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Citation	Mycologia, 1999, v. 91 n. 5, p. 885-892
Issued Date	1999
URL	http://hdl.handle.net/10722/53343
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Ascal ultrastructural study in *Annulatascus hongkongensis* sp. nov., a freshwater ascomycete

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Abstract: A new species of *Annulatascus*, *A. hongkongensis*, is described from freshwater habitats. This species differs from other *Annulatascus* species as ascospores are ellipsoidal, 3-septate, verruculose and are surrounded by a thick mucilaginous sheath. *Annulatascus hongkongensis* is described and illustrated with light and transmission electron micrographs, and compared with other *Annulatascus* species.

Key Words: Annulatascaceae, aquatic fungi, electron microscopy, systematics, taxonomy

Hyde (1995) from Australia, *A. palmietensis* K. D. Hyde et al (1997a) from S. Africa and *A. triseptatus* S. W. Wong et al (1998b) from Brunei. *Annulatascus bipolaris* was transferred to *Cateractispora* S. W. Wong et al primarily due to the presence of unfurling polar ascospore appendages (Wong et al unpubl). All *Annulatascus* species were described from submerged wood in tropical or subtropical streams. This genus was first placed in Lasiosphaeriaceae (Hyde 1992), and recently following ultrastructural data, it was erected as the type genus of the new family, the Annulatascaceae (Wong et al 1998a).

Annulatascus hongkongensis is described and compared with other *Annulatascus* species. The ultrastructure of the asci and ascospores of *A. triseptatus* and *A. velatisporus* has been examined by Wong et al (1998b). We illustrate the ultrastructure of different stages of development of the asci and ascospores of *A. hongkongensis*.

INTRODUCTION

During the past 6 yr, a group of novel ascomycetes, characterized by cylindrical, unitunicate asci having relatively massive apical rings has been described from wood submerged in tropical and subtropical freshwater habitats. This group includes genera such as *Annulatascus* K. D. Hyde (Hyde 1992, 1995, Wong et al 1998b), *Proboscispora* S. W. Wong & K. D. Hyde (1998), *Rivulicola* K. D. Hyde (Hyde et al 1997c), and *Tamsiniella* S. W. Wong et al (1998c). A new family, the Annulatascaceae, Sordariales (Wong et al 1998a), was erected to accommodate some of the freshwater genera, including *Annulatascus* and *Proboscispora*.

During our continuing studies on freshwater fungi (Hyde and Goh 1997, Hyde et al 1997a, Ho 1998), an additional taxon in the Annulatascaceae was found on wood submerged in a reservoir in Hong Kong. Close examination of the morphological characters revealed it to be a species of *Annulatascus*. *Annulatascus* was introduced by Hyde (1992) to accommodate two freshwater fungi, *A. velatisporus* K. D. Hyde (type species) and *A. bipolaris* K. D. Hyde, which were collected on submerged wood in Australia. Thereafter three additional species were described: *A. biatriisporus* K. D.

MATERIALS AND METHODS

Submerged wood was collected from Plover Cove Reservoir, Hong Kong in November 1996. Wood samples were returned to the laboratory and incubated in plastic boxes lined with moistened paper towels, and examined within one month. Squash mounts and sections of ascomata were prepared on slides, mounted in water for measurement and photographed with differential interference contrast microscopy. Procedures for the preparation of the material examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) follow those of Wong et al (1998b, c). Material examined using TEM were fixed in (i) 4% (v/v) glutaraldehyde with ruthenium red for 4 h, and postfixed in 2% (w/v) OsO₄ at 4 C overnight; (ii) 2% (w/v) KMnO₄ at 24 C for 10 min. Asci and released mature ascospores were examined with TEM.

TAXONOMY

KEY TO SPECIES OF ANNULATASCUS

- 1. Ascospores unicellular 2
- 1. Ascospores 3-septate 3
 - 2. Ascospores 40–58 × 8–10 μm, with swollen ends *A. biatriisporus*
 - 2. Ascospores 26–42 × 9–12 μm, without swollen ends *A. velatisporus*
- 3. Ascospores with rounded ends, 20–26 × 6–7 μm *A. palmietensis*

Accepted for publication April 29, 1999.

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3. Ascospores with acute ends 4
 4. Ascospores $35\text{--}37.5 \times 12.5\text{--}15$, ellipsoidal, with a thick mucilaginous sheath $6\text{--}8$ μm wide *A. hongkongensis*
 4. Ascospores $18\text{--}33 \times 6\text{--}12$ μm , fusiform, with a thin mucilaginous sheath ca 1 μm wide *A. triseptatus*

