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Ultrastructural studies of *Massarina ingoldiana* and *M. purpurascens*

K. M. Tsui[1]
S. W. Wong[2]
K. D. Hyde
I. J. Hodgkiss

Fungal Diversity Research Project, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong

Abstract: Ascus and ascospore appendage structures can be important characters in delineation of genera. We have therefore examined the ascus and ascospore ultrastructure of *Massarina ingoldiana* and *M. purpurascens,* as *Massarina* is thought to be a heterogeneous genus. The ascus wall of *M. ingoldiana* is two layered and comparable to *M. thalassiae.* Ascospores of both species comprise an electron-transparent mesosporium, an electron-dense episporium and an exosporial mucilaginous sheath. In this respect they are similar to the ultrastructure of other *Massarina* species, and should be maintained in *Massarina.*

Key Words: aquatic fungi, ascomycetes, fine structure, electron microscopy

INTRODUCTION

The genus *Massarina* Sacc. comprises 43 species of which eight are known from freshwater habitats (Aptroot 1998) and is thought to be heterogeneous. Most aquatic species have ascospores with polar appendages, e.g., *M. bipolaris* K. D. Hyde (Hyde 1995a), *M. fronsisubmersa* K. D. Hyde (Hyde 1994), or they are surrounded by a mucilaginous sheath, e.g., *M. ingoldiana* Shearer & K. D. Hyde (Shearer and Hyde 1997) and *M. purpurascens* K. D. Hyde & Aptroot (Hyde and Aptroot 1998). Since the ascospore appendage is significant in the delineation of genera (Jones 1995), this study was initiated to establish if there are any major differences between species at the ultrastructural level, and whether the differences can be used to place the *Massarina* species into different genera.

Investigation on the fine structure of *Massarina* species has been restricted to marine species, including *M. thalassiae* Kohlm. & Volkm.-Kohlm. and *M. armatispora* K. D. Hyde et al (Read et al 1994). *Massarina purpurascens* and *M. ingoldiana* are particularly common in streams in Hong Kong, and we have therefore examined the fine structure of the ascospores of these species at the ultrastructural level. The results are also compared with ultrastructural data from other bitunicate species.

MATERIALS AND METHODS

Submerged wood was collected from rivers and reservoirs in the New Territories, Hong Kong, and in Brunei and returned to the laboratory in plastic bags, where they were incubated for 2–3 wk on moist paper in sterile plastic boxes. Material was periodically examined under a dissecting microscope and fungal fruiting bodies present identified.

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), an ascospore suspension was prepared in distilled water using the ascomata content, and fresh ascomata were removed from wood. They were fixed and prepared following the methods described by Ho et al (1999). For cryoscanning electron microscopy (cryo-SEM), the polycarbonate membrane with specimens were air-dried for 5 min at room temperature mounted on aluminium stubs using carbon cement. The material was then frozen in an external chamber (Polaron LT7400 Cryopep) which was connected to the main chamber of Leica Stereoscan 440 SEM. The material was lowered to −150 C followed by coating with gold for 40 s using a built-in sputter coater. Subsequently, the specimen was transferred under vacuum into the main chamber and placed on the cryostage for low temperature (−150 C) observation using accelerating voltages of 10 kV.

RESULTS

Massarina purpurascens produces fusiform, two-celled ascospores that are constricted at the septum, and surrounded by a mucilaginous sheath (FIGS. 1, 2), although the appearance of the sheath depends on the condition of material. In a fresh specimen from Brunei, the sheath had an outer thin, undulating membrane and was examined in this study (FIGS. 3, 4). In dried specimens the sheath is often inconspicuous or lacking.

