Geastrum pleosporus sp. nov., a new species of Geastraceae identified by morphological and molecular phylogenetic data

Clovis DOUANLA-MELI[1,*], Ewald LANGER[1] & Francisco D. CALONGE[2]

An unusual species of *Geastrum* was found growing on decayed wood debris and leaves of *Triplochiton scleroxylon* in the Mbalmayo Forest Reserve, Cameroon. The species morphologically resembles *G. saccatum* and *G. fimbriatum* in having sessile endosperidium partly enclosed by the saccate base of the exoperidium. Microscopically, it is characterized by and distinguished from all other known species of the genus, in having subsmooth, punctate to moderately verruculose, slightly thick- to distinctly thick-walled polymorphous, constricted to eight-shaped, mostly oblong, ovoid, cylindrical, elliptic to club-shaped basidiospores. *G. pleosporus* was studied from a collection of about fifteen basidiomata covering different stages of development. It is described as new based on morphological analyses and phylogenetic inferences made from large ribosomal DNA sequence alignments. Phylogenetic relationship of *G. pleosporus* is investigated. In parsimony analyses of partial sequences of the large subunit rDNA from selected Gasteromycetes species, *G. pleosporus* is closely related to *G. saccatum* within the strongly supported clade of *Geastrum* species. The cluster of *G. pleosporus* and *G. saccatum* is well supported in parsimony analysis of the dataset with *Geastrum* species and related taxa using parsimony and maximum likelihood analysis.

Taxonomical novelty: *Geastrum pleosporus* Douanla-Meli

Keywords: Earthstar, fibrilose-gleba, LSU rDNA, Lycoperdales, mycosclereids, peridium, polymorphous spores, subiculum

The genus *Geastrum* was erected by PERSOON (1801) with *G. coronatum* Persoon as the type species. It is the most widely distributed and complex genus in the Puffballs and fungi with enclosed hymenophore. Species in this genus are obviously recognised by the distinctly star-like basidiomata at maturity. The young fruitbody buried under leaf litter resembles a small, pointed, globular or dome-shaped puffball, lacking typical narrow stem and cap of other common fungi; the peridium is composed of not less than four layers. As maturity approaches, the exoperidium splits from the apex downwards into several stellate lobes and exposes the endoperidium, which dehisces by a unique stoma. Within the family Geastraceae comprising two other genera, *Myriostoma* Desv. is distinguished from other earth stars by the multiple perforations on the spore sac and *Radiigera* Zeller is also similar to an earthstar except that it does not open. CUNNINGHAM (1979) estimated about 110 species of *Geastrum*, currently described are not more than 30 valid species in the genus. Up to 279 species have been described, basically using classical taxonomy. Morphologically identifying characteristics of *Geastrum* are the rays that curl back in a star-like manner, this clears a space around the endoperidium which remains seated in the cup thus formed, as it is the case in *G. sessile* (Sowerby) Pouzar and *G. saccatum* Fr., or the rays fold tightly over the spore sac and raise the latter like that in *G. coronatum* Pers., and typically in *G. fornicatum* (Huds.) Hook., with a peridium carried upon a pseudostem. In other species like *G. schweinitzii* (Berk. & M. A. Curtis) Zeller, the fruitbodies sit on a subiculum, a felt like mass of hyphae, which covers rotting wood. In some species the rays expand, but then close up over the endoperidium when they dry out. They are described as hygroscopic and resemble hygroscopic species like *Astraeus hygrometricus* (Pers.) Morgan. Significantly, however, several species of *Geastrum* having hygroscopic rays can be distinguished by the presence of a peristome, a columella, and generally smaller spores. Microscopically the striking features of the genus concern the spores. They are globose, or less frequently subglobose, some shade of brown, and almost invariably verrucose or echinulate, produced on 4–8 spored basidia.

Geastrum is the most common Gasteromycete genus in Cameroon (CALONGE & DANIËLS 1998, HJORTSTAM et al., 1993). In the framework of a research program aimed at the assessment of fungal biodiversity in South Cameroon, periodical collections were carried out. During 2002 we collected several specimens in the Mbalmayo Forest Reserve, belong-

[1] Universität Kassel, Fachbereich 18 Naturwissenschaften, Institut für Biologie, Fachgebiet Ökologie, Heinrich-Plett-Staße. 40, 34132 Kassel, Germany.
[2] Real Jardín Botánico, CSIC, Plaza de Murillo 2. E-28014 Madrid, Spain. E-mail <calonge@ma-rjb.csic.es>
[*] Corresponding author e-mail <cmeli@yahoo.com>, Tel.: (0049) 561 8044215, Fax: (0049) 561 8044115

ing to the genus *Geastrum*, and the study revealed well-known species such as *G. saccatum* and *G. fimbriatum*, representing the first records for Cameroon, while other did not match with any of the described species (PONCE DE LEÓN 1968; CALONGE 1998; CALONGE & DEMOULIN 1975; SUNHEDE 1989; SOTO & WRIGHT 2000, etc.). Besides the typical morphology reminiscent of *Geastrum* species, the spore patterns observed in the whole collection were unknown in the genus. In this case an exclusively morphological study appears not sufficiently reliable. Therefore molecular tools for their identification were combined. Very limited molecular data are available to infer phylogenetic relationships in *Geastrum*. In order to test the hypothesis that the fungus is related to *Geastrum* species, we sequenced a portion of the large subunit rDNA, and included it in two datasets of (a) Gasteromycete species and (b) *Geastrum* species together with related taxa. Its phylogenetic placement was investigated. Morphological and molecular studies suggested that this is a new species in *Geastrum*.

Material and methods

Basidiomata were collected and dried with a DÖRREX SIGG 6252.10 (Switzerland). The description was made based on the examination of the collection containing fifteen fruitbodies in all stages of development. Colour terms in parentheses are those of KORNERUP & WANSCHER (1978). Herbaria are cited according to HOLMGREN, HOLMGREN & BARNETT (1990). Holotype specimens are deposited at the herbarium of Cryptogamy Laboratory, Faculty of Science, University of Yaoundé I, Cameroon (HUYI), an isotype at MA-Fungi.

Line drawings, light and scanning electron microscopy

Morphological description was based on fresh material. Microscopic structures were mainly observed on dried material. Free-hand thin sections were mounted in KOH (5 %) for more detailed observations. Light microscopy studies were carried out at 400 and 1000 X using an Olympus BX51TF microscope. Line drawings were made using a grid as drawing aid representing a magnification of 52000. Size ranges of basidiospores were based on at least 50 basidiospores; light photography was done with a Nikon COOLPIX 4500 Digital, and processed in Photoshop (Adobe Photoshop 4.0). For the SEM photographs, just prior to examination with a Hitachi S 4000 Scanning Electron Microscope, dried spores were mounted on platinum stubs and gold coated to a thickness of 20 nm with a Balzers SCD 020 Coating Unit (Balzers, Liechtenstein).

