Chapter IV

Results

4.1 *Torpedospora* and *Swampomyces*

Introduction

The genus *Torpedospora* was first described on wood panels from marine habitats by Meyers in 1957. This genus is characterized by dark-colored, immersed or superficial ascomata, persistent or deliquescing paraphyses, thin-walled, clavate to ellipsoidal asci which deliquesce early, hyaline, and cylindrical or clavate ascospores, with several radiating appendages at one or both ends (Kohlmeyer and Kohlmeyer, 1979). The type species, *Torpedospora radiata* Meyers, has cylindrical or clavate, triseptate ascospores, with 3-5 radiating appendages at one end (Figure 3c, d). The other species, *Torpedospora ambispinosa* Kohlm., has cylindrical to elongate-ellipsoidal ascospores, that are triseptate and with 4-7 radiating subterminal appendages at both ends (Figure 3e-g). *Torpedospora radiata* is a cosmopolitan species, whereas *T. ambispinosa* has been reported in Denmark, Friday Harbor (USA) and Chile (Kohlmeyer, 1960; Kohlmeyer and Kohlmeyer, 1979; Jones, 1985; Shearer and Burgos, 1987; Koch and Peterson, 1996).

The taxonomic position of *Torpedospora* is unclear, and was not included in the Halosphaeriaceae by Kohlmeyer (1972) and Kohlmeyer and Kohlmeyer (1979) but rather referred to the Sphaeriales *incertae sedis* for the following morphological reasons: *Torpedospora* does not possess a central pseudoparenchymatous tissue within the centrum, paraphyses grow irregularly inside the ascoma venter and between the asci, and asci originating from a hymenial layer at the base of the centrum (Figure 3a, b). No paraphyses have been observed in the Halosphaeriales (Pang, 2002), and asci originally formed at the base of the ascomata (Kohlmeyer and Kohlmeyer, 1979).

Although *Torpedospora* possesses unique appendaged ascospores, their ontogeny does not fall into any one of the ten types identified by Jones (1995). *Torpedospora radiata* has 3-5 radiating terminal appendages (Figure 3c, d), which are fibrillar in appearance (Jones and Moss, 1978). Ascospores of *T. ambispinosa* are bright orange-colored in mass, the appendages are subterminal (Figure 3e, f), rigid, straight or curved, but do not appear to be fibrillar as in *T. radiata* (Kohlmeyer and Kohlmeyer, 1979; Johnson, 1980, Jones and Moss, 1978; 1980). Due to the differences in the ascospore appendage morphology between *T. radiata* and *T. ambispinosa,* Jones (1995) queried if both species should be assigned in the same genus.

Swampomyces was first described by Kohlmeyer and Volkmann-Kohlmeyer (1987) and assigned, with reservation, in the Polystigmataceae (currently Phyllachoraceae). Members of the Phyllachoraceae are characterized by stromatic tissue forming a clypeus, thin-walled peridium and asci with a narrow apical ring occasionally staining with iodine (Barr, 1990). *Swampomyces armeniacus* Kohlm. and Volkm.-Kohlm. (the type species) has immersed ascomata with a clypeus, cylindrical asci with an obscure apical structure and septate paraphyses (Figure 4a, b) (Kohlmeyer

and Volkmann-Kohlmeyer, 1987). Hyde and Nakagiri (1992) confirmed the presence of an inconspicuous apical ring in this species and described *S. triseptatus* K. D. Hyde and Nakagiri. Morphologically *S. triseptatus* is similar to *S. armeniacus* with the possible difference in the apical ascus structure (Hyde and Nakagiri, 1992). Two more species (*S. aegyptiacus* Abdel-Wahab, El-Sharouney and E. B. G. Jones, *S. clavatispora* Abdel-Wahab, El-Sharouney and E. B. G. Jones) were recently described from a mangrove in Egypt, bringing the total to four species. (Figure 4a-h) (Abdel-Wahab *et al*. 2001a).

Morphological characteristics of *Torpedospora* and *Swampomyces* species are compared in Table 6. Morphological features suggest the placement of *Swampomyces* in the Phyllachoraceae, but Kohlmeyer and Volkmann-Kohlmeyer (1987) expressed concern about the difference, particularly in its mode of life as most members of the family are biotrophic parasites on leaves (Table 7) (Müller and von Arx, 1962). Kirk *et al*. (2001) and Read *et al*. (1995) (after an ultrastructural investigation), suggested referral of *Swampomyces* to Ascomycota *incertae sedis* until further information is available.

Objectives for this chapter are:

1). to test whether *Torpedospora* and *Swampomyces* are monophyletic, and,

2). to determine the higher taxonomic position for *Torpedospora* and *Swampomyces* within the Sordariomycetes.

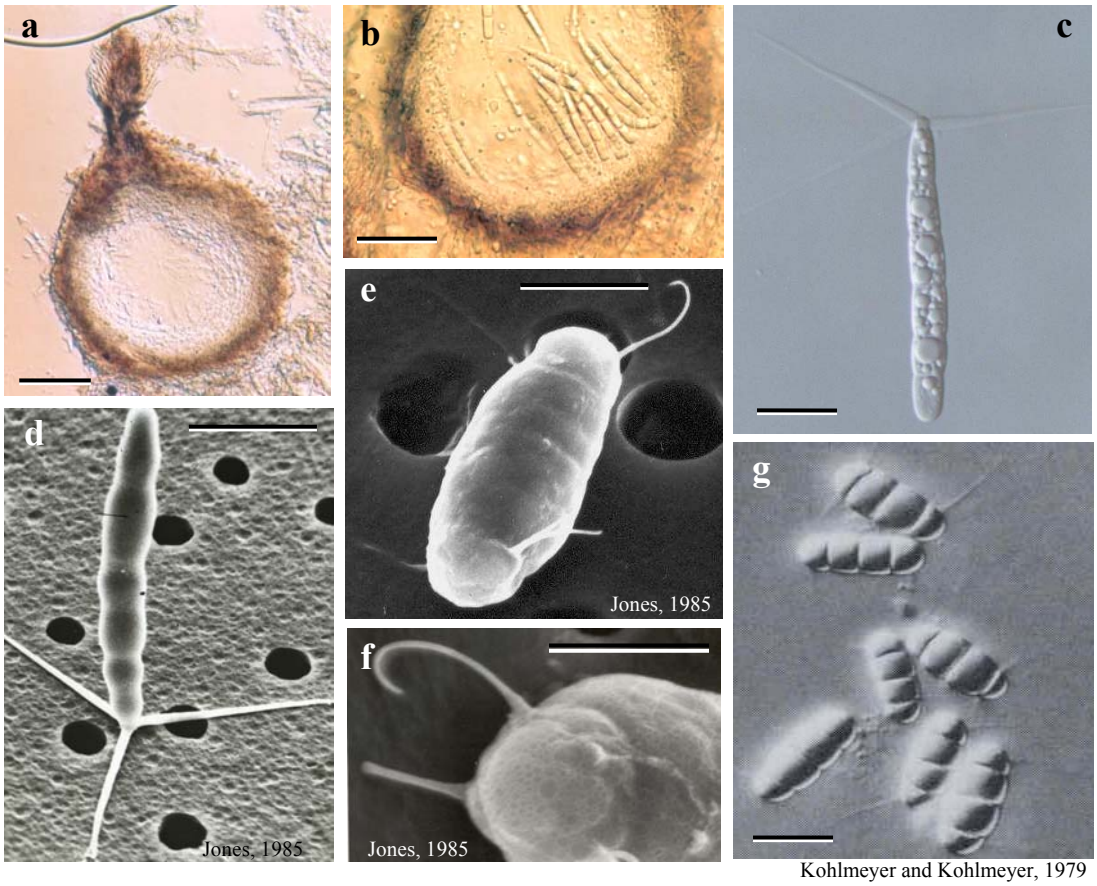
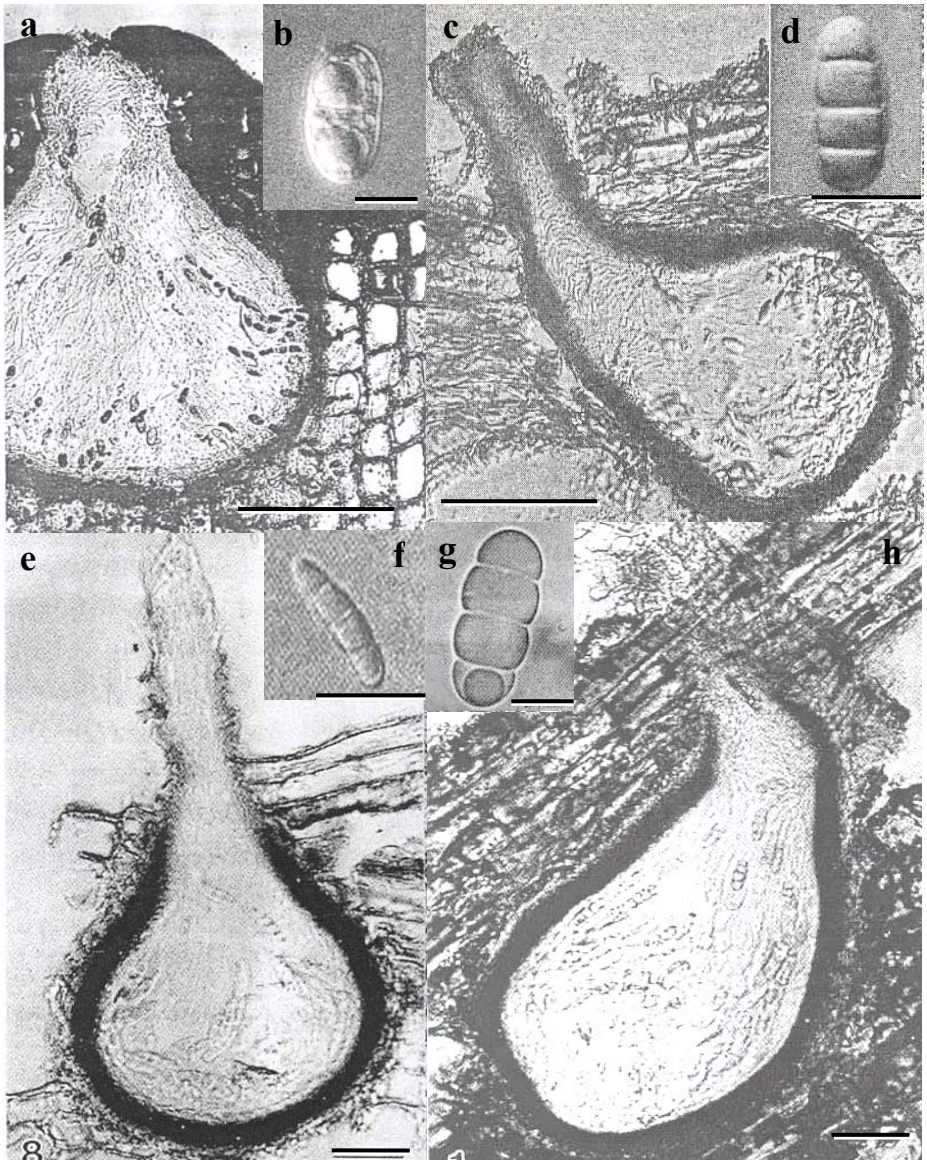


Figure 3. Morphological features of *Torpedospora* species

a-d: *Torpedospora radiata* a, b: Longitudinal section through ascomata;
c: Ascospore with radiating polar appendages; d: SEM of ascospore with appendages at terminal end
e-g *Torpedospora ambispinosa* e, f: SEM of ascospores with bipolar appendages at subterminal ends; g: Appendaged ascospores

Scale bars: a, b = 60 µm; c, d, g = 10 µm; e, f = 5 µm



Kohlmeyer and Volkmann-Kohlmeyer, 1987; Hyde and Nakagiri, 1992; Abdel-Wahab *et al*., 2001a

Figure 4. Morphological features of *Swampomyces* species

a, b *Swampomyces armeniacus* a: Longitudinal section through ascoma with a black clypeus; b: Ascospore; c, d *S. triseptatus* c: Longitudinal section through ascoma with long axis horizontal to the host surface and neck bending upwards; d: Ascospore; e, f *S. clavatispora* e: Longitudinal section through ascoma in wood; f: Ascospore; g, h *S. aegyptiacus* g: Ascospore; h: Longitudinal section through ascoma immersed in wood

Scale bars: a = 150 µm; b, d = 10 µm; c = 100 µm; e, h = 50 µm; f = 20 µm; g = 5 µm

Table 6. Comparisons of morphological characteristics of *Torpedospora* and *Swampomyces* species (After Meyers, 1957; Kohlmeyer, 1960; Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer and Volkmann-Kohlmeyer, 1987; Hyde and Nakagiri, 1992; Abdel-Wahab *et al*., 2001a)

Morphological characters	***T. radiata***	***T. ambispinosa***	***S. armeniacus***	***S. clavatispora***	***S. aegyptiacus***	***S. triseptatus***
Clypeus/ stroma	Absent	Absent	Present a clypeus	Present a superficial pseudostroma	Present a superficial pseudostroma	Present a superficial pseudostroma
Ascomata	Superficial or immersed, subglobose to pyriform, ostiolate	Superficial or immersed, subglobose, globose to ellipsoidal, ostiolate	Immersed, subglobose, ostiolate, periphysate	Immersed, globose, ostiolate, periphysate	Immersed, pyriform, ostiolate, periphysate	Deeply immersed with a neck leading to the surface, pyriform, ostiolate, periphysate
Paraphyses	Septate	Septate	Simple, rarely branched, anastomosing	Numerous, mostly unbranched	Numerous, mostly unbranched	Branched, anastomosing
Ascus shape	Clavate or oblong-ellipsoidal, sessile or short pedunculate, deliquescing early	Broadly clavate or subglobose, deliquescing early	Cylindrical, short pedunculate	Oblong, thin-walled, short pedunculate	Cylindrical, short pedunculate, longer, narrower than *S. triseptatus*	Cylindrical, short pedunculate

Table 6. (Continued)

Morphological characters	***T. radiata***	***T. ambispinosa***	***S. armeniacus***	***S. clavatispora***	***S. aegyptiacus***	***S. triseptatus***
Apical apparatus	Absent	Absent	Apical ring-like structure	Thickened apically Apical thickening	Thickened apically Apical thickening	Thickened apically Apical thickening
Ascospore shape	Cylindrical to clavate, rarely fusiform, broader at the apex, not or slightly constricted	Cylindrical to elongate-ellipsoidal, slightly constricted	Ellipsoidal, verruculose, not constricted	Clavate, weakly constricted	Ellipsoidal, deeply constricted	Ellipsoidal, spore wall with granular ornamentation, weakly constricted
Ascospore septation	3	3	1	3	3	3
Appendages/ sheath	3-4 radiating appendages on the lower end semirigid, straight or slightly curved, with a thick base, tapering toward the apex, fibrillar, attenuate	Subterminal crown of 4-7 radiating appendages at each end, rigid, attenuate, straight or slightly curved	Absent	Absent	Absent	Absent

Table 7. Comparisons of morphological features between *Torpedospora, Swampomyces* and other related orders in the Hypocreomycetidae, Sordariomycetes (After Meyers, 1957; Kohlmeyer, 1960; Kohlmeyer and Kohlmeyer; 1979; Kohlmeyer and Volkmann-Kohlmeyer, 1987; Alexopoulos *et al*., 1996; Rossman, 1996; Abdel-Wahab *et al*., 2001a; Barr, 2001; Samuels and Blackwell, 2001)

Morphological characters	***Torpedospora***	**Halosphaeriales**	**Hypocreales**	***Swampomyces***	**Phyllachorales**
Clypeus/ stroma	Absent	Absent	Present stroma in some genera	Present a clypeus in *S. armeniacus*, a pseudostroma in *S. clavatisporus, S. aegyptiacus S. triseptatus*	Present a clypeus
Ascomata	Superficial or immersed, subglobose to ellipsoidal	Superficial or immersed, globose to subglobose	Superficial or immersed, globose to subglobose	Immersed, subglobose	Superficial or immersed, subglobose
A pseudoparenchy-matous centrum	Absent	Present	Absent	Absent	Absent
Paraphyses	Present	Absent	Present	Present	Present

Table 7. (Continued)

Morphological characters	***Torpedospora***	**Halosphaeriales**	**Hypocreales**	***Swampomyces***	**Phyllachorales**
Paraphyses formation	Growing irregularly throughout the ascoma venter, ramose, deliquescing early		Growing from a meristem at the top of the locule and grow downward, persistent	Merging with periphyses, embedded in a gel and connected to the upper part of the ascomatal cavity, filamentous, persistent	Developing from among asci, scattered, narrowed, deliquesing at maturity
Asci origin	Developing along the wall in the lower half of the ascoma	Developing at the base of the centrum, formed in a hymenium	Formed in a hymenium at the base of the centrum	Developing at the base and along the side of locule, parallel to each other	Arranged basally or peripherally in a hymenium
Ascus shape	Clavate to ellipsoidal	Clavate, cylindrical, fusiform	Variable: cylindrical, elongate	Cylindrical, elongate	Oblong to cylindrical
Apical apparatus	Absent	Absent in most genera	Present and absent in some genera	Present, the tip of ascus is truncated, with an apical plate	Present a pore encircled by narrow ring

Table 7. (Continued)

Morphological characters	***Torpedospora***	**Halosphaeriales**	**Hypocreales**	***Swampomyces***	**Phyllachorales**
Ascospore shape	Cylindrical to elongate-ellipsoidal	Cylindrical to filiform	Basically ellipsoidal by varies from globose, allantoid to filiform	Ellipsoidal, fusiform, cylindrical to clavate	Variable; obovoid, ellipsoid, filiform
Appendages/ sheath	Present	Mostly present	Mostly absent	Absent	Appendages or ornamentation may be present in some genera
Anamorph	Unknown	Found in some genera	Mostly present	Unknown	Found in some genera
Mode of life	Saprobic on marine driftwood	Saprobic on marine driftwood	Saprobic, biotrophic on other plant or fungi	Saprobic on mangrove wood	Biotrophic on leaves and stems of herbaceous plants

Fungal strains studied are listed below. The genomic DNA for all fungi listed was extracted by using the CTAB lysis buffer as outlined in Chapter III. *Swampomyces* SSU and LSU sequences were generously provided for this study by Dr. Lai Ka Pang.

Fungal studied	**Original code**	**BCC code**	**Origin**	**GenBank accession No.**	
				SSU	**LSU**
Swampomyces aegyptiacus	CY2973	-	Egypt	AY858943	AY858950
Swampomyces armeniacus	CY2799	-	Egypt	AY858944	AY858951
Swampomyces clavatispora	LP83	-	Egypt	AY858945	AY858952
Swampomyces triseptatus	CY2802	-	Egypt	AY858942	AY858953
Torpedospora ambispinosa	CY3385	16003	Friday Harbor, USA	AY858940	AY858949
Torpedospora ambispinosa	CY3386	-	Friday Harbor, USA	AY858941	AY858946
Torpedospora radiata	JS77	11269	Narathiwat, Thailand	AY858938	AY858948
Torpedospora radiata	PP7763	-	Unknown	AY858939	AY858947

DNA amplification and sequencing

Every region of the rRNA gene (partial SSU, LSU and ITS1-5.8S-ITS2) and beta-tubulin gene were sequenced for *Torpedospora radiata* and *T. ambispinosa*. The pairs of primers used for PCR were: NS1/NS8, NS1/NS6, LROR/LR7, JS1/JS8, ITS5/LR7, ITS5/ITS4 and Bt2a/Bt2b. The amplification profiles were performed as outlined in Chapter III. PCR products were sequenced directly using forward and reverse primers (NS1, NS3, NS5, NS6, NS8, JS1, JS5, JS8, LROR, LR7, NL4R, ITS4, ITS5, Bt2a and Bt2b) (White *et al*., 1990; Bunyard *et al*., 1994; Glass and Donaldson, 1995; Landvik, 1996).

Phylogenetic analysis

The sequences of *Torpedospora* alone and *Torpedospora* combined with *Swampomyces* were analyzed using Clustal W 1.6 (Thompson *et al*., 1994) along with other sequences obtained from the GenBank (Appendix C). The alignments were refined manually in Se-Al v1.0a1 (Rambaut, 1999), BioEdit 5.0.6. and 6.0.7 (Hall, 2001; 2004). The tree construction procedure was performed in PAUP* 4.0b10 both in Macintosh and Window versions (Swofford, 2002).

***Torpedospora* sequence analyses**

Small subunit, LSU, combined SSU+LSU and combined beta-tubulin+ITS1-5.8S-ITS2 datasets were analyzed using equally weighted maximum parsimony method (heuristic searches with a stepwise starting tree, a random stepwise addition of 10 replicates and tree-bisection-reconnection (TBR) branch-swapping algorithm). Gaps were treated as missing data, the fifth state and gaps excluded. Finally, 1,000 replicates of bootstrapping analysis were performed on each dataset (full heuristic searches, stepwise addition of sequence, 10 replicates of random addition of sequence and TBR branch-swapping algorithm).

***Torpedospora* combined with *Swampomyces* sequences**

Small subunit and LSU were analyzed individually using equally weighted maximum parsimony method (heuristic searches with a stepwise starting tree, a

random stepwise addition of 10 replicates and TBR branch-swapping algorithm). Gaps were treated as missing data, the fifth state and gaps excluded. Combined SSU and LSU dataset was analyzed using equally weighted parsimony and weighted parsimony. Weighted parsimony analysis was performed using a step matrix to weight nucleotide transformations based on the transition:transversion (ti:tv) ratio estimated from the dataset using maximum likelihood score in PAUP* (Swofford, 2002). Finally, 1,000 replicates of bootstrapping analysis (Felsenstein, 1985) were performed on each dataset (full heuristic searches, stepwise addition of sequence, 10 replicates of random addition of sequence and TBR branch-swapping algorithm).

Results

Total taxa, tree length, numbers of the most parsimonious trees (MPTs), Consistency Index (CI) and Retension Index (RI) for all analyses are summarized in Table 8.

Table 8. Summary of results from *Torpedospora* and *Swampomyces* analyses

Torpedospora alone	**Figure**	**Total taxa included**	**Tree length (steps)**	**No. of MPTs**	**CI**	**RI**
SSU	5	48	1,214	6	0.696	0.773
SSU (with additional taxa)	6	43	930	12	0.690	0.798
LSU	7	54	1,409	4	0.427	0.642
SSU+LSU	8	30	1,647	10	0.541	0.577
beta-tubulin+ITS1-5.8S-ITS2	9	21	995	2	0.729	0.709
Torpedospora+Swampomyces						
SSU	10	48	1,542	48	0.640	0.752
LSU	11	81	1,856	147	0.413	0.717
SSU+LSU (equally weighted)	12	36	2,102	2	0.561	0.593
SSU+LSU (weighted parsimony ti:tv ratio= 1.6)	13	36	2,638.2	1	0.564	0.603

SSU result

To determine the placement of *Torpedospora*, the sequences of *T. radiata* and *T. ambispinosa* were initially searched through BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), with the nearest match in the Hypocreales. The SSU sequences of 44 taxa of the major orders of unitunicate ascomycetes (Diaporthales, Halosphaeriales, Hypocreales, Microascales, Ophiostomatales, Pleosporales, Sordariales and Xylariales) were included in the preliminary analysis. Six MPTs were obtained, gaps treated as missing characters, gaps treated as the fifth state, and gaps exclusion, had no effect on the overall topology and the position of *Torpedospora*. Therefore, gaps were treated as missing data for all analyses. The MPT number one is shown as a phylogram, representing the best topology (resulting from the K-H test, Kishino and Hasegawa, 1989) (Figure 5). The tree length, CI and RI are 1,214 steps, 0.696 and 0.773, respectively. The overall structure of this tree shows a reasonable support. The difference among these six MPTs is the minor swapping position of members in the Hypocreales. The two *Torpedospora* species group together with 98% bootstrap values, and form a sister clade to the Hypocreales, with weak support (Figure 5).

To get better resolution of the potential position of *Torpedospora* within the Hypocreales, additional taxa have been included (Rossman, pers. comm.). *Melanospora* Corda, species with a dark thin-walled ascomata, clavate, evanescent asci and absence of paraphyses; Hypocreales *incertae sedis* (*Scopinella* Lév., *Hapsidospora* Malloch and Cain, *Nigrosabulum* Malloch and Cain genera that do not fit into any families) and the order Phyllachorales, which falls between the

Microascales/Halosphaeriales, were incorporated into the SSU dataset, resulting 12 MPTs. The best topology estimated from the K-H test is shown in Figure 6 of the tree length, CI and RI of 930 steps, 0.690 and 0.798, respectively. The tree contains fewer number of orders, with the Xylariales as the outgroup. It clearly shows that *Torpedospora* has no affinity to these additional taxa. It forms a neighboring clade to *Hapsidospora irregularis* Malloch and Cain and *Nigrosabulum globosum* Malloch and Cain, with low support (Figure 6), whereas the Phyllachorales that represented by *Colletotrichum* Corda and *Glomerella* Spauld and H. Schrenk species, forms a basal clade to the Microascales and Halosphaeriales (Figure 6).

