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Ascomatal morphogenesis in *Myxotrichum arcticum* supports the derivation of the Myxotrichaceae from a discomycetous ancestor

A. Tsuneda[1]
R. S. Currah

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9 Canada

Abstract: Electron microscopy shows that ascomata of *Myxotricum arcticum* bear a striking resemblance to discocarps in morphogenesis and in previously overlooked aspects of gross morphology. Although mature ascomata of *M. arcticum* superficially resemble reticuloperidial cleistothecia common in the Onygenales, the bramble-like aggregation of thick-walled hyphae, previously considered to represent a closed peridium, forms a basket-like apothecium that overarches a distinct hymenium of stipitate, protunicate asci interspersed with paraphyses. There is no evidence of asci developing in chains and at different levels as is characteristic of the centrum of many Eurotiomycetes. Instead, more or less globose, stipitate and evanescent asci arise individually from penultimate cells of croziers and develop almost synchronously across a distinct hymenial layer derived from a richly branched network of crozier-bearing hyphae. After dissolution of the ascus wall, ascospores adhere to a membranous sheath that underlies the hymenium. These observations provide strong support for prior suggestions based on molecular phylogenetic comparisons that the Myxotrichaceae recently are derived from a helotialean ancestor. Observations of conidiogenesis show that the typical *Oidiodendron* anamorph is accompanied by a second conidiogenous form with ampullae and botryose clusters of blastic conidia.

Key words: conidiogenesis, discocarps, hymenium, Onygenales, ultrastructure

INTRODUCTION

The Onygenales (Eurotiomycetes, Eriksson et al 2003) traditionally has encompassed four cleistothecial families, Arthrodermataceae, Gymnoascaceae, Myxotrichaceae and Onygenaceae (Currah 1985). Many taxa are keratinophilic, produce morphologically simple arthroconidial states, and all four families host some genera in which the ascocarp possesses a mesh-like reticuloperidium (Currah 1985, Greif and Currah 2003) that encloses at maturity a mass of single-celled, hyaline, ascospores. The Myxotrichaceae was considered an atypical family in the Onygenales because of their cellulolytic abilities, more elaborate dendritic arthroconidial states in the genera *Oidiodendron* and *Geomyces,* and ascospores that are fusoid to navicular and usually markedly striate, rather than oblate, globose or allantoid and smooth or pitted. In addition to *Myxotrichum,* the Myxotrichaceae includes *Pseudogymnoascus* and *Gymnostellatospora* (Sigler et al 2000).

Based on some ecological and morphological similarities between the Myxotrichaceae and *Hymenoscyphus ericae* (Helotiales), Currah (1994, 1995) hypothesized that the family, in spite of having cleistothecial ascomata (and to some extent, arthroconidial anamorphs), might have stronger affinities to the inoperculate discomycetes than to the Eurotiomycetes. Some support for this hypothesis subsequently came from comparisons of 18S DNA sequence data from these and other ascomycetous taxa (Hambleton et al 1998a, Sugiyama et al 1999, Sugiyama and Mikawa 2001), but strong morphological and morphogenetic evidences were missing. Light microscopy (LM) shows that, in species of *Myxotrichum* at least, asci are subclavate and borne on a stipe thus differing from the more or less globose asci of Eurotiomycetes. Studies of the ascomata of the Myxotrichaceae using electron microscopy have been done with *Myxotrichum deflexum* (Rosing 1985) and *Pseudogymnoascus roseus* (Tsuneda 1982), but these investigations neither looked for nor revealed potential similarities to apothecial forms. There are no published developmental studies of the species of *Gymnostellatospora.*

During a search for additional morphological clues to the ancestry of the Myxotrichaceae, we re-investigated the ultrastructural aspects of ascomatal morphogenesis using a strain of *Myxotrichum arcticum* (Udagawa et al 1994). This species was selected because its ascomata and conidial state are easily produced in culture, are generally typical of other members of the genus and its affiliation with other species in *Myxotrichum* had been confirmed on the basis of DNA sequence analysis (Hambleton et al 1998b).

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[1] Corresponding author. E-mail: atsuneda@ualberta.ca

