Mycoscience 42: 255–260, 2001

Halosarpheia unicellularis sp. nov. (Halosphaeriales, Ascomycota) based on morphological and molecular evidence

Mohamed Ahmed Abdel-Wahab[1], Ka Lai Pang[1], Hassan Mohamed El-Sharouny[2] and E. B. Gareth Jones[1]

[1] Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong Special Administrative Region, People's Republic of China.
[2] Department of Botany, Faculty of Science, Sohag, South Valley University, Egypt.

Received 28 February 2000
Accepted for publication 28 March 2001

***Halosarpheia unicellularis* sp. nov. is described from decayed attached wood of *Avicennia marina* collected from two mangrove sites in the Red Sea, Egypt. The ascomycete is compared with other marine taxa having ascospores with polar unfurling appendages. It is also compared with other marine genera with oval, round or ellipsoid, hyaline, unicellular ascospores, but for which appendages have not been reported. Molecular data confirms the assignment of the fungus to *Halosarpheia* which forms a clade with the type species *Halosarpheia fibrosa*.**

Key Words——Halosphaeriaceae; marine fungi; molecular phylogeny; taxonomy.

Examination of decayed attached wood of *Avicennia marina* (Forsk.) Vierh. for higher marine fungi, in a Red Sea mangrove in Egypt, resulted in collections of a taxon with unicellular, hyaline, ascospores having bipolar unfurling polar appendages. Thirty-three marine fungi were recorded from the Red Sea by El-Sharouney et al. (1998) with three unidentified species. In the present study twenty-one species were collected from three mangroves (Abdel-Wahab, unpublished). Since this species cannot be accommodated in any described taxon with unicellular ascospores with bipolar unfurling appendages, a molecular analysis was undertaken. A new species of *Halosarpheia* Kohlm. & E. Kohlm. is described on the basis of morphological and molecular results.

Materials and Methods

Collection of material Attached decayed branches of *Avicennia marina* were collected from the intertidal zone in two mangrove stands (Abu-Mingar, Safaga) in the Red Sea coast of Egypt. Material was examined on return to the laboratory and after 4-6 wk incubation in sterile moist chambers (Jones and Hyde, 1988). Single ascospore isolates were made and the fungus maintained on 2% malt extract or corn meal extract seawater agar. Growth on both media was extremely slow (approximately 1 mm/wk).