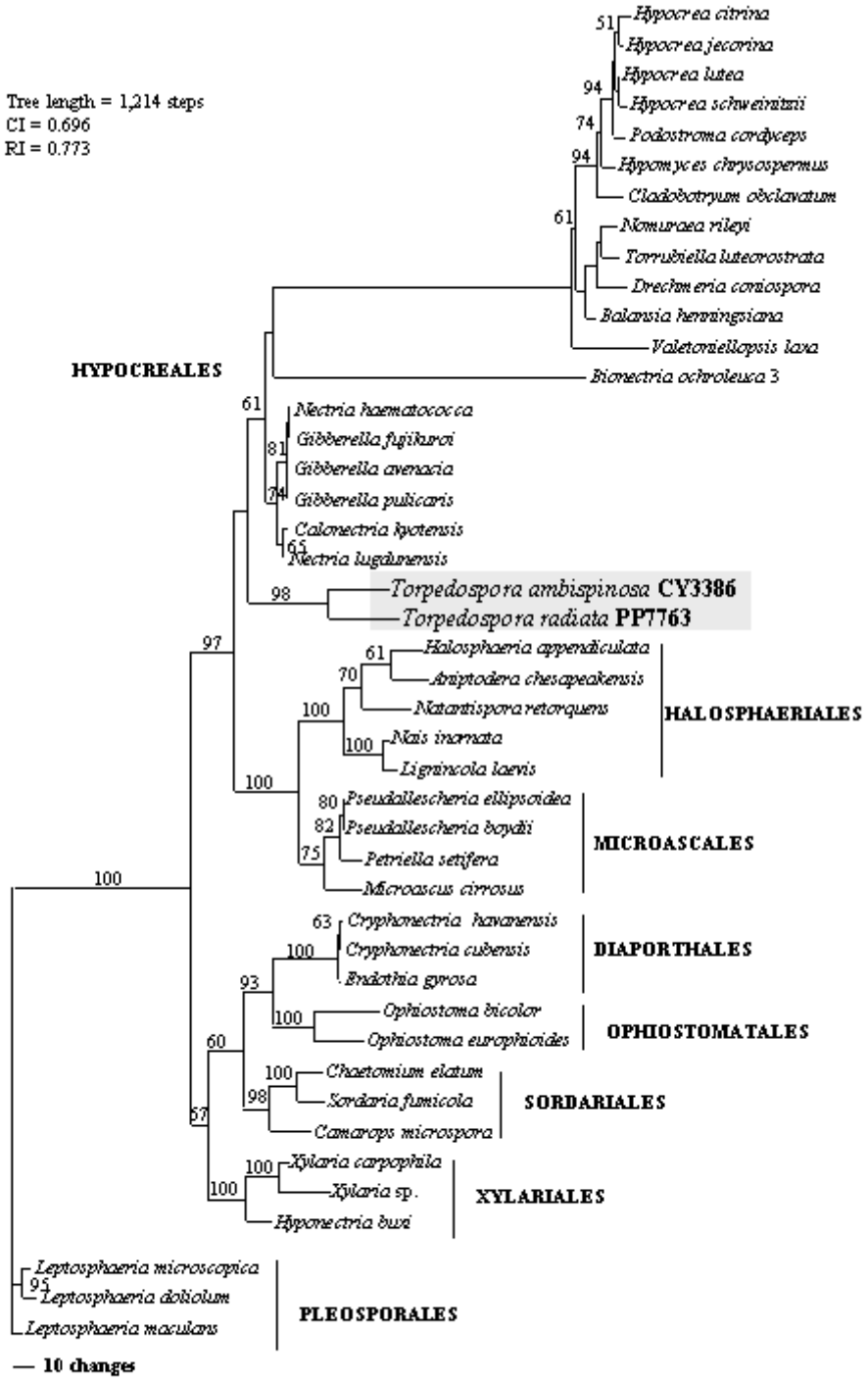


Figure 5. One of six MPTs inferred from SSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* alone). Bootstrap values greater than 50% are given above branches.

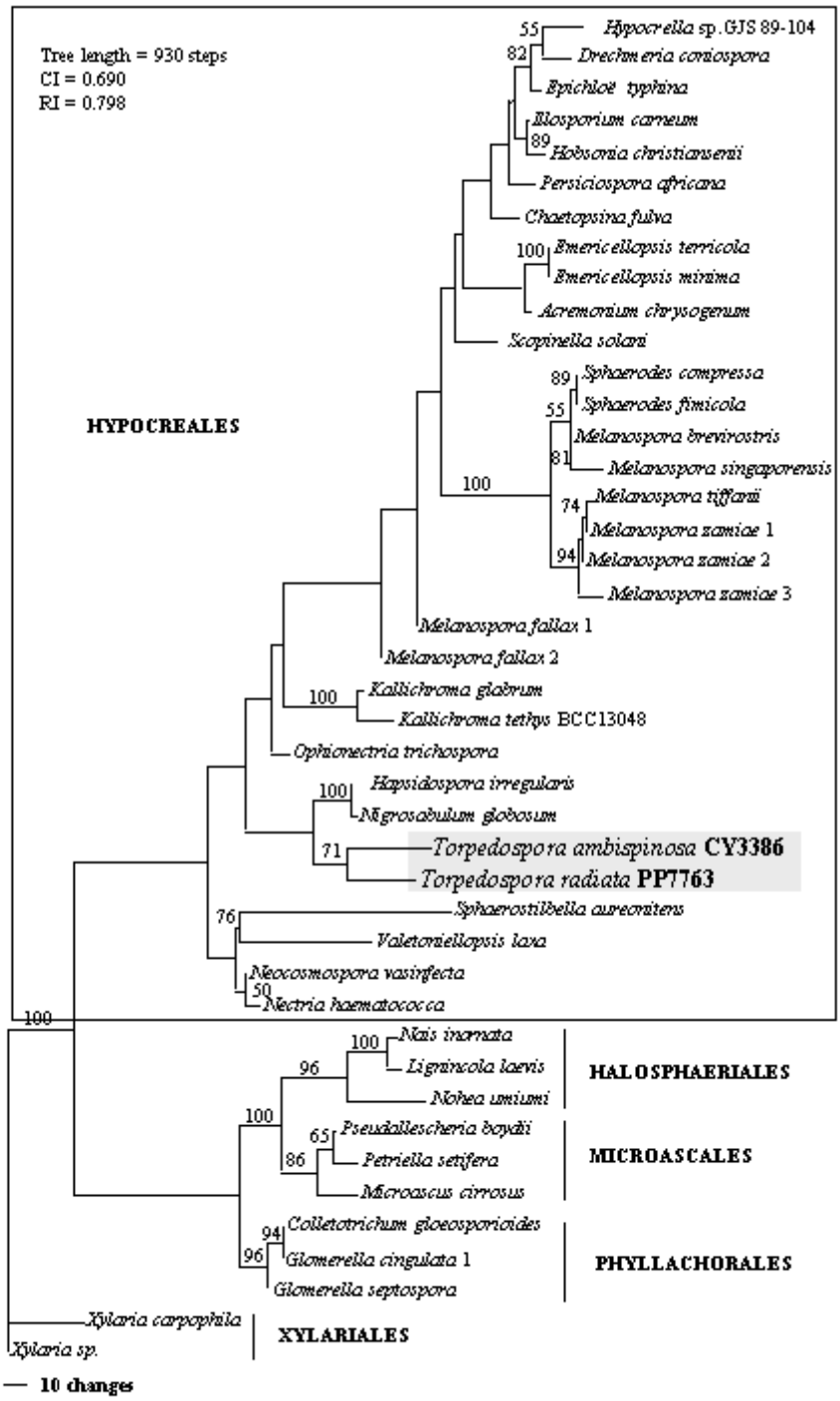


Figure 6. One of 12 MPTs inferred from SSUrRNA sequences with additional taxa, generated with maximum parsimony analysis (*Torpedospora* alone). Bootstrap values greater than 50% are given above branches.

LSU result

A LSU dataset comprises further taxa from the Hypocreomycetidae, in order to determine their affinity with *Torpedospora*. More taxa of the Halosphaeriales, Hypocreales, Microascales and Phyllachorales were included. Four MPTs were obtained. The best tree, after comparison by the K-H test, is shown as a phylogram with tree length of 1,409 steps, CI of 0.427 and RI of 0.642 (Figure 7). The difference among these four MPTs is in the minor interrelationships of members within the Hypocreales. For each MPT, the position of *Torpedospora* is stable as the basal clade to the Hypocreales with weak support, and two *Torpedospora* species are closely related with 100% bootstrap support (Figure 7).

SSU+LSU result

The combined SSU and LSU analysis consisted of 30 taxa with the Pezizales as an outgroup, gaps treated as missing data, and resulting in ten MPTs. Figure 8 is shown as a phylogram, and representing the best topology for the dataset estimated from the K-H test. Each of the ten MPTs differs in the position of members within the Hypocreales. *Torpedospora radiata* groups consistently with *T. ambispinosa* (79% support). Their grouping is nested as a sister clade to the Hypocreales (Figure 8).

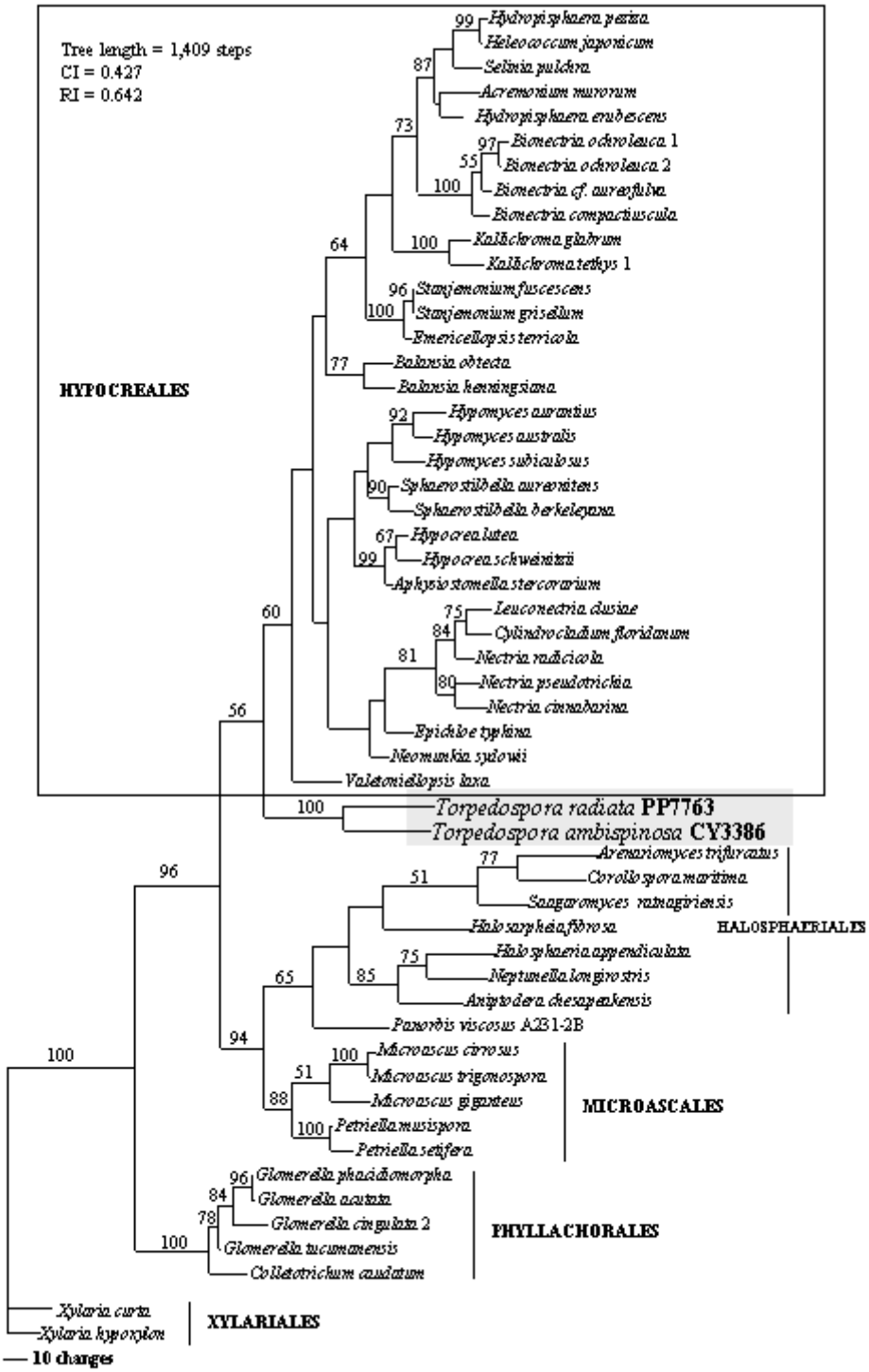


Figure 7. One of four MPTs inferred from LSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* alone). Bootstrap values greater than 50% are given above branches.

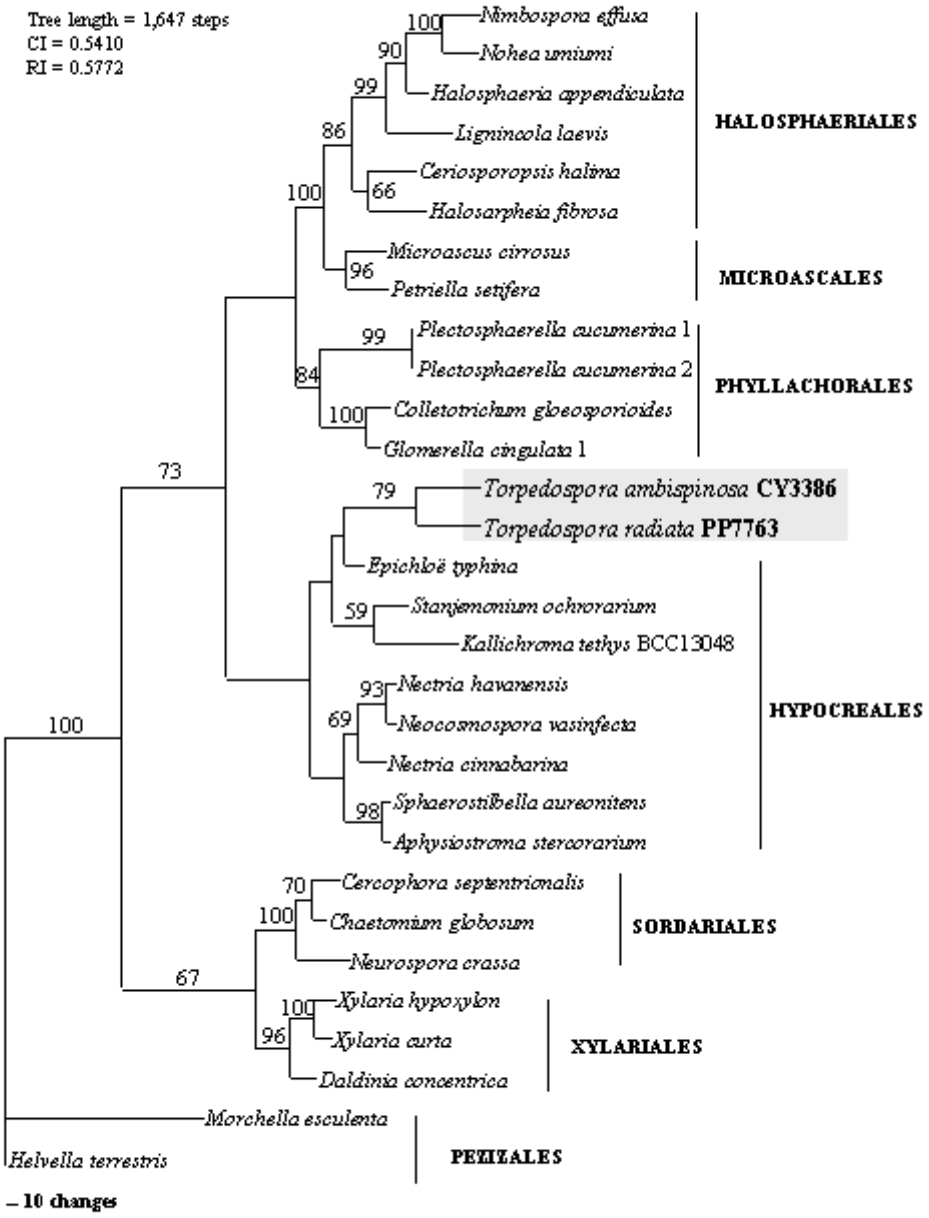


Figure 8. One of ten MPTs inferred from combined SSU+LSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* alone). Bootstrap values greater than 50% are given above branches.

Beta-tubulin+ITS1-5.8S-ITS2 result

To determine the interrelationships between *Stachybotrys* Corda and *Torpedospora* species, another gene was sequenced (Rossman and Spatafora, pers. comm.). An ITS1-5.8S-ITS2 region was combined with the beta-tubulin gene. One of the two MPTs gave a tree length, CI and RI of 995 steps, 0.729 and 0.709, respectively (Figure 9). The two *Torpedospora* species are closely related with robust support (100%) as the basal clade of the tree, adjoining the Phyllachorales and Sordariomycetes *incertae sedis* (*Gaeumannomyces graminis* var. *tritici* (Sacc.) Arx and Olivier and *Phialophora* Medlar, Magnaporthaceae). *Stachybotrys* species form a distant relationship with *Torpedospora*, along with *Myrothecium* Tode and the Hypocreaceae as its relatives (Figure 9).

***Torpedospora* combined with *Swampomyces* sequences**

SSU result

Major orders of the unitunicate ascomycetes (Diaporthales, Erysiphales Halosphaeriales, Hypocreales, Meliolales, Microascales, Ophiostomatales Phyllachorales, Pleosporales, Sordariales and Xylariales) were included in the analysis (Figure 10). Maximum parsimony resulted 48 MPTs, tree length, CI and RI of 1,542 steps, 0.6407 and 0.7527, respectively. The 50% majority consensus tree showed that *Torpedospora* and *Swampomyces* form a monophyletic clade close to the Phyllachorales, Microascales, Halosphaeriales and Hypocreales in the

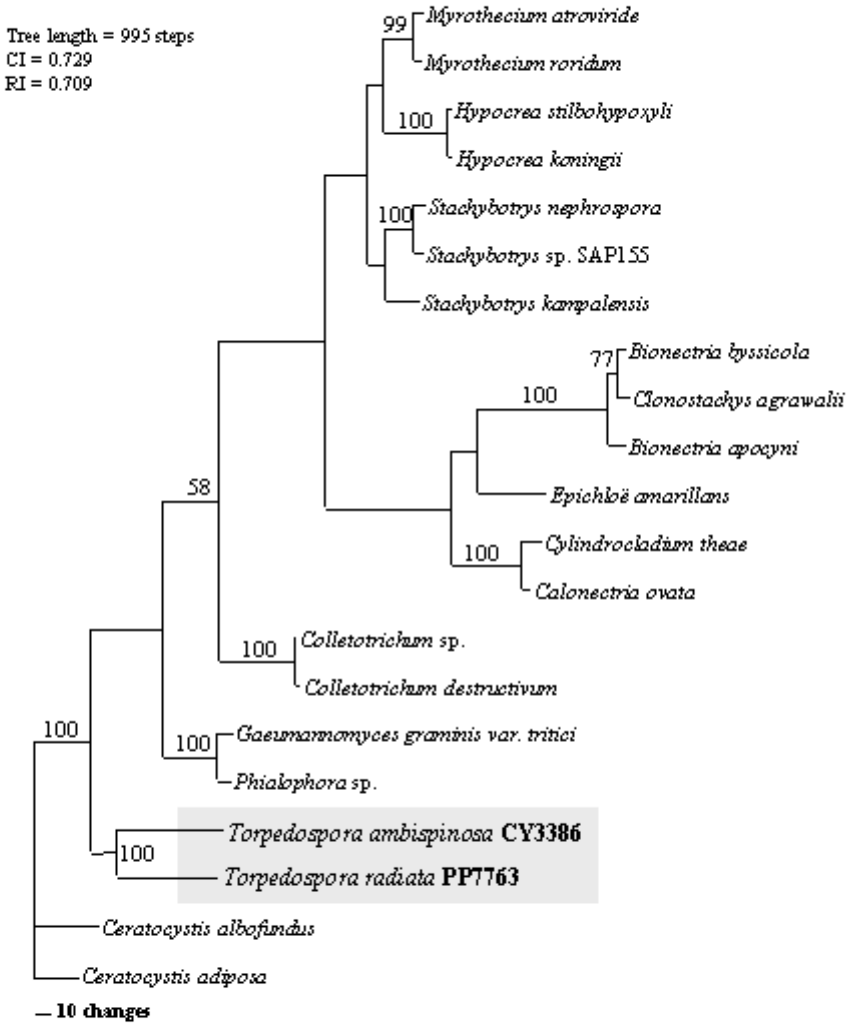


Figure 9. One of two MPTs inferred from combined beta-tubulin+ITS1-5.8S-ITS2 sequences, generated with maximum parsimony analysis (*Torpedospora* alone). Bootstrap values greater than 50% are given above branches.

Hypocreomycetidae, Sordariomycetes (61%) (Figure 10). The support between the *Torpedospora* species is 78%. *Swampomyces aegyptiacus, S. clavatispora* and *S. armeniacus* group strongly with high bootstrap values, whereas *S. triseptatus* forms a sister group with lower support (68%).

LSU result

Further taxa from the Hypocreomycetidae were added to the analysis of the LSU dataset, with two *Xylaria* species as the outgroup. Maximum parsimony resulted 147 MPTs, tree length, CI and RI of 1,856 steps, 0.413 and 0.717, respectively. The 50% majority consensus tree is shown in Figure 11 with a better resolved phylogeny. *Swampomyces* and *Torpedospora* cluster together with 87%, whereas the support with the Hypocreales is below 50% (Figure 11). However, the branches leading to the major orders Phyllachorales, Microascales/Halosphaeriales and Hypocreales are reasonably stable. The grouping of *Torpedospora radiata* and *T. ambispinosa* is 81%, while *S. triseptatus* forms as a basal sister taxon (67%), while the three *Swampomyces* species are well grouped with strong support (Figure 11).

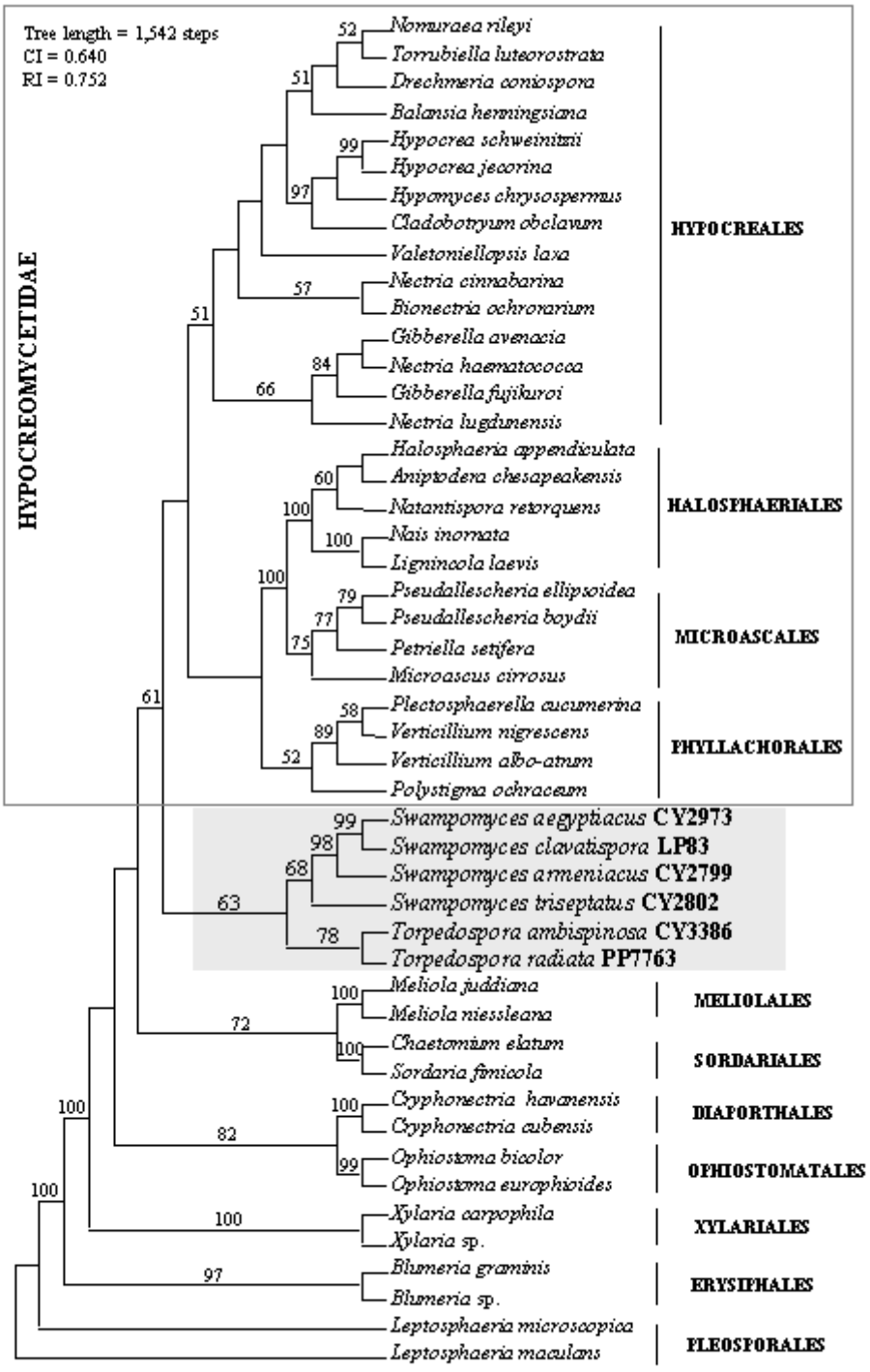


Figure 10. The 50% majority consensus tree inferred from SSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* + *Swampomyces*). Bootstrap values greater than 50% are given above branches.