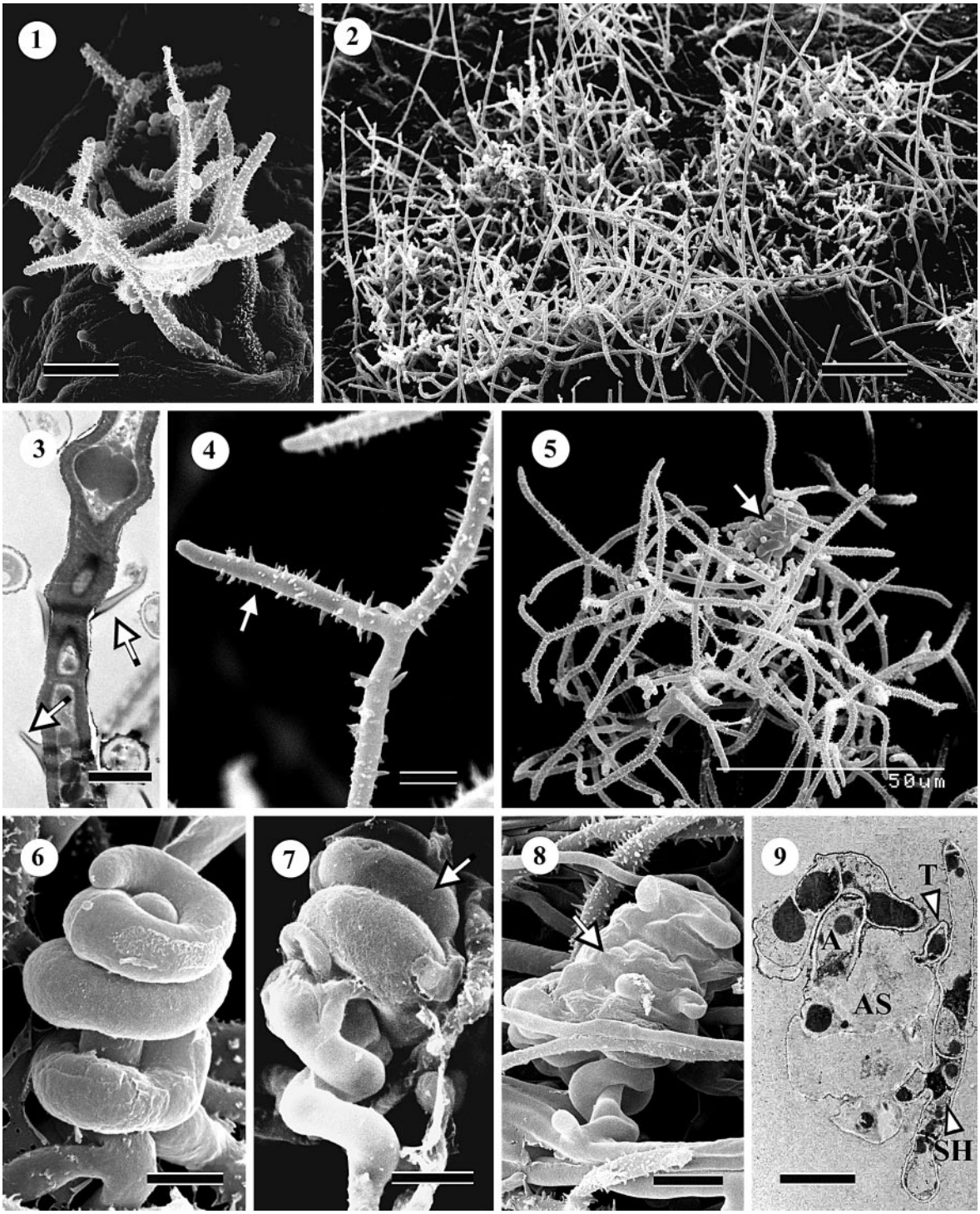
MATERIALS AND METHODS

Myxotrichum arcticum (UAMH 7565, GenBank No. AF062810) was grown on cornmeal agar (CMA; Difco, Detroit, Michigan) or cornmeal agar with dextrose (CMAD, Difco) at 20 C for 2–8 wk in the dark. For scanning electron microscopy (SEM), 5 mm agar disks containing ascomata were cut from cultures of different ages and either air-dried or critical-point dried. In the former method, samples were fixed overnight with OsO_4 vapor in a glass Petri dish at room temperature and gradually dried in a fume hood to prevent spores from dislodging. In critical-point drying, the agar disks were washed in phosphate buffer (pH 7.0) and fixed in 2% glutaraldehyde in buffer for 2 h at room temperature. After rinsing with buffer, these disks were immersed in 2% tannic acid-2% guanidine hydrochloride solution for 4–5 h, rinsed thoroughly in distilled water and postfixed overnight in 2% OsO_4 at 5 C. The fixed material was dehydrated in an ethanol series, taken to amyl acetate and critical-point dried in a Polaron E-3000 dryer using carbon dioxide. The dried samples were coated with gold 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), ascomata and conidiogenous structures of different developmental stages were fixed overnight in a solution of 2% glutaraldehyde and 2% OsO_4 in phosphate buffer (pH 7.3). The fixed samples were dehydrated in an ethanol series and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs of samples were taken at 75 kV with a Hitachi H-7000 electron microscope.

