

Short title: Trierveiler-Pereira et al.: *Cystangium* in Patagonian *Nothofagus* forests

Sequestrate fungi from Patagonian *Nothofagus* forests: *Cystangium* (Russulaceae,

Basidiomycota)

Larissa Trierveiler-Pereira¹

PPGBOT, Department of Botany, Universidade Federal do Rio Grande do Sul, Porto

Alegre, Brazil

Matthew E. Smith

Department of Plant Pathology, University of Florida, Gainesville, USA

James M. Trappe

Department of Forest Ecosystems and Society, Oregon State University, Corvallis, USA

Eduardo R. Nouhra

Instituto Multidisciplinario de Biología Vegetal (CONICET), Universidad Nacional de

Córdoba, Argentina

Abstract: Six species of *Cystangium*, a genus of sequestrate taxa related to *Russula*, were collected in Patagonia (Argentina and Chile) during autumn 2001. Two species, *C. depauperatum* Singer & A.H. Sm. and *C. nothofagi* (E. Horak) Trappe, Castellano & T. Lebel, were already known from this region, while four new species, *C. domingueziae*, *C. gamundiae*, *C. grandihyphatum* and *C. longisterigmatum*, are described, illustrated and a key to the species is provided. In addition, sequences of the ITS (rDNA) region were obtained to explore the phylogenetic relationships of our South American *Cystangium* species.

Key words: hypogeous fungi, mycorrhiza, Russulales, truffle-like fungi

INTRODUCTION

Patagonia is a region of southern South America with temperate subantarctic forests mostly dominated by southern beech (*Nothofagus* spp.). Ten native species of

Nothofagus are present in this region, according to Promis et al. (2008). This unique forest type has biogeographic ties with southern Australia and New Zealand (Gondwanan connection) and includes environments with great biodiversity and high rates of endemism (Bertonatti and Corcuera 2000). The macrofungal diversity of the Patagonian *Nothofagus* forests has not been adequately explored: this is particularly true for the truffle-forming fungi.

Ectomycorrhizal, hypogeous fungi in the Basidiomycota and Ascomycota are important components of the forest soil environment. Not only do they function as nutrient absorbing organisms for their tree hosts, these fungi also improve soil conditions (Perry et al. 1989) and interact with a variety of forest organisms (Trappe and Luoma 1992). In particular, they are an important food source for animals in ectomycorrhizal forests (Maser et al. 1978, Claridge et al 2002, Vernes et al. 2004, Claridge and Trappe 2005, Trappe et al. 2006, Vernes 2010, Kataržytė and Kutorga 2011, Schickmann et al. 2012), including those of Argentina (Perez Calvo et al. 1989, Nouhra et al. 2005). Because these fungi are integral to the function and conservation of forest communities, it is important that we document their diversity and distribution (Claridge et al. 2000a, b, 2009; North et al. 2002; Smith et al. 2002; Trappe and Bougher 2002; Meyer and North 2005; Hosaka et al. 2007; Desjardin et al. 2008; Abell et al. 2012; Bonito et al. 2013.).

Knowledge of sequestrate fungi in southern South America is currently scanty but improving. The first records from Argentina were by Spegazzini (1887, 1909): *Hymenogaster australis* (Speg.) Speg., *Tuber australe* Speg. and *Tuber argentinum* Speg. vars. *argentinum* and *pamparum* Speg., in Patagonia. *Tuber australe* since has been synonymized with *T. maculatum* Vittad. (Trappe and Cázares 2000) and *Tuber argentinum* var. *pamparum* with *Stouffera longii* (Gilkey) Kovács & Trappe (Kovács et

al. 2011). Since the time of Spegazzini several mycologists have conducted taxonomic studies of hypogeous fungi in South America (Singer 1960 a, b, 1962, 1963, 1964, 1969, 1971; Horak 1964a–d, 1973, 1979; Horak and Moser 1965, 1966; Calvelo and Lorenzo 1989; Gamundi et al. 1991; Castellano and Muchovej 1996; Giachini et al. 2000; Romero and Blumenfeld 2001; Nouhra et al. 2005, 2008; Cortez et al. 2011; Sulzbacher et al. 2010). However none of these studies entailed a systematic sampling of fungi in the vast *Nothofagus* forests.

Nouhra et al. (2012) estimated the diversity and ecological significance of hypogeous fungi within two *Nothofagus* forest types (*N. pumilio*, *N. dombeyi*) in Patagonia. Their data show that hypogeous species richness and sporocarp biomass fluctuate according to rainfall, altitude and forest type.

The hypogeous fungi associated with *Nothofagus* include numerous sequestrate relatives of the epigeous mushroom-forming genus *Russula*. These were apportioned among five genera by Singer and Smith (1960): *Cystangium* Singer & A.H. Sm., *Gymnomyces* Masee & Rodway, *Elasmomyces* Cavara, *Martellia* Mattir. and *Macowanites* Kalchbr. Morphological studies of type species of these genera led Lebel and Trappe (2000) to synonymize *Martellia* with *Gymnomyces* and *Elasmomyces* with *Macowanites* and to redefine the boundaries of *Cystangium* and *Gymnomyces*.

Molecular studies provided a different perspective in the classification of Russulales, in that hypogeous russuloid species appear scattered and nested within various clades of epigeous *Russula* (Miller et al. 2001), indicating a need to reexamine generic nomenclature within the group. For example, new species of *Macowanites* from Australasia were placed in the genus *Russula* (Lebel and Tonkin 2008).

However we here retain the genus *Cystangium* sensu Lebel & Trappe (2000), because phylogeny alone reflects only part of the evolutionary process; the other part is

the interaction of genotypes with the environment (Bruns et al. 1989, Hörandi and Stuessy 2010). The genes involved in morphological changes from epigeous forms with forcible spore charge to hypogeous forms with passive spore dispersal have not been defined or included in present phylogenies. Placing our Patagonian species in *Cystangium* does not detract from their known relationship to the genus *Russula*. Instead it is nomenclature that reflects an ecological and possibly functional trajectory fundamentally different from that of the epigeous species.

Perhaps more important, *Russula* sensu lato is a huge genus. Presently available phylogenies include only a small proportion of the total number of species in the genus analyzed by a limited number of genes that could well be inadequate to achieve stable typologies (Rokas et al. 2003). Furthermore we expect that there will be more nomenclatural changes within Russulaceae (Buyck et al. 2008). Accordingly we see no need to rush in assigning hypogeous species to the genus *Russula* until these issues are resolved.

Cystangium species are widespread and morphologically characterized by an epithelial peridiopellis. Thirty-one species have been described in the genus (Trappe et al. 2002, Lebel 2003, Trappe and Claridge 2003), only two from South America: *C. depauperatum* (Singer and Smith 1960) and *C. nothofagi* (Horak 1964a).

In this paper we describe new species of *Cystangium* from Patagonia, the first contribution of a series of taxonomic studies planned to reveal the diversity of sequestrates fungi associated with *Nothofagus* species in the region. New studies are aimed at describing new taxa and their phylogenetic affinities in the orders Russulales, Cortinariales and Pezizales.

MATERIALS AND METHODS

Field collecting.—Basidiomata were collected Apr 2001 in forests of *Nothofagus pumilio* (Poepp. & Endl.) Krasser, *N. alpina* (Poepp. & Endl.) Oerst., *N. obliqua* (Mirb.) Oerst., and *N. dombeyi* (Mirb.)

Oerst. in northern Patagonia within the Lanin and Nahuel Huapi national parks in Neuquén and Rio Negro provinces of Argentina and Region X of Chile. In most cases specimens were obtained by uncovering the basidiomata by raking the forest litter layer and upper soil or by hand for basidiomata emergent from the soil. Field notes included location and associated hosts for each species along with descriptions of fresh macroscopic characters. Specimens were cut in half along the vertical axis, photographed and dried on an electric forced-air dehydrator at ± 40 C.

Herbarium material.—In addition to our collections, types of previously described species from Fundación Miguel Lillo, Tucumán, Argentina (LIL) and Geobotanisches Institut, Eidgenössische Technische Hochschule Zürich, Switzerland (ZT), also were studied and their characters are included in the species revisions and keys. Our collections have been deposited in Museo Botánico, Universidad Nacional de Córdoba, Argentina (CORD), Herbario Fitopatológico de Valdivia, Universidad Austral de Chile (HFV), and Herbarium of the Botany and Plant Pathology Department, Oregon State University, Corvallis (OSC).

Basidioma identification and description.—Color, size, shape, type of hymenophoral structure, presence of stipe and other macro-characters were recorded for fresh collections. Color names of fresh and dry specimens are in general terms. Basidioma sections for describing microscopic characters were mounted in 5% KOH, phloxine and Melzer's reagent. Spore dimensions, including ornamentation, are based on 15 spores for each basidioma, including exceptional dimensions in parentheses. Microscopic characters were observed with a Nikon light microscope at 400–1000 \times magnification. Scanning electron microscopy (SEM) of spores was with a Zeiss LEO 1450VP. Identification of taxa was facilitated by keys and specific references (Singer and Smith 1960, Horak 1964a, Beaton et al. 1984, Castellano et al. 1989, Lebel and Trappe 2000, Lebel and Castellano 2002, Trappe et al. 2002, Lebel 2003).

Molecular protocols and analyses.—Basidioma fragments were ground with liquid nitrogen in 1.5 mL Eppendorf tubes and then DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN, Valencia, California) following the protocols of the manufacturer. The internal transcribed spacer region (ITS, including ITS1, 5.8s, ITS2) was PCR-amplified with forward primer ITS1F (Gardes and Bruns 1993) in combination with reverse primer ITS4 (White et al. 1990). If this was not successful, internal primer pairs were used (ITS1F and ITS2 for the ITS1 region and ITS3 and ITS4 for the ITS2 region). Thermo-cycler conditions during PCR followed the protocol for sporocarps used by Smith et al. (2007). Successful amplicons were electrophoresed on 1.5% agarose gels and stained with SYBR Green I (Molecular Probes,

Eugene, Oregon). Amplicons were cleaned with EXO (Exonuclease I) and SAP (shrimp alkaline phosphatase) enzymes (Werle et al. 1994) and sequenced by the University of Florida ICBR (<http://www.biotech.ufl.edu/>).

