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Multi-gene phylogeny of the Halosphaeriaceae: its ordinal status, relationships between genera and morphological character evolution

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Abstract The halosphaeriaceous fungi constitute the largest group of marine *Ascomycota* found predominantly in marine environments, with few transitional species found in freshwater and brackish water habitats. It has been one of the most intensively studied groups of marine fungi, with 126 species in 53 genera. The classification of the halosphaeriaceous fungi is contentious with one school maintaining that they should be regarded as a family in the *Microascales*, while others continue to retain the order *Halosphaeriales*. To refine the phylogenetic inter-relationships among the halosphaeriaceous fungi, 36 taxa were sequenced and analysed based on three loci [nuclear small and large subunit (SSU, LSU), the second largest RNA polymerase II subunit (RPB2)]. The halosphaeriaceous fungi constitute a monophyletic group and share a common ancestor with the *Microascaceae* however, they share few morphological characters. In the *Halosphaeriaceae* the centrum tissue comprises catenophyses; asci are clavate to fusiform; ascospores are hyaline, unicellular to many septate, usually with appendages, and most are saprobic in aquatic habitats. Whereas, the peridium of the *Microascaceae* is carbonaceous, frequently bearing hyphal appendages or setae; asci are globose or ovoid; ascospores are reniform, often bear ornamenting ridges or wings. Genera in the *Microascaceae* are mainly saprobic from soil to rotting vegetation and occasionally found as pathogens, and primarily terrestrial. Based on morphological data the halosphaeriaceous taxa might be considered as a group warranting ordinal status (*Halosphaeriales*) and this issue is discussed in this study. Sequence data also show clearly that the genera *Remispora* and *Ceriosporopsis* are polyphyletic and we propose the erection of three new genera to accommodate *C. tubulifera* (*Toriella*), *R. crispa* (*Kochiella*) and *R. galerita* (*Tubakiella*). *Bovicornua intricata* is referred to the genus *Ceriosporopsis*. The molecular data indicate that different phylogenies based on DNA sequences support a hypothesis that ascospore appendage developments e.g. unfurling bipolar appendages, have evolved and been lost several times.

Keywords Evolution · *Halosphaeriaceae* · *Kochiella* · *Microascales* · New genera · *Toriella* · *Tubakiella*

Introduction

The *Halosphaeriaceae* represents the largest and most diverse lineage of marine ascomycetes to date, proposed by Müller and von Arx (1962) and Eriksson (1984) with *Halosphaeria* as the type genus (Barghoorn and Linder 1944). Members of the *Halosphaeriaceae* are amongst the most intensively studied ascomycetes at the morphological, ultrastructural and molecular level, with 53 genera (of which 35 are monotypic) and 126 species (Pang 2002; Jones et al. 2009). Most of the taxa referred to the order are marine, with few transitional species found in freshwater and brackish water habitats e.g. *Aniptodera*, *Ascosacculus*, *Halosarpheia*, *Lignincola*, *Luttrellia*, *Magnisphaera*, *Nais*,

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Natantispora, *Oceanitis* and *Tirispora* (Jones 1995; Pang et al. 2003; Jones et al. 2009).

Morphological characters used to distinguish the genera in the *Halosphaeriaceae* include: perithecioid ascomata, necks (usually with periphyses), presence of catenophyses that generally deliquesce, unitunicate, thin-walled asci that deliquesce early, asci lacking an apical apparatus and appendages on ascospores (Jones 1995). While studies at the scanning and transmission electron microscope level (SEM, TEM) of ascospore appendage morphology and ontogeny, yielded useful characters for delineation of certain genera in the *Halosphaeriaceae* (*Ceriosporopsis, Corollospora, Marinospora*) (Jones et al. 1983b, 1984; Jones 1995), however it fails to distinguish other genera e.g. *Halosarpheia*, *Lulworthia* (Campbell et al. 2003, 2005).

DNA sequence analysis to infer phylogenies became common place in fungal systematics, from a single locus to multi loci. Recently, the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) has been accepted in order to recognize fungal species concept based on multiple gene genealogies (Taylor et al. 2000). It provides a powerful tool to investigate fungal evolutionary relationships that cannot be determined using morphological or biological characters alone.

There have been attempts to clarify the phylogeny of the *Halosphaeriaceae* by examining selected genera and related taxa. For example, Spatafora et al. (1998) proposed a terrestrial origin of this order supported by phylogenetic analysis. Subsequent studies have been undertaken by many authors mainly with a narrower focus on the taxonomic revision of specific genera e.g. *Halosarpheia* species (Campbell 1999; Kong et al. 2000; Abdel-Wahab et al. 2001b; Anderson et al. 2001; Pang 2001); *Halosphaeria* (Pang et al. 2004a); *Lulworthia* and *Lindra* (Campbell et al. 2003; Pang et al. 2003; Koch et al. 2007); *Torpedospora* and *Swampomyces* (Abdel-Wahab et al. 2001a; Sakayaroj 2005; Sakayaroj et al. 2005a); *Antennospora* (Pang et al. 2008b) and reviewed by Jones et al. (2009). Nevertheless, the higher level classification of the halosphaeriaceous fungi remains contentious with one placing them as a family within the *Microascales* (Hibbett et al. 2007; Schoch et al. 2007), while others continue to retain the ordinal name *Halosphaeriales* (Zhang et al. 2006; Tang et al. 2007; Jones et al. 2009).

So far, 97 halosphaeriaceous strains have been sequenced in several studies and their DNA sequences deposited in GenBank, with the following breakdown by gene: 127 LSU, 53 SSU, 7 RPB2, 1 largest subunit of RNA polymerase II (RPB1), 3 elongation factor 1-alpha (EF1-alpha), 1 beta-tubulin and 26 sequences from other genes (data retrieved 4 August 2010). However, resolution of the classification and inter-relationships of many genera within the *Halosphaeriaceae*, such as *Bathyascus*, *Ceriosporopsis*, *Lignincola* and *Remispora* remains poor.

The genus *Remispora* was described by Barghoorn and Linder (1944) (type species: *R. maritima*) and characterized by mostly hyaline ascomata, 1-septate hyaline ascospores, with pleomorphic polar appendages which arise as fragmentation of an exosporial layer (Johnson et al. 1984). The assignment of this genus has been revised several times based on morphological characteristics with Kohlmeyer (1972) referring it to *Halosphaeria* (Jones and Moss 1978, 1980; Johnson et al. 1984). Johnson et al. (1984) and Manimohan et al. (1993a, b) demonstrated that the polar appendages of *Remispora* species were formed by fragmentation of the ascospore exosporium, which consists of two components, a fibrillar material in an amorphous matrix. Currently eight *Remispora* species are accepted: *R. maritima*, *R. stellata*, *R. quadri-remis*, *R. pilleata*, *R. galerita*, *R. crispa, R. minuta* and *R. spitsbergenensis* (Jones et al. 2009).

The genus *Ceriosporopsis* is characterized by light brown to black, subglobose to cylindrical ascomata, clavate, thin-walled asci, ascospores ellipsoidal, 1-septate, hyaline with polar appendages, with the exception of *C. tubulifera*, which has polar and equatorial appendages. In *Ceriosporopsis* species, polar appendages uncoil to form long drawn out structures, while *C. tubulifera* has end chambers or tubes containing mucilage that is released when mounted in water to form long thread-like appendages. There are five *Ceriosporopsis* species (*C. caduca*, *C. cambrensis*, *C. capillacea*, *C. halima* (type species), *C. tubulifera*), but species delineation is unclear due to the variation in the morphology of ascospore appendages (Johnson et al. 1987; Jones et al. 2009). The classification of other genera remains unresolved e.g. *Bathyascus*, *Halosarpheia*-like species, *Lignincola*, as no fresh collections have been available for a molecular study.

The goals of this study were 1) to combine morphology and DNA sequence based phylogenetic analyses of multiple loci (LSU, SSU rDNA and RPB2 sequences) to elucidate the phylogeny and taxonomy of key genera in the *Halosphaeriaceae*, 2) to consider the evolution of the family and 3) to review its ordinal status based on morphological and biological evidence.

Materials and methods

Collection and growth of fungi

Decaying driftwood was collected from various countries: China, Denmark, Norway, Taiwan, Thailand, USA and Wales (Table 1). Single-spore isolations were made on

Table 1 Fungi sequences generated from this study

	Genus, species	Code	Source	GenBank Accession number		
				LSU	SSU	RPB2
1	*Aniptodera longispora*	BBH28304	Thailand	HQ111008	–	–
2	*Ceriosporopsis halima*	BCC15553	Wales	HQ111016	HQ111004	–
3	*Ceriosporopsis (Bovicornua) intricata*	BCC33465	Denmark	HQ111029	–	–
4	*Ceriosporopsis (Bovicornua) intricata*	BCC34299	Denmark	HQ111030	–	–
5	*Ceriosporopsis (Bovicornua) intricata*	BCC33466	Denmark	HQ111031	–	–
6	*Haligena elaterophora*	PP4705	USA	AY864845	–	HQ111040
7	*Halosarpheia kandeliae*	CY1492	Hong Kong	HQ111025	–	–
8	*Havispora longyearbyenensis*	CY5278	Norway	HQ111023	–	–
9	*Kochiella (Remispora) crispa*	BCC33502	Denmark	HQ111020	–	–
10	*Kochiella (Remispora) crispa*	BCC33504	Denmark	HQ111018	–	–
11	*Kochiella (Remispora) crispa*	BCC33507	Denmark	HQ111019	–	–
12	*Marinospora calyptrata*	CY3491	Wales	HQ111036	–	–
13	*Marinospora calyptrata*	BBH28307	Wales	HQ111035	–	HQ111042
14	*Morakotiella salina*	BCC12781	Wales	AY864844	–	HQ111039
15	*Naufragella spinibarbata*	BCC33508	Wales	HQ111033	–	–
16	*Naufragella spinibarbata*	BCC33482	Taiwan	HQ111034	–	–
17	*Naufragella spinibarbata*	PP6886	Unknown	HQ111032	–	–
18	*Nautosphaeria cristaminuta*	BBH28308	Unknown	HQ111009	–	–
19	*Ocostaspora apilongissima*	CY3399	Denmark	HQ111007	–	–
20	*Ocostaspora apilongissima*	LP32	Denmark	HQ111006	–	–
21	*Ocostaspora apilongissima*	LP53	Denmark	HQ111005	–	–
22	*Ondiniella torquata*	BCC34303	Taiwan	HQ111038	–	–
23	*Ondiniella torquata*	BCC33480	Taiwan	HQ111037	–	–
24	*Remispora maritima*	BCC33517	Denmark	HQ111013	–	–
25	*Remispora maritima*	BBH28309	Denmark	HQ111012	HQ111002	HQ111041
26	*Remispora pilleata*	BBH28305	Denmark	HQ111021	–	–
27	*Remispora pilleata*	BBH28306	Denmark	HQ111022	–	–
28	*Remispora quadri-remis*	BCC15555	Unknown	HQ111010	–	–
29	*Remispora spitsbergenensis*	CY5279	Norway	HQ111011	–	–
30	*Remispora stellata*	JS150	Denmark	HQ111017	–	–
31	*Sablecola chinensis*	BCC22809	China	HQ111024	–	–
32	*Toriella (Ceriosporopsis) tubulifera*	BCC33511	Denmark	HQ111026	–	–
33	*Toriella (Ceriosporopsis) tubulifera*	BCC33512	Wales	HQ111027	–	–
34	*Toriella (Ceriosporopsis) tubulifera*	BCC33513	Denmark	HQ111028	–	–
35	*Tubakiella (Remispora) galerita*	BCC33500	Denmark	HQ111014	HQ111003	–
36	*Tubakiella (Remispora) galerita*	PP5577	Unknown	HQ111015	–	–

seawater cornmeal agar (CMA) with added antibiotics following the method of Choi et al. (1999). Axenic cultures and specimens were kept at BIOTEC Culture Collection (BCC, JS) and BIOTEC Bangkok Herbarium (BBH). Additional cultures were obtained from City University of Hong Kong (CY), University of Portsmouth, UK (LP, PP). Fungi were cultured into seawater potato dextrose agar (PDA) before growing in seawater glucose yeast extract peptone (GYP) broth (4 g/l glucose, 4 g/l yeast extract, 2 g/l peptone).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from both mycelia and ascomata (Pang et al. 2003; Sakayaroj et al. 2005b). Mycelium (~100 mg) was harvested by filtration, washed twice with sterile distilled water, blotted dry by filter paper and immediately frozen in liquid nitrogen. Mycelial pellets were ground into fine powder using a mortar and pestle, and DNA was extracted using a NucleoSpin^{R} Plant DNA extraction kit (Macherey-Nagel). Additional DNA was extracted using 1–2 ascomata following the microwave technique (Pang et al. 2003). Fruiting bodies were picked up with fine forceps or needles, transferred to a tube containing 400 µl lysis buffer (O'Donnell et al. 1997). The tube was sealed with a plastic wrap, and microwaved for 20 s (10 s-5 s-5 s) at maximum power, and then incubated at 70°C for 30 min. The mixture was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). The upper liquid phase was precipitated with 7.5 M ammonium acetate and absolute ethanol and kept at 20°C for at least 30 min. Extracted DNA was washed twice with 70% ethanol, air-dried, and the DNA resuspended in 20 µl TE buffer.