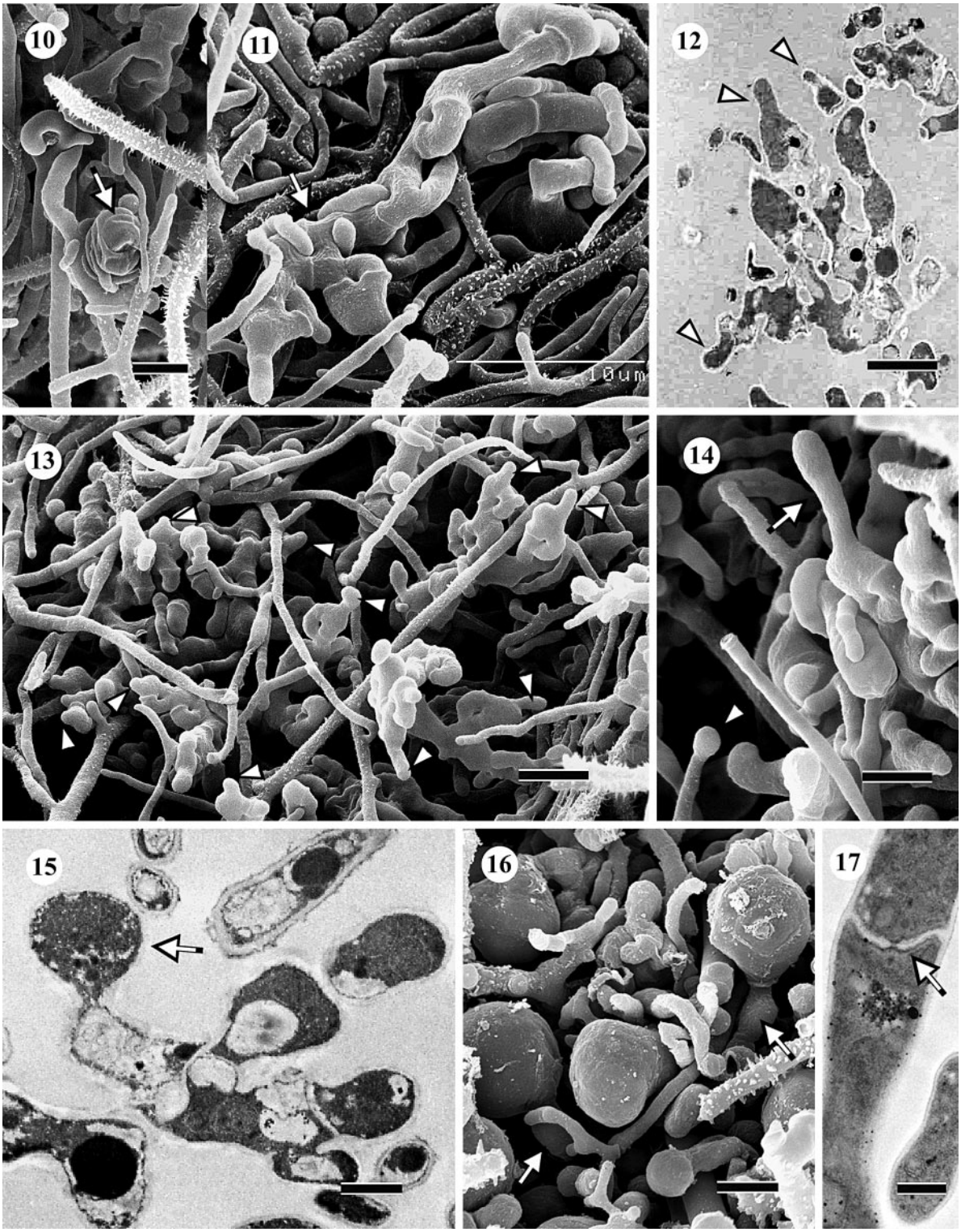
RESULTS

Ascomatal development.—The earliest signs of ascoma development occurred when hyphae, typical of the reticuloperidial elements of mature cleistothecia, arose from submerged vegetative hyphae (FIG. 1) and accumulated in more or less spherical masses that developed singly or in clusters (FIG. 2). These elements were branched, thick-walled, septate, sometimes swollen at the nodes, and often bore minute, spine-like projections (FIG. 3, arrows). Short appendages, which also were asperulate, extended slightly beyond the periphery of the individual hyphal masses (FIG. 4). Each spherical mass was associated with one ascogonium (more when spherical masses were in clusters) (FIG. 5, arrow), although they often were absent at very early stages of accumulation (FIG. 1). The gametangial apparatus consisted of paired hyphae that originated from different vegetative hyphae. One hypha of each pair wrapped around a club-shaped partner to form a coil (FIG. 6) that enlarged at its distal end (FIGS. 7, 8, arrows) to become an ascogonium. TEM of the gametangial apparatus shows what appears to be the antheridial cell (FIG. 9, A) and a trychogyne-like terminal cell (FIG. 9, T) arising from the ascogonium, but their roles in fertilization could not be confirmed. Slender sterile hyphae often arose from the basal cells of ascogonia (FIG. 9, SH) or from the parent hypha below the ascogonium, but these were not involved in peridium formation. Ascogenous hyphae radiated from the ascogonium and gave rise to croziers that grew out and branched to form additional croziers until an elaborate and distinct hymenial network was in place (FIGS. 10 and 11). Ascus mother cells arose from the penultimate cells of the croziers in this network (FIGS. 12 and 13, arrowheads), elongated (FIG. 14) and developed into short-stipitate, subglobose to ovoid asci (FIGS. 15 and 16); this process proceeded more or less synchronously throughout the hymenium. Smooth, slender, paraphyses