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Conservation of cytoplasmic organization in the cystidia of *Suillus* species

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Abstract: Cystidia of *Suillus americanus* and *S. granulatus* (Boletales) were examined cytochemically and ultrastructurally with cells prepared by freeze substitution. We present the first study showing ultrastructural details and cytological functions of the cystidium to be conserved in two closely related species. The results are presented for inclusion in the AFTOL Structural and Biochemical Database <http://aftol.umn.edu> to aid in the application of morphological characters to phylogenetic studies. The cystidia of these *Suillus* species appear to be united by a series of conserved characters, including specialized secretion mechanisms, smooth tubular endoplasmic reticulum and abundant free ribosomes. The conservation of these subcellular traits among members of this genus suggests that ultrastructural details of cystidia may provide a suite of phylogenetically informative characters. Inclusion of such characters in phylogenetic analyses might resolve or provide support for monophyletic groups at the level of family or genus.

Key words: basidiocarp, cell wall, cytology, Golgi apparatus, phylogeny, septa

INTRODUCTION

The cystidia of Agaricomycetes have been the object of scrutiny and curiosity since their revelation by Micheli in the 18th century (Singer 1986). In the 1920s Buller (1922, 1924) introduced a classification scheme for cystidia based on their position within the sporocarp, wall thickness, ornamentation and other characters. His nomenclature has proven to be useful in describing distinct microscopic features for taxonomic identification. Subsequent cystidial types have been described, such as chrysocystidia, both with light and electron microscopy. Clémençon (1997, 2004) proposed two basic classifications: deutero- and aleuthocystidia, defined by endo- or exosecretion. The problem with these classifications is that the classification schemes have not been based on phylogenetic data. For example the classification deuterocystidia includes the phylogenetically unrelated chysocystidia of the *Strophariaceae* (Agaricales) and gloeocystidia of the *Russulales*.

At present much remains unknown regarding the function, physiology and evolution of these highly derived cells. Although hypotheses abound, concrete conclusions as to the function of secretory products, supposed nutrient/waste storage structures and curiously modified cystidia are rare. The only ultrastructural examinations of cystidia from members of the Boletales are of *Tylopilus felleus* and *Suillus americanus* (Clémençon 1975). The advent of new imaging technology and the scarcity of data on these cells demand that they be reexamined.

The great degree of specialization observed in cystidial cells suggests that they are highly adaptive structures. Although convergent evolution of function and gross morphology are evident in cystidia, ultrastructural observation reveals that cellular mechanisms, such as secretion, cytoplasmic organization, septal pore condition and patterns of wall differentiation, are likely to be conserved. With the voluminous output of molecular information over the past decade a renaissance of interest in subcellular morphological characters to supplement and provide guidance in the interpretation of molecular-based phylogenetic hypotheses has gained momentum. The organization of the septal pore and the structure of the spindle pole body have been shown to be phylogenetically informative characters in resolving phylum and subphylum level relationships of the Fungi (McLaughlin et al 1995, Celio et al 2006). In their report on the progress of the AFTOL project Lutzoni et al (2004) foreshadowed that highly specialized cell types (such as cystidia) are likely to be of systematic use at lower taxonomic levels.

To establish the utility of cystidial organization as a phylogenetic trait two closely related species must be examined to determine which character states are homologous and might be informative in a phylogenetic analysis. In this study we have attempted the first comprehensive, parallel characterization of cystidial cytology in two species of *Suillus* using cryofixation techniques with the intent of determining homologies that might be scored for phylogenetic analysis. Physiology and function, especially of secretion,