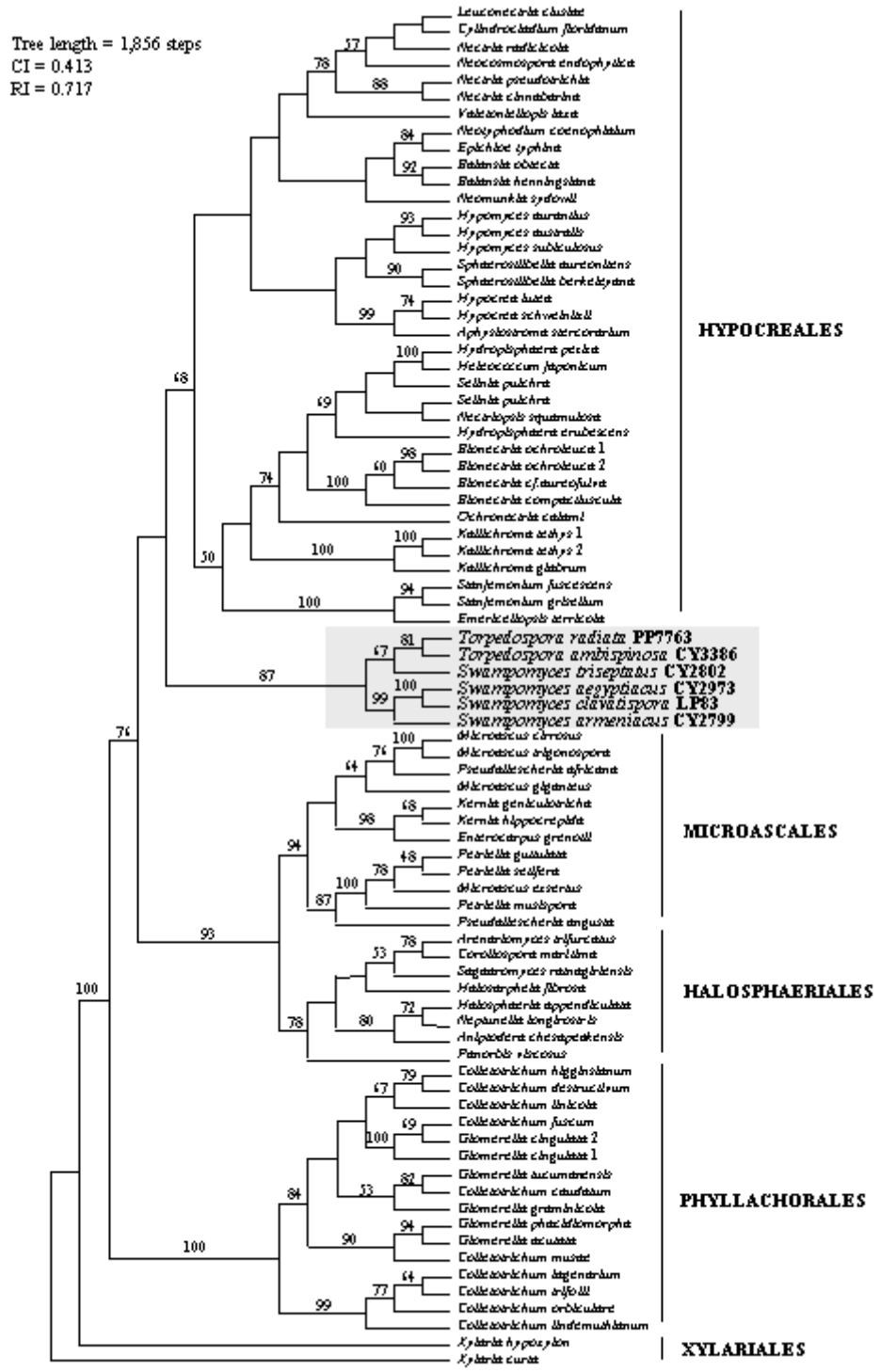


Figure 11. The 50% majority consensus tree inferred from LSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* + *Swampomyces*). Bootstrap values greater than 50% are given above branches.

SSU+LSU result

Combined analysis of the SSU and LSU sequences (equally weighted parsimony) suggests a similar result to the SSU phylogeny, the major difference being in the inter-ordinal relationship within the Hypocreales (Figure 12). The best tree from two MPTs compared by the K-H test is shown in Figure 12, resulted in tree length, CI and RI of 2,102 steps, 0.561 and 0.593, respectively. Two strains of *T. radiata* and *T. ambispinosa* were included to confirm their monophyly. *Torpedospora* group consistently with *Swampomyces*, although with low support (50%). The *Torpedospora* species group consistently with 72% bootstrap support, while *Swampomyces armeniacus*, the type species, groups strongly within a subclade that includes *S. clavatispora* and *S. aegyptiacus*. However, *S. triseptatus* appears in the subclade of *Torpedospora*, but with weak support (below 50%) (Figure 12). The *Torpedospora/Swampomyces* clade is adjacent to the Hypocreales and Halosphaeriales/Microascales, within the Hypocreomycetidae, Sordariomycetes.

Moreover, weighted parsimony analysis was performed using a step matrix to weight transversion 1.6 times higher than transition (ti:tv ratio estimated from the dataset using maximum likelihood score in PAUP*). The resulting tree is shown in Figure 13 with tree length of 2,638.2 steps, CI of 0.564 and RI of 0.603. This tree gives a higher support value between *Torpedospora* and *Swampomyces* (57%) *Swampomyces triseptatus* groups with the three *Swampomyces* species in the subclade, but with weak support. However, the position of *Torpedospora/Swampomyces* clade remains between the Hypocreales and Halosphaeriales/Microascales clade, within Hypocreomycetidae.

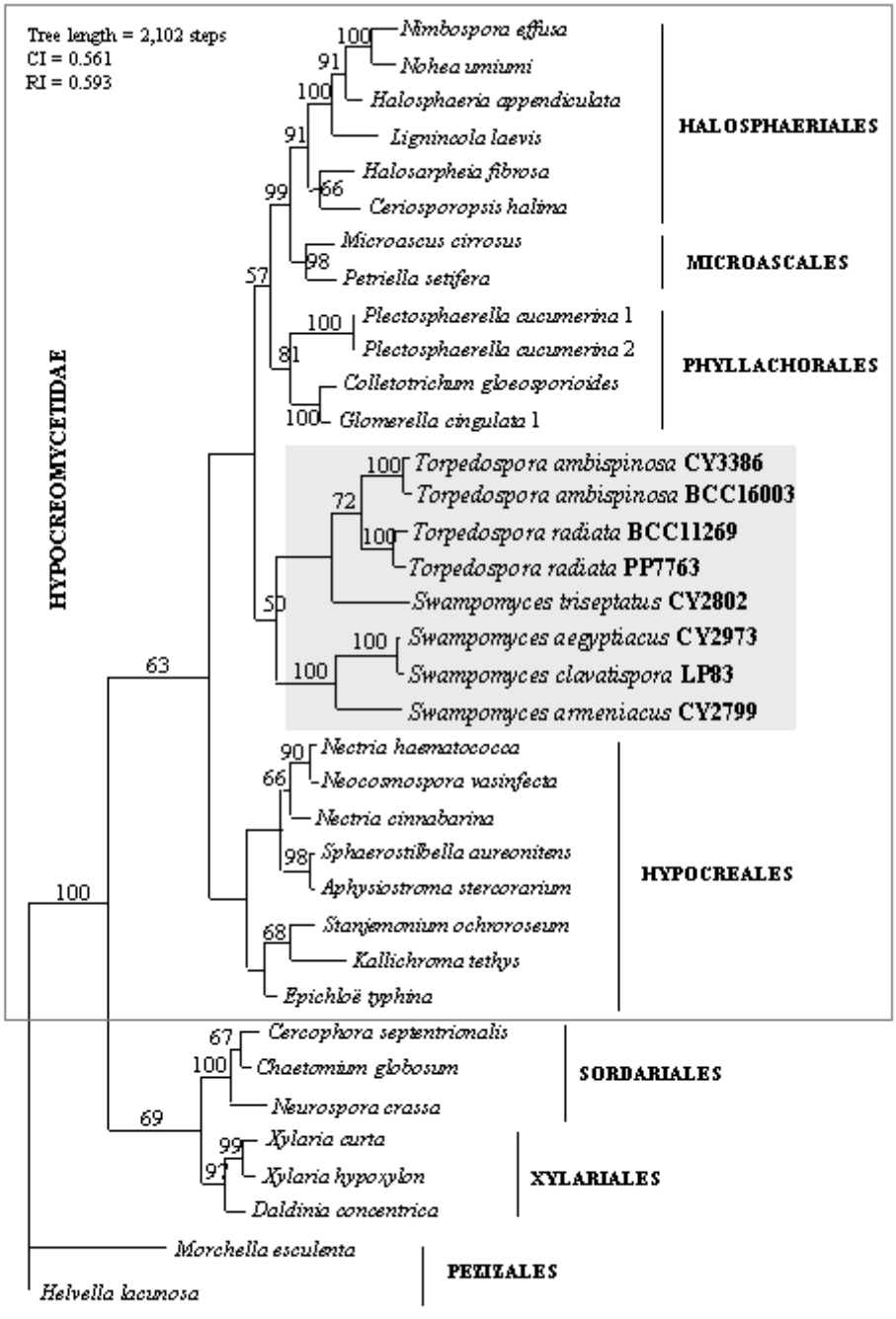


Figure 12. One of two MPTs inferred from combined SSU+LSU rRNA sequences, generated with maximum parsimony analysis (*Torpedospora* + *Swampomyces*). Bootstrap values greater than 50% are given above branches.

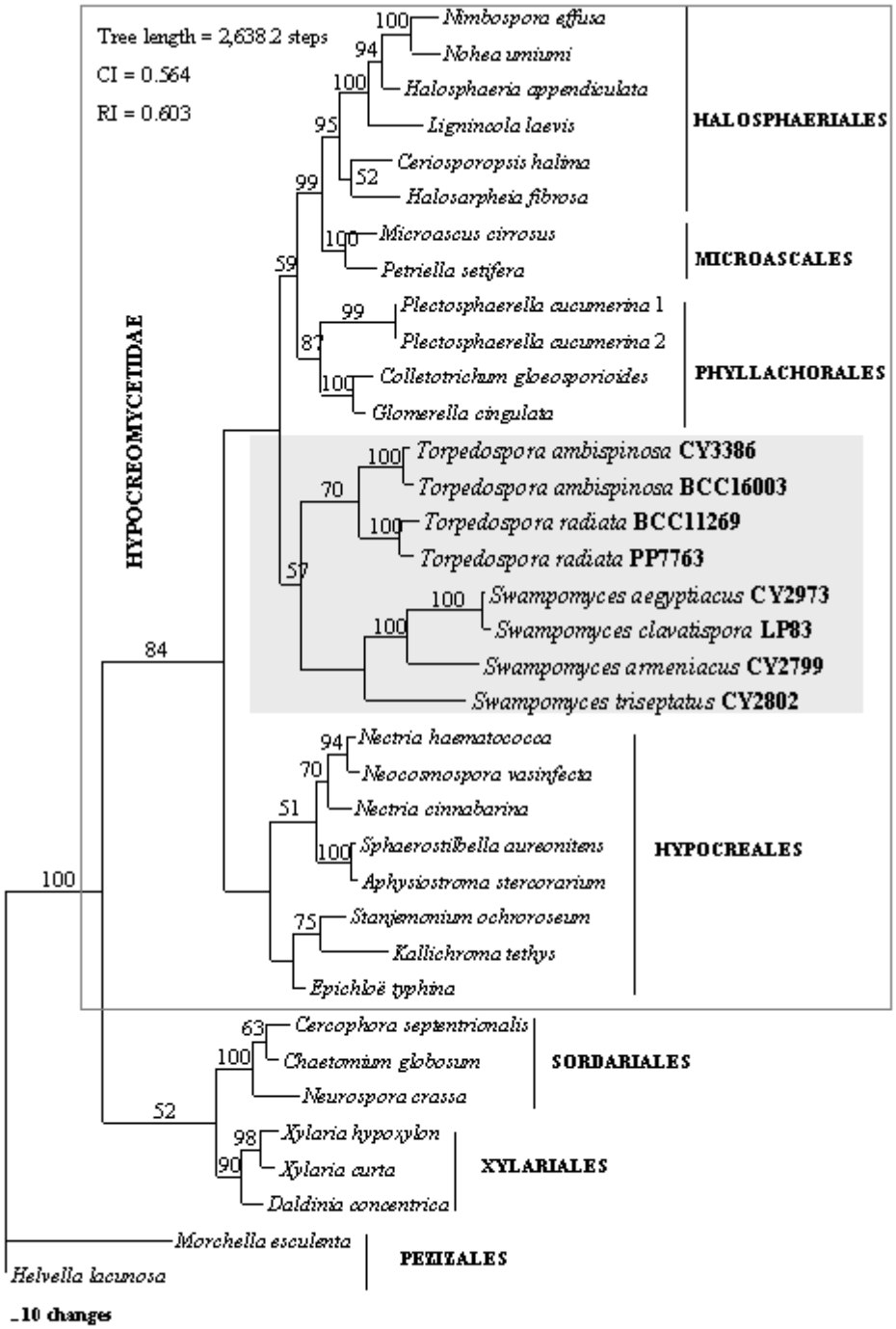


Figure 13. A single MPT inferred from combined SSU+LSU rRNA sequences, generated with weighted maximum parsimony analysis (*Torpedospora* + *Swampomyces*). Bootstrap values greater than 50% are given above branches.

Discussion

1). Phylogenetic relationships

A number of ascomycetes morphologically resemble *Torpedospora*: *Lanspora coronata* K. D. Hyde and E. B. G. Jones, with its crown-like appendages at both ends of the ascospores, resembles *Torpedospora* and was referred to the Halosphaeriales by Hyde and Jones (1986). This species has an uncertain placement within the Hypocreomycetidae, and molecular data suggest a distant relationship with *Torpedospora* (tree not shown). *Halonectria* E. B. G. Jones might also share some common characteristics with *Torpedospora*, with its long necked ascomata and elongate needle-shaped ascospores (Jones, pers. comm.). This genus is similar to *Nectria* (Fr.) Fr., but differentiated by the immersed perithecia with long necks (Jones, 1965). These morphological characters differ from most hypocrealean fungi, although, the ascomal wall surface and KOH reaction indicate it might be best included in the Bionectriaceae, Hypocreales (Rossman *et al*., 1999). However, this relationship needs to be further investigated.

In some analyses, *Torpedospora* forms a sister clade to the Hypocreales, but with weak support. Therefore, further taxa with characteristics of the Hypocreales such as *Melanospora, Stachybotrys* and *Kallichroma* Kohlm. and Volkm.-Kohlm. were included in the analysis. Our molecular results demonstrate that these genera do not show any affinities to *Torpedospora* (Figures 5-7).

Morphologically *Torpedospora* appears to share few characters in common with the Hypocreales (Table 7). *Torpedospora* has dark-colored ascomata, asci and paraphyses growing irregularly inside the ascoma venter, which are atypical of the Hypocreales, in which the ascomata are mostly bright-colored without a clypeus and paraphyses growing downwards to the base of the fruiting body (Kohlmeyer and Kohlmeyer, 1979; Rossman *et al*., 1999). Furthermore, phialidic anamorphs have been reported for a number of the Hypocreales, while none has been found in *Torpedospora.*

The monophyly of *T. radiata* and *T. ambispinosa* is supported in all analyses, indicating the taxonomic importance of ascoma characters, type of centrum development and the hamathecium arrangement as generic characters, although the spore shape, appendage nature and morphology are quite different (Table 6). The branching in the sequences of these two species suggests that they may have evolved at the same evolution rate, giving rise to the long branch lengths for rRNA phylogeny (Figures 5-8) and beta-tubulin gene (Figure 9).

Our results clearly show that *Torpedospora* does not have an affinity with the Halosphaeriales morphologically and phylogenetically, and supports the views of Kohlmeyer (1972) and Kohlmeyer and Kohlmeyer (1979) that this genus should be included elsewhere. Although members of the Halosphaeriaceae often possess unique appendaged or sheathed ascospores, this character may be the result of convergence with modification or adaptation to the marine environment (Shearer, 1993; Jones, 1995). Appendages may aid in the entrapment and attachment to suitable substrata by their sticky nature and in increasing the surface area for attachment (Jones, 1994). Moreover, marine ascomycetes have adapted to life in the

marine environment in a number of ways: great diversity in morphology, such as loss of the ascus apical apparatus, passive release of ascospores as the result of deliquescing asci and degeneration of the pseudoparenchymatous tissue inside the centrum (Shearer, 1993; Jones, 1995). Therefore, it clearly shows that the presence of appendaged ascospores does not guarantee a unique character defining the placement of *Torpedospora* in the Halosphaeriales.

Swampomyces forms part of a clade with *Torpedospora* among the unitunicate orders. Individual SSU and LSU phylogenies and combined SSU and LSU analysis suggest that *Swampomyces* also belongs to the subclass Hypocreomycetidae, Sordariomycetes. However, they do not group within well-defined orders, but form a basal clade to the orders Halosphaeriales, Hypocreales, Microascales and Phyllachorales (Figures 10-13).

Three *Swampomyces* species are monophyletic, but *S. triseptatus*, groups occasionally with weak support with the *Torpedospora* species (Figures 10-13). The three species; *S. aegyptiacus, S. armeniacus* and *S. clavatispora*, share some morphological characters in common, e.g. ascoma morphology, ascospores apricot-colored in mass, branched paraphyses in a gel and the asci with a thickened apex (Table 6). *Swampomyces triseptatus* differs from the type species with its deeply immersed ascomata with a neck leading to the surface without a clypeus (Kohlmeyer and Volkmann-Kohlmeyer, 1987). The ascus of *S. triseptatus* is different in its dehiscence and in the structure of the ascus apex at the ultrastructural level (Hyde and Nakagiri, 1992).

Molecular data conclusively indicates that *Swampomyces* does not belong in the Phyllachorales, as suspected by Kohlmeyer and Volkmann-Kohlmeyer (1987),

although they share some morphological similarities in the possession of a clypeus, the presence of paraphyses and the apical apparatus to the ascus (Table 7). These morphological characters may be the result of convergence during evolution, and the loss and rapid modification of characters (Alexopoulos *et al*., 1996; Samuels and Blackwell, 2001). Samuels and Blackwell (2001) opinionated that such changes could be very common. However, the presence of a clypeus is a unifying ordinal character for the Phyllachorales. A distinct difference between the Phyllachorales and *Swampomyces* is their mode of life. The Phyllachorales are strict biotrophs on leaves and stems of herbaceous plants (Samuels and Blackwell, 2001), whereas *Swampomyces* is saprobic on mangrove wood.

Hyde and Nakagiri (1992), in comparing *Swampomyces* with the marine genus *Marinosphaera*, noted a similarity in ascospore morphology (Read *et al*., 1995). In *Marinosphaera*, the ascomata are immersed or superficial with long bushy necks, absence of a clypeus, paraphyses are wide and evenly septate, unbranched and lack a gel, asci possess a subapical plate, ascospores initially unicellular, becoming 3-septate later, the spore wall distinctly ornamented, the spores are usually full of oil globules (Hyde, 1989b; Read *et al*., 1995).

At the ultrastructural level, they differ with respect to ascus structure and morphology of the paraphyses. In *S. armeniacus*, the asci possess an apical fibrous thickening which is not apparent in *M. mangrovei*. Paraphyses in *M. mangrovei* are chain-like, simple, regularly septate and not embedded in a gel, while in *S. armeniacus* they branch profusely, filamentous and in a gel (Kohlmeyer and Volkmann-Kohlmeyer; 1987; Hyde and Nakagiri, 1992; Read *et al*., 1995; Abdel-Wahab *et al*.,

2001a). Moreover, the ascospore wall is different in the two genera; *M. mangrovei* lacks an exosporium, as shown at the TEM level, while the origin of the wall in *S. armeniacus* remains unresolved (Read *et al*., 1995).

Torpedospora and *Swampomyces* share few morphological features in common (Tables 6, 7). *Swampomyces armeniacus* forms a clypeus, while the other three species form a pseudostroma, whereas *Torpedospora* has neither of these features. Presence of persistent paraphyses in *Swampomyces* is observed, while they degenerate early in *Torpedospora.* They also differ in ascal morphology, although the ascospore shape is similar but lack appendages.

2). Marine lineages within the Ascomycota

A number of marine pyrenoascomycetes lineages have evolved from terrestrial counterparts. The major lineage is that of the Halosphaeriales, with the largest number of marine taxa, which is characterized by ellipsoidal, mostly appendaged ascospores, clavate asci that deliquesce early and a pseudoparenchymatous centrum (Spatafora *et al*., 1998). The Lulworthiales is another major marine lineage, comprising two genera: *Lulworthia* and *Lindra*, with *Kohlmeyeriella* and *Spathulospora* that should be transferred to this order based on the molecular evidence. The Lulworthiales is characterized by variously shaped ascospores with apical chambers and deliquescing asci (Campbell *et al*., 2002; Inderbitzin *et al*., 2004). Both the Halosphaeriales and Lulworthiales lineages, have been confirmed by molecular results to be derived from terrestrial counterparts (Spatafora *et al*., 1998; Kohlmeyer *et al*., 2000), although the stability of different

structures has not yet to be fully evaluated. In evaluating the phylogeny of ascomycetes, we do not know how much the environment conditions modify their morphology. That environmental conditions bringing about changes is well demonstrated, for example the deliquescent asci of the Halosphaeriales and Lulworthiales that are phylogenetically distant to one another. Other marine genera with deliquescent asci are also well documented (Hypocreales: *Kallichroma*; cleistothecial: *Amylocarpus* Curr., *Biflua* J. Koch and E. B. G. Jones, *Dryosphaera* J. Koch and E. B. G. Jones; Eurotiales: *Eiona* Kohlm.). In all cases, we do not know the structure of the asci in their terrestrial counterparts, although in *Marisolaris* J. Koch and E. B. G. Jones there is a suggestion that the ascus is bitunicate (Koch and Jones, 1989). So are *Swampomyces* and *Torpedospora* a lineage of marine ascomycetes, whose ancestral morphological character states cannot be predicted at this time.

Some other minor lineages within the Ascomycota that have been documented, comprising the genera: *Kallichroma, Heleococcum* Jørg. (Hypocreales) (Kohlmeyer, 1986, Kohlmeyer and Volkmann-Kohlmeyer, 1993; Rossman *et al*., 1999), while other genera remain to be confirmed by molecular data, such as *Halonectria*. *Torpedospora* and *Swampomyces* lineage has been suggested by the reviewer to be compare with Ascomycota *incertae sedis* which are derived from terrestrial counterparts (*Ascotaiwania* Sivan. and H. S. Chang, *Carpoligna* F. A. Fern. and Huhndorf, *Gondwanamyces* G. J. Marais and M. J. Wingf.), and it shows distinct grouping (Figure 36). Moreover, the marine bitunicate ascomycetes are another distinctive lineage that has evolved successfully to the marine environments, especially the mangrove habitat, and they need to be further studied.

Our study has revealed a new lineage of marine fungi comprising the genera *Torpedospora* and *Swampomyces* within the Hypocreomycetidae, Sordariomycetes. Although the true affinities of these two genera may not be resolved at this time, until more genes need to be sequenced and combined with rRNA data. Further taxa (e.g. *Halonectria, Mangrovispora* K. D. Hyde and Nakagiri, *Marinosphaera*) or new taxa from other habitats or substrata are required to be collected, described and sequenced.

Summary

Torpedospora is distantly related, morphologically and phylogenetically, to the Halosphaeriales despite similarities in ascus and ascospore appendage morphology. The type species, *Torpedospora radiata*, groups consistently with *T. ambispinosa* with high bootstrap support. *Swampomyces* also shows no affinities with the Phyllachorales despite earlier assignment to that order. *Swampomyces armeniacus*, the type species, groups strongly within the subclade with *S. clavatispora* and *S. aegyptiacus*, while *S. triseptatus* is located within the clade but with weak support. *Torpedospora* and *Swampomyces* form a monophyletic clade and group within the subclass Hypocreomycetidae, Sordariomycetes with the Halosphaeriales, Hypocreales, Microascales and Phyllachorales with moderate bootstrap support. Our findings demonstrate a new lineage of marine ascomycetes invaded the sea from terrestrial counterparts.

4.2 *Haligena*

Introduction

Haligena was described by Kohlmeyer (1961), with the type species *H. elaterophora* Kohlm. The unique characteristic of the species was the long bipolar strap-like appendages and multi-septate ascospores (Figure 14a-e), which characterize and clearly distinguish the genus from other members of the Halosphaeriaceae (Kohlmeyer, 1961). Later, a number of species were assigned to the genus: *H. amicta* (Kohlm.) Kohlm. and E. Kohlm., *H. spartinae* E. B. G. Jones, *H. unicaudata* E. B. G. Jones and Le Camp.-Als. and *H. viscidula* Kohlm. and E. Kohlm. (Jones, 1962; Kohlmeyer and Kohlmeyer, 1965; Jones and Le Campion-Alsumard, 1970). Shearer and Crane (1980) transferred *H. spartinae*, *H. unicaudata* and *H. viscidula* to *Halosarpheia* because of their hamate polar appendages that uncoil to form long thread-like structures. Recent phylogenetic studies showed that they are not related to *Halosarpheia*; and were transferred to *Magnisphaera* and *Ascosalsum* (Anderson *et al.*, 2001; Campbell *et al*., 2003). *Haligena amicta* is distinct from *Haligena* in having appendages that arise from the episporium at various points from the spore wall (Johnson *et al*., 1987). In *Haligena,* appendages are polar, arising as outgrowths of the ascospore wall. Therefore, a new genus *Appendichordella* R. G. Johnson, E. B. G. Jones and Moss was introduced to accommodate *H. amicta* (Johnson *et al*., 1987).

Another species that has been accepted in *Haligena* is *H. salina* C. A. Farrant and E. B. G. Jones, which was originally identified as a *Remispora*-like species (Jones, 1985; Farrant and Jones, 1986). *Haligena salina* differs from the type species in ascospore size, septation and especially appendage morphology; appendages spoon-shaped at the base, initially coiled and attached closely to the spore wall and separating to form a long thread-like filament (Figure 14f-j) (Farrant and Jones, 1986). Thus there are only two species; *H. elaterophora* and *H. salina* still retained in *Haligena*.

The objectives for this chapter are:

1). to test whether the genus *Haligena* is monophyletic,

2). to verify whether *H. elaterophora* and *H. salina* are correctly assigned to the Halosphaeriales,

3). to test if the appendage morphology can aid in the delineation of genera, and,

4). to examine their relationships to other taxa within the Halosphaeriales.