that sometimes had swollen apices (FIGS. 14, arrowhead; 16) were interspersed among the asci. Paraphyses were thin-walled, possessed typical simple septa associated with Woronin bodies (FIG. 17), and were derived from the basal cells of the ascogonia (FIG. 9, SH) and their parental hyphae, as well as from surrounding peridial and smooth-walled vegetative hyphae. Under a dissecting microscope, hymenia at this stage were grayish white and the peridial hyphae above them frequently were sparse; thus ascomata looked like discocarps (FIG. 18). By SEM, numerous immature asci, interspersed with paraphyses, were visible throughout the hymenium (FIG. 19). Another striking feature was the presence of membranous sheath, revealed as an electron-dense line by TEM (FIG. 20, arrow), underlying the hymenium (FIG. 19, arrow). Immature ascospores were navicular and surrounded only by the primary wall, which was thick and smooth (FIG. 21, arrow). As the ascospores matured, a ridged secondary wall layer was deposited on the primary wall (FIG. 22). Epiplasm remained until late stages of ascosporogenesis (FIG. 22, E). As the asci evanesced, the released ascospores accumulated on the exposed hymenium (FIG. 23). Paraphyses also deliquesced. The released ascospores had longitudinal striations characteristic of the genus and were supported en masse on the underlying membranous sheath (FIG. 24). This sheath was clearly visible in ascomata in which ascospores had been washed free of the hymenium during preparation for critical-point drying (FIG. 25, arrow).

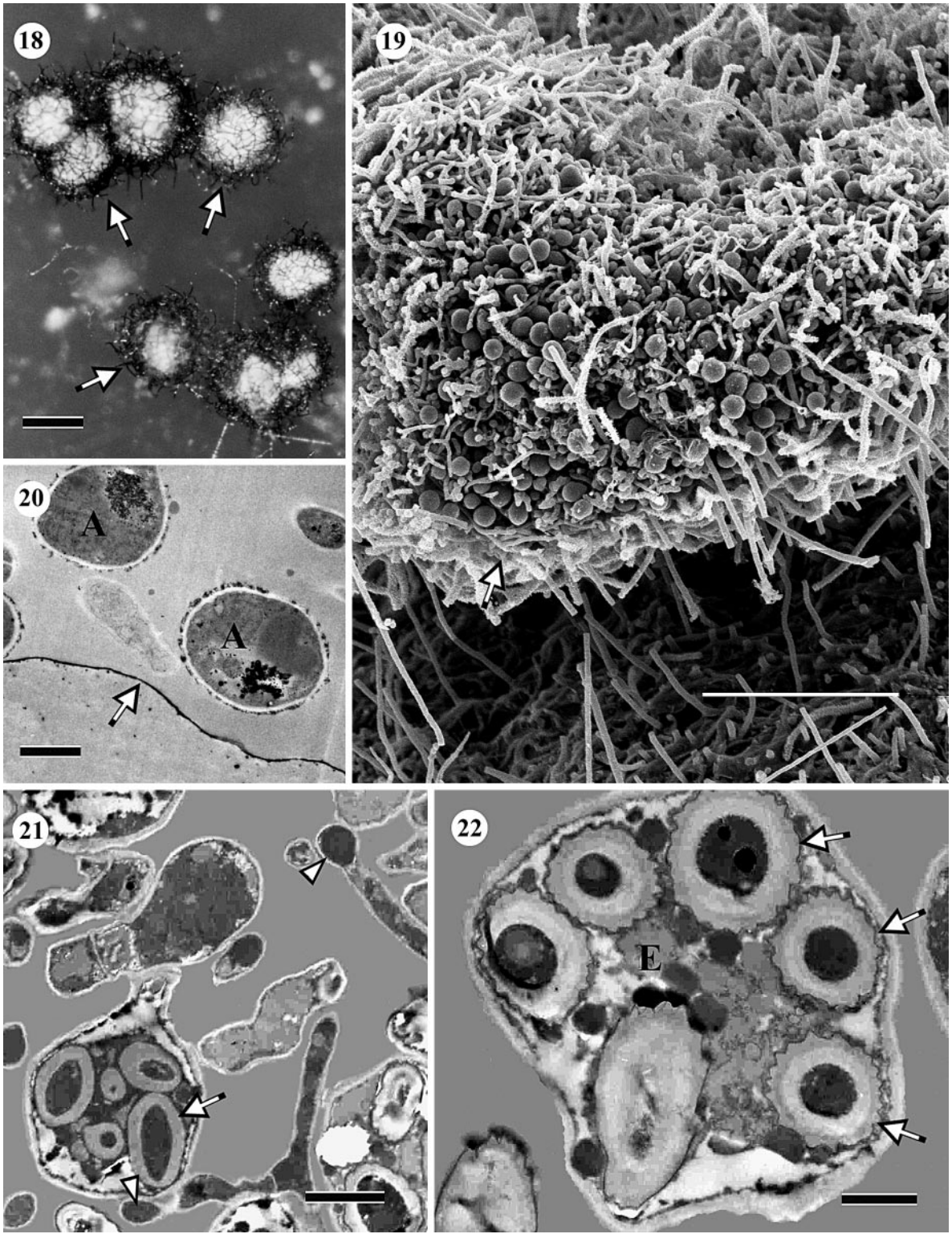
Conidiogenesis.—The primary mode of conidiogenesis was characteristic of *Oidiodendron*. Apices of dematiaceous conidiophores terminated in a complex branching head of fertile hyphae that subsequently segmented basipetally into arthroconidia (FIG. 26). Arthroconidia were slightly verrucose and often had distinct connectives between them (FIG. 27). Intercalary cells of conidiophores occasionally swelled to