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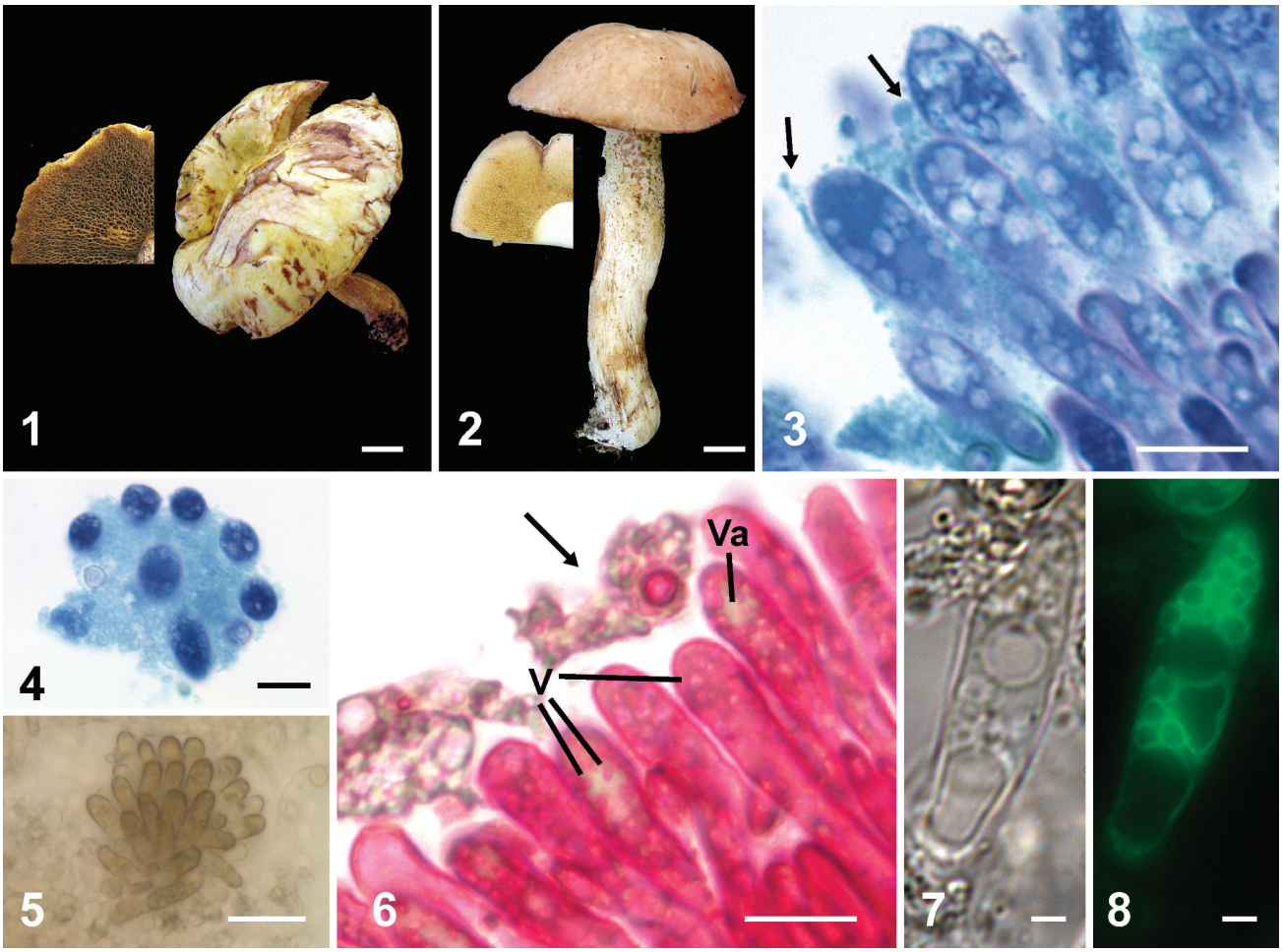


FIGS. 1–8. Macro- and microscopic images of *Suillus americanus* and *S. granulatus*. 1, 2. Sporocarps of *S. americanus* (BD303) and *S. granulatus* (BD301), respectively. 3, 4. Light micrograph (LM) of clustered cystidia in longitudinal (3) and cross (4) section of *S. americanus* stained with toluidine blue. Note the differential staining of the exudate (arrows). 5. Bright field micrograph of clustered cheilocystidia of *S. granulatus*, unstained. 6. LM of the hymenium layer of *S. americanus* stained with periodic acid/Schiff's reagent. Extracellular exudate (arrow) stains lightly and incompletely and has light to dark-staining spherical inclusions. Vesicles (V) also are stained, but vacuoles (Va) remain unstained. 7, 8. Cystidium from *S. americanus* observed with bright field and fluorescence microscopy stained with $DiOC_6$ showing the diffuse tubular distribution of the endomembrane system. Bars: 1, 2 = 1 cm; 3, 4, 6 = 10 µm; 5 = 50 µm; 7, 8 = 2 µm.

appear to be homologous characters uniting these two species.

MATERIALS AND METHODS

All sporocarps were collected in Minnesota. *Suillus americanus* (Peck) Snell (FIG. 1) was collected 23 Sep 2004 from Afton State Park, Washington County, and *Suillus granulatus* (Fr.) Kuntze (FIG. 2) was collected 16 Sep 2004 from Cedar Creek Natural History Area, Anoka County. Both collections were transported to the laboratory in wax paper to prevent desiccation. Voucher specimens BD301 (*S. granulatus*) and BD303 (*S. americanus*) were deposited in the University of Minnesota Herbarium, Bell Museum of Natural History (MIN).

Light microscopy.—Small samples of mature pileus tissue were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4 C. Fixed tissue was washed three times with 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series and embedded in plastic with a JB-4 embedding kit (Polysciences Inc., Warrington, Pennsylvania). Two micrometer thick sections were cut with a glass knife on a Reichart-Jung Ultracut E microtome and collected on glass slides. Thick sections were stained in 0.05% tolouidine blue O (TB) in benzoate buffer (pH 9) gently heated over an alcohol flame and rinsed with dH_2O, or blocked in dimedone solution and stained with 1% periodic acid followed by Schiff's reagent (PAS) (Ruzin 1999).

