially aligned with Muscle v.3.6 (Edgar 2004a, b), followed by manual alignment in the data editor of BioEdit ver. 7.0.1 (Hall 1999). Maximum parsimony (MP) analysis was done with TNT ver. 1.1 (Goloboff et al. 2008). “Traditional” heuristic searches were carried out with a total of 1000 random addition sequences and submitted to tree-bisection reconnection (TBR) branch swapping, holding 50 trees, followed by a more extensive TBR holding 50000 trees. The most parsimonious trees found were collected and a strict consensus was calculated using the “Nelsen” option in TNT. Support was estimated by bootstrap as implemented in TNT, resampling 1000 times with TBR set to 100 replications holding 20 trees, followed by a more extensive TBR holding 5000 trees, and saving the consensus for each resampling matrix.

Bayesian analyses were conducted using MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). Models for the molecular data were evaluated using ModelTest v. 3.06 (Posada and Crandall 1998) to find the best fit according to the Akaike information criterion (Akaike 1974). The GTR+G + I model was found to be the most appropriate and Bayesian analyses were run with 20 million Metropolis-coupled Markov chain Monte Carlo (MCMCMC) generations with four chains, sampling trees every 100th generation. Stationarity was determined based on the convergence of likelihood scores using TRACER version 1.5 (Rambaut and Drummond 2009), and sample points generated prior to

stationarity were eliminated as burn-in (25%). The posterior probabilities (PP) of the clades were determined by a 50% majority-rule consensus of the trees retained.

After final alignment of 32 sequences with a total of 699 characters, 389 were parsimony uninformative, 310 were parsimony informative, and only parsimony informative sites, were considered for parsimony analysis. Under a heuristic search, three most parsimonious trees were obtained with a length of 2350 steps, CI = 0.85, RI =0.80 and RC=0.75. The Bayesian analysis shows that the average standard deviation of split frequencies reaches 0.005 after 20 million generations. The four runs converged with a potential scale reduction factor (PSRF) tending to 1.001 (estimated marginal likelihood = −9,837.66). The 50% majority-rule consensus tree from the Bayesian analysis of the combined data is shown in Fig. 1. Both Parsimony and Bayesian analyses based on the ITS1, ITS2 and 5.8S rRNA gene loci showed similar topologies throughout the general phylogeny and similar relationships between *G. mirabile* Mont., *G. saccatum* Fr., *G. minutisporum* and *G. velutinum* Morgan clade (Fig. 1). These topologies show *G. minutisporum* clustered with species with saccate exoperidium and fibrillose peristome. Thus we present only the consensus tree resulting from Bayesian analyses and on their branches are included posterior probability (PP) and bootstrap support (BS). Both Parsimony and Bayesian analyses showed strong support for the monophyly of the *G. minutisporum* clade and *G. velutinum* as the sister taxa.

Geastrum minutisporum Hern. Caff. & L.S. Domínguez, sp. nov. Figs. 2, 3.
MycoBank no.: 805023.

Diagnosis: Basidioma up to 25 mm diam., exoperidium saccate to planar, non-hygroscopic; endoperidium globose to subglobose, sessile, greyish brown; peristome silky fibrillose, lighter or darker than the endoperidium; basidiospores globose, slightly verrucose, 2–2.5–3 µm in diam., with a short apiculum up to 0.5 µm long.

Type: Argentina. Córdoba, Departamento San Javier, Quebrada Los Molles, 31º58'33"E, 64º57'42"W, growing on *Polylepis australis* organic soil, 31 March 2003, leg. L. S. Domínguez (holotype, CORD-MLHC 14; isotype, CORD-MLHC 12).

ITS sequences: Ex-holotype: KM260664 (CORD-MLHC 14). Ex-paratypes: KM260665 (CORD-MLHC 15), KM260666 (CORD-MLHC 16).

Etymology: The epithet refers to the spore size.

