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Ultrastructure of asci and ascospores of the mangrove ascomycete *Dactylospora haliotrepha*

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Ultrastructure of the marine Loculoascomycete *Dactylospora heliotrepha* is presented and compared with *Marinosphaera mangrovei*, *Swampomyces armeniacus* and other marine species. Ascospores are bi-celled and ridged. The ridges are outgrowths of the outer mesosporial layer and formed later in ascosporogenesis. The exosporial layer fragments to release mucilaginous material present between the spore wall ridges. Asci and pseudoparaphyses are held together by a fibrillar mucilaginous network. The endoascus is thicker than the ectoascus. Comparisons are made of the diameter of ascomata, size of asci and ascospores of *D. heliotrepha* collected from mangroves in Hong Kong, Malaysia, the Philippines and Taiwan.

Key Words—bitunicate ascus; *Dactylospora haliotrepha*; Loculoascomycetes; mangrove fungi; mucilage; ultrastructure.

Dactylospora haliotrepha (Kohlm. et Kohlm.) Hafellner is a frequent ascomycete on decaying wood in mangroves, both tropical and subtropical (Kohlmeyer and Kohlmeyer, 1979). The fungus forms dark brown to black, flat disk-like ascomata on the surface of substrata (Hyde, 1986; Nakagiri, 1993). Asci are bitunicate, apically thickened, while the ectoascus is reported to secrete a gelatinous sheath (Nakagiri, 1993) which stains blue with iodine. Ascospores are obovoid, one-septate in the lower third, constricted at the septum, light brown (sometimes appearing green or blue in colour) and with a striate spore wall. Hyde (1986) and Nakagiri (1993) showed, at the scanning electron microscope (SEM) level, that these striae are longitudinally or oblique woven ribs, with remnants of a sheath or mucilage attached to them. Pseudoparaphyses are swollen at the apex, septate, hyaline, and occasionally branching.

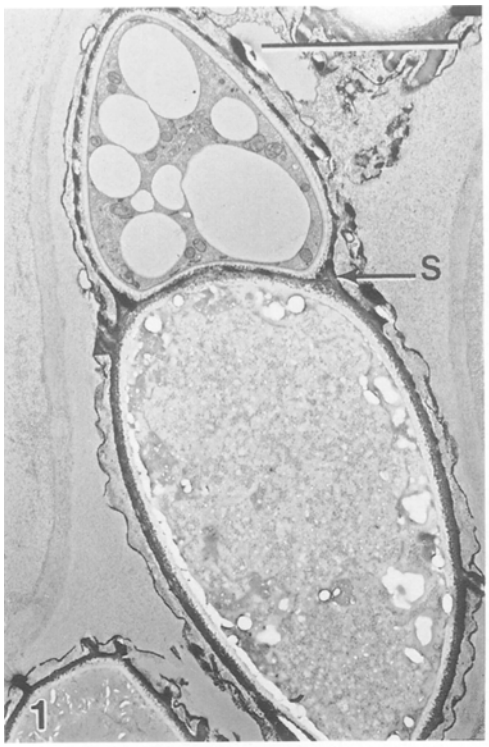
This species was originally described as *Buellia haliotrepha* Kohlm. et Kohlm. (Kohlmeyer and Kohlmeyer, 1965) but later transferred to a new genus *Kymadiscus* Kohlm. et Kohlm. More recently the species has been assigned to *Dactylospora* by Hafellner (1979). The species has been variously assigned to the Patellariaceae (Kohlmeyer and Kohlmeyer, 1979), Lecanorales (Hafellner, 1979), the Physciaceae, Lecanorales (Kohlmeyer, 1986), and the Dactylosporaceae Bellem. et Hafellner, Lecanorales incertae sedis (Hafellner in Hawksworth, 1994).

Although there are extensive studies on the ultrastructure of marine unitunicate ascomycetes (Moss, 1990; Jones, 1995), only a few marine Loculoascomycetes have been examined at the transmission electron microscope level (TEM): e.g. *Paraliomyces lentiferus* Kohlm. (Read et al., 1992), *Pleospora gaudefroyi* Pat. (Yusoff et al., 1994); and *Massarina thalassiae* Kohlm. et Volkm.-Kohlm. (Read et al., 1994).