Sequences were edited with Sequencher 5 software (Gene Codes Corp., Ann Arbor, Michigan) and compared with sequences from GenBank with the BLAST-N algorithm. Sequences generated in this study were compiled with similar sequences from GenBank into an alignment of 655 nucleotides from 20 unique sequences with the software package MESQUITE (<http://mesquiteproject.org>). Automated alignment was conducted with the MUSCLE software package (Edgar 2004) followed by manual adjustments. To explore the phylogenetic relationships of our South American *Cystangium* species in relation to publicly available DNA sequences, we conducted maximum likelihood (ML) analysis with the GTR+I+G algorithmic model in the software package GARLI (Zwickl 2006) and maximum parsimony (MP) analysis with default settings in PAUP* (Swofford 2002). Consistency of the placement of these taxa was assessed based on 500 bootstrap replicates with default settings in GARLI, and for the MP analysis clade stability was assessed based on 1000 bootstrap replicates with default settings in PAUP*.

RESULTS

We were able to generate at least partial ITS rDNA barcode sequences from four of the six Patagonian *Cystangium* species treated here, but were unable to obtain sequences for the remaining two species. PCR amplification and DNA sequencing was challenging; PCR of the large subunit (LSU) rDNA failed in all cases and in all but one *Cystangium* species the ITS1 and ITS2 regions had to be amplified and sequenced separately. The reason for the molecular difficulties is not yet clear but may be due to a long ITS region (>700 nucleotides) and/or samples that were dried using heat under field conditions.

Although our dataset is incomplete, the ITS rDNA sequences suggest that the four analyzed *Cystangium* species from Patagonia are close relatives that probably diverged recently. These taxa have similar ITS rDNA sequences (ca. 97% similarity), indicating that the degree of morphological divergence is likely greater than the molecular divergence. The four species that were able to sequence are not well separated in the ITS phylogeny based on either Maximum Likelihood (FIG. 1, -ln L 2318.98) or

Maximum Parsimony analysis (268 steps, data not shown). The South American *Cystangium* species form a single monophyletic clade.

Based on comparisons of sequence homology with taxa deposited in GenBank, the Patagonian *Cystangium* species are closely related to *Nothofagus* – and Myrtaceae – associated Russulaceae from Australasia. These relatives include epigeous agaricoid forms (e.g. *Russula tawai* JX178491 and *Russula atroviridis* GU222285), hypogeous sequestrate forms (*Russula tapawera* EU019935, *Cystangium sessile* EU019948, and *C. seminudum* EU019947), environmental DNA sequences from soil (DQ388847 from Queensland, Australia) and ectomycorrhizal roots of *Nothofagus* (JX316375, JX316483).

TAXONOMY

Cystangium depauperatum Singer & A.H. Sm., Mem. Torrey Bot. Club 21:69, 1960.

FIGS. 4a–b

Etymology: Latin, *depauperatum* = reduced, possibly in reference to it being small and morphologically reduced from other *Russula*-related species.

Basidiomata 4–15 × 9–24 mm, pulvinate to subglobose; peridium margin incurved and loosely appressed to the stipe-columella or seceded a few millimeters to slightly expose the gleba. Peridial surface glabrous, white to pale yellow, often with pale yellow to pink, deep red, vinaceous or purple patches or those colors overall. Gleba white to ivory or pale yellow, sometimes with a pinkish blush, indented around the stipe-columella, with elongate to irregular locules or contorted, fused, crowded lamellae. Stipe-columella percurrent, the surface white to reddish or pale yellow, the context white to pale yellow, simple, 1.5–3 mm broad, sometimes slightly exceeding the gleba and enlarged at the base. Odor mild, flavor mild to acrid.

Peridiopellis two-layered: suprapellis thin, of repent to ascendant, hyaline hyphae, some with yellowish refractive content, 2–6 μm broad, cylindrical or clavate at the apex; pellis an epithelium 72–140 μm thick, an irregular, agglutinated, pseudoparenchyma with cells 5–67 μm broad; peridial context 30–120 μm wide, heteromerous, of loosely interwoven, hyaline hyphae 2.5–5 μm broad, and scattered nests of sphaerocysts. Stipitipellis of repent to ascendant hyaline hyphae, mostly with yellowish refractive content, overlying an epithelial subpellis. Stipe-columella context heteromerous, of simple-septate, hyaline hyphae interwoven with sphaerocysts. Hymenophoral trama 35–80 μm wide, lacking sphaerocysts, of interwoven, hyaline hyphae 2–4 μm broad; subhymenium of pseudoparenchyma, the cells 5–28 μm broad.

Basidia 25–37 \times 7–16 μm , clavate to broadly clavate, broadly cylindrical or ventricose, hyaline, mostly four-spored, the sterigmata up to 10 μm long. Basidiospores 10.5–12.5 \times 9–12 μm including ornamentation, 8–10 \times 7.5–9 μm excluding ornamentation, globose, subglobose to broadly ellipsoid, hyaline, slightly thick-walled, ornamentation strongly amyloid, of isolated, crowded spines 1.0–1.5 μm tall; hilar appendix usually conspicuous, 1–1.5 \times 1 μm , hyaline, central, cylindrical, straight or slightly curved. Hymenial cystidia arising from the subhymenium and projecting 19–49 μm beyond the palisade of basidia, scanty to abundant, 58–87 \times 7–19 μm , broadly cylindrical to fusoid, hyaline, some with yellowish refractive content, with rounded, mucronate or rostrate apices, sometimes branching at the rostrum, rostrum when present up to 35 \times 5 μm .

Distribution, habitat and season: Widely distributed in *Nothofagus* forests of Patagonia of both Argentina and Chile; March and April.

Examined specimens: CHILE. LLANQUIHUÉ: Lake Todos los Santos, above Peulla. 20 Mar 1959, *R. Singer M1921* (LIL, HOLOTYPE); 52 Km north of Valdivia, 40°7'403"S, 72°51'945"W. 28 Apr 2001, *M. Castellano, Trappe 26283* (CORD, HFV); ibidem. 28 Apr 2001, *M. Castellano, Trappe 26292*

(CORD 26292); south of Valdivia, 39°52'037"S, 73°9'988"W. 28 Apr 2001, *L. Domínguez*, *Trappe* 26306 (CORD, HFV); North exit of Valdivia, 39°42'744"S 73°6'388"W. 28 Apr 2001, *R. Godoy*, *Trappe* 26309 (CORD, HFV); *ibidem*. 28 Apr 2001, *L. Domínguez*, *Trappe* 26315 (CORD, HFV); *ibidem*. 28 Apr 2001, *Trappe* 26323 (CORD, HFV). ARGENTINA, NEUQUÉN: 40°42'414"S, 71°42'084"W. 24 Apr 2001, *Trappe* 26184 (CORD, OSC 145985); *ibidem*. 24 Apr 2001, *Trappe* 26185 (CORD, OSC 145986); *ibidem*. 24 Apr 2001, *L. Domínguez*, *Trappe* 26192 (CORD); *ibidem*. 24 Apr 2001, *L. Domínguez*, *Trappe* 26193 (CORD, OSC 145987); NEUQUÉN: Parque Nacional Nahuel Huapi, 40°43'298"S, 71°47'915"W. 29 Apr 2001, *E. Cázares*, *Trappe* 26332 (CORD, OSC 145988).

Commentary: Cystangium depauperatum is the first South American species of the genus to be described (Singer and Smith 1960) and the most frequent species among our *Cystangium* collections. It is characterized by pulvinate basidiomata, peridium surface with pale yellow to pink, red, vinaceous or purple patches or sometimes overall; stipe-columella percurrent, gleba usually sublamellate and exposed near the stipe, echinulate basidiospores and the presence of hymenial cystidia. Its original description states that hymenial cystidia are absent or inconspicuous; however, our analysis of the holotype revealed that cystidia are rare to abundant, depending on the glebal zone examined. Also, the wide range of peridial color is more striking than reported by Singer and Smith (1960). The shape and size of the basidiospore ornamentation resemble those of *C. nothofagi* and *C. grandihyphatum* (see below).

Cystangium domingueziae Nouhra & Trierv.-Per., sp. nov.

FIGS. 2a–d, 5a

MycoBank MB805941; GenBank KF819811

Holotypus: CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S, 73°6'388"W. 28 Apr 2001, *C.*

Barroetaveña, *Trappe* 26311 (CORD).

Etymology: In honor of Argentine mycologist, Dr Laura S. Domínguez.

Basidiomata 8–11 × 8–11 mm, globose to pulvinate; peridium margin appressed to the stipe. Peridial surface glabrous, yellowish white with some brown patches. Gleba white, with locules up to 1 mm broad, often smaller near the apex. Stipe-columella

percurrent, simple, not exceeding the gleba, 1.5–2 mm broad. Odor mild, flavor not recorded. Latex absent.

Peridiopellis two-layered, a thin suprapellis of repent to ascendant, hyaline hyphae 2.5–5 μm broad with oily, refractive content overlying an epithelial subpellis 45–190 μm thick, of irregular, agglutinated, pseudoparenchymatous hyphae 5–36 μm . Peridial context 130–285 μm thick, heteromerous, of tightly interwoven, hyaline hyphae, 2–4 μm broad, simple-septate, and scattered nests of sphaerocysts. Stipitipellis of ascendant to repent hyaline hyphae 4–10 μm broad, with refractive content, the apices rounded or acute. Stipe-columella context heteromerous, of tightly interwoven, hyaline, tortuous hyphae, simple-septate, 1–5 μm broad, sphaerocysts present. Hymenophoral trama 17–45 μm wide, lacking sphaerocysts, of interwoven, hyaline hyphae 2–4.5 μm broad, simple-septate; subhymenium of isodiametric cells 6.5–12 μm broad.

Basidia 22–35 \times 10–12 μm , clavate, tapering, hyaline, four-spored, with sterigmata 1–6 μm long. Basidiospores 7.5–10.5 \times 7–10 μm including ornamentation, 7–10 \times 6.5–9 μm excluding ornamentation, globose to subglobose or broadly ellipsoid, hyaline, slightly thick-walled; ornamentation amyloid, of isolated or connected short rods, usually 0.5 μm high; hilar appendix usually conspicuous, 1–1.5 \times 0.5–1 μm , hyaline, central, conical. Hymenial cystidia abundant, 61–90 \times 13–22 μm , broadly cylindrical, hyaline, usually short-rostrate or more rarely with rounded apices, the rostrum 2–10.5 \times 1–2 μm , arising from the subhymenium, projecting 22–49 μm beyond the palisade of basidia.

Distribution, habitat and season: Known only from the type locality north of Valdivia, Chile; hypogeous in a *Nothofagus obliqua* forest; April.

Examined specimens. CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S, 73°6'388"W. 28 Apr 2001, C. Barroetaveña, Trappe 26311 (CORD).