Macroscopic features: Usually one or two basidiomata, up to 25 mm diam. (Figs. 2A, B, 3A) (fresh specimens photographs are not available). Exoperidium saccate to planar, splitting in 6–8 unequal and non-hygroscopic rays recurved under the exoperidial disc (Figs. 2A, 3A); in dry specimens the pseudoparenchymatous layer is dark brown (7.5YR3/3) to very dark brown (7.5YR2.5/3), easily peeling off, sometimes developing a pseudoparenchymatous collar (Fig. 2A); the fibrous layer is reddish yellow (7.5YR7/8) and the mycelial layer is reddish yellow (7.5YR7/8), slightly cracked, encrusting debris, a rhizomorph scar, with miceliar cords (up to 25 µm in diam.) and without subiculum (Fig. 3A). Endoperidial body sessile, globose to subglobose and 11–15 mm in diam.

Endoperidium papery, grey (10YR5/1) to light yellowish brown (2.5Y6/4). Peristome often distinctly delimited, 4–5 mm diam., silky fibrillose, surrounded by a light-coloured ring, darker or lighter in colour than the endoperidium (Fig. 2A, B). Gleba very dark brown (7.5YR2.5/1).

Microscopic features: Endoperidium wall up to 62.5 µm thick, made of thick walled hyphae of up to 2.5 µm diam. (Fig. 3B). Exoperidium three layered: a pseudoparenchymatous layer up to 1000 µm thick made of globose hyphae of up to 30 µm diam. (Fig. 3C); a fibrous layer up to 400 µm thick, made of thick walled hyphae of up to 6 µm diam. (Fig. 3D); and a mycelial layer with a compact inner part of tightly adhered hyphae and an outer part of loosely arranged hyphae with hyphae cords formed by aggregated clampless hyphae of up to 3 µm diam. (Fig. 3E). Basidiospores globose, 2–2.5–3 µm diam. (Fig. 2C), thick-walled and asperulate, with small planar to rounded tips verrucae (Fig. 2D, E) and an apiculus up to 0.5 µm long (Figs. 2E, 3F). Capillitium hyaline to dark brown, 2.5–10 µm broad, walls evenly thickened, with smooth surface, with or without debris adhered; flexuous and with rounded tips (Figs. 2C, D, 3G).

Habitat and distribution: Solitary or in groups of two on *P. australis* organic soil, among fallen leaves, currently known only from the type locality: Dpto. San Javier, Quebrada Los Molles, Córdoba, Argentina.

Additional specimens examined: Argentina. Córdoba, Departamento San Javier, Quebrada Los Molles, 31º58'33"E, 64º57'42"W, 31 March 2003, leg. Domínguez, MLHC 15 (CORD). Ibid., MLHC 16 (CORD).

From the morphological analysis of 80 *Geastrum* collections from *P. australis* woodlands (Hernández Caffot et al. 2013) four of them turn out to be different and we described *G. minutisporum* as a new species for science. *Geastrum minutisporum* is characterized by a saccate to planar exoperidium; a globose to subglobose, sessile, greyish brown endoperidium; a silky fibrillose peristoma and globose and slightly verrucose basidiospores of 2–2.5–3 µm in diam., with a short apiculus up to 0.5 µm long.

Morphological features in addition to its endemic nature (so far) and phylogenetic analysis support the description of *G. minutisporum* as a new species. In the phylogram *G. minutisporum* is placed together with *G. mirabile*, *G. saccatum* and *G. velutinum.* Besides the phylogenetic analysis, these species share some morphological characteristics with *G. minutisporum* such as a saccate exoperidium, a silky fibrillose peristome and some other macro- and microscopic features. Differences between these species are stated in the identification key.