Materials and Methods

Material of *D. haliotrepha* was collected on intertidal wood from Three Fathoms Cove mangrove, Hong Kong; Boracay and Takalong mangroves, the Philippines; Morib mangrove, Malaysia; and from a mangrove community in Taiwan. The diameter of the ascomata on wood; the size (length and width) of asci and ascospores (30–50 samples) were measured under the light microscope (Table 1).

Three fixation methods were used for the TEM. Fresh material was 1) embedded in 2% ion agar No. 2 (Oxoid), fixed in 2% (w/v) aqueous potassium permanganate for 15 min; 2) primary fixed in 4% (v/v) glutaraldehyde in filtered seawater for 3 h at room temperature, secondarily fixed in 2% (w/v) osmium tetroxide for 2 h at 4°C, and post-fixed in 1% (w/v) uranyl acetate at 4°C overnight; 3) to stain for polysaccharides, material was primary fixed in glutaraldehyde as in (2), ruthenium red was added to 2% (w/v) osmium tetroxide to a final concentration of 0.04% (w/v) in 0.1 M sodium cacodylate buffer for secondary fixation at 4°C overnight. Fixed material from methods (2) and (3) were embedded in 2% ion agar No. 2 before dehydration. Dehydration was carried out through a graded ethanol series, then transferred to acetone and embedded in Spurr's resin (Sigma). Ultrathin sections were stained with lead citrate, post-stained with uranyl acetate and examined at 80 kV in a JEOL 100SX TEM or Philips CM20 TEM.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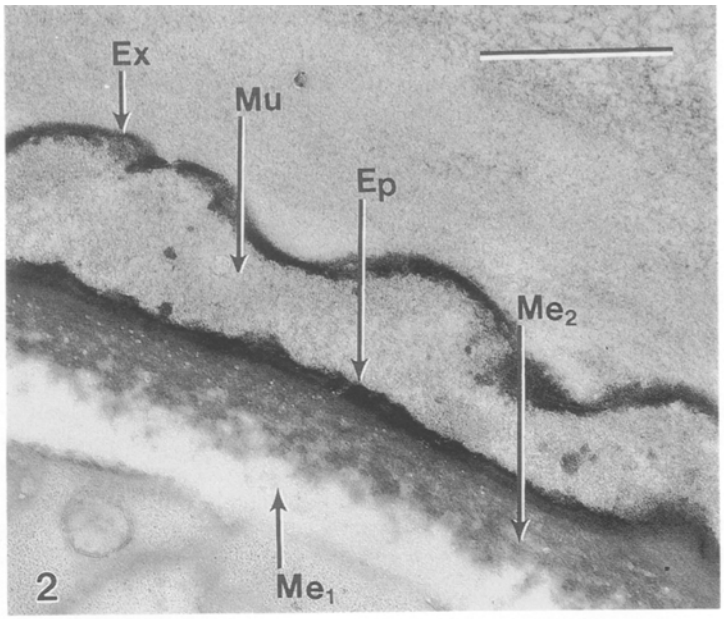
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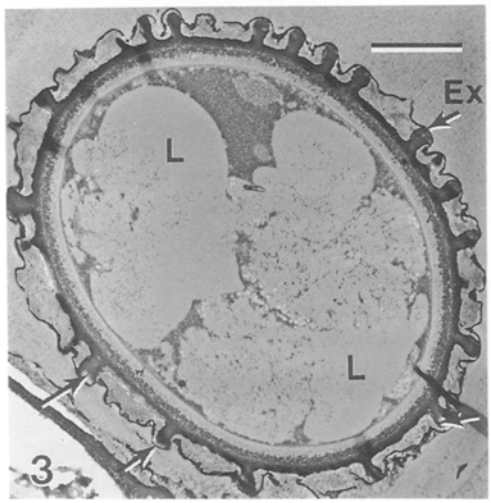
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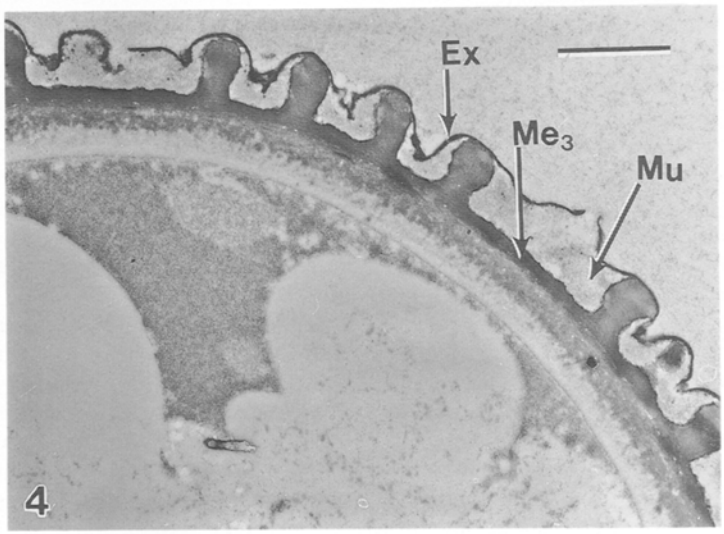
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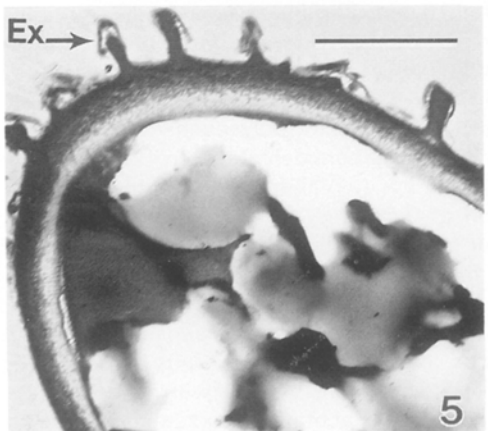
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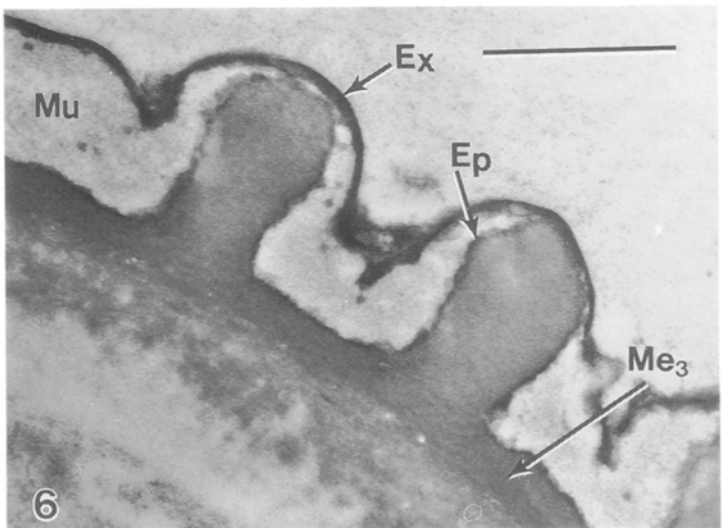
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Table 1. Measurements (μm) (average and range) of ascomata, asci and ascospores of *Dactylospora haliotrepha* collected from mangroves at different geographical locations.