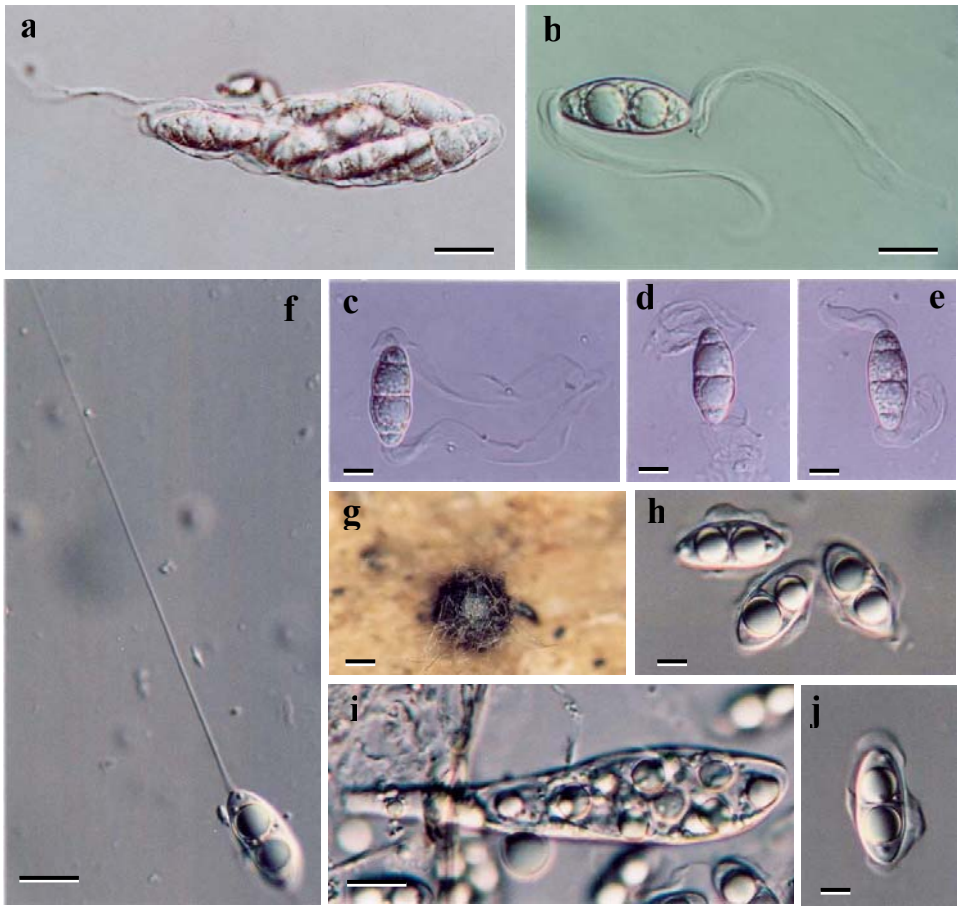


Figure 14. Morphological features of *Haligena* species

a-e *Haligena elaterophora* (JS147) a: Ascus containing ascospores; b-e: Ascospores multi-septate with bipolar, long, strap-like appendages
f-j *Haligena (Morakotiella) salina* (BCC12781) f: Ascospore forms a long thread-like appendage after released into water; g: Black, globose ascomata: h,j: Ascospores one-septum with tightly coiled appendages around the ascospores; i: Cylindrical-clavate ascus

Scale bars: a-e = 20 µm; g = 100 µm; f, h-j = 10 µm

Materials and methods

The genomic DNA for all fungi listed below were extracted by using the the CTAB lysis buffer as outlined in Chapter III.

Fungal studied	**Original code**	**Origin**	**GenBank accession No.**
Haligena elaterophora	PP4705	Friday Harbor, USA	AY864845
Haligena elaterophora	JS147	Portsmouth, England	AY864846
Haligena salina	CY3437	Friday Harbor, USA	AY864843
Haligena salina	BCC12781	Marloes, South Wales	AY864844

DNA amplification and sequencing

The LSU rRNA region was amplified using the primers: LROR/LR7 and JS1/JS8, amplification profiles performed as outlined in Chapter III. PCR products were directly sequenced using the forward and reverse primers: JS1, NL4, JS5, JS8, LROR, NL3 and NL4R (Bunyard *et al*., 1994; Landvik, 1996).

Phylogenetic analysis

The consensus sequences for each species were multiple aligned by Clustal W 1.6 (Thompson *et al*., 1994) along with other sequences obtained from the GenBank database (Appendix C). The dataset was refined visually in Se-Al v1.0a1 (Rambaut, 1999) and BioEdit 5.0.6, 6.0.7 (Hall, 2001; 2004). *Daldinia concentrica* (Bolton) Ces. and *Xylaria hypoxylon* (L) Grev. were chosen as the outgroup for all

analyses. Two insertion regions were observed, one at a position 835-1035 of *Halosarpheia trullifera*, *H. unicellularis*, *H. salina* (AY094182), *H. salina* (CY3437) and *H. salina* (BCC12781) and the other at a position 1148-1243 of *Halosarpheia unicellularis*, *H. fibrosa* and *Saagaromyces ratnagiriensis* (S. D. Patil and Borse) K. L. Pang and E. B. G. Jones. Inclusion and exclusion of all insertion regions had no effect on the tree topology for all analyses. Therefore, the insertion regions were included for all analyses.

The phylogenetic analyses were performed with PAUP 4.0b10 (Swofford, 2002) using maximum parsimony analysis applying heuristic searches with the following setting: 100 replicates of random stepwise addition of sequence and TBR branch-swapping algorithm. Gaps were treated as missing data, the fifth state and gaps excluded had no affect to overall topology. Therefore, all gaps treated as missing data, and all characters were given equal weight.

Weighted parsimony analysis was performed using a step matrix to weight nucleotide transformations based on the reciprocal of the observed transition to transversion (ti:tv) ratio, which was estimated by maximum likelihood score setting in PAUP* (Swofford, 2002). Moreover, characters were reweighted according to their Rescaled Consistency Index (RC) using PAUP* default setting for reweighting character. Bootstrap analysis (Felsenstein, 1985) was performed for all analyses using full heuristic search on 1,000 replicates (10 replicates of random stepwise addition of sequence and TBR branch-swapping algorithm).

Bayesian phylogenetic inference was calculated using MrBayes 3.0b4 with 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 from random starting trees for 2,000,000 generations and sampled every 100 generations. The first 100,000 generations were discarded as burn-in of the chain. A majority rule consensus tree of all remaining trees, as well as the posterior probabilities, was calculated. The alignments were deposited in TreeBase: study accession number = S1228, matrix accession numbers = M2135, M2136.

Results

The dataset consists of 1,705 total characters, 1,081 characters are constant, 322 characters are parsimony informative and 302 variable characters are parsimony uninformative. The maximum parsimony analysis (unweighted) resulted in two MPTs of 1,399 steps long (CI = 0.602, RI = 0.607). The difference between these two MPTs is in the branching pattern of *Magnisphaera spartinae* (E. B. G. Jones) J. Campb., J. L., Anderson and Shearer (tree not shown). The weighted parsimony (step matrix of 1.38) resulted in two MPTs, which gave the same topology as unweight maximum parsimony, of 1,641.06 steps long, CI = 0.607, RI = 0.612. The best topology estimated from the K-H test is presented in Figure 15. Moreover, weighted parsimony (characters reweighted) resulted in a single MPT with the same topology as the other two analyses (tree not shown). The bootstrap values higher than 50% from reweighted parsimony analysis is given above branches of Figure 15.

Bayesian inference, with the posterior probabilities above 95%, gave a topology similar with other analyses. Although there was a minor difference in the position of *Nohea umiumi*, however, this difference does not affect the overall topology of the tree and the conclusions drawn.

The position of *Haligena* within the Halosphaeriales was clearly supported by all analyses, and shown to be polyphyletic. Two isolates of *H. elaterophora*, and three isolates of *H. salina* were shown as separate but monophyletic clades with high bootstrap values. *Haligena elaterophora* was always shown on a basal branch to the rest of the Halosphaeriales with 82% bootstrap values and 100% posterior probabilities support in weighted parsimony and Bayesian inference, respectively (Figures 15, 16). All three *H. salina* sequences grouped together with high bootstrap support as a sister clade to *Neptunella longirostris* (Cribb and J. W. Cribb) K. L. Pang and E. B. G. Jones with 75% bootstrap obtained from weighted parsimony analysis and below 95% posterior probabilities from Bayesian analysis

Discussion

Ascospore appendage morphology and ontogeny are significant characters used to delimit genera of marine ascomycetes. Campbell *et al.* (2003), in their treatment of *Halosarpheia* species with bipolar unfurling appendages, stated that "transmission electron microscope and scanning electron microscope studies on a limited number of species to date do not indicate any heterogeneity in structure or ontogeny", and that "all the appendages are reported to develop the same way, by extrusion through pores in the episporium wall". These statements do not consider the diversity in structure of the polar appendages. For example, the development of ascospore appendages from a pore in *Magnisphaera spartinae* differs significantly from that of the pore fields in *Saagaromyces ratnagiriensis* (Jones and Moss, 1980; Baker *et al*., 2001).

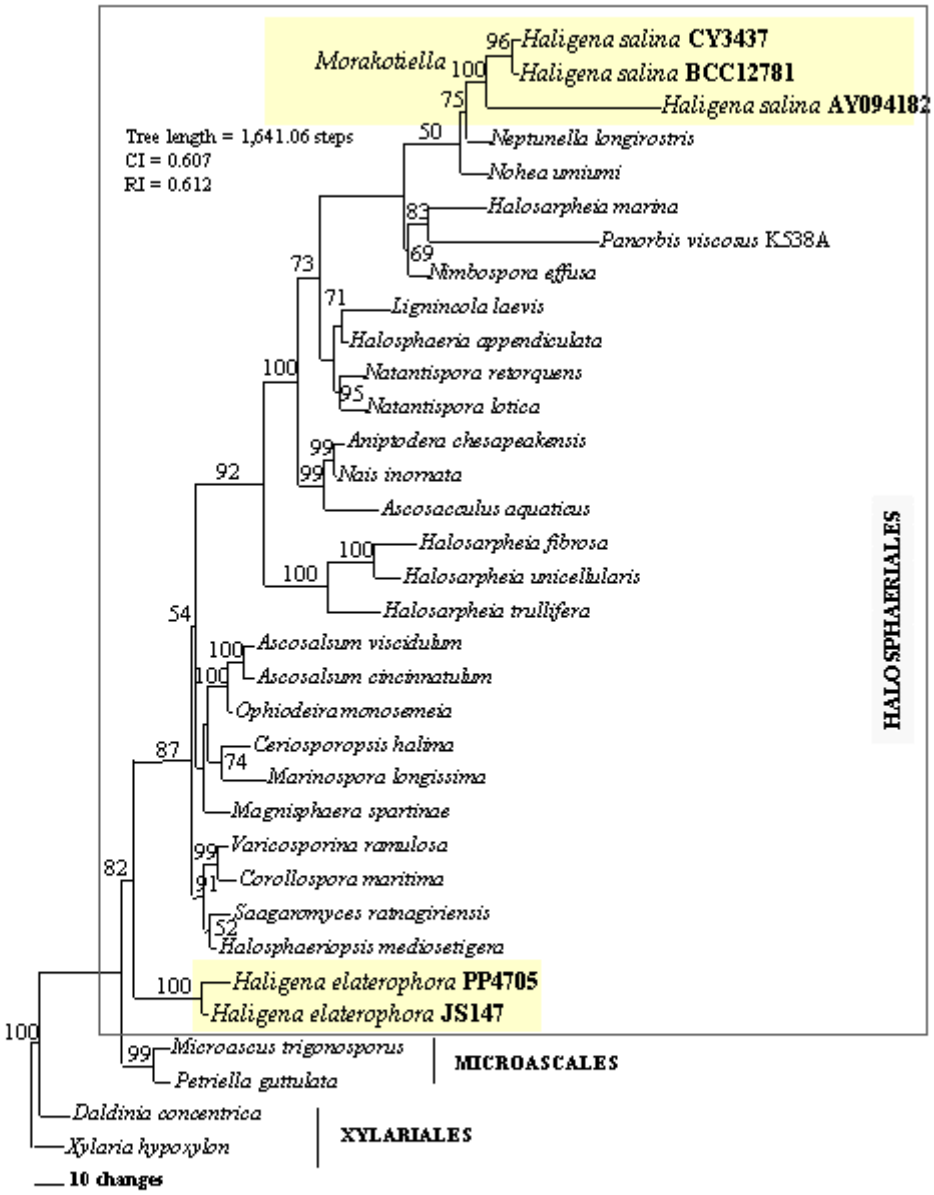


Figure 15. One of two MPTs inferred from LSU rRNA sequences of the genus *Haligena*, generated with weighted maximum parsimony analysis (step matrix). Bootstrap values higher than 50% from weighted Parsimony (characters reweighted) are given above branches. Scale bar indicates 10 character state changes.

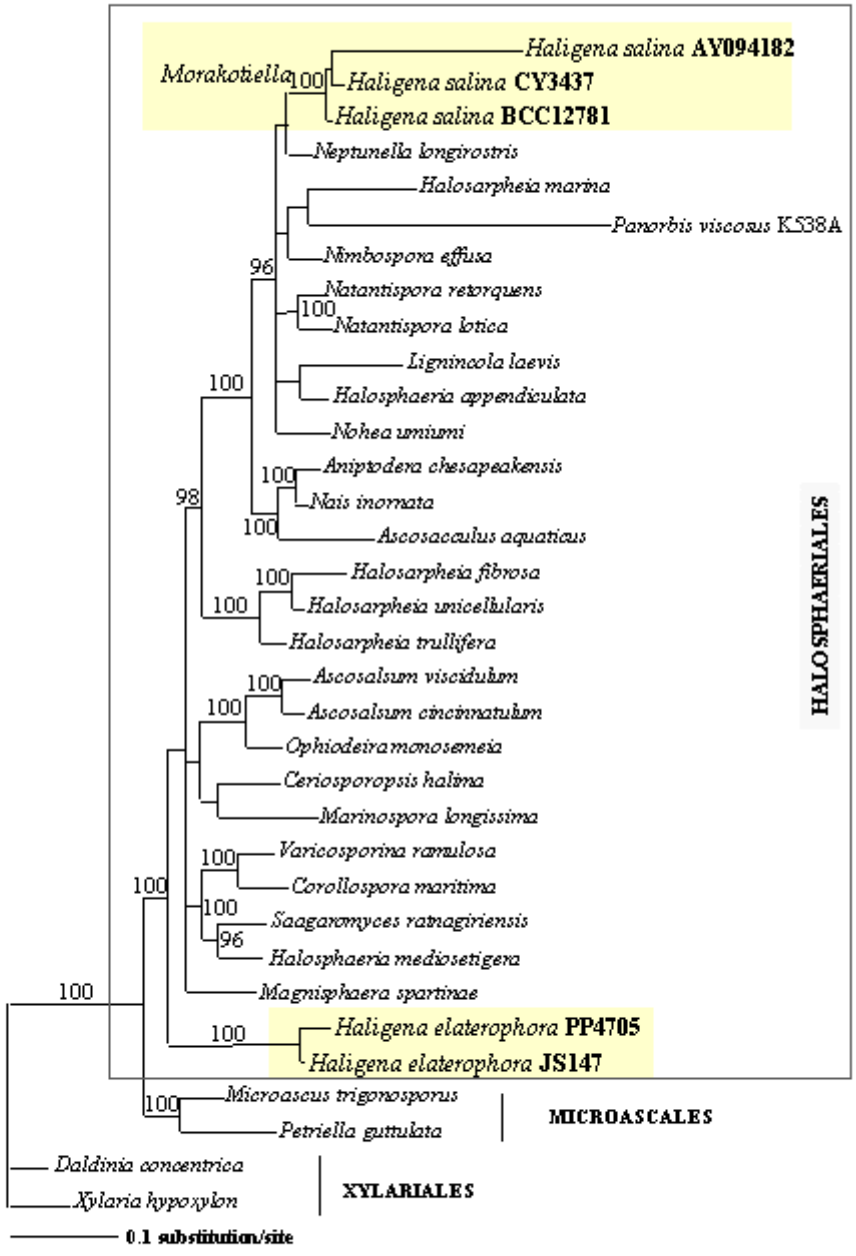


Figure 16. Bayesian analysis of partial LSU rRNAsequences of the genus *Haligena*. Posterior probabilities higher than 95% are indicated above the branches. Scale bar indicates 0.1 substitution/site.

In *Cucullosporella mangrovei* (K. D. Hyde and E. B. G. Jones) K. D. Hyde and E. B. G. Jones, the substructure of the appendage comprises two distinct elements: folded fibro-granular electron-dense material and fine fibrils (Alias *et al*., 2001). However, we have much to learn about how appendages in species with bipolar unfurling appendages are formed, as appreciated by Campbell *et al.*, (2003). While distinct fibers are apparent in the appendages within the delimiting membrane in asci in some species, in others the appendage may be extruded as a gel-like material, only later to aggregate to form fibers (Nakagiri and Ito, 1994; Jones, unpublished). Another consideration is the condition under which appendages are formed as it was shown for the appendages of *Aniptodera salsuginosa* Nakagiri and Tad. Ito (Nakagiri and Ito, 1994). In this case, the salinity of the water exerts a profound influence on the morphology of the appendages.

Likewise, the appendage structure of *Haligena* species differ. In *H. elaterophora* and *H. salina*, the appendages are initially wrapped around the ascospores and then uncoil to form long polar appendages (Figure 14). These characteristics are relevant when comparing the appendages of *H. salina* and *Panorbis viscosus* (I. Schmidt) J. Campb., J. L. Anderson and Shearer. However, *H. salina* and *P. viscosus* differ at the ultrastructural level and molecular results (Figures 15, 16). *Haligena salina* was originally identified as a *Remispora*-like species (Farrant and Jones, 1986), however, it cannot be referred to *Remispora* as morphologically and phylogenetically they are distantly related (see Chapter 4.3). Appendages of *H. salina* also resemble the appendages of *Halosphaeria appendiculata* Linder but they differ at the ultrastructural level: in *H. salina* equatorial appendages are lacking and appear amorphous, while in *H. appendiculata*

they are present and are reticulate, with a substructure composed of both electron-dense and less electron-dense material (Hyde *et al.,* 1994). Our recent molecular results confirm that they are distantly related (Figures 15, 16).

Marine ascomycetes have adapted to life in the marine environment in a number of ways: as indicated by their great diversity in morphology, such as early deliquescing asci, lack of apical apparatus and variously appendaged ascospores (Jones, 1995). Polar and equatorial ascospore appendages aid in the entrapment and attachment to suitable substrata by their sticky nature and in increasing the surface area for attachment (Jones, 1994).

The deliquescent nature of the asci of many marine ascomycetes was considered as an unifying character of the Halosphaeriales by many authors (Cain, 1972; Berbee and Taylor, 1992; Blackwell, 1994; Spatafora and Blackwell, 1994). With the transfer of *Lulworthia* and *Lindra* to a new order, the Lulworthiales, this character can no longer be considered as a unifying feature. Spatafora *et al*., (1998) argued that evanescent asci are a continuation of the lineage arising from entomopathogenic ascomycetes. However, deliquescent asci are common in a number of unrelated taxa that have made the transition from terrestrial to the marine environment: e.g. *Halonectria* (Bionectriaceae); *Amylocarpus* (Leotiomycetidae) and *Dryosphaera* (Sordariomycetes).

Delineation of genera within the Halosphaeriales has relied heavily on the ascospore and appendage ontogeny, due to their great variation in morphology (Jones, 1994). This similarity is often the result of environmental adaptation to life in the marine milieu (Shearer, 1993; Jones, 1995). This has led to many marine genera being incorrectly circumscribed, for example, the inclusion of such species as

Arenariomyces trifurcatus Höhnk and *Nereiospora comata* (Kohlm.) E. B. G. Jones, R. G. Johnson and S. T. Moss in *Corollospora*. Ultrastructural studies clearly demonstrated distinct differences in ascospore appendage ontogeny, observations later supported by molecular sequences of the SSU analysis (Campbell *et al.*, 2002).

Haligena was shown to be clearly delineated within the Halosphaeriales, but is polyphyletic, with *H. salina* distantly related to the type species, *H. elaterophora*. *Haligena elaterophora* constitutes the basal clade to the order with high support for all analyses. The molecular study by Campbell *et al*., (2003) and Pang *et al*. (unpublished) support the exclusion of *Magnisphaera spartinae* and *Ascosalsum unicaudatum* from *Haligena*, as does our recent molecular observations.

Haligena elaterophora can be differentiated from *H. salina* by both morphological and molecular evidence. *Haligena elaterophora* possess smooth, multiseptate ascospores with constriction and the appendages are wider, strap-like, and polymorphic (Figure 14b-e). Ascospores of *H. salina* are smaller than those of the former species; they have a warty ascospore wall surface (in the original collection) composed of an electron-transparent mesosporium and an electron-opaque episporium, continuous beneath the appendages (Figures 6, 10 in Farrant and Jones, 1986). Its appendages are long, narrower, drawn out and attenuated at their tips and distinctly spoon-shaped at their point of attachment (Figure 14f, h, j). Pseudoparenchymatous cells in the ascoma centrum of *H. elaterophora* break up to form catenophyses while these cells might be absent or present in some collections of *H. salina*. However, we did not observe catenophyses in material from which our isolates were derived.

The three isolates of *H. salina* formed a monophyletic clade and showed a high number of base substitutions that caused a long branch length in isolate AY094182. The closest sister taxon to *H. salina* is *Neptunella longirostris*, but they are not congeneric and with weak phylogenetic support (Figures 15, 16). They significantly differ in the morphology of the ascomata and ascus structure: in *H. salina* the ascus deliquesce early while in *N. longirostris* the ascus is persistent and with an apical pore.

Haligena salina differs from other genera with uncoiling appendages by the mode of attachment of the appendage to the ascospore wall; appendages are coiled around the spore, spoon-shaped at the point of attachment, channelled along its length, amorphous with distinct striations running along the whole length of the appendage (visible at the SEM level), and arising as an outgrowth of the spore wall. Based on these morphological features and molecular data, a new genus *Morakotiella*, is proposed for *H. salina*.

Taxonomy

Morakotiella Sakayaroj, gen.nov.

Typus generis: *M. salina* (C. A. Farrant and E. B. G. Jones) Sakayaroj

Ascomata globosa, subglobosa, immersa vel partim immersa, ostiolata, papillata, coriacea, nigra, collo hyaline. Catenophyses praesentes. Asci clavati vel fusiformis, pedicellati, unitunicati, leptodermi, pristine deliquescentes. Ascosporae 1-septatae, ellipsoidalis, hyalina, ad septa constrictae, verrucosus pagina, appendices bipolares. Appendages filum, denique, polares, as basim cochleariformes, attenuatae, canaliculatae, ad extensionem apicis, sporae affixae.

Ultrastructurally spore wall composed of two layers: electron-dense episporium and a wide electron transparent mesosporium. Appendage fibrillar, bounded by a delimiting membrane.

Ascomata immersed or partly immersed or superficial, globose, subglobose, ostiolate, black, perithecial wall coriaceous. Neck short, cylindrical and periphysate. Asci thin-walled, unitunicate, pedunculate, fusiform to clavate, deliquescing early. Catenophyses present or absent. Ascospores 1-septate, ellipsoidal, slightly constricted, hyaline, appendaged. Appendages polar, initially wrapped around the ascospore wall, later separating to form long filaments that are spoon-shaped at the place of attachment to the spore wall, attenuate, channelled, over 50 μm long.

Ascospore wall at TEM level two-layered, outer episporium electron-dense, inner wall layer mesosporium less electron-dense, at each pole wall bulging outward with electron material within the mesosporium and beneath the episporium. Appendage origin not determined, but bounded by a thin electron-dense delimitating membrane attached to ascospore apices by fine threads. Appendage substructure fibrillar to amorphous, electron-dense. At the SEM level, appendage comprising fine fibrillar material running the length of the appendages becoming amorphous and sometimes deliquescing.

Typus: *Morakotiella salina* (C. A. Farrant and E. B. G. Jones) Sakayaroj

Etymology: "Morakot" refers to Professor Morakot Tanticharoen, Director BIOTEC Thailand, for her continued support of fungal taxonomy in Thailand and "ella" = diminutive

Morakotiella salina (C.A. Farrant and E.B.G. Jones) Sakayaroj comb. nov.

Figure 14f-g.

Basionym: *Haligena salina* C. A. Farrant, E. B. G. Jones. Botanical Journal of the Linnean Society 93: 405, 1986.

Holotype: IMI 297765

Key to the genera in the Halosphaeriales with polar unfurling appendages

1a. Parasitic on crabs, appendages coiled around ascospore ……..... *Trichomaris*

1b. Saprobes on marine plants, wood ……...…..2

2a. Ascospores with a single polar appendage ……3

2b. Ascospores with bipolar appendages …… ……....4

3a. Asci persistent, with retraction of the cytoplasm, at the apex, apical pore present ….. ……..……..*Tirispora*

3b. Asci deliquescing early, no apical pore ……*Ophiodeira*

4a. Asci persistent with retraction of cytoplasm …….5

4b. Asci deliquescing early without retraction of cytoplasm ……..…….6

5a. Ascospore dimensions wider than 20 μm, asci with long pedicels……..... ……..…….S*aagaromyces*

5b. Ascospore dimensions narrower than 20 μm, ascus pedicel short ….. *Aniptodera*

6a. Ascospores hyaline …….…….……..7

6b. Ascospores brown ……...…..*Phaeonectriella*

7a. Ascospores 2 or more septate …….……...8

7b. Ascospores 1-septate ……..……...9

8a. Ascospores cylindrical narrow less than 5 μm wide, wall smooth…..…... ……..…….…….*Ascosalsum*

8b. Ascospores broad wider than 20 µm, shorter, wall verrucose ……..…

…….*Magnisphaera*

9a. Ascospore appendages coiled and arise as outgrowths of spore wall ……...…10

9b. Ascospore appendages hamate, arising from a pore field ……....……..….11

10a. Appendages wide (wider than 20 µm) strap-like ….……..*Haligena*

10b. Appendages narrow (width less than 10 µm) thread-like …..*Morakotiella*

11a. Ascospore appendages arise through a cup-like structure ……*Cucullosporella*

11b. Appendages arise through a pore field ……...12

12a. Asci persistent ……....…..… *Halosarpheia*

12b. Asci deliquesce early …….…...…….….13

13a. Ascospores fusoid to ellipsoidal, mostly over 25 µm long, catenophyses present

……..…….….*Natantispora*

13b. Ascospores ellipsoidal, mostly under 25 µm long, catenophyses present or absent…….…….……*Panorbis*

Keys to the halosphaeriaceous taxa with unfurling polar appendages (Jones, 1995, Campbell *et al*., 2003) are unsatisfactory because of the overlapping characters of many of the genera. Also ultrastructural studies of spore appendage ontogeny are only available for a few of these genera (Alias *et al*., 2001, Baker *et al*., 2001).

