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ULTRASTRUCTURE OF THE ASCUS APICAL APPARATUS IN LEOTIA LUBRICA AND SOME GEOGLOSSACEAE (LEOTIALES, ASCOMYCOTINA)

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The ultrastructure of the apical apparatus and lateral ascus wall is compared in *Leotia lubrica* and five species currently placed in the Geoglossaceae. The lateral ascus wall consists of two layers, of which the inner one increases in thickness in the apical apparatus. Considerable differences in substructure of both layers are described. On the basis of general morphology of the apical apparatus, structure and PA-TCH-SP reactivity of the apical thickening and annulus, and occurrence of an annular protrusion four main categories are distinguished. A reactive annulus is demonstrated in the apical apparatus of all species, including *L. lubrica*.

The species studied are arranged as follows: Category 1a. *Geoglossum nigritum* and *G. cookeianum*; 1b. *Trichoglossum hirsutum*; 2. *Leotia lubrica*; 3. *Microglossum viride*; 4. *Mitrula paludosa*. Most fundamental is considered the position of the annulus in the apical thickening, either fully (category 1) or partly (2–4) occupying the apical thickening, either associated with an annular protrusion (3, 4) or not (1, 2). The data on the ultrastructure of the ascus apical apparatus and lateral wall, and mode of dehiscence indicate that *L. lubrica* takes an isolated position, distant from the other Leotioideae (including Ombrophiloideae) and the Geoglossaceae. *Geoglossum*, *Trichoglossum*, and *Microglossum* can best be maintained as separate genera in the family Geoglossaceae. The ultrastructural data of *M. paludosa* indicate closer affinity with Sclerotiniaceae than with Geoglossaceae.

INTRODUCTION

The family Geoglossaceae Corda accommodates some of the largest and most conspicuous forms of the inoperculate discomycetes (Leotiales S. Carp.). It has been the subject of several monographical studies and most of its representatives are relatively well-known (Durand, 1908, 1921; Imai, 1941, 1955, 1956; Nannfeldt, 1942; Mains, 1954, 1955; Eckblad, 1963). Before the fundamental significance of the structure of the ascus apical apparatus became fully appreciated, clavate and capitate inoperculate discomycetes were treated as close relatives of operculate discomycetes like *Helvella* s.l. (Rehm, 1896).

The macromorphology of the ascocarp has always been a character of major importance to the taxonomy of the Geoglossaceae. But other characters were investigated more closely by workers like Corner (1930), who compared the ontogeny and microanatomy of stipitate, clavate, and capitate (pileate) ascocarps. Imai (1941) emphasized the shape of spores and the fleshy vs. gelatinous consistency of ascocarps. The Leotioideae, erected by Imai for *Leotia* Pers. and *Neocudoniella* Imai, were transferred to the Helotiaceae by Korf (1958). He did so on the ground of similarities in anatomy and gelatinization of excipulum to certain Ombrophiloideae, particularly *Neobulgaria* Petr. Later on, Korf (1973) merged

the Ombrophiloideae into the Leotioideae. Maas Geesteranus (1964) emphasized the taxonomic importance of the structure of the stipe and the transitional region of stipe and hymenium. Under the influence of these and other relevant studies, most of the capitate genera were transferred from the Geoglossaceae to the Leotiaceae Corda (Helotiaceae sensu auct.) or Sclerotiniaceae Whetz. But many problems concerning the delimitation of genera, the position of the transferred genera, and the boundary between the Geoglossaceae on the one hand and the Leotiaceae and Sclerotiniaceae on the other remained unsolved (Benkert, 1983).

The structure of the ascus, another important source for informative characters, has hardly been exploited. In particular the ultrastructure of the apical apparatus may contribute to solving some of these problems. This study aims to determine how the ultrastructural characters of the present Geoglossaceae relate to those of *Leotia lubrica* (Scop.) Pers. and other Leotiaceae (Verkley, 1992, 1993b) and Sclerotiniaceae (Verkley, 1993a). Because the study mainly focuses on the relationships within the large family Leotiaceae, the number of taxa had to remain limited.

Most of these taxa have been the subject of earlier ultrastructural studies (Bellemère, 1975, 1977; Bellemère et al., 1987; Honegger, 1983). But, as explained in previous reports, the results are difficult to compare with those on Leotiaceae and Sclerotiniaceae obtained by Verkley (1992, 1993a, 1993b), and some important data are not yet clarified. For example, Bellemère (1977) and Honegger (1983) studied the apical apparatus in *Leotia lubrica*, but it remained unclear whether it contains an annulus like the apical apparatus in most other Leotiaceae. Bellemère (1977) and Bellemère et al. (1987) also studied the apical apparatus in selected Geoglossaceae, *Geoglossum* spec., *Microglossum viride* (Pers.) Gillet, *Spathularia flavida* Pers.: Fr., *Mitrula paludosa* Fr., and in *Heyderia abietis* (Fr.) Link, now residing in the Leotiaceae.

New and additional data are presented on ascus wall ultrastructure in *Leotia lubrica*, *Geoglossum nigritum* Cooke, *G. cookeianum* Nannf., *Trichoglossum hirsutum* (Pers.) Boud., *Microglossum viride*, and *Mitrula paludosa*. *Trichoglossum hirsutum* (Pers.) Boud. is investigated in this way for the first time. The implications of the data for the taxonomy of these fungi are discussed.

MATERIALS AND METHODS

Fresh material was collected in the field. Specimens were fixed and embedded in Epon as described earlier by Verkley (1992, 1993a). Ultrathin sections were cut using a diamond knife. Sections were either treated for PA-TCH-SP as described earlier (Verkley, 1992), or contrasted with uranyl acetate and lead citrate. Preparations were examined using a Philips EM 300 or Jeol JM 1010 electron microscope at 60 kV.

In the following list details are given about the origin of the collections, deposited in Leiden (L).

Geoglossum nigritum Cooke. Eiland van Rolfers, Amsterdamse Waterleidingduinen, prov. Noord-Holland, the Netherlands, in grassland, Oct. 1992, *G. Verkley 153*; Ruiterplaat, Noord-Beveland, prov. Zeeland, the Netherlands, Oct. 1992, *G. Verkley 141*.

Geoglossum cookeianum Nannf. Ruiterplaat, Noord-Beveland, prov. Zeeland, the Netherlands, in grassland with moss, Oct. 1992, *G. Verkley 140.*

Trichoglossum hirsutum (Pers.) Boud. Eiland van Rolfers, Amsterdamse Waterleidingduinen, prov. Noord-Holland, the Netherlands, in grassland, Oct. 1992, *G. Verkley 152.*

Leotia lubrica (Scop.) Pers. Fôret de St. Prix, Morvan, dép. de Côte d'Or, France, on the ground in mixed forest, Oct. 1990, *J. van Brummelen 7974.*

Microglossum viride (Pers.) Gillet. Payolle, dép. Hautes Pyrénées, France, on the ground amongst mosses, Oct. 1991, *J. van Brummelen 8020.*

Mitrula paludosa Fr. Roode Beek, Vlodrop, prov. Limburg, the Netherlands, on plant debris in running water, May 1990, *H. Huyser s.n.*; Smuddebos, Losser, prov. Overijssel, the Netherlands, June 1990, *F. Ligtenberg s.n.*

A detailed clarification of the terms used for wall structure and stages in ascus development including the corresponding terms used by Bellemère (1977) and Bellemère et al. (1987) has been given elsewhere (Verkley, 1992).

The circumscription of the apical chamber is extended as follows.

Apical chamber: amount of epiplasm enclosed to a variable extent by an annular protrusion, or enclosed by the most protruding part of an apical thickening which is fully occupied by an annulus (e.g. Fig. 4).

