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Morphology and development of the reticuloperidial ascomata of *Auxarthron conjugatum*

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Abstract: Light and electron microscopy showed that the reticuloperidium of thick-walled hyphae, characteristic of the mature ascoma of *Auxarthron conjugatum*, originated from branches that grew from the broad, gyre-like hyphal loops making up the ascomatal initials. Within the developing peridium, short, acropetally proliferating chains of prototunicate asci each arose from a single crozier and matured from base to tip. The walls of young asci were two-layered but evanesced as they matured with the outer layer dissolving before the inner one. Distal asci in some chains retained the inner wall, detached from adjacent asci by septum schizolysis and when transferred to fresh media produced germ tubes and mycelium. Ultraviolet epifluorescent staining with a DNA intercalator (Hoechst) indicated that these spore-like asci probably contained diploid nuclei. In normal asci, ascospores had an inner, electron lucent primary wall and a three-layered secondary wall. The deposition pattern of the middle layer of the secondary wall created the distinctive array of pits and ridges characteristic of the ascospores in this taxon. The production of ascospores, spore-like asci and arthroconidia, along with the tendency of ascospores to adhere in a mass, is interpreted as contributing to the reproductive flexibility and inoculum potential of *A. conjugatum*. In all respects the ascomata of *A. conjugatum* differed substantially from the morphologically similar taxon, *Myxotrichum arcticum*. These findings underscore the benefit of using DNA-based phylogenies in concert with cytological and ultrastructural observations for exploring selective pressures behind homoplasious characters and revealing novel structural features.

Key words: ascogenesis, ascospore development, gymnothecia, morphogenesis, Onygenales, reticuloperidial cleistothecia

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INTRODUCTION

Auxarthron, with approximately 12 described species (Currah 1985, Solé et al 2002a, Sigler et al 2002), is a relatively large genus in the Onygenaceae (Eurotiomycetes). At maturity the cleistothecial ascomata are more or less globose and have a mesh-like peridium of rigid, thick-walled hyphae (= reticuloperidium) surrounding a mass of minute, single-celled ascospores that form in globose, evanescent asci. Ascomata are strikingly similar to those of species of *Myxotrichum* (Leotiomycetes) which have a similar reticuloperidium (Apinis 1964), even though the two taxa are phylogenetically distant (Sugiyama et al 1999). Greif and Currah (2003) provided evidence supporting the hypothesis that the similar reticuloperidia represent a convergent adaptation that allows attachment to an arthropod carrier through impalement of ascomata on its setae, thereby improving the chances of dispersal of meiospores to appropriate new habitats and substrata.

A developmental study of the ascomata of *Myxotrichum arcticum* Udagawa, Uchiy. & Kamiya (Tsuneda and Currah 2004) showed clearly that the apparently cleistothecial ascoma in this taxon is apothecial in structure and therefore the reticuloperidium represents excipular tissue. Centrum characteristics (e.g. synchronous development of asci in a hymenial layer) were compatible with this interpretation, and both sets of observations supported phylogenies, based on DNA sequence analyses, which had placed *Myxotrichum* among the inoperculate discomycetes (Sugiyama et al 1999).

A study similar to Tsuneda and Currah (2004) showing the development of the ascoma in *Auxarthron* was needed for comparative purposes. Kuehn (1955a, b) studied some early stages in the ascoma formation in two *Auxarthron* species but his observations were made without the use of electron microscopy and lack details concerning the origin and development of the reticuloperidium and the centrum. Consequently, we examined stages in ascoma development in two strains of a representative species, *A. conjugatum*, with a combination of light and electron microscopy and here describe the origin and development of the reticuloperidium and hitherto unreported characteristics of the centrum in this taxon.

MATERIALS AND METHODS

Electron microscopy.—*Auxarthron conjugatum* (UAMH 3156) was grown on cornmeal agar (CMA; Difco, Detroit, Michigan) or CMA with dextrose (CMAD, Difco) at 20 C for 2–8 wk in the dark. For scanning electron microscopy (SEM), agar disks bearing developing ascomata were cut from cultures of different ages, and either critical-point or air-dried. After fixation (see Tsuneda and Currah 2004) specimens were gold coated and examined with a JEOL JSM-6301 FXV field-emission or a Hitachi S-510 scanning electron microscope at 10 or 15 kV. For transmission electron microscopy (TEM), specimens were fixed in glutaraldehyde, postfixed in OsO_4, dehydrated, and embedded in Spurr's resin before sectioning and staining with uranyl acetate and lead citrate (Tsuneda and Currah 2004). Photomicrographs were taken with a Hitachi H-7000 electron microscope at ~75 kV.