4.3 *Remispora* and *Naufragella*

Introduction

The genus *Remispora* was erected by Linder (Barghoorn and Linder, 1944). It is characterized by mostly hyaline ascomata (except for *R. pilleata* Kohlm.), 1-septate hyaline ascospore, 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 (Kohlmeyer, 1972; Jones and Moss, 1978; 1980; Johnson, 1980; 1982; Johnson *et al*., 1984). All *Remispora* species were referred to *Halosphaeria*, based on similarities in ascomata and ascospore appendages morphology (Kohlmeyer, 1972). However, scanning and transmission electron microscope studies supported the acceptance of *Remispora* as a genus distinct from *Halosphaeria* (Jones and Moss, 1978; 1980; Johnson, 1980; 1982; Johnson *et al*., 1984). *Remispora* differs from *Halosphaeria* by the following characters: 1) perithecia usually hyaline (although Cavaliere and Johnson (1966) mentioned repeatedly that ascoma color is not important at the generic level in most Halosphaeriaceae); 2) the ascocarp wall is composed of 6-9 layers of elongate, hyaline cells; 3) catenophyses are present; 4) polar appendages are pleomorphic; 5) appendages are formed by fragmentation or dissolution of an exosporium which initially totally surrounds the spore, and 6) appendages composed of an amorphous component dissolving in water at maturity (Johnson *et al*., 1984).

Six species have been referred to *Remispora*: *R. maritima* Linder (the type species)*, R. stellata* Kohlm.*, R. quadriremis* (Höhnk) Kohlm., *R. pilleata* Kohlm.*, R. galerita* Tubaki and *R. crispa* Kohlm. (Johnson *et al.,* 1984; Manimohan *et al*.,

1993a). Their morphological features at the light microscope level are illustrated in Figure 17, and the ascospore appendage ontogeny at the ultrastructural level are compared in Table 9. The assignment of all six species to *Remispora* was universally accepted, although it has been suggested that *R. stellata* and *R. quadriremis* are not distinct species (Manimohan *et al*., 1993a).

Naufragella spinibarbata (J. Koch) Kohlm. and Volkm.-Kohlm. was originally described as *Remispora spinibarbata* by Koch (1989). Nevertheless, its apical gelatinous cap at each pole and long polar appendages clearly differ from other *Remispora* species (Figure 17h), however, the ascoma structure conforms to that of *Remispora* (Manimohan *et al*., 1993a). Therefore, the fungus was only tentatively placed in *Remispora* and an ultrastructural study is required to resolve ascospore appendage ontogeny (Manimohan *et al*., 1993a; Jones, 1995; Kohlmeyer and Volkmann-Kohlmeyer, 1998).

All *Remispora* species differ in the substructure of the mesosporium (one or two layered), the degree of loss of the fibrillar matrix, and the number and rearrangement of the strands in the appendage (Manimohan *et al*., 1993a). Jones and Moss (1978) commented that *R. maritima, R., pilleata, R. stellata* and *R. quadriremis* have a similar mode of appendage development by fragmentation of a sheath. Nevertheless, differences in their morphology has been noted: wing-like appendages are found in *R. maritima* (Figure 17d), *R. pilleata* has a unique thick-walled ascospores that are rhomboidal in shape (Figure 17g); in *R. quadriremis* each polar appendage is composed of 4 segments (Figure 17k), with 5-9 segments in *R. stellata* (Figure 17i, j). Ascospore appendages in *R. galerita* appear to be more compact than in other species and the exosporial wall does not readily separate from the spore

wall, they are still attached to one another (Figure 17f) (Hyde *et al*., 1992), while *R. crispa* possesses a wide cap that initially envelops the spore, but later is orientated to one side of the middle of each cell (Figure 17e) (Kohlmeyer, 1981).

The objectives for this chapter are:

1). to verify whether the genus *Remispora* is monophyletic,

2). to test whether the genus has been correctly assigned to the Halosphaeriales,

3). to test if the unique pleomorphic polar appendages can aid in the delineation of *Remispora* species, and,

4). to examine its relationships to other taxa within the Halosphaeriales.

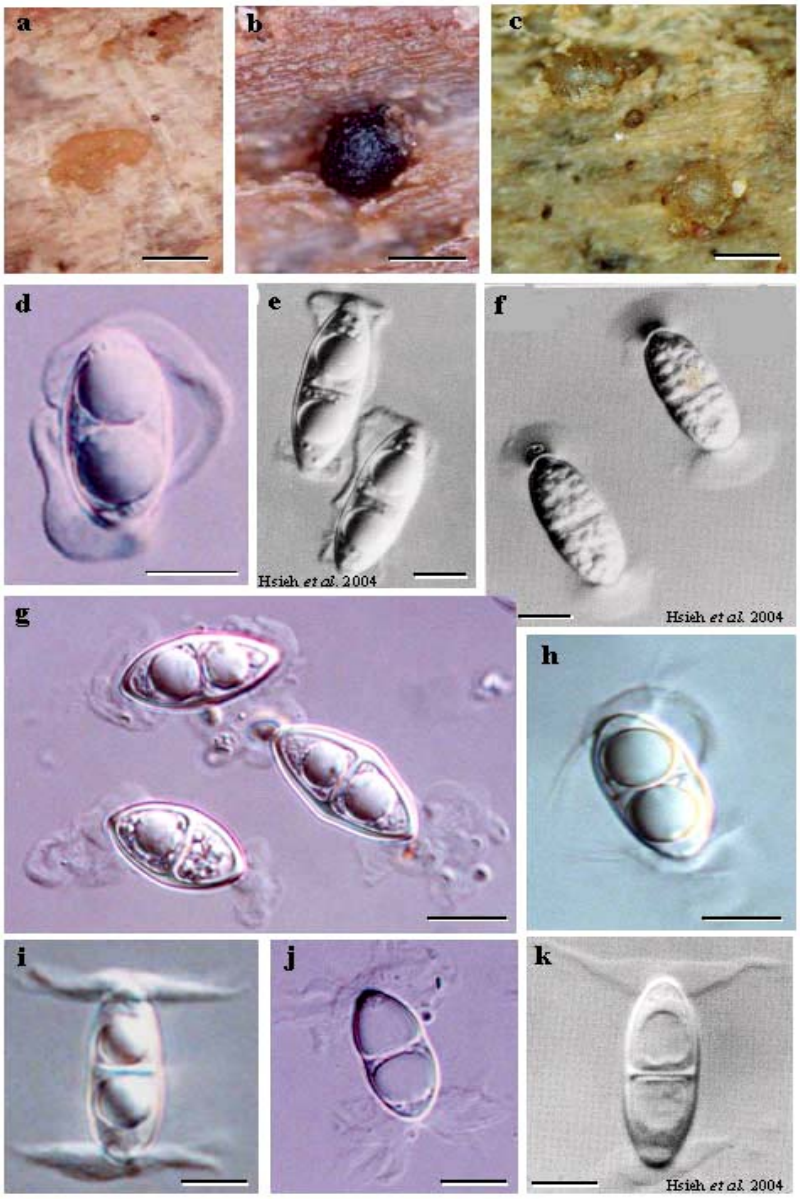


Figure 17. Morphological features of *Naufragella* and *Remispora* species

a: Hyaline ascomata of *Remispora stellata* immersed in wood; b: Superficial black ascomata of *R. pilleata* on wood; c: Superficial hyaline ascomata of *Naufragella spinibarbata* on wood; d: Wing-like polar appendages of *R. maritima*; e: Wide cap polar appendages that orientated to one side of the middle of *R. crispa*; f: Subglobose compact cap-like polar appendages of *R. galerita*; g: Thick-walled rhomboidal ascospores with subgelatinous appendages of *R. pilleata*; h: Ascospore of *N. spinibarbata* with long polar appendages and soft spines; i, j: Ascospores of *R. stellata* with 5-9 radiating segments; k: Ascospore of *R. quadriremis* with 4 discrete radiating segments

Scale bars: a = 200 µm; b = 150 µm; c = 300 µm; d-k = 10 µm

Table 9. The morphological characteristics of six *Remispora* species at light and ultrastructural levels

Characters	***R. maritima***	***R. stellata***	***R. quadriremis***	***R. pilleata***	***R. galerita***	***R. crispa***
Ascomata measurement in diameter	180-570 (-670) μm	226-471 μm	135-400 μm	209-364 μm	100-280 μm	200-350 μm
Ascomata shape	Globose, subglobose or ovoid, immersed or superficial	Subglobose to ovoid, immersed or become exposed	Globose, subglobose, immersed or superficial	Globose, subglobose, immersed or partly immersed	Globose, subglobose, or pyriform, immersed or become exposed	Subglobose to ellipsoidal, immersed or superficial
Ascomata color	Almost hyaline, cream-colored or smoke-gray, sometimes the upper part darker than the lower part	Cream-colored, yellowish or brownish	Cream-colored, grayish	Almost black above, grayish below	Hyaline, yellowish or light brown	Cream-colored to gray, darker around the base of the neck

Table 9. (Continued)

Characters	***R. maritima***	***R. stellata***	***R. quadriremis***	***R. pilleata***	***R. galerita***	***R. crispa***
Necks	Subcylindrical or truncate-conical, sometimes forked or two on one ascocarp	Conical	Cylindrical	Elongate-subconical	Cylindrical	Cylindrical
Catenophyses	Present	Present	Present	Not reported	Not reported	Not reported
Ascospore measurement	18-30x8-13 µm	24-30.5x8.5-12.5µm	18-30x8-12 µm	24-34x12.5-19 µm	20-28x7-12.5 µm	22-34x8-12 µm
Ascospores shape	Ellipsoidal, ovoid or broadly ellipsoidal, not constricted, hyaline	Ellipsoidal , not or slightly constricted, hyaline	Ellipsoidal, not or slightly constricted, hyaline	Rhomboid or rarely subellipsoidal, not constricted, thick-walled, hyaline	Ellipsoidal, not or slightly constricted, thick-walled at the apices, hyaline	Ellipsoidal, not or slightly constricted, hyaline

Table 9. (Continued)

Characters	***R. maritima***	***R. stellata***	***R. quadriremis***	***R. pilleata***	***R. galerita***	***R. crispa***
Appendages morphology and ontogeny	At first surrounded by subgelatinous, exosporic sheath that unfolds, remaining attached at both ends of the ascospores; pleomorphic, yoke-shaped, apices attenuate and irregularly streched out, developing by fragmentation	At each end generally six (rarely more or fewer) radiating appendages, developing by fragmentation of the exosporium; at the base, terminal, subclavate, curved, attenuate, semirigid, slightly channeled in the inner side,	At each end four radiating appendages, developing by fragmentation of the exospore; at the base, terminal, obclavate, curved, attenuate, semirigid, inconspicuously striate by fiber-like elements embedded in the subgelatinous matrix	At first surrounded by an exosporic, subgelatinous cover that unfolds, remaining attached at the apices, finally stretched out and becoming veil-like, pleomorphic, inconspicuous striations in the appendage close to the ascospore wall becomes evident by	At first a gelatinous sheath covers ascospores completely; later only a subglobose, faintly striate, subgelatinous appendages, covers each apex, at SEM level the exosporium ruptures and rolls back towards the spore apices	Initially subgelatinous enveloping the spore, later spread out from the wall but remains attached at the polar regions, the lower part swells fibers are straight near the point of attachment, the whole appendage is transformed into fibers, except for the part that was initially

Table 9. (Continued)

Characters	***R. maritima***	***R. stellata***	***R. quadriremis***	***R. pilleata***	***R. galerita***	***R. crispa***
	of the exosporium	inconspicuously striate by fiber-like elements embedded in subgelatinous matrix, fibrous with age		stains, fibrous with age	forming a sticky disc	attached to the side of spore, this portion becomes spoon-shaped tip of the appendage which remains smooth and uniform
References	Kohlmeyer and Kohlmeyer, 1979	Manimohan *et al*., 1993a; Hyde *et al*., 1992	Manimohan *et al*., 1993a	Kohlmeyer and Kohlmeyer, 1979; Hyde *et al*., 1992	Manimohan *et al*., 1993b; Tubaki, 1968	Kohlmeyer, 1981; Manimohan *et al*., 1993a, Hyde and Jones, 1989

Materials and methods

The genomic DNA of all fungi listed below (except for *R. pilleata* and *R. stellata*) was extracted by using CTAB lysis buffer. Since difficulty was encountered in the germination of *R. pilleata* and *R. stellata* spores, their ascomata were picked from wood and the DNA from the spore mass extracted by the microwave genomic DNA extraction method (see Chapter III).

Fungal studied	**Original code**	**BCC code**	**Origin**
Naufragella spinibarbata	PP6886	BCC16004	Unknown
Naufragella spinibarbata	JS75	-	Wales, UK
Remispora crispa	PP415	BCC15556	Unknown
Remispora galerita	PP5577	-	Unknown
Remispora maritima	LP64	-	Strandegarad, Denmark
Remispora pilleata 2	DEJ10_2	-	Jutland, Denmark
Remispora pilleata 1	DEJ10_1	-	Jutland, Denmark
Remispora quadriremis	JS196	BCC15555	Hong Kong
Remispora stellata	DEJ09	-	Jutland, Denmark

DNA amplification and sequencing

Large subunit rRNA gene was amplified using primers: LROR/LR7, JS1/JS8 and ITS5/LR7. The amplification profiles were performed as mentioned in Chapter III. PCR products were directly sequenced using the forward and reverse primers: JS1, JS5, JS8, LROR, LR7 and NL4R (Bunyard *et al*., 1994; Landvik, 1996).

Phylogenetic analysis

The LSU dataset was initially analyzed using all phylogenetic criteria: maximum parsimony, maximum likelihood and neighbor joining in PAUP* 4.0b10. However, all criteria gave the same topology, therefore in this chapter we present only the maximum parsimony analysis. The parameters for maximum parsimony were set: heuristic searches with a stepwise starting tree, a random stepwise addition of 10 replicates and TBR branch-swapping algorithm. Gaps were treated as missing data, fifth character state and excluded from the analysis. All gaps treatments gave the identical overall tree pattern, thus we included all gaps as the missing data in this chapter. Finally, bootstrapping analysis for maximum parsimony (Felsenstein, 1985) was performed using full heuristic search on 1,000 replicates (10 replicates of random stepwise addition of sequence and TBR branch-swapping algorithm).

Results

The dataset comprised 50 taxa from the Halosphaeriales and Microascales with the Xylariales as the outgroup. There are a total 1,831 characters, of which 1,013 characters were constant, 407 characters were parsimony informative and 411 variable characters were parsimony uninformative. Three insertion regions were observed, one at a position 835-1055 of *Halosarpheia trullifera*, *H. unicellularis*, *H. fibrosa, Morakotiella salina* (AY094182), *M. salina* (CY3437), *M. salina* (BCC12781), *Remispora stellata* (DEJ09) and *R. quadriremis* (JS196), the other at a position 1169-1264 in *H. fibrosa, Saagaromyces ratnagiriensis* and *H. unicellularis*, and the last region at a position 1271-1346 of *R. stellata* (DEJ09). Inclusion and

exclusion of these insertion regions had no effect on the tree topology for all analyses. Therefore, the insertion regions were included for all analyses.

The maximum parsimony yielded two MPTs with tree length, CI and RI of 1,924 steps, 0.590 and 0.648, respectively. The overall topology for all two MPTs was identical. One of the MPTs obtained from the K-H test was shown as the phylogram, with the bootstrap values above 50% (Figure 18). Our molecular results show that all six *Remispora* species are well placed in the Halosphaeriales. *Remispora maritima* (LP64), the type species of the genus, forms a clade with the two *R. pilleata* sequences with high support (100%). Two strains of *R. pilleata* are monophyletic (93%), and *R. quadriremis* (JS196) and *R. stellata* (DEJ09) are closely related as a basal group to the *R. pilleata/R. maritima* clade. The *R. maritima, R. pilleata, R. quadriremis* and *R. stellata* grouping forms a well supported subclade within the subclade comprising *Periconia prolifica/Okeanomyces cuculata/Aniptodera cheasapeakensis* and *Ascosacculus* taxa (89%) (Figure 18).

Remispora crispa (PP415) nestles with the *Morakotiella salina* sequences, *Panorbis viscosus* and *Nimbospora effusa* Jørgen Koch with weak statistical support. This group is distantly placed from the *Remispora* clade comprising the type species. *Remispora galerita* (PP5577) has no affinity to other *Remispora* species and constitutes a basal branch to the *Haligena elaterophora* sequences with moderate support (67%) (Figure 18). The two *Naufragella spinibarbata* isolates are well placed in the Halosphaeriales and are monophyletic with 90% bootstrap values. This genus is located in subclade comprising a number of genera but not with any closely related taxa (Figure 18).

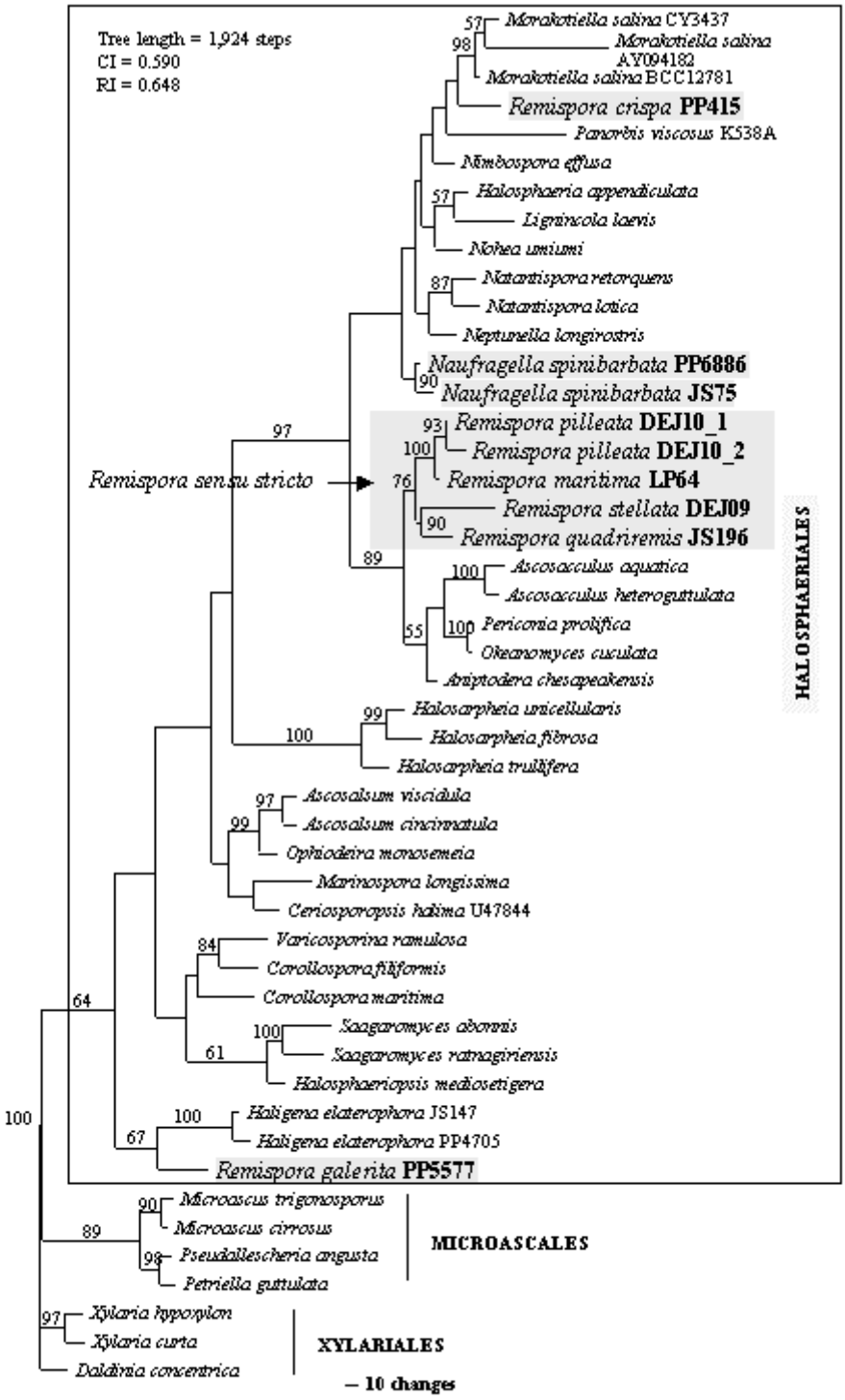


Figure 18. One of two MPTs inferred from LSU rRNA sequences of the genera *Naufragella* and *Remispora*, generated with maximum parsimony analysis. Bootstrap values higher than 50% from weighted parsimony are given above branches. Scale bar indicates 10 character state changes.

Discussion

The genomic DNA of *R. pilleata* and *R. stellata* extracted from the ascomata using the microwave technique provided enough template for a PCR reaction. The DNA sequencing results were satisfactory and provided enough informative characters for the phylogenetic analysis. I may therefore recommend this method as suitable for DNA extraction of slow-growing or unculturable fungi, however, care should be taken in the identification of the fungus, picking up the spore mass from the ascomata and the extraction process.

The molecular results demonstrate that the genera *Remispora* and *Naufragella* are well placed and correctly assigned to the Halosphaeriales. Both morphological and molecular data clearly confirm the removal of *Naufragella spinibarbata* from the genus *Remispora*, as it does not have any affinity with *Remispora* species. The unique ascospore appendage morphology of *N. spinibarbata* with two types of appendages: 1) long polar appendages stretching to form a band-like undulating appendage and 2) a mucilaginous sheath that fragments at the central septum into subpolar soft spines (Koch, 1989), can be used in the delineation of this genus.

Our molecular data confirms the distant phylogenetic relationships of *Remispora* with the genus *Halosphaeria* where it was previously referred to. *Halosphaeria appendiculata* always constitutes as a sister taxon with *Lignincola laevis*, while *Remispora* species are polyphyletic in origin. The type species, *R. maritima*, and its sister taxa, *R. pilleata, R. stellata* and *R. quadriremis* (Figure 18) can be regarded as *Remispora sensu stricto*. This grouping forms a well supported subclade with the *Periconia/Okeanomyces/Aniptodera/Ascosacculus* subclade (Figure

18). *Remispora* species *sensu stricto* share little features in common with this subclade of only cylindrical ascospore shape, but differ in appendage structure and its mode of development.

Although *Remispora sensu stricto* is a diverse assemblage of species with varation of ascospore appendage substructure and arrangement, they share similar morphological characteristics at the light microscope level: globose or subglobose, cream-colored to yellowish ascomata (except for *R. pilleata*), well-developed periphysate necks, clavate asci, pedunculate that deliquescing early, catenophyses present (not reported in *R. pilleata*), ellipsoidal, thin-walled ascospore (except thick-walled and rhomboid shape in *R. pilleata*), the polar pleomorphic appendages that are initially wrapped around the ascospores. The studies by Johnson *et al.* (1984) and Manimohan *et al*. (1993a) confirmed that the appendages in *R. maritima, R. stellata* and *R. quadriremis* (including *R. crispa*) consist of two elements a strand-like component embedded in a fibrillar matrix. Moreover, recent ultrastructural observations of *R. stellata* and *R. quadriremis* (including *R. crispa*) revealed that the strands are exuded through pores in the episporium, and it is unlikely that the appendages are exosporial in origin, while the origin of the fibrillar (mucilaginous) matrix is unresolved (Manimohan *et al*., 1993a).

Remispora pilleata appears to be different morphologically from other species by having dark ascomata. However, the ascoma color may be a variable character caused by the environmental conditions as Cavaliere and Johnson (1966) mention repeatedly that ascomata color is not important at the generic level in most Halosphaeriaceae. Although *R. pilleata* possesses rhomboid ascospores which are thick-walled and distinct from other species, this character may not be

phylogenetically important. *Remispora pilleata* is closely related to *R. maritima* with a high percentage of DNA similarity, estimated from pairwise comparison of the first 650 bases of the LSU sequences. The branch length of *R. pilleata* (DEJ10_2) is longer than another strain (DEJ10_1), which may be accounted for its shorter sequence.

Remispora stellata and *R. quadriremis* are closely related with high bootstrap support and 98% DNA similarity estimated by pairwise comparison. The ascospore appendages of these two species are very similar, particularly at the light microscope level, however, they differ ultrastructurally. In *R. stellata* each polar appendage is composed of 5-9 fibrous strands, each strand is 20-40 nm thick, appendages consisted of persistent strands embedded in a fibrillar matrix, relative straight and discrete near the spore poles but fused distally into bundles of radiating arms (Jones and Moss, 1978; Manimohan *et al*., 1993a). Polar appendages of *R. stellata* appear to be more fibrillar at both light microscope and ultrastructural levels due to the loss of the amorphous components (Johnson *et al.,* 1984). While, in *R. quadriremis* four fibrous strands in each polar appendage are observed, each strand is 30-70 nm thick, more compact and persistent in comparison with *R. stellata*, initially radiating from the pore pole, interconnected basally and separate from each other by the fibrillar matrix. Moreover, the fibers of both species formed in the same way by extrusion through pores in the polar episporium (Manimohan *et al*., 1993a).

