Ultrastructure of *Annulatascus aquaticus* sp. nov., a freshwater ascomycete on submerged wood from Hong Kong

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A new species of *Annulatascus*, *A. aquaticus*, is described from freshwater habitats in Hong Kong and is illustrated at the light microscope and scanning and transmission electron microscope levels. It differs from other species in the genus as its ascospores are aseptate, ellipsoidal with acute ends, thin-walled, and contain large lipid globules at maturity. Its asci have a bilamellate ascus wall and bipartite apical ring. The upper part of the apical ring differentiates from the inner ascus wall layer, and the lower part elongates downwards during maturation. Ascospore walls are bilamellate, with an electron-transparent mesosporium and an electron-dense episporium.

Key words: Annulatascaceae, electron microscopy, saprobic, taxonomy.

Introduction

In a study of freshwater mycota in the tropics (Ho, Hyde and Hodgkiss, 1997; Goh, Hyde and Ho, 1998; Hyde, Ho and Tsui, 1998b), an undescribed ascomycete was found on submerged wood in a small stream in Hong Kong. This ascomycete is characterized by immersed, brown, thin-walled, and beaked ascomata; wide, tapering, septate paraphyses; long, cylindrical unitunicate asci which possess a relatively massive, J- apical ring; and hyaline, fusiform ascospores. Close examination of the morphological characters revealed it to be a species of *Annulatascus* K.D. Hyde, and here it is described as *A. aquaticus* sp. nov.

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 from freshwater habitats: *A. biatriisporus* K.D. Hyde (1995) from Australia, *A. palmietensis* K.D. Hyde,

Goh et T.D. Steinke (1998a) from S. Africa and *A. triseptatus* S.W. Wong, K.D. Hyde, E.B.G. Jones et S.T. Moss (1998c) from Brunei. *Annulatascus bipolaris* was transferred to *Cateractispora* S.W. Wong, K.D. Hyde et E.B.G. Jones, primarily due to the presence of unfurling polar ascospore appendages (Wong, Hyde and Jones, 1998b). This genus was first placed in Lasiosphaeriaceae (Hyde, 1992), and recently following ultrastructural data, it was transferred to a new family, the Annulatascaceae, Sordariales (*sensu* Barr, 1990; Wong, Hyde and Jones, 1998a).

Annulatascus aquaticus is described and illustrated at the light microscope and scanning and transmission electron microscope levels in this paper and is compared with other *Annulatascus* species.

Materials and methods

Light microscopy

Submerged wood was collected from Tai Po Kau Forest Stream, Hong Kong in June, 1996. Wood samples were brought to the laboratory and incubated in plastic boxes lined with a moistened paper towel, and examined within one month. Immersed ascomata were identified by cutting the surface tissue of the wood using razor blades. Squash mounts of ascomata were mounted in water for measurement of asci, ascospores and hamathecia, and photographed using an interference contrast microscope. Apical rings of the asci were stained by Melzer's reagent.

Scanning electron microscopy

Ascospore suspensions were settled onto polycarbonate membranes (Nucleopore) with a pore size of 5 μm. Subsequently the membranes were fixed in 2 % (w/v) aqueous OsO_4 at 4 C overnight. Fixed material was dehydrated through a graded ethanol series from 10 to 90 %, in 10 % steps, followed by 95 % and three changes of absolute ethanol. Each of the above changes were for 15 min. After critical point drying in carbon dioxide and sputter coating with gold-palladium, the material was examined in a Leica Cambridge Stereoscan 440 scanning electron microscope operated at 20 kV.

Transmission electron microscopy

Suspensions of asci and ascospores were embedded in 2 % (w/v) Ion agar and subsequently fixed in 4 % (v/v) glutaraldehyde with added ruthenium red in 0.1 M sodium cacodylate buffer at pH 7.2 for 4 hours at room temperature. They were then post-fixed in 2 % (w/v) aqueous OsO_4 with added ruthenium red at 4 C overnight. The dehydration process was the same as that described

for scanning electron microscopy, but the material was finally transferred to absolute acetone (three times, 15 min each), and embedded in Mollenhauer's resin (Mollenhauer, 1964). Sections were stained with lead citrate (Reynolds, 1963) for 20 min and uranyl acetate solution for 40 min. Finally the sections were examined using a JEOL 100SX transmission electron microscope operated at 80 kV.