Light microscopy.—Ascomata of UAMH 3156 grown on CMA were fixed, dehydrated, embedded in araldite, sectioned (about 1 μm) and stained with a slightly alkaline solution of toluidine blue (1%) in borax (1%) (Meek 1970, Tsuneda et al 2004). Ascomata of UAMH 10597, grown on CMA (Acumedia, Baltimore, Maryland), were used to make wet mounts for bright field and fluorescence microscopy. For bright field, ascomata were stained with acid fuchsin, mounted in polyvinyl alcohol and examined with an Olympus BX50 light microscope fitted with an Olympus UPlanFl 100× oil immersion objective and photographed with an Olympus DP12 digital camera. For fluorescence microscopy, material was stained with 0.5 μg/mL Hoechst 33258 (Sigma-Aldrich, Canada) (a bisbenzimide DNA intercalator that excites in the near UV and emits in the blue region) in phosphate buffered saline (1.37 M NaCl, 26 mM KCl, 100 mM Na_2HPO_4, 17.6 mM KH_2PO_4, pH 7.4), sealed under a cover slip with nail polish and kept refrigerated for less than 96 h before viewing with a Leica DMRXA fluorescence microscope fitted with a Leica HCX PL Fluotar 100×/1.30 oil immersion objective. Images were prepared with an Optronics DXM digital camera using UV laser illumination with emission filters set at 425–475 nm (blue region). Images were captured with a 4× digital zoom. Image quality was improved with Adobe Photoshop 5.0.