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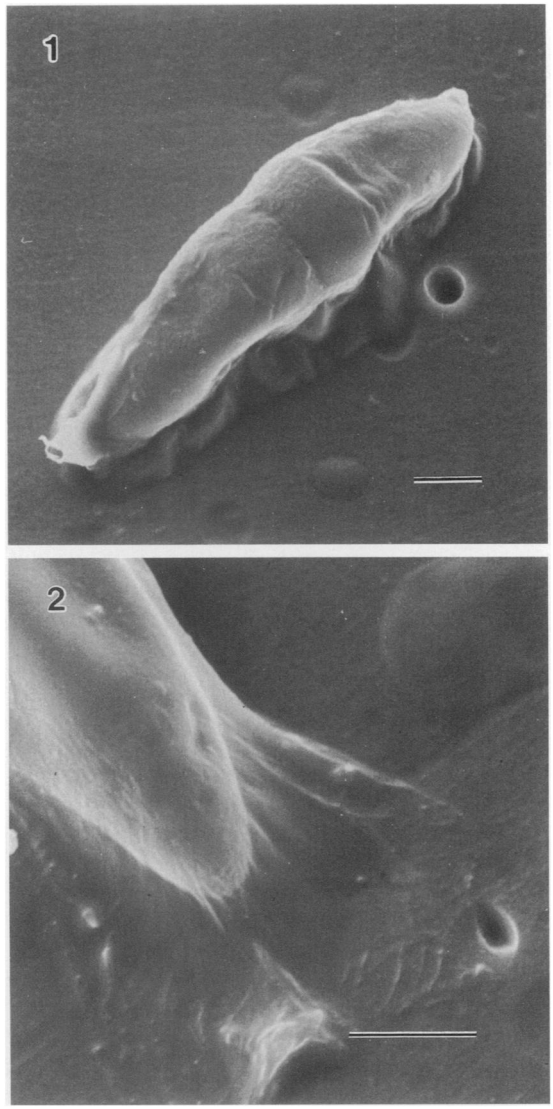
[1] Email: h9308167@hkusua.hku.hk

[2] Present Address: Singapore Eye Research Institute Laboratory, MD9 Physiology Building, #01–15, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260.



FIGS. 1, 2. *Massarina purpurascens,* SEM of ascospore surrounded with a characteristic sheath. Scale bars: 5 μm.

Mature ascospores of *M. purpurascens* were surrounded by a thick electron-transparent mucilaginous sheath (0.86–1.35 μm thick) with an outer thin electron-dense layer (ca 90 nm thick) (FIGS. 3, 4). This outer layer was electron dense, undulating and composed of compacted fibrillar material. Remnants of the membrane complex were also present (FIG. 4). In addition, the outer layer was connected to the ascospore wall at some regions e.g., ascospore apex (not illustrated here) and appeared to agree with the characteristic morphology of the ascospore sheath at the SEM level (FIGS. 1, 2). The thick mucilaginous sheath comprised diffuse material (FIG. 4) and some electron dense inclusions (FIG. 4). Both the mucilaginous sheath and the outer layer were exosporial. The mature ascospore wall comprised an outer electron dense amorphous episporium (ca 30 nm) and an inner electron-transparent mesosporium (ca 130 nm) (FIG. 4).

Ascospores of *Massarina ingoldiana* were also two-celled, fusiform, slightly constricted at midseptum, hyaline, guttulate and surrounded by an inconspicuous mucilaginous sheath that could only be observed following the addition of India ink (Shearer and Hyde 1997).

When viewed by scanning and cryoscanning electron microscopy, mature ascospores were fusiform and surrounded by wide elongate mucilaginous sheath (FIGS. 5, 6). The drying process in conventional SEM dehydrated the mucilaginous sheath (FIG. 5), whereas the mucilaginous sheath was in a condensed form when examined by cryo-SEM (FIG. 6). This sheath was closely adpressed to the polycarbonate membrane and may aid in ascospore attachment.