Two *Remispora* species, *R. crispa* and *R. galerita*, differ at the molecular level from the main *Remispora sensu stricto* clade, both species are distantly placed in relation to the *Remispora* type species. Although appendages of *R. crispa* developed in the same way as for other species in exuding through pores in the polar

episporium (Manimohan *et al*., 1993a), but it differs from other *Remispora* species in the nature and arrangement of the strands in the appendages. Appendages of *R. crispa* initially subgelatinous envelop the ascospore, later spread out from the wall but remain attached at the polar regions, the lower part swells and parallel fibers become apparent that emerge fountain like from the thickened tip of the spore wall, the whole appendage is transformed into fibers, except for the part that was initially attached to the side of the spore, this portion becomes spoon-shaped at the tip of the appendage (Kohlmeyer, 1981; Hyde and Jones, 1989).

Remispora crispa constitutes a sister taxon to *Morakotiella* sequences and *Panorbis viscosus*, but they are not congeneric. Narrow, thread-like appendages of *M. salina* that formed as the outgrowth of the spore wall (Farrant and Jones, 1986) is different from *R. crispa*. Moreover, *Panorbis viscosus* also markedly differs from *R. crispa* in having hamate, uncoiling, thread-like appendages (Campbell *et al.,* 2003). Therefore, *R. crispa* can not be placed into any taxa, and new genus is proposed for this fungus.

Remispora galerita differs from other *Remispora* species in the distinct subglobose cap-like appendages that appear to be more compact, contains a greater number of strands than other species. At the SEM level, the exosporial polar appendages do not appear to separate from the spore wall, they do not completely ruptured and remain attached to one another (Manimohan *et al.,* 1993b). Moreover, the extremely sticky nature of the appendages of *R. galerita* was observed, and they adhere to any object they come to contact with (Hyde and Jones, 1989).

Remispora galerita groups in the same subclade as *Haligena elaterophora*, but they are not congeneric. *Haligena elaterophora* possesses long, wide, strap-like

appendages (Johnson *et al*., 1987), while pleomorphic, compact, fibrillar appendages are present in *R. galerita* (Manimohan *et al.,* 1993b). Therefore, *R. galerita* can not be placed into any taxon currently described, and the erection of new genus to accommodate it under consideration.

4.4 *Marinospora, Lautisporopsis, Ocostaspora, Nautosphaeria,* and *Carbosphaerella*

Introduction

4.4.1 *Marinospora and Lautisporopsis*

Marinospora was described by Kohlmeyer (1960), and two species have been described, *M. calyptrata* (Kohlm.) Cavaliere (the type species) and *M. longissima* (Kohlm.) Cavaliere (Kohlmeyer, 1962). This genus possesses 1-septate, hyaline ascospores with distinct primary polar and secondary equatorial appendages with cup-like structures at their apices (Johnson *et al.,* 1984). 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 (Figure 19a). In *M. calyptrata,* the length of primary and secondary appendages are equal (Johnson *et al.,* 1984).

Marinospora was reduced to synonymy with *Ceriosporopsis* Linder by Kohlmeyer and Kohlmeyer (1979), as the possession of a stromatic ascomata was not

considered sufficient for separating it from *Ceriosporopsis* (Kohlmeyer, 1971). However, Johnson *et al.* (1984) retained the genus, as ascospore appendage ontogeny at the ultrastructure level was distinct from that of *Ceriosporopsis*.

The ascospore walls of *Marinospora* species comprise 3 layers, and have both polar and equatorial appendages (Figure 19 a, b). These arise as outgrowths of the spore wall, the exosporium fragments remnants as cup-like at their tips (Johnson *et al.,* 1984; Jones, 1995). *Ceriosporopsis* species (*C. halima* Linder*, C. capillaceae* Kohlm.*, C. caduca* E. B. G. Jones and Zainal) 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*.

Lautisporopsis circumvestita (Kohlm.) E. B. G. Jones, Yusoff and S. T. Moss*,* initially described as *Halosphaeria circumvestita* Kohlm. (Kohlmeyer and Kohlmeyer, 1979), was later transferred to *Ceriosporopsis* (Kohlmeyer, 1972). Ascospore appendage ontogeny at the ultrastructural level is different from *Ceriosporopsis*, as the polar and equatorial appendages are not formed as outgrowths of the spore wall (Johnson *et al*., 1987; Yusoff *et al*., 1994). *Lautisporopsis circumvestita* posesses polar sticky, pad-like appendages, and equatorial appendages (Figure 19h, i), the sheath is mesosporial and episporial in origin with no collar at the base of the appendages (Yusoff, *et al.,* 1994). No exosporium has been observed for this species.

4.4.2 *Ocostaspora*

Ocostaspora apilongissima E. B. G. Jones, R. G. Johnson and S. T. Moss, a monotypic genus, was described and placed in the Halophaeriales by Jones *et al*. (1983a). This fungus possesses a unique ascospore appendage morphology with long irregular amorphous polar appendage (greater than 5 μm); with many equatorial appendages, spoon-shaped, with a fibrillar component (Figure 19c-e). The appendage ontogeny has been referred to as type III: appendages formed by fragmentation of the outer exosporial layer of the spore wall. The appendages are ontogenetically similar to those of *Haligena, Lanspora* and *Remispora* (Jones and Moss, 1987; Hyde and Jones, 1989; Jones, 1995).

4.4.3 *Nautosphaeria*

Nautosphaeria cristaminuta E. B. G. Jones, a monotypic genus, was described and referred to the Halophaeriales by 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 (Figure 19f). Ascospores are one-celled, ellipsoidal, hyaline, possess a tuft of bristle-like appendages at each end and four tufts around the equator (Figure 19g). 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 (Jones, 1995; Hughes, unpublished). 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).

4.4.4 *Carbosphaerella*

Carbosphaerella was described by Schmidt (1969a, b), and with two species: *C. pleosporoides* I. Schmidt (the type species) and *C. leptosporioides* I. Schmidt (Kohlmeyer and Kohlmeyer, 1979). The two species can be distinguished from each other by ascospore septation: *C. pleosporoides* with muriform ascospores, and *C. leptosporioides* with 3-septate ascospores. The central large cells are dark brown, with small apical cells that are hyaline or light brown. Ascospores are surrounded by a persistent gelatinous-like sheath (Figure 19j), which is reticulate or net-like in appearance at the SEM level. The sheath becomes fibrillar by the loss of the cross connections, except at the ascospore poles where it remains intact. The type of appendage ontogeny falls into type III: appendages formed by fragmentation of an exosporic layer that remains attached at the poles with the appendages becoming thread-like (Jones and Moss, 1978; Johnson *et al*., 1984; Jones and Moss, 1987; Jones, 1995; Borse and Pawar, 2001).

The objectives for this chapter are:

1). to test whether the genus *Marinospora* is monophyletic,

2). to verify whether *Marinospora, Lautisporopsis, Ocostaspora, Nautosphaeria* and *Carbosphaerella* are correctly assigned to the Halosphaeriales,

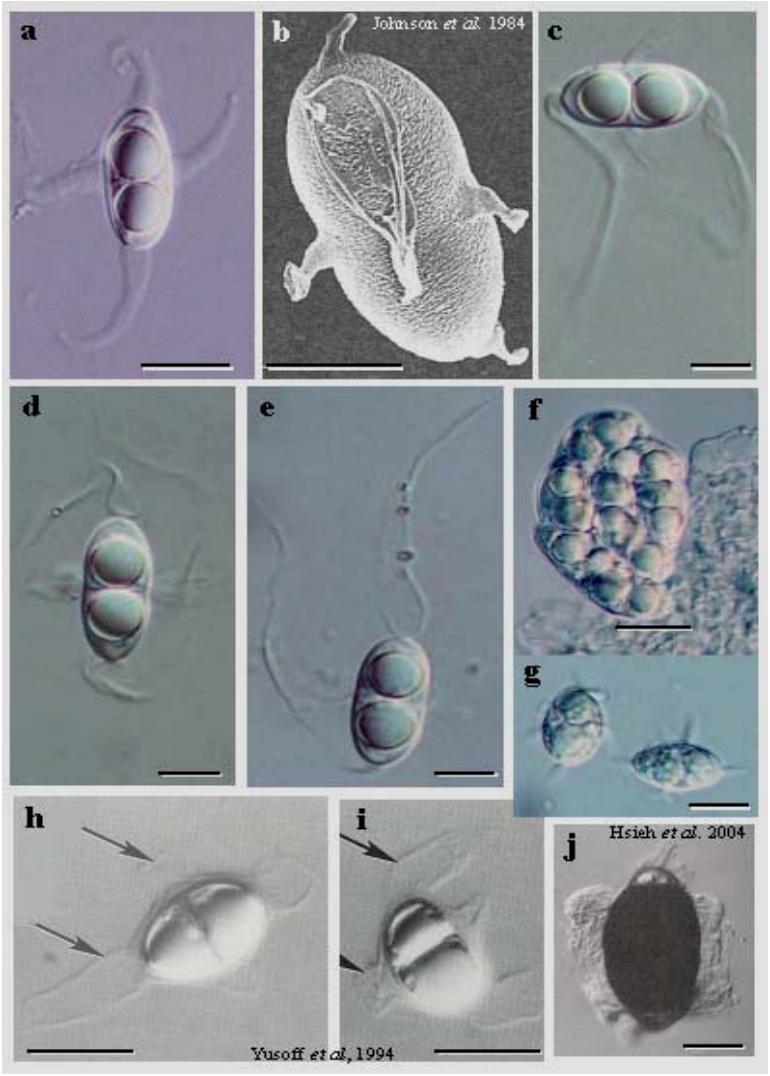


Figure 19. Morphological features of *Marinospora, Ocostaspora, Nautosphaeria*, *Lautisporopsis* and *Carbosphaerella*

a: Ascospore of *Marinospora longissima* with polar and equatorial appendages;
b: SEM of *M. calyptrata* ascospore with polar and equatorial appendages;
c-e: Ascospores of *Ocostaspora apilongissima* with spoon-shaped polar appendages with equatorial spines; f: Broad clavate ascus of *Nautosphaeria cristaminuta*;
g: Ascospores of *N. cristaminuta* with tufts of appendages; h,i: Ascospores of *Lautisporopsis circumvestita* with polar and equatorial appendages (arrowed);
j: Dark ascospore of *Carbosphaerella leptosporioides* with striate gelatinous-like sheath

Scale bars: a-j = 10 µm

3). to test if the appendage morphology can aid in the delineation of genera, and,

4). to examine their relationships to other taxa within the Halosphaeriales.

Materials and methods

The genomic DNA for all fungi listed below was extracted by using the CTAB lysis buffer as outlined in Chapter III.

Fungal studied	**Original code**	**BCC code**	**Origin**
Carbosphaerella leptosphaerioides	PP1774	-	Unknown
Carbosphaerella leptosphaerioides	JS183	BCC15532	China
Lautisporopsis circumvestita	CY3461	-	Friday Harbor, USA
Lautisporopsis circumvestita	LP8	-	Jutland, Denmark
Lautisporopsis circumvestita	LP49	-	Jutland, Denmark
Lautisporopsis circumvestita	LP27/1	-	Strandegarad, Denmark
Marinospora calyptrata	CY3491	-	Friday Harbor, USA
Marinospora calyptrata	JS207	-	Falington, Denmark
Nautosphaeria cristaminuta	JS121	-	Wales, UK
Ocostaspora apilongissima	LP53	-	Jutland, Denmark
Ocostaspora apilongissima	LP32	-	Jutland, Denmark
Ocostaspora apilongissima	CY3399	-	Friday Harbor, USA
Ocostaspora apilongissima	LP31/2	-	Strandegarad, Denmark

DNA amplification and sequencing

The LSU rRNA region was amplified using the primers: LROR/LR7, JS1/JS8 and ITS5/LR7, amplification profiles performed as outlined in Chapter III. PCR products were directly sequenced using the forward and reverse primers (JS1, JS5, JS8, LROR, LR7 and NL4R; Bunyard *et al*., 1994; Landvik, 1996).

Phylogenetic analysis

The LSU dataset was initially analyzed using all phylogenetic criteria: maximum parsimony, maximum likelihood and neighbor joining in PAUP* 4.0b10, and all gave the same topology. Therefore, in this chapter only the maximum parsimony analysis result are presented. The parameters for maximum parsimony were set: heuristic searches with a stepwise starting tree, a random stepwise addition of 10 replicates and TBR branch-swapping algorithm. Gaps were treated as missing data, fifth character state and excluded from the analysis. All gaps treatments gave an identical overall tree pattern, thus I include all gaps as the missing data in this chapter. Finally, bootstrapping analysis for maximum parsimony (Felsenstein, 1985) was performed using a full heuristic search on 1,000 replicates (10 replicates of random stepwise addition of sequence and TBR branch-swapping algorithm).

Results

The LSU dataset comprised 57 taxa representing taxa from the Halosphaeriales and Microascales, with the Xylariales as an outgroup. Four isolates of *Lautisporopsis circumvestita* and two isolates of *Carbosphaerella leptosporioides* were sequenced, but the result was not satisfactory. Two *L. circumvestita* strains (LP8, LP49) contained a long unalignable insertion (700 bases). The other two strains (CY3461, LP27/1) were not monophyletic, thus the culture identity is suspect. Sequencing result of two *C. leptosporioides* strains yielded many ambiguous bases. Therefore, these six sequences were excluded from the analysis. Only data for

Marinospora calyptrata, Ocostaspora apilongissima and *Nautosphaeria cristaminuta* are discussed here.

A total 1,834 characters, of which 987 characters are constant, 419 characters are parsimony informative and 428 variable characters are parsimony uninformative. The taxa sampling for the analysis in this chapter are the same as in the *Remispora* dataset. Two insertion regions were found in *O. apilongissima* sequences (LP32, LP53, LP31/2) at a position 835-1055 and 1169-1264.

Maximum parsimony analysis resulted in two identical MPTs with 2,137 steps long, CI = 0.568 and RI = 0.662. The best topology representing the highest likelihood scores estimated from the K-H test, with the bootstrap values above 50% is shown as a phylogram in Figure 20. Our molecular result shows that *Marinospora, Ocostaspora* and *Nautosphaeria* are well placed in the Halosphaeriales. Two isolates of *O. apilongissima* (LP53 and LP31/2) are monophyletic (93% support), and form a sister group to the *Morakotiella salina* strains with good support (86%). *Morakotiella/Ocostaspora* subclade is adjacent to *Remispora crispa* (PP415) with 58% bootstrap support (Figure 20).

Two *Marinospora calyptrata* sequences (JS207 and CY3491) are monophyletic (86%) and form a stable clade with *M. longissima* with moderate support (72%). These two species always form a consistent group to two *Ceriosporopsis halima* isolates without other related taxa. There is weak support for the two subclades comprising *Ascosalsum*/*Ophiodeira* and the *Marinospora*/*Ceriosporopsis* subclade (below 50%) (Figure 20).

Nautosphaeria cristaminuta forms a monophyletic group to *Remispora galerita* (PP5577) with strong support (98%), together with *Haligena elaterophora* as

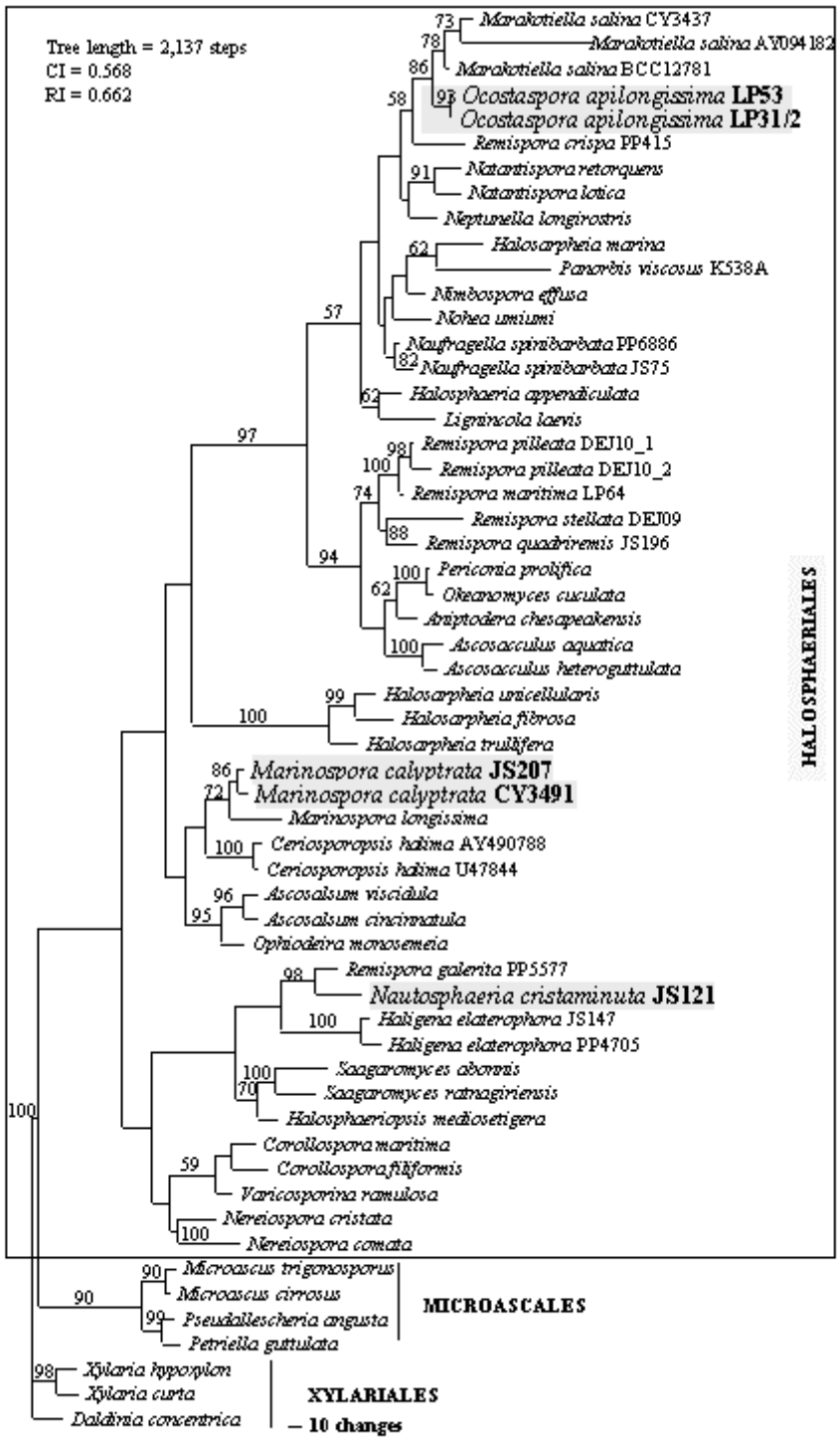


Figure 20. One of two MPTs inferred from LSU rRNA sequences *Marinospora*, *Ocostaspora* **and** *Nautosphaeria*, **generated with maximum parsimony analysis. Bootstrap values higher than 50% from weighted parsimony are given above branches. Scale bar indicates 10 character state changes.**

a sister subclade. The *Nautosphaeria/Remispora/Haligena* clade has *Saagaromyces abonnis* (Kohlm.) K. L. Pang and E. B. G. Jones*, S. ratnagiriensis* and *Halosphaeriopsis mediosetigera* (Cribb and J.W. Cribb) T.W. Johnson as a sister group but with a weak relationship. It clearly shows that *Nautosphaeria cristaminuta* has no affinity to any of the *Nereiospora* species, which are placed in a lower subclade of *Corollospora* species and *Varicosporina ramulosa* Meyers and Kohlm. (Figure 20).

Discussion

Marinospora calyptrata

Molecular results demonstrate that the genus *Marinospora* is well placed and correctly assigned to the Halosphaeriales, and supports the removal of this genus from *Ceriosporopsis* by Johnson *et al.* (1984). The morphological characteristics of these two genera are different. *Marinospora* differs in the stromatic ascomata, the ascospore walls composed of many layers and the distinct primary polar with a cup-like structure at the tip of the polar and the equatorial appendages (Johnson *et al.,* 1984).

The consistent adjoining subclade to *Marinospora* is *Ceriosporopsis halima,* but with low support (Figure 20). This may reflect their ascospore appendage ontogeny formed by a combination of a wall outgrowth and elaboration of the outer exosporial wall layer (Jones, 1995). Therefore, this appendage ontogeny type may be a stable character in the delineation of *Marinospora* and *Ceriosporopsis*. The

ascospore appendages of *Bovicornua intrica* Koch and Jones resemble those of *Ceriosporopsis* and *Marinospora* (Yosoff *et al*., 1993), and requires study at the molecular level. Currently no isolates are available of the species.

Marinospora is shown to be monophyletic in origin, and *M. calyptrata* and *M. longissima* are closely related. The only characters that have been used to distinguish these two species are ascospore size and the length of primary polar appendages (Johnson *et al.*, 1984). However, these characters may be variable as they depend on the maturity of the ascospores, the intermediate length of ascospores and appendages may cause a problem in species delineation. Whether these two taxa are conspecific remains to be further evaluated when more strains become available.

Ocostaspora apilongissima

Four strains of *Ocostaspora apilongissima* were sequenced, and all were monophyletic in the initial analysis. This confirms their culture identities. I include only two isolates as representatives in the final tree (Figure 20), in order to decrease conflict in the analysis. *Ocostaspora apilongissima*, is a monotypic genus, as are *Halosphaeriopsis* and *Halosphaeria* that also possess polar and equatorial appendages. However, they do not share morphological characteristics with *O. apilongissima*, particularly in appendage development and their molecular phylogeny.

Halosphaeriopsis mediosetigera has three lunate-shaped equatorial and cap-like appendages with a distinct substructure (Jones *et al.,* 1984; Jones and Moss, 1987; Kohlmeyer and Kohlmeyer, 1979), while *Ocostaspora* appendages have little or no substructure (Jones *et al*., 1984). The phylogenetic position of *H. mediosetigera* is

distantly placed from *O. apilongissima*, forming a basal clade within the Halosphaeriales, and a sister clade of *Saagaromyces* species.

Ascospores of *Halosphaeria appendiculata* possess polar and equatorial appendages, initially wrapped around the spore surface, they separate, extend and become spoon-shaped at the point of attachment, with a reticulate substructure. (Jones, *et al*., 1984; Jones and Moss, 1987). Phylogenetic it has no affinity with *O. apilongissima*, and forms a clade adjacent to *Lignincola laevis* (Figure 20).

Phylogenetically the only genus that shares the clade with *O. apilongissima* is *Morakotiella salina*, but they are not congeneric (Figure 20). The appendages of these two genera arise as the fragmentation of an exosporial layer of the spore wall (Jones, 1995), but *M. salina* has only polar appendages, while *O. apilongissima* has both polar and equatorial appendages. The polar appendages of both genera appear to be spoon-shaped at the point of attachment, but it is more distinct in *O. apilongissima* (Figure 19d, e). However, the unfurling polar appendages of *O. apilongissima* are not thread-like as in *M. salina*, but it is more ribbon-like. Although *Remispora crispa* and *O. apilongissima* nestle in the same clade, they are not congeneric and differ markedly in the arrangement of their appendages, and in particular their substructures.

Nautosphaeria cristaminuta

Our molecular result confirms that *Nautosphaeria cristaminuta* is well assigned to the Halosphaeriales. The appendage ontogeny of this genus falls into the same type as *Nereiospora* (Jones, 1995). However, Hyde and Jones (1989) opinioned that they were distinctly different and this is supported by the sequence data presented here.

Although *N. cristaminuta* groups consistently with *Remispora galerita* with good support, they are not congeneric. Morphologically they differ with one-celled ascospores, with tufts of polar and equatorial appendages that arise as the outgrowths of the spore wall in *N. cristaminuta* (Jones, 1995), while *R. galerita* has pleomorphic polar appendages that arise by the fragmentation of an exosporial layer (Johnson *et al.,* 1984). Thus morphological and molecular evidence confirm their placement in different genera.

Therefore, the unique appendage morphology of monotypic genera, *Ocostaspora* and *Nautosphaeria*, may represent the diversity of appendage morphology as the result of adaptation to aquatic habitats. Both morphological and molecular evidence confirm the independent placement of these fungi as monotypic genera. *Ocostaspora* may have recently evolved from common ancestors as opinionated by Jones (1995), and closely related to *Morakotiella salina* sequences, as indicated by short branch length (except for *M. salina* AY094182). A distinct long branch length of *Nautosphaeria* may refer to unique character state changes or lack of closely relatives which have not yet been evaluated at the molecular level.

4.5 Other fungi: *Bathyascus*, *Marinosphaera* and *Pedumispora*

Introduction

Bathyascus sp.

The genus *Bathyascus* is characterized by having hyaline, guttulate, one-celled ascospores that are filiform, straight or curved, often apically attenuate and thick-walled, without septa or apical chambers, unitunicate asci that are thin-walled, without apical apparatus, and deliquescing early (Hyde and Jones, 1987). Four species have been described: *B. vermisporus* Kohlm. (the type species), *B. avicenniae* Kohlm., *B. tropicalis* Kohlm. and *B. grandisporus* K. D. Hyde (Hyde and Jones, 1987). *Bathyascus* species differ morphologically in ascomata size, peridium thickness, the presence of periphyses, ascospore demensions and the substrata they grow on (Hyde and Jones, 1987).

Kohlmeyer and Kohlmeyer (1979) and Kohlmeyer (1986) referred *Bathyascus* to the Halosphaeriales because ascomata are ostiolate, the centrum with pseudoparenchymatous tissue, and asci that are thin-walled, unitunicate, lacking an apical pore and deliquescing early. However, its placement in the order has been questioned (Jones, 1995), and its taxonomic position in this respect needs to be evaluated with sequence analysis.