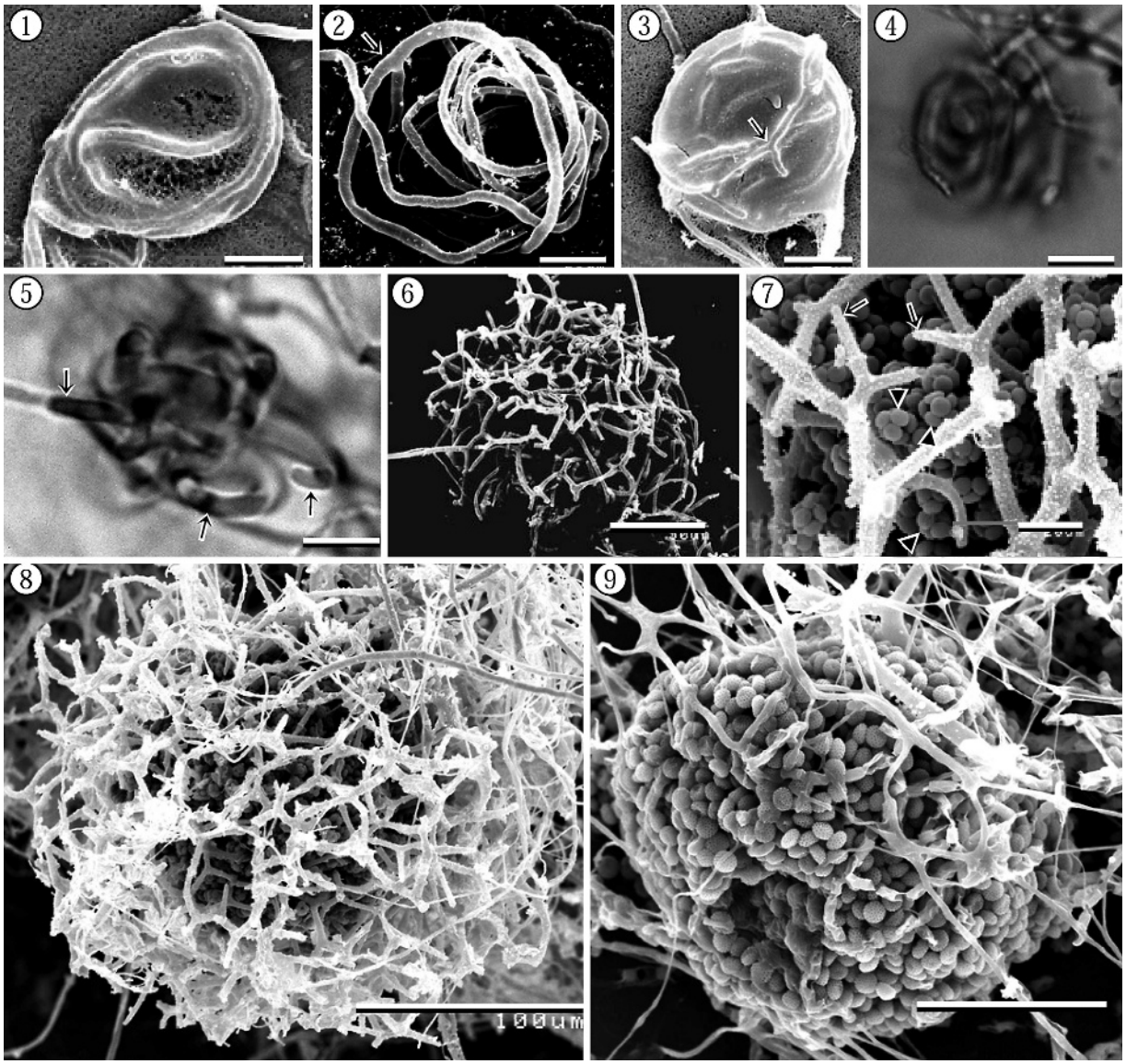
RESULTS

The first indication of ascoma initiation was the formation of 2–4 concentric hyphal coils, or gyres, 25 μm diam (FIG. 1). Subsequent gyres formed obliquely to these to create a loose, more or less globose enclosure (FIG. 2). Hyphae arising from the perimeter of the enclosure elongated and branched within and adjacent to the cavity (FIG. 3). Gyres of hyphae forming the perimeter were still easily discernable in subsequent stages of primordium development (FIG. 4) and, through branching and elongation (FIG. 5), gave rise to the mesh-work characteristic of the mature peridium (FIG. 6).

Some branches extended beyond the perimeter of the developing ascocarp (<650 μm) to form smooth-walled, hooked appendages. At maturity hyphae making up the peridial network were 3 μm broad, septate, thick-walled and asperulate and were more or less straight although abundant dichotomous branching created the characteristic broad angles and zigzag pattern of interwoven hyphae (FIGS. 7, 8). In many of the dichotomies, one branch would cease to elongate and form a short, peg-like spine, while the other would extend and branch again (FIG. 7, arrows). Peridial hyphae encompassed the developing ascogenous interior. Mature cleistothecia were 200–500 μm diam (FIG. 8). In some instances the peridium of mature ascocarps degenerated and exposed the interior collection of ascospores (FIG. 9).

Concurrent with development of the peridial elements, which separated easily from centrum tissue during early stages of ascoma formation (FIG. 10), globose ascus initials (4–7 μm) developed asynchronously from the penultimate cells of croziers, which in turn were borne singly on ascogenous hyphae (FIGS. 11–14). Blastically proliferating, straight (FIGS. 11, 15, 16) or branching chains of up to five globose asci (FIGS. 17, 18) formed from ascus initial cells and matured from the base to the tip. Interphase nuclei in ascospores, hyphae, and postmeiotic, tetranucleate asci were sharply defined when stained with Hoechst and viewed with fluorescent microscopy. Nuclei were either bilobed with an indistinct isthmus (FIG. 19), or not apparent in younger, more distal asci in a chain.