Nucleic acid extraction, PCR amplification and sequencing

Genomic DNA was isolated from dried herbarium specimens using a modified version of SDS method of EDWARDS, JOHNSTONE & THOMPSON (1991) and HENRION, LE TACON & MARTIN (1992). Material for DNA isolation (30–40 mg) was taken from the gleba tissue (mass of spore with capillitium) and the exoperidium. Serial dilutions (1:100, 1:1000) were used as template for the polymerase chain reaction (PCR). Reaction for PCR amplification were performed in 50 µl mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 75 mM $MgCl_2$, 10 mM of each of the four deoxynucleotide triphosphates, 25 pM of each primer, and 1 unit of *Taq* polymerase. The 5' end domain of the nuclear DNA coding for the large ribosomal subunit (LSU rDNA) was amplified using the polymerase chain reaction (MULLIS & FALOONA 1987; WHITE et al. 1990) with NL1 and NL4 as primers (O' DONNELL 1993). The PCR reactions were performed with the TGradient Thermocycler 96, Biometra of Whatman Company. The cycle parameters were an initial denaturation at 94 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 60 s, and a final elongation step of 7 min at 72 °C was included. PCR products were checked on 0.7 % agarose gel, and purified with the QIAquick™ kit (QIAGEN) according to the manufacturer's instructions. The DNA products were sequenced in both directions with the respective primers NL1 and NL4, using the ABI PRISM™ Bigdye Terminator Cycle Sequencing Kit, Version 3.1, Applied Biosystems, and analysed using an automated DNA sequencer ABI 3100, Perkin Elmer.

Sequence alignment and phylogenetic analysis

The contiguous nucleotide sequences were edited and assembled using the programs EditSeq and MegAlign of Lasergene (DNASTAR 2000) software for Macintosh, and manually corrected with Se-Al version 2.0a11 (RAMBAUT 2002). Gaps (insertions/deletions) in the alignment were treated as missing data. All positions were included in the final alignment. The three sequences of *Geastrum* we determined have been deposited in Genbank with the accession numbers AY566241 and AY566242 for *G. pleosporus* and AY714318 for *G. fimbriatum*. Additional sequences used for comparative analyses were obtained from GenBank (Tab. 1). We assembled two data sets of LSU sequences by selecting species of Gasteromycetes, and a selection of *Geastrum* species together with related species. The aligned sequences are deposited in TreeBase in NEXUS format, with accession numbers SN1798.

Phylogenetic analysis was performed with PAUP* (Phylogenetic Analysis Using Parsimony) version 4b10 (SWOFFORD 2002) for Macintosh. Nucleotide substitutions were treated as unordered, equally weighted characters. Gapped positions were included to improve alignment and treated as missing data or were excluded in separate analysis. Maximum parsimony trees were inferred using heuristic search option, Starting tree(s) obtained via stepwise addition, tree-bisection-reconnection (TBR) branch-swapping algorithm was used, MAXTREES reset to 10 000. MULTRESS option (saving of all optimal trees) effective, Steepest descent option in effect,

Tab. 1: List of taxa included in the phylogenetic analysis, GenBank numbers, strain, sequence data and sequence length. Annotated with (*) the sequences generated in this study.

Species	Accession no.	Strain	Sequence data	SeqLength
Anthurus archeri	AJ406479	GEL5392	LSU	921
Astraeus hygrometricus	AF336238	Ashy3	LSU	906
Auricularia delicata	AF291290	USJ 54470	LSU	585
Auricularia mesenterica	AF291292	FO 25132	LSU	566
Bovista paludosa	AJ237630	BPRU	LSU, SSU, ITS1&2	695
Bovista plumbea	AJ237629	DA-54	LSU, SSU, ITS1&2	694
Calvatia rubro-flava	AF485064	TENN59078	LSU	673
Clathrus ruber	AF213127	T-9354	LSU	615
Clavariadelphus pistillaris	AF213133	OSC-69446	LSU	608
Cyathus stercoreus	AF261583	T815	LSU	938
Cyathus striatus	AF336247		LSU	895
Gautieria gautierioides	AF213123	OSC-48547	LSU	608
Gautieria parksiana	AF213126	OSC-49803	LSU	606
Geastrum fimbriatum	AY714318*	DMC 290	LSU	633
Geastrum nanum	AF336250		LSU	908
Geastrum pleosporus	AY566241*	DMC 224a	LSU	617
Geastrum pleosporus	AY266242*	DMC 224b	LSU	628
Geastrum rufescens	AF336251		LSU	911
Geastrum saccatum	AF287859		LSU	964
Geastrum sessile	GSE406480	GEL5319	LSU	922
Gomphus floccosus	AF287862		LSU	969
Hysterangium clathroides	AF213121	SZEMORE	LSU	618
Hysterangium coriaceum	AF213122	OSC-69448	LSU	612
Lycoperdon echinatum	AJ237622	LEVB	LSU, SSU, ITS1&2	690
Lycoperdon foetidum	AJ237623	DA-100	LSU, SSU, ITS1&2	691
Lycoperdon perlatum	AY264919		LSU, SSU, ITS1&2	698
Phallus impudicus	AY152404	FO 46622	LSU	605
Pseudocolus fusiformis	AF213128	ASM-4705	LSU	616
Scleroderma bovista	AF336264	W#1149	LSU	903
Ramaria rainierensis	AF213115	M-231	LSU	612
Rhizopogon pannosus	AY177254	Rpa1	LSU	878
Rhizopogon pumilionus	AY177252	Rp1	LSU	894
Rhizopogon subareolatus	AY177250	Rsa1	LSU	882
Tulastoma brumale	AF336272		LSU	890
Tulostoma simulans	AF261486		LSU	1131

and zero length branches collapsed. A neighbour-joining (NJ) tree was constructed using the Kimura 2-parameter model (KIMURA 1980). Relative robustness of internal branches was assessed by 1000 bootstrap replications (FELSENSTEIN 1985; HILLS & BULL 1993). Other indices for the generated topology, included tree length, consistency index (CI), and retention index (RI). As outgroup for the two data sets *Auricularia delicata* (Fr.) Henn. and *Auricularia mesenterica* (Dicks.) Pers. were chosen based on the results of many phylogenetic studies involving species of Gasteromycetes and related taxa (HIBBETT et al. 1997; HUMPERT et al. 2001). In addition, for the two datasets, parsimony analysis was also performed by maximum likelihood (ML) analysis using starting trees for TBR branch swapping with one random taxon addition sequence. Empirical nucleotide frequencies were used: A = 0.25646, C = 0.21262, G = 0.29862 and T = 0.23229. The transition/transversion ratio was set to 2.0, and the Hasegawa-Kishino-Yano (HKY85) distance model was selected, the ML bootstrap values were generated using a stepwise sequence addition with 100 replicates.