Commentary: This species is characterized by the combination of heteromerous peridial context, basidiospores ornamented with short rods and mostly short-rostrate hymenial cystidia. It can be distinguished from *Cystangium longisterigmatum* (see below) due to the presence of a stipe-columella, short sterigmata and heteromerous peridial context. The species also resembles the Australian *C. pisiglarea* T. Lebel, which has much smaller basidia, 15–20 µm long.

Cystangium gamundiae Nouhra & Trierv.-Per., sp. nov.

FIGS. 2e–h, 5b

MycoBank MB805942; GenBank KF819810

Holotypus: CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S, 73°6'388"W. 28 Apr 2001, *E. Cázares*, *Trappe 26316* (CORD, ISOTYPES in OSC, VALD).

Etymology: In honor of Argentine mycologist, Dr Irma J. Gamundí.

Basidiomata 5–15 × 6–18 mm, pulvinate to subglobose; peridium margin appressed to the stipe, almost entirely enclosing the gleba. Peridial surface glabrous, white with pink, pale yellow, vinaceous, or brown patches. Gleba white, sublamellate to loculate, locules round to elongated, up to 1 mm long. Stipe absent. Columella white, percurrent, simple, not exceeding the gleba, 1.5–2 mm broad. Odor and flavor mild.

Peridiopellis two-layered, a suprapellis of scarce to abundant, repent, ascendant or emergent, hyaline hyphae 3–9.5 µm broad, with yellowish refractive content and cylindrical, clavate or rostrate apices; underlying epithelial subpellis 40–132 µm thick, of irregular, agglutinated, pseudoparenchymatous hyphae 4–51 µm broad. Peridial context 24–160 µm thick, heteromerous, of loosely interwoven, hyaline hyphae 2–6 µm broad, simple-septate; and scattered nests of sphaerocysts, sometimes difficult to observe. Stipitipellis of ascendant to repent hyaline hyphae with refractive content, 5–9 µm broad, the apices clavate. Stipe-columella context heteromerous, of interwoven, hyaline hyphae 2–6 µm broad, simple-septate, and scattered nests of sphaerocysts.

Hymenophoral trama 12–54 µm thick, lacking sphaerocysts, of interwoven, hyaline hyphae 2–5 µm broad, simple-septate; subhymenium of inflated hyphae 3–18 µm broad.

Basidia 22–40 × 11–16 µm, oblong-clavate to broadly cylindrical or ventricose, hyaline, 2–(3) spored, the sterigmata up to 8 µm long. Basidiospores 14.5–19.5 × 14–19 µm including ornamentation, (9–)10.5–14 × 10–14 µm excluding ornamentation, globose to subglobose or broadly ellipsoid, hyaline, slightly thick-walled, the ornamentation strongly amyloid, of robust, isolated crowded spines 1.5–4 µm tall, some curved at the apex; hilar appendix usually conspicuous, 1–3.5 × 1 µm, hyaline, central, conical to cylindrical, straight or slightly curved. Hymenial cystidia frequent to abundant, 51–79 × 7–11 µm, broadly cylindrical to cylindrical or fusoid, hyaline, with apices rounded or long-rostrate, rostrum when present 11–26 × 2–2.5 µm, arising from the subhymenium, projecting 20–46 µm beyond the palisade of basidia.

Distribution, habitat and season: Known only from the type locality north of Valdivia, Chile; hypogeous in a *Nothofagus obliqua* forest; April.

Examined specimens: CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S, 73°6'388"W. 28 Apr 2001, E. Cázares, *Trappe 26316* (CORD, ISOTYPES in OSC, VALD); ibidem. 28 Apr 2001, C. Barroetaveña, *Trappe 26283A* (CORD); 52 km north of Valdivia, 40°7'403"S, 72°51'945"W. 28 Apr 2001, C. Barroetaveña, *Trappe 26278* (CORD); ibidem. 28 Apr 2001, M. Castellano, *Trappe 26291* (CORD).

Commentary: This species is characterized by its two-spored basidia and large basidiospores ornamented with robust spines that differentiate it from *C. depauperatum* and *C. nothofagi*. *Cystangium bisporum* T. Lebel, described from *Nothofagus* stands in southeastern Australia and Tasmania, closely resembles *C. gamundiae* in several ways. However *C. bisporum* lacks the pink to vinaceous hues common on *C. gamundiae*, has much larger cells in the suprapellis and subhymenia and much longer and broader hymenial cystidia. Other *Cystangium* spp. with basidiospores with elongated spines (1–

3 µm) include *C. megasporum* (Rodway) T. Lebel & Castellano and *C. seminudum* (Masse & Rodway) T. Lebel & Castellano (Lebel and Castellano 2002, Lebel 2003). In *C. megasporum*, the cystidia are absent and basidiospores are ornamented with warts, rods and wedges, instead of robust spines. *Cystangium seminudum* has smaller basidiospores with shorter ornamentation (1–2.5 µm tall) and lacks a stipe-columella.

Cystangium grandihyphatum Nouhra & Trierv.-Per., sp. nov. FIGS. 3a–e, 5c

MycoBank MB805943

Holotypus: CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S 73°6'388"W. 28 Apr 2001, M. Castellano, Trappe 26325 (CORD).

Etymology: Latin, *grandihyphatum* = with large hyphae, in reference to the exceptionally broad hyphae of the peridium and context.

Basidiomata 10–14 mm broad, subglobose to irregular, the peridium entirely covering the gleba. Peridium irregular, glabrous, yellowish white with brown stains. Gleba white, often with brownish stains, with rounded to elongated locules 0.2–1 mm broad. Columella white, not percurrent and not exceeding the gleba, simple, vestigial or 1.5–2 mm broad. Odor and flavor not recorded.

Peridiopellis two-layered, a thin suprapellis of scattered, hyaline to yellowish, ascendant to repent hyphae, rarely with refractive content, 2.5–5 µm broad, the rounded or acute apices overlying an epithelial pellis 40–97 µm thick of irregular, agglutinated cells 6–57 µm broad. Peridial context 75–180 µm thick, lacking sphaerocysts, of thick, interwoven, tortuous, branched, hyaline hyphae 5–12 µm broad, some of these interwoven with the basal pseudoparenchymatous hyphae of the pellis. Hymenophoral trama 30–113 µm thick, heteromerous, of interwoven, hyaline hyphae 2–6 µm broad and scattered large hyphae similar to those of the peridium context; sphaerocysts present; subhymenium of isodiametric, hyaline to yellowish cells 8–25 µm broad.

Basidia 27–35 × 9.5–12 µm, clavate to broadly cylindrical, hyaline, some with yellowish refractive content, four-spored; sterigmata 3–5(–7) µm long. Basidiospores 10–12.5 × 9–12 µm including ornamentation, 8–10.5 × 7–10 µm excluding ornamentation, globose, subglobose to broadly ellipsoid, hyaline, slightly thick-walled, ornamentation strongly amyloid, of isolated, crowded spines 1–2.5 µm tall, some curved at the apex; hilar appendix usually conspicuous, 1–1.5 × 1 µm, hyaline, central, cylindrical. Hymenial cystidia frequent, 69–87 × 18–32 µm, ventricose, hyaline, the apex rounded or acute, rarely with a small rostrum, 2–10 × 1 µm, arising from the subhymenium, projecting 19–42 µm beyond the palisade of basidia.

Distribution, habitat and season: Known only from the type locality north of Valdivia, Chile; hypogeous in a *Nothofagus obliqua* forest; April.

Examined specimens: CHILE. REGIÓN X: north exit of Valdivia, 39°42'744"S 73°6'388"W, 28 Apr 2001, M. Castellano, Trappe 26325 (CORD).

Commentary: *Cystangium grandihyphatum* is characterized by subglobose to irregular basidiomata, columella not percurrent, a loculate gleba and peridial context with large hyphae, and basidiospores ornamented with crowded spines. Hymenial cystidia are frequent and usually not rostrate. Basidiospores of *C. grandihyphatum* are somewhat similar to those of *C. gamundiae*, but spines are smaller (1–2.5 µm tall). Moreover *C. gamundiae* has two-spored basidia and lacks large hyphae in the peridium context.

Cystangium longisterigmatum Nouhra & Trierv.-Per., sp. nov. FIGS. 3f–i, 5d

MycoBank MB805944; GenBank KF819808

Holotypus: CHILE. OSORNO: Parque Nacional Puyehue, 40°46'546"S, 72°12'174"W. 27 Apr 2001, M. Castellano, Trappe 26265 (CORD).

Etymology: Latin, *longisterigmata* = with long sterigmata, named for its sterigmata, which are unusually long for this genus.

Basidiomata 3–5 × 5–10 mm, depressed-globose to hemispherical; peridium margin covering the gleba. Peridial surface irregular, glabrous, white. Gleba white to brownish, loculate, locules labyrinthiform to subglobose, locules small, rounded, up to 0.5 mm, some elongated, up to 1 mm. Stipe absent. Columella absent or vestigial, when present a narrow strand ≤ 1 mm thick, brownish gray, percurrent, simple, connected to a basal, sterile pad. Odor and flavor not recorded. Latex absent, but scanty, watery exudates were observed.

Peridiopellis two-layered, a thin suprapellis of repent, hyaline hyphae, with oily, refractive content, 3–8 μm broad, overlying an epithelial subpellis 42–90 μm thick, of irregular, agglutinated cells 8–53 μm broad. Peridial context 60–135 μm wide, lacking sphaerocysts, of interwoven, branched, hyaline hyphae 2–7 μm broad. Hymenophoral trama 16–40 μm broad, of interwoven, hyaline hyphae 2–5 μm broad, sphaerocysts absent; subhymenium of isodiametric hyphae 2.5–10 μm broad.

Basidia 26–42 × 8.0–11 μm, clavate, hyaline, four-spored, the sterigmata 6.0–15 μm long. Basidiospores (7.5–)8.5–10 × 7.5–9 μm including ornamentation, 7–9 × 7–8 μm excluding ornamentation, subglobose to broadly ellipsoid, hyaline, slightly thick-walled; ornamentation strongly amyloid, of isolated or connected short spines and rods up to 0.5 μm tall, some connected by irregular ridges; hilar appendix usually conspicuous, 1–2 × 1–1.5 μm, hyaline, central, conical. Hymenial cystidia common except rare in locules near the peridium, 53–90 × 9.5–13 μm (including rostrum), hyaline, with oily refractive content, ventricose to lanceolate, long-rostrate, the rostrum 11–24.5 × 2.0–5.0 μm, some young cystidia not rostrate, arising from the subhymenium, more rarely from the hymenophoral trama, projecting 7–39 μm beyond the palisade of basidia.

Distribution, habitat and season: Known only from the type locality in Parque Nacional Puyehue, Chile; hypogeous in a *Nothofagus dombeyi* forest; April.