The *Bathyascus* isolate used in this study was isolated from a mangrove twig, collected at Three Fathom Cove, Hong Kong, and possessed elongate-fusiform ascospores. This species cannot be identified with confidence due to insufficient material for study.

Marinosphaera mangrovei

Marinosphaera mangrovei has been found commonly on decayed mangrove wood. It is characterized by immersed thin-walled perithecia with long bushy necks extending beyond the substratum surface, absence of a clypeus, paraphyses that are wide and evenly septate, unbranched and lacking a gel, cylindrical asci possess a subapical structure but no ascus pore illustrated, and ascospores initially unicellular, becoming 3-septate later, the spore wall distinctly ornamented, and the spores usually full of oil globules (Figure 21) (Hyde, 1989a; Read *et al*., 1995).

Hyde and Nakagiri (1992) in comparing *Marinosphaera* with the marine genus *Swampomyces* noted similarities in ascospore morphology (Read *et al*. 1995). However, at the ultrastructural level, they differ with respect to ascus structure and morphology of the paraphyses. In *S. armeniacus* (the type species), the asci possess an apical fibrous thickening which is not apparent in *M. mangrovei*. Paraphyses in *M. mangrovei* are chain-like, simple, regularly septate and not embedded in a gel, while in *S. armeniacus* they branch profusely, are filamentous and in a gel (Kohlmeyer and Volkmann-Kohlmeyer, 1987; Hyde and Nakagiri 1992; Read *et al*., 1995; Abdel-Wahab *et al*., 2001a). Moreover, the ascospore wall is different; *M.*

mangrovei lacks an exosporium, as demonstrated at the TEM level, while the origin of the spore wall in *S. armeniacus* remains unresolved (Read *et al*., 1995).

The taxonomic placement of *Marinosphaera mangrovei* is difficult, as no suitable family can be found. The genus was placed, with reservation, in the Phyllachoraceae. However, it differs from most of the Phyllachoraceae members in having four-celled ascospores, wide paraphyses, no clypeus and a marine mode of life (Hyde, 1989a), while the Phyllachoraceae mostly posses one-celled ascospores, narrow paraphyses, presence of a clypeus and are biotrophic on plants (Barr, 2001; Samuels and Blackwell, 2001).

Pedumispora rhizophorae

Pedumispora rhizophorae K. D. Hyde and E. B. G. Jones, an intertidal marine ascomycete frequently occurring on decayed fruits, prop roots and twigs of *Rhizophora apiculata* Tamil Nadu (Hyde and Jones, 1992). This genus is characterized by brown to black pseudostroma covering the wood surface (Figure 22a), with erumpent pustules containing 1-4 immersed ascomata, unitunicate asci, without an apical apparatus and filiform ascospore with wall striations which run along the length of the ascospores (Figure 22b-d), tapering toward each end, 13-17 septate, bearing non-septate tips, which are curved or hook-shaped and inflated (Hyde and Jones, 1992). *Pedumispora rhizophorae* resembles other marine ascomycetes with scolecosporous ascospores, such as *Lindra*, *Lulworthia* and *Linocarpon* Syd. and P. Syd. (Hyde and Jones, 1992).

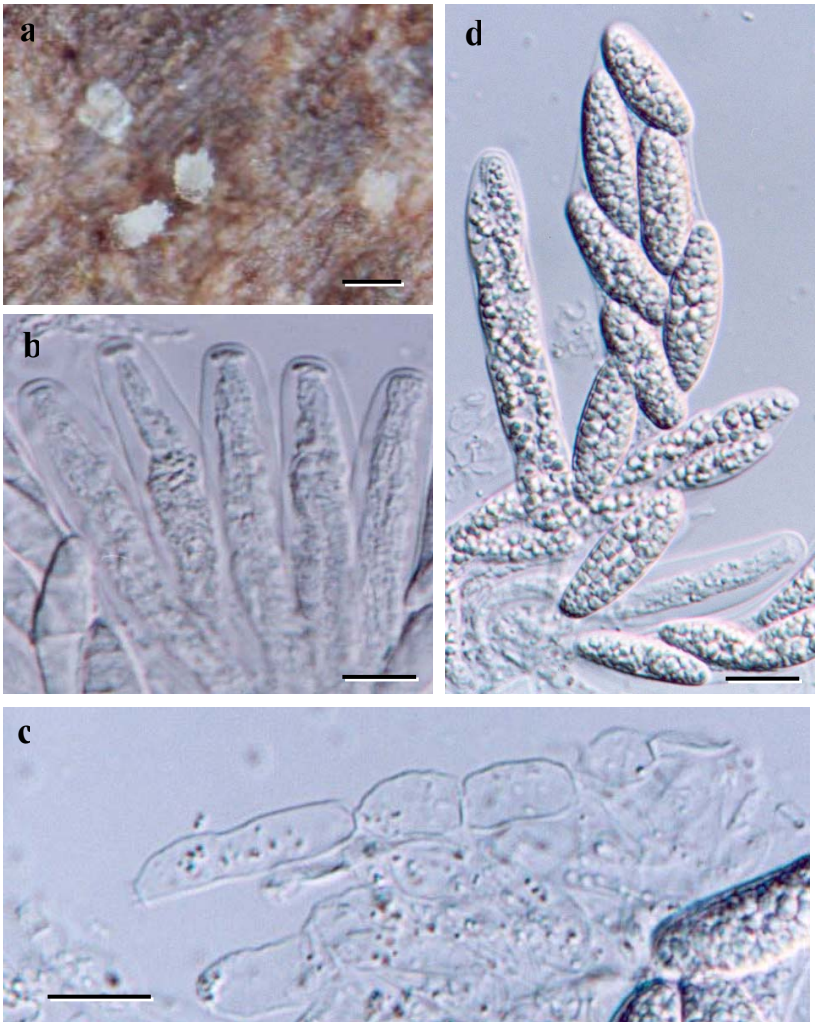


Figure 21. Morphological features of *Marinosphaera mangrovei*

a: The immersed ascomata with long necks on the wood surface; b: The cylindrical asci with subapical structures; c: The ornamented ascospores full of oil globules; d: Wide-septate, chain-like paraphyses

Scale bars: a = 200 µm; b = 20 µm; c, d = 10 µm

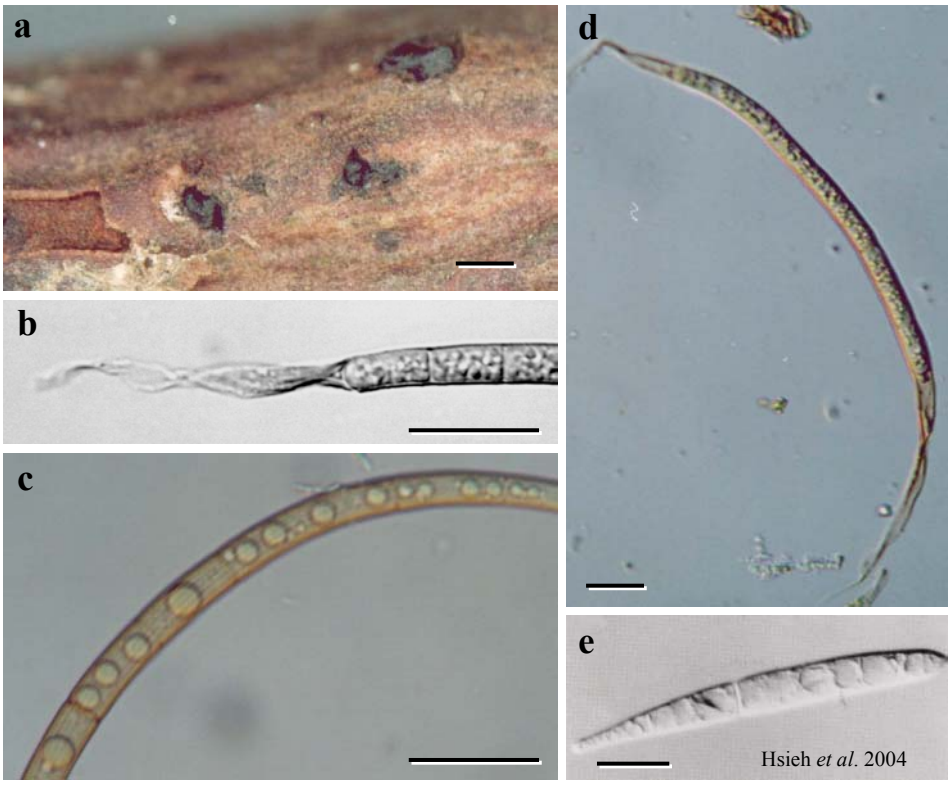


Figure 22. Morphological features of *Pedumispora rhizophorae* and *Bathyascus tropicalis*

a-d *P. rhizophorae* a: The exposed region of ascomata on wood surface; b, d: The filiform ascospores tapering towards both ends; c: The longitudinal striations of the ascospore; e: Ascospore of *B. tropicalis*

Scale bars: a = 1 mm; b-d = 20 µm; e = 15 µm

However, *Lindra* and *Lulworthia* differ from *Pedumispora* in the immersed ascomata and not in a group beneath raised pustules, while the ascospores lack pigmentation, wall striations and a crook-like apical region (Kohlmeyer and Kohlmeyer, 1979). *Linocarpon* also differs in the immersed ascomata that are lenticular in section and cylindrical asci with a ring-like apical apparatus (Hyde, 1988). Thus the taxonomic placement of *Pedumispora* is unclear, with Hyde and Jones (1992) assigning it to the Melanconidaceae, Diaporthales.

The objective for this chapter is to evaluate the taxonomic relationships of the genera: *Bathyascus*, *Marinosphaera* and *Pedumispora*.

Materials and methods

The genomic DNA of all fungi listed below was extracted by using CTAB lysis buffer.

Fungal studied	**Original code**	**BCC code**	**Origin**
Bathyascus sp.	JS206	-	Hong Kong
Marinosphaera mangrovei	JS172	BCC16549	Ranong, Thailand
Pedumispora rhizophorae	JS205	-	Guam, Micronesia, USA

DNA amplification and sequencing

The small and large subunit rRNA gene were amplified and sequenced with the primers listed below (White *et al*., 1990; Bunyard *et al*., 1994; Landvik, 1996).

	PCR primers	Sequencing primers
Bathyascus sp.	NS1/NS6, LROR/LR7, JS1/JS8	NS1, NS3, NS5, LROR, JS1, JS5
Pedumispora rhizophorae	NS1/NS6, LROR/LR7, JS1/JS8	NS1, NS2, NS3, NS4, NS5, NS6, LROR, JS1, JS5, LR5, LR7
Marinosphaera mangrovei	NS1/NS6, JS1/JS8	NS1, NS3, NS5, JS1, JS5, LR5

Phylogenetic analysis

All datasets were initially analyzed using all phylogenetic criteria: maximum parsimony, maximum likelihood and neighbor joining in PAUP* 4.0b10, and all gave the same topology. Therefore, in this chapter only the maximum parsimony analysis results are presented. The parameters for maximum parsimony were set: heuristic searches with a stepwise starting tree, a random stepwise addition of 10 replicates and TBR branch-swapping algorithm. Gaps were treated as missing data, fifth character state and excluded from the analysis. All gaps treatments gave an identical overall tree pattern, thus I include all gaps as the missing data in this chapter. Finally, bootstrapping analysis for maximum parsimony (Felsenstein, 1985) was performed using a full heuristic search on 1,000 replicates (10 replicates of random stepwise addition of sequence and TBR branch-swapping algorithm).

Results

Bathyascus sp.

The overall position of *Bathyascus* sp. was initially evaluated by SSU sequences, the dataset comprised taxa from 14 pyrenomycetes orders with

Sporobolomyces blumeae M. Takash and Nakase as an outgroup. A total of 2,696 characters were included, of which 1,687 were constant, 564 were parsimony uninformative and 445 characters were parsimony informative. The maximum parsimony resulted in ten identical MPTs of 2,855 steps long, CI = 0.504 and RI = 0.699. One of the MPT representing the best topology estimated from the K-H test with bootstrap higher than 50% is presented in Figure 23.

Bathyascus sp. is distantly placed from the Halosphaeriales and Lulworthiales. It nestles in the *Ophioceras* clade (a freshwater ascomycetes with scolecosporous ascospores) with 100% support (Figure 23). The cluster of *Ophioceras* species and *Bathyascus* sp. shows high step changes as can be seen from a long branch length. The taxa sampled from *Ophioceras* comprise two related freshwater genera, *Pseudohalonectria* Minoura and T. Muroi and *Gaeumannomyces graminis*, which form one major clade as a sister group of the Ophiostomatales (Figure 23).

The large subunit rRNA gene was sequenced and combined with the SSU data. This dataset contained more parsimony informative sites (total 3,374 characters, of 2,158 characters are constant, 602 parsimony uninformative sites and 614 parsimony informative sites). Maximum parsimony yielded two identical MPTs of 3,208 steps long, CI = 0.538, RI = 0.583, with the best topology estimated from the K-H test and presented in Figure 24. For all analyses, *Bathyascus* sp. is distantly placed from the Halosphaeriales and Lulworthiales. It groups consistently with *Ophioceras* sequences (100% support) together with *Pseudohalonectria* in the same major clade, and as a basal clade of the tree.

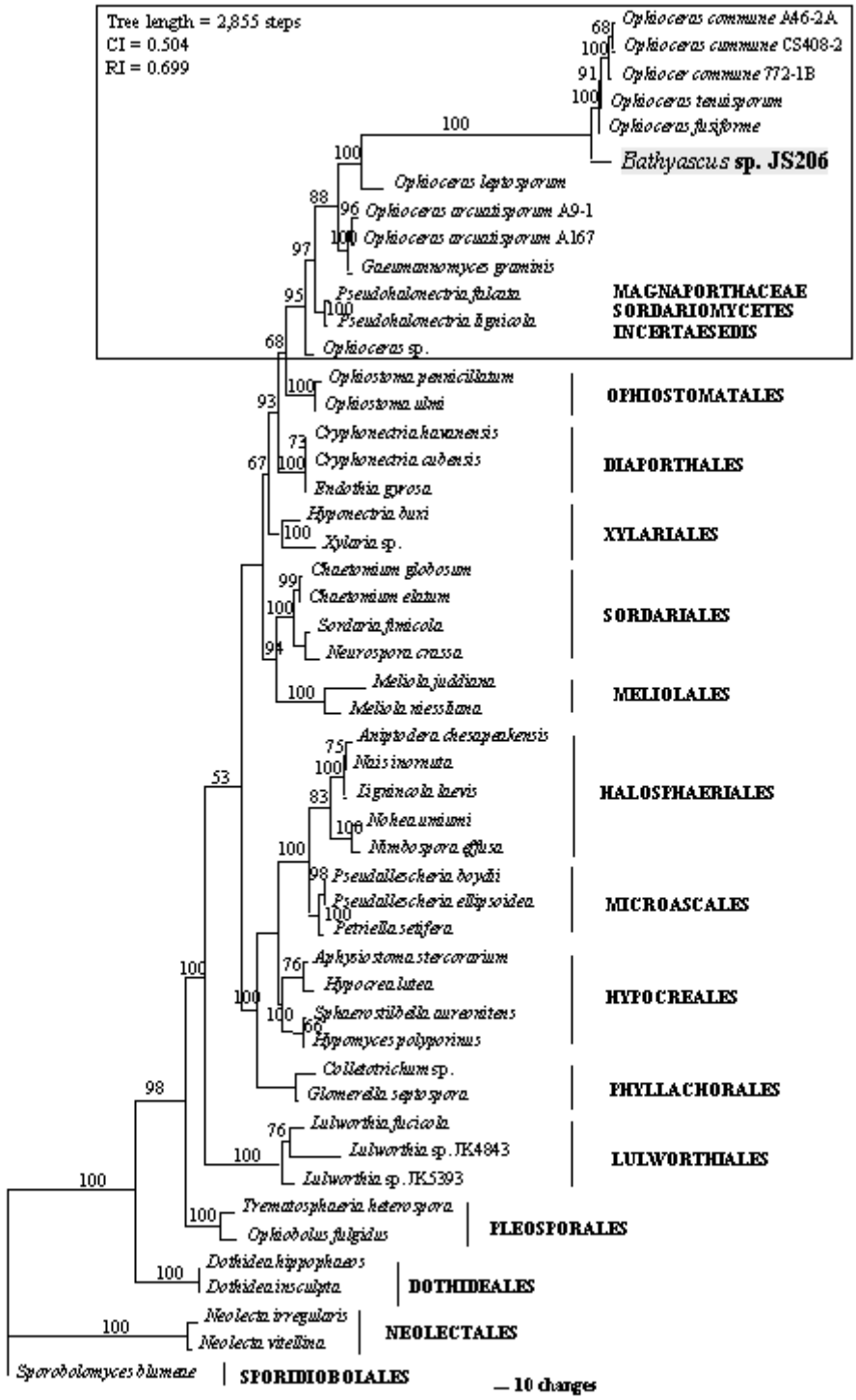


Figure 23. One of ten MPTs inferred from SSU rRNA sequences of *Bathyascus* sp., generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

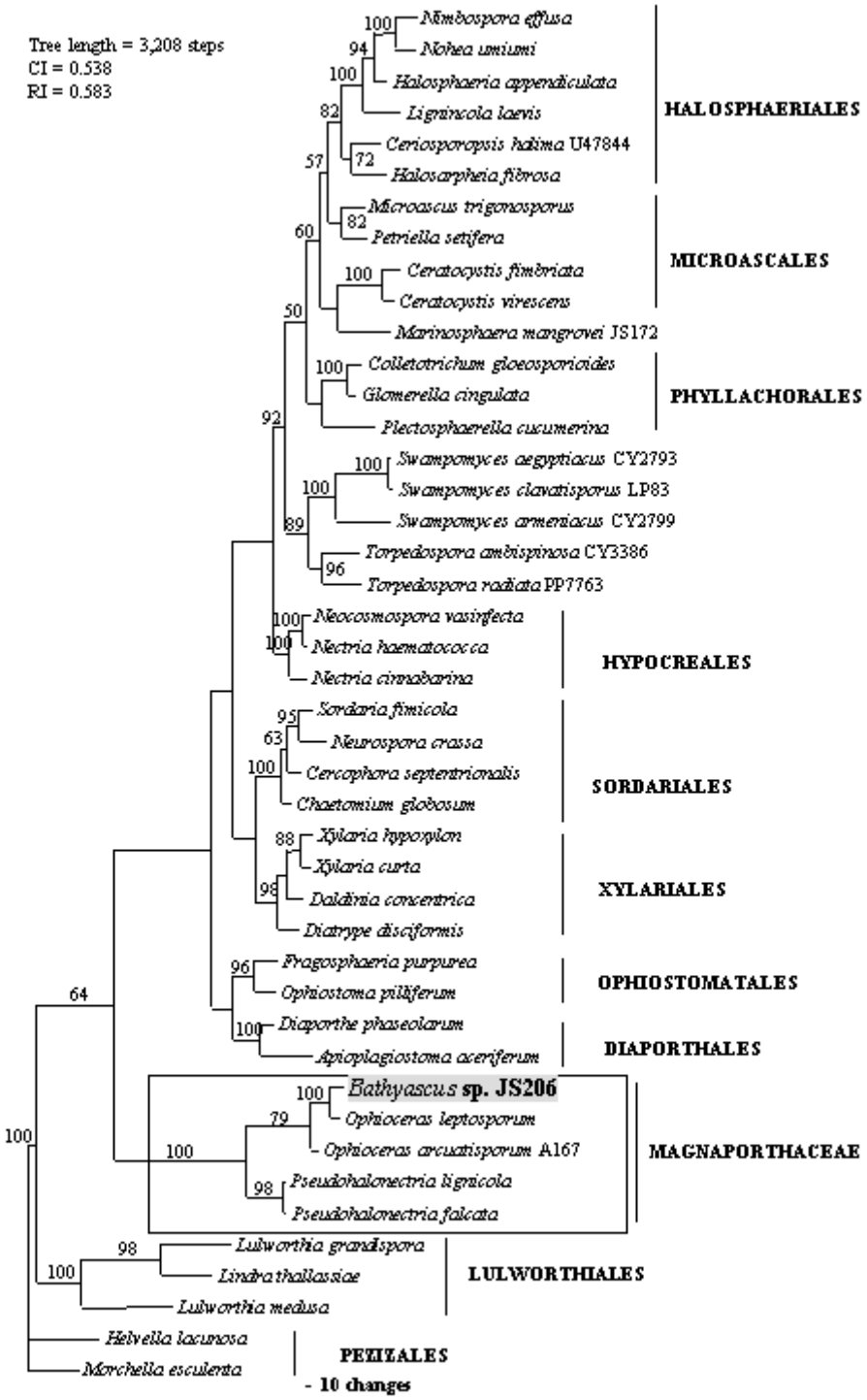


Figure 24. One of two MPTs inferred from combined SSU+LSU rRNA sequences of *Bathyascus* sp., generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

Marinosphaera mangrovei

Sequences of the SSU region were aligned with 63 taxa from ten pyrenomycete orders, in order to examine its relationship within the Ascomycota. The Pleosporales was chosen as the outgroup. A total of 2,729 characters were included, of which 1,501 were constant, 845 were parsimony uninformative and 383 characters were parsimony informative. Maximum parsimony analysis produced 36 equally MPTs of 2,108 steps long, CI = 0.687 and RI = 0.745, and the best topology compared from the K-H test is presented in Figure 25. The position of *Marinosphaera mangrovei* is unstable with weak bootstrap support (below 50%), and is basal to the Halosphaeriales. *Marinosphaera* also forms a long branch length as a sister clade to the Microascales. It is distantly related to the Phyllachorales (as initially thought) and to *Swampomyces*, a genus it morphologically most closely resembles.

Analysis of the LSU data with an increased number of sequences for the Phyllachorales yielded two equally MPTs with tree length of 858 steps, CI= 0.589 and RI= 0.696. One of the MPTs presented as the best evolutionary hypothesis from the K-H test is shown in Figure 26. The result supports the earlier data that *Marinosphaera mangrovei* is distantly placed from the Phyllachorales. It groups as a sister taxon to the Halosphaeriales and the *Swampomyces*/*Torpedospora* clade, with moderate support (69%).