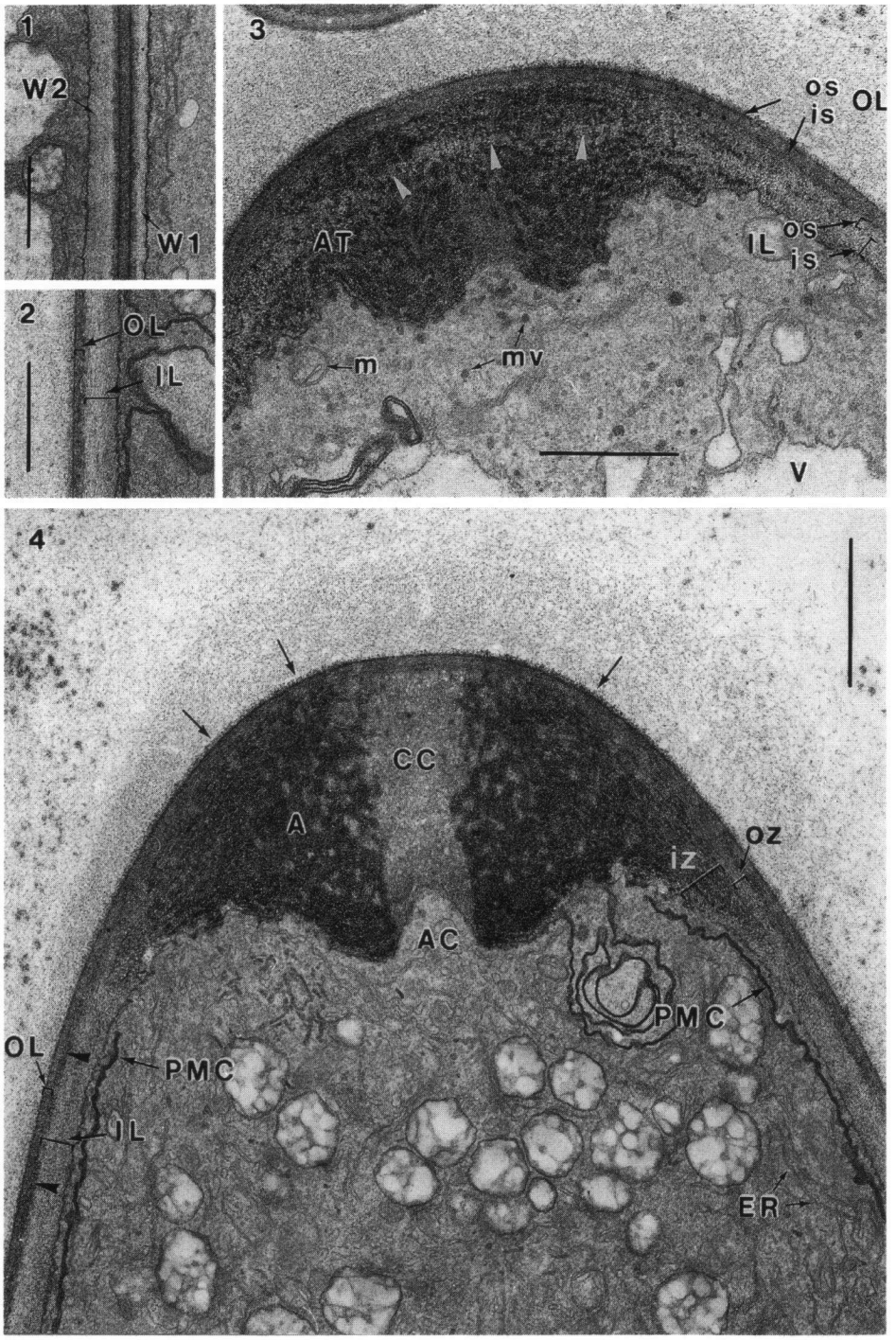
RESULTS

For reasons mentioned in earlier work (Verkley, 1992, 1993a, 1993b) the PA-TCH-SP technique was preferred over conventional staining of ultrathin sections for the study of wall substructure. The contrast obtained with this technique is based on the presence in the walls of PAS-positive (periodic acid-Schiff) polysaccharides. Free, vicinal hydroxyl groups of these polysaccharides are oxidized to aldehyde groups by PA. The addition of TCH to these aldehydes and the subsequent reduction of SP by thiocarbohydrazones results in a fine deposit of metallic silver on the thin sections, referred to as 'PAS-reactivity', or, simply, 'reactivity' in this paper. Since most cytoplasmic structures are insufficiently contrasted by this technique, post-staining with uranyl and lead salts was applied for closer study (Figs. 17, 18). Series of longitudinal median sections of young, immature, mature, and dehisced asci were studied. The lateral ascus wall, the apical apparatus, and some special features of the epiplasm are described.

GENERAL OBSERVATIONS

In the young, elongating ascus initial the apical cytoplasm contains a spherical area (circular in thin section) of microvesicles, surrounded by an area with a variable number of larger apical vesicles. During apex formation, when the apical apparatus is formed (Verkley, 1992), a large concentration of microvesicles containing reactive material is found in the cytoplasm in the direct vicinity of the apical wall (mv, Fig. 3).

The species studied all develop two layers in their ascus walls, of which the inner one increases in thickness at the apex. But the substructure and reactivity can differ considerably between species.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SPECIFIC DESCRIPTIONS

Geoglossum nigritum — Figs. 1–6, 38

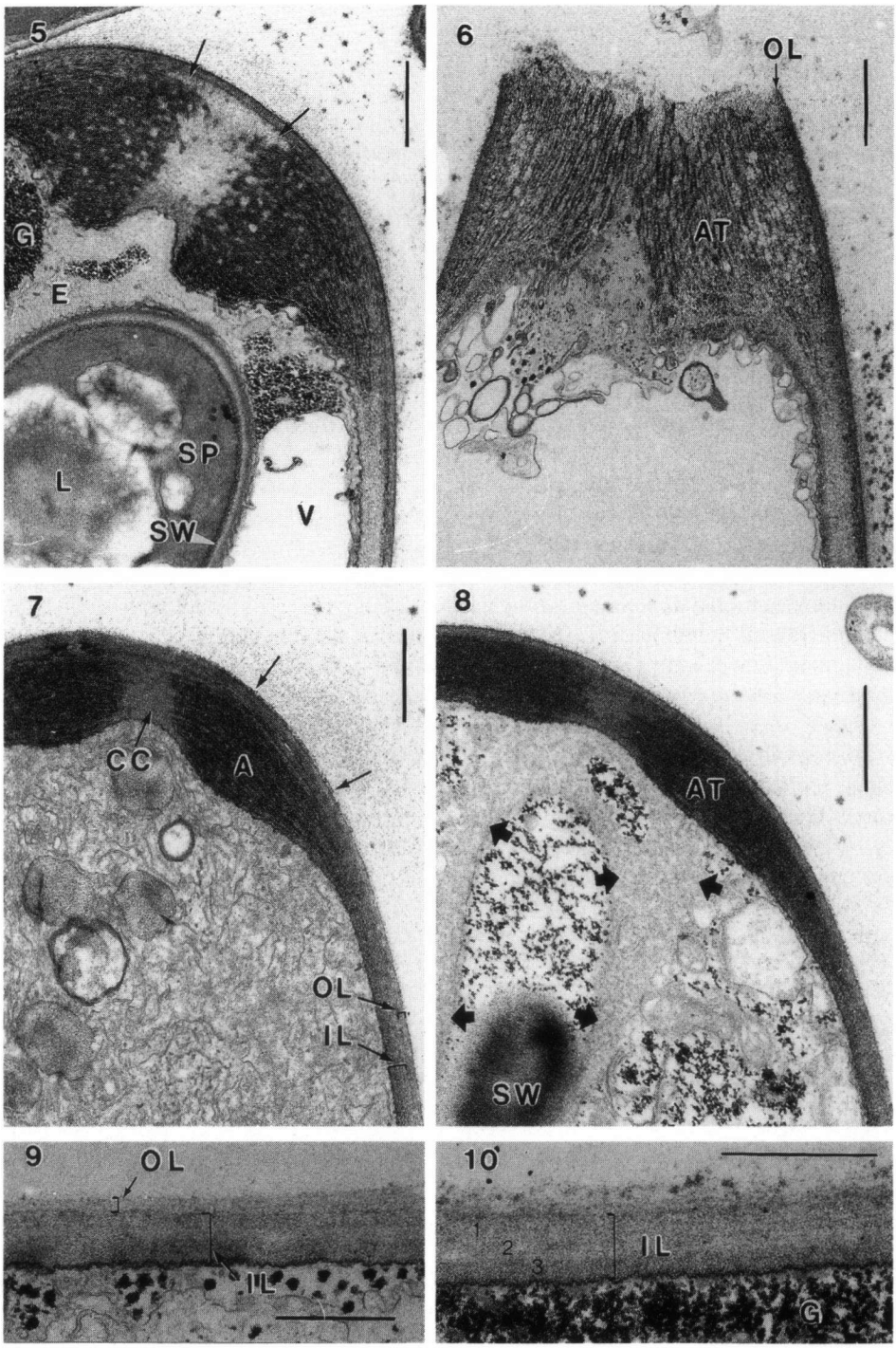
Lateral ascus wall – The ascus gradually tapers over a relatively extensive area in the upper part, and is characterized by a rounded apex. The outer layer, 90–110 nm thick, consists of a very thin, strongly reactive outer stratum and a much thicker, moderately to strongly reactive inner stratum (Figs. 2–4). Towards its inner side this stratum is locally delimited by a discontinuous line of more reactive material in cross section (arrowheads, Fig. 4). Over long distances of the ascus the outer stratum is closely associated with strongly reactive extra-mural material, which, especially over the apical apparatus, extends in bristle-like fibrils from the surface of the wall (arrows, Fig. 4). It represents a very thin layer, even at the apex. The diffuse, granular cap-like layer that covers this material over the upper part of most asci is also found over the ends of paraphyses. In between the hymenial and subhymenial elements a diffuse granulo-fibrillar matrix occurs, containing patches of more concentrated, granular reactive material.

In the young elongating ascus, before the start of apex formation, the outer layer is fully developed, both in reactivity and thickness (W1, Fig. 1). During the following phase of apex formation, when elongation caused by growth is minimized, the inner layer is thickened considerably (W2, Fig. 1). By the time the apex formation is fully completed and a continuous peripheral membrane cylinder (PMC, Fig. 4) is found over an extensive area near the plasma membrane, the inner layer reaches its maximum thickness of about 320–360 nm. The inner layer shows two strata (Figs. 2–4), of which the inner one is the more reactive.

Young ascus – The inner layer increases gradually in thickness over a relatively small area in the apical apparatus. Throughout the inner layer in the apical thickening strongly reactive material is found which constitutes a broad annulus. This annulus completely occupies the apical thickening. The reactivity pattern gradually changes towards the central cylinder (Fig. 4). In the part of the apical thickening most distant from the central cylinder discontinuous layers of strongly reactive material are oriented parallel to the inner face of the wall in a moderately reactive matrix (Fig. 4). Towards the central cylinder this layered pattern is transformed into a pattern in which the strongly reactive material is found in

Abbreviations used in Figures 1–42. **A**, annulus; **AC**, apical chamber; **af**, apical funnel; **AP**, annular protrusion; **AS**, ascospore; **AT**, apical thickening; **AW**, ascus wall; **CC**, central cylinder; **E**, epiplasm; **ER**, endoplasmatic reticulum; **G**, glycogen; **IL**, inner layer; **im**, investing membrane; **is**, inner stratum; **iz**, inner zone; **L**, lipid body; **M**, gelatinous matrix; **m**, mitochondrion; **ms**, middle stratum; **mv**, microvesicles; **N**, nucleus; **OL**, outer layer; **os**, outer stratum; **oz**, outer zone; **Pa**, paraphysis; **PMC**, periferal membrane cylinder; **SP**, sporoplasm; **SW**, ascospore wall; **tr**, tractus; **V**, vacuole. **Ws**, wall of seta.