There are several species of *Geastrum* with spore size less than 4 micro meters or similar to *G. minutisporum*, but they are morphologically distinguishable. The mean spore size of *G. minutisporum* is 2.5 ± 0.2 µm, with the minimum of 2 µm diam. and the maximum of 3 µm diam. In first place we can mention *Geastrum hirsutum* Baseia and Calonge with a spore size range that goes from 1.8–2.2 µm diam. (Fazolino Perez 2009) to 3.5–4 µm diam. (Trierveiler Pereira et al. 2011) differs from the new species on having a densely velvety mycelial layer, a columnar spores ornamentation and its lignicolous habit.

In second place, spore range of *G. lloydianum* (Rick) P. Ponce de León is 2.2–3.2 µm diam. but differs from *G. minutisporum* on having a sulcate peristome and arched exoperidium (Ponce de León 1968; Fazolino Perez 2009). Finally, *G. velutinum* Morgan with spore size of 2.5–4.2 µm (Dring 1964) and *G. javanicum* Lév. with spore size 2–7 µm in diam. (Fazolino Perez 2009; Trierveiler Pereira et al. 2011), have slightly larger spores, but the fleshy layer gets partially to entirely separate from the fibrous layer. In both species the spores ornamentation is made of columnar processes while in *G. minutisporum* spores ornamentation is made of small verrucae and the fleshy and the fibrous layers remain adhered.

As many species within *Geastrum*, *G. minutisporum* was difficult to distinguish macroscopically from other *Geastrum* species. Thus, a combination of both, spores characteristics and macroscopic features are necessary to differentiate *G. minutisporum* from its closest species. Phylogenetic analyses suggest a close relationship of the species in the clade containing *G. minutisporum* supporting the affinities observed in their basidiomata; and together with microscopic characteristics of these species the molecular analysis supports proposing *G. minutisporum* as a new species.

Even though more sampling data are needed from *P. australis* woodlands and from Argentina in general, *G. minutisporum* could be another endemic species from this ecosystem and from Argentina. Increasing the sampling area in this endangered ecosystem might reveal some new and endemic species.

Key to species of Geastrum *within* G. minutisporum *clade*

1a. Mycelial layer tomentose 2
1b. Mycelial layer smooth..... 3

2a. Button spherical (0.8–1.2 cm) attached to a thick subiculum, expanded basidiome 1.4–2.5 cm wide, exoperidium not splitting in two layers, capillitial threads 3.4–5 µm in diam., unbranched and tapering tips ..... *G. mirabile*
2b. Button ovate and bluntly pointed (2–5 cm) with apoorly developed subiculum, expanded basidioma 3–6 cm wide, exoperidium splitting in two layers, capillitial threads up to 11 µm in diam., often branched near the tapering tips..... *G. velutinum*

3a. Expanded basidiome 1–5 cm in diam., spores 3.2–5.5 µmin diam., ornamented with columnar processes up to 0.6 µm high.....*G. saccatum*
3b. Expanded basidiome 2–2.5 cm in diam., spores 2–3 µm in diam., ornamented with low verrucae up to 0.2 µm high .....*G. minutisporum*

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of Argentina.

Acknowledgments

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Legend to figures

Fig. 1 – Fifty percent majority rule Bayesian consensus of the molecular phylogenetic analyses. Posterior probabilities are indicated below the branches. Bootstraps supports are indicated above the branches. *Bar*: 0.1 substitutions per site. GenBank accession numbers in parentheses.

Fig. 2 – *Geastrum minutisporum* (KM260664). A, B: Basidioma. C: Basidiospores and capillitium at light microscope. D, E: Spores and capillitium at SEM. *Bars*: A, B 5 mm; C 5 μm; D, E 1 μm.

Fig. 3 – *Geastrum minutisporum* (KM260664). A: Basidioma. B: Endoperidial hyphae. C: Pseudoparenchymatic layer hyphae. D: Fibrous layer hyphae. E: Mycelial layer hyphae and hyphal cords. F: Basidiospores at light microscope. G: Capillitial threads and spores at light microscope. *Bars*: A 10 mm; B, F 2 μm; C, E, G 10 μm; D 5 μm.