Nuclear small and large subunit ribosomal rDNA and RBB2 were amplified using fungal universal primers (White et al. 1990; Bunyard et al. 1994; Landvik 1996; Liu et al. 1999; Lutzoni et al. 2004). PCR reactions were performed in 50 µl using FINNZYMES, DyNAzyme II DNA Polymerase Kit (Macherey-Nagel) in a Perkin Elmer thermal cycler. The PCR products were analyzed by agarose gel electrophoresis and purified using a NucleoSpin Plant DNA Purification Kit (Macherey-Nagel) according to the manufacturer's instructions. PCR products were sent to Macrogen Inc., Korea, for direct sequencing.

Sequence alignment and phylogenetic analyses

Sequences were defined ambiguity and assembled using BioEdit 7.0.9.0 (Hall 2007). These sequences obtained from GenBank (Table 2) were aligned in Clustal W 1.6 (Thompson et al. 1994), MUSCLE 3.6 (Edgar 2004) and manually adjusted in BioEdit 7.0.9.0. The tree construction procedure was performed in PAUP* 4.0b10 Macintosh and Window versions (Swofford 2002).

The congruence of the three gene regions was tested with the partition homogeneity test (PHT, Farris et al. 1995; Swofford 2002). The LSU dataset was analyzed individually then the combined 3-gene sequences (LSU+RPB2+SSU) were analyzed using maximum parsimony (MP: heuristic searches with a stepwise starting tree, a random stepwise addition of 100 replicates and TBR branch-swapping algorithm) and neighbor joining (NJ) approaches. All characters were equally weighted. Gaps were treated as missing data. Tree lengths, Consistency Indices (CI), Retention Indices (RI) and Rescaled Consistency Indices (RC) were calculated for each Maximum Parsimonious Tree (MPT) generated. Finally, 1000 replicates of bootstrapping analysis for MP (full heuristic searches, stepwise addition of sequence, 10 replicates of random addition of sequence and TBR branch-swapping algorithm) and NJ analyses were performed (Felsenstein 1985).

MODELTEST v. 2.2 (Nylander 2004) was used to determine the best-evolution for each data set for Bayesian inference (BY). Bayesian inference was calculated with MrBayes v. 3.1.2 with a general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites (Huelsenbeck and Ronquist 2001). Four Markov chains were run for 2 runs from random starting trees for 5 million generations and sampled every 100 generations. The first 5 thousand generations were discarded as burn-in. A majority rule consensus tree of all remaining trees was calculated. The statistical bootstrap support for maximum parsimony (MPBS), neighbor joining (NJBS) and Bayesian posterior probabilities (BYPP) greater or equal than 50% (MPBS, NJBS) and 0.95 (BYPP) are shown above the tree branches, respectively.

Results

DNA sequence alignments and phylogenetic analyses

Forty-one new sequences from 36 taxa were generated in this study (34 LSU, 4 RPB2 and 3 SSU sequences, Table 1). They were analysed along with halosphaeriaceous fungi and other reference sequences from the *Hypocreomycetidae* (*Hypocreales*, *Coronophorales*, *Microascales*, *Melanosporales* and TBM lineage, Schoch et al. 2007) sampling from the GenBank (Table 2).

Two introns for LSU sequences, one at position 824−1026 and another at 1131−1225 for *Halosarpheia trullifera* (AF396875), *H. unicellularis* (AF396876), *H. fibrosa* (U46886)**,** *H. marina* (AY227125), *Panorbis viscosus* (AY227133), *Remispora quadri-remis* (HQ111010), *R. stellata* (HQ111017), *R. crispa* (HQ111018, HQ111019, HQ111020), *Morakotiella salina* (AY864844), *Ocostaspora apilongissima* (HQ111005, HQ111006, HQ111007), *Saagaromyces ratnagiriensis* (AF539470), *S. abonnis* (AF539469), *Ascosacculus heteroguttulata* (AY227136), *Corollospora portsaidica* (AB361016), *Okeanomyces cullulatus* (AY490787) and *Etheirophora blepharospora* (EF027723). Among the SSU sequences, three introns were observed at position 3193−3254, 3322−3397 and 4115−4525 for *Natantispora lotica* (AF352080), *H. fibrosa* (AF352078) and *H. marina* (AF352082). Inclusion and exclusion of all insertion regions had no effect on the tree topology in all analyses. Therefore, the insertion regions were included in all analyses.

Phylogenetic analyses of two datasets, the individual LSU and the combined 3-gene (LSU+RPB2+SSU) datasets, were performed. *Xylaria hypoxylon* and *Daldinia concentrica* were chosen as the outgroups. The MAXTREES parameter was specified, a MPT limit of 2000 for each replicate was set. The LSU dataset contained 122 taxa of 1691 bases of which 638 were constant (38%) and 524 were parsimony informative (31%). The heuristic searches

Table 2 Fungi sequences from GenBank used in the analyses

Orders/Genus/Species	Original code	LSU	SSU	RPB2
Halosphaeriaceae				
Alisea longicolla	CP2464b	EU118365	EU118370	–
Aniptodera chesapeakensis	A236	U46882	AF050483	DQ470896
Aniptodera lignatilis	A460-1	AY227115	–	–
Antennospora quadricornuta	–	AY090890	–	–
Arenariomyces trifurcatus	PP2747	AF491277	–	–
Ascosacculus heteroguttulata	A1087D	AY227136	AY227138	–
Ascosacculus aquatica	–	AY227136	–	–
Ceriosporopsis halima	LP45	AY490788	U47843	–
Corollospora intermedia	PP3910	AF491258	–	–
Corollospora portsaidica	MD832	AB361016	–	–
Corollospora lacera	PP2509	AF491259	–	–
Corollospora quinqueseptata	–	AF491262	–	–
Corollospora angusta	AFTOL5010	AF491254	FJ176845	–
Corollospora filiformis	PP3905	AF491256	–	–
Corollospora maritima	CBS264.59, AFTOL5011	AF491260	FJ176846	DQ368632
Corollospora pulchella	PP4206	AF491274	–	–
Cucullosporella mangrovei	–	AY150219	–	–
Haiyanga salina	GR85	EF383131	–	–
Haligena elaterophora	PP4705	AY864845	–	HQ111040
Halosarpheia unicellularis	–	AF396876	–	–
Halosarpheia trullifera	–	AF396875	–	–
Halosarpheia marina	JK5103B	AY227125	AF352082	–
Halosarpheia fibrosa	JK5132	U46886	AF352078	–
Halosigmoidea marina	AFTOL5008	AY762985	FJ176843	–
Halosigmoidea parvula	TUB407	AY762987	–	–
Halosphaeriopsis mediosetigera	–	U46887	U32420	–
Halosphaeria appendiculata	–	U46885	U46872	–
Humicola siamensis	IT110	DQ237875	DQ237874	–
Lignincola laevis	AFTOL-ID 737	U46890	U46873	DQ836886
Lignincola tropica	PP7777	AF539474	–	–
Magnisphaera stevemossago	A4091B	AY227135	AY227140	–
Magnisphaera spartinae	A2211	AY150221	AF352076	–
Marinospora longissima	PP0868	AF491266	–	–
Monodictys pelagica	PP3283	AF491275	–	–
Morakotiella salina	BCC12781	AY864844	–	HQ111039
Nais inornata	J1155, ATCC200453	AF539476	AF050482	–
Natantispora retorquens	A231-1D	AY227128	AF352086	–
Natantispora lotica	A333-1A, ATCC56668	AF396873	AF352080	–
Neptunella longirostris	PP4563	AF539472	–	DQ368633
Nereiospora comata	PP2520	AF491267	–	–
Nereiospora cristata	PP5988	AF491268	–	–
Nimbospora effusa	AFTOL-ID 761	U46892	U46877	DQ836887
Nohea umiumi	–	U46893	U46878	–
Oceanitis cincinnatula	A3181	AY150220	AF352077	–
Oceanitis scuticella	CP2464a	EU118367	EU118369	–
Oceanitis unicaudata	CY1333	AY150222	–	–
Oceanitis viscidula	ATCC 24310	AY150223	–	–
Okeanomyces cucullatus	LP67	AY490787	–	–

Table 2 (continued)

Orders/Genus/Species	Original code	LSU	SSU	RPB2
Ophiodeira monosemeia	–	U46894	U46879	–
Panorbis viscosus	JK5433	AY227133	AF352083	–
Periconia prolifica	–	AY090891	–	–
Phaeonectriella lignicola	PP7008, A157-1D	AY150224	AF050484	–
Pseudolignincola siamensis	IT41	DQ237873	DQ237872	–
Saagaromyces ratnagiriensis	CY 0732	AF539470	–	–
Saagaromyces abonnis	K5304	AF539469	AY227137	–
Saagaromyces glitra	PP4672	AF539475	–	–
Thalespora appendiculata	IT200	DQ237877	DQ237876	–
Tirispora unicaudata	CY2370	AY150225	–	–
Varicosporina ramulosa	–	U44092	–	–
Microascales incertae sedis				
Ceratocystis adiposa	CBS600.74, CCFC212707	AY283562	EU984263	–
Ceratocystis fimbriata	CBS374.83	AF221009	U32418	DQ368641
Ceratocystis virescens	–	U47824	U32419	–
Microascaceae				
Enterocarpus grenotii	–	AF275533	–	–
Kernia geniculotricha	–	AF275532	–	–
Kernia hippocrepida	–	AF275531	–	–
Microascus singularis	–	AF400869	–	–
Petriella guttulata	CBS362.61	AF027662	–	–
Petriella musispora	CBS745.69	AF027663	–	–
Petriella setifera	CBS110344	U48421	U43908	DQ368640
Pithoascus exsertus	–	AF275535	–	–
Pithoascus intermedius	–	AF400872	–	–
Pithoascus nidicola	–	AF400873	–	–
Pseudallescheria africana	CBS 311.72	EF151331	–	–
Pseudallescheria boydii	NCPF7236	AF027674	U43913	–
Pseudallescheria fusoidea	CBS106.53	AF027673	–	–
Scedosporium prolificans	CBS467.74	AF027680	–	–
Scopulariopsis brevicaulis	NCPF2177	AF027678	AY083220	–
Hypocreales				
Hypomyces polyporinus	–	AF543793	DQ368636	U32410
Hypomyces schweinitzii	–	U47833	DQ368635	–
Nectria cinnabarina	IFO30679	AF193237	DQ368637	AB003949
Coronophorales				
Bertia moriformis	SMH3344	AY695261	–	AY780151
Chaetosphaerella phaeostroma		AY346274	–	AY780172
TBM clade (*Hypocreomycetidae incertae sedis*)				
Etheirophora blepharospora	JK5397A	EF027723	–	EF027731
Swampomyces armeniacus	JK5325A	EF027729	–	EF027736
Melanosporales				
Melanospora singaporensis	ATCC38286	AY015629	–	–
Melanospora tiffanii	ATCC15515	AY015630	–	AY015637
Xylariales				
Daldinia concentrica	–	U47828	U32402	DQ368651
Xylaria hypoxylon	CBS499.80	U47841	U20378	DQ368652

maximum parsimony analysis gave tree length of 3662 step changes, CI=0.339, RI=0.642, RC=0.218 (tree not shown).

For the 3-gene region, the PHT revealed significant congruence. Based on the PHT, sequence data from the three DNA regions were combined for the phylogenetic analyses. The combined 3-gene dataset included 5095 characters for 122 taxa (1691 bases for LSU, 1136 bases for RPB2 and 2268 bases for SSU). Among these, 2595 were constant (51%) while 1444 were parsimony informative (28%). Parsimony analysis yielded tree length of 8498 step changes, CI=0.465, RI=0.575, RC=0.267 (Fig. 1). All absent genes were coded as missing data: RPB2 and SSU forming 79% and 71% missing data of the total characters, respectively.