Figs. 1–4. *Geoglossum nigritum*, electron micrographs of longitudinal median sections of ascus lateral walls and apices, treated with PA-TCH-SP (bar represents 1 μm). – 1. Lateral ascus wall of young, elongating ascus (before apex formation, W1) and neighbouring young ascus during apex formation (W2); 2. lateral ascus wall of young ascus after apex maturation has been completed; 3. young ascus, advanced stage of apex formation; 4. young ascus, apex formation completed.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patches. These patches are most concentrated in the part of the apical thickening that protrudes the deepest into the ascoplasm. This part is not an annular protrusion (Verkley, 1992), since the whole area of strongly reactive material is interpreted as the annulus. This changing pattern is so gradual that an annular and a non-annular region in the apical thickening cannot be distinguished. In mounts of material treated with Melzer's or Lugol's iodine solution (IKI) the whole apical thickening appears blue under the light microscope. The inner zone of the apical thickening, i.e. the part that is continuous with the inner stratum of the inner layer in the lateral wall (iz, Fig. 4), is conspicuous in the transitional region between subapical wall and apical thickening. In this region this zone contains strongly reactive material, while the outer zone (oz, Fig. 4), which is continuous with the outer stratum of the outer layer, does not. Such zonal differentiation is not observed further upwards within the inner layer material.

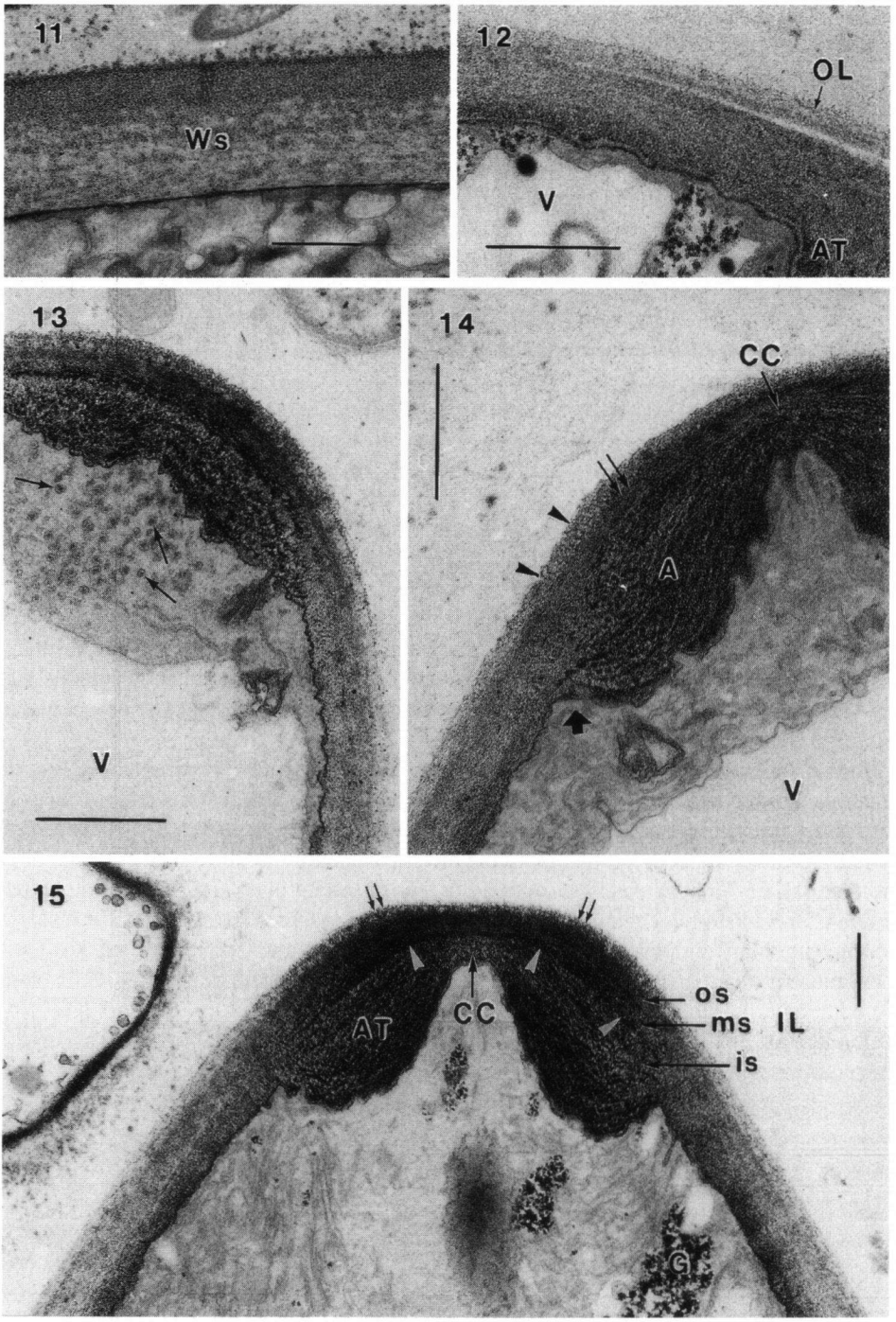
The central cylinder is moderately reactive, but may contain a variable amount of small patches of less reactive material. The boundary between inner and outer layer is particularly conspicuous here (Fig. 4). The invagination of the ascoplasm into the central cylinder represents an apical chamber (for adapted definition see 'materials and methods').

During apex formation there is still a single pattern of randomly distributed patches of strongly reactive material throughout the apical thickening. Only in asci at this stage a conspicuous zone of decreased reactivity is observed throughout the apical apparatus that is being formed (white arrowheads, Fig. 3). It appears to be in exact continuity with the inner stratum of the inner layer in the lateral wall, although it is somewhat thinner. This zone corresponds with the non-blueing zone which can be readily observed when living asci at this stage of development are treated with Lugol's iodine solution and studied with light microscopy (in *G. nigritum* and *G. cookeianum*). In the later stages of development this zone is not observed with TEM or LM.

Immature ascus – At first little change is observed. In some asci the reactivity in the upper part of the inner layer material of the central cylinder tends to decrease. Later on, particularly during formation of the secondary ascospore wall, the reactivity of the central cylinder decreases markedly, but not in the lower part delimiting the apical chamber or in the outer layer. The decrease in reactivity is also observed in a small zone in the upper region of the apical thickening that delimits the central cylinder (arrows, Fig. 5). It seems to be in continuity with the outer zone of the apical thickening mentioned earlier. The reactivity pattern of the apical thickening shows no further significant change. No erosion of the outer layer is observed.

Mature ascus – The apical apparatus is considerably compressed. No further change is observed.

Figs. 5–10. Electron micrographs of longitudinal median sections of ascus apices and lateral ascus walls, treated with PA-TCH-SP (bar represents 1 μm). – 5, 6. *Geoglossum nigritum*. 5. Immature ascus, advanced stage; 6. dehisced ascus. – 7, 8. *Geoglossum cookeianum*. 7. Young ascus, before ascospore delimitation; 8. immature ascus, advanced stage; the wall of the uppermost ascospore (SW) has been grazed; bold arrows indicate boundaries of a strand in the epiplasm (see specific description for details). – 9, 10. *Trichoglossum hirsutum*. 9. Immature ascus, lateral wall, showing intact outer layer; 10. idem, showing disintegrated outer layer.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Dehisced ascus – After dehiscence the apical thickening is everted over about a right angle. It appears as a rule that some remnants of the central cylinder remain attached to the wall (Fig. 6).

Geoglossum cookeianum — Figs. 7, 8

Lateral ascus wall – The ascus apex shows a shape similar to that in *G. nigritum*. The outer layer, 70–90 nm thick, consists of a very thin, strongly reactive outer stratum and a much thicker, less reactive inner stratum. The inner layer, 200–230 nm thick, also consists of two strata, of which the inner one is the more reactive (Fig. 7). Some reactive material is closely associated with the outer face of the apical wall (arrows, Fig. 7). The extra-mural matrix is similar to the one in *G. nigritum*.