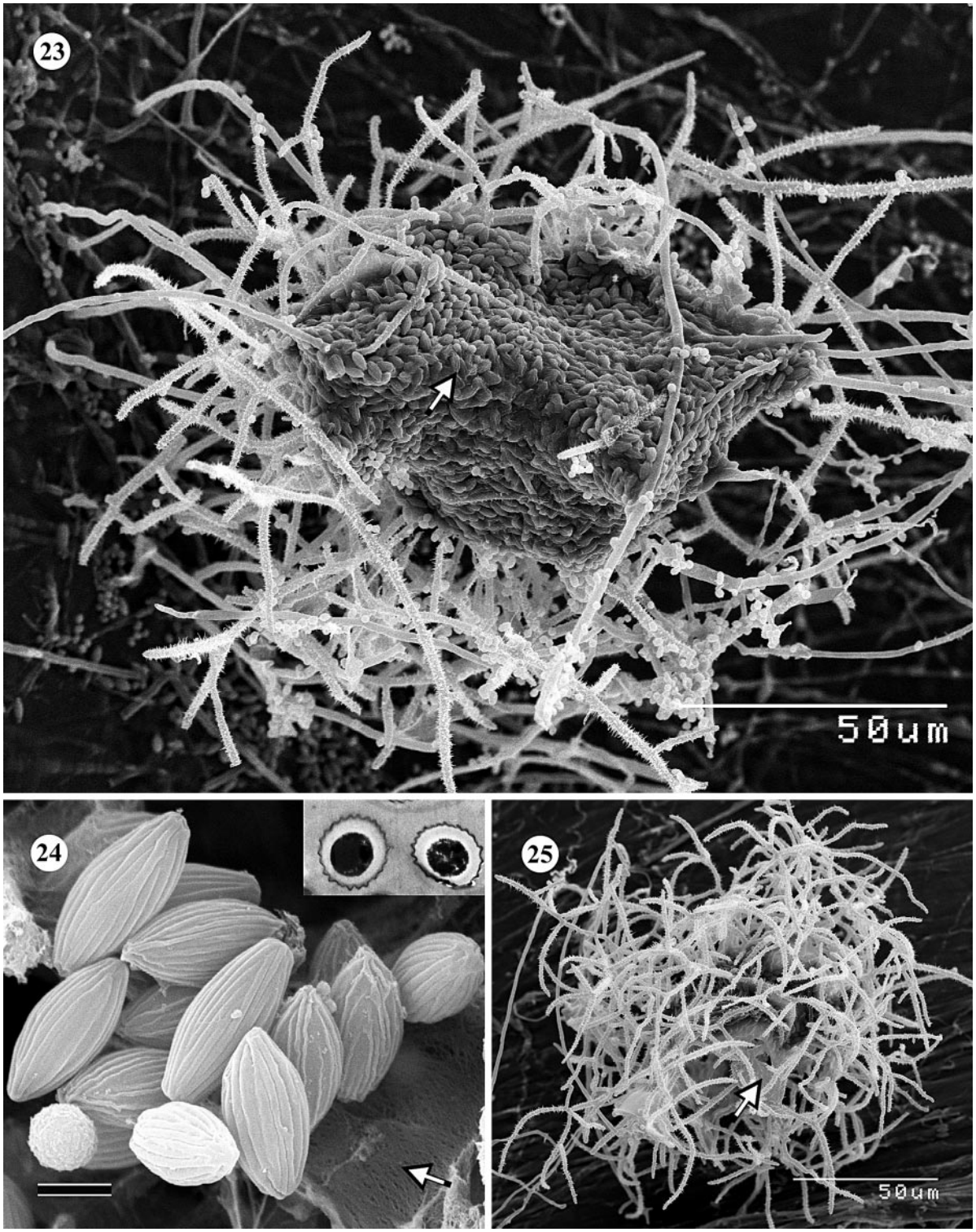
FIGS. 1–9. Early stages of ascomatal development in *Myxotrichum arcticum* on CMA. 1. Reticuloperidial hyphae arising from submerged vegetative hyphae. 2. Accumulating peridial hyphae. 3. TEM micrograph showing spine-like minute projections (arrows) on a peridial hypha. 4. Asperulate appendage (arrow). 5. Ascomatal initial with an ascogonium (arrow). 6. Gametangial apparatus. 7–9. Stages of ascogonial differentiation. Arrows in 7 and 8 indicate enlarging upper portions of gametangial structures. A = antheridium-like cell, AS = ascogonium, SH = Sterile hypha developed from the basal cell of ascogonium, T = trychogyne-like cell. Scale bars: 1 = 10 µm, 2, 5 = 50 µm, 3, 6 = 2 µm, 4, 7–9 = 3 µm.