For fluorescence microscopy, free-hand sections of fresh hymenium were stained with a 50 µg/mL solution of 3,3′-dihexyloxacarbocyanide iodine ($DiOC_6$) in dimethylsulfoxide for 20 min and mounted on glass slides with H_2O

(Duckett and Read 1991). Samples were examined with a Zeiss Axioskop with a 450–490 nm excitation filter, a 510 nm chromatic beam splitter, 520 nm barrier filter and plan-Neofluor 40× and 100× lenses, the latter with 1.3 NA. All images were recorded with a SPOT® Insight QE digital camera system. Digital micrographs were processed in Adobe® Photoshop® CS2 (Adobe Systems Inc., San Jose, California) with the Smart Sharpen filter at 100% with a radius of 3 pixels.

Electron microscopy.—Mature hymenophore tissue was fixed within 4–6 h of collection. Thin samples were sliced from the mature pileus with a scalpel and plunged into ca. −182 C liquid propane within 1 min of excision (Hoch 1986). Frozen sections were transferred to substitution fluid consisting of 2% osmium tetroxide and 0.1% uranyl acetate in 100% anhydrous acetone at −80 C and stored at −80 C for 48 h. Samples gradually were brought to room temperature (−20 C, 2 h; 0 C, 2 h; room temperature, 1 h), then washed three times with 100% HPLC-grade acetone amended with 1% acidified 2, 2-dimethoxypropane. Fixed tissue was microwave-infiltrated under 20–25 mm Hg pressure at 42 C for 2 min with a solution of Quetol 651 and hardeners (nonenyl sussinic anhydride and nadic methyl anhydride) with an accelerator (2,4,6-tri[dimethylaminoethyl]phenol) equal to 1% of the total solution weight (Abad et al 1988).

The Quetol-infiltrated hymenophore of each specimen was separated from the remaining pileus tissue with a stereomicroscope, dissected into smaller samples consisting of two or three tubes and polymerized in plastic resin at 74 C for 48 h. Embedded cystidia were selected with a Zeiss Axioskop at 800× magnification, cut from the plastic with a jeweler's saw and mounted on resin blocks for sectioning. Ultrathin sections (ca. 98 nm) were cut with a diamond knife-equipped Reichart-Jung Ultracut E microtome and collected on slot grids (Rowley and Moran 1975). Sections were poststained in 3% uranyl acetate followed by triple lead citrate (Sato 1968) and examined with a Philips CM 12 transmission electron microscope (TEM) operating at 60 kV. Detailed protocols for specimen preparation are available at <http://aftol.umn.edu>.