Results

Sequence alignment and phylogenetic analysis of the LSU rDNA datasets

BLAST and FASTA searches using the two LSU rDNA sequences of *G. pleosporus* (DMC 224a 617 bp and DMC 224b 628 bp fragments) recovered nrDNA sequences of species of *Geastrum*, *Sphaerobolus*, *Gomphus*. Highest scoring matches were with species of *Geastrum.* The sequences of partial LSU DNA ranged from 608 to 1131 bp. All positions were unambiguously alignable among the LSU rDNA of Gasteromycetes, only representative taxa from each group were included in the analysis along with available sequences of *Geastrum*. The LSU ribosomal DNA data set of 32 taxa was subjected to parsimony analyses. The length of aligned sequences including inserted gaps was 911 characters. Parsimony analysis of the LSU sequences in which indels were treated in various ways, generated MPT (most parsimonious trees) that differed in length, number of trees retained, consistency index and retention index. The MPT generated using different indel treatments were similar in topology, with minor variation at the tips of trees. Analysis with indels excluded and gaps treated as newstate produced eight MPT of shorter length (363 steps) with the same topology and the placement of *G. pleosporus*. In the parsimony analysis with indels coded with multistate characters and gaps treated as missing, 509 characters were variable, 428 of which were parsimony informative. Parsimony analyses using heuristic search resulted in three equally MPT of 1109 steps with the following scores consistency, retention, and rescaled consistency indices of 0.622, 0.836 and 0.520 respectively. The maximum likelihood analysis of the same sequence alignment yielded a tree with a very similar topology (not shown). The main difference was the lower bootstrap values obtained at different internodes. Phylogenetic analysis identified two major lineages of rDNA as measured by bootstrapping (Fig. 1). The larger lineage was weakly supported at bootstrap value 70 %, showing the monophyly of the rest of Gasteromycetes representatives without *Geastrum* species, further topology resolved two main groups with bootstrap support 64 % and 76 %. The former characterised the branch leading to the Phallales and Lycoperdales species and the latter supported the monophyly comprising representatives of Hymenogastrales, Sclerodermatales, Tulostomatales and Nidulariales. The clade of Phallales was confirmed with weak bootstrap support 63 % and the two species of Gautieriales nested within this clade, whereas the clade of Lycoperdales was supported at 100 % bootstrap value. The resolved position of Lycoperdales revealed by the analyses corroborates their phylogenetical separation from Geastrales demonstrated by KREISEL (1969) and reaffirmed by KRÜGER (2001). This study focussed on the placement of *G. pleosporus* has asserted its generic assignment. All species of *Geastrum* included in the analysis nested within a unique clade sister to the rest of Gasteromycetes representatives, and strongly supported with 99 % bootstrap value, but failed to be resolved in monphyly. *G. sessile* was basal in the clade, and the remainder species were moderately supported with 75 % bootstrap value. Further resolutions for each sub-clade alone were high, 89 % and 91 % bootstrap value respectively. *G. fimbriatum* was sister group to the sub-clade comprising *G. rufescens* and *G. nanum*. *G. pleosporus* clustered together with *G. saccatum* in a sub-clade with 89 % bootstrap support. In a neighbour-joining analysis (not shown) that yielded similar topology, *G. pleosporus* and *G. saccatum* occupied the same relative position as shown in Fig. 1.

The second alignment consisted only of sequences of *Geastrum* and closely related species, using the sequence of *Auricularia mesenterica* as outgroup. The available sequence of *G. triplex* could not be unambiguously aligned with the rest, and was excluded from the phylogenetic analysis. This alignment of 11 taxa included 634 characters; there were 148 variable characters of which 92 were parsimony-informative. Parsimony analysis resulted in a single MPT of 230 steps in length and consistency, retention, and rescaled consistency indices of 0.752, 0.731 and 0.550 respectively. The best fitting ML model selected by Modeltest is the Hasegawa-Kishino-Yano model with gamma distributed site-to-site rate variation. In analysis with one of the most parsimonious trees as starting tree for branch swapping, the optimal MLT was found with a log likelihood score of –2106.07633. The ML analysis recovered the grouping found by the MP analysis with some minor changes in bootstrap values. In the MPT (Fig. 2) and MLT (Fig. 3), *Geastrum* spp. and related taxa form monophyletic clades with bootstrap values of 100 % and 99 % (100 %) respectively, although the sister relationship was not supported by bootstrap analysis. The topology within the clade of *Geastrum* species did not differ from the first analysis, despite minor fluctuations of bootstrap values.

Taxonomy

Geastrum pleosporus Douanla-Meli sp. nov. **Figs. 4-7**

Carposoma iuvene epigaeum, adhuc clausum globulare vel depresso-globulare, 1.2–1.5 cm latum, aurantio-ruber vel fusco-ruber. Endoperidium globulare, sessile, 0.5–0.8 cm lato, avellaneus vel spadiceus, tenuis. Exoperidium apertum, non hygroscopicum, in 5–6 radios orbicularis, recurvos fissum, stratum myceliale fusco-ruber, persistens, stratum medium aurantio-ruber, tenuis; stratum internum carneum, ad 2 mm crassus, persistens, peristomio determinato, fibrilloso. Gleba brunnea, columella parva manifesta. Basidiospora laevis in microscopio optico, vel leviter punctulatus in microscopio electronico, polymorpha; globularis, cylindricus, ellipticus, reniformis, clavulatus, leviter crassus vel parva manifesta crassus tunicatus, (3.5) 4–6 (8) × (3) 4–5 (6) µm.

Ad Mbalmayo silva nova, Ekombitie-Cameroon, m 500–650 a.s.l., on emortuus lignum *Triplochiton scleroxylon*, leg. C. Douanla-Meli, 16.X.2002. Holotypus: herb. HUYI DMC 224. Isotypus: MA-Fungi 56971.

Etymology: Latinised form of polymorphic, referring to the polymorphous spores.