Examined specimens: CHILE. OSORNO: Parque Nacional Puyehue, 40°46'546"S, 72°12'174"W. 27 Apr 2001, *M. Castellano, Trappe 26265* (CORD).

Commentary: The most striking characteristics of *Cystangium longisterigmata* are the basidiospores ornamented with small spines and rods and the unusually long sterigmata. Other important features are: basidiomata small, the columella percurrent, much reduced, connected to a basal pad, peridial context lacking sphaerocysts, hymenial cystidia frequent, long-rostrate.

Cystangium nothofagi (E. Horak) Trappe, Castellano & T. Lebel, *Mycotaxon* 81: 198, 2002.

FIGS. 4c–d

≡ *Elasmomyces nothofagi* E. Horak, *Sydowia* 17:211, 1964 (basionym).

Etymology: Latin, *nothofagi* = of *Nothofagus*.

Basidiomata 10–11 × 12–16 mm, pulvinate to subglobose; peridium margin convoluted but not or only slightly seceded from the stipe, covering the gleba. Peridial surface glabrous, completely white or white with some pale yellowish brown patches. Gleba white, with minute, round, elongated to irregular locules up to 0.5 mm broad. Stipe-columella white, percurrent, cylindrical to slightly tapered toward the base, 2 mm broad, exceeding or not the gleba, fragile, easily separable. Odor mild, flavor not recorded.

Peridiopellis two-layered: suprapellis of repent to ascendant, hyaline hyphae 2–4 µm broad, some with yellowish refractive content, overlying an epithelial subpellis 49–87 µm thick, of irregular, agglutinated, pseudoparenchyma with cells 6–37 µm broad. Peridial context 100–225 µm wide, compact, heteromerous, of interwoven, hyaline hyphae 2.5–5 µm broad, simple-septate and slightly thick-walled, and a few scattered nests of sphaerocysts, usually near the subhymenium of the outermost locules.

Endocystidia absent. Stipitipellis a turf of ascendant hyaline hyphae, mostly with yellowish refractive content overlying an epithelial subpellis. Stipe-columella context heteromerous, of interwoven, simple-septate, hyaline hyphae, and groups of sphaerocysts. Hymenophoral trama 11–30 μm wide, lacking sphaerocysts, of interwoven, hyaline hyphae 2–4.5 μm broad, simple-septate, sphaerocysts absent; subhymenium of inflated hyphae 6–14 μm broad.

Basidia 28–36.5 \times 8–14 μm , broadly cylindrical, hyaline, some with yellowish refractive content, mostly four-spored, sterigmata up to 6 μm long. Basidiospores 10–13.5 \times 9.5–13 μm including ornamentation 8–11 \times 8–11 μm excluding ornamentation, globose to subglobose or broadly ellipsoid, hyaline, slightly thick-walled; ornamentation amyloid, of isolated spines 1–1.5 μm tall; hilar appendix usually conspicuous, 1–2 \times 1 μm , hyaline, central, conical to cylindrical, straight or slightly curved. Hymenial cystidia absent.

Distribution, habitat and season: Andes of Chile and Argentina, hypogeous under *Nothofagus pumilio*, *N. betuloides*, *N. dombeyi*, *N. antartica*, *Saxegothaea conspicua* and *Chusquea montana*; April.

Examined specimens: CHILE, OSORNO: Vulcan Antillancan. 15 Apr 1963, *E. Horak* (ZT, HOLOTYPE). ARGENTINA, NEUQUÉN: near the Chilean border, 40°40'672"S, 71°44'864"W. 29 Apr 2001, *M. Castellano*, *Trappe 36342* (CORD); ibidem. 29 Apr 2001, *L. Domínguez*, *Trappe 26350* (CORD). ITS GenBank: KF819809 (from 26350).

Commentary: *Cystangium nothofagi* is characterized by pulvinate basidiomata with a percurrent stipe-columella, finely loculate gleba exposed near the stipe, echinulate basidiospores, and a lack of hymenial cystidia. This species originally was described as *Elasmomyces* (Horak 1964) due to the lack of sphaerocysts. However the material examined has scattered nests of sphaerocysts in the peridial context, near the subhymenial layer of the outermost locules. In any event Lebel and Trappe (2000)

demonstrated that the boundaries between *Elasmomyces* and other sequestrate russuloid genera were not sustainable and allocated the species assigned to it to *Cystangium* or *Gymnomyces*, depending on peridial structure.

KEY TO *CYSTANGIUM* SPECIES FROM PANTAGONIAN *NOTHOSFAGUS* FORESTS

- 1a. Basidiospores ornamented with isolated or confluent rods or spines up to 0.5 µm tall; peridium white to yellowish white, some with brown patches 2
- 1b. Basidiospores ornamented with isolated spines 1–4 µm tall; peridium white to yellowish white, some with brown, pink, red or vinaceous patches or those colors overall..... 3
- 2a. Peridial context with scattered nests of sphaerocysts; sterigmata up to 10 µm long; hymenial cystidia 13–22 µm broad, short-rostrate; columella percurrent, up to 2 mm broad.....*C. domingueziae*
- 2b. Peridial context lacking sphaerocysts; sterigmata 6–15 µm long; hymenial cystidia 9.5–13 µm broad, long-rostrate; columella absent or < 1 mm broad*C. longisterigmatum*
- 3a. Basidiospore ornamentation up to 4 µm tall, basidia 2(–3)-spored; basidiospores 14.5–19.5 × 14–19 µm (including ornamentation) *C. gamundiae*
- 3b. Basidiospore ornamentation 1–2.5 µm tall, basidia mainly four-spored, basidiospores 9–13.5 × 9–12.5 µm (including ornamentation) 4
- 4a. Gleba distinctly loculate, peridial context with hyphae 5–12 µm broad
..... *C. grandihyphatum*
- 4b. Gleba loculate, sublamellate or lamellate, peridial context with hyphae 2.5–5 µm broad 5
- 5a. Gleba sublamellate to lamellate, cystidia rare to abundant; peridium white to pale yellow, often with yellow, to pink or vinaceous patches or those colors overall
.....*C. depauperatum*
- 5b. Gleba loculate, cystidia absent; peridium white with some pale yellowish brown patches ..*C. nothofagi*

DISCUSSION

Species of *Cystangium* are micromorphologically distinguished from other sequestrate genera related to *Russula* by the presence of a peridial epithelium. Important features to distinguish species within the genus are the type of gleba (locular, sublamellate or lamellate), presence or absence of a stipe-columella and its characteristics, presence of hymenial cystidia, basidial features, basidiospore size and ornamentation.

The type of gleba may vary considerably in basidiomata of the same species but in some cases is constant, as in *C. nothofagi* (always minutely loculate). A stipe-columella is often present but is reduced to a narrow strand in *C. longisterigmata*. The presence of hymenial cystidia is crucial to separate *C. depauperatum* from *C. nothofagi*. Singer and Smith (1960) described *C. depauperatum* with rare cystidia, but in the holotype we found that the amount of cystidia varies considerably from one place in the gleba to another. Because of this variation, it is important to examine different regions of the gleba.

Important basidium features include size and shape, number of spores formed, and length of sterigmata. *Cystangium* species may have long or short basidia (up to 42 μm in *C. longisterigmatum* and as short as 20 μm in *C. pisiglarea* from Australia) and in sterigma length (up to 15 μm in *C. longisterigmatum*). The number of spores formed on each basidium also distinguishes species; for example the Australian *C. bisporum* consistently forms only two spores per basidium.

The basidiospores of *Cystangium* species from Patagonia have only two kinds of ornamentation: tall, isolated spines as in *C. gamundiae* or short, connected spines or columns as in *C. domingueziae*. However other kinds of spore ornamentation are found in species of the genus, for example finely verrucose (*C. sparsum* T. Lebel), narrow wedges and rods connected by low ridges (*C. theodori* T. Lebel), and isolated warts (*C. phymatodisporum* G.W. Beaton, Pegler & T.W.K. Young).

The four analyzed *Cystangium* species from Patagonia are close relatives that probably diverged relatively recently. Their anatomical and morphological characters do not suggest conspecificity. In addition studies have shown that ITS analysis often fails to provide lower level resolution among close related species in various Basidiomycota genera (Vellinga 2003, Peintner et al. 2004, Wang et al. 2004, Frøslev et al. 2005) and

that underestimates the number of species predicted by other nuclear loci (Gazis et al. 2011), especially in rapidly evolving or highly diverse genera or species complexes (Lacap et al. 2003, Hoffman and Arnold 2008)

Based on comparisons of sequence homology with taxa deposited in GenBank, the Patagonian *Cystangium* species are closely related to *Nothofagus*—and Myrtaceae—associated Russulaceae from Australasia. Of interest, despite the putative separation of the Australasian and Patagonian Russulaceae taxa since the Oligocene (ca. 32 million y ago) (Markgraf et al. 1996), the ITS regions within this group are relatively conserved and differ only by ca. 2–5%. More complete field sampling in combination with further morphological and molecular analyses of the sequestrate Russulaceae are needed to elucidate the evolutionary relationships within this ecologically important group of ectomycorrhizal fungi. However, considering that *Cystangium* is a diverse and widespread genus, with species in Patagonia, Australasia, U.S.A, México and Africa (Lebel et al. 2003), and based on the diversity of anatomical characters of the species we think that members of *Cystangium* have probably evolved independently in each region (Lebel and Tonkin, 2007).

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LEGENDS

FIG. 1. Maximum-likelihood phylogeny of Patagonian *Cystangium* species and related sequences in GenBank based on internal transcribed spacer (ITS) ribosomal DNA. Note weak bootstrap support throughout the tree showing low differentiation in the ITS region among taxa in this group whether they are from southern South America or Australasia. All nodes collapse in the majority-rule consensus tree except for the node to *Russula vinosa*, the only northern hemisphere taxon included in the analysis.

FIG. 2. *Cystangium domingueziae* (a-d) and *C. gamundiae* (e-h): a, e. basidiomata; b, f. basidia; c, g. basidiospores; d, h. hymenial cystidia. Bars: 10 μ m.

FIG. 3. *Cystangium grandihyphatum* (a-e) and *C. longisterigmatum* (f-i): a, f. basidiomata; b, g. basidia; c, h. basidiospores; d, i. hymenial cystidia; e. hyphae from the peridium context (arrow). Bars: 10 μ m.

FIG. 4. Scanning electron micrographs of basidiospores: a, b. *Cystangium depauperatum* (holotype); c, d. *C. nothofagi* (Trappe 26342). Bars = 2 μ m.