For both datasets, all analyses (MP, NJ and BY) resulted in identical trees, except for the minor difference of the unsupported taxa in the *Halosphaeriaceae*. These differences do not affect the overall topology of the tree and the conclusions drawn. Therefore, the 3-gene tree is provided in Fig. 1.

Phylogenetic inference from the 3-gene tree

The 3-gene phylogeny provides support for the major ordinal lineages in the *Hypocreomycetidae*. The *Coronophorales* is well placed in an early diverging clade with the *Melanosporales* and the TBM clade. The placement is strongly supported by statistical values for MP and BY (Fig. 1), while the *Hypocreales* is a sister group to the *Microascales*. This order split into three groups: the *Microascaceae* and the *Halosphaeriaceae and Ceratocystis* species. The *Microascaceae* comprised key genera such as *Petriella, Pithoascus, Pseudallescheria, Scedosporium, Kernia, Scopulariopsis, Enterocarpus* and *Microascus*, while *Ceratocystis* species always formed a sister group to the *Microascaceae* and *Halosphaeriaceae* and well supported by statistical values of 96/75/0.99 for MP, NJ and BY, respectively (Fig. 1).

In every analysis, the phylogeny confirmed congruence of the newly sequenced taxa within the *Halosphaeriaceae* with other generated trees (Jones et al. 2009). Although major nodes in the *Halosphaeriaceae* have relatively low support, 10 well resolved subclades can be distinguished (Fig. 1). Our molecular results clearly indicate that the genus *Remispora* is not monophyletic. The type species, *R. maritima,* is well placed within a subclade comprising *R. pilleata, R. spitsbergenensis, R. stellata* and *R. quadri-remis*. Two isolates of *R. maritima* are closely related to *R. pilleata*, while *R. spitsbergenensis* groups well with *R. stellata* and *R. quadri-remis*, respectively. This subclade is strongly supported by the various analyses used and should be referred to as *Remispora sensu stricto*.

Another two species, *R. crispa* and *R. galerita*, are distantly placed in relation to the type species. Three isolates of *R. crispa* group well within subclade ii comprising *Ocostaspora apilongissima* and *Morakotiella salina*, with relatively high support and a short branch length. *Remispora galerita* strains form a basal subgroup in subclade x within the *Halosphaeriaceae*, and cluster with the monotypic genera *Nautosphaeria* and *Haligena* with high statistical support.

The genus *Ceriosporopsis* has been shown to be polyphyletic based on our molecular results. Two strains of the type species, *C. halima*, are well delineated in subclade ix, clustering with the monotypic genus *Bovicornua*. However, *C. tubulifera* is distantly placed from *C. halima*, grouping with the genera *Ondiniella* and *Marinospora* in subclade viii. Phylogenetically, *M. longissima* and *M. calyptrata* proved to be closely related and monophyletic, forming a sister group to *C. tubulifera* and *Ondiniella torquata*. The placement of subclade viii in the *Halosphaeriaceae* was statistically always well supported.

Our study also showed other stable entities within the *Halosphaeriaceae*, for example, subclade iii, which comprises the well-supported genera *Okeanomyces* (and its anamorph *Periconia prolifica*)*, Thalespora, Ascosacculus*, adjacent to *Aniptodera* and *Nais.* Four *Oceanitis* species form a monophyletic group in subclade iv with the monotypic genus *Ophiodeira monosemeia* as a sister group. Another stable entity within the family is the placement of *Halosarpheia sensu stricto* and *Lignincola tropica* in a well supported subclade v which clustered with a group comprising *Humicola siamensis, Pseudolignincola siamensis, Antennospora quadricornuta, Arenariomyces trifurcatus, Haiyanga salina, Cucullosporella mangrovei* and a new sequence of *Aniptodera longispora*. Based on our molecular analyses, *A. longispora* is distantly placed from the type species *A. chesapeakensis*. It is highly supported by various statistical values as a monophyletic group with *C. mangrovei*.

Subclade vi comprises various *Corollospora* species and their anamorphs: *Halosigmoidea parvula, H. marina* and *Varicosporina ramulosa*. The molecular results support their monophyly and placement within the *Halosphaeriaceae*. Subclade vii shows the placement of the genus *Saagaromyces* and confirms the monophyly of *S. ratnagiriensis, S. glitra* and *S. abonnis* with high statistical values.

The inter-relationship of other halosphaeriaceous taxa was not resolved by this analysis. A newly sequenced fungus *Sablecola chinensis* was positioned as a separate taxon and a basal branch of subclade i. However, it is not closely related to *Remispora sensu stricto*, and the data supports the decision to describe it as new genus. Three strains of *Naufragella spinibarbata* were located basal to subclade ii without any closely related taxa.

Finally, our molecular data show that a monotypic genus *Havispora* clustered as a sister taxon with *Nereiospora comata*, *N. cristata* and *Monodictys pelagica*. This grouping formed a weak relationship with *Magnisphaera* species for all analyses.

Discussion

The data presented above, with 41 new sequences, enable a reevaluation of the taxonomy of this group of unitunicate marine fungi at the generic level, as well as consideration of their evolution. We will address the taxonomic changes necessary as a result of this extensive study and review the position of other taxa whose phylogenetic position remains unresolved. Three other areas will be explored: the evolution of marine fungi, their morphological character evolution and the ordinal status of this group of ascomycetes.

Phyletic inference of key genera in the Halosphaeriaceae

Remispora and *Ceriosporopsis* were inferred to be polyphyletic genera and the following taxonomic changes are proposed to accommodate these displaced species. The taxonomic position of some genera/species with unfurling polar appendages and a number of monotypic genera (*Havispora*, *Ocostaspora*, *Ondiniella*, *Nautosphaeria* and *Sablecola*) is reviewed.

Taxonomy

Remispora Linder, Farlowia 1: 409, 1944

R. maritima Linder, Farlowia 1: 410, 1944 (Types species) Fig. 2a, b

Synonym: *Halosphaeria maritima* (Linder) Kohlm., Can J Bot 50: 1956, 1072

Anamorph: Not observed.

Mode of life: saprobic.

Habitat: wood.

Widely distributed in temperate waters.

Accepted species:

R. maritima Linder, Farlowia 1: 410, 1944

R. pilleata Kohlm., Nova Hedw 6: 319, 1963 Fig. 2c–e

R. quadri-remis (Höhnk) Kohlm., Nova Hedw 2: 3332, 1960 Fig. 2j–l

R. spitsbergenensis K.L. Pang & Vrijmoed, Mycologia 101: 531, 2009 Fig. 2m

R. stellata Kohlm., Nova Hedw 2: 334, 1960 Fig. 2f–i

Notes: D.H. Linder (in Barghoorn and Linder 1944) established the genus with *R. maritima* as the type species. It was characterized by globose, hyaline to brown ascomata, clavate, thin-walled, deliquescing asci, and hyaline, 1-septate ascospores, with wing-like appendages. Kohlmeyer (1972) reduced *Remispora* to synonymy with *Halosphaeria* as the ascospores of the "type species of *Halosphaeria* and *Remispora* did not differ substantially from each other in morphologic respect". This was based on the view that the appendages were formed by fragmentation of an epispore. However, SEM and TEM studies supported the acceptance of *Remispora* as a distinct genus from *Halosphaeria* (Jones and Moss 1980, 1987; Johnson et al. 1984). Currently eight species have been described with ascospore appendages varying in their morphology, although all appear to be the result of fragmentation of an exosporic sheath (Johnson et al. 1984; Manimohan et al. 1993a, b). Molecular studies indicated that *Remispora* was polyphyletic (Sakayaroj 2005; Sakayaroj et al. 2004) and this is confirmed by the present study.

Fig. 1 One of the most parsimonious tree resulted from maximum ▶ parsimony analysis from the combined SSU, LSU rDNA and RPB2 sequences. Bootstrap values greater than 50% from maximum parsimony, neighbor joining and Bayesian Posterior Probabilities are given above the branches. Bar indicates 100 character state changes. **Appendage ontogeny**: ✖ Fragmentation of exosporium; △ Outgrowth of exosporium; ▲ Outgrowth and fragmentation; ◉ Wall outgrowth and elaboration of exosporium; ● Direct outgrowth from one or more spore wall layers; ★ Unfurling, exuded through pore; ♥ Other type; **Catenophyses and periphyses:** ✱ Presence of catenophyses; ✚ Presence of periphyses; **Asci structure**: ■ Persistent asci; □ Deliquescing asci; ▣ Persistent or deliquescing asci; ☑ Apical structures; **Distribution:** ***Te***=Temperate; ***Tr***=Tropical/Subtropical; ***STr***=Subtropical; ***W***=Widespread; **Habitats**: ***Oc***=Oceanic; ***Mg***=Mangrove; ***Ds***=Deep sea; ***Fw***=Freshwater; **NA**=Not applicable

Remispora sensu stricto all possess polar appendages that are derived by fragmentation of an exosporial layer of the ascospore cell wall (Johnson et al. 1984). Appendages in *R. maritima* and *R. pilleata* are wing-like (Fig. 2a, b, d, e), while those of *R. spitsbergenensis, R. quadri-remis*, and *R. stellata*, form a crown of radiating appendages (Fig. 2g, h, i, k, l, m). All these species have appendages that comprise fibrous material in an amorphous matrix (Johnson et al. 1984; Pang et al. 2004b, 2009). *Remispora sensu stricto* species form a monophyletic group with *Sablecola chinensis* as a sister taxon with good support. *Sablecola chinensis* differs from *Remispora* species in having both polar and equatorial appendages (Pang et al. 2004b). *Remispora maritima* and *R. pilleata* formed a well supported group while the three other species formed a sister group. These groupings also reflect the differences observed in ascospore appendage morphology.

Two other *Remispora* species have been described: *R. lobata* (Höhnk 1955) and *R. spinibarbata* (Koch 1989). Meyers (1957) reduced *R. lobata* to synonymy with *R. maritima*, yet they differ in ascospore measurements (17.5)21.5−27(31.5)×(8)10−11.5(12) µm in *R. maritima* and