Results

Annulatascus aquaticus W.H. Ho, K.D. Hyde et I.J. Hodgkiss, sp. nov.

(Figs. 1-17)

Etymology: *aquaticus* meaning 'aquatic', referring to the habitat of the fungus.

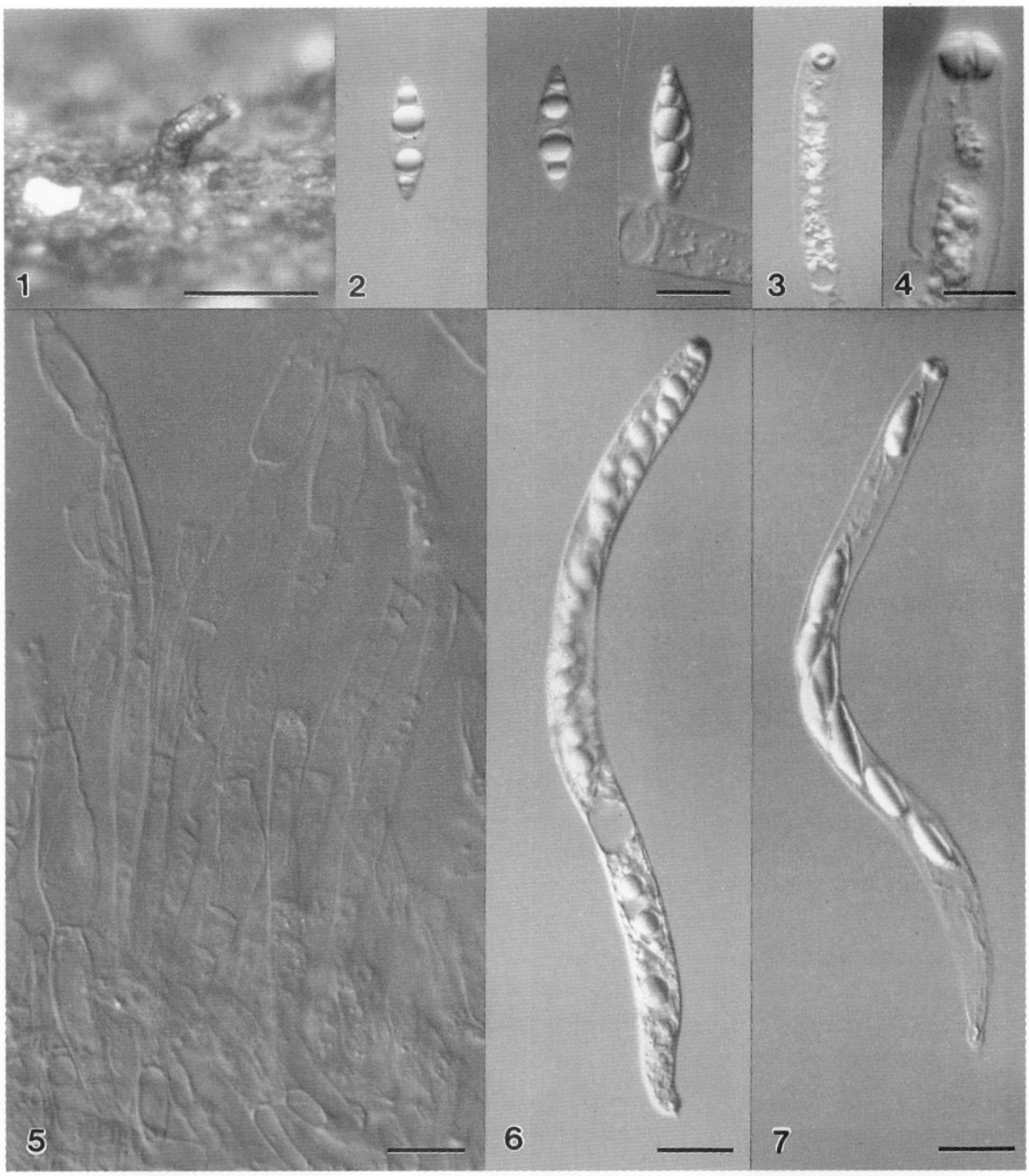
Ascomata 240-600 µm diam., 220-600 µm alta, subglobosa vel ellipsoidea, immersa vel superficiales, coriacea, atrobrunnea, solitaria, ostiolata, papillata. *Papilla* 400-800 × 60-80 µm, cylindrica, atrobrunnea, periphysata. *Peridium* textura angulatum. *Paraphysibus* 3-4.5 µm latis, septatis, angustatis. *Asci* 150-175 × 10-12 µm, octospori, cylindrici, unitunicati, persistenti, pedicellati, apparatu apicale praediti, J-. *Ascosporae* 19-24 × 6-7 µm, imbricatae, hyalinae, ellipsoideae, unicellulae, guttulatae, laeviae, tenuitunicatae.