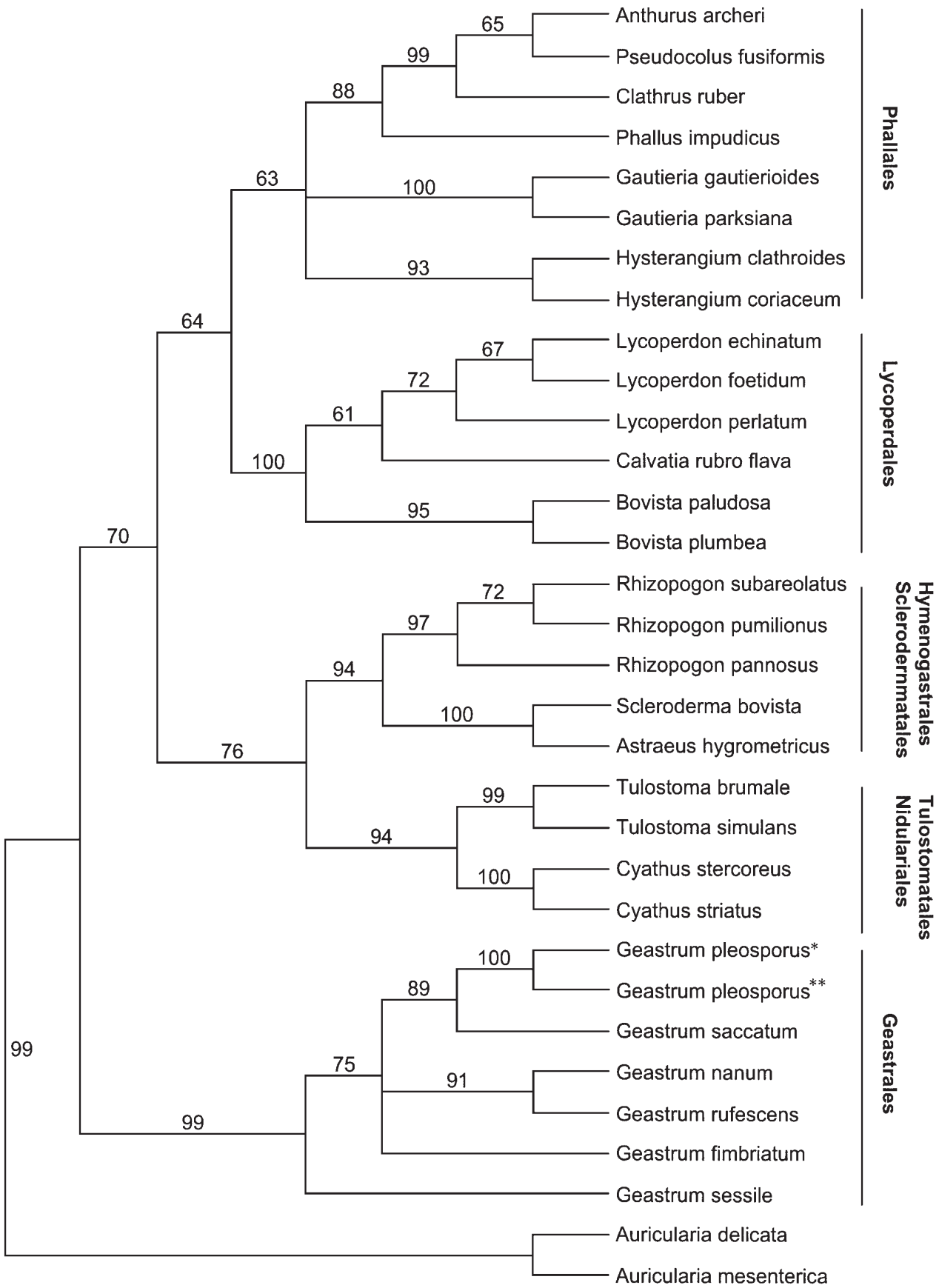

Fig. 1: One of the three equally parsimonious trees generated from heuristic search in PAUP 4b10 based on LSU rDNA sequences from 30 taxa of Gasteromycetes (L = 1109 steps, CI = 0.622, RI = 0.836 and RC = 0.520). Bootstrap values from 1000 replicates greater than 50 % are shown at the respective internodes. *Auricularia delicata* and *Auricularia mesenterica* were used as outgroup. * = DMC 224a; ** = DMC 224b.

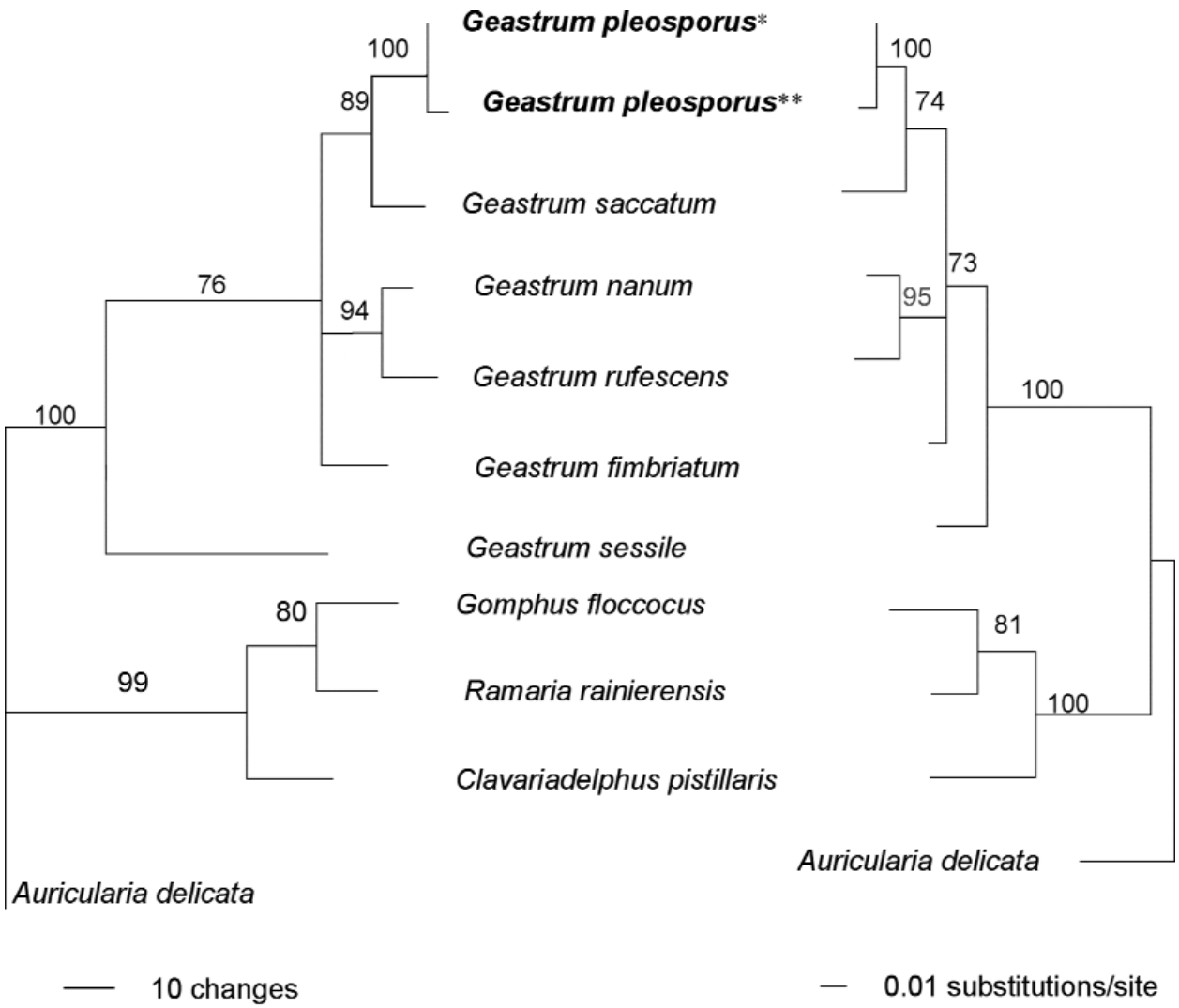

Fig. 2: Phylogenetic placement of *G. pleosporus*. Strict consensus phylogram of the single MPT recovered from maximum parsimonious (MP) analysis of LSU rDNA sequences of *Geastrum* spp. and related taxa (Lenght = 230 steps, CI = 0.752, RI = 0.731, RC = 0.550). Bootstrap greater than 50 % are indicated at the respective nodes. Length is given under each branch. *Auricularia delicata* was used as outgroup.