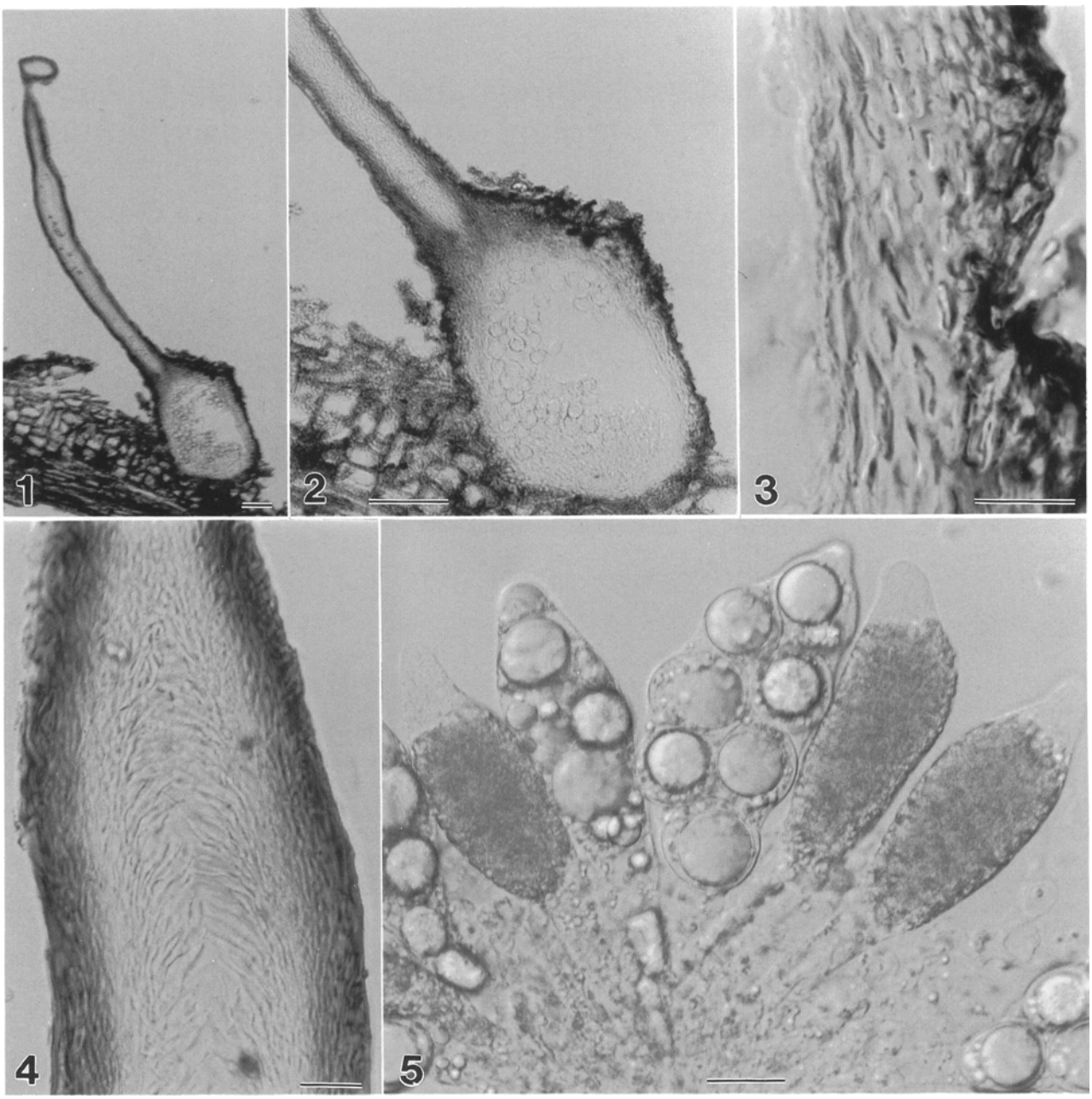
Isolation of DNA For the molecular study, five *Halosarpheia* species were sequenced (Table 1). The isolates were grown in GYP broth (10 g/l glucose, 10 g/l yeast extract and 5 g/l peptone) at 25°C in shake culture until sufficient mycelium (25–100 mg) could be used for DNA extraction. QIAGEN DNeasy Plant Mini Kit (Catalogue no. 69104) was employed for genomic DNA extraction. One region (~2 kb) from the 18S to 28S of the rRNA gene was amplified by PCR with the primers ITS5 (White et al., 1990) and JS8 (Landvik, 1996). Amplified product was purified by QIAGEN QIAquick PCR Purification Kit (Catalogue no. 28104). One part (~1.3 kb) of the 28S rDNA of the purified PCR product was used directly for sequencing with the primers JS1 and JS5 (Landvik, 1996) using Perkin-Elmer dRhodamine Terminator Cycle Sequencing Kit (Catalogue no. 406044) in a Perkin-Elmer ABI PRISM® 377 DNA sequencer.

Sequence analysis The region of the 28S rDNA sequenced from the taxa in this study was first checked for sequence ambiguity. Together with the sequences from the Genebank (Table 2), they were aligned using the program Clustal W 1.6 and refined manually in the program Se-Al v1.0a1. After adjustment, the sequences were entered into PAUP* 4.0b4a for maximum parsimony analysis. Based on the result of Spatafora et al. (1998) and Kohlmeyer et al. (2000), members of the order Xylariales were chosen as the outgroup taxa. With a total of 19 taxa, branch-and-bound search was used to find the most parsimonious cladogram. Characters were equally weighted and gaps were excluded from the analysis. Finally, 1000 bootstrap replicates (PAUP* 4.0b4a) and decay index (Autodecay 3.0.3) were performed to test the support of the clades.