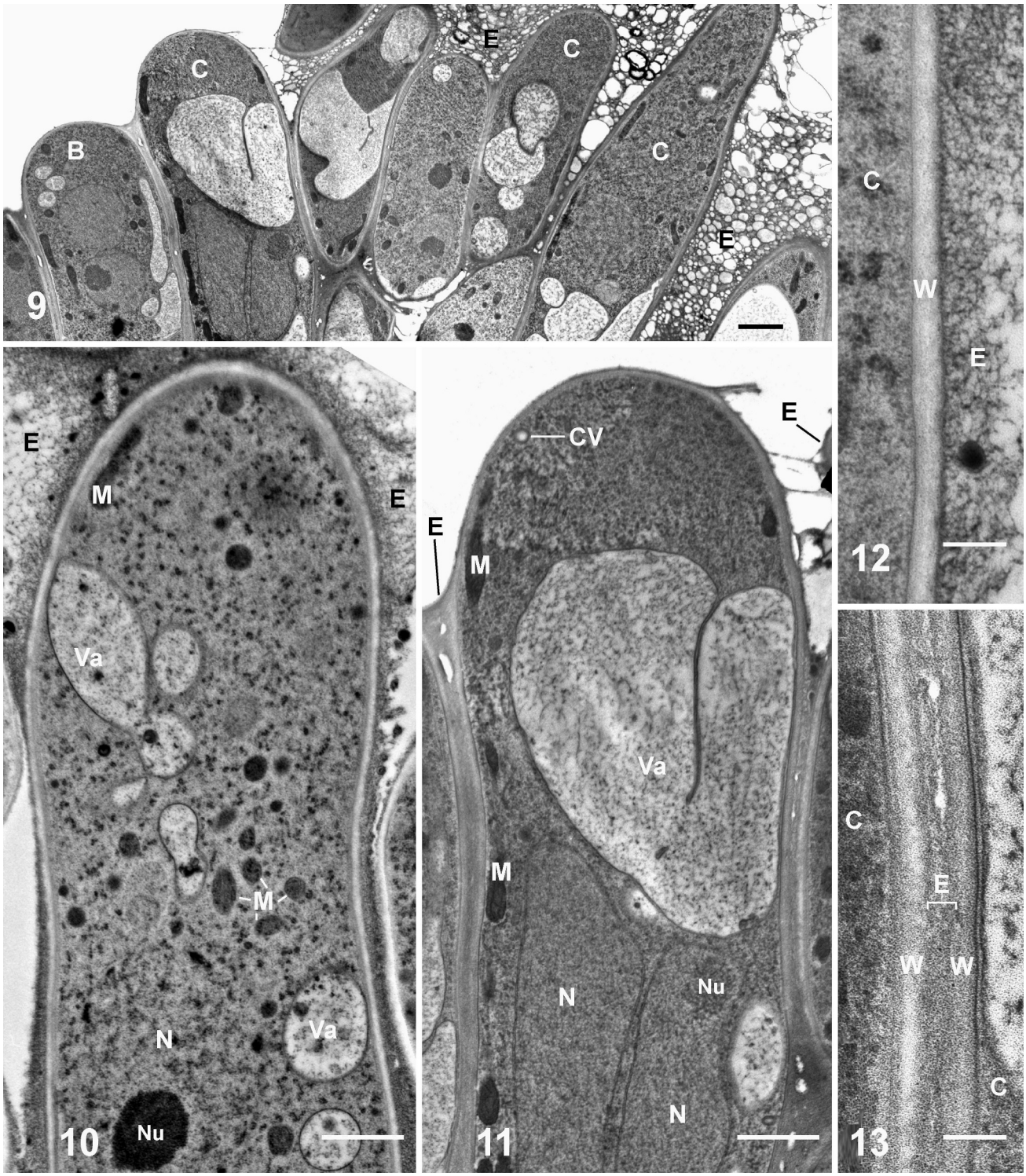
RESULTS

Cytoplasmic organization.—The cheilocystidia of *Suillus americanus* (FIGS. 1, 3, 4, 6–8, 10, 12, 14, 16, 18) and *S. granulatus* (FIGS. 2, 5, 9, 11, 13, 15, 17, 19–24) were well preserved by freeze substitution, although ice damage was observed in the base of some cells. These cheilocystidia occurred in clusters on the hymenium (FIGS. 3–6, 9). Cystidia of both species exhibited a binucleate cytoplasm with scattered small to large vacuoles and numerous long, branched mitochondria distributed throughout the length of the cell (FIGS. 9–11). The cystidia became increasingly vacuolated with age, whereupon the vacuoles produced a distinct lobed appearance (FIGS. 10, 11). PAS stained membrane bound vesicles within the cell, but vacuoles remain unstained (FIGS. 6, 10). Dimedone treated control slides for the PAS reaction showed unstained cytoplasm, cell wall and exudate. Glycogen granules were not evident in PAS-stained cystidia, nor were they observed by TEM. Lipid droplets were not observed. Clustered free ribosomes and tubules of smooth endoplasmic reticulum (ER) were homogeneously distributed throughout the cell (FIGS. 14, 15). Fluorescence microscopic examination of cystidia stained with $DiOC_6$, which localizes in endomembranes (Duckett and Read 1991), confirmed a loose, homogenous distribution of the endomembrane system throughout the cell in both species (FIGS. 7, 8).