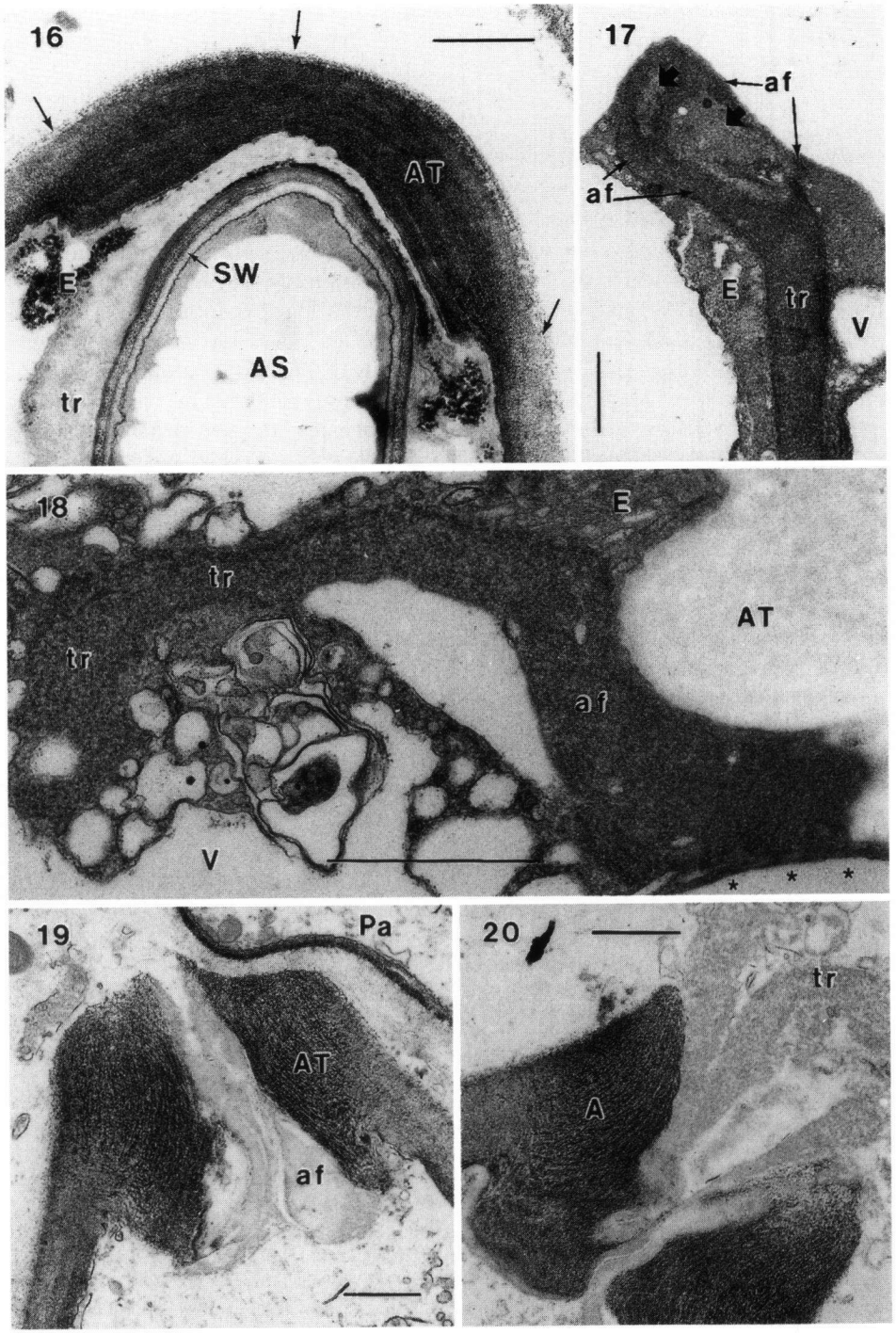
Young ascus – The inner layer increases gradually in thickness at the apex over a relatively small area (Fig. 7). In the apical thickening throughout the inner layer numerous layers of strongly reactive material occur within a moderately reactive matrix. These layers are oriented parallel to the wall surfaces. This reactive material, which constitutes a broad annulus, occurs in a single pattern. When treated with Melzer's or Lugol's iodine solution it appears blue under the light microscope. Randomly distributed patches of strongly reactive material are sometimes found throughout the annulus. The part of the apical thickening directly surrounding the central cylinder protrudes into the ascoplasm less profoundly than in *G. nigritum*. There is no annular protrusion, and the invagination of the inner face of the wall at the central cylinder represents a weakly developed apical chamber (Verkley, 1992). In the inner zone of the apical thickening, which is continuous with the inner stratum of the inner layer in the lateral wall, the reactive annular material occurs further downwards than in the outer zone. The central cylinder is moderately reactive.

Immature and mature ascus – Upon ripening of the ascus the apical apparatus is increasingly compressed (Fig. 8). No further change is observed.

In some asci, a strand was observed filled with moderately reactive tubular to granular material (e.g. the one indicated by bold arrows in Fig. 8). It is a continuous and branched strand from the inner face of the apical wall down to at least the second ascospore from the top. Over a relatively large area it is in close contact with the investing membrane, which separates the spore wall from the epiplasm. It seems to correspond to the refractive strand sometimes observed in water mounts with light microscopy. This strand, which seems to connect the apical apparatus and the upper one or two spores, is observed in mature or advanced immature asci only. The use of cotton blue in lactic acid or iodine solutions does not enhance its rather inconspicuous appearance.

Dehisced ascus – After dehiscence the apical thickening is everted over about a right angle.

Figs. 11–15. *Trichoglossum hirsutum*, electron micrographs of longitudinal sections of setal wall (Fig. 11), ascus apices and subapical wall, treated with PA-TCH-SP (bar represents 1 μm). – 11. Lateral wall of a seta in the hymenium (Ws); 12. immature ascus, advanced stage, outer layer coming loose from subapical and apical wall; 13. young ascus, early stage of apex formation, the central cylinder has not been grazed; 14. young ascus, semi-median section (central cylinder has not been grazed fully), apex formation completed; 15. immature ascus, middle stage, median section.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Trichoglossum hirsutum — Figs. 9–20, 39

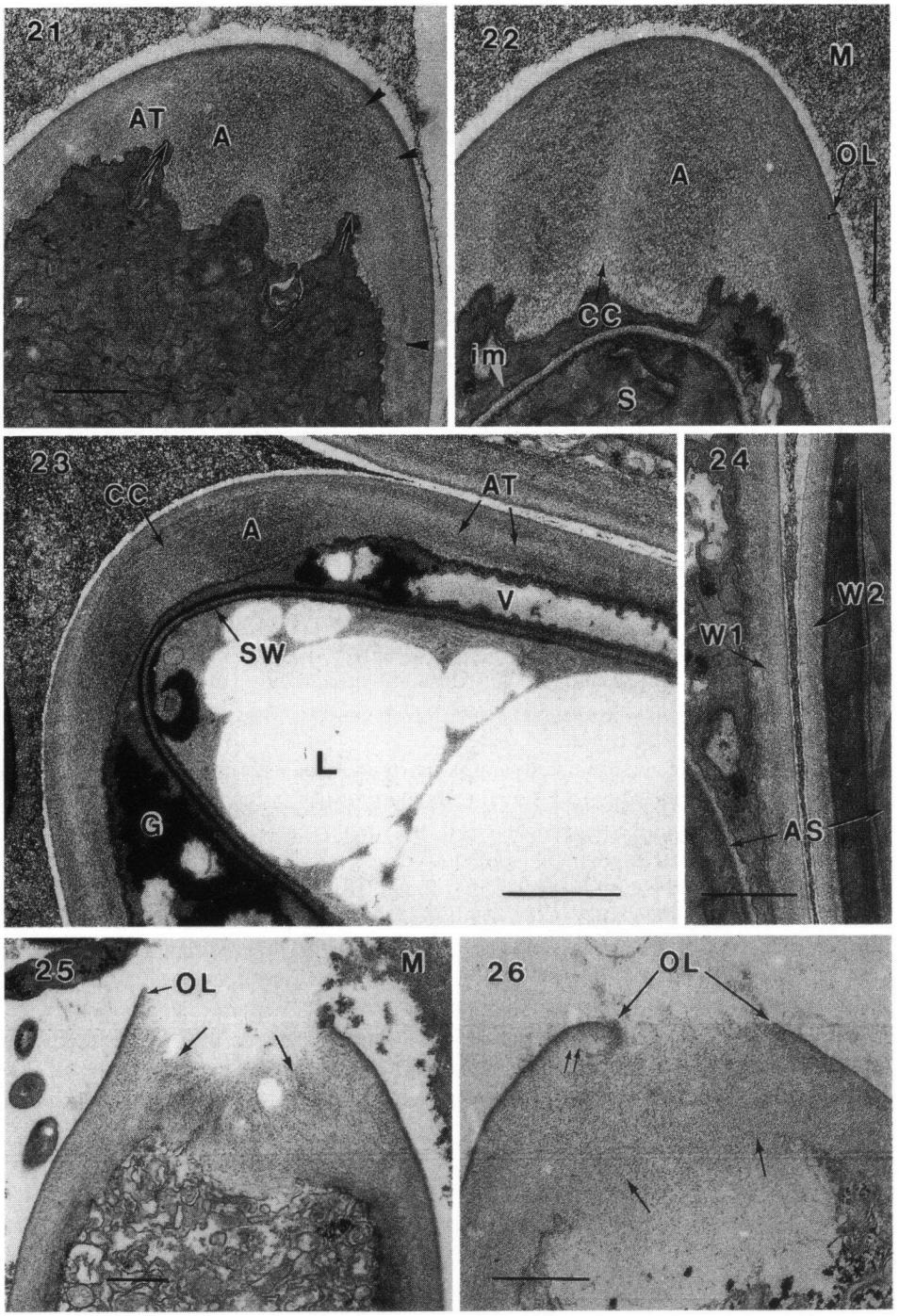
Lateral ascus wall – The ascus gradually tapers over a relatively extensive area in its upper part, and is characterized by a rounded to slightly flattened apex. The ascus wall consists of two layers. The outer layer, 160–200 nm thick, consists of a homogeneous, moderately to weakly reactive granular matrix (Fig. 9), reminding of a gelatinous matrix (semi-gelatinous). In general, the reactivity of this layer decreases during the development of the ascus. In large areas along the lateral wall it may disintegrate (Fig. 10) or, in the subapical region, come loose from the inner layer (Fig. 12). At the base of the ascus the outer stratum of the inner layer shows an increased reactivity, contrasting with the weakly reactive outer layer here, which does not participate in the basal septum of the ascus. Thus, it can be ascertained that the interpretation of the boundary of both layers in the lateral and apical wall is correct. In its more disintegrated form the outer layer misleadingly resembles an extra-mural gelatinous layer.

The inner layer, 650–750 nm thick, seems to consist of three strata, of which the middle one is the weakest, and the inner one usually the most reactive (Figs. 9, 10). A diffuse, fibrillar to fibrillo-granular matrix occurs between the elements of the hymenium and subhymenium. Throughout, small patch-like concentrations of reactive material are observed. These patches are contrasted significantly by uranyl acetate and lead citrate, especially when compared with the walls.

Young ascus – The young elongating ascus shows a rounded apex. The shape of the apex changes into the one described above when the ascus has reached about 90% of its ultimate length at maturity, and apex formation is started. During apex formation, numerous microvesicles containing strongly reactive material are observed in the ascoplasm near the apex (arrows, Fig. 13).