Taxonomy

Halosarpheia unicellularis Abdel-Wahab & E. B. G. Jones, sp. nov. Figs. 1–13

Ascomata solitaria, 216–296 μm diam, 320–400 μm alta, subglobosa, ad apicem collum longum formantia, omnino vel partim immersa, coriacea, nigra, singularia;



Figs. 1–5. Interference contrast micrographs of *Halosarpheia unicellularis* (from holotype). 1,2. Longitudinal sections of the ascomata on wood. 3. Section of the peridial wall composed of an inner and outer layer. 4. Section of the periphysate neck. 5. Squash of immature and mature asci, note the beaked appearance of the ascus apex. Scale bars: 1,2 = 100 μm; 3–5 = 20 μm.

peridium 26–34 μm crassum, texturam angularem formans. Catenophyses praesentes. Colla cylindrica, ca 1 mm longa, 72–104 μm diam, apice ostiolata, periphysata. Asci unitunicati, octospori, 100–122 × 28–34 μm, clavati, pedunculati, ad apicem umbonati, apparatu apicali carentes, tunica tenuiter membranacea tandem, deliquescenti. Ascosporae 15–25 × 14–22 μm, globosae vel subglobosae, unicellulares, hyalinae, apice utrinque appendice, parieti sporae cohaerenti et in aqua in taeniam longam transmutata praeditae.

Ascomata solitary, black, 216–296 μm diam, 320–400 μm high, subglobose to broadly ellipsoidal, immersed to erumpent, coriaceous and with a long neck (Figs. 1, 2). Peridium two layered forming *textura angularis*: outer stratum 6–30 μm consisting of 2–4 layers of polygonal, melanized cells that are black in colour and an inner stratum 20–34 μm, consisting of 5–6 layers of elongated thick-walled, hyaline cells (Fig. 3). Neck ca. 1 mm long and 72–104 μm in diam, periphysate (Figs. 1, 4), periphyses 25–34 × 1 μm (Fig. 4). Catenophyses present (Figs. 5, 7). Asci 8-spored 100–122 × 28–34 μm ($\bar{X}$ = 108.5 × 31.5 μm, n = 12), broadly clavate but

Table 1. Species of *Halosarpheia* sequenced.

Species	Culture no.[a)]	Origin	Habitat	Genebank Accession no.
Halosarpheia fibrosa	PP5159	Taiwan	Marine	AF396872
Halosarpheia lotica	ATCC56668	U.S.A.	Freshwater	AF396873
Halosarpheia retorquens	ATCC200259	Canada	Freshwater	AF396874
Halosarpheia trullifera	PP4268	U.K.	Marine	AF396875
Halosarpheia unicellularis	CP2980	Egypt	Marine	AF396876

[a)]ATCC-American Type Culture Collection, CP-City University Culture Collection, PP-Portsmouth University Culture Collection

with a distinct beak-like apical region, pedicellate, unitunicate, thin-walled, deliquescing, and without an apical apparatus (Fig. 6). Ascospores 15–25 × 14–22 μm ($\bar{X}$ = 20.8 × 17 μm, n = 50), one-celled, globose, subglobose to broadly ellipsoidal, with bipolar apical appendages, initially closely depressed to the ascospore wall becoming indistinct, and unfurling in water to form long thin filaments (Figs. 8–13).

Holotype: on decayed attached intertidal wood of *Avicennia marina* from Safaga mangrove, 29 January 1999, Red Sea, Egypt. IMI No. 381444.