***Annulatascus hongkongensis* W. H. Ho, Ranghoo, K.**

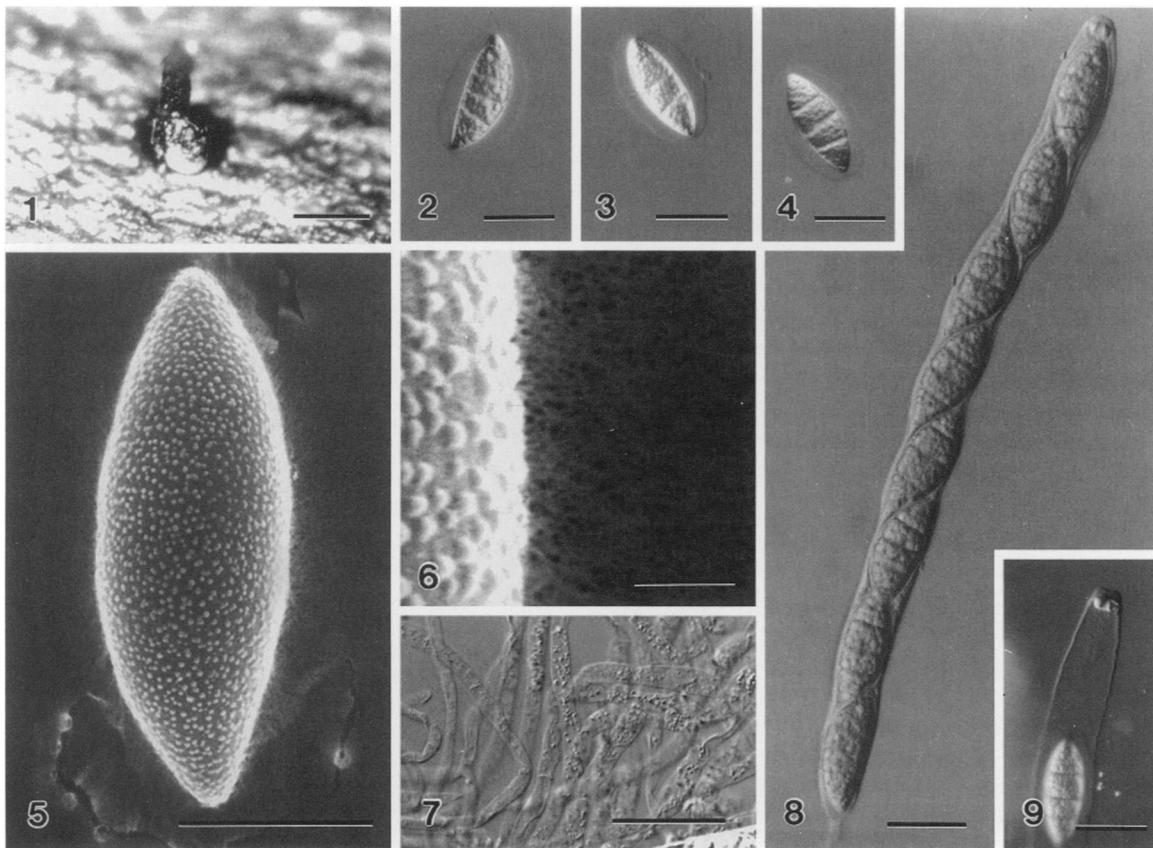
D. Hyde et I. J. Hodgkiss, sp. nov. FIGS. 1–27

Ascomata $250\text{--}280$ μm diameter, $210\text{--}250$ μm alta, subglobosa vel ellipsoidea, immersa ad superficialia, coriacea, atrobrunnea, solitaria, ostiolata, papillata. *Colla* $140\text{--}150 \times 35\text{--}40$ μm diameter, cylindrica, excentrica, curvata, 5–7 cellulis dermi, periphysata. *Peridium* $18\text{--}23$ μm crassum, textum angulatum. *Paraphyses* $200\text{--}250 \times 6.5\text{--}8.5$ μm , septatis, angustatis. *Asci* $250\text{--}275 \times 25\text{--}30$ μm , octospori, cylindrici, unitunicati, persistenti, pedicellati, apparato apicale praediti. *Ascosporae* $35\text{--}37.5 \times 12.5\text{--}15$ μm , uniseriatae vel imbricatae, hyalinae, ellipsoideae, 3-septatae, guttulateae, laeviae, tenuitunicatae, tunica gelatinosa praeditae.