i
Remispora sensu stricto
64/100/- Remispora maritima BBH28309 ✖ ✱ - ☐ - Te Oc
100/70/0.96 Remispora maritima BCC33517 ✖ ✱ - ☐ - Te Oc
98/100/0.96 Remispora pilleata BBH28305 ✖ ✱ ✚ ☐ - Te Oc
80/96/0.99 Remispora pilleata BBH28306 ✖ ✱ ✚ ☐ - Te Oc
100/100/0.97 Remispora spitsbergenensis CY5279 ✖ - - ☐ - Te Oc
100/100/0.96 Remispora stellata JS150 ✖ ✱ - ☐ - Te Oc
Remispora quadriremis BCC15555 ✖ ✱ ✚ ☐ - Te Oc
Sablecola chinensis BCC22809 ? - - ☐ - Tr Oc
71/95/- Halosarpheia marina ★ - ✚ ☐ ☑ Tr Oc,Mg
Panorbis viscosus ★ - ✚ ■ - Te, W Oc,Fw
-/58/- Phaeonectriella lignicola - ✱ ✚ ■ ☑ Te Fw
Nimbospora effusa ✖ - ✚ ☐ - Tr Oc
Tirispora unicaudata ♥ ✱ ✚ ■ ☑ Tr Mg
ii
-/79/1.00 Ocostaspora apilongissima LP53 ✖ - - ☐ - Te Oc
Ocostaspora apilongissima LP32 ✖ - - ☐ - Te Oc
Ocostaspora apilongissima CY3399 ✖ - - ☐ - Te Oc
99/89/0.97 Kochiella (Remispora) crispa BCC33502 ✖ - ✚ ☐ - Te,Tr Oc
100/100/1.00 Kochiella (Remispora) crispa BCC33504 ✖ - ✚ ☐ - Te,Tr Oc
Kochiella (Remispora) crispa BCC33507 ✖ - ✚ ☐ - Te,Tr Oc
Morakotiella salina BCC12781 △ ✱ ✚ ☐ - Te Oc
58/56/- Naufragella spinibarbata BCC33508 ♥ ✱ ✚ ☐ - Te Oc
Naufragella spinibarbata PP6886 ♥ ✱ ✚ ☐ - Te Oc
71/99/- Naufragella spinibarbata BCC33482 ♥ ✱ ✚ ☐ - Te Oc
Nohea umiumi ♥ ✱ ✚ ☐ - Tr Oc
89/55/0.99 Halosphaeria appendiculata ● - - ☐ - Te Oc
Lignincola laevis - ✱ ✚ ▣ - Tr,W Oc,Mg
100/63/1.00 Natantispora retorquens ★ ✱ ✚ ■ - W Oc,Fw
Natantispora lotica ★ ✱ ✚ ■ - W Oc,Fw
Neptunella longirostris - ✱ ✚ ▣ ☑ Tr Oc,Mg
99/100/-
iii
100/100/1.00 Periconia prolifica NA W Oc
73/75/0.98 Okeanomyces cucullatus - ✱ - ☐ - W Oc
50/56/0.99 Thalespora appendiculata ? - ✚ ☐ - Tr Oc
68/74/1.00 Ascosacculus heteroguttulata ★ ✱ ✚ ☐ - Tr,W Fw
82/63/0.99 Ascosacculus aquatica ★ ✱ ✚ ☐ - Tr,W Fw
66/52/- 82/80/0.99 Aniptodera chesapeakensis - ✱ ✚ ■ ☑ W Oc,Mg
Nais inornata - ✱ ✚ ☐ - Te Oc
79/74/- Aniptodera lignatilis - ✱ ✚ ■ ☑ Tr Fw
iv
100/91/1.00 Oceanitis scuticella ★ - ✚ ☐ - W Oc
Oceanitis viscidulum ★ - ✚ ☐ - Tr Oc
95/100/1.00 Oceanitis cincinnatulum ★ - ✚ ☐ - Tr Fw
Oceanitis unicaudatum ★ - ✚ ☐ - Tr Oc
100/100/1.00 Ophiodeira monosemeia ★ - ✚ ☐ - Tr Oc,Mg
100/100/1.00 Humicola siamensis NA Tr Oc
Pseudolignincola siamensis ? ✱ ✚ ■ ☑ Tr Oc
100/100/1.00 Antennospora quadricornuta ? - ✚ ☐ - Tr Oc
59/57/- Aniptodera longispora BBH28304 - ✱ ✚ ■ ☑ Tr Mg
86/70/- Cucullosporella mangrovei ★ ✱ ✚ ■ ☑ Tr Mg
Arenariomyces trifurcatus ● - - ☐ - W Oc
Haiyanga salina ● - - ☐ - W Oc
v
74/94/0.99 Halosarpheia fibrosa ★ ✱ ✚ ■ - Tr Oc,Mg
100/87/0.95 Halosarpheia unicellularis ★ ✱ ✚ ▣ - Tr Mg
97/84/0.99 Halosarpheia trullifera ★ ✱ ✚ ■ - W Oc
Lignincola tropica - ✱ ✚ ■ ☑ Tr Mg
Halosarpheia sensu stricto
vi
82/-/0.97 Corollospora pulchella ▲ - - ☐ - Tr Oc
Corollospora lacera ▲ - - ☐ - Te Oc
92/92/1.00 Corollospora filiformis ▲ - - ☐ - Te Oc
71/-/- Corollospora intermedia ▲ - - ☐ - Te Oc
52/74/0.97 Corollospora maritima ▲ - - ☐ - Tr,W Oc
77/55/0.99 Corollospora quinqueseptata ▲ - - ☐ - Te Oc
74/77/- Halosigmoidea parvula NA Te Oc
Varicosporina ramulosa NA W Oc
Halosigmoidea marina NA Te Oc
Corollospora angusta ▲ - - ☐ - Te Oc
65/78/1.00 Corollospora portsaidica ▲ - - ☐ - STr Oc
vii
57/97/1.00 Saagaromyces ratnagiriensis ★ ✱ ✚ ■ ☑ Tr Mg
99/97/0.99 Saagaromyces glitra - ✱ ✚ ■ ☑ Tr Mg
-/54/- Saagaromyces abonnis ★ ✱ ✚ ■ ☑ Tr Mg
Halosphaeriopsis mediosetigera ✖ ✱ - ☐ - W Oc
Halosarpheia kandeliae CY1492 ? ✱ ✚ ☐ - Tr Mg
-/56/- Nereiospora cristata ● - - ☐ - Te Oc
-/64/- Nereiospora comata ● - - ☐ - Te Oc
53/64/- Monodictys pelagica NA W Oc
60/60/- Havispora longyearbyenensis CY5278 ? ✱ - ■ - Te Oc
91/90/- Magnisphaera stevemossago ★ ✱ ✚ ☐ - Te Fw
Magnisphaera spartinae ★ ✱ ✚ ☐ - Te Fw
Halosphaeriaceae
viii
90/73/0.99 Toriella (Ceriosporopsis) tubulifera BCC33511 ★ - - ☐ - Te Oc
56/100/0.99 Toriella (Ceriosporopsis) tubulifera BCC33512 ★ - - ☐ - Te Oc
99/98/1.00 Toriella (Ceriosporopsis) tubulifera BCC33513 ★ - - ☐ - Te Oc
100/100/1.00 Ondiniella torquata BCC34303 ○ ✱ - ☐ - Te Oc
80/84/0.99 Ondiniella torquata BCC33480 ○ ✱ - ☐ - Te Oc
95/99/0.97 Marinospora calyptrata BBH28307 ○ ✱ ✚ ☐ - Te Oc
89/85/1.00 Marinospora calyptrata CY3491 ○ ✱ ✚ ☐ - Te Oc
-/55/0.99 Marinospora longissima ○ ✱ ✚ ☐ - Te Oc
ix
-/93/- Ceriosporopsis (Bovicornua) intricata BCC33465 ○ - - ☐ - Te Oc
-/96/1.00 Ceriosporopsis (Bovicornua) intricata BCC34299 ○ - - ☐ - Te Oc
Ceriosporopsis (Bovicornua) intricata BCC33466 ○ - - ☐ - Te Oc
Ceriosporopsis halima ○ - - ☐ - W Oc
71/-/- Ceriosporopsis halima BCC15553 ○ - - ☐ - W Oc
Alisea longicolla - - ✚ ☐ - Te Oc,Ds
x
100/100/1.00 Tubakiella (Remispora) galerita PP5577 ✖ - ✚ ☐ - Te Oc
93/91/0.99 Tubakiella (Remispora) galerita BCC33500 ✖ - ✚ ☐ - Te Oc
93/52/- Nautosphaeria cristaminuta BBH28308 ● - - ☐ - Te Oc
Haligena elaterophora PP4705 △ ✱ ✚ ☐ - Te Oc

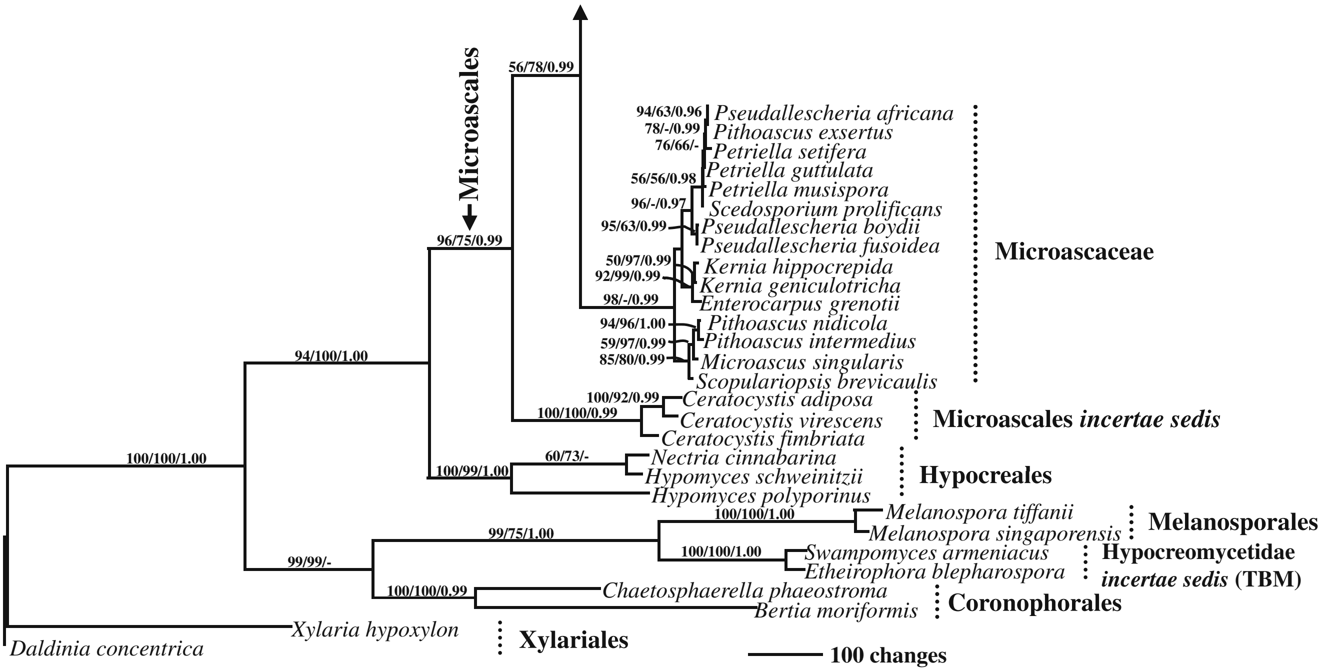


Fig. 1 (continued)

16−21.6(25.7) μm in *R. lobata.* They also differ in the morphology of the ascospore appendages. Recent collections made in Denmark, showed that *R. maritima* and *R. lobata* are distinguishable and so the taxonomic position of *R. lobata* should be revisited (J. Koch personal communication). However this observation can not be resolved until collections of *R. lobata* are sequenced and compared with other *Remispora* species.

Kohlmeyer and Volkmann-Kohlmeyer (1998) noted that ascospore appendage morphology in *R. spinibarbata* differed from those of other *Remispora* species. The former has two types of appendages: gelatinous polar appendages that become strap-like or band-like and up to 90 μm long and subpolar fine bristle-like appendages (Fig. 2o) (Koch 1989), however, *Remispora sensu stricto* species possess only polar appendages. Subsequently, Kohlmeyer and Volkmann-Kohlmeyer (1998) assigned *R. spinibarbata* to a new genus *Naufragella* (type species: *N. delmarensis*) based on the differences in appendage morphology as described above. Our molecular data confirm that *N. spinibarbata* does not belong in *Remispora sensu stricto* with three strains forming a monophyletic group, with *Ocostaspora apilongissima* and *Remispora crispa* as a sister group.

Kochiella Sakayaroj, K.L. Pang & E.B.G. Jones **gen. nov.**

MycoBank MB518769

Etymology: from Koch, in recognition of Dr. Jorgen Koch's outstanding contribution to Danish marine mycology, and the Latin diminutive suffix—*ellus*.

Ascocarpis subglobosis, ellispoides, superficialibus vel immersis, ostiolatis, papillatis, coriaceis; *asci* octosporis, clavatis, leptodermis, deliquescentibus; *ascoporis* ellispodeis, uniseptatis, hyalines, appendiculatis, pleomorphis.

Typus: Kochiella crispa (Kohlm.) Sakayaroj, K.L. Pang & E.B.G. Jones

Ascomata subglobose to ellipsoidal, superficial to immersed, ostiolate, papillate, periphysate, coriaceous, thin-walled, hyaline to cream coloured; *asci* ellipsoidal to clavate, thin-walled, deliquescing at maturity; *ascospores* 1-septate, hyaline, appendages pleomorphic.

Kochiella crispa (Kohlm.) Sakayaroj, K.L. Pang & E.B. G. Jones **comb. nov.**

MycoBank MB518770

Basionym: *Remispora crispa* Kohlm., Can J Bot 59: 1317, 1981

Anamorph: Not observed.