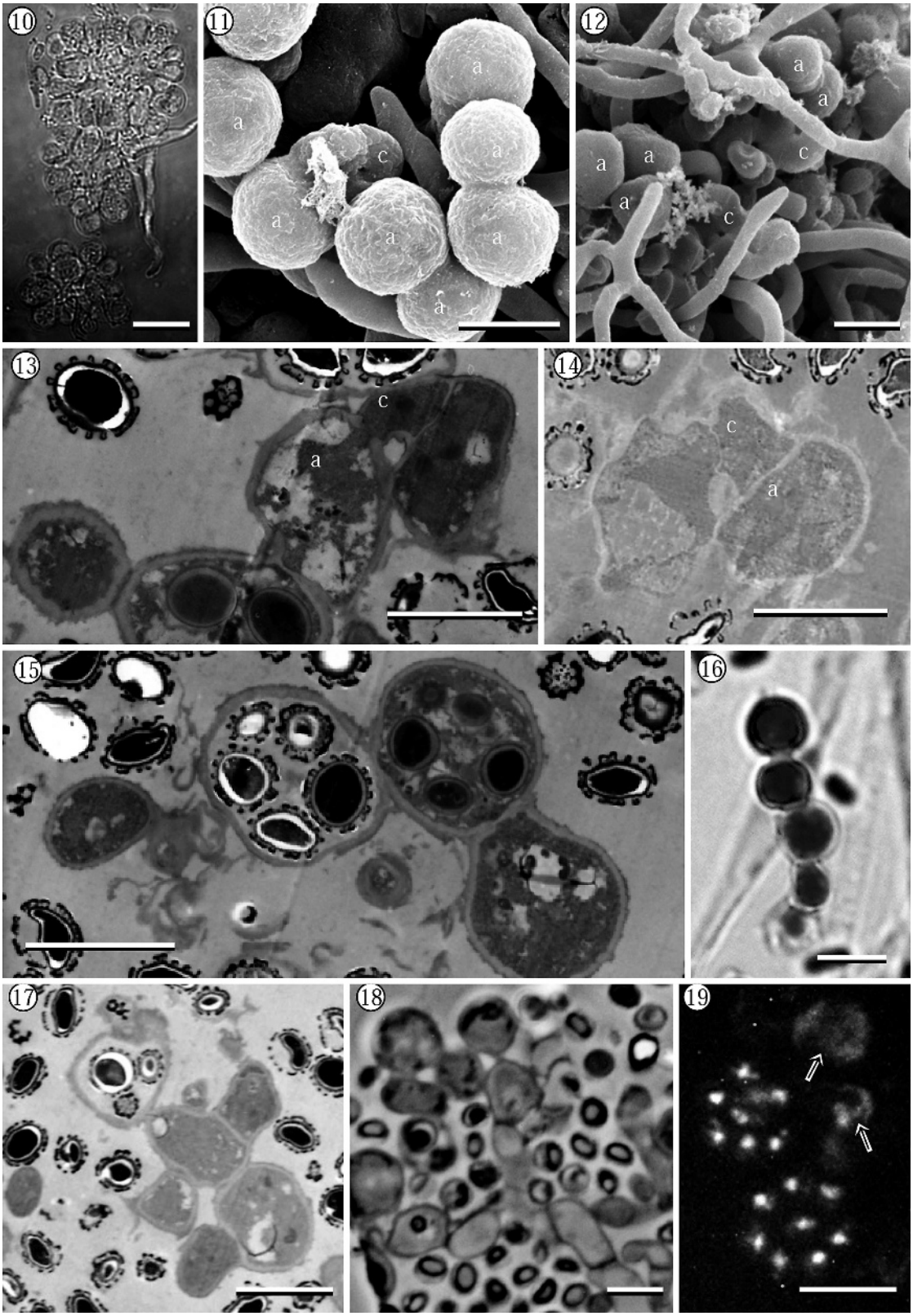
Ascogenous hyphae were thin-walled with simple septa (FIG. 20). Immature asci had a bilaminar wall consisting of an outer layer, which degenerated as the ascus matured, and a thicker, more persistent inner layer that formed a double septum with the inner wall layers of adjacent asci (FIG. 21). At maturity the inner ascus wall evanesced to expose eight, oblate, punctate-reticulate ascospores (2.5–3 × 1.6 μm) in a conglobate cluster. Some cells in otherwise normal looking chains of asci retained the inner wall and dehisced by septum schizolysis rather than producing ascospores. The resulting spore-like bodies, 6 μm diam, were most prevalent in immature ascocarps and bore prominent, circular dehiscence scars (FIGS. 22, 23). These spore-like bodies germinated by forming one or two germ tubes (FIGS. 24, 25). With Hoechst staining, germ tubes initially were anucleate (FIG. 26) and later had 2–3 nuclei (FIG. 27). More developed germ tubes did not have nuclei in multiples of four (FIG. 28). When transferred to CMA, germlings produced mycelia bearing fruiting bodies