The inner layer in the apical apparatus firstly increases abruptly in thickness (bold arrow, Fig. 14), and then narrows again gradually towards the central cylinder. In all strata of the inner layer the matrix reaches its maximum reactivity in the apical apparatus (Fig. 14). The outer stratum is homogeneously and strongly reactive in the apical region and shows no layered pattern of reactivity (double arrows, Fig. 14; see also os, Fig. 15). The matrix of the middle stratum is less reactive. The inner boundary of the middle stratum is clearly delimited by a continuous thin layer of strongly reactive material in the inner stratum (white arrowheads, Fig. 15). The middle stratum may contain some discontinuous layers of strongly reactive material. But most of this material, which constitutes a broad annulus, is confined to the inner stratum of the inner layer. In this stratum the layered pattern of strongly reactive material is found throughout, and, especially towards the inner face of the wall, this pattern becomes more profound as the layers become thicker

Figs. 16–20. *Trichoglossum hirsutum*, electron micrographs of longitudinal sections of ascus apices, treated with PA-TCH-SP or post-stained with uranyl acetate and lead citrate (U/L) (bar represents 1 μm). – 16. Immature ascus, advanced stage, semi-median section (central cylinder has not been grazed); 17. mature ascus, showing apical funnel (af) and tractus (tr, U/L); due to very low contrast the ascus wall is not visible in this micrograph; 18. mature ascus; asterisks indicate the position of the ascospore during fixation; afterwards, this spore has considerably shrunken during dehydration (U/L); 19. dehisced ascus, showing remains of apical funnel (af); 20. idem, also showing remains of tractus (tr) outside ascostome.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and continuous over longer distances (Fig. 14). The annulus thus fully occupies the apical thickening, and there is no annular protrusion.

The central cylinder is a narrow structure, and is thinner than the lateral wall. Its reactivity pattern is much like the one found in the apical thickening. But in the inner stratum of the inner layer the strongly reactive material seems less densely arranged than in the apical thickening.

The material of the outer layer in the subapical and apical parts differs from that in the lateral part of the wall. It mainly consists of reactive fibrils oriented perpendicularly to the outer face of the inner layer (arrowheads, Fig. 14). Overall reactivity of this layer increases in the apical apparatus.

Immature and mature ascus – The outer layer becomes thinner over the apical apparatus as the spores mature (double arrows, Fig. 15). Only a diffuse layer of remnants of the outer layer is observed in advanced immature asci (arrows, Fig. 16). The outer face of the inner layer often appears disintegrated also. During secondary ascospore wall formation the apical apparatus becomes increasingly compressed, while the reactivity pattern of distinct, rough layers changes into a pattern of much finer layers. The apical thickening thus appears more homogeneously reactive and the strata less evident (Fig. 16).

During the formation of the primary ascospore wall numerous mitochondria, arrays of endoplasmatic reticulum and glycogen particles are observed in the epiplasm. From the beginning of secondary ascospore wall formation onwards, the organelles rapidly disintegrate and the number of glycogen particles decreases. The epiplasm becomes increasingly vacuolated, and a single, broad plasma strand, or a few of these, can be observed pending from the apical apparatus downwards (Figs. 16, 17), then branching again and making contact with the spores.

With light microscopy the epiplasma strands are also readily visible in water mounts of living or rehydrated herbarium material. The core of the epiplasma strands is highly light-refractive and stains very deep blue when cotton blue (0.2%) in lactic acid is added to the mounts. Thus, in the apical region a funnel-like structure is evident, which broadens towards the inner face of the apical wall and encloses one large or a few smaller circular areas which stain much less intensely. Further downwards it becomes a tractus, which branches to reach the surface of the spores.

With electron microscopy a tractus is demonstrated in the epiplasmic strands, consisting of relatively electron dense material in sections contrasted with uranyl acetate and lead citrate, most likely structural proteins (Figs. 17, 18). Its upper part, indicated as 'apical funnel' (af), is in close association with the inner face of the extremely electron-transparent apical thickening and central cylinder. The apical funnel includes one or a few areas of non membrane-bound epiplasm (bold arrows, Fig. 17). This epiplasm does not differ from that surrounding the tractus and apical funnel. In mature asci, the most apical spore presses against the funnel. Areas of close contact with the tractus are located at the proxi-

Figs. 21–26. *Leotia lubrica*, electron micrographs of longitudinal median sections of ascus lateral walls and apices, treated with PA-TCH-SP (bar represents 1 μm). – 21. Young ascus, shortly before ascospore delimitation; 22. immature ascus, early stage; 23. immature ascus, advanced stage; 24. lateral wall of the early immature ascus shown in Fig. 22 (W1) and a neighbouring, mature ascus (W2); 25. dehisced ascus, outer layer torn at the ascan length axis; 26. idem, but outer layer torn next to the ascan length axis.

mal end or somewhere at the lateral wall of the ascospores, and this appears not to be dependent on the position of the spore within the ascus.

Dehisced ascus – After dehiscence the apical thickening (annulus) is only slightly everted, as it is already at a small angle to the ascan length axis before dehiscence (Figs. 19, 20). The ascus opens at the central cylinder. Remnants of the apical funnel are found still attached to the inner face of the apical thickening, and also remnants of the tractus (tr, Fig. 20), which protrude through the opening. This can also be seen with light microscopy.

Leotia lubrica — Figs. 21–26, 40

Lateral ascus wall – The ascus tapers gradually over a relatively extensive area, and is characterized by a rounded apex. The thickness of the two layers of the ascus wall depends strongly on the stage of development. Mature asci are on average 30% longer than early immature asci when fixed, and about as long as the paraphyses. In mature asci the wall is on average 40% thinner than in early immature asci. Apex formation is started when the asci have reached about 50–60% of their ultimate length at maturity, as could be determined in 1 µm sections of embedded material.

The thickness of the outer layer varies considerably, from 50 up to 80 nm, depending on the stage of development (Fig. 24). The outer layer consists of a very thin, moderately to strongly reactive outer stratum, and a thicker, rather variably reactive inner stratum. The inner layer varies in thickness from 170 up to 300 nm, depending on the stage. The reactivity in the inner layer is even more variable than in the outer one and the results are therefore inconclusive as far as the internal stratification of the inner layer in the lateral wall is concerned. There seems to be an inner zone of increased reactivity, at least in the apical thickening.

A dense, strongly reactive, extra-mural matrix occurs up to the tips of immature asci. This strongly gelatinous substance appears to consist of an even denser granular matrix than the ascus wall inner layer. Mature asci may, in the fixed state, protrude beyond the irregular edge of the gelatinous matrix.

Young ascus – The inner layer thickens at first gradually over a relatively extensive area in the apical apparatus. Then, it increases in thickness more abruptly, forming a fierce protrusion of the wall in the annular region of the apical thickening at this stage (arrows, Fig. 21). The annulus consists of concentrations of reactive material that seem to form a striated pattern within a moderately reactive, granular matrix (Fig. 21). The annular region does not occupy the whole apical thickening, and it is restricted to the inner zone of the thickening (arrowheads, Fig. 21). With light microscopy no blueing is observed with iodine solutions. The central cylinder is moderately reactive in PA-TCH-SP. The outer layer is considerably thinner over the annulus and central cylinder, but in the young and following stages no significant erosion occurs.

Immature ascus – At first little change is observed (Fig. 22). But during secondary ascospore wall formation, the apical thickening is considerably compressed leaving at best an inconspicuous protrusion of the wall into the ascoplasm in the annular region (Fig. 23). The reactive material in the annulus becomes more distinctly arranged in layers.

Mature ascus – The apical apparatus is further compressed and it becomes evident that there is no persistent annular protrusion or apical chamber. The annular reactivity

decreases, apparently only shortly before dehiscence. Neither with LM nor with TEM level strand-like structures were observed.

Dehisced ascus – After dehiscence the apical thickening shows only little remnants of annular material. The angle of eversion is variable. The inner layer disintegrates rapidly due to excessive swelling (arrows, Figs. 25, 26), while the outer layer keeps its integrity. Over an area of variable extent the inner layer may become loosened from the outer layer (double arrows, Fig. 26). The opening is usually located at the central cylinder (Fig. 25), but in some asci it seems to be located at least partly in the annular region as well, thus next to the ascan length axis (Fig. 26).

Microglossum viride — Figs. 27–31, 41

Lateral ascus wall – The ascus is characterized by a rounded apex. Over the central cylinder a more or less distinct, rounded protuberance is observed (between bold arrows, Fig. 28). The outer layer, 50–70 nm thick, consists of a strongly reactive outer stratum and a considerably less reactive inner stratum (Fig. 27). The inner layer, 175–200 nm thick, consists of two strata, of which the inner one is more reactive than the outer one. In the outer stratum, however, the reactivity increases from the inner boundary outwards, the outermost part being more reactive than the inner stratum of the outer layer, especially in the subapical region of the wall (Fig. 28).

A diffuse to moderately dense matrix of reactive fibrillar material occurs between the elements of the hymenium, and nearly reaches the tips of mature asci.