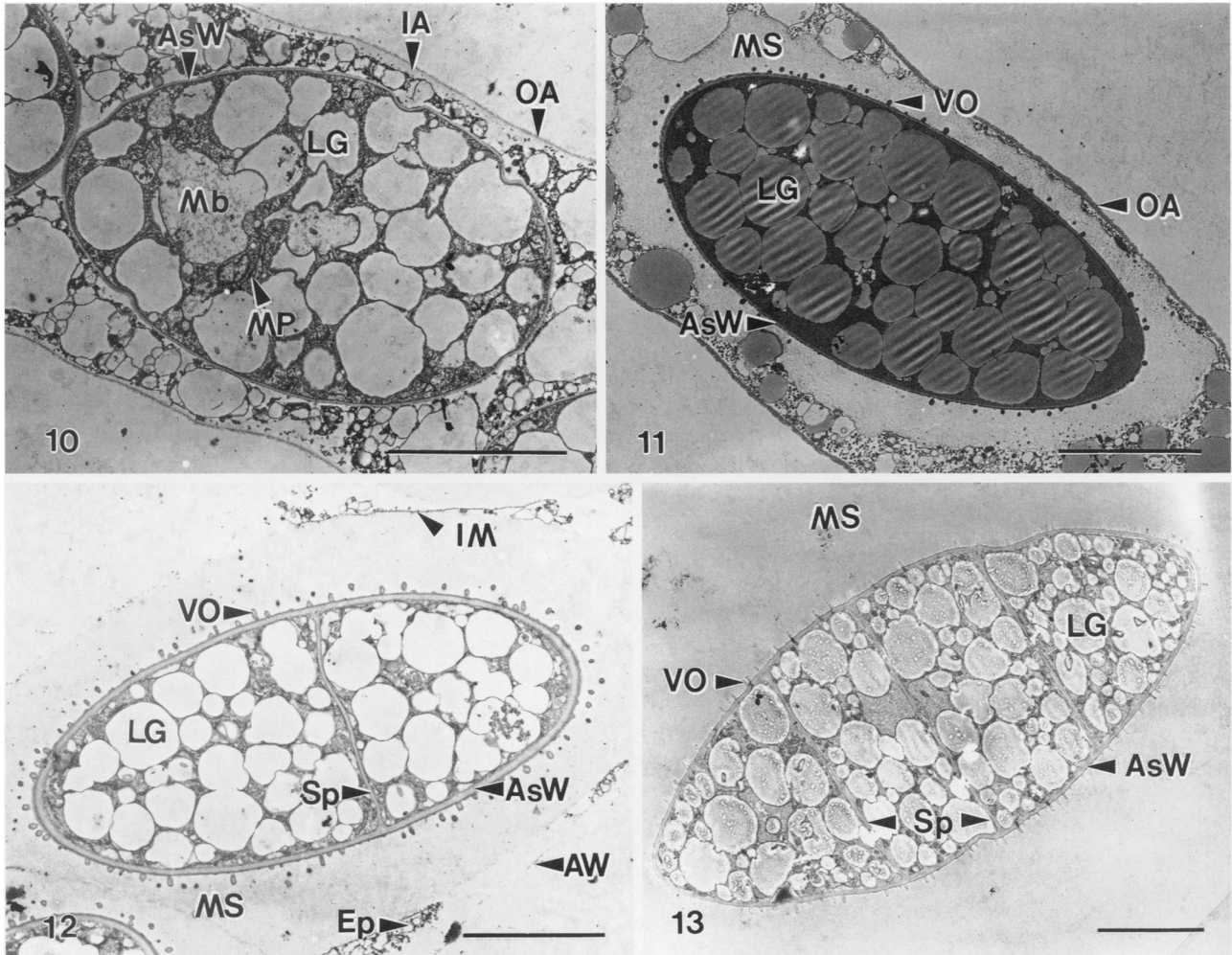
Ascomata $250\text{--}280$ μm diameter, $210\text{--}250$ μm high, subglobose to ellipsoidal, mostly immersed, occasionally superficial, coriaceous, dark brown, soli-

tary, ostiolate, papillate (FIG. 1). *Neck* $35\text{--}40$ μm diameter, $140\text{--}150$ μm long, cylindrical, eccentric, slightly curved, composed of 5–7 layers of brown globular cells, perpendicular to the surface of the substratum, periphysate. *Peridium* $18\text{--}23$ μm thick, composed of layers of brown elongated cells, *textura angularis* in surface view. *Paraphyses* $200\text{--}250 \times 6.5\text{--}8.5$ μm , septate, unbranched (FIG. 7), tapering towards the apex. *Asci* $250\text{--}275 \times 25\text{--}30$ μm ($\bar{x} = 257 \times 26$ μm , $n = 20$), 8-spored, cylindrical, unitunicate, persistent, pedicellate, with a large and refractive apical ring ($4.7\text{--}6.0$ μm diameter, $3.5\text{--}4.5$ μm long) (FIGS. 8, 9). *Ascospores* $35\text{--}37.5 \times 12.5\text{--}15$ μm ($\bar{x} = 36 \times 14$ μm , $n = 25$), uniseriate or overlapping uniseriate, hyaline, ellipsoidal, 3-septate, guttulate, smooth (verruculose at SEM level), thin-walled, with thick mucilaginous sheath ($6\text{--}8$ μm thick) (FIGS. 2–6).

Materials examined. HONG KONG. Plover Cove Reservoir, in ligno putredinis submersis, 15 November 1996, K. D. Hyde & M. Wong PC 21 [HOLOTYPE: HKU(M) 4702]. Plover Cove Reservoir, on submerged wood, 15 November 1996, K. D. Hyde & M. Wong PC 54 [HKU(M) 4701].



FIGS. 1–9. *Annulatascus hongkongensis*. 1. Semi-immersed ascoma on wood substratum. 2–4. Triseptate ascospores with thick mucilaginous sheaths. 5, 6. Ascospores with verruculose wall ornamentations. Note the fibrillar, interwoven texture of the mucilaginous sheath shown at higher magnification in FIG. 6. 7. Septate paraphyses. 8. Ascus with mature ascospores. 9. Ascus tip showing the refractive ring. 1. Stereo micrographs. 2–4, 7–9. Differential interference contrast micrographs. 5, 6. Scanning electron micrographs. Scale bars: 1 = 100 μm ; 2–4, 8, 9 = 20 μm ; 5 = 10 μm ; 6 = 1 μm ; 7 = 50 μm .



FIGS. 10–13. Transmission electron micrographs of *Annulatascus hongkongensis*. Longitudinal sections of ascospores at various developmental stages are in numerical sequence; AsW = ascospore wall, AW = ascus wall, IA = inner ascus wall layer, OA = outer ascus wall layer, Ep = epiplasm, IM = investing membrane, LG = lipid globules, Mb = microbodies, MP = membrane profiles, MS = mucilaginous sheath, Sp = central septum, VO = verruculose wall ornamentations. 10. Immature aseptate ascospore. 11. Later stage immature aseptate ascospore. 12. Later stage immature 1-septate ascospore. 13. Mature, released ascospore. 10, 12, 13. Fixed with KMnO_4 ; 11. Fixed with glutaraldehyde and OsO_4 . Scale bars: 5 μm .