FIGS. 1–9. Ascomatal developmental sequence of *Auxarthron conjugatum*. 1–4. Branched (arrows) and unbranched gyres of early primordia. 5. Primodium with peridium-forming elongating branches (arrows). 6. Mature peridial hyphae (appendages extend beyond photo). 7. A view through the mesh-like peridium. Note peg-like spines (arrows), ascospores still in groups of eight (arrowheads) and asperulate peridial hyphae. 8. Mature ascoma. 9. Nearly naked ascospores after degeneration of peridium. 1–3, 6–9 by SEM. 4, 5 by bright field. Bars: 1–5, 7 = 10 µm; 6, 8 = 100 µm; 9 = 25 µm.

that superficially resembled ascomata but lacked typical peridial elements and recognizable asci (FIG. 29) and produced instead swollen, spherical to pyriform terminal cells, 3–5.5 µm diam that occasionally had knob-like protrusions (FIGS. 30, 31). Croziers were not associated with these cells. These fruiting bodies were not observed in cultures inoculated with ascospores, hyphal fragments, or conidia and did not appear to be degenerate.

During the development of normal asci, ascospores first were delimited by an electron lucent primary wall and an outer, more electron dense, thinner layer (FIG. 21 P and S1 respectively). A third layer, thicker than the first and composed of an electron opaque material, was deposited in a reticulate pattern over the second and created the system of ridges and pits characteristic of the taxon (FIGS. 21 S2; 32). Finally, a fourth, electron dense layer was deposited over the outer surface (FIG. 21 S3). Ascospore size did not change after the appearance of the first two wall layers although the second layer became thinner and more electron dense as the third and fourth layers developed and matured (FIG. 33). Pits became more pronounced as the ascospores matured.



FIGS. 10–19. Ascus development of *Auxarthron conjugatum*. 10. Centrum tissue including ascus initials. 11–14. Immature asci (a) with croziers (c). 11, 15, 16. Unbranched chains of asci. 17, 18. Branched chains of asci. 19. Two near-mature asci clearly containing eight nuclei, adjacent to two immature asci containing one dividing nucleus (arrows point to isthmi). 10. DIC (Hoechst stained material). 11, 12 by SEM. 13–15, 17 by TEM. 16 by bright field (with acid fuchsin stain). 18. bright field of fixed, embedded and stained tissue. 19. by fluorescence microscopy. Bars: 10 = 10 μm; 11–19 = 5 μm.

Mature cleistothecia (UAMH 10597), with intact peridia as well as large clumps of exposed ascospores floated intact when placed in a drop of water. When placed in a drop of an organic solvent (2:1:1 of 95% ethanol:acetone:85% lactic acid) the central collection of ascospores dispersed and floated freely through the spaces in the reticuloperidium.

DISCUSSION

Cleistothecial ascomata with reticuloperidia, often referred to as gymnothecia (Novák and Galgóczy 1966), are found in both the Eurotiomycetes and Leotiomycetes and are exemplified respectively in these lineages by *Auxarthron* and *Myxotrichum*. These genera are also similar in producing single-celled, pale-colored ascospores in evanescent asci and in having arthroconidial anamorphs. Kuehn (1955a, b), in a search for taxonomic characters of use in distinguishing among a growing number of isolates of ''gymnothecial fungi'', examined the development of some ''*Myxotrichum*'' species in culture. Three of these later were transferred to *Auxarthron* (Orr et al 1963).