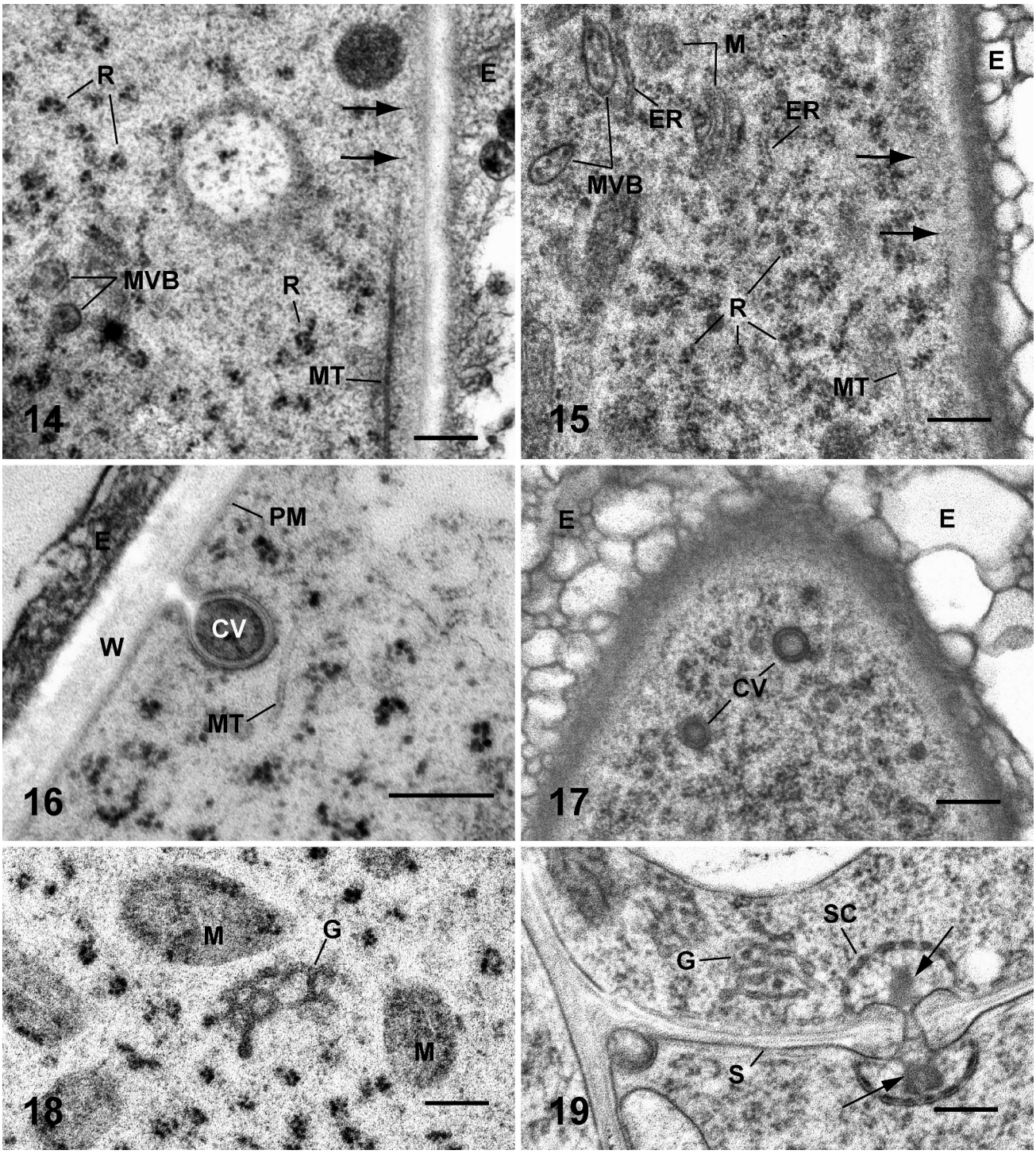
Conspicuous coated vesicles were observed numerous times near the cell periphery, mostly in the apical region, but also present throughout the cell near the plasma membrane (FIGS. 11, 16, 17). The coated vesicles contained an electron-dense core 150 nm diam, with a layered margin bordered by an electron-light layer 10 nm wide. In both species some vesicles were observed undergoing fusion with the plasma membrane (FIG. 16). A thin organelle-free zone 100–200 nm wide was present along the cell periphery (FIGS. 12, 14–17). Microtubules also were observed along the periphery, oriented parallel to the longitudinal axis of the cystidium (FIGS. 14–16). Golgi apparatus composed of unstacked cisternae (FIGS. 18, 19) and multivesicular bodies (FIGS. 14, 15) were scattered throughout the cytoplasm but were observed with less frequency.

Cell wall differentiation and extracellular secretion.—In both species the cell wall was differentiated into an electron translucent middle region surrounded by somewhat electron opaque outer and inner areas (FIGS. 12, 13, 16). A pellicle was absent. Cystidia of both species produced a voluminous exudate consisting of electron opaque threads and spherical inclusions in an electron translucent matrix (FIGS. 9, 10, 12, 14–17). The exudate appeared brownish in bright field microscopy (FIG. 6). TB reaction for polyphenolic compounds (O'Brien and McCully 1981) stained the threads, spheres and matrix greenish blue but the matrix was less intensely stained (FIGS. 3, 4). PAS reaction for carbohydrates stained the spherical inclusions and fine threads within the exudate (FIG. 6).

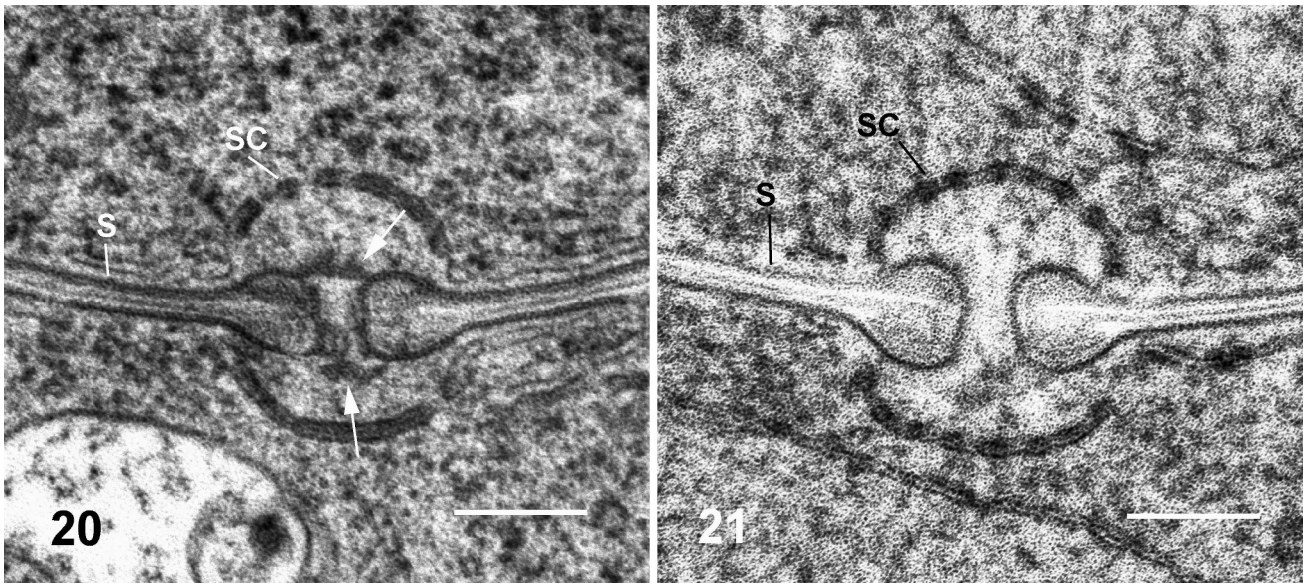
Septum and septal pore apparatus.—Septal pore apparatus were examined in the cystidial and subhymenial cross walls of *S. granulatus.* Multiperforate septal pore caps accompanied all septa (FIGS. 19–21). Most pore caps were contiguous with endoplasmic reticulum. A lower density of cytoplasmic staining was seen within the adseptal region of the pore cap in