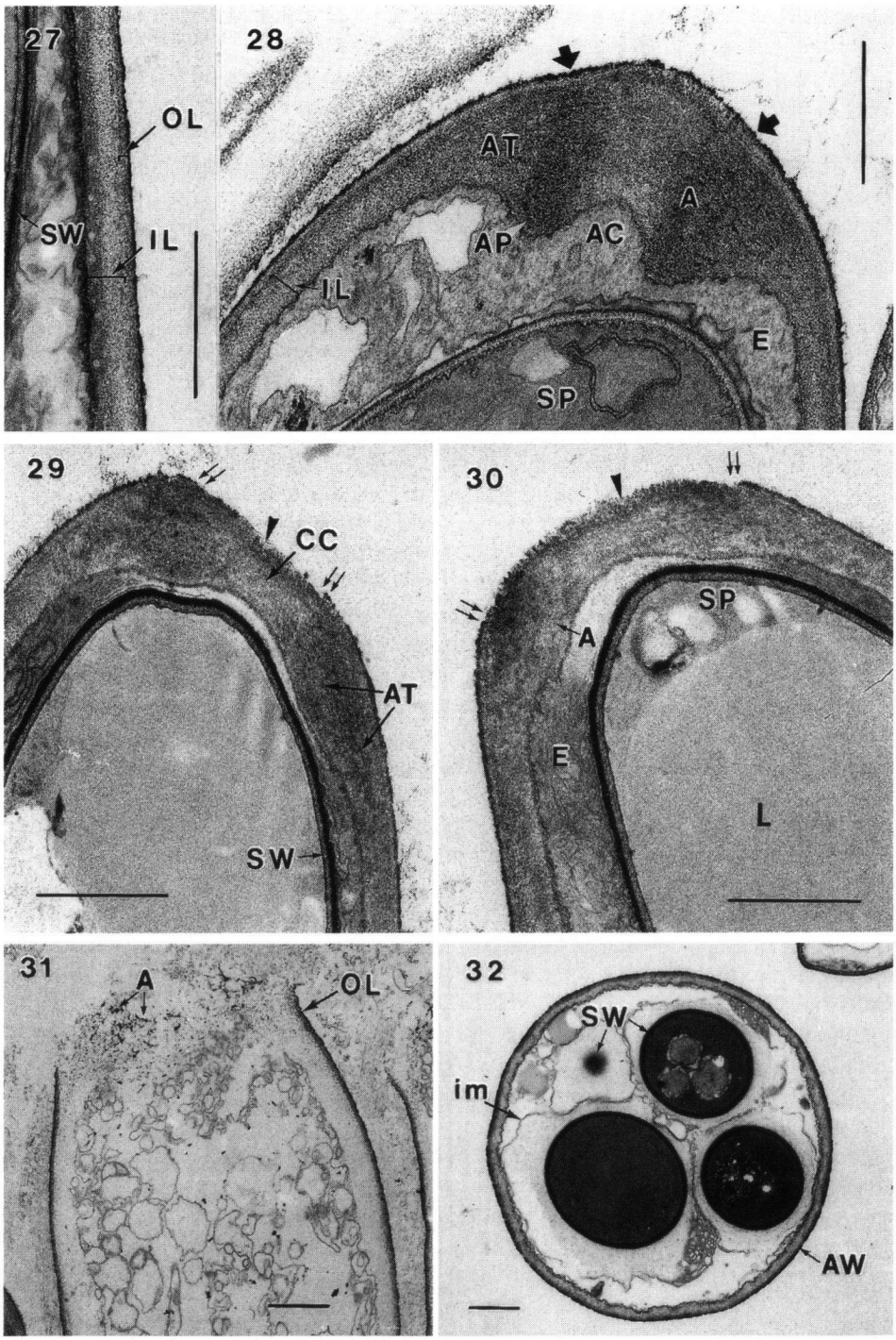
In the collection used for TEM, blueing of the apical apparatus by Melzer's reagent is quite variable, both in the non turgescent, fresh and rehydrated state. The upper part of the annulus blues weakly or not at all, irrespective of the stage of development. The lower part blues weakly in relatively mature and more strongly in young asci.

Young ascus – Not observed with TEM.

Immature ascus – The inner layer of the ascus wall increases gradually in thickness over a relatively small area in the apical apparatus (Fig. 28). The apical thickening consists of a moderately reactive, granular matrix. It is not fully occupied by the annulus, which consists of roughly granular, strongly reactive material. This material is arranged in a finely striated pattern to a greater or lesser degree. It is often the most concentrated in a narrow region directly surrounding the central cylinder and in the part of the apical thickening that is most protruding into the epiplasm. The latter part is interpreted as an annular protrusion, because the annulus is narrowing into it (Fig. 28). There is an apical chamber. The central cylinder consists of a moderately reactive, granular matrix.

The outer layer is present over the apical apparatus. In the protuberance above the central cylinder (between the bold arrows in Fig. 28), this layer is not more developed or differentiated in structure or reactivity than elsewhere in the apical apparatus. No special structural feature is observed in the inner layer which could be related to this protuberance. In advanced immature asci the apical apparatus is increasingly compressed, and in some of them the outer layer is already partly eroded over the central cylinder and annulus.

Mature ascus – The outer layer is eroded over the annulus and central cylinder (double arrows, Figs. 29, 30). The apical end of the uppermost ascospore is positioned against the central cylinder, which is bulging out. The annular protrusion, not a rigid but a flexible structure, can still be observed at this stage. It is more or less pushed aside and the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apical thickening as a whole is stretched. A further erosion of the exposed part of the inner layer seems to occur (arrowhead, Figs. 29, 30). The reactivity in the lower part of the annulus decreases.

Neither with light microscopy nor with electron microscopy strand-like structures were observed.

Dehisced ascus – After dehiscence the annulus is everted over about a right angle (Fig. 31).

Mitrula paludosa — Figs. 32–37, 42

Lateral ascus wall – The ascus tapers gradually over a relatively extensive area in the upper part, and is characterized by a rounded to truncate-rounded apex. The outer layer, 55–60 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, 145–165 nm thick, shows no stratification (Figs. 32, 33). Only in the subapical wall the reactivity of the inner layer increases, which makes it difficult to observe the boundary line of the two layers. The thickness of the wall decreases to a greater or lesser degree in the subapical region (Figs. 34, 35).

Reactive, fibrillo-granular material covers the apices of most asci. It is found to be continuous with similar material, which covers the ends of paraphyses throughout the hymenium. Further downwards between the elements of the hymenium a similar, but much more diffuse matrix is observed.

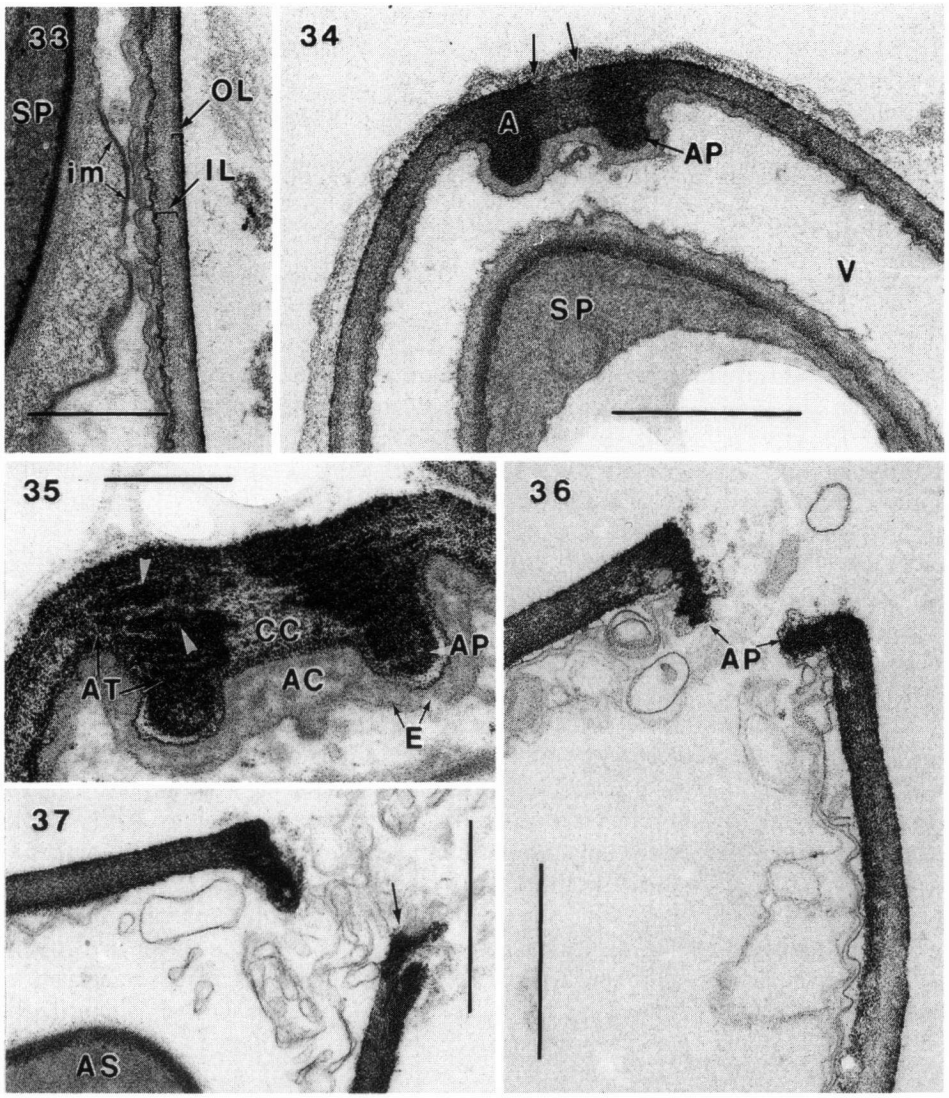
Young ascus – Not observed.

Immature ascus – The inner layer increases in thickness abruptly in the relatively small apical apparatus. An important part of the apical thickening consists of a well-developed annular protrusion, which surrounds an apical chamber. Here, the annulus consists mainly of a homogeneous, strongly reactive matrix. More upwards the annulus broadens to occupy the larger part of the apical thickening. There, it usually consists of densely packed layers of strongly reactive material in a moderately reactive matrix (white arrowheads, Fig. 35). However, in some asci a homogeneous pattern is observed throughout the annular region.

The central cylinder is much thicker than the lateral wall and consists of a moderately reactive granular matrix. In most asci the annular protrusion takes an abaxially oriented position, that is, in median sections pointing away from the axis (Figs. 34, 35), rather than pointing downwards or towards the axis, as observed in Sclerotiniaceae (Verkley, 1993a). In some asci, the outer layer is partly eroded over the central cylinder (arrows, Fig. 34).

Mature ascus – No significant change is observed. The annular protrusion is obviously a persistent and rigid structure. No strand-like structures were observed, neither with LM nor with TEM.