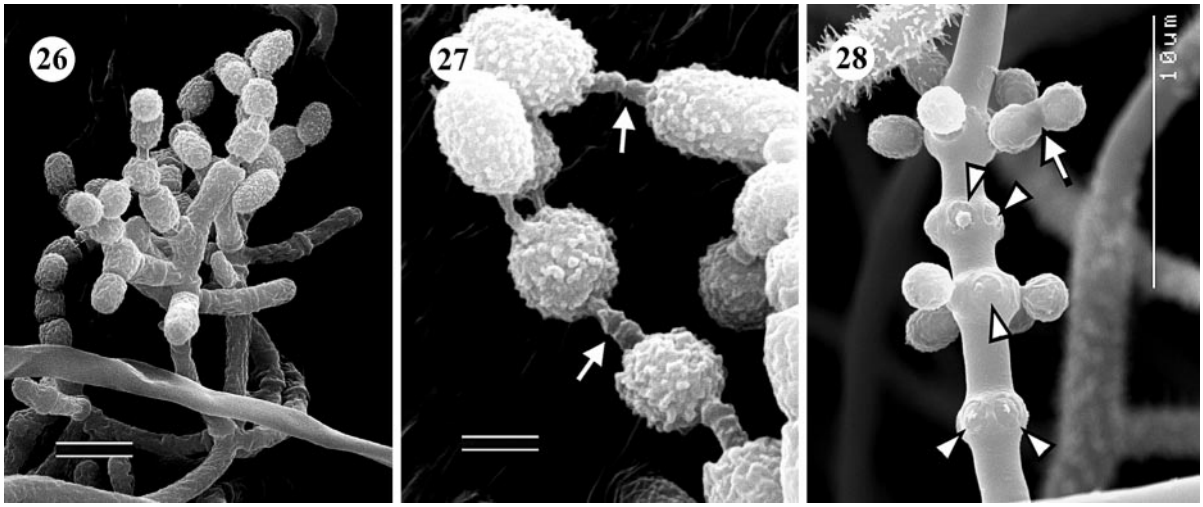
FIGS. 10–17. Developmental stages of ascogenous system in *Myxotrichum arcticum* on CMA. 10. Ascogonium (arrow) giving rise to ascogenous hyphae. 11. Ascogenous hypha extending horizontally with repeated formation of croziers and branching (arrow). Note that no asci have arisen from croziers at this stage. 12. TEM micrograph showing ascal cells arising from penultimate cells of croziers (arrowheads). 13. Ascogenous hyphae bearing many croziers. Ascal cells are arising almost synchronously from croziers (arrowheads). 14. Elongating ascal cell (arrow) and swollen apex of a paraphysis (arrowhead). 15. Enlarging ascus (arrow). 16. Near mature, stipitate asci (arrows) interspersed by paraphyses. Asperulate peridial hyphae are also seen. 17. Transverse section of thin-walled, paraphyses with a simple septum (arrow). Scale bars: 10, 12, 15, 16 = 5 µm, 11, 14 = 10 µm, 13 = 15 µm, 17 = 1 µm.