Ascomata 240-600 µm diam., 220-600 µm high, globose to subglobose, mostly immersed, occasionally superficial, coriaceous, solitary, dark brown, solitary, ostiolate, and beaked (Fig. 1). *Beak* 400-800 × 60-80 µm, cylindrical, dark brown, and periphysate. *Peridium textura angularis* in surface view, comprising a few layers of brown walled compressed cells. *Paraphyses* 3-4.5 µm wide at the base, hypha-like, numerous, septate, hyaline and tapering distally (Fig. 5). *Asci* 150-175 × 10-12 µm ($\bar{x}$ = 165 × 11 µm, n = 10), 8-spored, cylindrical, thin-walled, persistent, pedicellate, with a relatively massive refractive, J- apical ring (4.5-5 µm diam., 3.5-4 µm long; Figs. 3, 4, 6, 7). *Ascospores* 19-24 × 6-7 µm ($\bar{x}$ = 22 × 6.5 µm, n = 25), overlapping uniseriate, hyaline, ellipsoidal, with acute ends, unicellular, containing 2(-4) large lipid globules at maturity, smooth, thin-walled (Fig. 2), with a mucilaginous sheath observed at the ulstrastructural level.

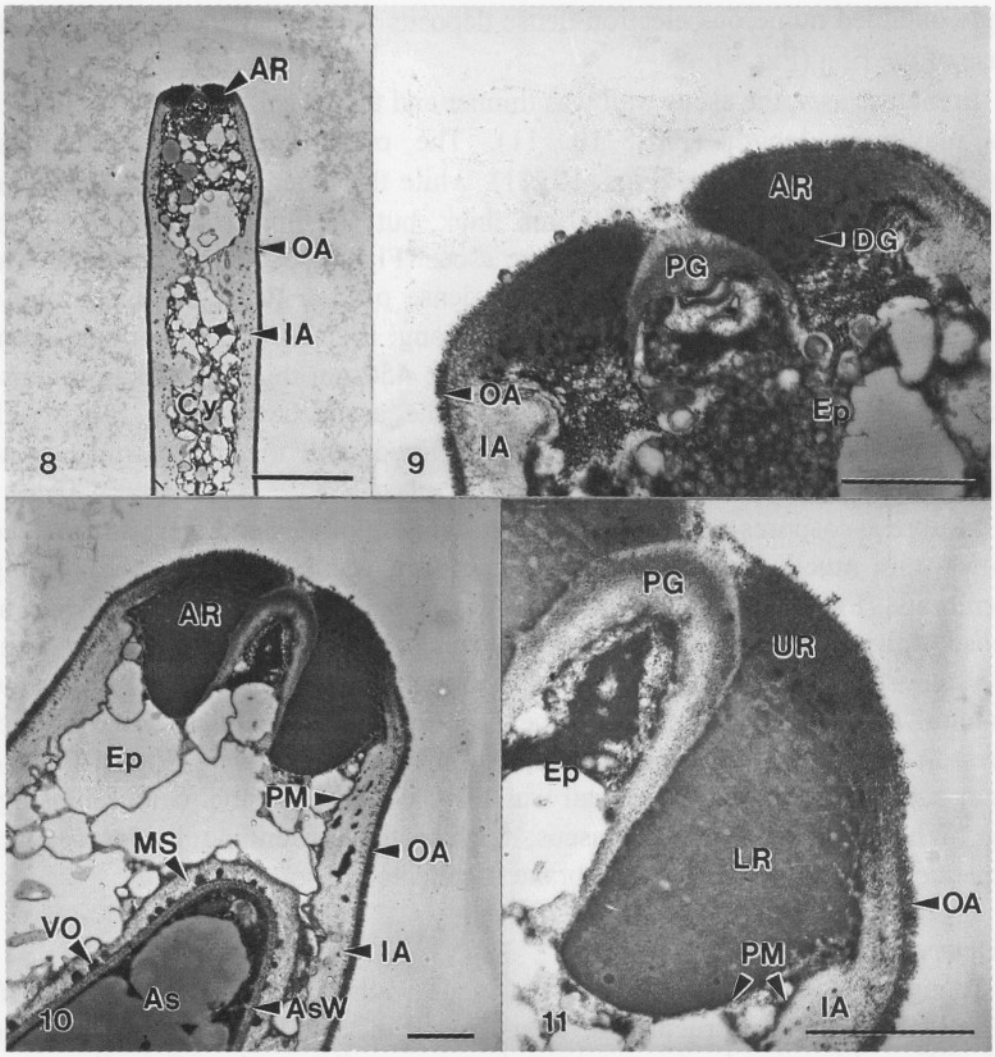
Holotype: HONG KONG, Tai Po Kau Forest Stream, on submerged decaying wood, 27 June 1996, K.D. Hyde WH238 (HKU(M) 4526).