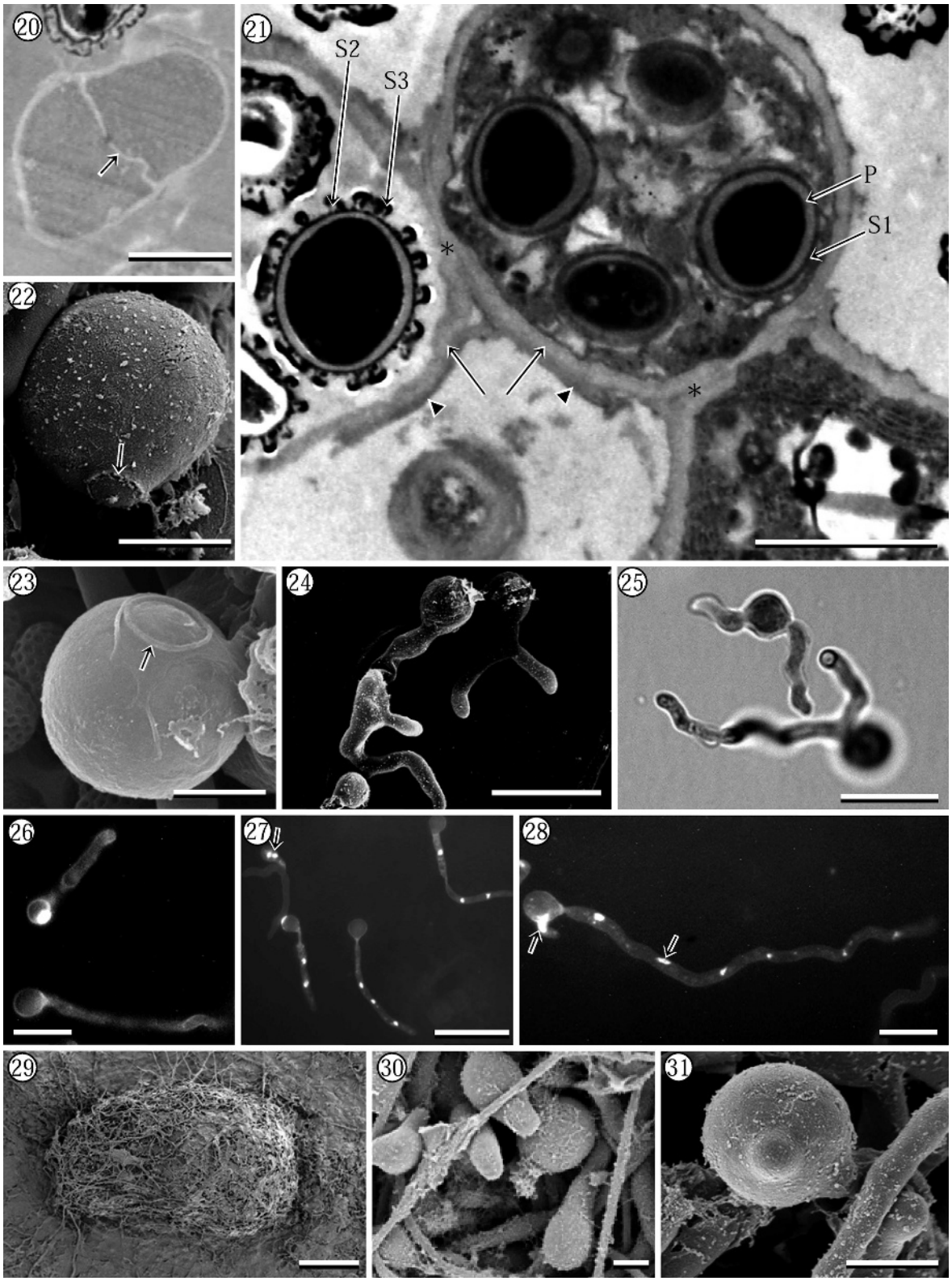
With light microscopy, Kuehn noted differences in gametangial, ascospore and appendage morphology and recorded these details as drawings (Kuehn 1955a, b). Since the redisposition of several of his studied species of *Myxotrichum* in the genus *Auxarthron* (Orr et al 1963), more species have been described in this genus and, although these descriptions have been supported by both light and electron micrographs, developmental aspects have been overlooked and Kuehn's original developmental observations have never been substantiated.

The multiple, gyre-like hyphae that indicated initial stages in ascoma development and the curving hyphae arising as branches from the perimeter of the initials also were observed by Kuehn (1955a, b) although in his work the appearance of these structures followed contact between two relatively undifferentiated gametangia and the formation of short, irregularly branched hyphae. In contrast, early stages in the development of *Myxotrichum arcticum* ascomata involved the formation of peridial elements before gametangial initials could be distinguished (Tsuneda and Currah 2004). Tzean et al (1992) also describe coiled ascogonial initials of *Talaromyces unicus* Tzean, Chen & Shiu (Eurotiales) that branch profusely before forming ascogenous hyphae.