FIGS. 18–22. Maturing ascomata and ascosporogenesis in *Myxotrichum arcticum* on CMA (except 19, CMAD). 18. Dissecting-microscope view of well-expanded ascomata (arrows). Only sparse peridial hyphae overarch the hymenium. 19. Fully expanded ascoma showing hymenium consisting of horizontally arranged asci interspersed with paraphyses. Note the presence of a membranous sheath at the bottom of the hymenium (arrow). 20. Oblique section of a small area at the bottom of a hymenium showing immature asci (A) and an electron-dense line (arrow) that is equivalent to the membranous sheath in 19. 21. Developing ascospores with primary walls in an ascus (arrow). Arrowheads indicate paraphyses whose apices are swollen. 22. Ascus containing maturing ascospores on which ridged secondary walls have deposited (arrows). E = epiplasm. Scale bars: 18 = 100 μm, 19 = 50 μm, 20 = 3 μm, 21 = 2 μm, 22 = 1 μm.



FIGS. 23–25. Late stages of ascomatal development in *Myxotrichum arcticum* on CMA. 23. Ascoma bearing numerous released ascospores on the hymenium (arrow). 24. Released ascospores being held on the membranous sheath (arrow). Inset is a TEM micrograph of released ascospores. 25. Deserted ascoma. The membranous sheath persists (arrow). Scale bars: 23, 25 = 50 μm, 24 (including the inset) = 2 μm.



FIGS. 26–28. Conidiogenesis in *Myxotrichum arcticum* on CMA. 26. Typical dendritic conidiophores of the *Oidiodendron* anamorph bearing dry arthroconidia. 27. Connectives (arrows) between verrucose arthroconidia. 28. Conidiophore with ampullae bearing globose conidia. The arrow indicates two conidia developed from a conidiogenous locus. Distinct scars remain on conidiogenous cells where conidia have seceded (arrowheads). Scale bars: 26 = 3 μm, 27 = 1 μm, 28 = 10 μm.

form ampullae that gave rise to blastic, botryose clusters of conidia either singly or in short chains (FIG. 28, arrow). Secession scars remained on the conidiogenous cells where conidia had detached (FIG. 28, arrowheads).

DISCUSSION

Cleistothecial fungi, such as species of *Myxotrichum* and *Auxarthron,* with strikingly similar cage- or lattice-like peridia of rigid, thick-walled hyphal elements, once were placed in a single family, Gymnoascaceae sensu Benjamin (1956), the same subfamily, Gymnoasoideae (Benny and Kimbrough 1980) or even considered congeneric (Kuehn 1955a, b). Differences between these two genera in ascospore morphology and patterns in substrate degradation later were used as arguments to move the genera to separate families, i.e., the Myxotrichaceae and Onygenaceae, respectively, within the Onygenales (Currah 1985). A combination of morphological (Currah 1985, 1994) and molecular data (Hambleton et al 1998a, Sugiyama et al 1999, Sugiyama and Mikawa 2001) later indicated that the differences between these two families were substantial, with the Onygenaceae representing a clade among the true cleistothecial fungi and the Myxotrichaceae a group derived ostensibly from the inoperculate discomycetes through excipular modification and loss of forcible ascospore discharge. A rationalization of the similarity in peridial morphology based on developmental sequences between the representatives of these two lineages was sought, but few studies were available to allow the comparisons of ascocarps morphogenesis. The ascogonial apparatus of *M. arcticum* resembles that of *Emericellopsis microspora* (Hypocreales, incertae sedis, Eriksson et al 2003) in ultrastructure (see FIG. 2 in Wu and Kimbrough 1990) but differs in that vegetative hyphae arising from the base cells of ascogonia take part in the formation of peridium in *E. microspora,* whereas such hyphae become paraphyses of the hymenium in *M. arcticum.* In *Gymnoascus reessii* (Gymnoascaceae), a cleistothecial species with a reticuloperidium superficially similar to species of *Myxotrichum,* the peridial elements develop from the surrounding vegetative hyphae after the formation of gametangia, a sequence also observed in other cleistothecial fungi (Kuehn and Orr 1959, Wu and Kimbrough 1990). We found that the reticuloperidial hyphae of *M. arcticum* arise from vegetative hyphae and often are devoid of ascogonia in their initial stages of accumulation. This indicates that peridium formation in *M. arcticum* is induced by stimuli other than those associated with the presence of gametangial initials and may account in part for the ''sterile'' *Myxotrichum*-like cleistothecia formed by strains of *Oidiodendron maius* (Rice and Currah 2002).