Location/sites	Ascomata	Asci[a)]	Ascospores[a)]
Hong Kong/Three Fathoms Cove	90 (60-130)	L: 90 (60-120) W: 16 (10- 24)	L: 22 (18-24) W: 6 (5- 8)
Taiwan	70 (40-110)	L: 120 (110-135) W: 13 (10- 16)	L: 21 (18-23) W: 8 (7- 9)
Philippines/Boracay	110 (60-170)	L: 80 (70-100) W: 17 (10- 22)	L: 22 (20-24) W: 8 (6-10)
Philippines/Takalong	130 (80-170)	L: 120 (100-130) W: 18 (16- 20)	L: 24 (20-26) W: 8 (6-10)
Malaysia/Morib			L: 18 (14-23) W: 7 (6- 9)

a) L: length; W: width.

Results

Morphological measurements Measurements of ascomata (80-170 μm in diam), asci (100-130 × 16-20 μm) and ascospores (20-26 × 6-10 μm) of *D. haliotrepha* on wood collected from Takalong mangroves, the Philippines, were greater compared to those from Hong Kong, Taiwan and Malaysian mangroves (Table 1). No variation in morphology of these structures was observed among samples from different geographical locations. However, a new species of *Dactylospora* remains to be described (Jones, Alias and Vrijmoed, personal communication).