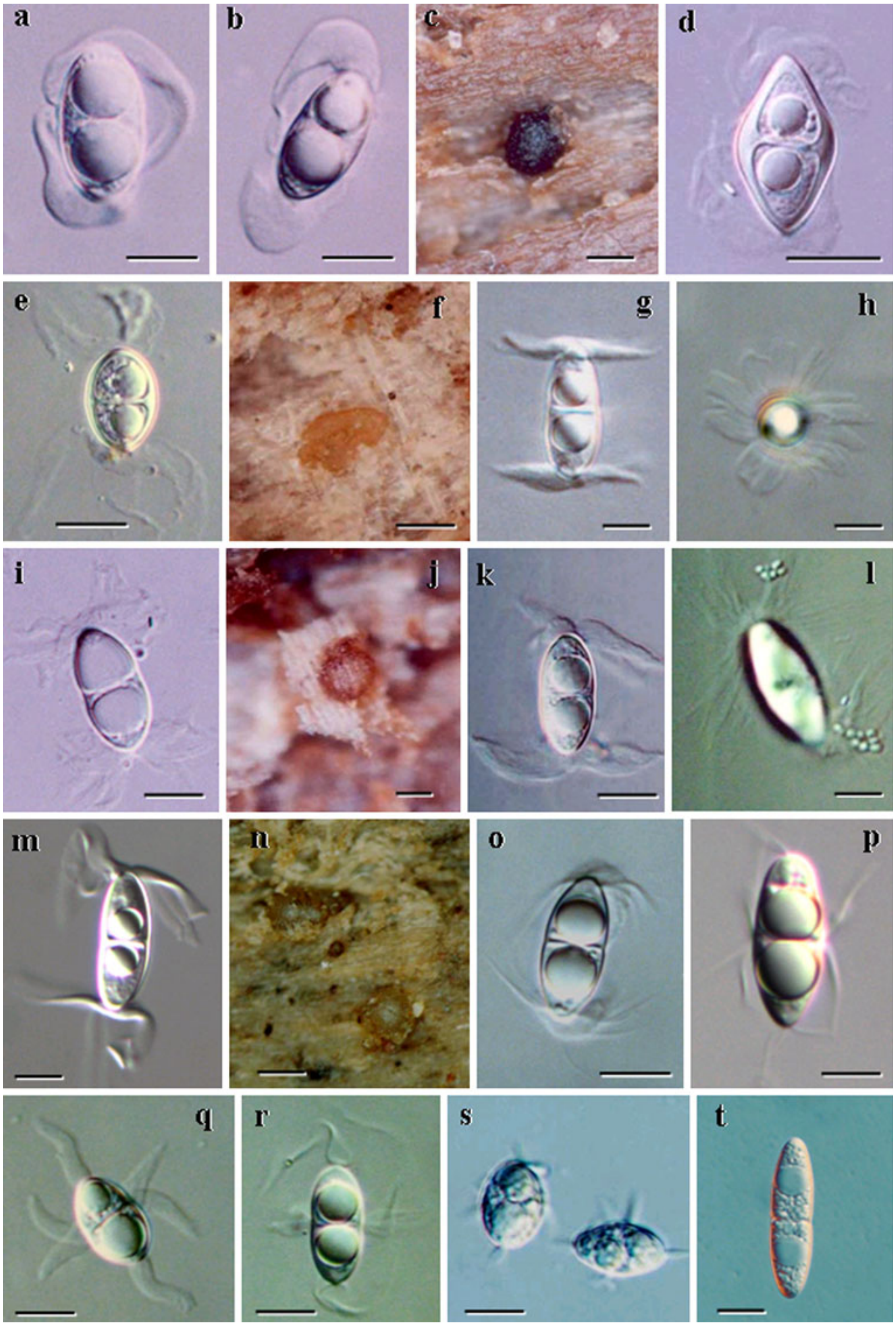
Mode of life: saprobic.

Habitat: wood.

Distribution: Denmark, Hawaii, Japan, Liberia, Martinique, Taiwan.

Notes: Kochiella crispa did not group with *Remispora* species *sensu stricto* but was placed in clade ii with three monophyletic strains of *Ocostaspora apilongissima* with high support. However these two fungi are not conspecific and differ markedly in ascospore appendage morphology. *Kochiella crispa* has polar appendages while *O. apilongissima* has polar and equatorial appendages. *Kochiella* differs from *Remispora* in morphology and substructure of the ascospore appendages. In *Remispora* species, catenophyses are present, the fibrous component of the appendages is wavy and appendages are wing-like or form a crown of radiating appendages. Conversely, catenophyses are lacking

Fig. 2 Morphological features of newly sequenced halosphaeriialean taxa. **a**, **b** Wing-like polar appendages of *Remispora maritima*; **c** Superficial black ascomata of *R. pilleata* on wood; **d**, **e** Thick-walled rhomboidal ascospores with subgelatinous appendages of *R. pilleata*; **f**: Hyaline-orange ascomata of *R. stellata* immersed in wood; **g** Ascospore of *R. stellata* with bipolar appendages; **h** Top view of polar radiating appendages of *R. stellata*; **i** Ascospore of *R. stellata* with 5–9 radiating segments; **j** Hyaline-light brown ascomata of *R. quadri-remis*; **k** Ascospore of *R. quadri-remis* with 4 discrete bipolar appendages; **l** Radiating segments of appendaged ascospore of *R. quadri-remis*; **m** A hyaline ascospore of *R. spitsbergenensis* with polar appendages; **n** Superficial hyaline ascomata of *Naufragella spinibarbata* on wood; **o** Ascospore of *N. spinibarbata* with long polar appendages and soft spines; **p** Ascospore of *Havispora longyearbyenensis* with bipolar and equatorial appendages; **q** Ascospore of *Sablecola chinensis* with with polar and equatorial appendages; **r** Ascospore of *Ocostaspora apilongissima* with spoon-shaped polar appendages with equatorial spines; **s** Ascospore of *Nautosphaeria cristaminuta* with tufts of polar and equatorial appendages; **t** Ascospores of *Aniptodera longispora*. Scale bars: a,b,d,e,g-i,k-m, o-t=10 μm; c,f=200 μm; j=100 μm; *n*=300 μm



in *K. crispa*, appendages are pleomorphic and do not completely separate from the ascospore cell wall, while the fibrillar component consists of an inner core continuous with the middle layer of the episporium and the outer sheath of the strand was continuous with the electron-dense layer of the episporium (Manimohan et al. 1993a, b). The species is regarded by Kohlmeyer (1981) as a tropical taxon, however collections have been made in Denmark and Taiwan (this study).

Tubakiella Sakayaroj, K.L. Pang & E.B.G. Jones **gen. nov.** Fig. 3a–c

MycoBank MB518772

Etymology: from Tubaki, in recognition of Professor Keiush Tubaki, distinguished mycologist and an early

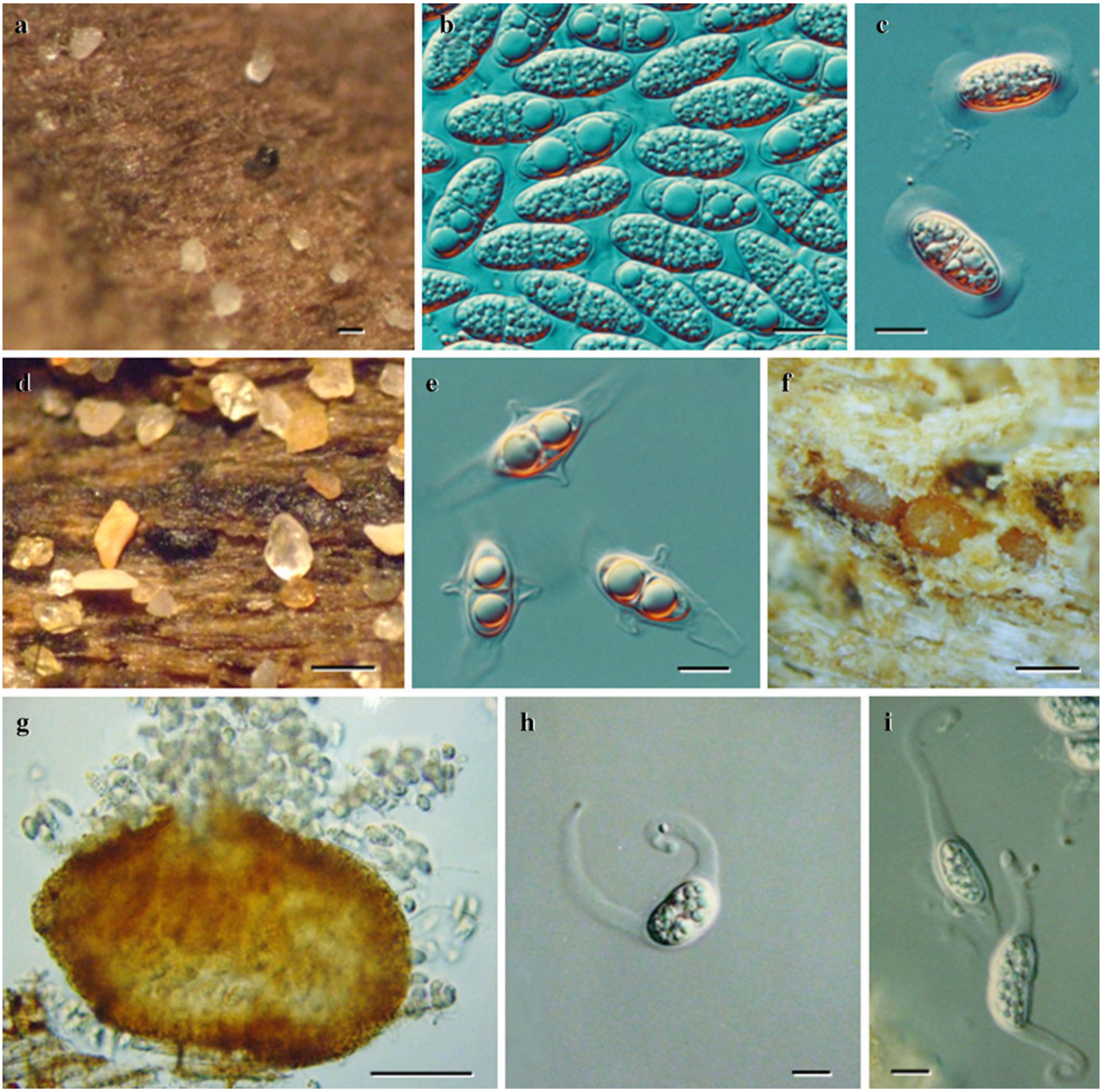


Fig. 3 Morphological features of new genera and new combination proposed from this study. **a** The immersed ascomata with hyaline spore mass of *Tubakiella galerita* oozing on the wood surface; **b** The ornamented ascospores of *Tu. galerita* full of oil globules; **c** Subglobose compact cap-like polar appendages of *Tu. galerita*; **d** Superficial black ascomata of *Toriella tubulifera*; **e** Ascospores of *To. tubulifera* with polar end chamber and equatorial appendages; **f** The immersed orange-creamy ascomata of *Ceriosporopsis intricata* on wood surface; **g** Ascospores of *C. intricata* released from the ascomata when mounted in seawater; **h**,**i** Ascospores of *C. intricata* full of oil globules with curved polar appendages. Scale bars: a,d,f=200 µm; g=50 µm; b,c,e,h,i=10 µm

contributer to marine mycology in Japan, and the Latin diminutive suffix—*ellus*.

Ascocarpis globosis vel subglobosis, superficialibus vel immerses, membranaceis, hylainis, flavidis vel pallide fuscis, rotrie elongate cylindricus, centricis, ostiolatis, leptodermis; *ascis* clavatis, leptodermis, octosporis, mox deliquescentibus; *ascoporis* ellipsoides, tegimentis mucosis tectis, uniseptatis, hyalines; *appendiculis* gelatinosis, plerumques ger germinatis galeriformibusque, deniques striates.

Typus: *Tubakiella galerita* (Tubaki) Sakayaroj, K.L. Pang & E.B.G. Jones

Ascomata globose, subglobose to pyriform, superficial to immersed, ostiolate, papillate, coriaceous, thin-walled, hyaline to yellowish or light brown; *asci* ellipsoidal or ovoid, thin-walled, deliquescing at maturity; *ascospores* ellipsoidal, 1-septate, hyaline, thick-walled at the apices; *ascospore appendages* at first a gelatinous sheath covers ascospores completely, later only a subglobose subgelatinous appendages.

Tubakiella galerita (Tubaki) Sakayaroj, K.L. Pang & E.B.G. Jones, **comb. nov.**

MycoBank MB518773

Basionym: *Remispora galerita* Tubaki, Publ Seto Mar Bio Lab 15: 362, 1967.

Synonym: *Halosphaeria galerita* (Tubaki) I. Schmidt in Kohlm. & E. Kohlm., In: Marine Mycology, The Higher fungi 291, 1979.

Anamorph: Not observed.

Mode of life: saprobic

Habitat: wood

Distribution: France, Denmark, Germany, Japan, Taiwan, USA, Yugoslavia

Notes: Tubakiella was distantly placed from *Remispora sensu stricto* with the two sequenced strains forming a monophyletic group with *Nautosphaeria cristaminuta* and *Haligena elaterophora* as a sister group with weak support. *Tubakiella* has appendages formed by fragmentation of an exosporic layer and morphologically has little in common with either of the associated species. *Haligena elaterophora* has strap-like polar appendages that initially are wrapped around the ascospore, while *Nautosphaeria* has both polar and equatorial tufts of hair-like appendages (Jones 1964; Farrant and Jones 1986, Fig. 2s).