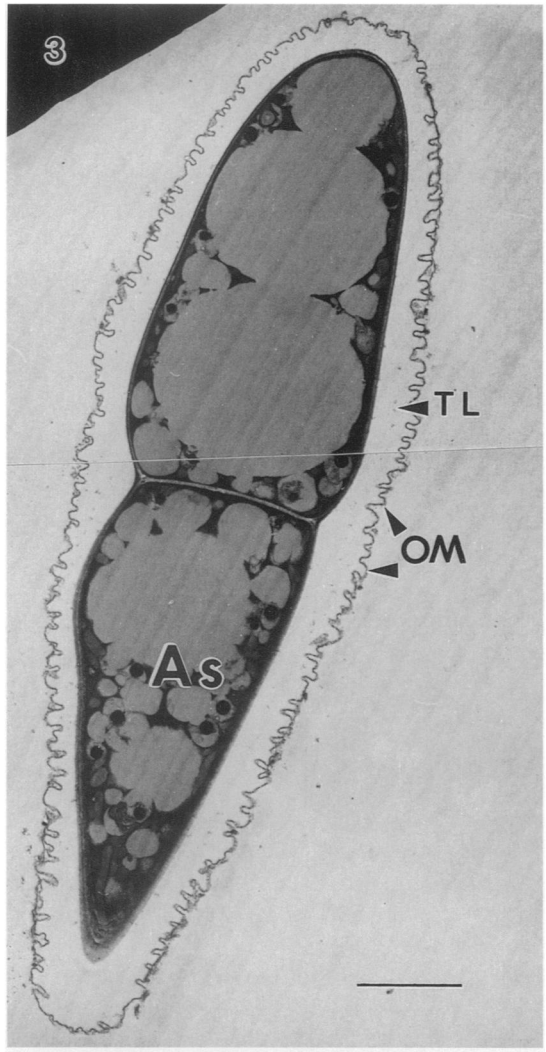
When viewed with transmission electron microscopy the bitunicate ascus of *Massarina ingoldiana* included an outer thin electron-dense ectoascus (ca 300 nm thick) and an inner electron-transparent endoascus (ca 770 nm thick) (FIGS. 7, 8).

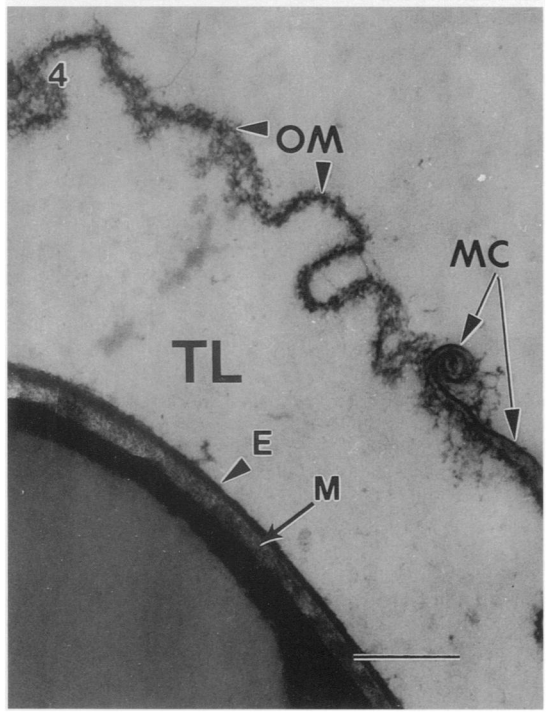
The walls of mature ascospores comprised an outer, thin, electron-dense episporium (ca 12 nm wide) and an inner thick electron-transparent mesosporium (ca 100 nm wide) (FIGS. 10, 12). Some electron-dense inclusions occurred within the mesosporium (FIG. 10). Condensed mucilaginous material aggregated on the outside of the ascospore wall, near to the septum (FIG. 11).

In the ascus, ascospores were surrounded by a thick, dense exosporial fibrillar layer (300–470 nm thick) with an outer discontinuous membrane (possibly the membrane complex) (25–55 nm) (FIG. 8). This membrane was irregular (FIG. 9) and in released ascospores, it coiled up at the edges where discontinuous (FIG. 8). The dense mucilaginous sheath (ca 1.85 μm thick) of released ascospores expanded (FIG. 7), which may have disrupted the outer membrane. The fibrillar component of the sheath became more diffuse and spread in water via the discontinuities of the outer membrane (FIGS. 9, 10). The outer membrane appeared to slough off or disintegrate, and the ascospore was then surrounded by a diffuse and expanded fibrillar sheath (FIGS. 11, 12).