Transmission electron microscopy

Immature asci had a bilamellate wall, an apical ring and epiplasm filled with numerous lipid globules and membrane bound vesicles (Figs. 8, 14). The outer ascus wall layer was electron-dense, 100-150 nm thick and discontinuous at the ascus apex (Figs. 9, 11). The inner ascus wall layer was electron-transparent, 800 nm thick and contained numerous electron-dense inclusions (Figs. 8, 10). The apical ring was electron-dense, continuous with the inner ascus wall layer,



Figs. 1-7. Interference light micrographs of *Annulatascus aquaticus*. **1.** Ascomatal neck on woody substrate. **2.** Ascospores which are hyaline, aseptate and mostly with four lipid globules. **3.** Relatively massive refractive ascal apical ring viewed from top. **4.** Ascal apical ring on side view. **5.** Asci with overlapping uniseriate ascospores. **7.** Paraphyses which are septate, wide and taper distally. Bars: 1 = 200 µm; 2, 3, 6, 7 = 20 µm; 4 = 5 µm; 5 = 10 µm.



Figs. 8-11. Transmission electron micrographs of longitudinal sections of mature and immature asci of *Annulatascus aquaticus*. AR = ascus apical ring, As = ascospore, AsW = ascospore wall, Cy = cytoplasm, DG = electron-dense granular deposits, Ep = epiplasm, IA = inner ascus wall layer, LR = lower portion of ascus apical ring, MS = mucilaginous sheath, OA = outer ascus wall layer, PG = plug, PM = plasma membrane, UR = upper portion of ascus apical ring, VO = verruculose wall ornamentations. **8.** An immature ascus with a thin outer ascus wall layer, a thick inner ascus wall layer and cytoplasm filled with lipid globules and membrane-bound structures. **9.** An immature ascus apical ring with an outer ascus wall layer which is discontinuous at the apical region; inner ascus wall layer which is continuous with the apical ring; and electron-dense granular deposits which accumulate in the lower region of the ring. **10.** A mature ascus with the relatively massive apical ring, and ascospore surrounded by a mucilaginous sheath and verruculose wall ornamentations. **11.** A mature ascus with distinct upper (UR) and lower portion (LR) of apical ring. Bars: 8 = 5 µm; 9-11 = 1 µm.

and contained numerous electron-dense deposits which had accumulated at the base of the ring (Fig. 9).