Ceriosporopsis Linder, Farlowia 1: 408, 1944

C. halima Linder, Farlowia 1: 408, 1944 (Type species).

Anamorph: Not observed

Mode of life: saprobic

Habitat: wood

Distribution: widely distributed in temperate waters

Accepted species:

C. caduca E.B.G. Jones & Zainal, Mycotaxon 32: 238, 1988

C. capillacea Kohlm., Can J Bot 59:1314, 1981

C. intricata (Jorg. Koch & E.B.G. Jones) Sakayaroj, K.L. Pang & E.B.G. Jones, Fungal Divers. this volume

Doubtful species:

C. cambrensis I.M. Wilson, Trans Br Mycol Soc 37:276, 1954

Rejected species: C. tubulifera (Kohlm.), P.W. Kirk ex Kohlm., Can J Bot 50: 1953, 1972

Notes: The genus *Ceriosporopsis* is characterized by subglobose to cylindrical ascomata, immersed, ostiolate, papillate, coriaceous or subcarbonaceous, light brown to black, catenophyses, deliquescing, asci clavate, pedunculate, unitunicate, thin-walled, deliquescing early, ascospores ellipsoidal, 1-septate, hyaline, with appendages. Appendage morphology variable depending on the species (Johnson et al. 1984, 1987; Jones et al. 1986; Yusoff et al. 1993).

Ceriosporopsis intricata (Jorg. Koch & E.B.G. Jones) Sakayaroj, K.L. Pang & E.B.G. Jones **comb. nov.** Fig. 3g–i

MycoBank MB518771

Anamorph: Not observed.

Mode of life: saprobic

Habitat: wood

Distribution: Denmark, Wales

Notes: Bovicornua intricata is characterised by globose to subglobose ascomata, ostiolate with short conical necks, grayish white, erumpent, membranous, gregarious, centrum pseudoparenchyma breaks down, no catenophyses, asci broadly clavate, pedunculate, unitunicate, thin-walled, early deliquescing, no apical pore, ascospores unequally 1-septate, slightly curved, constricted at the septum, hyaline and appendaged. At each pole there is a single appendage enclosed within an outer sheath which swells when mounted in seawater.

At the light microscope level, the ascospores of *Bovicornua intricata* with their polar appendages and enveloping exosporic sheath resemble those of *Ceriosporopsis* species, but differed in the complexity of the exosporium. This was considered sufficient evidence for the erection of a new genus *Bovicornua* (Yusoff et al. 1993). Ascospore walls of both genera consist of a mesosporium, episporium and exosporium, the polar appendage arising as an outgrowth of the spore wall. The exosporium also surrounds the polar appendage but in *C. halima* it ruptures and forms a cupule-like structure at the base of the polar appendage. However the structure of the ascospore wall and appendage in *B. intricata* is much more elaborate than that observed for any *Ceriosporopsis* species (Yusoff et al. 1993, 1994). Molecular data place *B. intricata* in the *Ceriosporopsis* clade ix with strong support, and so the species is transferred to *Ceriosporopsis*.

Toriella Sakayaroj, K.L. Pang & E.B.G. Jones **gen. nov.** Fig. 3d–f.

MycoBank MB518774

Etymology: *Tor* from the Thai for tube, in reference to the tube-like end chamber and the Latin diminutive suffix—*ellus.*

Ascocarpis globosis ad cylindricis vel elongatis, immersis, ostiolatis, papillatis, coriaceis, pallide brunea vel nigris, solitaris or gregarious, catenophyses absens; *asci* octosporis, clavatis, pedicellati, sine pori apicalibus, leptodermis, mox deliquescentibus; *ascoporis* ellispodeis, uniseptatis, hyalines, *appendiculis*, cum appendices ad polum et aequatorem quatuor adepisporium.

Typus: Toriella tubulifera (Kohlm.) Sakayaroj, K.L. Pang & E.B.G. Jones

Ascomata globose, cylindrical or elongate, immersed, ostiolate, papillate, coriaceous, brown to black, solitary or gregarious, no catenophyses; *asci* thin-walled, unitunicate, clavate, pedunculate, no apical pore, deliquescing early; *ascospores* hyaline, ellipsoidal, 1-septate, with equatorial and polar appendages. *Ascospore appendage ontogeny*: exosporium folds to form an annulus-like equatorial appendage while the polar appendages are formed inside and end chamber that consist of two electron-dense layers.

Toriella tubulifera (Kohlm.) Sakayaroj, K.L. Pang & E. B.G. Jones **comb. nov.**

MycoBank MB518775

Basionym: *Halosphaeria tubulifera* Kohlm., Nova Hedw 2: 312, 1960.

Synonym: Ceriosporopsis tubulifera (Kohlm.) P.W. Kirk ex Kohlm., Can J Bot 50: 1953, 1972

Anamorph: Not observed.

Mode of life: saprobic.

Habitat: wood

Distribution: Canada, Denmark, England, Germany, Iceland, Norway, Sweden, USA, Wales.

*Notes***:** *Ceriosporopsis tubulifera* was initially described as a *Halosphaeria* species (Kohlmeyer 1960), but transferred to *Ceriosporopsis* based on the development of the appendages: "an exosporic sheath is pierced apically (or also laterally) by the outgrowth of mucilaginous appendages" (Kohlmeyer 1972). However, in *C. tubulifera* the exosporium folds to form an annulus-like equatorial appendage and the polar appendage is formed inside an end chamber that consists of two electron-dense layers (Johnson et al. 1987). The polar appendage is not an outgrowth of the spore wall and the end chamber is persistent (Johnson et al. 1987; Jones 1995). Johnson et al. (1987) pointed out that a new genus might be required to accommodate *C. tubulifera* and this is supported by the molecular data which placed it distantly from the type species *C. halima* (Fig. 1, clade viii).

Toriella formed a sister group to *Ondiniella* with strong bootstrap support, a genus characterized by elongate-cylindrical or subglobose ascomata, immersed or superficial, ostiolate, papillate, membranous, ranges from hyaline to pale brown, necks short and cylindrical, no periphyses, catenophyses deliquescing, asci clavate to subfusiform, apiculate, pedunculate, unitunicate, thin-walled, no apical apparatus, deliquescing, ascospores broadly ellipsoidal, 1-septate, slightly constricted at the septum, hyaline with appendages. Two types of appendages: spine-like polar appendages and an annulus-like equatorial appendage, arise as outgrowths of the episporium, with amorphous material within the appendages and lacking an exosporium (Jones et al. 1984). Although *O. torquata* groups consistently with *C. tubulifera*, they are not congeneric.

Marinospora calyptrata was initially described as a *Ceriosporopsis* species (Kohlmeyer 1960), transferred to *Ceriosporella* (generic name occupied) (Cavaliere and Johnson 1966), to *Marinospora* (Cavaliere and Johnson 1966) and referred back to *Ceriosporopsis* by Kohlmeyer (1972) based on the structure of the appendages. Johnson et al. (1984) re-established the genus based on the ontogeny of ascospore appendage ontogeny. Appendages arise as outgrowths of the spore wall, the exosporium fragments leaving remnants as cup-like structures at their tips (Johnson et al. 1984; Jones 1995). *Ceriosporopsis* species (*C. halima, C. capillacea C. caduca*) also have appendages that arise as outgrowths of the spore wall, but the exosporial sheath persists around the spore body and forms a collar at the base of the polar appendages (Johnson et al. 1987; Yusoff et al. 1994). No cup-like exosporial remnants are found on the primary appendages in *Ceriosporopsis*. Two *Marinospora* species have been described, *M. calyptrata* (type species) and *M. longissima* (Kohlmeyer 1962). The two species can be distinguished by ascospore size and the length of the primary polar appendages. *Marinospora longissima* ascospores are always enveloped by a layer of mucilage and has polar appendages which are longer than the equatorial ones. In *M. calyptrata,* the length of primary and secondary appendages are equal (Johnson et al. 1984).

Ascomycetes with unfurling polar appendages

A number of marine and freshwater ascomycetes have ascospores with initially hamate appendages that are closely adpressed to the spore cell wall, later uncoiling in water to form long bipolar thread-like sticky appendages (Kohlmeyer and Kohlmeyer 1977). Kong et al. (2000) indicated that the genus *Halosarpheia* was polyphyletic, an observation confirmed by Anderson et al. (2001). Subsequently a number of species were transferred to new genera based on sequence data (Abdel-Wahab et al. 2001b; Campbell et al. 2003; Pang et al. 2003; Pang and Jones 2004). However, the taxonomic position of a number of *Halosarpheia* species remained unresolved: *H. bentotensis*, *H. culmiperda, H. kandeliae*, *H. marina, H. minuta* and *H. phragmiticola*, primarily because fresh material was not available for a molecular study (Jones et al. 2009). That this group is polyphyletic is well demonstrated by their placement in different positions in the *Halosphaeriaceae* (Fig. 1). Some formed well supported clades e.g. clade iv—*Oceanitis* species; clade v—*Halosarpheia sensu stricto*; clade vii—*Saagaromyces* species. However, the position of other taxa is not resolved e.g. *Aniptodera lignatilis*, *A. chesapeakensis* and *Ascosacculus* species in clade iii, although the clade has high support.

Another group that is unresolved includes the genera/species *Halosarpheia marina*, *Panorbis viscosus* and *Phaeonectriella* species. *Halosarpheia marina* and *H. kandeliae* are two species that clearly do not belong in *Halosarpheia sensu stricto*. *Halosarpheia marina* forms a sister group to *P. viscosus* with weak support, while *H. kandeliae* forms a sister group to a clade comprising *Saagaromyces* species and *Halosphaeriopsis mediosetigera* with low support. However further taxon sampling is required before they can be assigned to new genera.

Halosarpheia kandeliae Abdel-Wahab & E.B.G. Jones, Mycologia 103:1500, 1999

This species is characterized by ellipsoidal, membranous, ostiolate, papillate ascomata, lateral necks, periphysate, catenophyses present, asci 8-spored, thin-walled, unitunicate, with narrow long stalks, persistent, lacking an apical apparatus, ascospores ellipsoidal, 1-septate, not constricted at the septum, hyaline, thin-walled, with bipolar apical appendages (Abdel-Wahab et al. 1999). It differs from many *Halosarpheia* species in having asci with long drawn out stalks (tail-like), and polar appendages that initially appear amorphous and only later form the characteristic thread-like bipolar appendages (Abdel-Wahab et al. 1999).

Genera with bipolar unfurling appendages have evolved a number of times within the family and are found in both freshwater and marine habitats. Although morphologically similar they differ radically at the ultrastructural and molecular level. Marine taxa are distributed within six of the ten clades in our analysis of three genes. Species may possess one (e.g. *Tirispora unicaudata*, *Oceanitis scuticella*, *O. unicaudata*, *Nohea umiumi*) or two polar appendages (e.g. *Cucullosporella mangrovei*, *Panorbis viscosus*). Most have 1-septate ascospores, while others are multiseptate (e.g. *Oceanitis cincinnatula*, *Magnisphaera spartinae*), the majority are hyaline although *Phaeonectriella* species have brown spores. Ultrastructurally ascospore appendages are produced through a single apical pore (*Magnisphaera spartinae*, Jones 1962), a hood-like structure (*Cucullosporella mangrovei*, Alias et al. 2001) or through a series of discontinuities in the episporium layer (McKeown et al. 1996; Baker et al. 2001). Three basic ontogenetic processes have been reported for ascospores with uncoiling appendages: in *Trichomaris invadens* they arise from the episporium (Porter 1982); in *Tunicatispora australiensis* from the mesosporium (McKeown et al. 1996) or their origin is not resolved, but released through discontinuities in the episporium and probably derived from the mesosporium (*Saagaromyces* (as *Halosarpheia*) *ratnagiriensis*, *Aniptodera* (as *Halosarpheia aquadulcis*) (Hsieh et al. 1995; Baker et al. 2001). The appendage may initially be amorphous in appearance and later differentiate; or comprise a single tightly coiled thread or composed of two distinct components (Jones 2006). Some are a single stout thread while others comprise a series of threads.

Some eleven marine genera have ascospores with uncoiling polar appendages (Jones et al. 2009) while 9 genera (e.g. *Ascosacculus*) with similar spores are known from freshwater habitats (Cai et al. 2006). However, these are not confined to the *Halosphaeriaceae,* as the *Annulatascaceae* and *Lasiosphaeriaceae* have similar appendages: *Annulatascus*, *Cataractispora*, *Diluviocola*, *Proboscispora* (Wong et al. 1998a, b). *Cataractispora bipolaris* has a polar pad-like appendage that eventually forms long, drawn out strands, and *Diluviocola capensis* has a cone-shaped hood that separates to form narrow threads (Hyde et al. 1998; Hyde and Goh 2003).