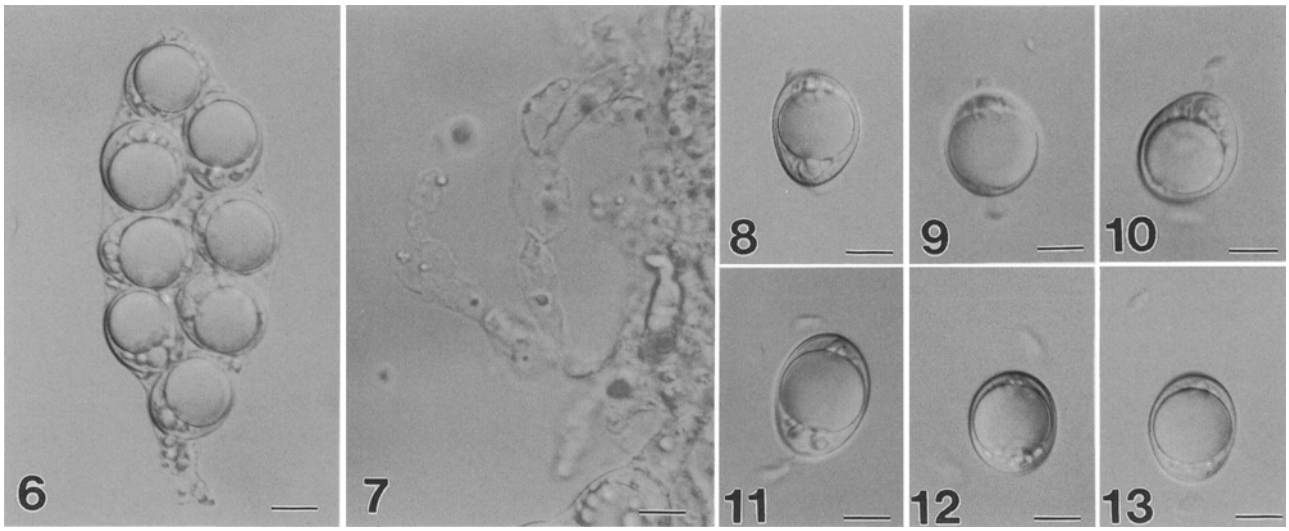
Other collections: five pieces of intertidal wood of *A. marina* from Abu-Mingar mangrove,14 April 1999, Red Sea, Egypt.

Results and Discussion

Phylogenetic relationship Out of the 1208 total characters used in the analysis, 208 characters were parsimony informative. A single most parsimonious tree of 604 steps (C. I. = 0.626, R. I. = 0.687) was produced by maximum parsimony analysis using branch-and-bound searching algorithm which is shown in Fig. 14. The Microascales forms a sister group with the Halosphaeriales receiving a supportive bootstrap value

Table 2. Sequences retrieved from the Genebank.

Taxa	Genebank Accession no.
HALOSPHAERIALES	
Aniptodera chesapeakensis Shearer & M. A. Mill.	U46882
Arenariomyces trifurcatus Höhnk	U46883
Corollospora maritima Werderm.	U46884
Halosarpheia fibrosa Kohlm. & E. Kohlm.	U46886
Halosphaeria appendiculata Linder	U46885
Lignincola laevis Höhnk	U46890
Nohea umiumi Kohlm. & Volkm.-Kohlm.	U46893
MICROASCALES	
Gondwanamyces capense (M. J. Wingf., & P. S. van Wyk) Marais & M. J. Wingf.	AF221012
Gondwanamyces proteae (M. J. Wingf., P. S. van Wyk & Marasas) Marais & M. J. Wingf.	AF221011
Microascus trigonosporus C. W. Emmons & B. O. Dodge	U47835
Petriella setifera (J. C. Schmidt) Curzi	AF043596
XYLARIALES	
Daldinia concentrica (Bolton : Fr.) Ces. & De Not.	U47828
Xylaria curta Fr.	U47840
Xylaria hypoxylon (L.: Fr.) Grev.	U47841



Figs. 6–13. Interference contrast micrographs of *Halosarpheia unicellularis* (from holotype). 6. Mature ascus. 7. Catenophyses. 8–13. Different stages in the ascospores, unfurling of the polar appendages. Scale bars: 6–13 = 10 μm.