DISCUSSION

Ascus wall.—The ascus wall in *M. ingoldiana* shares structural similarities to *M. thalassiae* (Read et al 1994) in having an outer narrow electron-dense ectoascus and an inner thick electron-transparent endoascus surrounded by mucilage. It is, however, different from that in *Pleospora gaudefroyi* Pat., which is characterized by a much narrower ectoascus and a





bipartite endoascus (Yusoff et al 1994). No partition of the endoascus has been observed in *Massarina* species.

The separation of the ascus wall into an inelastic ectoascus and an elongate elastic endoascus is a unique character of bitunicate fungi (Reynolds 1989). This separation occurs irrespective of the number of sublayers within the ectoascus and endoascus. The asci of *Massarina ingoldiana and M. purpurascens* are bitunicate, and only have two sublayers.

Ascospore wall and appendage.—The ascospore wall of *M. thalassiae* (Read et al 1994), *M. ingoldiana* and *M. purpurascens* include an electron-transparent mesosporium, an electron-dense episporium and an exosporial mucilaginous sheath with an outer electron-dense layer. *Massarina purpurascens* differs slightly from the other two species in having electron-dense inclusions within the mesosporium. The outer membrane of the mesosporial sheath is undulate in *M. purpurascens* and *M. ingoldiana,* whereas in *M. thalassiae* it is linear. The mucilaginous sheath of *M. thalassiae* and *M. purpurascens* is constricted in some regions, and this results in the characteristic shape of the sheath. In *M. ingoldiana,* the outer membrane is discontinuous, probably due to swelling of the exosporial sheath. Some aquatic Loculoascomycetes also possess similar sheaths. In *M. ramunculicola* K. D. Hyde, ascospores are at first surrounded by a thin membrane, and this subsequently breaks at the poles to form pads. The membrane then disintegrates and a wide mucilaginous sheath is formed (Hyde 1991). In *M. ricifera* K. D. Hyde the sheath is two-layered and is only visible in India ink (Kohlmeyer et al 1995). The inner layer is thought to rupture the outer membrane. In *Trematosphaeria confusa* K. D. Hyde and *Vaginatispora aquatica* K. D. Hyde, the sheath is formed by rupture of an outer membrane (Hyde 1995a, b). The outer membranes in ascospores of *M. thalassiae* and *M. purpurascens* are persistent even when the ascospore sheath has expanded in water,