Etymology. In reference to the occurrence in Hong Kong.

Known distribution. Hong Kong.

Habitat. Saprobic on decaying wood in streams.

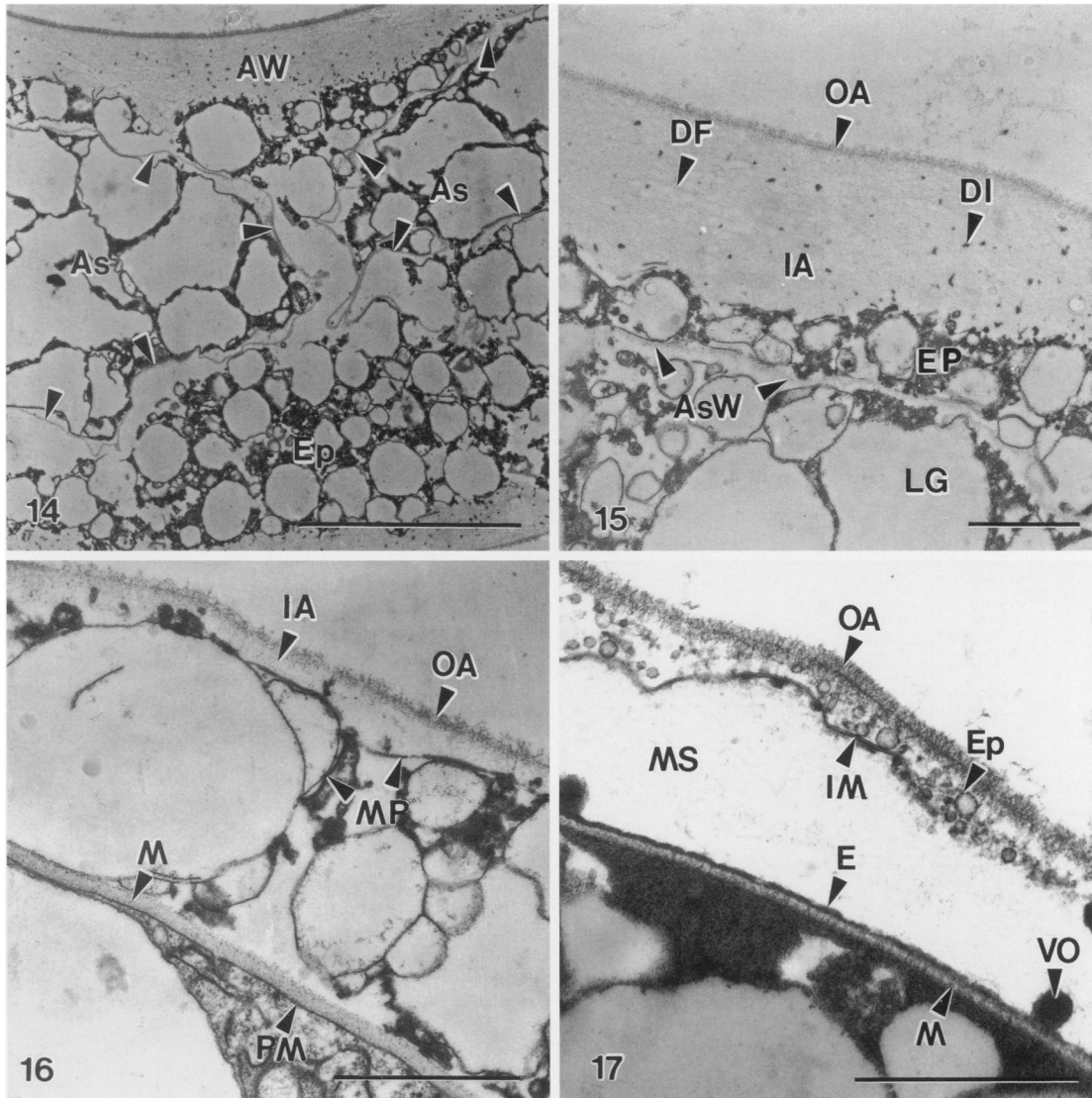
Anamorph. Unknown.

Electron microscopy.—Mature ascospores were densely verruculose and covered with mucilaginous sheaths. The verruculose wall ornamentations were 150–250 nm diameter, hemispherical and smooth-walled. The mucilaginous sheath contained inter-woven fibrils (Figs. 5, 6).

Developing ascospores were initially broadly ellipsoidal, aseptate, with numerous lipid globules (LG), microbodies (Mb) and membrane profiles (MP) in the cytoplasm (FIG. 10). Subsequently, a thick mucilaginous sheath (MS) and verruculose wall ornamentations (VO) were formed (FIG. 11), followed by the formation of a central septum (Sp) (FIG. 12) and two other septa (140–160 nm thick) (FIG. 13). The mucilaginous sheath (MS) was expanded in mature, released ascospores (FIG. 13).