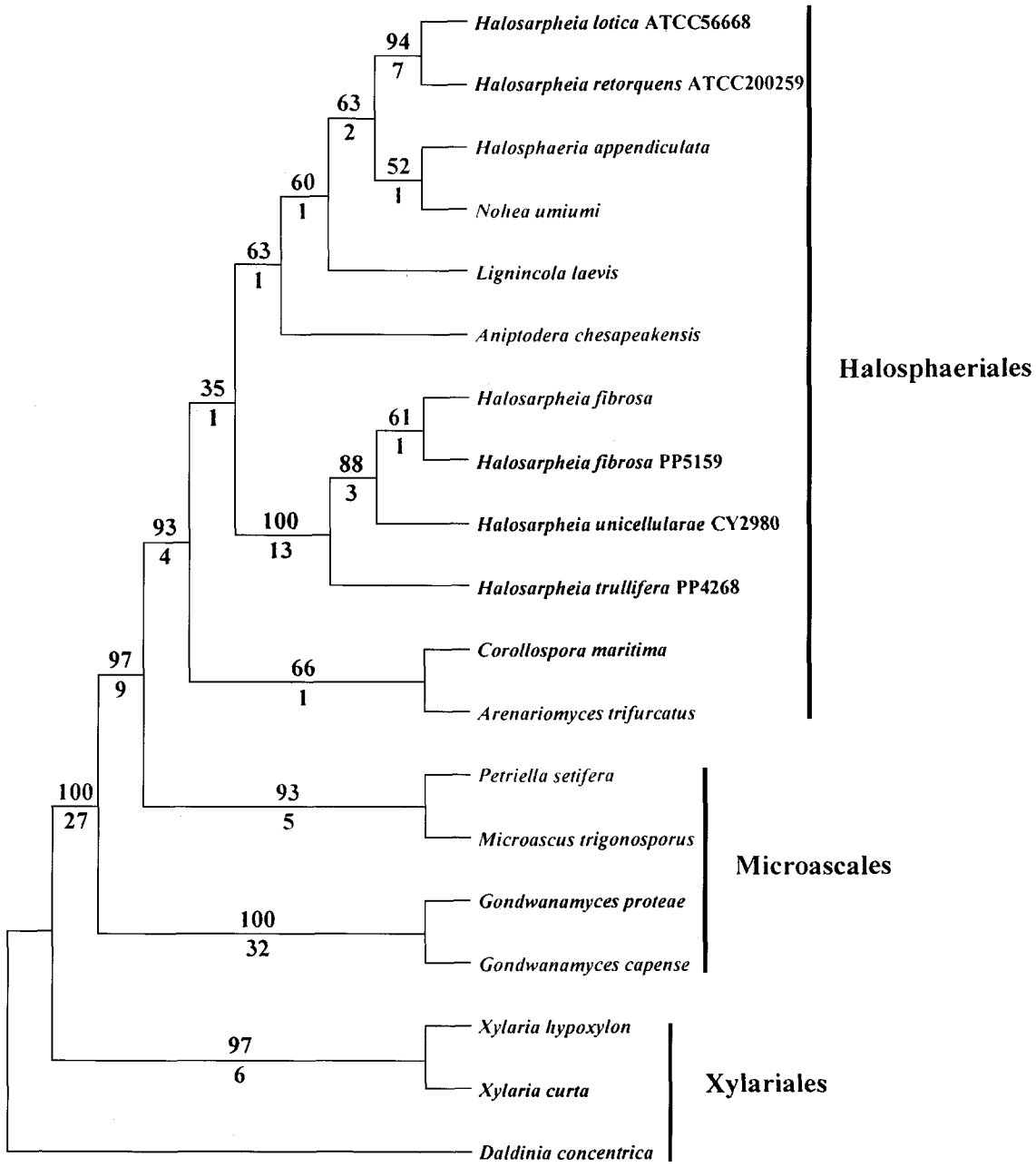


Fig. 14. A single most parsimonious tree inferred from the partial 28S rDNA sequence data. The tree was produced using branch-and-bound algorithm (tree length=604 steps, C. I.=0.626, R. I.=0.687). Boostrap values and decay indices are shown above and below the branches respectively.

(97%) and decay index (9 steps). This is in agreement with results of Spatafora et al. (1998) and Kong et al. (2000). *Halosarpheia unicellularis* constitutes a robust clade with *H. fibrosa* Kohlm. & E. Kohlm. (the type species) and *H. trullifera* (Kohlm.) E. B. G. Jones, S. T. Moss & Cuomo (bootstrap value=100%, decay index=13 steps). Moreover, both *H. unicellularis* and *H. fibrosa* share two very similar insertions in the 28S region of the rDNA used in this study which are 66 and 55 bases long, and 79 and 58 bases long respectively. Therefore, the new mangrove fungus can be best placed in *Halosarpheia* based on the molecular evidence.

Taxonomic evaluation Ascospore morphology differs widely within the genus *Halosarpheia*, the unifying feature is the possession of bipolar unfurling appendages (Kohlmeyer and Kohlmeyer, 1977; Shearer and Crane, 1980). Currently 16 marine and 4 freshwater *Halosarpheia* have been described while most species have bicelled ascospores, some are multiseptate (e.g. *H. unicaudata* (E. B. G. Jones & Camp.-Als.) R. G. Johnson, E. B. G. Jones & S. T. Moss) while *H. unicellularis* is the only species lacking septa. Spores are ellipsoid (e.g. *H.*

fibrosa) to filiform (e.g. *H. cincinnatula* Shearer & J. L. Crane), and all have bipolar appendages with the exception of *H. unicaudata* and *H. cincinnatula*.