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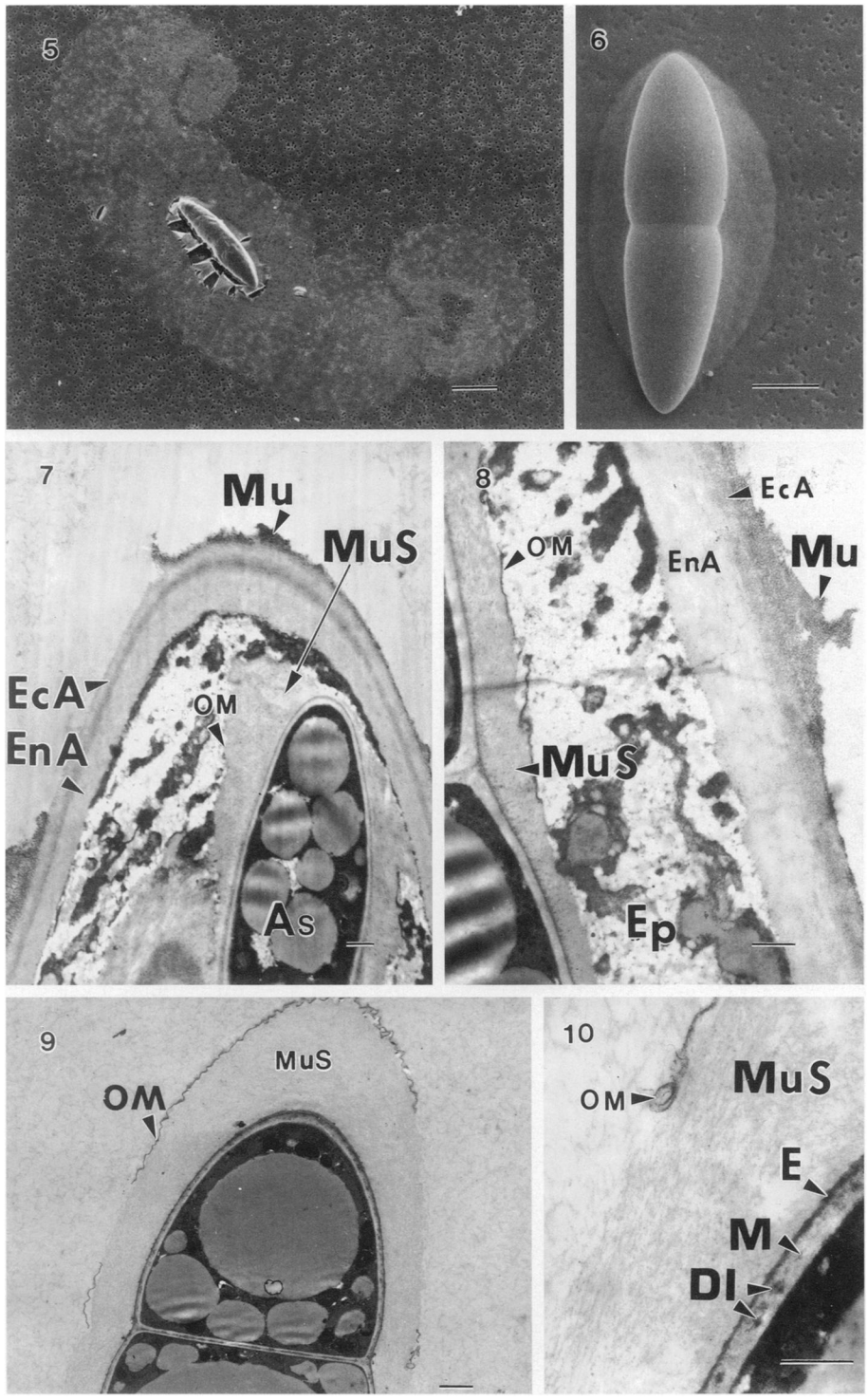
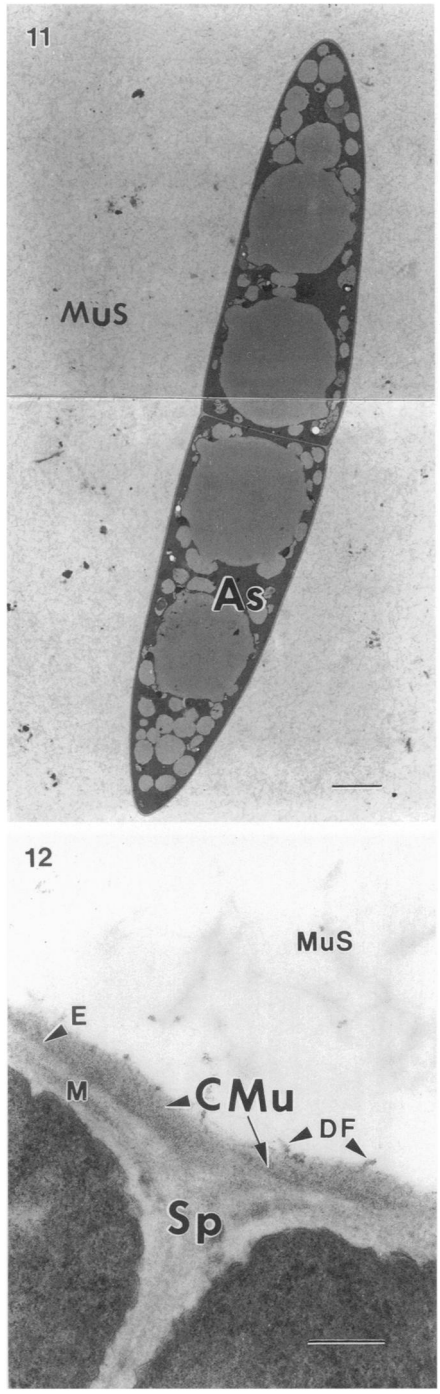
FIGS. 3, 4. *Massarina purpurascens,* TEM. 3. Oblique longitudinal section illustrating the whole ascospore surrounded by an outer irregular electron-dense layer (OM) and inner electron-transparent layer (TL). 4. Higher magnification of the ascospore sheath illustrating the outer layer (OM) composed of compact fibrillar material. Remnants of the discontinuous membrane complex (MC) are present to the outside undulating layer (OM). Longitudinal sections of ascospore wall also illustrating the outer electron-dense episporium (E) and the inner electron-transparent mesosporium. Scale bars: 3 = 5 μm, 4 = 0.5 μm.