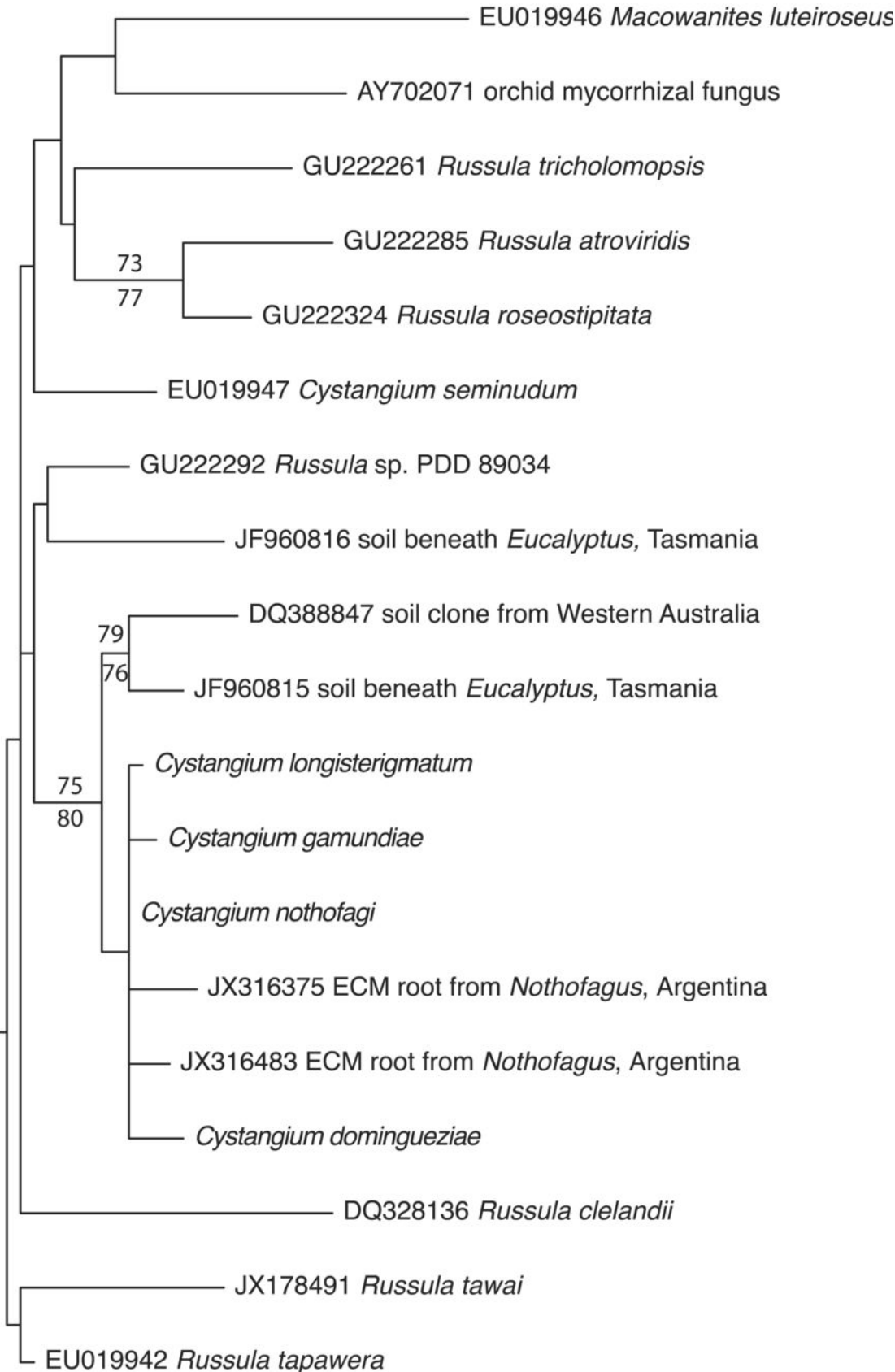
FIG. 5. Scanning-electron micrographs of basidiospores: a. *Cystangium domingueziae*; b. *C. gamundi*; c. *C. grandihyphatum*; d. *C. longisterigmatum*. Bars = 2 μ m.