A striking characteristic of *M. arcticum* ascomata is the presence of a distinct hymenial layer of paraphyses and stipitate asci that mature synchronously. During ascus development the penultimate cell of the first-formed croziers extend and branch to produce more croziers, each of which extends and branches repeatedly in the same way until a layer of richly branched ascogenous hyphae has formed. The penultimate cells of the most distal branches give rise to asci (FIGS. 10–14). This pattern of maturation and

disposition of mature hymenial elements is discomycetous (Henssen 1981). Prior reports of centrum development in *Myxotrichum* that diverge from this pattern were based on misidentified strains of *Auxarthron umbrinum* (as *M. emmonsii* and *M. thaxteri*), *A. conjugatum* (as *M. conjugatum*) and in *Gymnoascus uncinatus* (as *M. uncinatus*) (Kuehn 1955a, b) that had irregularly disposed asci as would be expected in true cleistothecial fungi. Other genera such as *Onygena, Talaromyces, Monascus* and *Auxarthron* (Tsuneda and Currah, unpubl data) form chains of asci that are randomly disposed in the centrum and which ripen consecutively rather than synchronously (von Arx 1981, Emmons 1935, Fennell 1973, Malloch and Cain 1971, 1972, Paden 1971, Spiltoir 1955, Wong and Chien 1986, Wu and Kimbrough 1990). Further studies of centrum morphogenesis in prototunicate taxa would refine and clarify these patterns and be useful to ascomycete systematics. Ascosporogenesis in *M. arcticum* is similar to that described in *M. deflexum* Berkeley (Rosing 1985) and in a number of Ascomycota (Read and Becket 1996, Wu and Kimbrough 1990).

It is unlikely that the striking similarity in the form of the mesh-like hyphae in the Myxotrichaceae and the Onygenaceae is an evolutionary coincidence, but its role in the reproductive fitness of these taxa is unclear. Currah (1985) suggested that the hooked or recurved appendages associated with many mesh-like peridial types in the Onygenales attach the ascocarps to the animal vector and the spaces between the peridial elements allow ascospores to sift out as the carrier travels from one habitat to the next. Summerbell (2000) suggested the mesh-like peridium, in species of *Arthroderma,* at least, could function as a deterrent to grazers. Greif and Currah (2003) proposed that the function of the lattice-like structure of the reticuloperidium in *Myxotrichum* and *Auxarthron* was to allow impalement of entire ascomata on arthropod setae and were able to demonstrate this mechanism in vitro. This model suggests that once impaled, and thus fastened to the surface of an arthropod, the adhering ascomata are carried away to new substrata and/or elicit a grooming response in the carrier that results in disruption of the peridium and more local dispersal of the meiospores. Our observations of ascoma development in *M. arcticum* suggest that water also might play a role in ascospore dispersal. Dehisced ascospores remain on the basal membranous sheath in mature undisturbed ascomata but are readily washed off with water. Ascospores in suspension or in water film might move or be moved very easily from one substrate to the next. The membranous sheath is reminiscent of pseudoepithecium occurring in discocarps of some Discomycetes (Tsuneda 1983).

Mitosporic states in *Oidiodendron* are the confirmed or putative anamorphs of species of *Myxotrichum* (Hambleton et al 1998b, Rice and Currah 2002). Morphologically similar taxa in the Onygenales, e.g., *Auxarthron,* are associated with *Malbranchea* states. If the reticuloperidium is a convergent feature in both onygenalean and helotialean lineages, why would both also have arthroconidial anamorphs? Have habitat and dispersal strategies also influenced the morphology of the mitosporic stages? Empirical evidence concerning the role of arthropods in the dispersal of arthroconidial states of the Onygenales is unavailable but some other unrelated arthroconidial taxa are known associates of insects (Tsuneda et al 1993). The role of airborne arthroconidia in the epidemiology of *Coccidioides immitis* (Onygenales) (Rippon 1988) is understood to be significant, and it is possible that air currents are effective means of dislodgement and carriage for these propagules. In *Myxotrichum* (and in species of *Pseudogymnoascus* and *Gymnostellatospora,* which also have arthroconidia) and in the Onygenales this conidial morphology simply may confer an advantage by letting the fungus exploit a variety of dispersal agents. In addition to the *Oidiodendron* anamorph, ampullae occurred as anomalous conidiogenous structures (referred to by Udagawa et al [1994] as a type of ''geniculate'' conidiogenesis). This type of pleomorphic conidiogenesis is distinct and is not found in other species of *Myxotrichum.*

In summary, ascomata of *M. arcticum,* in spite of their superficial resemblance to the cleistothecia of some onygenalean fungi, clearly are derived from a discomycetous ancestor in morphogenesis and in gross morphology. Unlike cleistothecia, fully expanded ascomata of *M. arcticum* are not entirely closed and possess a distinct, disk-like hymenium. Stipitate, protunicate asci and paraphyses constitute the maturing hymenium. Asci develop from croziers rather than in chains and mature almost synchronously throughout the hymenium. These results add to the argument that the Myxotrichaceae are derived from an inoperculate discomycetous ancestor (Currah 1994, Hambleton et al 1998b, Sugiyama et al 1999).

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