Fig. 3: Phylogenetic placement of *G. pleosporus*. The tree with the highest likelihood (lnL –2106.07633) obtained from maximum likelihood (ML) analysis of 11 LSU rDNA sequences of *Geastrum* spp. and related taxa. Branch lengths redrawn to scale correspond to genetic distance (expected nucleotide substitutions per site). Bootstrap values greater than 50 % are given above branches. *Auricularia delicata* was used as outgroup.

Unexpanded fruitbody epigeous, globose to depressed globose, up to 1.2–1.5 cm broad, orange red (8A8) to brown red (8C8), outer surface wrinkled, emerging from a white subiculose mycelium, which grows on plant debris. Expanded fruitbody (Fig. 4) up to 2 cm broad. Exoperidium non hygroscopic, splitting into 5-6 star-like, thick, round-tipped rays, up to 5 mm long that eventually roll downwards. Endoperidium globose, initially dusted, covered by a powdery or meal layer, soon glabrous, sessile, 0.5–0.8 cm broad, beige (4C3) to olive brown (4D3), thin, papery, dry. Peristome beak-like, up to 2 mm tall, with grey (2B2) soft silky fibrils extending radially from ostiole to a slight depression. Pseudoparenchymatous layer orange red (8A8) to brown red (8C8), up to 2 mm thick, persistent. Outer mycelial layer thin, felted and easily peeled off to reveal underlying fibrous layer of the peridium, slightly encrusted with debris. Gleba white, cottony when young, with radial fibrils, then turning dark brown (6F8) and powdery before outer the peridium begins to split open, pseudocolumella distinct.

Basidiospores dark brown (6F8) in mass, epispore light brown (6D8) to brown (6E8) in 5 % KOH, slightly thick to distinctly thickened (Fig. 6d), appearing smooth under light microscope (LM) (Figs. 6a-e) but subsmooth, punctate, minutely and irregularly verruculose under scanning electron micro-

Fig. 4: *G. pleosporus* (Holotype DMC 224): mature fruitbodies. Scale bar = 1 cm. Photo: C. Douanla-Meli.

scope (SEM) (Figs. 7a-d), very variable in size and shape, (3.5) 4–6 (8) × (3) 4–5 (6) µm, globose, cylindrical, elliptic, reniform, club-shaped, producing constricted spores (Figs. 5b, 6b, 6d), sometimes showing a mid-length to subapical constriction, that narrowing gradually and may be developed to a deep strangulation, thus the eight-shaped.

Basidia only observed in young gleba, 15.5–19 × 4–5.5 µm, clavate, ventricose to flask-shaped with a more or less long collar, (2) 4–8 spored, long pedunculate with basal clamp, continuing with hyaline fibrils (Fig. 5a). Capillitium threads (Figs. 5c and 6e) abundant, almost tinted, yellowish brown (5E8) to dark brown (6F8), 1.5–4.5 µm broad, thick-walled, lumen narrowing, much reduced, or even absent, fusiform to cylindrical, continuous, strongly attenuating towards the ends, unbranched or seldom with acute branches at apices, attached to the pseudo-columella and the inner wall of the endoperidium. Endoperidium of tightly interwoven hyphae, 2.5–6 µm broad, hyaline to pale yellow (3A3), thick-walled with large or narrow lumen, at times dichotomously branched, tapering towards the ends. Fleshy layer consisting of pale yellow (3A3) to yellow (2B8), thin- to slightly thick-walled cells, up to 35 µm broad. Exoperidium composed of tight hyphae (Fig. 5d), hyaline to light brown (5D7), 10-16 µm broad, thick-walled, walls up to 7 µm thick, with narrow lumen, stout, attenuating towards the base, intermixed with hyaline, interwoven hyphae up to 13 µm broad.

Habitat and ecology: Scattered to gregarious on decayed wood debris and leaves under *Triplochiton scleroxylon* K. Schum. (Sterculiaceae), Ayous, in semi-deciduous forest rich in Sterculiaceae and Ulmaceae.

Material studied: CAMEROON, Centre Province, Department of Nyong & So'o, in the Mbalmayo forest reserve, 47 km South east of Yaounde, 11° 14′–11° 54′ E and 3° 5′–3° 58′ N, 500-650 m asl., 16.X.2002, leg. C. Douanla-Meli. Holotype DMC 224 (HUYI), Isotype 56971 (MA- Fungi).

Discussion

Few studies are carried out on the species of *Geastrum* from the tropics. In 1962 Dissing & Lange published a work on the Gasteromycetes of Congo, where they distinguished 20 species. Later, Dring (1964) described 8 species from West Tropical Africa, Demoulin & Dring (1975) reported 8 species from Eastern Central Africa. Regarding the neotropics, Calonge, Moreno-Arroyo & Gómez (2000) have described a new species, *G. ovalisporum* from Bolivia, and recently Baseia & Milanez (2002) proposed *G. setiferum* as a new species from Brazil. In the absence of a comprehensive monograph of *Geastrum,* the most complete work on the world species of the genus *Geastrum* is that of Ponce De León (1968), and elsewhere the study of Northern European species by Sunhede (1989). The morphological characteristics and the sequence data