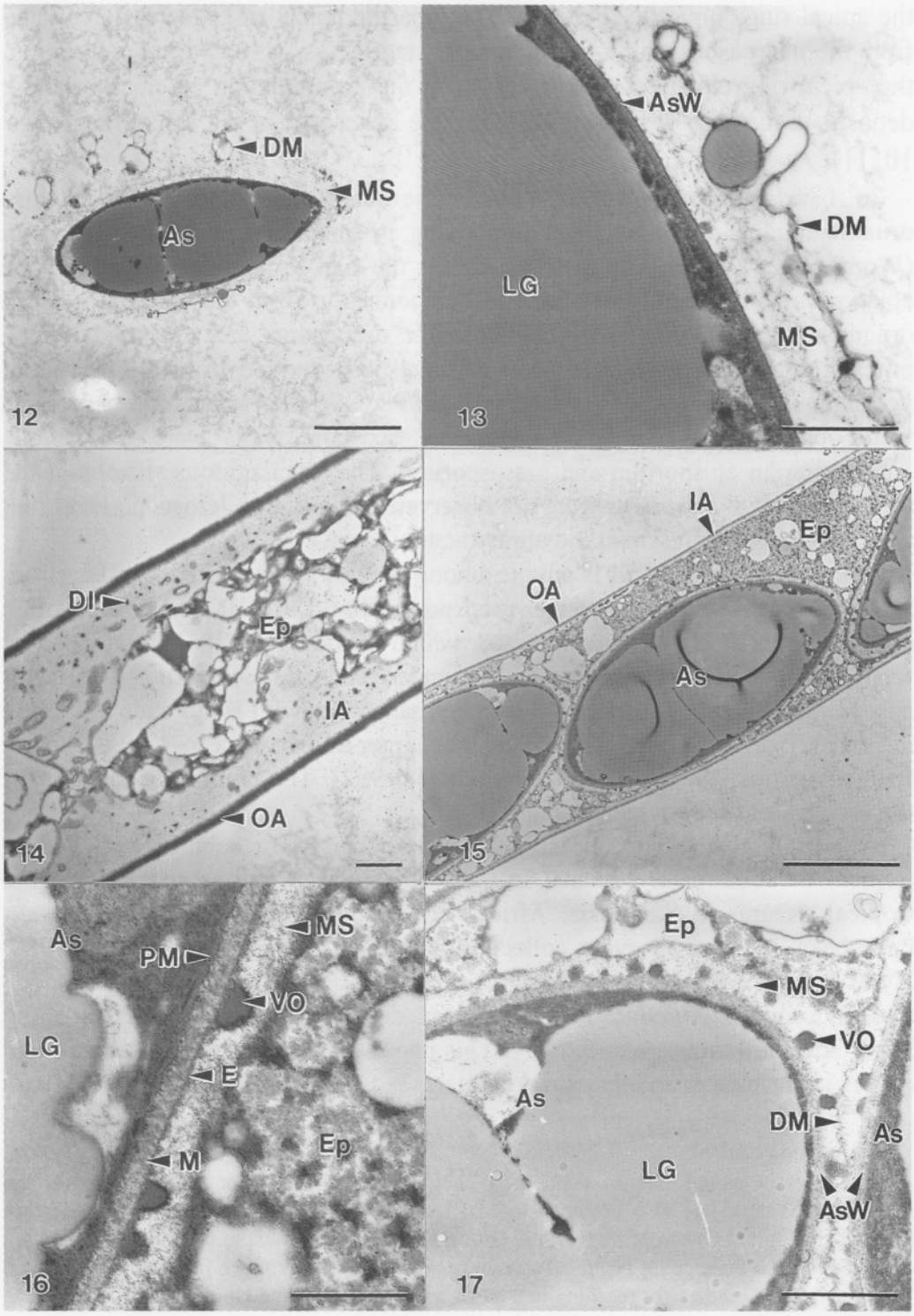
In mature asci, the ascus wall was thinner and the apical ring had extended to its maximum length (Figs. 10, 11). The outer ascus wall layer was discontinuous at the apex (Figs. 10, 11), while the inner ascus wall layer was much thinner (in general 150-300 nm thick, but 700 nm at subapical region; Figs. 10, 15) than that of the immature ascus (Figs. 8, 9). The apical ring was bipartite, comprising an upper, electron-dense portion (0.55 μm long) and a lower, less electron-dense portion (2.7 μm long; Fig. 11). The inner surface of the ring was plugged with a thin layer (up to 450 nm thick) of electron-dense material (Fig. 11). The epiplasm within the ascus cavity was filled with numerous membrane-bound vacuoles, and lined with the plasma membrane (Figs. 11, 15).