Ascospores (As) were initially irregular in shape (FIG. 14) and their wall (AsW) was electron-transparent, 1-layered and thin (40 nm thick) (FIG. 15). Subsequently, the ascospore wall thickened (70 nm thick) (FIG. 16). The mesosporium (M) appeared to be the first-formed wall layer (FIG. 16). A thin electron-dense layer (DL, 5–10 nm thick) was deposited on the outer surface of mesosporium (M) (FIG. 18) and formed the episporium (E, 25–30 nm thick) (FIG. 17). At maturity, the mesosporium was bilamellate: the outer layer of the mesosporium (M1, 60–80

nm thick) (FIG. 19) and the inner layer (M2, 20–30 nm thick) (FIG. 19). The outer layer of the mesosporium (M1) was electron-transparent and the inner layer (M2) was electron-dense. The outer layer of the mesosporium (M1) was 60–80 nm thick and the inner layer (M2) was 20–30 nm thick. The outer layer of the mesosporium (M1) was electron-transparent and the inner layer (M2) was electron-dense. The outer layer of the mesosporium (M1) was 60–80 nm thick and the inner layer (M2) was 20–30 nm thick.



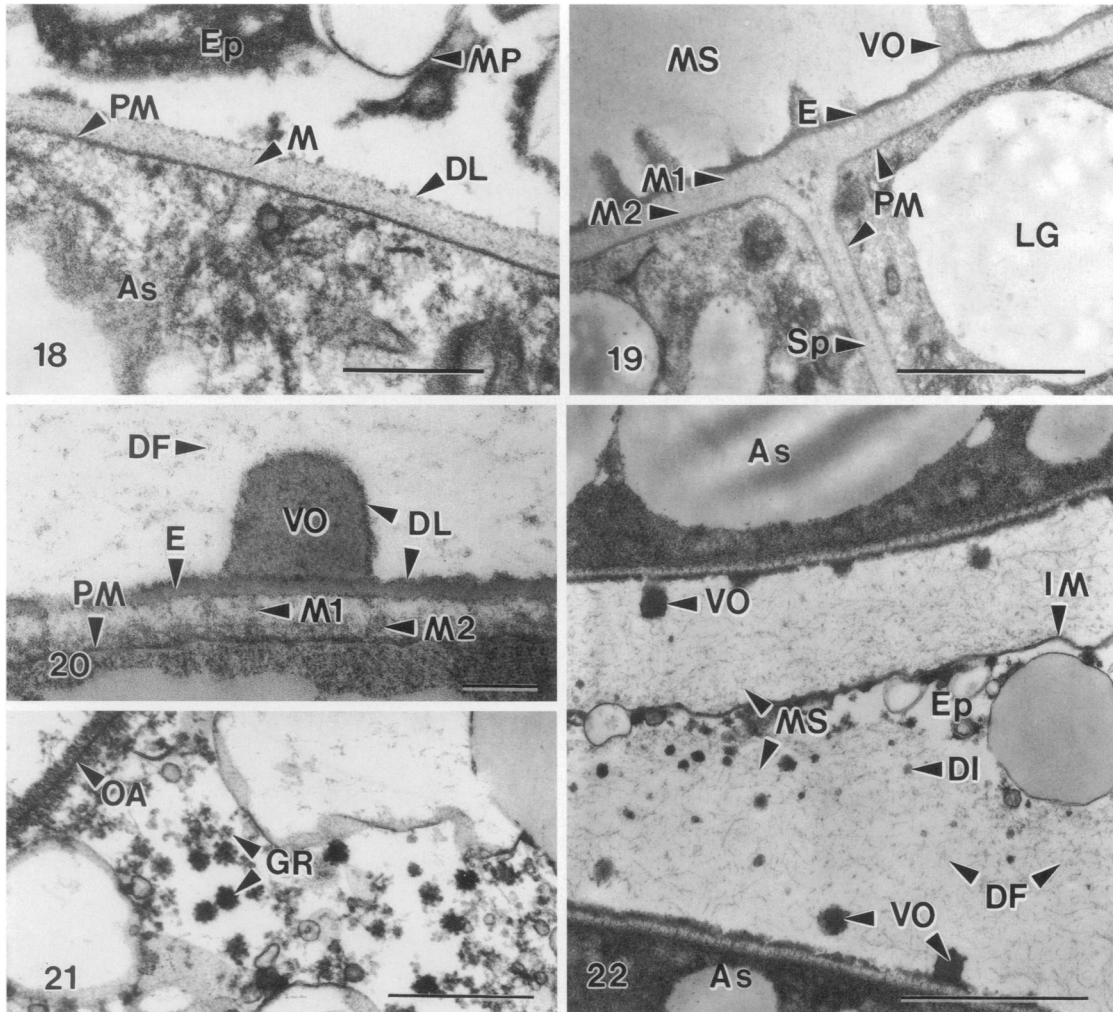
FIGS. 14–17. Transmission electron micrographs of *Annulatascus hongkongensis*. Longitudinal sections of asci and ascospores at various developmental stages are in numerical sequence. As = ascospores, AsW = ascospore wall, AW = ascus wall, DF = electron-dense fibrils, DI = electron-dense inclusions, E = episporium, Ep = epiplasm, IA = inner ascus wall layer, IM = investing membrane, LG = lipid globules, M = mesosporium, MS = mucilaginous sheath, MP = membrane profiles, OA = outer ascus wall layer, PM = plasma membrane, VO = verruculose wall ornamentations. 14. Immature ascus with thin-walled primordial ascospores (arrowheads). 15. Higher magnification of FIG. 14. 16. Later stage immature ascus. 17. Later stage immature ascus. 14–16. Fixed with KMnO_4 ; 17. Fixed with glutaraldehyde and OsO_4 with ruthenium red. Scale bars: 14 = 5 μm ; 15–17 = 1 μm .

nm thick) comprised parallel electron-dense striations that were perpendicular to the ascospore wall; while the inner layer (M2, 100–120 nm thick) was continuous with the septa (Sp, 120–140 nm thick) (FIGS. 19, 20).