Figure
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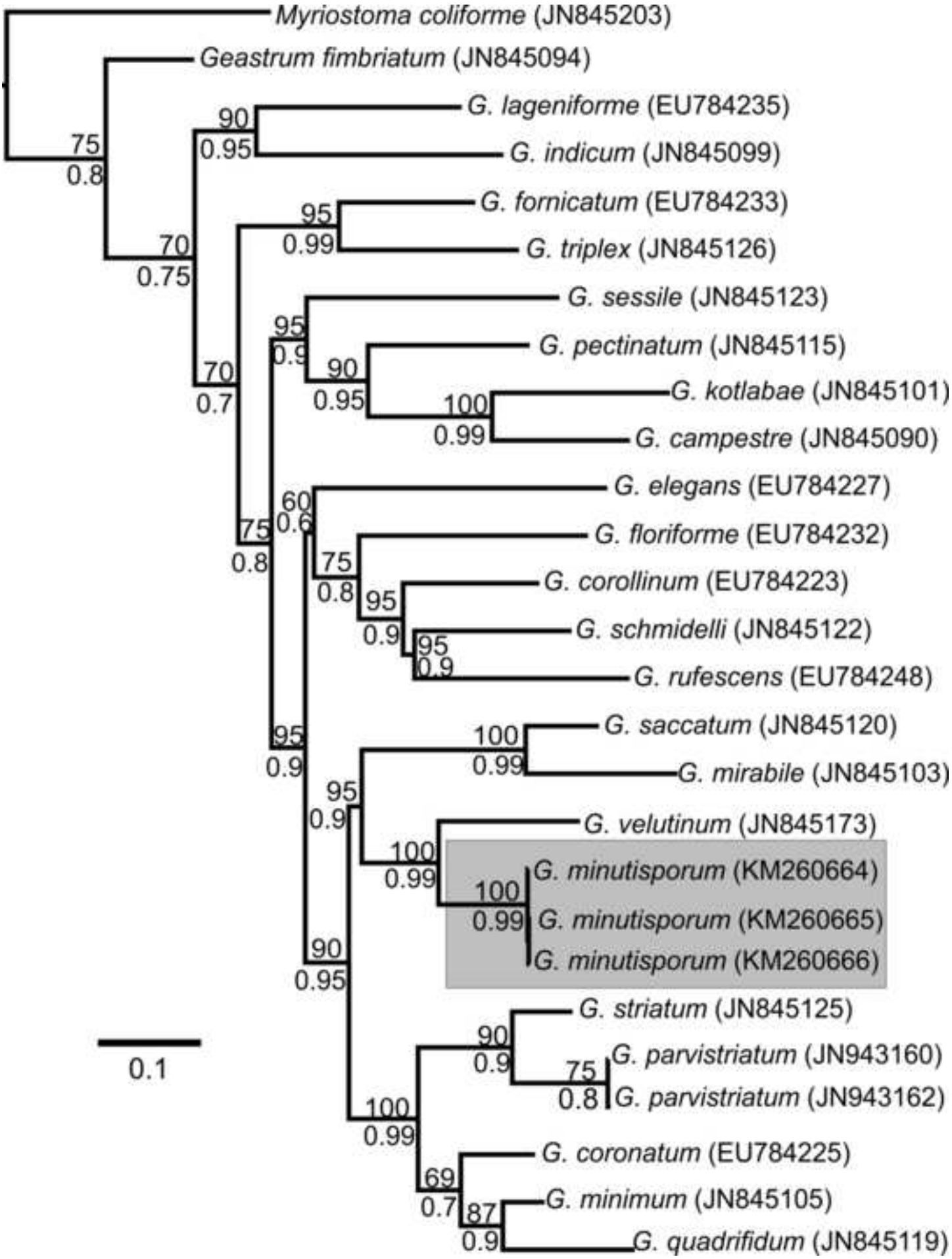