Ascospores Fully developed ascospores were obovoid, pale brown, one septate, the lower cell smaller, and constricted at the septum (Fig. 1). No appendage or mucilaginous sheath was observed at the light microscope level. Each cell in the ascospore possessed up to 6 layers. An outer membranous layer, the exosporium (30-60 nm) was attached to the spore wall ridges and mucilaginous material was present between the ridges (Figs. 2-6). An electron-dense episporial layer (40-60 nm) surrounded the ridges and separated the mucilage from the mesosporium (Figs. 2-6). The mesosporium comprises 3 distinct zones (Me_1: 200-220 nm; Me_2: 200-230 nm; Me_3: 100-160 nm) increasing in electron density from the inside to the outside (Figs. 2, 4, 6). Ascospore ridges arise as outgrowths of the outer mesosporium (Me_3) with the episporium surrounding the ridges which are 500-700 nm long and 220-400 nm wide (Figs. 3-6). Early in development the exosporium is sinuate and the spore wall ridges not yet formed (Fig. 2). Later, the exosporium comprises a membranous sheath, attached at the tips of the spore ridges (Figs. 3-6) but rupture at maturity with the loss of the mucilaginous material (Fig. 5). In between the ridges there is fibrillar mucilaginous material that is electron-dense and heterogeneous in texture (Fig. 6). All three mesosporial layers contribute to the formation of the septum, which is constricted at the centre to form a pore with associated Woronin bodies (Fig. 7).

Pseudoparaphyses Pseudoparaphyses are thick-walled, septate with numerous small vacuoles and extensive membraneous system (Figs. 9-10, 16-17). Pseudoparaphyses are swollen at their apex and are surrounded by a mucilaginous sheath which is heavily stained by ruthenium red, indicating it is polysaccharide in composition (Figs. 9, 10, 15).

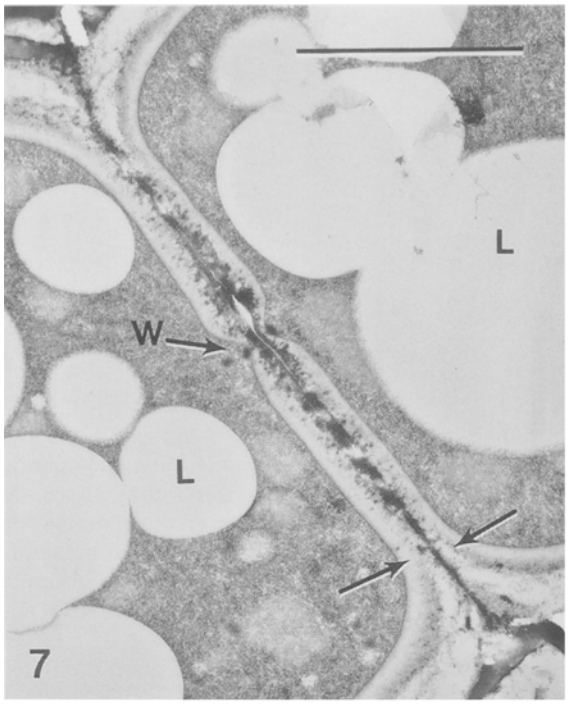
Asci Asci are 80-120 × 13-18 μm, eight spored, clavate, bitunicate, without an apical apparatus (Figs. 12, 13). The endoascus is thickened apically. Asci and pseudoparaphyses are held together by a fibrillar mucilaginous network (Figs. 14, 16, 17). The origin of the mucilaginous matrix cannot be determined, but is most likely to be derived from the pseudoparaphyses.

Discussion

This study confirms that the ascospores of *D. haliotrepha* are ridged as indicated by Hyde (1986) and Nakagiri (1993). It shows that the ridges arise as outgrowths of the outer electron-dense mesosporial layer and formed late in ascosporogenesis since they are absent in early stages of ascosporogenesis. The episporium follows the contours of the spore wall ridges and does not separate the ridges from the mesosporium. This study also

Figs. 1-6. *Dactylospora haliotrepha*. Transmission electron micrographs. Ascospores. $KMnO_4$ fixation.