Figs. 27–32. Electron micrographs of longitudinal median sections of ascus lateral walls and apices, and transverse section (Fig. 32), treated with PA-TCH-SP (bar represents 1 μm). – 27–31. *Microglossum viride*. 27. Lateral ascus wall of an advanced immature ascus; 28. immature ascus, early stage; 29. mature ascus, advanced stage; 30. idem; 31. dehisced ascus, rather deteriorated; a strong reduction of reactivity of the inner layer as shown here is typical for somewhat older, dehisced or severely damaged asci of any species. – 32. *Mitrula paludosa*, transverse section of mature ascus.



Figs. 33–37. *Mitrula paludosa*, electron micrographs of longitudinal median sections of ascus lateral wall and apices, treated with PA-TCH-SP (bar represents 1 µm, except in Fig. 35, bar equals 0.5 µm). – 33. Immature ascus, advanced stage, showing lateral ascus wall and ascospore; 34. immature ascus, advanced stage; 35. idem; 36. dehisced ascus; 37. idem, showing a partly damaged annular protrusion (arrow).

Dehisced ascus – After dehiscence the annulus is everted over an angle of about 45–60°. The annular protrusion is normally still intact (Fig. 36), but in a minority of the asci observed it is damaged to a lesser or greater degree (arrow, Fig. 37), or even partly detached from the rest of the apical thickening. No remnants of the central cylinder are found.

DISCUSSION

As in previous studies on Leotiales (Verkley, 1992, 1993a, 1993b) the lateral ascus wall is basically two-layered in the species studied at present.

The organization of the apical cytoplasm of young, elongating asci agrees with that observed in Sclerotiniaceae and most Leotiaceae studied (Verkley, 1993a, 1993b). After the apical apparatus has been formed, from ascospore delimitation onwards, a further increase in length during maturation of the ascus is observed in material processed for TEM. In the Sclerotiniaceae (Verkley, 1993a) an increase of approximately 5 to 20% was measured between young and mature asci in fixed and embedded material, depending on the species. The 30% increase observed in the asci of *L. lubrica* thus seems comparatively high. The amount of decrease in thickness of the lateral wall, which is observed at the same time, was not found before. The variability in reactivity, the lack of internal stratification and especially the extreme swelling occurring after dehiscence are indications for a highly gelatinous inner layer in this species. *Mitrula paludosa* also shows a non-stratified inner layer, but this one is very thin. It needs to be stated here, that these percentages of length increase are less important than the increase exhibited by turgescent, living asci. Shrinkage caused by loss of turgor and dehydration during processing is relatively strong and may be dependent on structure and composition of the wall.

The apical apparatus

Diagrammatic schemes of the apical apparatus and subapical wall are given in Figs. 38–42. In the left half of each scheme relative reactivity in PA-TCH-SP is depicted, while in the right half interpretation of the wall stratification and range of the annular region is shown. The general shape of the apex of fixed asci does not as a rule differ significantly from that observed in living asci with light microscopy.

On the basis of the general morphology of the apical apparatus, the structure of the annulus, and the presence of an annular protrusion four categories can be outlined. The most important characters of these categories are summarized below.

1. Apical thickening fully occupied by a (partly or throughout) distinctly layered reactive annulus, no annular protrusion:

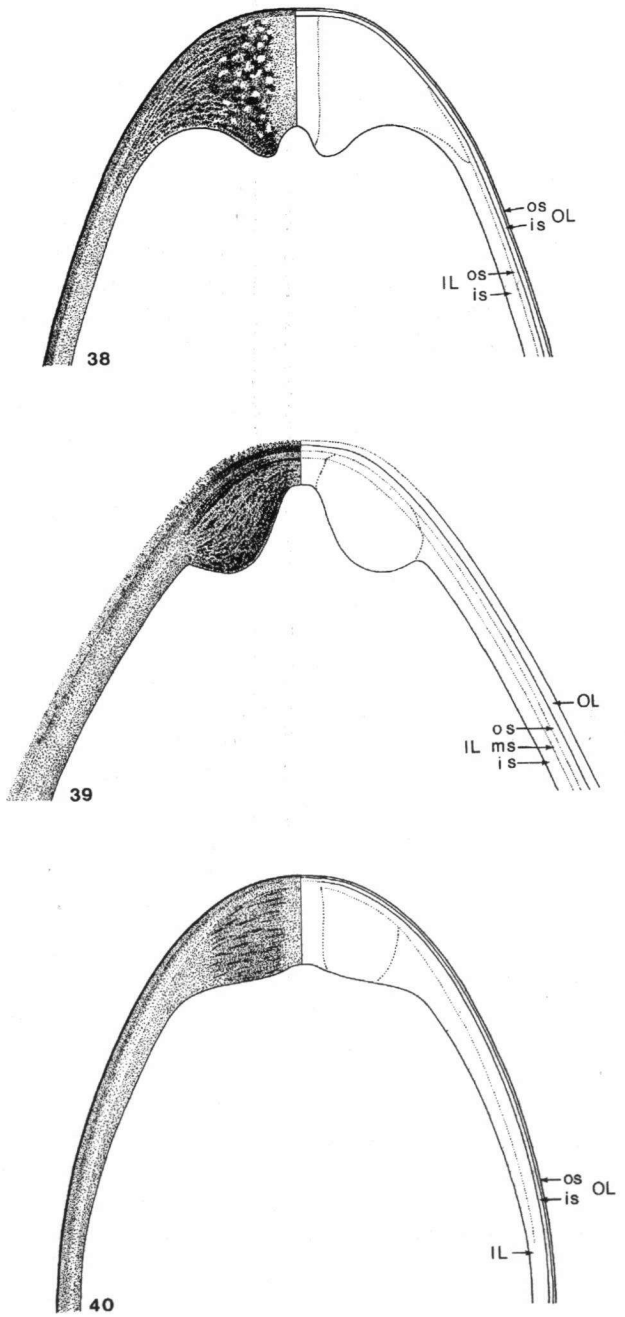
a. The thickest part of the apical thickening directly surrounding the central cylinder (proximal), and enclosing an apical chamber, outer layer consisting of two strata: *Geoglossum nigritum* (Fig. 38) and *G. cookeianum.*

b. The thickest part of the apical thickening distant from the narrow and relatively thin central cylinder (distal), outer layer semi-gelatinous, without internal stratification: *Trichoglossum hirsutum* (Fig. 39).

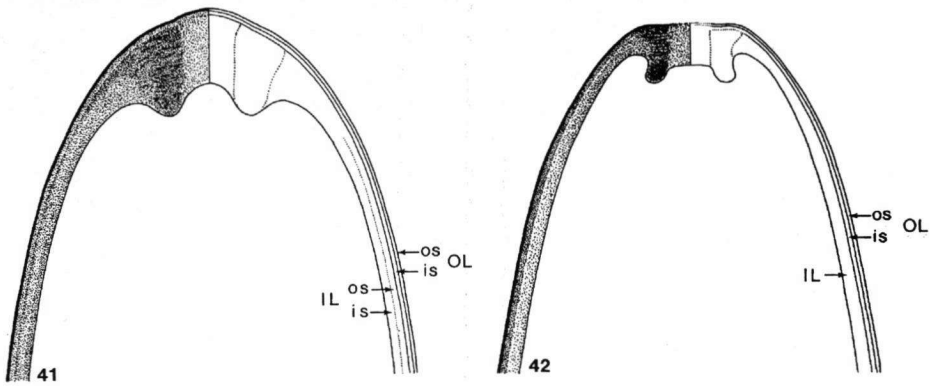
2. Apical thickening extending far downwards, only the larger part of the well-developed upper region of the thickening occupied by a broad, diffusely layered reactive annulus, no persistent annular protrusion: *Leotia lubrica* (Fig. 40).

3. Apical thickening well-developed and not fully occupied by a relatively broad, homogeneously and finely layered reactive annulus, which narrows into a flexible annular protrusion: *Microglossum viride* (Fig. 41).

4. Apical thickening mainly consisting of a strongly developed, rigid annular protrusion, the subapical wall thinner just under the apical apparatus, and annular reactivity usually differentiated: *Mitrula paludosa* (Fig. 42).



Figs. 38–40. Diagrammatic schemes of ascus apical apparatus and subapical wall, showing relative PATCH-SP reactivity in the left half, and interpretation of layers and strata, and range of the annular region in the right half of each scheme. – 38. *Geoglossum nigritum*, young ascus. – 39. *Trichoglossum hirsutum*, young ascus. – 40. *Leotia lubrica*, immature ascus.



Figs. 41, 42. Diagrammatic schemes of ascus apical apparatus and subapical wall. – 41. *Microglossum viride*, immature ascus. – 42. *Mitrula paludosa*, immature ascus.

The changes in apical wall structure and reactivity during the ripening of the ascus, which were indicated as 'apex maturation' (Verkley, 1992), seem to be of little diagnostic value for the group of species under study. The most important changes are summarized in Table I. Little change in reactivity occurs and there are no distinctive patterns to be recognized, as in 19 species of the Hymenoscyphoideae (Leotiaceae, Verkley 1993b). On the other hand, in most of the studied species of Ombrophiloideae (Verkley, 1992) and Sclerotiniaceae the apex maturation pattern has a diagnostic value (Verkley, 1993a).

Geoglossum

There are some differences between *G. nigritum* and *G. cookeianum* in the shape of the apical thickening, reactivity pattern of the annulus, and apex maturation (Table I). They confirm the separation at the level of the species, which is mainly based on morphology of the paraphyses. *Thuemenidium* Kuntze, which differs mainly by its colourless ascospores, may be closely related to *Geoglossum* (Eckblad, 1963; Benkert, 1983), but its apical apparatus has not been investigated with TEM.