Clearly taxa with ascospores with unfurling bipolar appendages are not monophyletic and have occurred many times during the evolution of the ascomycetes (Campbell et al. 2003; Pang and Jones 2004; Pang et al. 2003) and similar appendages also occur in the Trichomycetes (Moss 1979; Jones 1994). The possession of such appendages must confer an environmental advantage and the result of convergent evolution (Jones 1995, 2006). The long thread-like appendages are sticky and wrapped around debris/substrata or adhere the spore to surfaces (Jones 1994, 2006).

Monotypic and unresolved genera: *Havispora longyearbyenensis, Ocostaspora apilongissima, Ondiniella torquata, Nautosphaeria cristaminuta, Sablecola chinensis* and *Aniptodera longispora*.

Havispora longyearbyenensis K.L. Pang & Vrijmoed, Mycologia 100: 293, 2008 Fig. 2p

This genus was described from Longyearbyen, Norway with subglobose to ellipsoidal ascomata, immersed, coriaceous, solitary or gregarious, lacking periphyses, catenophyses present deliquescing, long perithecial necks, asci clavate, pedunculate, unitunicate, thin-walled, persistent, ascospores ellipsoidal, hyaline, thin-walled, 3-septate, constricted at the septa, with tufts of appendages (Pang et al. 2008a). Ascospores have a polar appendage and four at the central septum that are string-like and composed of intertwining strands (Pang et al. 2008a). Morphologically this genus has similar tufts of polar and equatorial appendages as *Nautosphaeria* and *Nereiospora* species (Jones 1964; Jones et al. 1983b). However the ascospores of *N. cristaminuta* are unicellular while *Nereiospora* species have ascospore with brown central cells and hyaline end cells. The phylogenetic position of *Havispora longyearbyenensis* is distantly placed from *Nautosphaeria*, but has affinities with the *Nereiospora/Monodictys* grouping.

Ocostaspora apilongissima E.B.G. Jones, R.G. Johnson & S.T. Moss, Bot Mar 26: 353, 1983 Fig. 2r

This genus was described and placed in the *Halosphaeriales* by Jones et al. (1983a). This fungus possesses unique ascospore appendage morphology with long irregular amorphous polar appendage, with many equatorial appendages, spoon-shaped, with a fibrillar component. The appendage ontogeny has been referred to as type III: appendages formed by fragmentation of the outer exosporial layer of the spore wall (Jones 1995). Morphological data supports that *O. apilongissima* is not congeneric with

Kochiella crispa, although they share a common ancestor in clade ii (Fig. 1).

Nautosphaeria cristaminuta E.B.G. Jones, Trans Br Mycol Soc, 47: 97, 1964. Fig. 2s

This species was described from material collected on wood in Wales and referred to the *Halosphaeriales* (Jones 1964). This fungus has spherical ascocarps, hyaline to cream-colored, and anchored with brownish hyphae to the substratum. The asci are broadly clavate or ellipsoidal, pedunculate and without an apical apparatus. Ascospores are one-celled, ellipsoidal, hyaline, possess a tuft of bristle-like appendages at each end and four tufts around the equator. Ascospore appendage ontogeny falls into type VII: formed as direct outgrowths of the spore wall, however, it has not been studied at the TEM level (G.C. Hughes personal communication; Jones 1995). Ascospore appendages resemble those of *Nereiospora*, with the tufts of polar and equatorial appendages. However, these two genera appear to be different at the SEM level (Jones and Moss 1987; Hyde and Jones 1989). Moreover, the molecular result confirms that *N. cristaminuta* and *Nereiospora* species are not related. Additionally *N. cristaminuta* has little in common with the sister taxa *Haligena elaterophora* and *Tubakiella galerita*.

Sablecola chinensis E.B.G. Jones, K.L. Pang & Vrijmoed, Can J Bot 82: 486, 2004. Fig. 2q

Pang et al. (2004b) described this genus from southern China growing on wood with ellipsoidal to subglobose ascomata, immersed to partly immersed, yellow to brown, ostiolate, papillate, necks short and periphysate, lacking catenophyses, asci clavate, pedunculate, unitunicate, thin-walled, no apical apparatus, persistent to deliquescing. This genus is characterized by its unique bipolar and quadri-equatorial appendages, which disintegrate when mounted in seawater. The appendages are obclavate, tapered to a fine point, appear striated when released from the ascus but later become fibrillar (Pang et al. 2004b). This species groups in clade i with various *Remispora* species with weak support and its phylogenetic position remains unresolved.

Aniptodera longispora K.D. Hyde, Bot Mar 33: 335, 1990 Fig. 2t

This species is characterized by pyriform to subglobose ascomata, ostiolate, papillate, hyaline to light-brown, catenophyses present, asci clavate to cylindrical, pedunculate, unitunicate, thin-walled, with a refractive pore or plug-like structure at the apex, with a retracting plasmalemma subapically, persistent asci, ascospores ellipsoidal, 1-septate, hyaline, thin-walled, longer than 35 μm (Fig. 2t). Ascospores are released from the asci through a fissure made at the refractive pore or plug-like structure (Hyde 1990). In our analysis, *Aniptodera longispora* was distantly placed from *A. chesapeakensis* (the type species) but formed a robust clade with *Cucullosporella mangrovei* with high support. These two genera share morphological similarities with clavate, pedunculate, unitunicate, thin-walled asci, thickened apically with a lens-shaped refractive region. They differ in that *C. mangrovei* has well developed bipolar appendages composed of two components, a fibrillar material in an amorphous matrix. Molecular data suggests *A. longispora* should be assigned to *Cucullosporella*, but this is not formally proposed here until a more detailed study of marine and freshwater *Aniptodera* species is undertaken.

The evolution of marine fungi

In considering the evolution and transition of fungi from land to the marine environment, a number of observations can be made based on their morphological adaptation and phylogenetic data. It is multifaceted, with many unique lineages now identified, ranging from the *Basidiomycota* (e.g. *Halocyphina villosa*, *Haloaleurodiscus mangrovei*, *Mycoaureola dilsea,* Binder et al. 2006); dothideomycetous *Ascomycota* (e.g. *Aigialus*, *Biatriospora*, *Halotthia*, *Verruculina,* Suetrong et al. 2009b); and sordariomycetous *Ascomycota* (most of the genera in the *Halosphaeriaceae*, *Groenhiella*, *Swampomyces*, *Torpedospora*). This transition from land to an aquatic milieu was accompanied by varying morphological changes, reduction in the size of the basidiome (*H. villosa, Calathella mangrovei,* Binder et al. 2006), and production of appendaged basidiospores (*Nia vibrissa*, *Digitatispora* species, Jones and Choeyklin 2008) in the basidiomycetes; loss of active spore dispersal in the ascomycetes (particularly in the unitunicate ascomycetes: *Halonectria milfordensis*, *Halosphaeriopsis mediosetigera*, *Torpedospora radiata*, Moss and Jones 1977), development of appendaged ascospores (*Eiona tunicata*, *Halosarpheia* spp., *Groenhiella bivestita*, Jones 1995), or multibranched or appendaged conidia for anamorphic fungi (*Orbimyces spectabilis*, *Varicosporina anglusa*, Abdel-Wahab et al. 2009; Jones et al. 2009).

Terrestrial ascomycetes were initially thought to have evolved from a marine ancestor as outlined in the Floridean hypothesis (Sachs 1874; de Bary 1887) and with the discovery of marine ascomycetes this theory gained nominal support for at least some taxa (Demoulin 1974, 1985; Kohlmeyer 1975; Kohlmeyer and Kohlmeyer 1979; Kohlmeyer 1986). However, molecular phylogenetic data clearly show that the *Halosphaeriaceae* evolved independently from a terrestrial origin (Spatafora et al. 1998). A plausible explanation of the origin of marine ascomycetes could be migration from terrestrial habitats to freshwater and brackish water, then to marine environments (Shearer 1993; Jones 1995). This gradual transition may have brought about morphological diversity and changes in response to environmental conditions. For example, *Savoryella* species are found in freshwater and marine habitats, and preliminary data indicates that phylogenetically the two groups separate into distinct subclades (Boonyuen et al. in press). Many morphological adaptations to aquatic habitats are pertinent and include: deliquescing asci, lack of an apical ascal structure, passive release of ascospores, and modification of pseudoparenchymatous tissue into catenophyses and presence of appendaged ascospores (Shearer 1993; Jones 1995; Pang 2002). The possession of appendaged ascospores may confer and enable ascospores to stick onto substrata, and remain attached often under turbulent water movement and also as an aide for flotation and dispersal of spores (Fazzani and Jones 1977; Hyde et al. 1986; Hyde and Jones 1989; Hyde et al. 1993; Jones 1994).

It is however apparent that marine ascomycetes have made the transition into the sea many times and bitunicate and unitunicate taxa may have followed different evolutionary pathways. The majority of unitunicate ascomycetes is found in temperate oceanic and coastal waters, and possesses deliquescing asci, passive spore release and often with elaborate appendaged ascospores. Many lineages can be identified: e.g. *Halosphaeriaceae*, *Xylariales*, *Hypocreales*, *Lulworthiales* and taxa that currently do not fall into any family or order e.g. TBM clade (Schoch et al. 2007; Jones et al. 2009). The TBM clade comprises the genera *Etheirophora*, *Juncigena*, *Swampomyces* and *Torpedospora*, all marine taxa, that constitute a clade that forms a sister group to the *Hypocreales* in the *Hypocreomycetidae* (Schoch et al. 2007). On the other hand bitunicate ascomycetes are predominantly tropical, in coastal waters, with active spore discharge and ascospores lacking appendages or with a mucilaginous sheath that swells in water (Suetrong et al. 2009a). Tropical species occur on mangrove wood and herbaceous plants (Alias and Jones 2009), while temperate taxa are found on maritime and monocotyledonous plants, e.g. *Atriplex*, *Halimione*, *Juncus*, and *Spartina* (Kohlmeyer and Kohlmeyer 1979; Kohlmeyer and Volkmann-Kohlmeyer 2002).

Morphological character evolution in the Halosphaeriaceae

Ascomatal and hamathecium structure

The morphological variables in the family are the possession of periphyses and catenophyses. Periphyses have been observed and well defined for a wide range of genera, in particular in clades ii, iii, iv, v, vii (Fig. 1). However, the possession of periphyses varies between some genera in these clades and even within a genus (e.g. *Remispora sensu stricto*). Taxa in clade vi, which are mostly associated with sand grains (*Corollospora, Nereiospora, Arenariomyces*), lack periphyses or these are degenerate at maturity.

Catenophyses are persistent chains of utricular, thin-walled cells formed by the vertical separation of the pseudoparenchyma in the centrum (Kirk et al. 2008). Presence of catenophyses has been observed in a wide range of genera e.g. *Aniptodera, Marinospora, Haligena, Halosarpheia, Remispora, Lignincola, Nais, Phaeonectriella, Tirispora, Naufragella* and *Morakotiella* (Fig. 1). This character can be used to distinguish between certain genera, such as, *Remispora* from *Halosphaeria, Marinospora* from *Ceriosporopsis*, but may not be used to characterize such genera as *Halosarpheia* from *Saagaromyces* and *Ascosacculus*. Catenophyses have not been reported in the arenicolous genera *Corollospora, Nereiospora,* and *Arenariomyces*. Therefore, presence or absence of periphyses and catenophyses, whilst informative, may not be indicators of phylogenetic relationships of taxa within the *Halosphaeriaceae*. Gain or loss of these morphological features may be the result of adaptation to different habitats or substrata, but observation of these features depends upon the age of specimens.

Ascus structure

The ancestral ascus may have been persistent, with or without an apical structure, and this variation is found in a number of genera in the *Halosphaeriaceae*: *Aniptodera*, *Halosarpheia* (selected species only), *Natantispora*, *Neptunella*, *Panorbis, Tirispora*, *Phaeonectriella, Saagaromyces* and *Halosarpheia kandeliae* (Pang 2002). In most of these genera the persistent ascus has retraction of the plasmalemma around the apical pore (e.g. *Aniptodera chesapeakensis*, *Saagaromyces abonnis*) (Baker et al. 2001; Pang et al. 2003). These appear in a number of different subclades within the family so is this a retained or reacquired character? while some may be more common on intertidal and freshwater substrata (e.g. *Aniptodera chesapeakensis*, *Natantispora retorquens*). Development of asci lacking an apical pore and active spore release may have followed but still with a persistent ascus (e.g. *Lignincola laevis*). Evanescent asci with early deliquescence of the centrum and the passive release of the ascopores are the predominant feature of the family. Genera found to be primarily submerged generally have deliquescent asci and passive release of the ascospores e.g. *Bovicornua (*as *Ceriosporopsis)*, *Ceriosporopsis, Corollospora, Marinospora* and most are in early diverging lineages in the tree (Fig. 1).