FIGS. 9–13. Transmission electron micrographs (TEM) of *S. americanus* and *S. granulatus*. 9. TEM of the hymenium layer of *S. granulatus* showing clustered cheilocystidia (C), basidium (B) and extracellular exudate (E). 10. TEM of a mature cystidium of *S. americanus* showing mitochondria (M), nucleus (N), nucleolus (Nu), vacuoles (Va), extracellular exudate (E) and characteristic cytoplasmic organization. 11. TEM of a mature cystidium from *S. granulatus* showing mitochondria (M), nuclei (N), nucleolus (Nu), extracellular exudate (E) and characteristic cytoplasmic organization. A coated vesicle (CV) is present in the apical region near the plasma membrane. 12. Cell wall (W) differentiation in cystidium (C) of *S. americanus* and extracellular exudate (E). 13. Cell wall differentiation in the walls of two adjacent cystidia (C) of *S. granulatus*; exudate (E). Bars: 9–11 = 1 μm; 12, 13 = 0.25 μm.



FIGS. 14–19. TEM of *S. americanus* and *S. granulatus*. 14. Cytoplasmic organization characteristic of cystidia in *S. americanus* showing clusters of loose ribosomes (R), multivesicular bodies (MVB), peripheral microtubule (MT) and organelle-free zone next to wall (arrows). Exudate (E). 15. Cytoplasmic organization characteristic of cystidia in *S. granulatus* showing clusters of loose ribosomes (R), multivesicular bodies (MVB), mitochondria (M), endoplasmic reticulum (ER), peripheral microtubule (MT) and organelle-free zone adjacent to wall (arrows). Exudate (E). 16. Coated vesicle (CV) undergoing fusion with plasma membrane (PM) in cystidium of *S. americanus*. Exudate (E), wall (W) and microtubule (MT). 17. Coated vesicles observed near the plasma membrane in the apex of a cystidium of *S. granulatus*. Exudate (E). 18. Cystidium of *S. americanus* with Golgi cisterna (G) in the process of vesicle formation. Mitochondria (M). 19. Cystidium of *S. granulatus* with Golgi cisterna (G). Cystidial septum (S) with occlusions (arrows). Septal pore cap (SC). Bar = 0.25 μm.

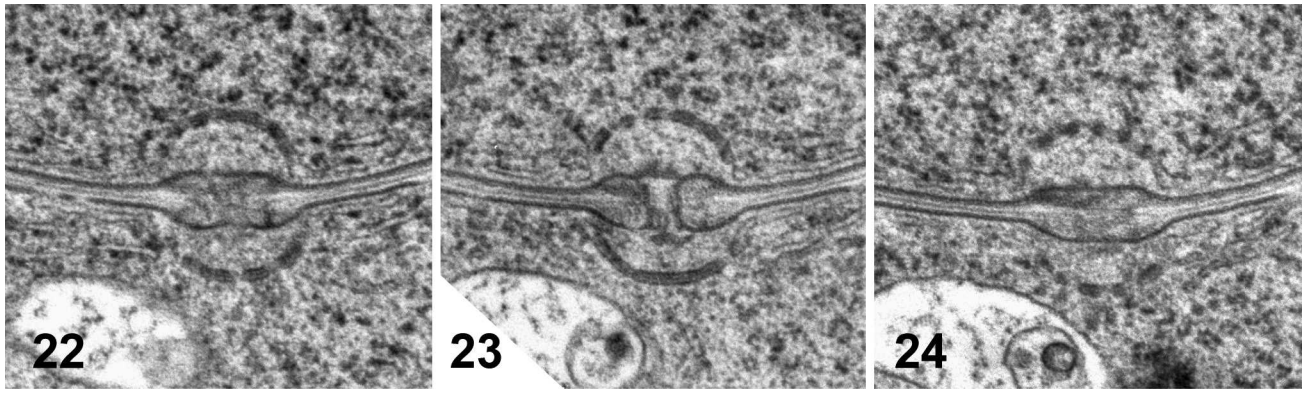


FIGS. 20, 21. TEM of septa in *S. granulatus*. Septum (S); septal pore cap (SC). 20. Basal septum from mature cystidium of *S. granulatus*. The pore is occluded with electron-dense material (arrow). 21. Subhymenial septum from hymenophore of *S. granulatus* with unoccluded pore. Bar = 0.25 μm.

electron micrographs of unoccluded septa (FIG. 21). The structure of the pore varied depending on septum location within the hymenophore. The pore at the base of mature cystidia in *S. granulatus* was occluded by granular electron-opaque structures near either pore orifice (FIGS. 19, 20). The occlusions lacked a layered structure. The continuity of the pore occlusion in cystidia was verified with serial sections through the septum. Subhymenial septal pores were unoccluded (FIG. 21–24).