FOOTNOTES

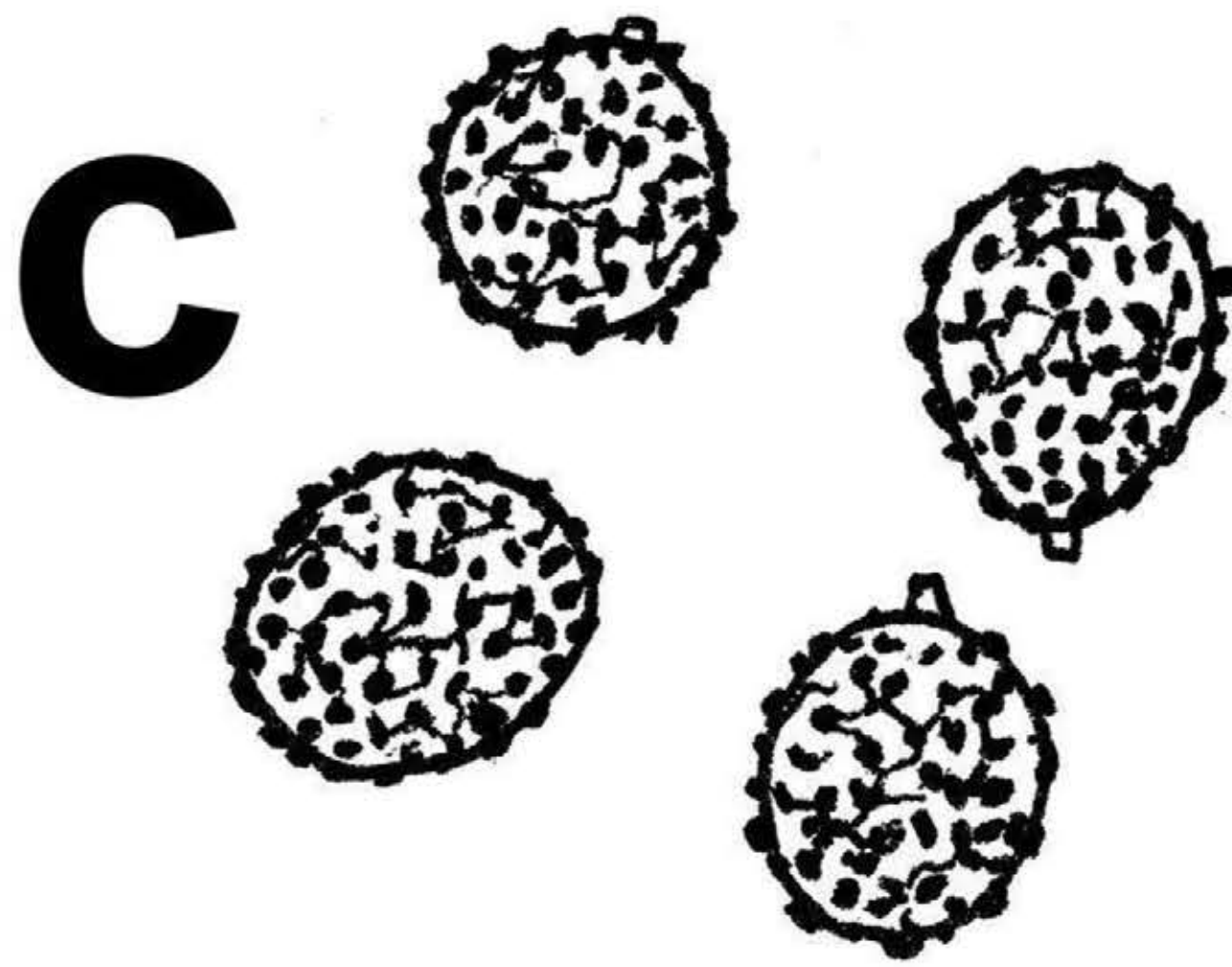
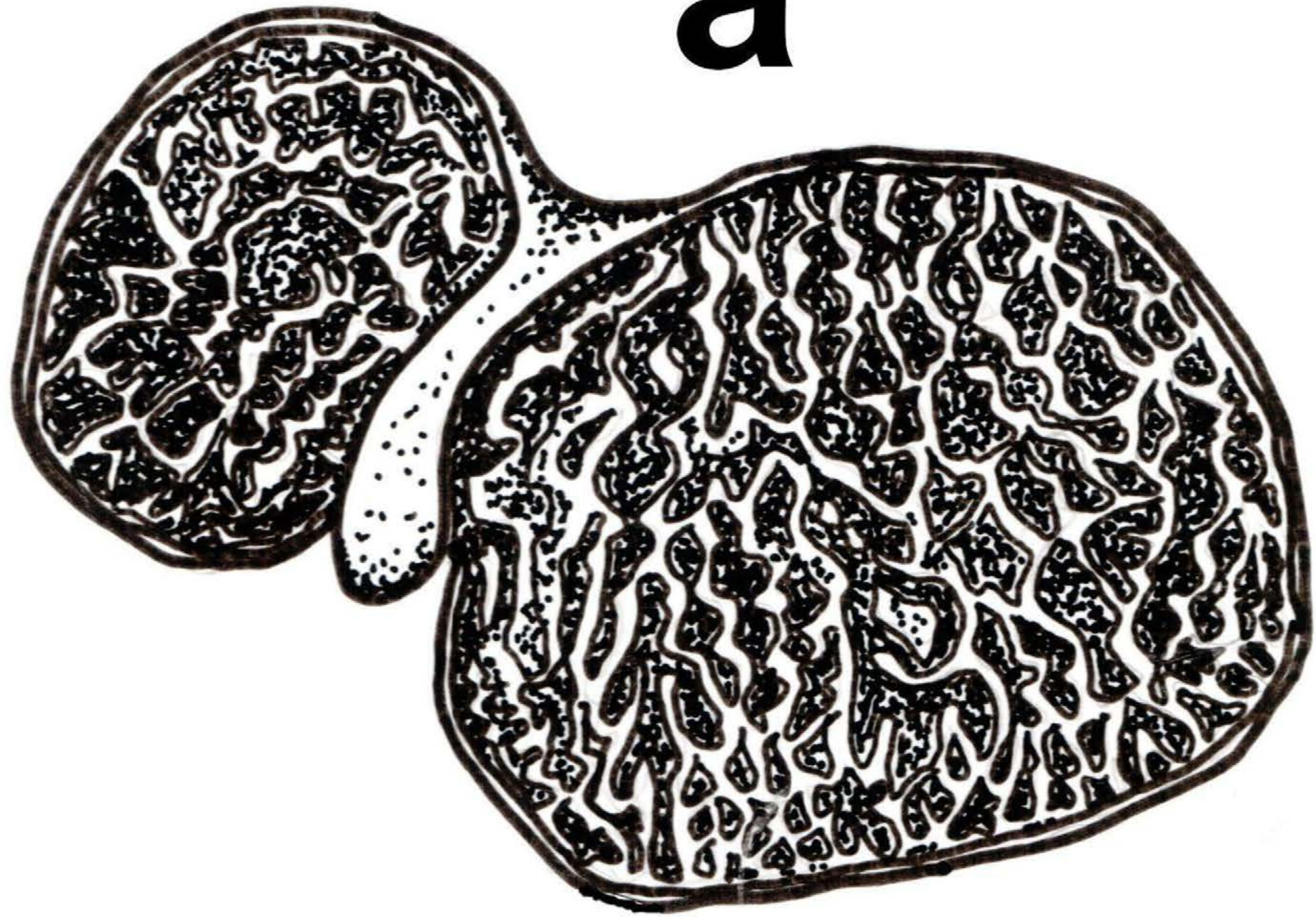
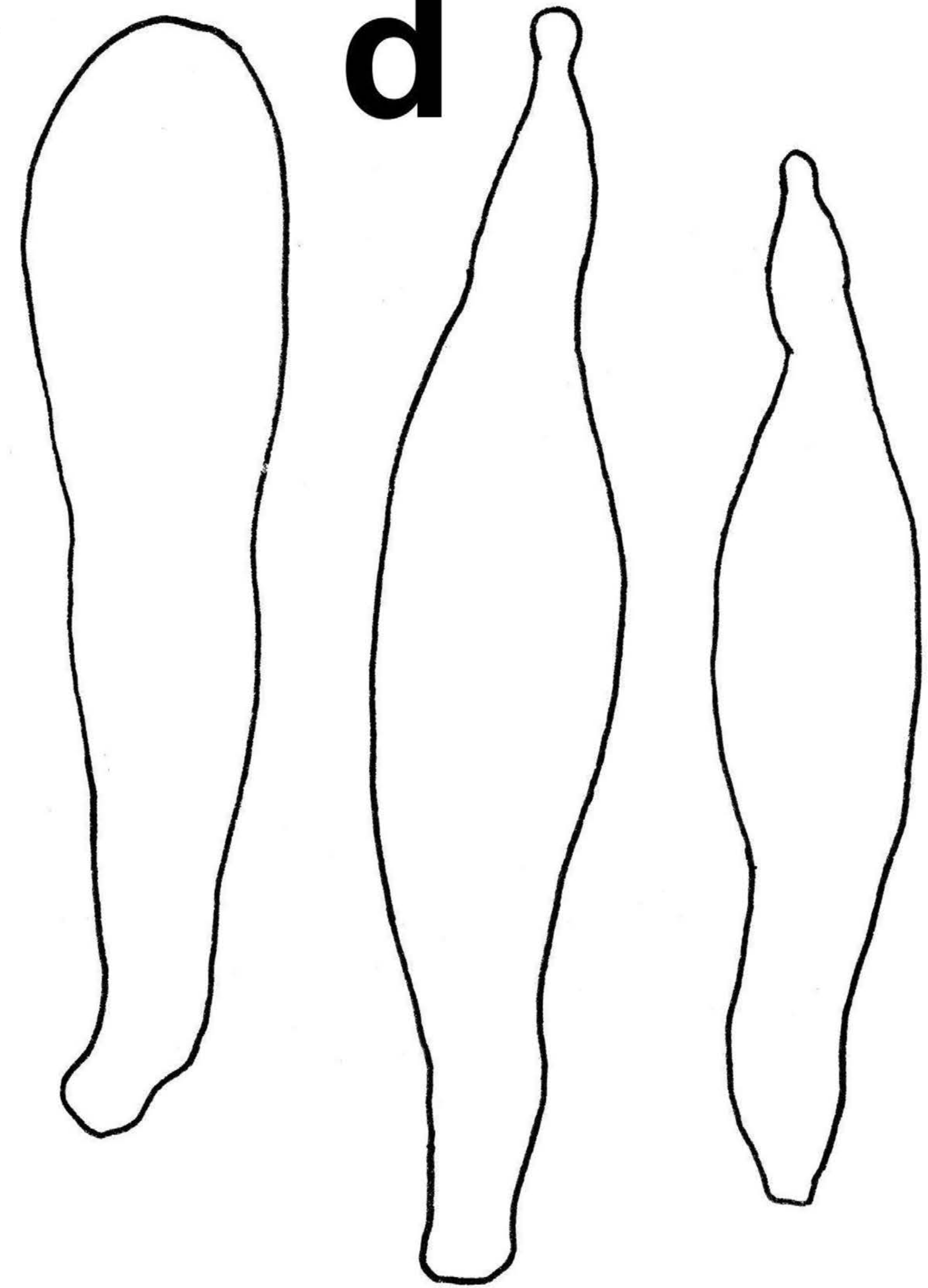
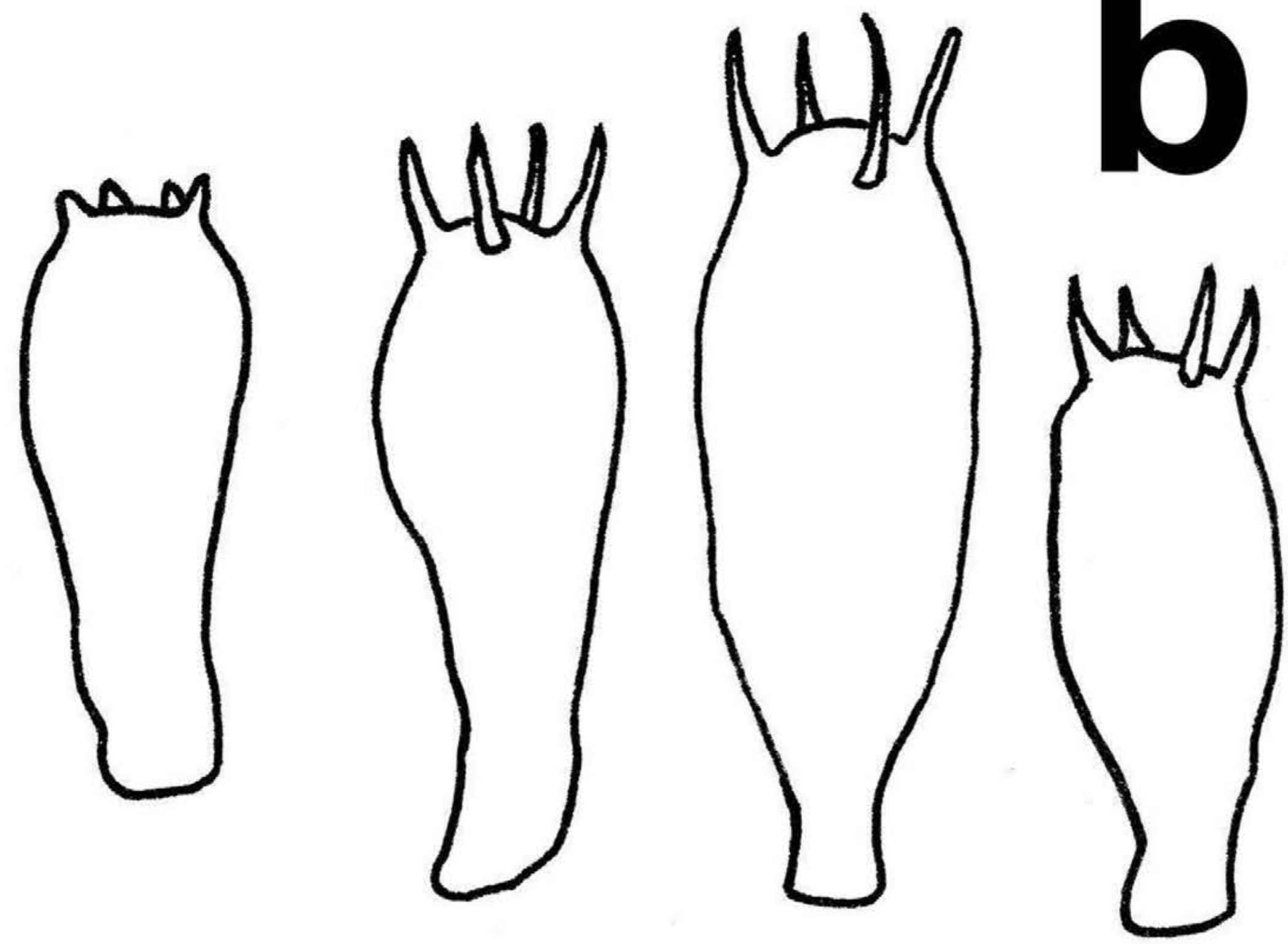