Kong et al. (2000) have suggested that the genus *Halosarpheia* is polyphyletic, with their phylogenetic analysis of 4 species (*H. fibrosa*, *H. trullifera*, *H. retorquens* Shearer & J. L. Crane, *H. viscosa* (I. Schmidt) Shearer & J. L. Crane). This is supported by data presented in Fig. 17 with the freshwater *H. retorquens* (ATCC200259) and *H. lotica* (ATCC56668) placed at a distance from the marine *Halosarpheia* clade. Species in the *H. fibrosa* clade have a number of features in common that distinguish them from other *Halosarpheia* species: large ascomata (greater than 250 μm diam), well developed necks (over 350 μm long), prominent periphyses, peridial wall is two-layered and brown to black, while the asci are clavate with a long peduncle (over 20 μm), lack an apical apparatus, and the tip of the asci are beaked. However, they differ in that *H. fibrosa*, and *H. trullifera* have bicelled ascospores while *H. unicellularis* lacks septa.

Although *H. trullifera* falls in the *H. fibrosa* clade, there are significant morphological features that separate them. The key features are the thick, slow to uncoil appendages of *H. trullifera* as opposed to the fine thread-like appendages of *H. fibrosa*. In the latter species, the ascospore wall has two layers (episporium and mesosporium) while in *H. trullifera*, an exosporic sheath is present (Baker, unpublished data). In this respect, *H. trullifera* resembles *Tunicatispora australiensis* K. D. Hyde (Hyde, 1990) which has a pronounced exosporic sheath that separates from the episporium (Mckeown et al., 1996). *Halosarpheia trullifera* is therefore retained within the *H. fibrosa* clade until isolates of other *Halosarpheia*-like species become available for molecular study (e.g. *T. australiensis*, *Anisostagma rotundatum* K. R. L. Petersen & J. Koch).

The ascospores of *H. unicellularis* resembles a number of other genera: *Anisostagma rotundatum* (Petersen and Koch, 1996), *Thalassogena sphaerica* Kohlm. & Volkm.-Kohlm. (Kohlmeyer and Volkmann-Kohlmeyer, 1987), *Iwilsoniella rotunda* E. B. G. Jones (Jones, 1991), and *Hapsidascus hardus* Kohlm. & Volkm.-Kohlm. (Kohlmeyer and Volkmann-Kohlmeyer, 1991) which have unicellular, nearly spherical, ascospores but all lack appendages. *Thalassogena sphaerica* also differs from *H. unicellularis* in that the peridial wall is composed of one tissue type, and the ascomata are cream in colour, it has persistent asci that are broadly clavate, with a flattened apex and a pore, while ascospores lack an appendage.

Anisostagma rotundatum is also similar to *Thalassogena sphaerica*, but differs in that the latter has an undifferentiated peridium (Petersen and Koch, 1996), and asci with an apical pore. *H. unicellularis* and *A. rotundatum* share features in common, namely a peridial wall composed of two layers, deliquescing asci with long stalks. *Hapidascus hardus* differs from *H. unicellularis*, in that the peridial wall is 3-layered, and paraphyses have a net-like apical apparatus (Kohlmeyer and Volkmann-Kohlmeyer, 1991). *Iwilsoniella rotunda* has dark brown ascomata, a two-layered peridial wall, deliquescing asci lacking an apical pore with unappendaged ascospores. Thus it differs from *H. unicellularis* in ascomatal appearance, shape of the asci and in having non-appendaged ascospores.

Jones (1995) posed the question: "are the Halosphaeriales a natural group?" He particularly signalled the lack of information on ascus structure. Indeed has too much emphasis been placed on the deliquescent nature of the ascus in the Halosphaeriales. This might be an adaptation to environmental pressures such as an aquatic habitat (Moss, 1990; Jones, 1995). Similarly, great significance has been placed on the appendaged nature of the ascospores. Kong et al. (2000) provide evidence that *Halosarpheia*, a genus with bipolar unfurling appendaged ascospores, is polyphyletic. This is further evidence for not assigning undue emphasis on ascospore appendage morphology as they may have evolved by parallel evolution (Moss, 1990).