1. Longitudinal section of an immature ascospore which is obovoid, one septate (arrowed S) and constricted the septum; 2. Higher magnification of ascospore wall in Fig. 1. Wall comprises: a sinuated exosporium (arrowed Ex), mucilaginous material (arrowed Mu), electron-dense episporium (arrowed Ep) and the bilayered mesosporium, Me_1 (electron-transparent) and Me_2 (electron-dense); 3. Transverse section of ascospore. Spore wall ridges (arrowed) are electron-dense and distributed evenly over the spore wall. The exosporium (Ex) remains intact and is attached to the ridges. Lipid bodies (L) are abundant in the cytoplasm; 4, 6. Higher magnification of the spore wall in Fig. 3. Spore wall ridges originate from the electron-dense, outer mesosporial layer (arrowed Me_3), with the sinuated exosporium (arrowed Ex) attached to the tip of the ridges and mucilaginous material between the ridges (arrowed Mu). Ep = episporium; 5. Ascospore wall. With remnant of the ruptured exosporium (arrowed Ex) remaining attached to the tip of spore wall ridges which are more elongate than in Figs. 3, 4. Scale bars: Fig. 1 = 5 μm; Fig. 2 = 0.5 μm; Fig. 3 = 2 μm; Fig. 4 = 1 μm; Fig. 5 = 2 μm; Fig. 6 = 0.5 μm.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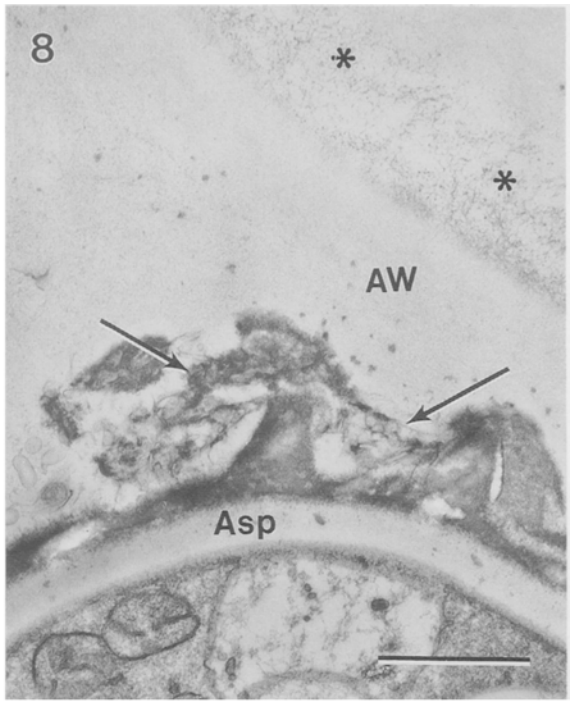
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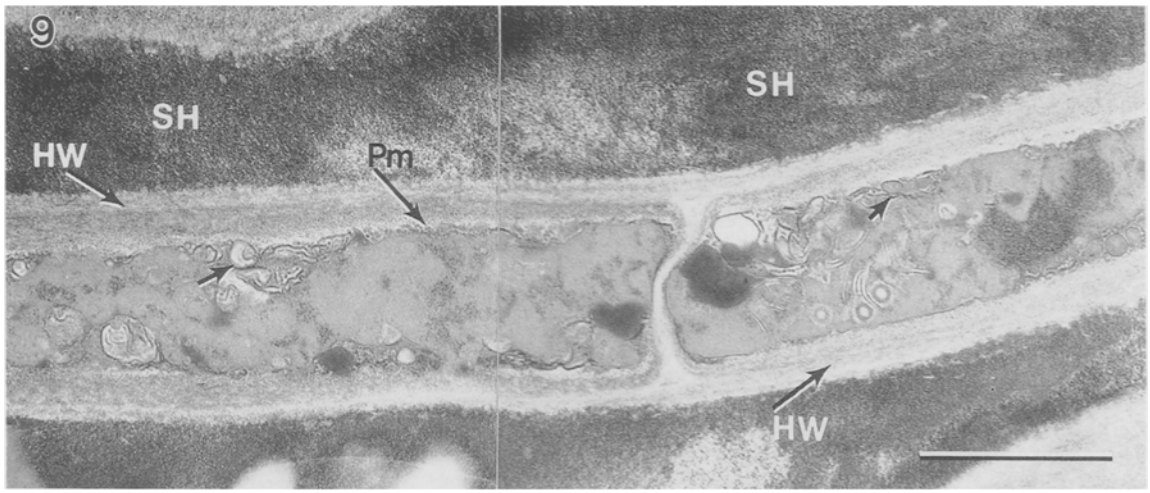
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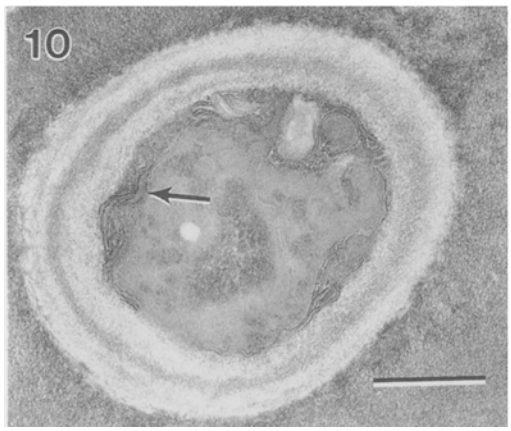
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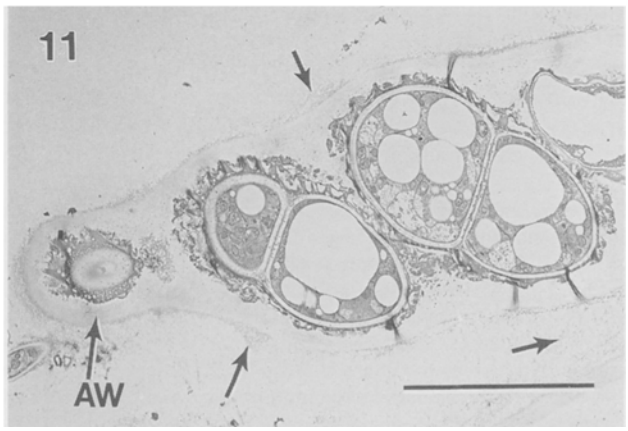
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demonstrate that the spore is surrounded by an electron-dense exosporium which is attached to the tips of the spore wall ridges. Mucilage separates the episporium from the exosporium. When the exosporium ruptures the mucilage is lost. While Nakagiri (1993) does not refer to the presence of mucilage on the spore wall, Hyde (1986) does and it is clearly visible in his SEM micrographs. We have been unable to determine the origin of the mucilage or its function. Unlike the ascospore appendages of members of the Halosphaeriaceae, the mucilage does not appear to play a role in ascospore adhesion (Hyde and Jones, 1989; Jones, 1994).