Verruculose wall ornamentations (VO) were hemispherical (150–200 nm diameter), with the same electron-density as the episporium (E) (FIG. 20). Both the verruculose wall ornamentations and episporium were covered by a thin electron-dense layer that appeared to form the electron-dense fibrils (DF,

ca 5 nm diameter) embedded in the mucilaginous sheath (FIG. 20). The electron-dense fibrils were more prominent when fixed with glutaraldehyde and osmium tetroxide with added ruthenium red (FIGS. 20, 22), in contrast to those fixed with potassium permanganate (FIG. 19).

The spore investing membrane and spore plasma membrane were observed in most samples. The spore plasma membrane (PM) was closely adpressed to the inner surface of ascospore wall (FIGS. 16, 18–20) while the investing membrane (IM) was closely



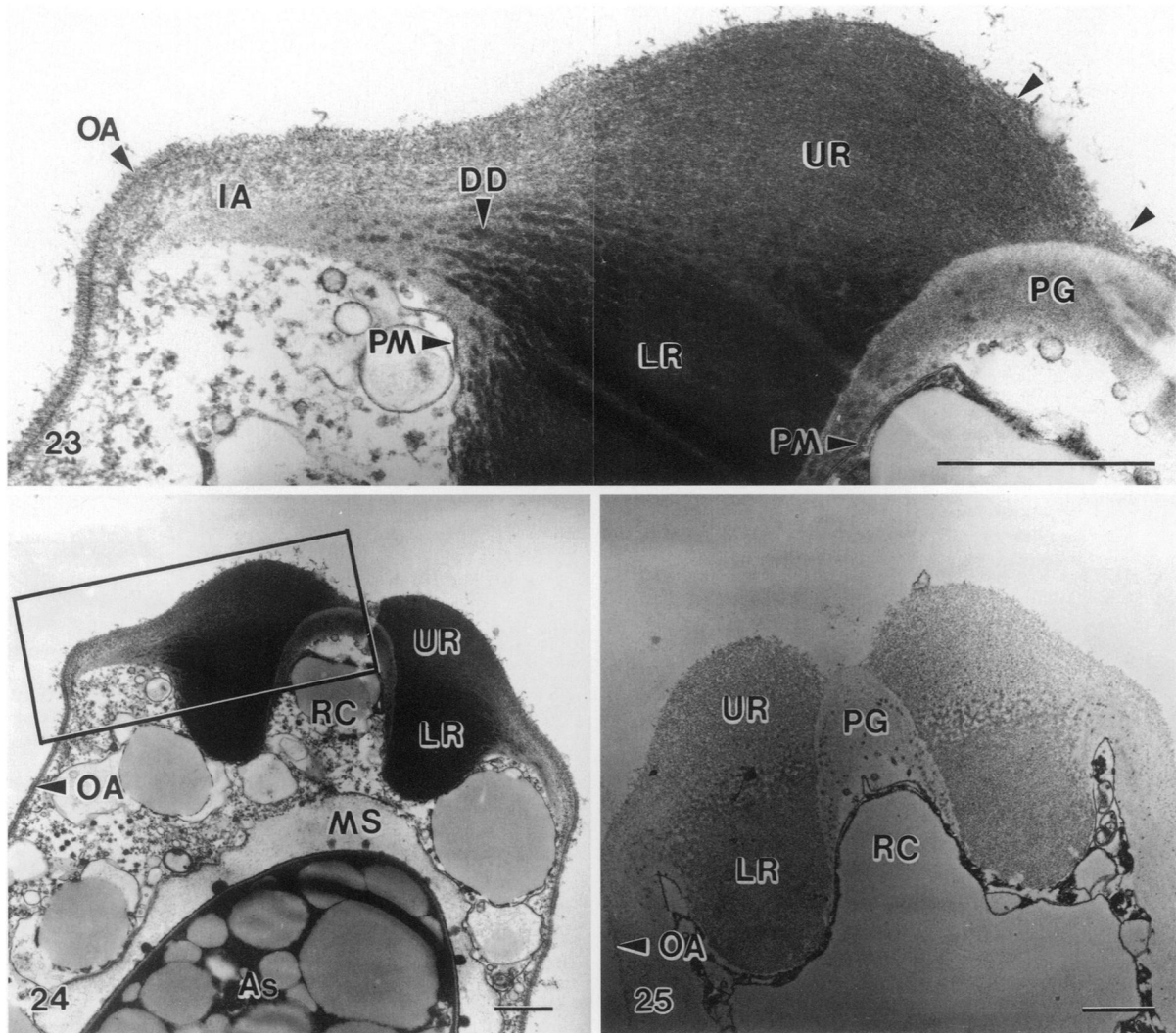
FIGS. 18–22. Transmission electron micrographs of *Annulatasacus hongkongensis*. Longitudinal sections of ascus wall and ascospore wall. As = ascospore, DF = electron-dense fibrils, DI = electron-dense inclusions, DL = electron-dense layer, E = episporium, Ep = epiplasm, GR = glycogen rosettes, IM = investing membrane, LG = lipid globules, M = mesosporium, M1 = outer layer of mesosporium, M2 = inner layer of mesosporium, MP = membrane profiles, MS = mucilaginous sheath, OA = outer ascus wall layer, PM = plasma membrane, Sp = septum, VO = verruculose wall ornamentations. 18. Ascus with immature ascospore. 19. Mature ascospore. 20. Mature ascospore wall. 21. Ascus. 22. Ascospores. 18, 19. Fixed with KMnO_4 ; 20–22. Fixed with glutaraldehyde and OsO_4 , with ruthenium red. Scale bars: 18, 21 = 0.5 μm ; 19, 22 = 1 μm ; 20 = 0.1 μm .

addressed to the mucilaginous sheaths (FIGS. 12, 17, 22). The absence of a spore investing membrane (IM) (FIGS. 15, 16, 18) and a spore plasma membrane (PM) (FIG. 15) in different samples examined might be an artifact of fixation.