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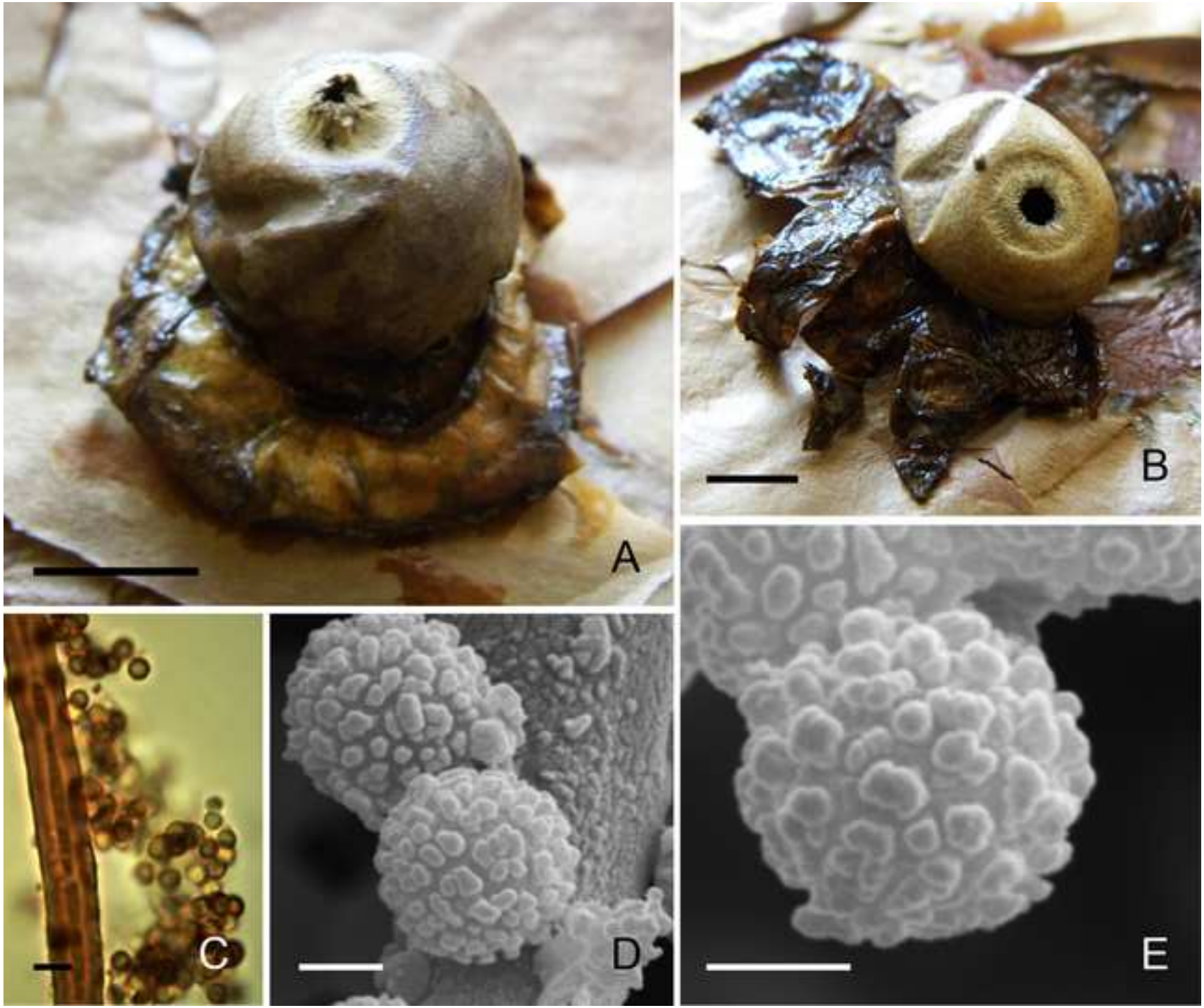

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