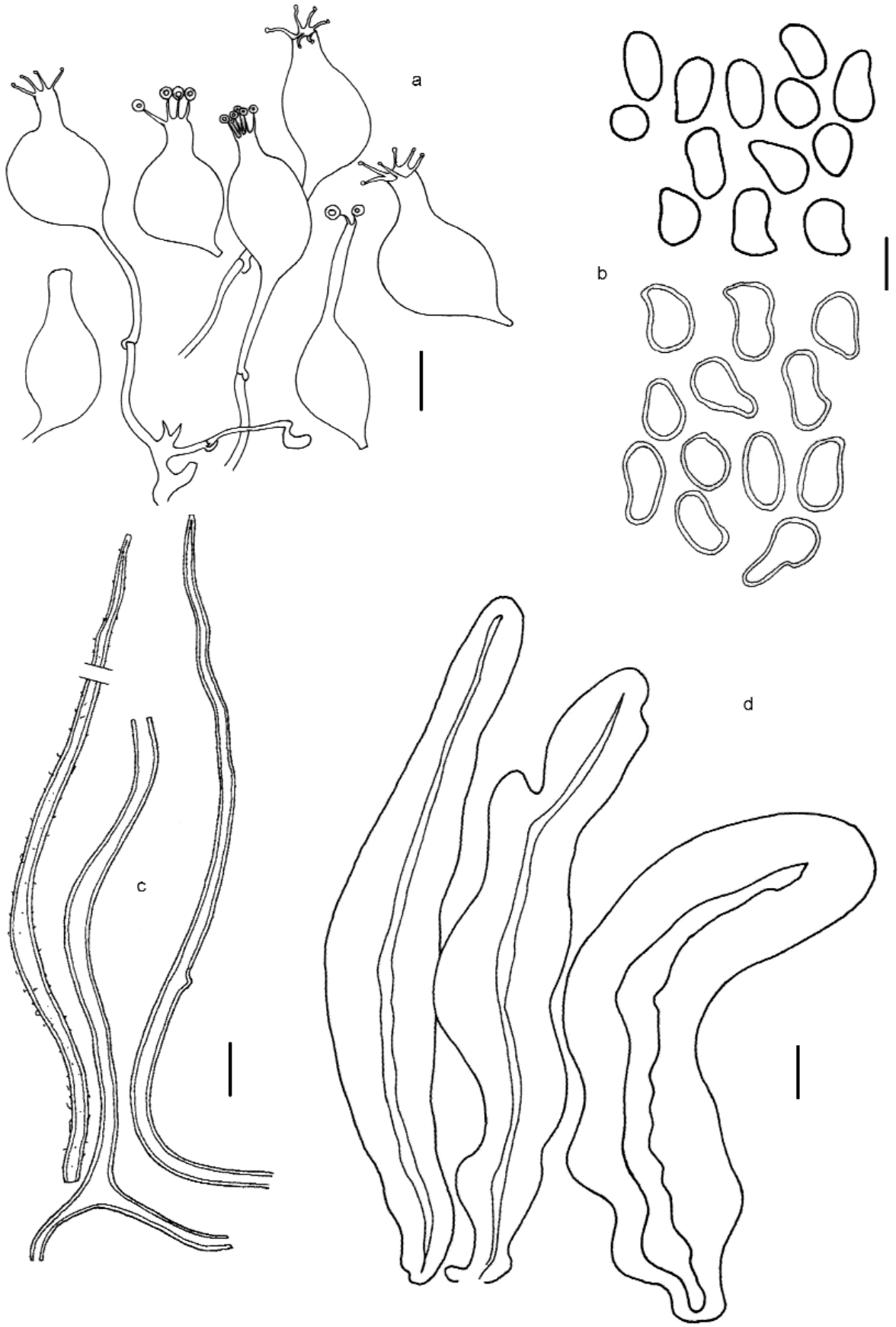

Fig. 5: *G. pleosporus* (Holotype DMC 224). – **a**. basidia ventricose, long pedunculate, with basal clamped, continuing with hyaline fibrils, only observed in young gleba. – **b**. basidiospores as seen by LM, smooth, very variable in shape and size, with slightly thick-to thickened walls. – **c**. thick-walled capillitium threads, strongly tapering with acute ends, may be finely incrusted with debris. – **d**. thick-walled hyphae of the exoperidium, cylindrical, with narrow lumen. Scale bars = 5 μm.

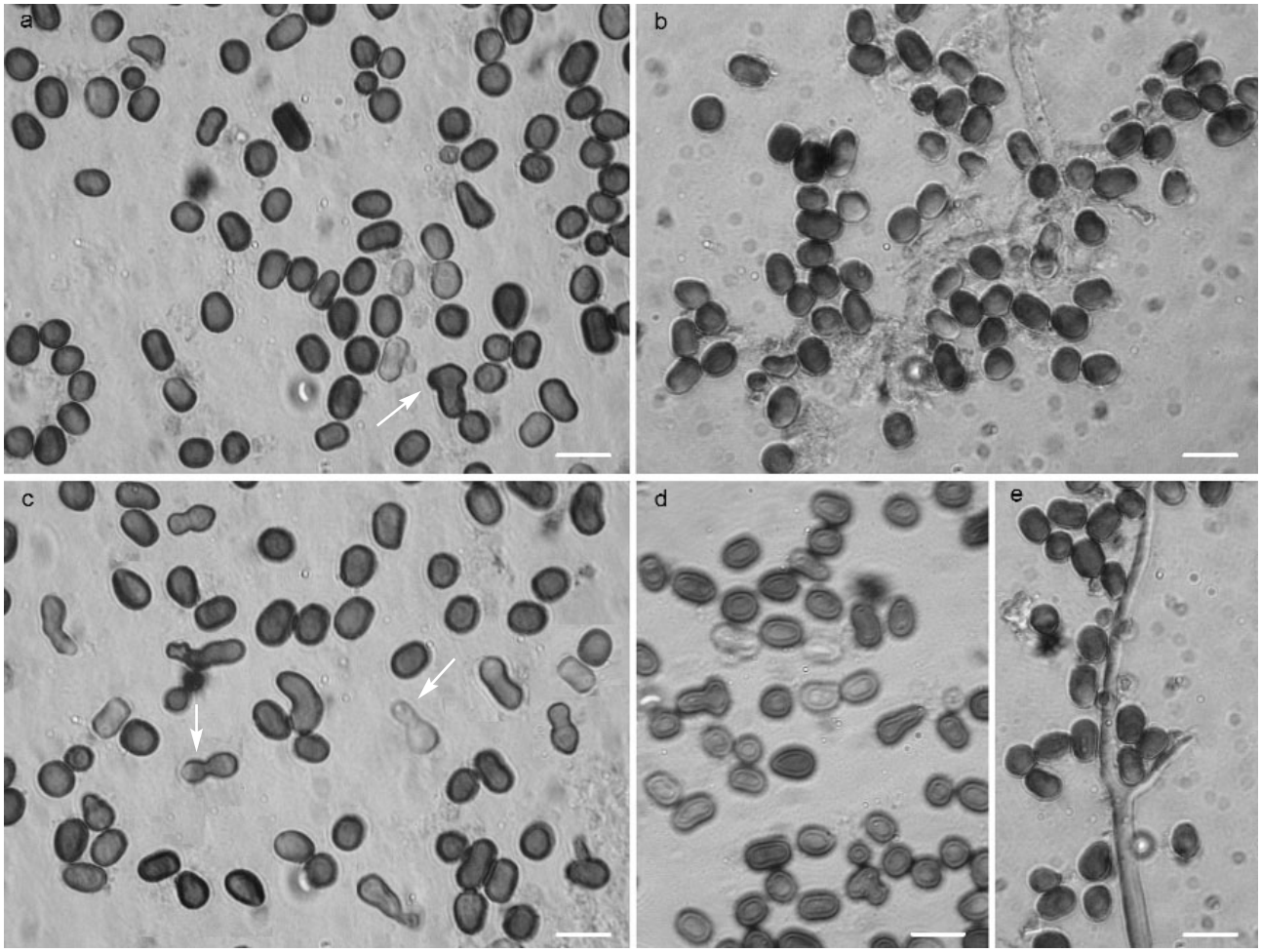

Fig. 6: *G. pleosporus* (Holotype DMC 224): Micrographs from LM. – **a-c**. basidiospores smooth, with variable forms, globose, elliptic, club-shaped to eight-shaped showing a mid-length to subapical constriction (arrows). – **d**. basidiospores showing thickened walls. – **e**. capillitium threads with acute, subapical branches. Scale bars = 5 µm.

of LSU rDNA demonstrated that our collection from Cameroon represented a new species of *Geastrum* in tropical Africa.