Mature ascospores contained 2(-4) large lipid globules and were surrounded with a thin mucilaginous sheath, which in turn was held within a delimiting membrane (Figs. 15, 17). The ascospore wall was thin, comprising an electron-dense episporium (20-30 nm thick) which was covered with numerous verruculose wall ornamentations (100-230 diam.) composed of the same material as the episporium, and a mesosporium (100 nm thick) with parallel electron-dense striations perpendicular to the ascospore wall (Fig. 16). The inner surface of the ascospore wall was lined with the plasma membrane (Fig. 16). When released from the ascus, the sheath surrounding the ascospores expanded and the delimiting membrane fragmented (Fig. 12).

Discussion

Wong *et al.* (1998c) illustrated the ascus walls of *A. velatisporus* and *A. triseptatus* at the ultrastructural level and found them to be bilamellate, and the apical rings bipartite. In *A. aquaticus*, the ascus walls are also bilamellate, and

Figs. 12-17. Transmission electron micrographs of oblique longitudinal sections of different stages of asci and ascospores of *Annulatascus aquaticus*. As = ascospore, AsW = ascospore wall, DI = electron-dense inclusions, DM = delimiting membrane, Ep = epiplasm, IA = inner ascus wall layer, LG = lipid globules, M = mesosporium, MS = mucilaginous sheath, OA = outer ascus wall layer, PM = plasma membrane, VO = verruculose wall ornamentations. **12.** Released ascospore with a mucilaginous sheath and a fragmenting delimiting membrane. **13.** Higher magnification of released ascospore in Fig. 12. **14.** An immature ascus with electron-dense inclusions. **15.** Mature ascus with delimited ascospores and inner ascus wall layer which is much thinner than that in Fig. 14. **16.** Mature ascospore with verruculose wall ornamentations and a mucilaginous sheath. **17.** Ascospore with epiplasm, delimiting membrane, mucilaginous sheath, verruculose wall ornamentations, ascospore wall, and lipid globules. Bars: 12, 15 = 5 μm; 13, 14, 17 = 1 μm; 16 = 0.5 μm.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the apical rings bipartite (Fig. 10). The bipartite apical rings are differentiated from the inner ascus wall layer at an early stage (Figs. 8, 9). During maturation, the apical ring elongates downwards with the accumulation of electron-dense deposits at the base of the ring, forming the lower part of the apical ring (Figs. 10, 11).

In *A. velatisporus* and *A. triseptatus*, the ascospore walls have verruculose ornamentations, are bilamellate, comprising an episporium and a mesosporium (Wong *et al.*, 1998c). The mesosporium is the first formed wall layer and the episporium subsequently develops and elaborates to form the verruculose wall ornamentations (Wong *et al.*, 1998c). The ascospores are surrounded by a mucilaginous sheath which appears to have been derived from the episporial verruculose ornamentations (Wong *et al.*, 1998c). Similar verruculose structures were observed in *A. aquaticus* and the ascospore walls were bilamellate, comprising an episporium and mesosporium. The mucilaginous sheaths of the ascospores of *A. aquaticus* were not observed at the light microscope level, but were shown to be present at the ultrastructural level.

Annulatascus aquaticus is unique among species in the genus. It differs from *A. biatriisporus* which has longer ascospores (40-58 × 8-10 μm) with swollen ends (Hyde, 1995); *A. palmietensis* which has larger (20-26 × 6-7 μm), fusiform, 3-septate ascospores with rounded ends (Hyde *et al.*, 1998a); *A. triseptatus* which has fusiform, 3-septate ascospores with acute ends (Wong *et al.*, 1998c); and *A. velatisporus* which has larger ascospores (26-42 × 9-12 μm) with numerous small lipid globules (Hyde, 1992). The composite diagram of the ascospores are provided in Figs. 18-22.

All *Annulatascus* species have been described from submerged wood in tropical or subtropical streams. In our collections of submerged wood from tropical streams in S.E. Asia, Africa and Australia, species of *Annulatascus* have been recorded in every collection (Hyde and Goh, 1997, 1998a, 1998b; Hyde *et al.*, 1998b; Ho, pers. observ.). Recently Umali (pers. comm.) recorded two undescribed *Annulatascus* species on decaying bamboo culms in Hong Kong and Philippines, respectively. This genus appears to be pan-tropical, and occurs in freshwater and terrestrial habitats.