DISCUSSION

The ultrastructural features of cystidia from two *Suillus* species are remarkably similar. This organization is markedly distinct from the cystidial cytology reported throughout the Agaricomycetes, particularly in ER morphology and the condition of secretory or storage products (Clémençon 1972, 1975, Thielke 1972, Gull and Newsam 1975, Thaler and Gailhofer 1981, Thielke 1983). The cytoplasmic organization characteristic of cystidia from these *Suillus* species includes a loose endomembrane system, which is more tubular rather than cisternally organized. This pattern is supported by $DiOC_6$ staining, which appears to show a continuous system of tubules appressed to other organelles. Based on its morphological appearance and position within the cell, this staining does not appear to be correlated with mitochondria, although they also may react with this reagent (Duckett and Read 1991). Exocytotic vesicles were observed fusing with the plasma membrane. Abundant mitochondria, clusters of free ribosomes and large central vacuoles dominate the cytoplasm of



FIGS. 22–24. Serial sections through the same septum (as in FIG. 20), confirming full occlusion of the pore.

these binucleate secretory cells. Storage products are inconspicuous or absent. Exudates show variation in morphology but may be characterized ultrastructurally as electron-light spheres in an electron-dense matrix with scattered electron-dense spheres. The exudates may be chemically complex as deduced from the brown pigments when unstained, the scattered positive reactions for carbohydrates with PAS and the more general reaction for polyphenolics with TB (O'Brien and McCully 1981, Ruzin 1999). The similarities in cytoplasmic organization among the cystidia of *Suillus* and the contrast of this organization with cystidia of other Agaricomycetes underscore the potential source of phylogenetically informative characters that ultrastructural details of cystidia may present.

Functional homologies.—The secretory nature of the cystidium first was investigated ultrastructurally by Clémençon (1972, 1975). In a 1975 study presenting ultrastructural observations on the cystidia of *Suillus americanus*, much of his attention was devoted to the composition and ontogeny of the conspicuous exuded incrustation. Clémençon (1972) proposed an interesting ''transparietal secretory'' pathway in the cystidia of *Baeospora myosura* (Agaricales), which he used to explain the secretory process in *S. americanus*. Clémençon hypothesized that the secretory product is deposited outside the plasma membrane at the cell wall interface via ER/plasma membrane fusion where discrete ''clumps'' of the product are incorporated into the wall as new wall material is deposited outside the plasma membrane. He goes on to speculate that the excreted clumps are liberated from within the wall as the outer surface undergoes autodissolution. The liberated clumps, along with ''slime'' derived from the digested face of the cystidial wall, are suggested to explain the staining heterogeneity of the exuded incrustation (Clémençon 1972, 1975).

The data we present do not support Clémençon's hypothesis. Distal wall surface dissolution was not observed. In addition the differentiations within the cell wall of our specimens do not appear as the discrete clumps reported by Clémençon (1975) but instead appear amorphous in both species. The secretory products of *Suillus* cystidia, which may be composed at least in part of glycoproteins or Golgi-glycosylated polysaccharides, must be soluble in the amorphous phase of the cell wall and translocated across it by the constitutive outward flow of the amorphous matrix through the dynamic microfilament wall skeleton.

Because Clémençon's 1972 and 1975 investigations used $KMnO_4$ fixation, it is difficult to directly compare our respective findings. With the development of freeze-substitution techniques conventional chemical fixation has been shown to be inferior in accurately preserving membrane bound organelles, especially Golgi bodies (Hoch 1986). Clémençon (1975) did not observe dictyosomes or Golgi equivalents in the cheilocystidia of *S. americanus* and therefore concluded that exocytosis is mediated by ER-derived vesicles. We observed Golgi bodies in the process of vesicle formation in this species on several occasions. A great body of research on the role of Golgi equivalents in fungal exocytosis has emerged since Clémençon's hypothesis, which he continues to maintain (Clémençon 1975, 1997, 2004) despite the fact that evidence for the function of the Golgi in hymenial cells of the Agaricomycetes had been documented as early as 1972 (McLaughlin 1972, 1974).