The ultrastructure of the apical apparatus was studied by Bellemère (1975, 1977) in a '*Geoglossum* sp.', and referred to the 'type *Pezicula*'. This type is characterized by a well-developed c-layer (= outer stratum of the inner layer?), a thinner d-layer, shown in scheme Fig. 1A (Bellemère, 1977), but described as 'epaisse' in the text, and a distinctly layered, strongly reactive annulus occupying a rather narrow part of the apical thickening, in contrast to the electron micrograph Fig. 1B (Bellemère, 1977), where it occupies the whole apical thickening. However, the present results show that the inner stratum is thicker than the outer one in the inner layer of the lateral wall and in the apical thickening of *G. nigritum* and *G. cookeianum*. The proportions of the annulus seem to be comparable to those in *Pezicula*, as is the presence of a thin extramural layer of perpendicularly oriented, reactive material, indicated as 'fine periascus' by Bellemère (1977).

Table I. Survey of the main changes in reactivity related to apex maturation of the species studied, and erosion of the outer layer over the apical apparatus.

Species	Changes in reactivity		Erosion
	apical thickening or annulus	central cylinder	OL
Geoglossum nigritum	decrease in upper, narrow region	decrease	o.s. only
Geoglossum cookeianum	none	none	no
Trichoglossum hirsutum	none	none	yes
Leotia lubrica	annulus, decrease (at mature stage)	none	no
Microglossum viride	annulus, decrease in lower part	none	yes
Mitrula paludosa .	none	none	yes/no

Bellemère presented two electron micrographs of dehisced asci in a *Geoglossum* sp., which seem to agree with the present results. He did, however, not give an image or detailed description of the dehisced ascus of *Pezicula*. As described by Ingold (1971), in *Geoglossum* each spore may stopper the ascus for a relatively long period of time prior to discharge. During passage of the spores the eversion may exceed an angle of 90°, as can be observed in asci shooting spores. After fixation some asci were found with an ascospore partly sticking out of the ascostome, much like in Bellemère's micrographs (1977, fig. 1D, E).

Beckett et al. (1974) found a similar way of discharge in *Lophodermella sulcigena* (Rostr.) Höhn. The apical apparatus of this inoperculate species seems to show resemblance in general morphology to that in *Geoglossum cookeianum*, rather than to that in *Hypoderma rubi* (Pers.) DC. ex Chev. (Bellemère, 1977).

Trichoglossum

The separation at the generic level of the setose from the other geoglossoid forms with brown-walled spores is supported by the differences in general morphology of the apical apparatus and structure of the lateral ascus wall. Nevertheless, *Geoglossum* and *Trichoglossum* form a separate group, which differs from other clavate and pileate forms in structure of the ascus apical apparatus and mode of dehiscence. The semi-gelatinous type of outer layer has not been encountered earlier in Leotiales. The lateral wall is comparatively thick, which may be related to the fact that the outer layer tends to disintegrate and weakens during ascus development.

Dehiscence occurs in the same way as in *Geoglossum* (Ingold, 1971). The apical thickening is able to make close contact over a large area with the wall of the spore that is temporarily stoppering the ascostome. This would prevent leakage of fluid and enable the ascus to restore the necessary pressure level after every discharge except the last one.

For the first time the presence of a tractus and apical funnel is recorded at the LM and EM-level in a species of the Leotiales. As pointed out by Beckett (1981), the strand-like structure may disintegrate during chemical fixation. This seems to have occurred in the material of *Geoglossum cookeianum* used for the present study. But it must be stated that the presence of a tractus was not observed beyond doubt with light microscopy in *G. cookeianum*. The fact that a tractus is found in reasonable shape in *T. hirsutum*, a species with thick ascus walls, is probably due to the firmness of the structure. Chadefaud (1944) reported a tractus in *L. lubrica*, but evidence for its presence has neither been obtained at LM nor at EM-level.

Possibly homologous structures have been recorded in Sphaeriales and Pezizales, e.g. recently in certain freeze-substituted and chemically fixed species of *Scutellinia* (Cooke) Lamb. (van Brummelen, 1993), and in freeze-substituted *Thelebolus crustaceus* (Fuckel) Kimbr. (Czymmek & Klomparens, 1992).

The tractus actually connects the uppermost spore to the apical wall, and the spores to each other. It thus effectively ensures a smooth dehiscence in *T. hirsutum*. While it is discharged, each spore pulls its successor into the ascostome, and a new pressure built-up is ensured. There is, however, also some negative effect to be expected as some energy must be spent to break the tractus-spore connection.

Leotia

The general morphology of the apical apparatus, the structure of the annulus and the lateral wall, and the mode of dehiscence suggest an isolated position of *Leotia* within the Leotiales. *Cudonia* Fr. and *Spathularia* Pers., although not staining blue with iodine in the apical apparatus, do not seem to be closely related to *Leotia*. A cladistic analysis of sequences of 18S rDNA placed *Cudonia confusa* Bres. and *Spathularia flavida* in one sister group of *Leotia lubrica* (Landvik et al., 1993).

Spathularia flavida was studied by Bellemère (1977), who referred it to his 'type *Hypoderma*'(based on *H. rubi*). The genera in the Leotioideae sensu Korf (1973) that have been studied so far, *Bulgaria* Fr., *Ombrophila* Fr., *Neobulgaria* Petr. (Verkley, 1992), and *Ascocoryne* J.W. Groves & D.E. Wils. (Bellemère, 1977) do not show affinity to *Leotia* in the characters investigated. In the Leotiaceae the characters of excipular anatomy and gelatinous consistency of the ascocarp correlate meagrely with those of the structure of the ascus wall and apical apparatus.

The present results on the ultrastructure of the apical apparatus in *L. lubrica* differ considerably from those obtained by Bellemère (1977). Firstly, a reactive annulus is demonstrated. Bellemère reported on some fibrillar structures within the 'pendentif' at the moment of dehiscence, but found no annular PA-TCH-SP reactivity. Secondly, the most protruding part of the apical thickening ('pendentif') appears well-developed ('bien développé') at the young and early immature stages, but is not persistent in the mature stage (while the annulus is still very distinct), which is considered relevant, but this was not mentioned by Bellemère. Thirdly, no internal stratification is observed in the lateral wall, while in Bellemère's interpretation a d-layer was found, which is thicker than the c-layer in the lateral wall, and which is the only one increasing in thickness. Fourthly, the present results show that the apical thickening extends much further downwards than reported by Bellemère. Honegger (1983) also investigated the ascus apex of *L. lubrica*, but used a different fixation (acroleïn-glutaraldehyde) and did not apply the PA-TCH-SP technique.

The present results agree with the observations of dehisced asci by Bellemère (1977) and Honegger ('onset of dehiscence', 1983). Chadefaud (LM, 1944) used the term 'hémioperculé' for *L. lubrica*, putting emphasis on the observations that opening of the ascus wall occurs occasionally next to the ascus length axis in such a way, that it resembles the dehisced state of an operculate ascus. This variable way of dehiscence in *L. lubrica* is, however, merely the result of the diffuse structure of the annulus.

Microglossum

Bellemère et al. (1987) reported the presence in the apical apparatus of two separate rings ('anneau inférieur' and 'anneau supérieur'), differing in reactivity pattern. They placed *Microglossum* in the 'type *Pezicula*' of Bellemère (1977), close to *Geoglossum*. The present results show a change in level rather than in pattern of annular reactivity, and such a gradual change that there is no reason to designate two annuli in the apical apparatus of *M. viride*. The intensity of blueing with iodine of the annulus correlates positively with this level in reactivity. The upper part of the annulus blues much weaker than the lower part.

Unfortunately, detailed information on the way the spores are discharged is not available. At this point it is difficult to place *Microglossum*, but its position in the Geoglossaceae is not an unacceptable one. Benkert (1983) stated that many characters would support a fusion of *Geoglossum* and *Microglossum* (and *Thuemenidium*), but the ultrastructural data clearly oppose to this. The main difference between *Microglossum* and *Geoglossum* is that in the former the annulus occupies a restricted area of the apical thickening, reaching the inner boundary of the wall only in the annular protrusion. In this respect, and in the structure of the outer layer *Microglossum* also differs from *Bulgaria* (Verkley, 1992). Baral (1987) designated *Microglossum* and *Mitrula paludosa* to his apex 'typ *Bulgaria*' (LM). Although the annular protrusion is flexible and appears compressed in the mature stage, it remains a true protrusion, in contrast of that in *L. lubrica*. It is, however, different in structure from that found in *M. paludosa*.

Mitrula

Bellemère (1977) regarded the apex of *Mitrula paludosa* as a variety of his 'type *Bulgaria*'. There is certainly a resemblance at the LM-level (Baral, 1987). But in contrast to that in *Bulgaria* (Verkley, 1992), the dehisced ascus shows a limited, but distinct eversion of the very compact annular protrusion. Moreover, electron microscopy made it clear that the outer layer lacks any perpendicularly oriented constituents, and the annulus does not occupy the apical thickening completely, although close observation is necessary in the weakly developed distal part of the apical thickening. Only the thinning of the subapical wall reminds of *Bulgaria inquinans*

Well-developed annular protrusions like the one found in *M. paludosa*, occur frequently in the family Sclerotiniaceae (Schoknecht, 1975; Bellemère, 1977; Verkley, 1993a). Perhaps the genera which develop similar ascocarps, like *Mitrulinia* Spoon. or *Scleromitrula* Imai are related to *M. paludosa*. Their species have ascus apices which blue only indistinctly or not at all with iodine, at least in herbarium material (Spooner, 1987), which is uncommon in the family. This does not necessarily mean that the structure of the apical apparatus is essentially different. Investigations of this structure in *Mitrulinia* and *Scleromitrula* could provide useful information.

CONCLUSIONS

The ultrastructural data on the apical apparatus and lateral ascus wall suggest that:

(1) *Leotia lubrica* takes an isolated position, rather distant from other Leotioideae sensu Korf (1973, including Ombrophiloideae, Leotiaceae) and the Geoglossaceae investigated;

(2) *Geoglossum*, *Microglossum*, and *Trichoglossum* should be maintained as separate genera of the family Geoglossaceae;

(3) *Mitrula paludosa* is more closely related to members of the Sclerotiniaceae than to members of the Geoglossaceae.

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