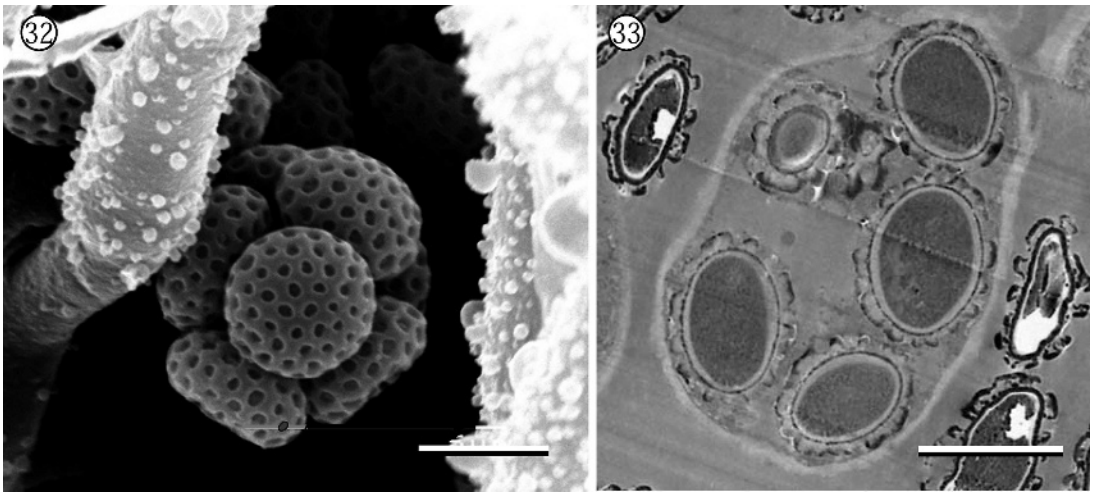
The asynchronous development of catenulate asci in this species differs markedly from ascogenesis in *M. arcticum* in which a branched network of croziers formed a hymenium of a single layer of asci that went on to mature synchronously. In his figures showing ascus formation in *A. conjugatum*, Kuehn (1955b) depicted two pyriform asci borne singly below the tip of a hypha terminated by a crozier that bears a younger developing ascus. The three asci, each at different developmental stages, are not connected to each other but are illustrated in a row with the most mature, spore-bearing ascus in the most proximal position and the most recently formed ascus at the distal end. It is possible that Kuehn saw catenulate asci but misinterpreted their ontogeny. Solé et al (2002b) illustrate what appear to be catenulate asci for both *A. concentricum* Solé, Cano & Guarro and *A. chlamydosporum* Solé, Cano & Guarro but make no explicit reference to ontogeny. Catenulate asci have been reported in other Eurotiomycetes including *Eupenicillium* (Emmons 1935), *Cephaloascus* (Dixon 1959, Wilson 1961), and *Talaromyces* (Stolk and Samson 1972, Tzean et al 1992) and in the mazaedial centrum of ascomata of the Caliciales (Tibell 2001). The evolutionary or adaptive significance of this mode of ascus formation is unknown.

There are few studies of crozier-free ascogeny (Read and Beckett 1996). However the mechanism of ascogeny of *Cephaloascus* proposed by Dixon (1959) and Wilson (1961) might be similar to that in *Auxarthron*. As in *Cephaloascus*, croziers mediate nuclear sorting at the base of ascus chains. Because croziers were not associated with the catenulate asci and because monokaryons (and not dikaryons) were observed in immature asci, the acropetal propagation of diploid nuclei by mitosis to acropetally forming asci (Dixon 1959, Wilson 1961) seems probable. Unlike Dixon (1959) and Wilson (1961), we observed that the asci matured from the base to the tip and that more distal asci were either tetranucleate and ostensibly postmeiotic or uninucleate and either premitotic or premeiotic (FIG. 19). Some terminal cells in chains of asci sometimes lacked nuclei, possibly because they had not received a diploid daughter nucleus from older, adjacent cells.