Studies investigating secretory mutants of *Saccharomyces cerevisiae* in the 1980s and 1990s have revealed not only the physiological steps in the fungal secretory pathway but also demonstrate the conserved mechanism of endomembrane-directed exocytosis across the Eukaryota (Alberts 2002, Novick 1981). The core oligosaccharide glycosylation motif that initiates asparagine-linked glycosidic modification and Golgi targeting is identical throughout the eukaryotes (Esmon et al 1981). While the majority of fungal Golgi bodies lack stacked cisternae, immunolocalization studies of Golgi-associated proteins involved in secretory processing show that fungal Golgi bodies indeed are differentiated into early (cis) and late (trans) Golgi compartments based on the role of their respective resident enzymes in the hierarchy of glycosyl transfer (Franzusoff 1991).

Based on what is known about the function of late Golgi bodies in the fungi it is possible that the major secretory products are heavily mannosylated glycoproteins. The carbohydrate nature of these molecules, which is suggested by their PAS reaction (Ruzin 1999), would allow their solubility in the amorphous phase of the cystidial wall and provide the possible mechanism of transparietal secretion. Of note, the green-blue reaction of the exudate when stained with TB also suggests the presence of polyphenols (O'Brien and McCully 1981). It is clear that chemical analysis of the cystidial secretory products is needed before a definite conclusion can be reached on this matter, but we believe that the process and chemical composition of the secretion might be an important synapomorphy uniting this group.

The basal septal pores were occluded in all mature cystidia examined in *S. granulatus*, but subhymenial septa were clearly unoccluded. Flegler et al (1976) studied the septal pore complexes in five species of Agaricomycetes and one species of Dacrymycetes.

They concluded that the septal channels of vegetative mycelium are characteristically occluded with ''nonperforate'' plugs, while the channels are unoccluded or partially occluded in the hymenial tissue of the Basidiomycota. In a study of septation in hymenial and tramal hyphae of *Agaricus bisporus* Craig et al (1977) observed that complete septal occlusions occur randomly over the lamellae and are ''apparently not correlated with cell type, septal position or cell state.'' Our data suggest that additional studies are needed before such firm conclusions can be drawn about the septal pore condition in specialized cells.

Histochemical observations of Flegler et al (1976) agree with our suspicion that the pore occlusion is proteinaceous and not membranous and that the pore canal is a site of higher than average metabolic activity. We suggest that the occlusion of the basal pore is initiated at a determined stage of cystidial maturation. It would be advantageous for this specialized secretory cystidium to control cytoplasmic streaming as a way to sequester the oxidation product, adenosine triphosphate, of its abundant mitochondria to meet the high energy needs of vesicular exocytosis. This condition, discussed by Clémençon (1975), also might be a shared derived trait applicable to phylogenetic analysis.

Phylogenetic considerations.—We present the first evidence that cystidia of *Suillus* are united by a series of conserved morphological and physiological traits. Our group also has observed certain aspects of a ''boletoid'' cystidial organization that appear common to multiple genera in the Boletales including *Suillus, Chalciporus* (McLaughlin et al unpubl) and *Tylopilus* (Clémençon 1975). These shared characters include the dispersed tubular smooth ER, numerous free ribosome clusters and the relatively organelle-free cytoplasm along the cell periphery. It is likely that subtle variations exist within this order and have yet to be elucidated. For example *Chalciporus rubinellus* forms distinctive fibrous, spherical cytoplasmic inclusions (McLaughlin et al unpubl). Of interest, *C. rubinellus* is continually shown to occupy the basal branch of the Boletineae with strong support in multilocus molecular analyses (Kretzer and Bruns 1999, Binder and Hibbett 2007). Likewise our group has tentatively identified distinct russuloid cystidial organizations; that is, unique vesicles in young cystidia of members of the *Russulales* that precede the development of their characteristic gloeocystidia (McLaughlin et al 2008). This preliminary data suggests that conservation of cystidial subcellular features is not unique to the Boletales. Further investigation of cystidial ultrastructure from the Agaricomycetes is needed to determine the potential application of cystidial ultrastructural characters in phylogenetic analyses.

A major obstacle to assessing the homology of subcellular cystidial characters arises from the variety of fixation methods employed. Cryofixation techniques developed in the 1980s are believed to yield the most accurate subcellular data. Because these protocols are more technically demanding their widespread use might have been discouraged. This study is the first report to employ freeze substitution in describing the ultrastructural organization of cystidia. Our observations have been entered in the AFTOL Structural and Biochemical Database <http://aftol.umn.edu>. Twenty-seven taxa currently are listed in the cystidial dataset, with 19 structural characters defined. These characters include descriptions of secretory products, wall differentiation, nuclear condition and the differentiation of specific cytoplasmic organelles such as Golgi and ER. The marked degree of ER differentiation among cystidia of species of Agaricomycetes has been stressed by many investigators (Setliff et al 1972, Thielke 1972, Thaler and Gailhofer 1981, Clémençon 2004). Because the number of taxa entered in the Database is small, full delineation of cystidial character states is far from resolved. We have attempted to describe the basic cellular organization of *Suillus* cystidia and highlight cystidial organization as a potentially informative set of phylogenetic characters. It is our expectation that the characters in the AFTOL Structural and Biochemical Database will open the door to a more in-depth analysis of the evolution of cystidia in the Agaricomycetes.

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