Morphologically, *G. pleosporus* is characterised by having minute basidiomata, orange red to brown red, thick-walled capillitium occasionally with apical acute branches, pleomorphous, and smooth to subsmooth spores. The characteristic simple or freely branched capillitium threads in *G. pleosporus* remind of species of Lycoperdaceae (KRÜGER et al. 2001), but based on the number of layers of the fruitbody, more than two, the new species is assigned to the Geastrales that includes the genera *Geastrum*, *Myriostoma* and *Ragdiigera*. It further shows a spore sac with a unique stoma distinguishing the genus *Geastrum*. *G. pleosporus* belongs to a group that is characterised by having a subiculose mycelium, with only four taxa (PONCE DE LEÓN 1968). Two of them, *G. javanicum* Lév. and *G. javanicum* var. *welwitschii* can be separated by an exoperidium whose external layer splits into two fibrous persistent layers; while the other two, *G. schweinitzii* and its variety *G. schweinitzii* var. *stipitatum* (Solms ex Fischer) P. Ponce, differ from *G. pleosporus* by having bigger basidioma (2–5 cm broad) and always globose and smaller (3.2–3.8 µm diameter) spores. *G. subiculosum* (Cooke & Massee) G. H. Cunningham also grows crowded upon a white subiculum covering the surface of decaying vegetable debris; it is however easily identified by the globose and small, almost smooth to delicately verruculose spores. Another species morphologically similar to *G. pleosporus* is *G. velutinum*. It has a sessile endoperidium, non-hygroscopic exoperidium similar to *G. pleosporus*. Both species also have a shallow depression at the base of the peristome. However, the peristome of *G. velutinum* is pubescent, non sulcate, surrounded by a dark ring. It also differs in the well-developed, dark brown, velutinous ornamentation covering the external surface. Concluding from the characters of the endoperidium and the slightly depressed disc surrounding the apical pore, *G. pleosporus* could also be related to *G. sessile* and *G. saccatum*, but it is easily distinguished by its colour. It becomes obvious that the species placed in the genus *Geastrum* resemble one another so closely that separation is frequently a difficult matter, and the taxonomic value of morphological features is not any more efficient to warrant the erection of new taxa. Nevertheless, the best features to separate *G. pleosporus* from other known species is

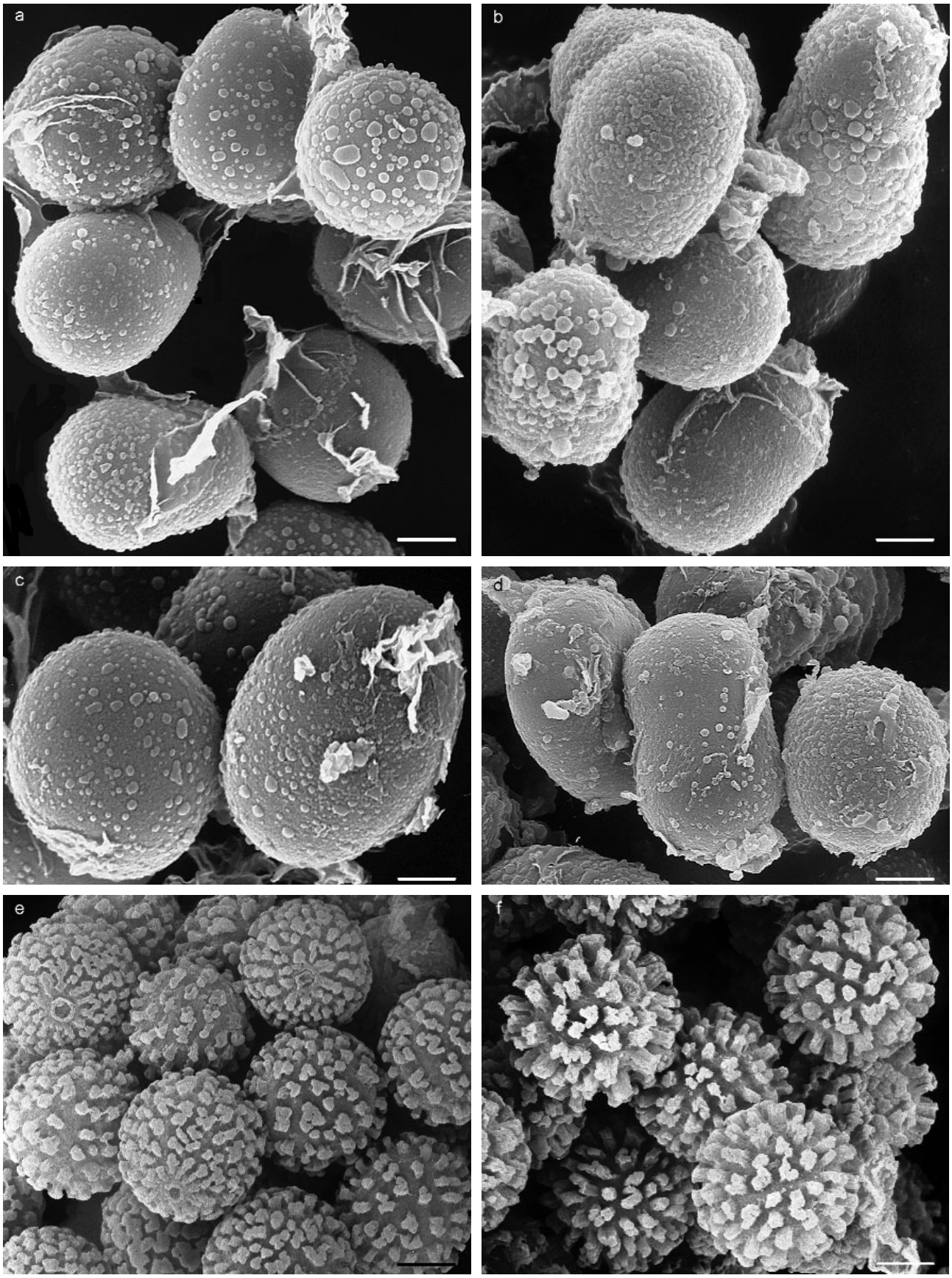

Fig. 7: SEM micrographs of basidiospores. – **a-d**. *G. pleosporus* (Holotype DMC 224); basidiospores subsmooth to minutely and irregularly verruculose, variable in shape. – e. *G. saccatum* (DMC 227); basidiospores globose, densely warted. – **f**. *G. fimbriatum* (DMC 290); basidiospores globose, with coarsely, densely arranged column-shape warts. Scale bars =1 μm.

based on spores. They are less subject to variation than other features of this variable genus, and their size and markings afford useful specific characters (CUNNINGHAM 1979, DRING 1964). *G. pleosporus* does not display the globose to subglobose, warted spores common in the genus *Geastrum*, but shows spores with uncommon patterns in shape, mostly ellipsoid, reniform, ovoid, elongate to constricted, thick-walled and widely ranging in size. Such unusual spores have not been found in other described species of *Geastrum*. They were observed from all individual basidiomata of the collection. *G. pleosporus* shows a combination of characters, such as tiny orange-red basidiomata, presence of a subiculose mycelium and polymorphic subsmooth to verruculose spores, which confer this taxon a unique identity.