There are pronounced differences in ascospore morphology between *Auxarthron*, which has oblate to spherical, punctate ascospores, and *Myxotrichum*, which has fusiform to navicular, longitudinally ridged ascospores. At the ultrastructural level ascospore walls in *A. conjugatum* consist of four distinct layers with an innermost electron lucent primary wall enclosed by three secondary wall layers (terminology follows Read and Beckett [1996]). Ascospore walls in *M. arcticum* have a similar electron lucent primary wall but the secondary wall, which is furrowed to form a series of longitudinal ridges, comprises a single layer of electron dense material. There are relatively few ultrastructural studies of the ascospores of cleistothecial fungi (Garrison et al 1973) and it is unknown



FIGS. 20–31. Ascus dehiscence and germination of *Auxarthron conjugatum*. 20. A simple septum in an ascogenous hypha (arrow). 21. A chain of asci featuring inner (arrows) and outer (arrowheads) walls and a double septum (*). The rightmost ascus has not yet formed ascospores, the middle ascus contains immature ascospores with only their primary and first secondary spore walls (P, S1), while the leftmost ascus contains ornamented nearly mature ascospores with all spore walls (including S2, S3). 22, 23. Dehisced asci with dehiscence scars (arrows). 24, 25. Germinating spore-like bodies. 26–28.



FIGS. 32, 33. Details of ascosporogenesis of *Auxarthron conjugatum*. 32. Group of eight mature ascospores with characteristic reticulate pattern. 33. Ascus containing immature ascospores with all layers of spore wall but still without full pit development. 32 by SEM. 33 by TEM. Bars = 2.5 μm.

whether wall structural characteristics have any taxonomic or ecological significance.

The uninucleate, spore-like bodies produced in otherwise normal-looking chains of asci might represent a developmental aberration or a hitherto undescribed type of propagule. Three-nucleate stages were common in germlings and indicated mitotic rather than meiotic divisions were occurring during germination. If our scenario regarding ascus ontogeny is correct, the parent nucleus in the spore-like bodies would be diploid and resultant mycelia would be diploid. Mycelia arising from germinated spore-like bodies produced structures analogous to yet distinct from normal ascomata (FIG. 29). A similar phenomenon was reported by Elliot (1960) who observed that ascoma ontogeny in diploid strains of *Emericella nidulans* (Eidam) Vuillemin resulted in the production of a few abnormal asci dispersed among a large number of sterile elements.

The role these ostensibly diploid cells might play in the life cycle of this fungus is unclear although having three spore types (i.e. arthroconidia, ascospores and the larger, more robust and diploid spore-like bodies) would be expected to improve reproductive versatility under varying conditions. The tendency for the hydrophobic ascospores to adhere together in a relatively large group, thus forming an even larger dispersal unit, would offer still more versatility along with an increase in potency for dissemination (inoculum potential *sensu* Garrett [1970]). This would be especially effective if the reticuloperidial covering on this large propagule did indeed function as an impalement/attachment device for affixing the reproductive mass to an animal vector (Greif and Currah 2003).

In contrast to the similar appearance of the mature ascomata in species of *Auxarthron* and *Myxotrichum*, substantial differences can be observed during development. The distinctive reticuloperidial elements in both differentiate before the ascogenous tissue gives rise to asci. In *Auxarthron* the peridial elements encompass an ascogenous interior that gives rise to asynchronously developing asci that develop in short branching chains, each of which is subtended by a single crozier at its base. In contrast, in *Myxotrichum arcticum* the peridium is excipular in disposition, surrounding and overarching to some degree a hymenium containing synchronously developing asci, each of which is subtended by a crozier. The ascospore walls of *Auxarthron* are more complex than those of *Myxotrichum* but the significance of this difference is unclear in the absence of ultrastructural data for other Eurotialean and Leotialean species. In both cases, regardless of origin or developmental se-

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Germinating spore-like bodies; images are in order of germ tube maturity. Nuclei are clearly stained. Dividing nuclei are marked with an arrow. 29. Fruiting body of a culture derived from a spore-like body. 30, 31. Terminal swellings found on such a fruiting body. Bars: 20–23, 30, 31 = 2.5 μm; 24, 25, 27, 28 = 10 μm; 26 = 20 μm; 29 = 100 μm.

quence, the reticuloperidium and ascogenous tissues in each taxon function in a similar manner, at least in vitro (Greif and Currah 2003).

When forearmed with phylogenies based on DNA sequence comparisons, studies of development and morphology of ascomycetes using cytological and ultrastructural techniques have the potential to reveal valuable information about the selective pressures behind homoplasious characteristics. This approach also might uncover previously overlooked structural features, such as the spore-like bodies reported here, that might have considerable significance to the life history of these organisms.

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