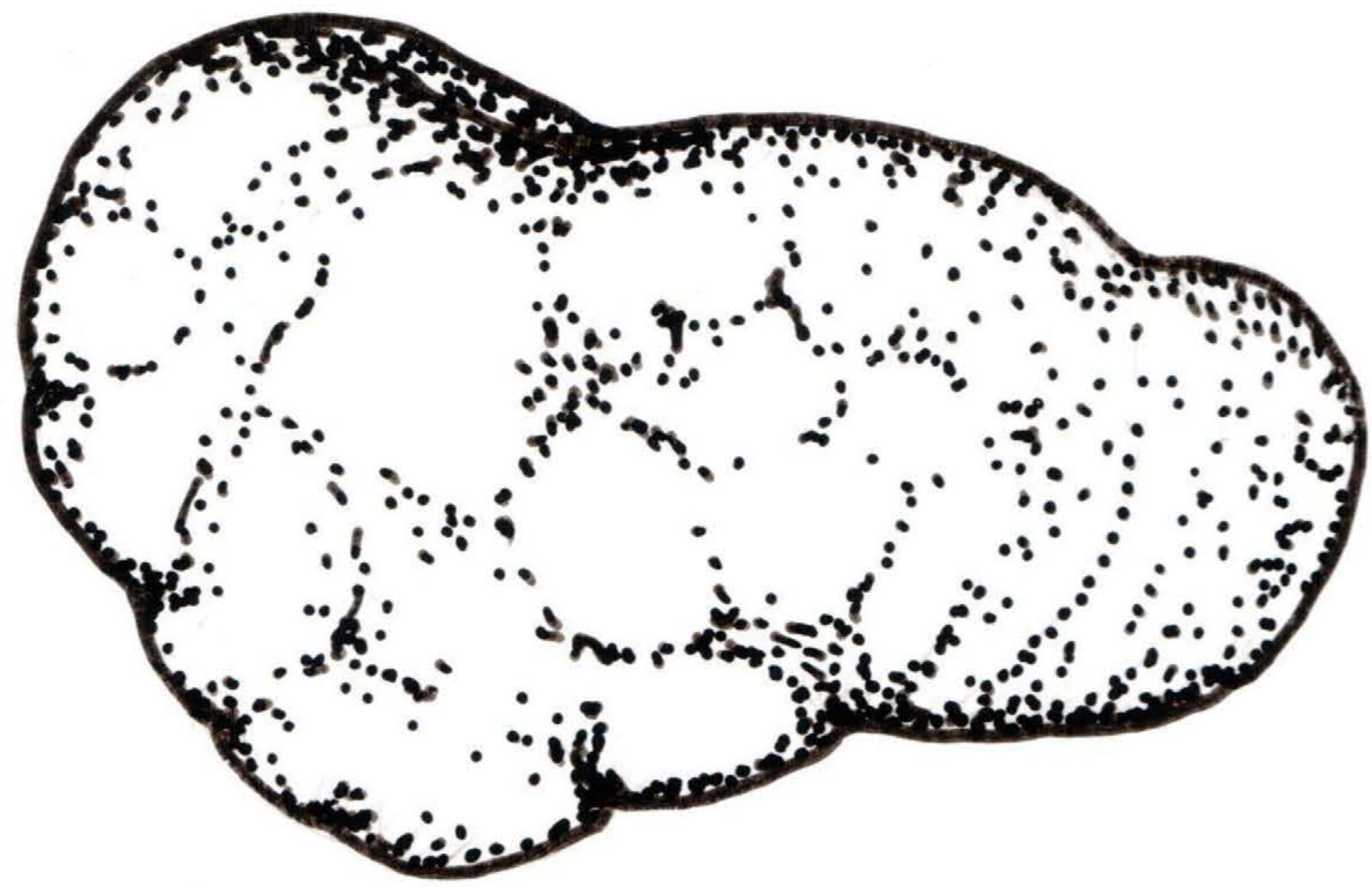
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¹Corresponding author. E-mail: Lt_pereira@yahoo.com.br