We employed rDNA sequence analysis to clarify the generic placement of *G. pleosporus*. Analysis of LSU rDNA placed *G. pleosporus* in the Gasteromycetes in a clade among other *Geastrum* species. Analysis of two datasets with representatives of different Gasteromycetes groups and *Geastrum* species together with related taxa clearly associated *G. pleosporum* with *G. saccatum*, as initially indicated by the results of BLAST search using the two LSU rDNA sequences generated from *G. pleosporus*. In both parsimony and maximum likelihood analyses, *G. pleosporus* and *G. saccatum* formed a cluster with high bootstrap support. Results of the parsimony analysis based on LSU rDNA warrant its placement in the genus *Geastrum*. *G. sessile* and *G. fimbriatum* however are distantly related to this cluster. The sessile spore sac does not appear to be a reliable indicator of relatedness among *Geastrum* species. Two of the *Geastrum* species within the clade have slightly raised spore sacs, and are more related to *G. pleosporus* than *G. sessile* and *G. fimbriatum*. Indeed, *G. pleosporus* has morphological similarities with *G. saccatum* and other species of the genus. Phylogenetic analysis showed relationships and differences with the former species. On the other hand it is recognised as a distinct species based on spores patterns and smaller size of the fruitbodies.

Acknowledgements

The authors appreciate the help of Mr. Harald Rühling of Cellular Biology Department with SEM photography and Dr. Alexandra Riethmüller for a technical management in the laboratory. Furthermore, Mr. Zambo Robert is thanked for facilitating fieldwork. The comments of three anonymous reviewers were very helpful for improving the manuscript. This research was financially supported by the grant A/01/20502 from the German Academic Exchange Service (DAAD) to the senior author.

References

BASEIA IG, MILANEZ AI (2002) *Geastrum setiferum* (Gasteromycetes): A new species with a setose endoperidium. – Mycotaxon **84**: 135-139.

CALONGE FD (1998) Gasteromycetes. I. Lycoperdales, Nidulariales, Phallales, Sclerodermatales, Tulostomatales. – Flora Mycologica Iberica **3**: 1-271.

CALONGE FD, DANIËLS PP (1998) Fungi of the Dja Biosphere Reserve (Cameroon). Notes on some Gasteromycetes. – Boletín de la Sociedad Micológica de Madrid **23**: 135-138.

CALONGE FD, DEMOULIN V (1975) Les Gasteromycètes d'Espagne. – Bulletin de la Société Mycologique de France **91**: 247-292.

CALONGE FD, MORENO-ARROYO B, GÓMEZ J (2000) Aportación al conocimiento de los Gasteromycetes, Basidiomycotina, de Bolivia (América del Sur). *Geastrum ovalisporum* sp. nov. – Boletín de la Sociedad Micológica de Madrid **25**: 271-276.

CUNNINGHAM GH (1979) The Gasteromycetes of Australia and New Zealand. – Bibliotheca Mycologica **67**: 1-227.

DEMOULIN V, DRING DM (1975) Gasteromycetes of Kivu (Zaïre), Rwanda and Burundi. – Bulletin Jardin Botanique National de Belgique **45**: 339-372.

DISSING H, LANGE M (1962) Gasteromycetes of Congo. – Bulletin Jardin Botanique de l´Etat, Bruxelles **32**: 325- 416.

DRING DM (1964) Gasteromycetes of West Tropical Africa. – Mycological Papers **98**: 1-60.

EDWARDS K, JOHNSTONE C, THOMPSON C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. – Nucleic Acids Research **19**: 1349.

FELSENSTEIN J (1985) Confidence limits on phylogenies: an approach using the bootstrap. – Evolution **39**: 783-791.

GREUTER W, BARRIER FR, BURDET H-M, DEMOULIN V, FILGUERIAS TS, MCNEILL J, NICOLSON DH, SILVA PC, SKOG JE, TREHANE P, TURLAND NJ, HAWKSWORTH DL, eds. (2000) International Code of Botanical Nomenclature. (St. Louis Code). – Regnum Vegetabile **137**. Koeltz Scientific Books, Königstein.

HENRION B, LE TACON F, MARTIN F (1992) Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. – New Phytologist **122**: 289-298.

HIBBETT DS, PINE EM, LANGER E, LANGER G, DONOGHUE M.J (1997) Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. – Proceedings of National Academy of Science USA, **94**: 12002-12006.

HILLS DM, BULL JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. – Systematic Biology **42**: 182-192.

HJORTSTAM R, RYVARDEN L, WATLING R (1993) Preliminary checklist of non-Agaricoid Macromycetes in the Korup National Park, Cameroon and surrounding area. – Edinburgh Journal of Botany **50**: 105-119.

HOLMGREN PK, HOLMGREN NH, BARNETT LC (1990) Index Herbariorum. Part I. The Herbaria of the World 8th edition. Regnum Vegetabile vol. 120. New York Botanical Garden.

HUMPERT AJ, GIACHINI AJ, MUENCH EL, GASTELLANO MA, SPATAFORA JW (2001) Molecular phylogenetics of *Ramaria* and related genera: evidence from nuclear large subunit and mitochondrial small subunit rDNA sequences. – Mycologia **93**: 465-477.

KIMURA M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. – Journal Molecular Evolution **16**: 111-120.

KORNERUP A, WANSCHER JH (1978) Taschenlexikon der Farben, 1400 Farbnuancen und 600 Farbnamen. Zürich-Göttingen. 242 pp.

KRÜGER D, BINDER M, FISCHER M, KREISEL H (2001) The Lycoperdales. A molecular approach to the systematics of some gasteroid mushroom. – Mycologia **93**: 947-957.

MULLIS KB, FALOONA FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. – Methods Enzymologic **155**: 335-350.

O'DONNELL K (1993) *Fusarium* and its near relatives. In Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorph speciation in fungal systematic, pp 225-233. CAB International, Wallingford.

PERSOON CH (1801) Synopsis Methodica Fungorum, 706 p.

PONCE DE LEÓN P (1968) A revision of the family *Geastraceae.* – Fieldiana Botany **31**: 302-349.

POUZAR Z (1971) *Geastrum sessile* (Sow.) Pouzar, the correct name for *Geastrum fimbriatum* Fr. (Gasteromycetes). – Folia Geobotanica et Phytotaxonomica Bohemoslovaca. **6**: 93-97.

RAMBAUT A (2002) Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, South Parks Road, Oxford OXI 4JD, U.K.

SOTO MK, WRIGHT JE (2000) Taxonomía del género *Geastrum* (Basidiomycetes, Lycoperdales) en la provincia de Buenos Aires, Argentina. – Boletín de la Sociedad Argentina de Botánica **34**: 185-201.

SUNHEDE S (1989) *Geastraceae* (Basidiomycotina). Morphology, ecology and systematic, with special emphasis on the North European species. Synopsis Fungorum **1**. Fungiflora. Oslo.

SWOFFORD DL (2002) PAUP: Phylogenetic Analysis Using Parsimony, version 4.b10. Illinois Natural History Survey: Champion, Illinois.

WHITE TJ, BRUNS T, LEE S, TAYLOR J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications. Edited by M.A. Innis, H. Gelfand, J.S. Sninsky, and T.J. White. Academic Press, New York, pp. 315-322.

Accepted: 15.9.2004