Acknowledgements——K. L. Pang is grateful to the City University of Hong Kong for the award of a research studentship. We thank Drs. R. Y. C. Kong and L. L. P. Vrijmoed for their continued support and to Dr J. I. Mitchell for his invaluable comments on a draft of the manuscript.

Literature cited

El-Sharouny, H. M., Raheem, A. M. and Abdel-Wahab, M. A. 1998. Manglicolous fungi of the Red Sea in Upper Egypt. Microbiol. Res. **153**: 81–96.

Hyde, K. D. 1990. Intertidal fungi from warm temperate mangrove of Australia, including *Tunicatispora australiensis* gen. et sp. nov. Aust. Syst. Bot. **3**: 711–718.

Jones, E. B. G. 1991. *Iwilsoniella rotunda*, a new pyrenomycete genus and species from wood in a water cooling tower. Syst. Ascomyc. **10**: 7–12.

Jones, E. B. G. 1995. Ultrastructure and taxonomy of the aquatic ascomycetous order Halosphaeriales. Can. J. Bot. **73**: S790–S801.

Jones, E. B. G. and Hyde, K. D. 1988. Methods for the study of marine mangrove fungi. In: Mangrove microbiology; role of microorganisms in nutrient cycling of mangrove soils and water. (ed. by Agate, A. D. Subramanian C. V. and Vannucci M.), pp. 9–27. UNDP/UNESCO.

Kohlmeyer, J. and Kohlmeyer, E. 1977. Bermuda marine fungi. Trans. Br. Mycol. Soc. **68**: 207–219.

Kohlmeyer, J., Spatafora, J. W. and Volkmann-Kohlmeyer, B. 2000. Lulworthiales, a new order of marine Ascomycota. Mycologia **92**: 453–458.

Kohlmeyer, J. and Volkmann-Kohlmeyer, B. 1987. *Thalassogena*, a new ascomycetous genus in the Halosphaeriaceae. Syst. Ascomyc. **6**: 223–228.

Kohlmeyer, J. and Volkmann-Kohlmeyer, B. 1991. *Hapsidascus hardus* gen. et sp. nov. (Ascomycotina) from mangroves in the Caribbean. Syst. Ascomyc. **10**: 113–120.

Kong, R. Y. C., Mitchell, J. I., Chan, J. Y. C., Vrijmoed, L. L. P. and Jones, E. B. G. 2000. Relationships of *Halosarpheia*, *Lignincola* and *Nais* inferred from partial 18S rDNA. Mycol. Res. **104**: 35–43.

Landvik, S. 1996. *Neolecta*, a fruit-body-producing genus of the basal ascomycetes, as shown by SSU and LSU rDNA sequences. Mycol. Res. **100**: 199–202.

McKeown, T. A., Moss, S. T. and Jones, E. B. G. 1996. Ultrastructure of ascospores of *Tunicatispora australiensis*. Mycol. Res. **100**: 1247–1255.

Moss, S. T. 1990. The relevance of scanning electron microscopy in the taxonomy of marine ascomycetes. In: Scanning electron microscopy in taxonomy and functional morphology (ed. by Claughter, D), pp. 149–170. Oxford Science Publishing, Oxford, U. K.

Petersen, K. R. L. and Koch, J. 1996. *Anisostagma rotundatum* gen. et sp. nov., a lignicolous marine ascomycete from Svanemollen Harbour in Denmark. Mycol. Res. **100**: 209–212.

Shearer, C. A. and Crane, J. L. 1980. Fungi of the Chesapeake Bay and its tributaries. VIII. Ascomycetes with unfurling appendages. Bot. Mar. **23**: 607–615.

Spatafora, J. W., Volkmann-Kohlmeyer, B. and Kohlmeyer, J. 1998. Independent terrestrial origins of the Halosphaeriales (marine Ascomycota). Amer. J. Bot. **85**: 1569–1580.

White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J. W. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal DNA genes. In: PCR protocol: a guide to methods and applications. (ed. by Innis, M. A. Gelfand, D. H. Sninsky, J. J. and White, T. J.), pp. 315–322. Academic Press, New York.