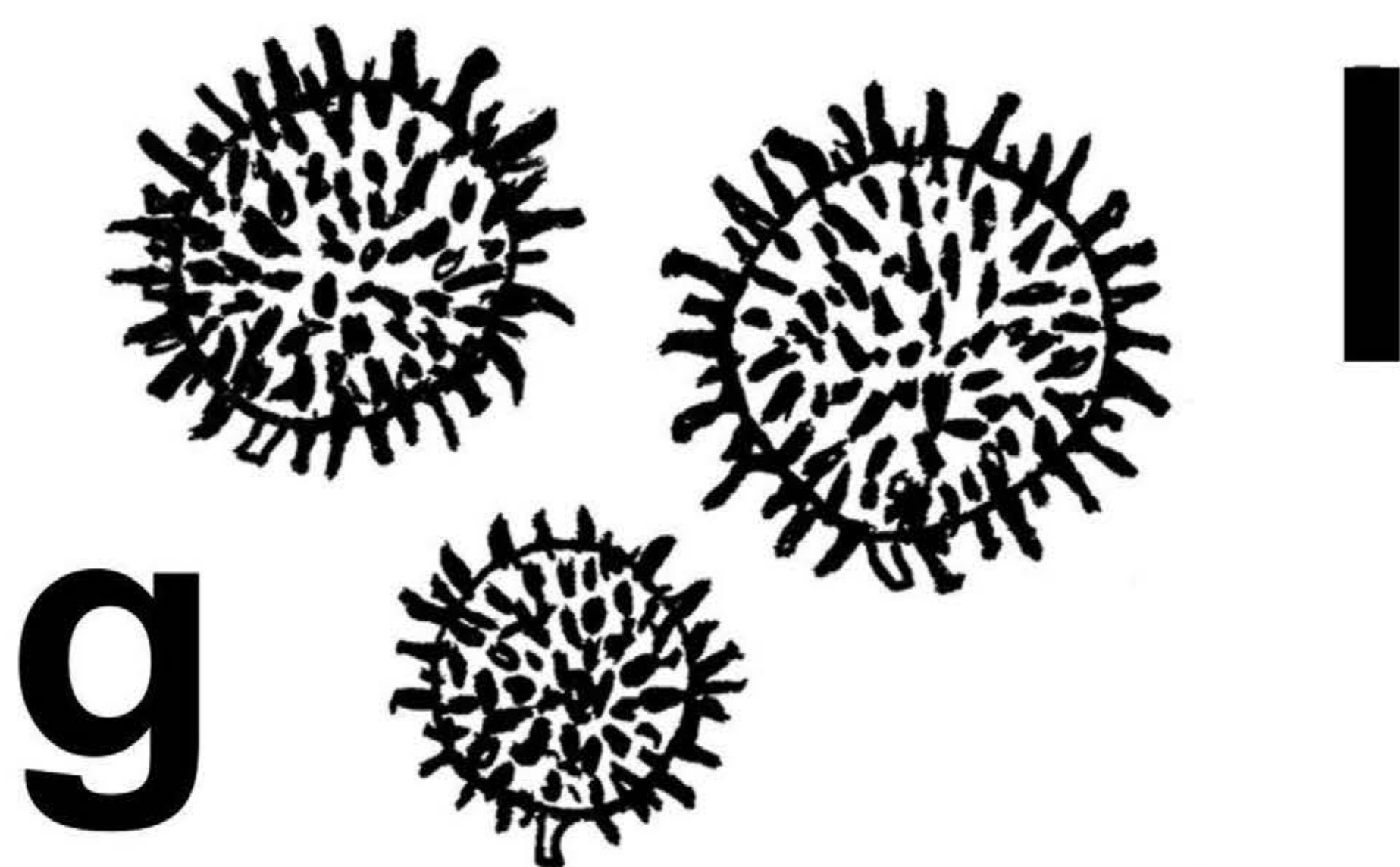
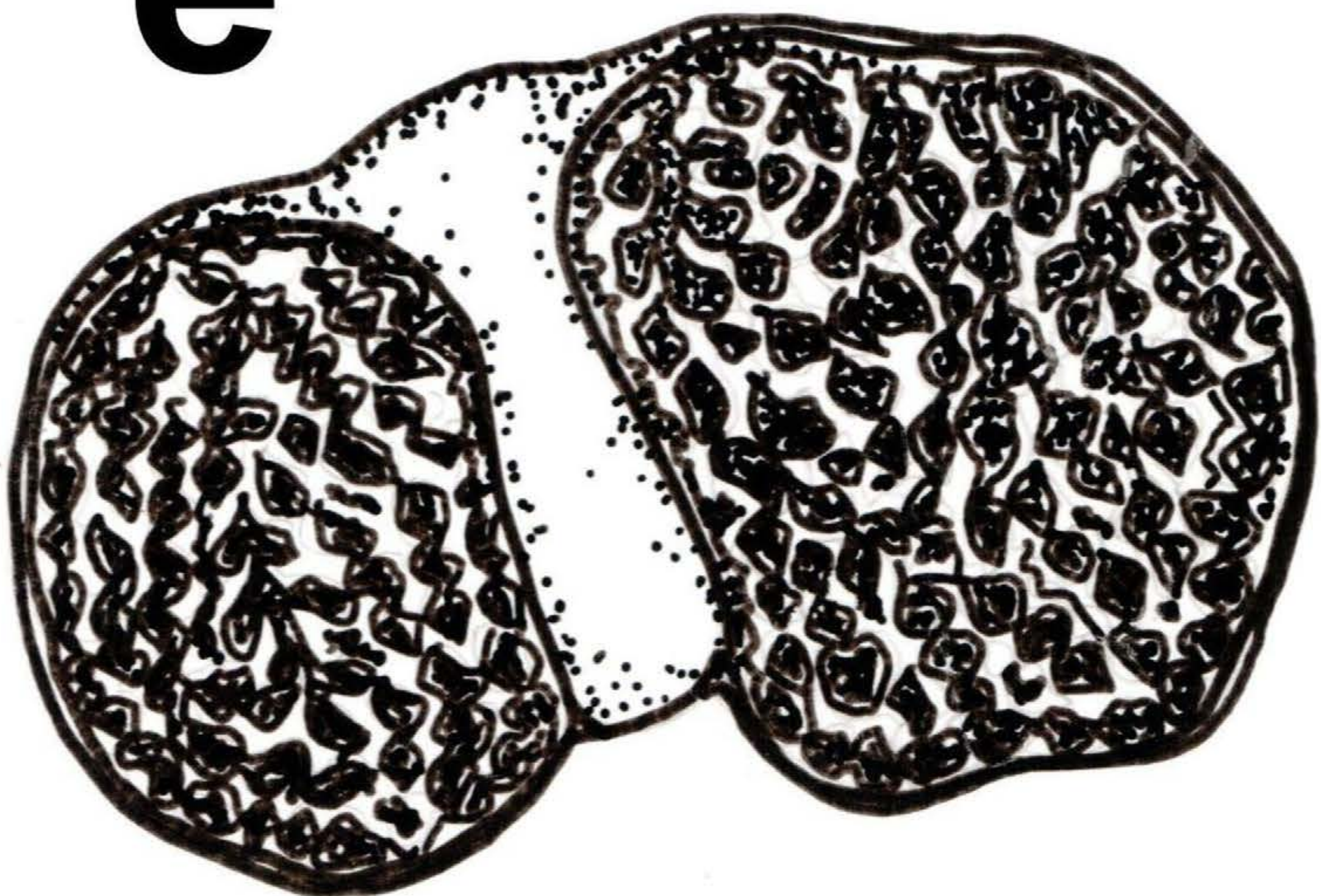
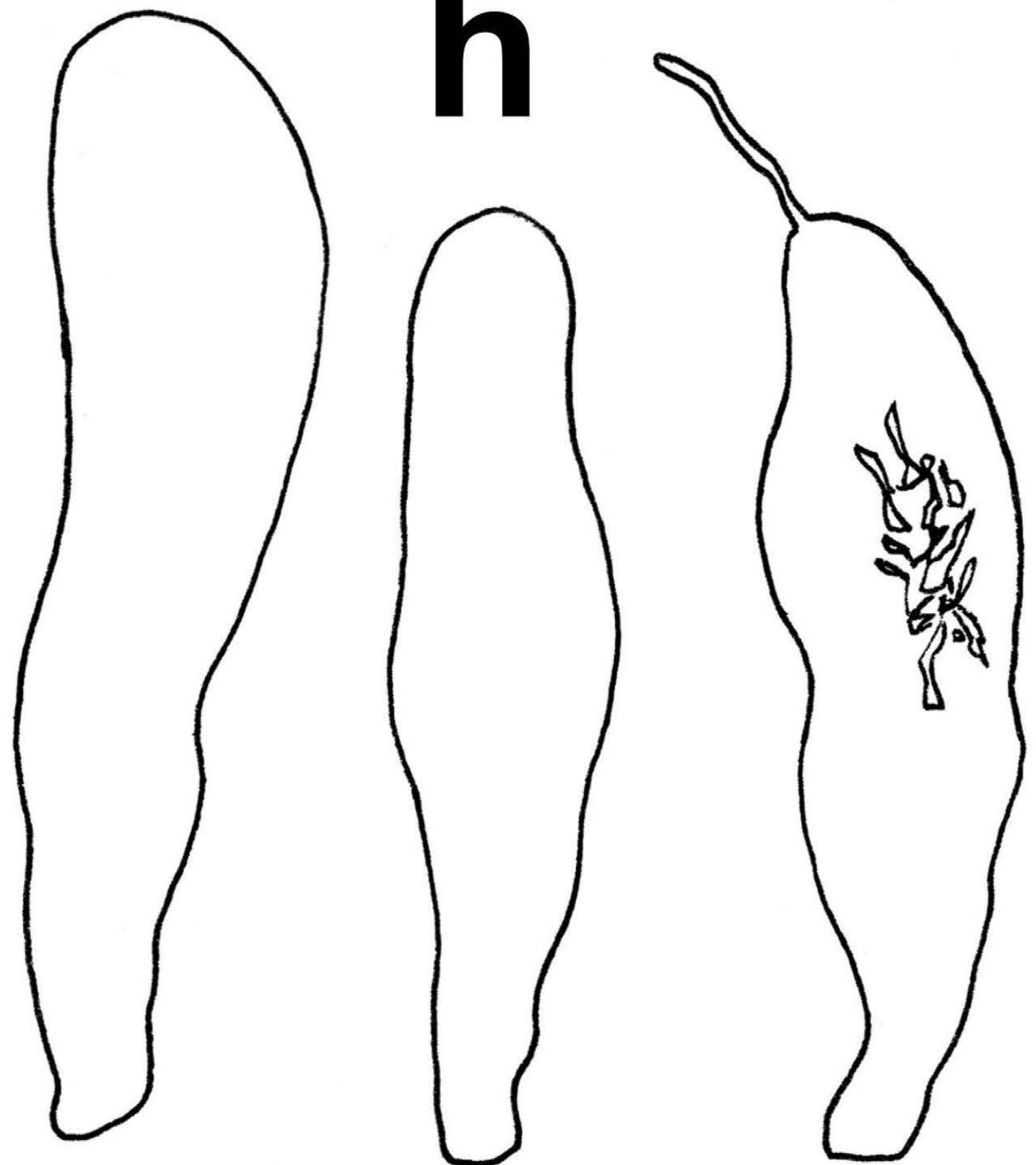
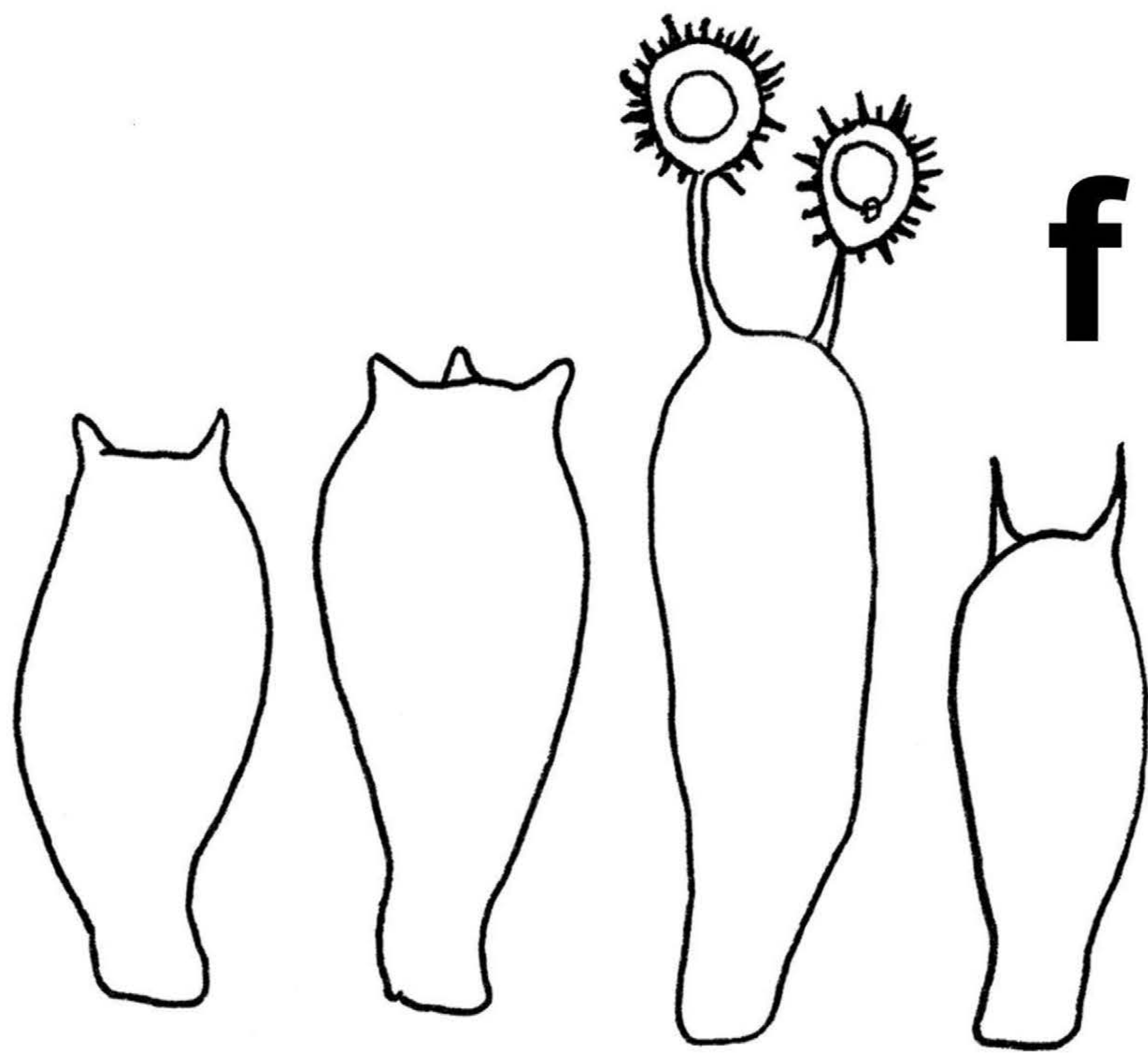
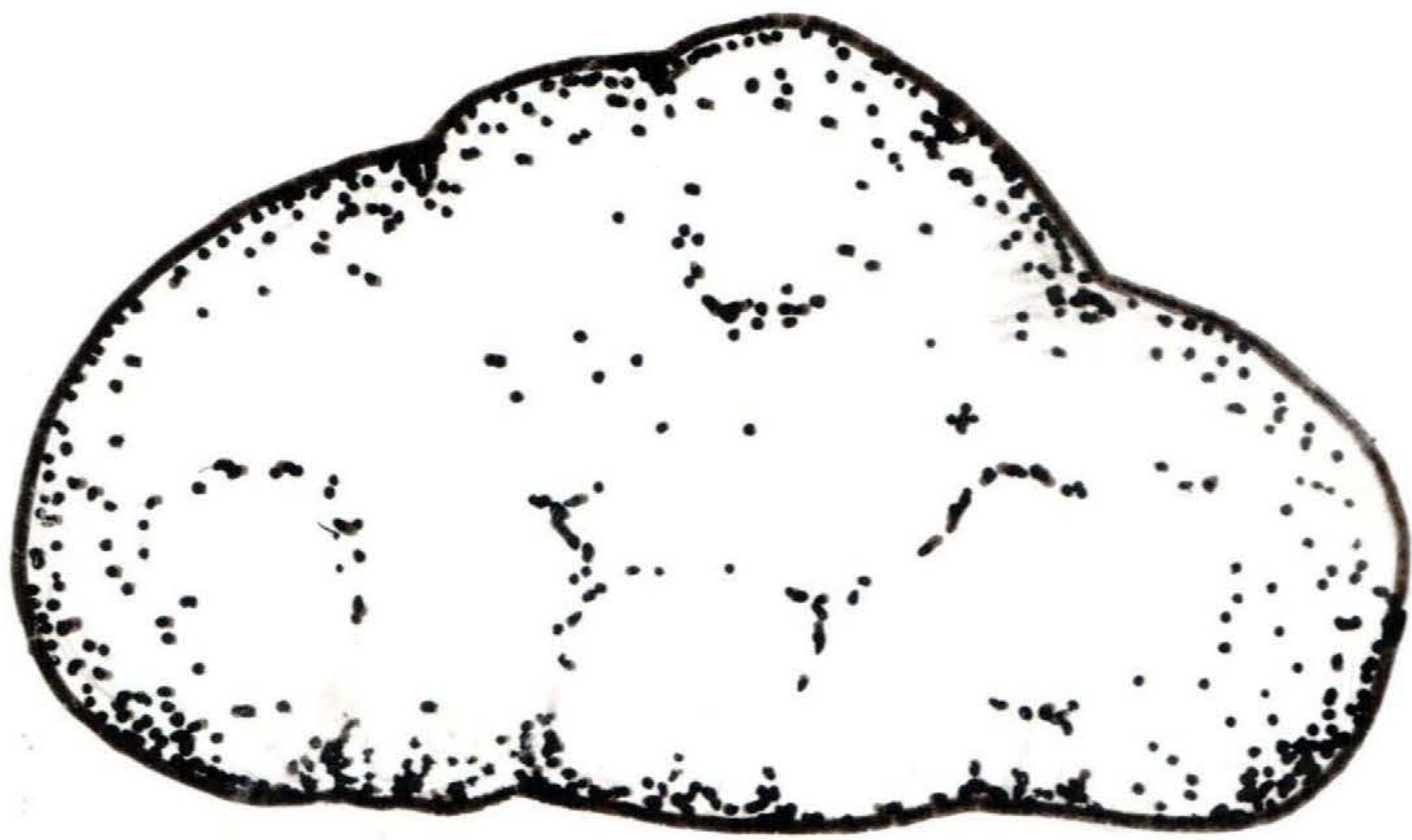
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