In immature asci, the epiplasm was filled with numerous lipid globules (LG), membrane profiles (MP) and glycogen rosettes (GR) (FIGS. 14–17, 21). The wall of the immature ascus comprised two layers: an outer, electron-dense layer (OA, 60–80 nm thick); and an inner, electron-transparent layer (IA, 1–1.5 μm thick) containing electron-dense fibrils (DF) and inclusions (DI) (FIG. 15). During maturation the thickness of the inner ascus wall layer decreased (IA) (FIG. 16), except at the apical region (FIG. 26). At

maturity the inner ascus wall layer appeared to be absent throughout (FIGS. 17, 21), while the outer ascus wall layer (OA) appeared to dissolve, and was thus discontinuous at the apex (FIGS. 23, 24, arrowheads). FIGS. 12 and 27 illustrate an ascus and ascus apical ring with apparently ‘dissolved’ ascus wall that might be an artifact of fixation or processing of the material.

The two different fixatives had no effect on the appearance of the ascus apical ring. The rings measured 5.5–6 μm diameter, 3.5–4.5 μm long, with the rim measuring 1.8–2 μm diameter. The ring comprised an upper portion (UR, 1.3–2 μm long) that contained horizontally orientated electron-dense fibrils; and a lower portion (LR, 2.2–2.5 μm long) that



FIGS. 23–25. Electron micrographs of *Annulatascus hongkongensis*. Longitudinal sections of asci at the apical region. As = ascospore, DD = electron-dense desposits, IA = inner ascus wall layer, LR = lower portion of ascus ring, MS = mucilaginous sheath, OA = outer ascus wall layer, PG = plug, PM = plasma membrane, RC = ring channel, UR = upper portion of ascus ring. 23. Higher magnification of the marked region at the ascus apex in FIG. 24. Note that the outer ascus wall layer (OA) is discontinuous and dissolving at the apical regions (arrowheads). 24, 25. Ascus apex. 23, 24. Fixed with glutaraldehyde and OsO_4 ; 25. Fixed with KMnO_4 . Scale bars: 23–25 = 1 μm .

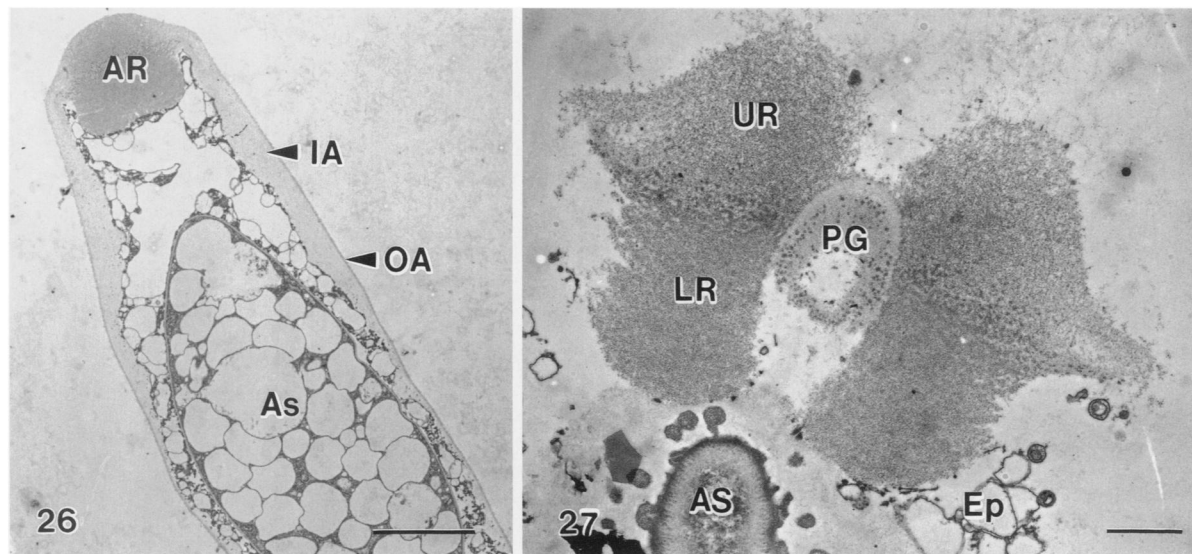
contained compact electron-dense granular deposits (DD) (FIGS. 23–25). The lower portion (LR) of the ascus ring was more electron-dense than the upper portion (UR) (FIGS. 23–25). The channel within the ascus ring was plugged with a layer of amorphous material (PG) (FIGS. 23–25, 27).