The ridged ascospore wall, at the TEM level, resembles those of *Swampomyces armeniacus* Kohlm. et Volkm.-Kohlm., a marine ascomycete which has hemispherical ornamentations up to 130 nm thick. A 20–25 nm thick exosporium was closely adpressed to the raised regions of the ornamentations. In some regions, the exosporium was intact, whereas in others it was discontinuous, fragmented or deliquesced (Read et al., 1995). In ascospores of *Marinosphaera mangrovei* Hyde, block-like outgrowth of the episporium formed surface ornamentations. These were cuboidal in longitudinal section and measured up to 120 nm high and 140 nm across. Between the electron-dense cuboidal ornamentations was a less electron-dense fibrillar matrix. However, no exosporium was present in this species (Read et al., 1995).

Many marine bitunicate fungi have extensive mucilaginous sheaths that are exosporial in position (e.g. *Ascocratera manglicola* Kohlm., *Julella avicenniae* (Borse) Hyde, *Leptosphaeria avicenniae* Kohlm. et Kohlm., *L. pelagica* Jones, *Lophiostoma mangrovei* Kohlm. et Vittal, *Massarina acrostichi* Hyde, *M. lacertensis* Kohlm. et Volkm.-Kohlm., *M. thalassiae*, *M. velatospora* Hyde et Borse, and *P. gaudefroyi*. These sheaths may aid in spore adhesion as demonstrated for *Leptosphaeria* sp. and *P. gaudefroyi* (Hyde et al., 1986). However, in none of the species examined at the TEM level has a membraneous, fragmenting exosporium been reported.