Spatafora et al. (1998) drew attention to terrestrial ascomycetes with evanescent asci, e.g. *Ceratocystis*, *Microascus* and *Petriella* species; however, in the *Microascaceae* ascospores are insect dispersed. This is not the only group with deliquescing asci, as this feature has evolved several times in the ascomycetes. Cain (1972) opined that “the plasticity of the ascus morphology and dehiscence is consistent with a character that is strongly affected by selection pressures”. This may well account for the great variation observed in the occurrence of this character within the family.

Presence of apical structures (apical pore, apical plate, apical thickening and retraction of the plasmalemma) evolved independently from different common ancestors (Fig. 1), and is a difference between the *Ceratocystis* spp. and the *Halosphaeriaceae*. Genera possessing these structures include *Phaeonectriella, Tirispora, Neptunella, Halosarpheia marina, Aniptodera, Cucullosporella* and *Saagaromyces*, but these characters do not give any clue of their phylogenetic relationships (Fig. 1). However many of these taxa are common in intertidal habitats, e.g. *Cucullosporella mangrovei*, *Tirispora unicaudata*, *Saagaromyces glitra*, suggesting the retention of a primary character. Ascal apical structures are thought to be characteristic of terrestrial ascomycetes that serve as an aide for active discharge of ascospores (Ingold 1975). Within terrestrial ascomycetes they can be quite distinctive at the ordinal/family level, e.g. J+ and well developed rings in the *Xylariales* and J- with huge rings in the *Annulatascaceae* (Wong et al. 1998a).

Although the ascus shape in the *Halosphaeriaceae* is commonly clavate, it can vary from fusiform, ellipsoidal, saccate, clavate-broadly fusoid, elongate clavate, clavate to ellipsoidal and cylindrical. Pang (2002) suggested that the length to width ratio of the asci should be closely considered in the delineation of genera. Ascus stalk length should also be examined for a relationship between different taxa e.g. long stalks are present in *Halosarpheia kandeliae, Saagaromyces abonnis* and *S. ratnagiriensis*, while short stalks are a feature in *Halosarpheia sensu stricto.*

Ascospore appendages

Jones et al. (1986) and Jones (1995) suggested that ascospore shape and ascospore appendage ontogeny are of primary importance as taxonomic characters in the delineation of genera in the *Halosphaeriaceae.* Halosphaeriaceous ascospores are usually hyaline (with the exception of *Phaeonectriella, Carbosphaerella* and *Nereiospora*), with ascospore morphology varying from ellipsoidal (*Morakotiella, Remispora, Neptunella)*, cylindrical to ellipsoidal (*Haligena*), or long cylindrical or fusiform (*Ascosalsum, Magnisphaera*). Ascospore septation is also variable: unicellular (*Nautosphaeria*), 1-septate (*Morakotiella, Remispora, Neptunella*), or multi-septate (*Haligena, Ascosalsum, Magnisphaera*). Therefore, there is no clear pattern for ascospore morphology within the *Halosphaeriaceae.*

Although it has been shown that marine ascomycetes evolved from a terrestrial ancestor (Spatafora et al. 1998), no hypothesis has been proposed for the evolution of appendaged ascospores within the *Halosphaeriaceae*. The basic ascospore wall in marine fungi has three layers: epi- (outermost), meso- (central) and endo- (inner) sporium (Kirk 1976, 1986; Johnson et al. 1984). Spore appendages are in turn derived from these three wall layers by different processes: fragmentation of one or more layers (e.g. *Corollospora* species, *Halosphaeriopsis mediosetigera*, *Remispora* species); outgrowths of one or more layers and elaboration of the exosporium (e.g. *Ceriosporopsis*, *Halosphaeria*) and wall outgrowth (e.g. *Ondiniella torquata*).

Speculation as to which of these appendage types are primitive or derived characters have been voiced (Kirk 1986; Jones 2006). It is assumed that the ancestral ascospore lacked appendages or any spore wall ornamentation (Jones 2006). Kirk (1986) proposed a scheme representing genera that might share a close relationship, based on ascospore appendage morphology and histochemistry of the cell wall layers. He suggested they evolved along two separate lines: 1. a group where the exosporium is absent (e.g. *Ondiniella torquata*) and 2. those with an exosporium (e.g. *Remispora* spp.). However, he cautioned that “because selection is most rigorous where a prototype crosses an adaptive threshold, a few intermediate forms remain and close homologies are difficult to find”.

Our DNA sequence analyses of various taxa suggest that appendages have evolved and then been lost several times during evolutionary times, or has a primitive character been retained in some genera? (Fig. 1). Examples of non-appendaged fungi include: *Aniptodera sensu stricto* species, *Aniptodera longispora, Lignincola laevis, L. tropica, Pseudolignincola siamensis*, *Neptunella longirostris, Okeanomyces cucullatus, Nais inornata* and *Saagaromyces glitra*. Of the 77 species sequenced here (Fig. 1), only 8 lack appendages, and some also of these have persistent asci. *Aniptodera chesapeakensis* has been reported with and without polar appendages and molecular data is required to determine if this constitutes one taxon (Shearer 1989). *Saagaromyces glitra* and *Lignincola tropica* are the only species in a clade-genus lacking appendages, and may suggest the retention of a primitive character or the loss of a character. Ascospores of *Thalespora appendiculata* on release passively from the ascus lack appendages but when mounted in seawater produce apical appendages (Jones et al. 2006), but not in freshwater. A similar condition has been noted for the aquatic species *Savoryella appendiculata* and *Halosarpheia aquatica* (Jones and Hyde 1992; Hyde 1992).

Appendages that arise as an outgrowth of the exosporium (*Haligena, Morakotiella*); direct outgrowth from one or more spore wall layers (*Nereiospora, Arenariomyces*, *Nautosphaeria*); or exuded through pores (*Halosarpheia* *sensu stricto, Ascosacculus, Ophiodeira, Magnisphaera*) are present in number of genera throughout the family. The appendage type that seems to be stable for certain genera, and include appendages that arise as an outgrowth and fragmentation of the spore wall (*Corollospora*), or as an outgrowth of the spore wall and with the elaboration of an exosporium (*Marinospora, Ceriosporopsis*).

Marine fungi were thought to be unusual in that they possessed ascospores with elaborate appendages, however with better microscopes and mounting material in water, other groups have been shown to be equally endowed with appendaged spores (Jones 2006). Hyde et al. (2000) documented 100 palm fungal genera, the majority of which (67) have some kind of appendages or sheaths. Ascospores with mucilaginous sheaths predominated (52), 20 genera had appendages and 10 genera produced polar mucilaguinous pads or a drop of extruded mucilage from the spore wall. Also coelomycetes have conidia that show infinite variation in the type of appendages produced and have been well documented by Nag Raj (1993). Of some 7000 coelomycetes described, 420 have appendaged conidia and were characterised into 11 developmental patterns by Nag Raj (1993). Appendaged spores are produced by a wide range of taxa, freshwater and terrestrial ascomycetes, hyphomycetes, coprophilous fungi and Trichomycetes (Jones 2006). So development of appendages or sheaths is not confined to fungi in aquatic habitats.

Review of the ordinal status of the Halosphaeriaceae

The monophyly of the *Halosphaeriaceae* has been confirmed and our results are concordant with other studies (Spatafora et al. 1998; Chen et al. 1999; Kohlmeyer et al. 2000; Kong et al. 2000), with the family closely related to the terrestrial *Microascales*. Members of the *Halosphaeriaceae* and *Microascales* share certain features in common e.g. perithecial ascomata, ascomal position, absence of paraphyses, evanescent asci and passively discharged ascospores (Table 3). Unlike the *Halosphaeriaceae*, however, the *Microascaceae* is

Table 3 Comparison of the morphological characters used for delineation the *Halosphaeriaceae* and the *Microascales* (Barr 2001; Samuels and Blackwell 2001; Tang et al. 2007; Zhang et al. 2006; Kirk et al. 2008)

	Halosphaeriaceae	*Microascales*
Trophic condition	Saprobic on decaying woody and herbaceous substrata in marine and freshwater habitats	Saprobic from soil or rotting vegetation to plant pathogens, many species are associated with insect, with a few as human and animal pathogens
Ascomal position	Superficial or immersed, non-stromatic	Superficial, non-stromatic and form filiform beaks
Ascomal type	Perithecioid, membranous, carbonaceous, presence or absence of periphysate necks, centrum cavity initially filled with deliquescing thin-walled pseudoparenchymatous cells	Perithecioid or cleistothecioid, carbonaceous, sometimes with well-developed setae
Ascomal shape, size, color	Clavate or ellipsoid, 50–500 μm in diameter, hyaline, white to dark brown and black	Globose, pyriform or irregular, 50–350 μm in diameter, usually black
Paraphyses	Absent	Absent
Hamathecium formation	Typically catenophyses and periphyses	Interascal tissue absent or rarely undifferentiated hyphae
Asci formation	Unitunicate, formed in a basal hymenium, persistent or deliquescing early	Unitunicate, randomly arranged, deliquescing early
Apical apparatus	Presence or absence of apical structures e.g. apical ring, apical plate, apical thickening, retraction of plasmalemma	Absence
Ascus shape	Varied in shape e.g. clavate, fusiform, ellipoidal, saccate, clavate-broadly fusoid, clavate to ellipsoidal, cylindrical	Globose or ovoid, formed in chain
Ascospore morphology	Typically hyaline, but also brown-colored, unicellular, one-septum to many septate, ellipsoid or fusiform	Typically hyaline or reddish brown, unicellular, reniform or heart-shaped triangular, often bear ornamenting ridges or wings
Appendages/sheath formation	Present or absent with different types of development	Absence
Anamorph	Chlamydospore-like anamorphs and I ngoldian hyphomycetes	Percurrently proliferating conidiogenous cells (annellides), chlamydospore-like or aleurioconidial synanamorphs

mainly saprobic in terrestrial habitats (e.g. soil, plant debris, rotting vegetation, dung) while a few are plant (e.g. *Ceratocystis fimbriata*) or animal (*Pseudallescheria*) pathogens. There are few reports of the *Microascales sensu lato* from marine habitats (Jin and Jones in press). The halosphaerialean fungi are saprobic on decaying woody and herbaceous substrata in aquatic environments. The *Microascales sensu lato* is also associated with insects, whereas the *Halosphaeriaceae* may have lost their arthropod association ascospore dispersal on migration to the sea (Spatafora et al. 1998). Moreover, the ascomal type in the *Halosphaeriaceae* is typically perithecioid, while many genera in the *Microascales sensu lato* possess cleistothecia, sometimes with well developed setae. Ascus apical structures are present in many genera in the *Halosphaeriaceae*, while there is none in the *Microascales sensu lato*. Moreover, the asci and ascospore morphology are different: in the *Microascales sensu lato* asci are globose or ovoid, often produced in chains; with reniform ascospores, which often bear ornamenting ridges or wings. Halosphaeriaceous genera possess clavate to fusiform asci; hyaline ascospores, unicellular to many septate, usually with appendages (Table 3). Another feature that separates them is the paucity of anamorphs in the *Halosphaeriaceae* (*Cirrenalia macrocephala*, *Clavatospora bulbosa*, *Varicosporina anglusa*, *Trichocladium achrasporum*, *Monodictys pelagica*, *Humicola siamensis*) all possessing dematiaceous conidia (Shearer and Crane 1977; Mouzouras and Jones 1985). In the *Microascales sensu lato* anamorphs include *Scopulariopsis*, *Graphium* and *Scedosporium*, with hyaline annellidic or aleuro-conidia (Abbott et al. 1998).

Sequence data support three entities in the order *Microascales*: the *Halosphaeriaceae*, *Microascaceae* and a group of *Ceratocystis* species, all in a well supported clade (Fig. 1). However, the morphological differences noted above between the *Halosphaeriaceae* and *Microascaceae* raise the issue should they be referred to the same order?

In conclusion, this study of all the major genera in the *Halosphaeriaceae* has shown that while most are well resolved (e.g. *Corollospora, Oceanitis, Halosarpheia sensu stricto, Saagaromyces*), others require further investigation (e.g. *Aniptodera, Nereiospora, Alisea*). Taxa with unfurling polar appendages have evolved many times within the family and may indicate a special adaptation to an aquatic exsistence. While appendages have evolved in response to environmental conditions, they may also have been lost (e.g. *Saagaromyces glitra*). Sequence data showed that the genera *Ceriosporopsis*, *Remispora* were polyphyletic and the genera *Kochiella*, *Torriella* and *Tubakiella* are introduced to accommodate the displaced taxa.

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