DISCUSSION

Annulatascus hongkongensis differs from *A. biatriisporus*, which has longer (40–58 μm), unicellular ascospores with swollen ends; *A. palmietensis*, which has smaller (20–26 \times 6–7 μm), fusiform, 3-septate ascospores with rounded ends, and *A. velatisporus*, which

has mostly unicellular, fusiform ascospores. *Annulatascus hongkongensis* is closest to *A. triseptatus*, where both species have ascospores that are 3-septate, filled with numerous lipid globules, and are surrounded by mucilaginous sheaths. These species differ in that the ascospores of *A. triseptatus* are fusiform and smaller (18–33 \times 6–12 μm vs 35–37.5 \times 12.5–15), with a thin mucilaginous sheath, while the ascospores of *A. hongkongensis* are ellipsoidal, with a much thicker mucilaginous sheath.

At ultrastructural level, the ascus walls of *A. triseptatus* and *A. velatisporus* are bilamellate and the apical rings are bipartite (Wong et al 1998b). In *A. hongkongensis*, the ascus walls are also bilamellate and the



FIGS. 26, 27. Electron micrographs of *Annulatascus hongkongensis*. Longitudinal sections of asci at the apical region. AR = apical ring, As = ascospore, Ep = epiplasm, IA = inner ascus wall layer, LR = lower portion of ascus ring, OA = outer ascus wall layer, PG = plug, UR = upper portion of ascus ring. 26. Ascus with inner ascus wall layer (IA) thickened around the apical region. 27. Apex of an ascus with 'dissolved' wall layers. Fixed with KMnO_4 . Scale bars: 26 = 5 μm ; 27 = 1 μm .

apical rings are bipartite (FIG. 15, OA/IA, UR/LR). In addition, the inner ascus wall layer of *A. velatisporus* becomes thinner during maturation (Wong et al 1998b). In *A. hongkongensis*, the inner ascus wall layer also becomes thinner during maturation (FIGS. 10, 16, IA), and may be absent in mature asci (FIGS. 11, 19), except at the apical region (FIGS. 24, 26, IA).

Numerous terms have been used in describing the different layers of the ascospore wall. Bellemère (1994) proposed the terms "proper wall," "perispore," "intermediary wall" and "endospore" in a review of ultrastructural studies of ascus and ascospore. Furtado and Olive (1970) used the terms "endospore," "episporium" and "perispore" in describing species such as *Leptosphaerulina australis* McAlpine and *Placoasterella baileyi* (Berk. & Br.) Arx apud Müller & Arx. In a recent review on ascospore morphogenesis, Read and Beckett (1996) adopted the terminology proposed by Beckett et al (1968): "primary wall layer" which is composed of undifferentiated wall material that is initially deposited between two investing membranes; and "secondary wall layer" which is composed of wall material formed following the primary wall, either by modification of, or addition to the primary wall. In this study we adopt the terminology, "episporium," "mesosporium" and "exosporium," proposed by Kirk (1966) in describing species of the Halosphaeriaceae. His terminology is widely used in marine (e.g., Yusoff et al 1994, Jones 1995, Hyde et al 1997b) and freshwater ascomycetes (e.g., Hsieh et al 1995, Hyde et al 1997c, Wong et al 1998c).

The components of the ascospore walls, and their developmental sequence, of *A. hongkongensis* are similar to those of *A. triseptatus* and *A. velatisporus* (Wong et al 1998b). In these three species, the ascospore walls comprise verruculose ornamentations and are bilamellate, comprising an episporium and a mesosporium (Wong et al 1998b, FIG. 19, VO, E, M1/M2). The mesosporium is the first formed wall layer and the episporium subsequently develops and appears to elaborate to form the verruculose wall ornamentations (Wong et al 1998b, FIGS. 15, 18, 19, 20, M, E, VO). The mesosporium itself becomes bilamellate at maturity, with the outer mesosporium layer comprising parallel electron-dense striations that were perpendicular to the ascospore wall (Wong et al 1998b, FIG. 19, M1, M2). The ascospores are surrounded by a mucilaginous sheath which is embedded with electron-dense fibrils. These electron-dense fibrils are shown to have been derived from the verruculose ornamentations in *A. triseptatus* and *A. velatisporus* (Wong et al 1998b) and this appear to be the same in *A. hongkongensis* (FIG. 20, DF, VO). The ascospore septa found in *A. hongkongensis* and *A. triseptatus* are derived from the inner mesosporium layer (Wong et al 1998b, FIG. 19, Sp, M2).

The ascus apical rings of *A. hongkongensis* are similar to those of *A. triseptatus* and *A. velatisporus* at ultrastructural level. The rings are bipartite and occluded with a plug (Wong et al 1998b, FIGS. 23–27, UR, LR, PG). The upper portion of the ascus apical ring is differentiated from the inner ascus wall layer, and contains horizontally orientated electron-dense

fibrils; while the lower portion of the ring is closely associated with the upper portion, and comprises densely packed electron-dense granules (Wong et al 1998b, FIGS. 23–27, UR, LR, IA, DD).

ACKNOWLEDGMENTS

The authors thank G. J. Samuels, O. E. Eriksson and an anonymous reviewer giving valuable comments on the manuscript. Thanks are extended to the staff of the Electron Microscopy Unit at Queen Mary Hospital, the staff of the Photographic Unit of the Faculty of Science, The University of Hong Kong, and Ms. Helen Y. M. Leung for technical assistance. W. H. Ho is very much indebted to Drs. S. W. Wong and S. J. Saunders for teaching him TEM techniques. V. M. Ranghoo and W. H. Ho would also like to thank The University of Hong Kong for the award of postgraduate studentships.

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