A number of bitunicate marine ascomycetes have been shown to have ornamented (*Belizeana tuberculata* Kohlm. et Kohlm., *Caryosporella rhizophorae* Kohlm., *Didymosphaera lignomaris* Strongman et Miller, *Verruculina enalia* (Kohlm.) Kohlm. et Volkm.-Kohlm.) or ridged ascospores (*Lineolata rhizophorcea* (Kohlm. et Kohlm.) Kohlm. et Volkm.-Kohlm. and *Trematosphaeria striatispora* Hyde (Kohlmeyer and Volkmann-Kohlmeyer, 1991). However, none have been examined at the TEM level (Nakagiri, 1993). The ascospore wall of bitunicate ascomycetes vary in their degree of complexity. In *M. thalassiae*, 3 layers are recognized: a mucilaginous exosporial sheath with an outer electron-dense layer, an electron-dense episporium and an inner mesosporial layer. In most marine ascomycetes these layers are single entities of varying width (Jones et al., 1986; Jones and Moss, 1987; Jones, 1995). However, recent studies of the ultrastructure of spore walls indicates that many species have a mesosporium composed of two (e.g. *Carbosphaerella leptosphaerioides* I. Schmidt) or three layers (e.g. *P. gaudefroyi*) (Read et al., 1992; Read et al., 1993; Read et al., 1994; Yusoff et al., 1994).

Hafellner (1979) suggested that the ascus in *D. haliotrepha* was one layered (at the light microscope level) and covered apically by a thick gelatinous cap that turns blue in iodine. Kohlmeyer and Kohlmeyer (1979) suggested that the ectoascus secretes a gelatinous sheath and this has also been reported by Nakagiri (1993). In the present study we have shown that the pseudoparaphyses are surrounded by a hyphal sheath which stains intensively with ruthenium red. Organelles within the pseudoparaphyses are well preserved and not degenerate as in many species (Read et al., 1992). It is suggested that the mucilage enrobing the asci and pseudoparaphyses is more likely to be produced by the pseudoparaphyses. It is unlikely that the ectoascus or organelles within the ascus are the source of this material. There are no secretory organelles present in the ectoascus, while internally in the ascus ascosporogenesis is the major activity.

In conclusion, this study confirms that the endoascus in bitunicate ascomycetes is thicker than the ectoascus, that the ridges on the ascospores observed at the light microscope level are outgrowths of the mesosporium and demonstrates for the first time the presence of an outer electron-dense layer that fragments to release a fibrillar mucilaginous material present between the spore wall ridges.