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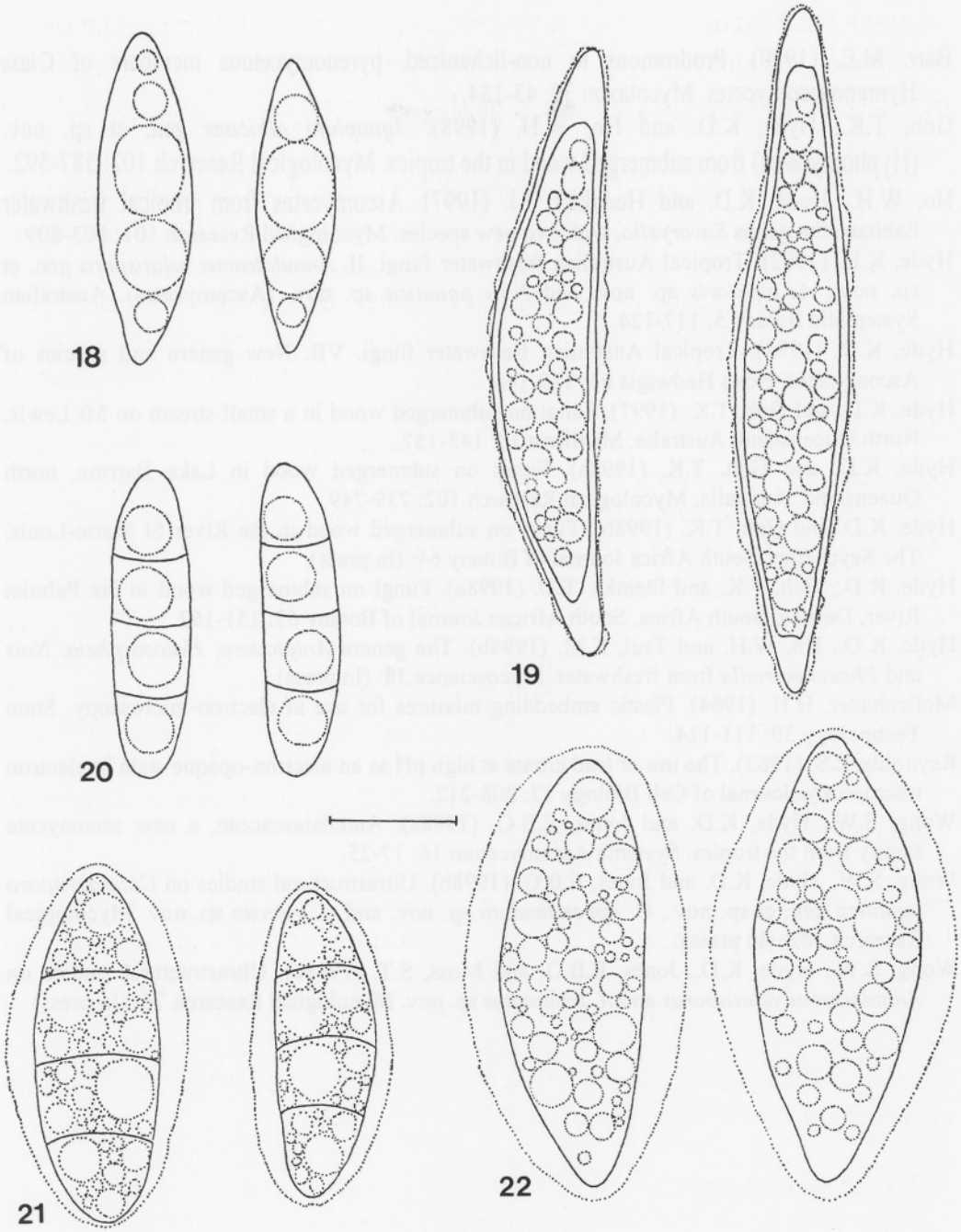

Figs. 18-22. Comparative illustrations of ascospores of *Annulatascus* species. Dotted lines indicate shape of mucilaginous sheaths. **18.** *A. aquaticus*. **19.** *A. biatriisporus*. **20.** *A. palmietensis*. **21.** *A. triseptatus*. **22.** *A. velatisporus*. Bar = 10 µm.

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