FIGS. 5–10. *Massarina ingoldiana*. 5. Ascospore surrounded by a wide elongated sheath, SEM. 6. Condensed form of mucilaginous sheath surrounding the ascospore, cryo-SEM. 7, 8. Longitudinal sections of mature asci illustrating the ectoascus (EcA) and endoacus (EnA), TEM. Some mucilaginous material (Mu) occurs on the ascus wall. Ascospores in the ascus are surrounded by a mucilaginous sheath (MuS) with an outer membrane (OM). 9. Oblique longitudinal sections illustrating released ascospores surrounded by a mucilaginous sheath (MuS) with outer membrane (OM), TEM. Note the discontinuities in the membrane (arrowed). 10. Longitudinal section illustrating the outer membrane (OM) coiled at the discontinued edge, TEM. The sheath comprises fibrillar material, which remains condensed against the episporium (E). Some electron-dense inclusions (DI) occur within the mesosporium (M). Scale bars: 5 = 20 μm, 6 = 10 μm, 7, 8, 10 = 0.5 μm, 9 = 0.1 μm.



FIGS. 11, 12. *Massarina ingoldiana,* TEM. 11. Longitudinal sections of ascospores illustrating the lack of outer membrane and dense mucilaginous sheath surrounding the ascospores (As), which is more diffuse. 12. Higher magnifications of the ascospore wall illustrating condensed mucilaginous material (CMu) which has accumulated on the ascospore wall at the septum (Sp). Electron-dense fibrillar material (DF) appears to be associated with this condensed mucilaginous material (CMu) and the episporium (E) of the released ascospore. Scale bars: 11 = 5 μm, 12 = 0.1 μm.

although a discontinuity may occur occasionally at the ascospore tip in *M. thalassiae* (Read et al 1994).

Electron-dense granular inclusions occur in the exosporial sheath of immature ascospores of *M. thalassiae* and *M. armatispora* (Read et al 1994). These inclusions may be condensed mucilaginous material, which absorbs water to form the extensive sheath. We have only examined mature ascospores of *M. ingoldiana* and *M. purpurascens,* however, electron-dense inclusions have been found in the exosporial sheath of *M. purpurascens.* Episporial polar chambers containing concentrated fibrillar material occur at the apices of ascospores of *M. thalassiae,* whereas no chambers have been observed in other *Massarina* species.

Although both *Pleospora* and *Massarina* are placed in the Pleosporales (sensu Barr 1987), their ascospore walls differ. In *Pleospora gaudefroyi* the ascospore wall comprises an electron-transparent mesosporium (W1) enclosing each cell (Yusoff et al 1994). All cells are embedded in a common layer with electron-dense inclusions (W2), and the whole ascospore is surrounded by a layer containing compact melanin bodies (W3) with an outer thin episporium (W4). No partition of mesosporium has been observed in *Massarina* species. In addition, although the episporium of ascospores of *Massarina* species are thick and electron dense, no melaninlike bodies have been observed, although they may be present in older ascospores.

The data presented here indicated that ascospores in both *M. ingoldiana* and *M. purpurascens* have similar wall structure, including a mesosporium, an episporium and an exsosporial sheath with an outer layer. These characters are similar to those found in ascospores of *M. thalassiae* (Read et al 1994), and the similarities imply they have a similar appendage ontogeny. As the type species *M. eburnea* has not yet been studied, it is premature to separate *Massarina* species into further genera.

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