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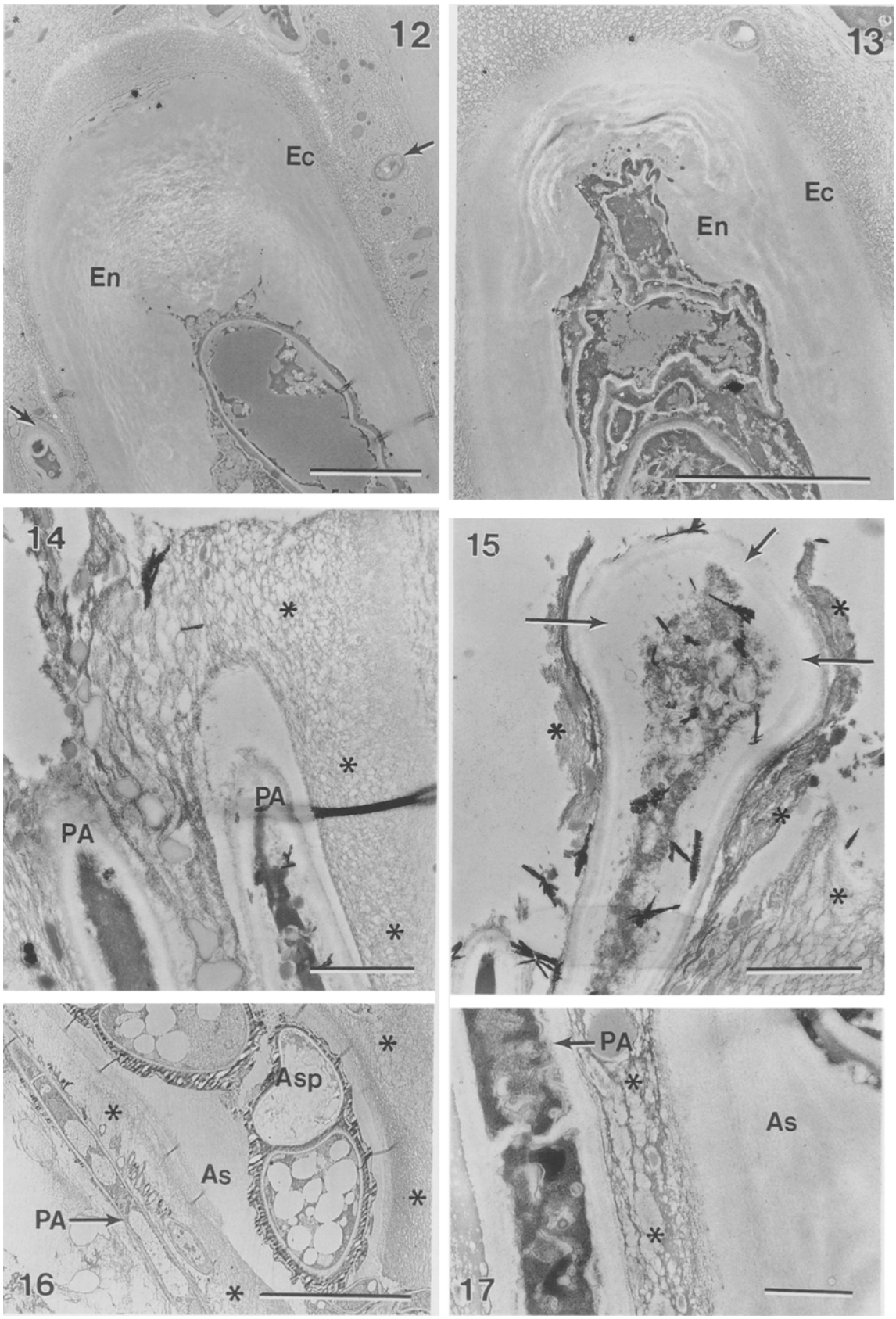
Figs. 7–11. *Dactylospora haliotrepha*. Transmission electron micrographs. 7. Septum of ascospore formed by the electron-transparent inner mesosporial layer (arrowed). Electron-dense deposits are present in the septal wall. L=lipid bodies; W=Woronin bodies; 8. Remnants of epiplasm (arrowed) attached to the ascospore (Asp) within an ascus. Ascus wall (AW) is covered with electron-dense, amorphous material (*); 9, 10. Pseudoparaphyses. Ruthenium red stained: 9. Longitudinal section. A muciliginous sheath (SH), is heavily stained by ruthenium red, is electron-dense, and enrobes the pseudoparaphyses. Hyphal wall is multilayered comprising alternate electron-dense and electron-transparent layers (arrowed HW). The plasma membrane (Pm) is highly convoluted and associated with numerous vesicles and vacuoles (arrowed); 10. Transverse section of pseudoparaphyses. Hyphal wall is multilayered, as Fig. 9, with an extensive membranous cytoplasmic system (arrowed); 11. Longitudinal section of an ascus showing four (part) ascospores, enclosed in the electron-transparent ascus wall (arrowed AW), which externally bears electrondense amorphous and fibrillar material (arrowed). Scale bars: Fig. 7=2 μm; Figs. 8, 9=1 μm; Fig. 10=0.5 μm; Fig. 11=10 μm.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Figs. 12-17. *Dactylospora haliotrepha*. Transmission electron micrographs. Glutaraldehyde, osimum tetroxide and uranyl acetate fixation. 12, 13. Apex of immature asci. The electron-transport ectoascus (Ec) and an extensive network of fibrillar mucilage in which the asci and pseudoparaphyses (arrowed) are embedded. The endoascus (En) is thick in the apical region and comprises numerous electron-transparent and electron-dense layers; 14. Pseudoparaphyses (PA) are embedded in a mucilaginous matrix (*); 15. Pseudoparaphysis swollen in the apical region (arrowed) and covered with copious mucilage (*); 16. Pseudoparaphyses (PA) and ascus (As) embedded in an electron-dense mucilaginous matrix (*). Asp=ascospore; 17. High magnification of the pseudoparaphyses (PA) and ascus (As) with the electron-dense, fibrillar network of mucilage (*).
Scale bars: Figs. 12, 13=5 μm; Figs. 14, 15=2 μm; Fig. 16=10 μm; Fig. 17=1 μm.