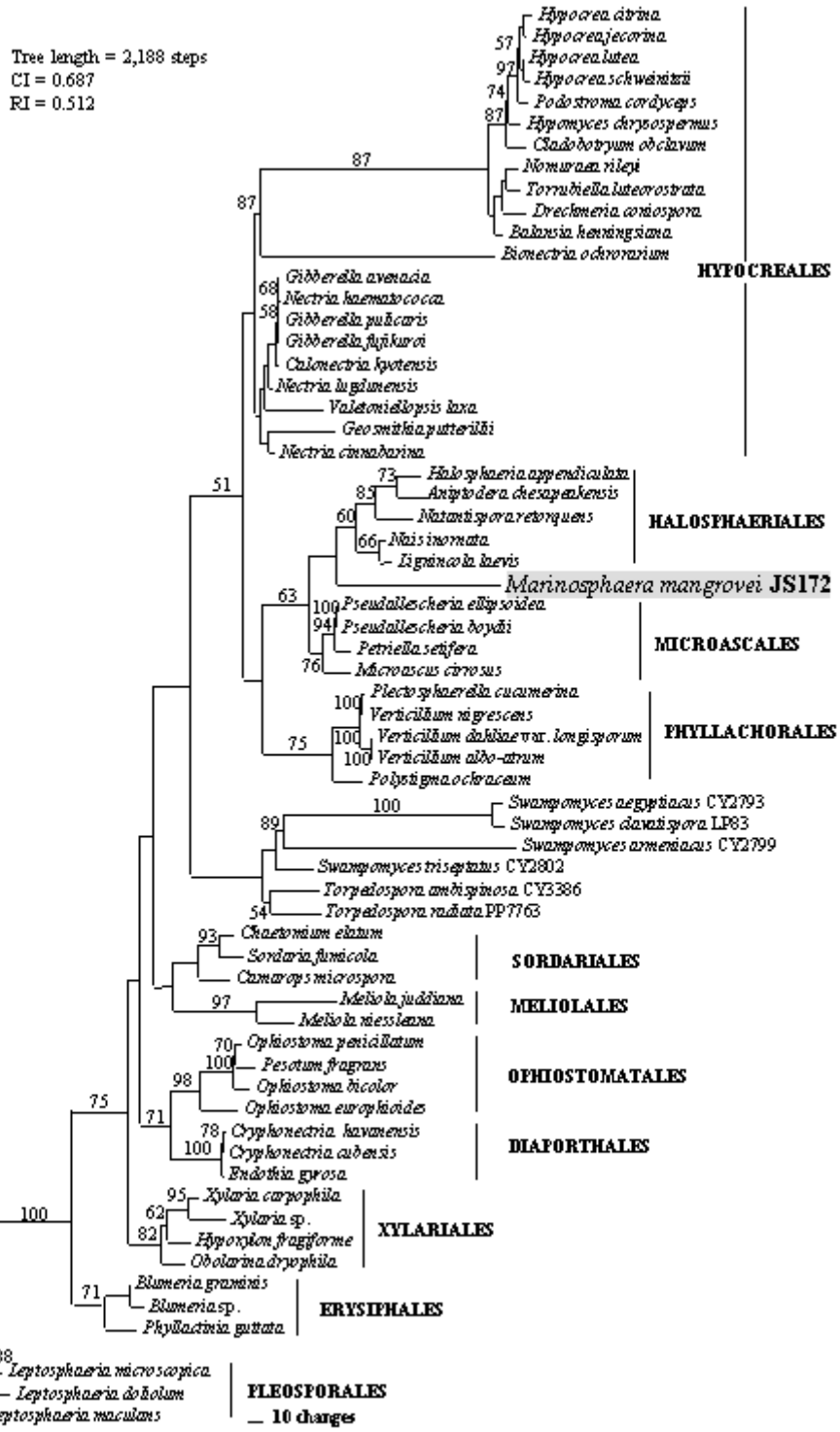


Figure 25. One of 36 MPTs inferred from SSUrRNA sequences of *Marinosphaera mangrovei*, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

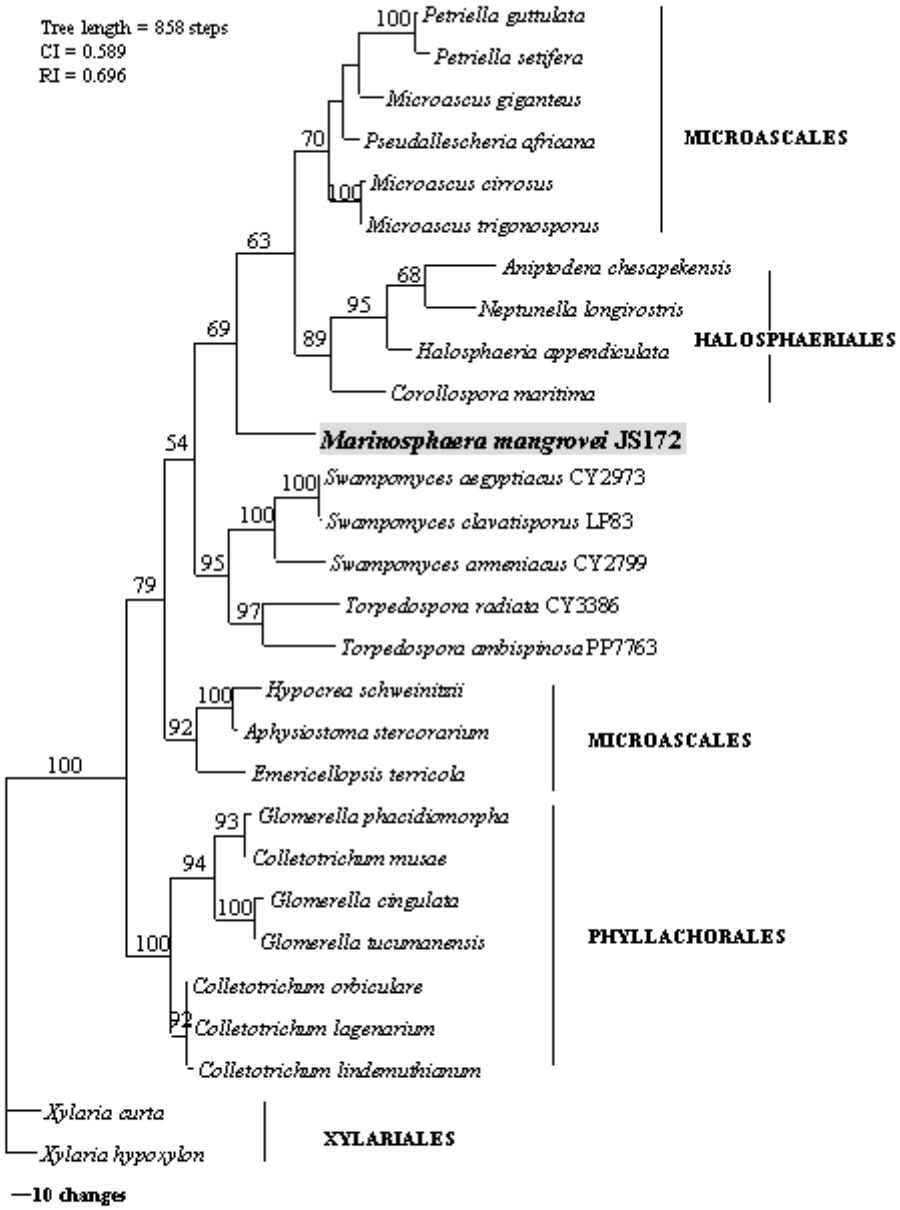


Figure 26. One of two MPTs inferred from LSU rRNA sequences of *Marinosphaera mangrovei*, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

An analysis of the combined SSU and LSU sequences with a total 3,374 characters, of which 2,158 are constant, 602 are parsimony uninformative and 614 are parsimony informative. Two MPTs were produced (tree length = 3,208 steps, CI = 0.538, RI = 0.583), and the best tree resulted from the K-H test is shown in Figure 27. *Marinosphaera mangrovei* forms a distinct branch with *Ceratocystis fimbriata* and *C. virescens* but without any supportive bootstrap values, and groups between the Microascale and Phyllachorales. Exclusion of the *Ceratocystis* sequences from the analysis resulted in a similar topology to the SSU phylogeny that *M. mangrovei* settles as a sister clade to the Halosphaeriales and Microascales (Figure 28).

Pedumispora rhizophorae

Small and large subunit rRNA of *Pedumispora rhizophorae* were sequenced, but the quality of the SSU sequence was not satisfactory with many ambiguous bases. Therefore, only the LSU phylogeny is discussed in this Chapter. The nearest matches from a BLAST search for the LSU of *Pedumispora rhizophorae* was with xylariaceous taxa. *Pedumispora* sequence was incorporated into a dataset comprising representatives of the Diaporthales, Halosphaeriales, Hypocreales, Microascales and Xylariales, with the Pleosporales as an outgroup. The first dataset analyzed comprised many xylariaceous taxa and resulted in equally 20 MPTs, 1,888 steps long, CI = 0.392, RI = 0.623. One of the MPT s representing the best topology obtained from the K-H test is shown in Figure 29. *Pedumispora rhizophorae* forms a clade with *Libertella blepharis* A. L. Sm. (a mitosporic fungus) with 62% support, within the family Diatrypaceae, Xylariales.

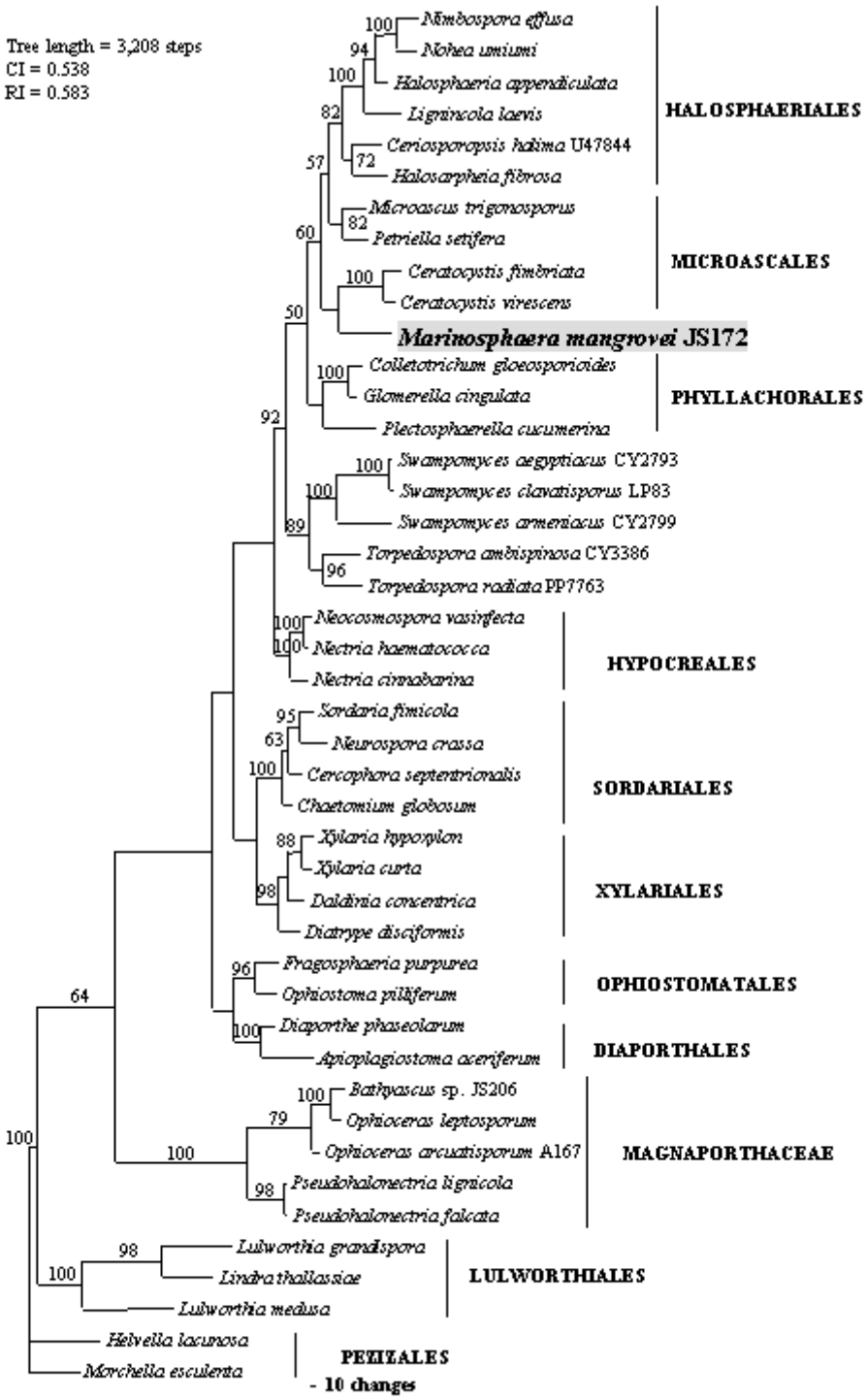


Figure 27. One of two MPTs inferred from combined SSU+LSU rRNA sequences of *Marinosphaera mangrovei* with *Ceratocystis* sequences, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

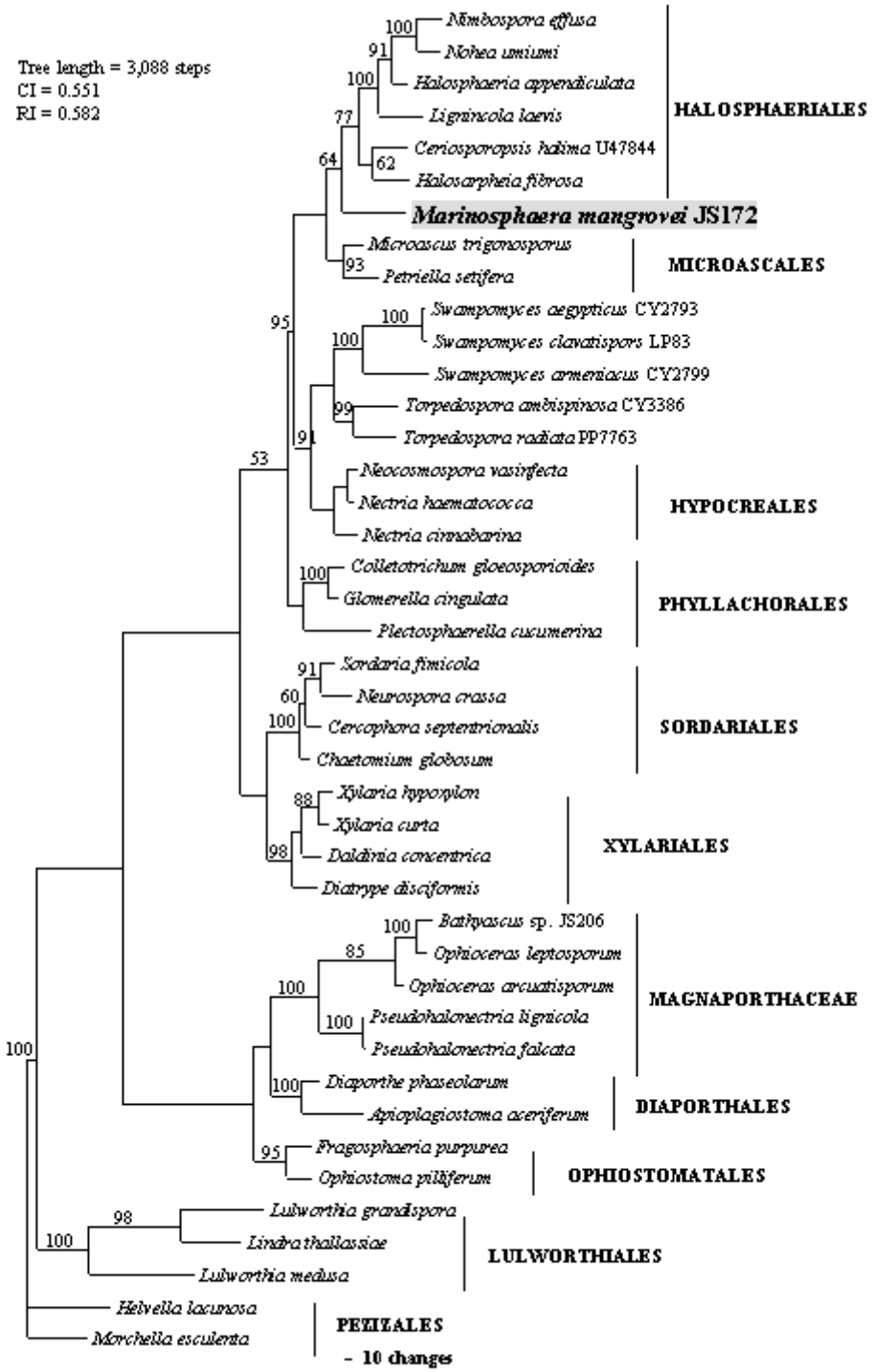


Figure 28. One of two MPTs inferred from combined SSU+LSU rRNA sequences of *Marinosphaera mangrovei* without *Ceratocystis* sequences, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

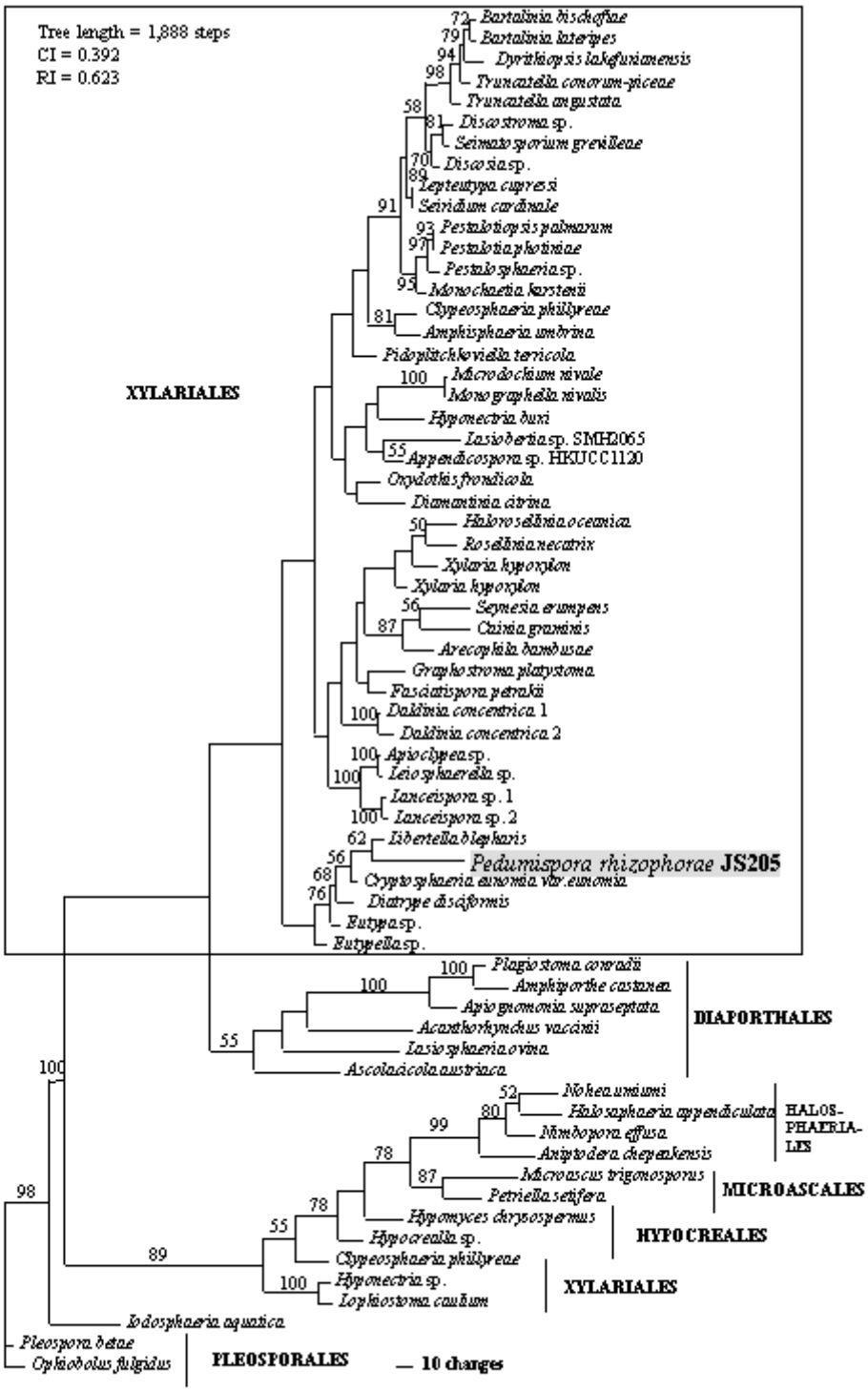


Figure 29. One of 20 MPTs inferred from LSUrRNA sequences of *Pedumispora rhizophorae*, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

There are too many xylariaceous taxa in the dataset with weak support, as indicated by the low CI of only 0.392, therefore the number of unrelated taxa were reduced in the subsequent analysis. The resulting shortened dataset gave a single MPT (Figure 30) with higher statistical support (tree length = 819 steps, CI= 0.573, RI = 0.685). *Pedumispora rhizophorae* still groups as a long branch length with *Libertella blepharis* but with low support (51%). From this LSU phylogeny, *P. rhizophorae* has no affinity with the Diaporthales or any of the other marine ascomycetes, e.g. the Halosphaeriales or Lulworthiales (Figures 36, 37).

Discussion

Bathyascus sp.

Bathyascus sp. was isolated from Hong Kong, and cannot be fully identified due to the lack of material for morphological investigation. From the molecular results, this isolate of *Bathyascus* sp. does not have affinity with the Halosphaeriales where it was initially referred to. It has no affinities with the Lulworthiales, although both have scolecosporous ascospores. However, *Bathyascus* lacks end chambers which is a feature of the genus *Lulworthia.* It is well placed within the *Ophioceras* cluster comprising *Ophioceras, Pseudohalonectria* and *Gaeumannomyces*.

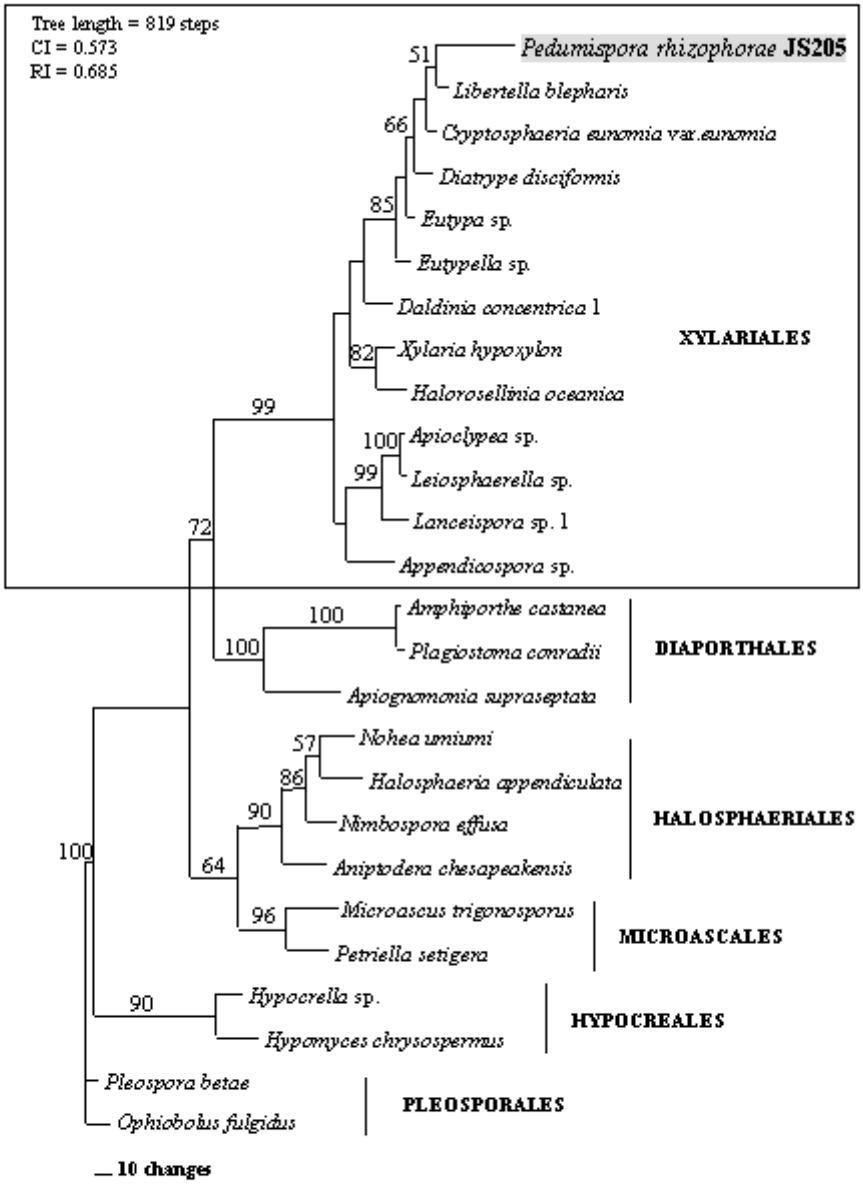


Figure 30. A single MPT inferred from LSU rRNA sequences of *Pedumispora rhizophorae* with less taxa of the Xylariales, generated with maximum parsimony analysis. Bootstrap values higher than 50% are given above branches. Scale bar indicates 10 character state changes.

Ophioceras and *Pseudohalonectria* have morphological characters of both the Diaporthales (perithecia with long beak, deciduous asci: asci which separate from the hymenial layer) and Sordariales (peridium of *textura angularis* and hypersaprobic habit) (Shearer *et al*., 1999). Conway and Barr (1977) and Shearer (1989) favored placement of these two genera in the Sordariales, however, the molecular study by Chen *et al.* (1999) indicated that both genera are more related to the Ophiostomatales than to species in the Diaporthales and Sordariales. At present *Ophioceras, Pseudohalonectria* and the closely related *Gaeumannomyces graminis* are tentatively placed within a newly-established family, Magnaporthaceae, Sordariomycetes *incertae sedis* based on morphological and molecular data (Cannon, 1994; Chen *et al.,* 1999).

Bathyascus sp. shares common feature with *Ophioceras* species of having filiform or scolecosporous ascospores. However, they differ as the genus *Ophioceras* has deciduous asci with an apical apparatus, presence of long tapering paraphyses, whereas *Bathyascus* sp. has thin-walled asci without apical apparatus, early deliquescing and absence of paraphyses (Hyde and Jone, 1987).

All *Bathyascus* species share common morphological characters with the Halosphaeriales (Kohlmeyer and Kohlmeyer, 1979; Hyde and Jones, 1987). However, there are differences particularly in the size of ascocarps, peridium thickness, ascus, ascospore dimensions, their substrata and habitats (Hyde and Jones, 1987). Therefore, the placement and affinity of these four *Bathyascus* species requires further investigation.

Marinosphaera mangrovei

The results presented here confirms that *Marinosphaera mangrovei* does not have affinities with the Phyllachorales, or the *Swampomyces/Torpedospora* clade. Phylogenetically *M. mangrovei* is located between the Halosphaeriales and Microascales but without any closely related taxa. However, *M. mangrovei* is clearly distinguished from the Halosphaeriales and Microascales by the presence of paraphyses, persistent cylindrical asci that possess a subapical plate (Hyde, 1989a).

Although *Ceratocystis* sequences groups with *M. mangrovei*, they differ in possessing delequescent asci, ovoid, rectangular or hat-shaped ascospores, and are insect associated (Alexopoulos *et al*., 1996), while *M. mangrovei* possesses persistent asci with a subapical structure and 3-septate ascospores (Hyde, 1989a). Nevertheless, the phylogenetic position of *Ceratocystis* species in this study is in concordant with Spatafora *et al.* (1998) result, as it is placed as a basal clade to the Microascales. *Marinosphaera* may have shared common ancestors or given rise from *Ceratocystis*, this result is consistent with the hypothesis that the arthropod-associated ascomycete may have given rise to fungi that adapt to marine environments (Spatafora *et al.*, 1998).

The true affinities of this fungus may not be resolved at this time, as there are no further closely related taxa to compare it with. Thus, more ascomycete taxa from other habitats especially from the mangrove environments should be collected, studied, described and sequenced. Further sequences of other DNA regions should be studied along with beta-tubulin or RPB2 genes.

Pedumispora rhizophorae

From the LSU phylogeny, *Pedumispora rhizophorae* is distantly placed from the major marine ascomycetes, Halosphaeriales and Lulworthiales. The morphological characteristics outlined earlier supports this conclusion. Similarly *P. rhizophorae* cannot be assigned to the Diaporthales, as initially suggested by Hyde and Jones (1992). *Pedumispora rhizophorae* shows affinity with the Xylariales and in particular with the Diatrypaceae. However, *P. rhizophorae* shares a few morphological features in common with the Xylariales: the presence of a pseudostroma covering the wood surface, presence of paraphyses and the pigmented ascospores.

There are many differences between *Pedumispora rhizophorae* and the Xylariales. Xylariaceous taxa, especially the Diatrypaceae, mostly possess dark perithecia embedded in a stroma, persistent asci usually with an apical ring, the asci are sometimes polysporous, the club-shaped to cylindrical unicellular colored ascospores (Alexopoulos *et al*., 1996), whereas the asci of *Pedumispora rhizophorae* are irregularly fusiform lack of an apical apparatus and ascospores are multiseptate long with inflated tip. No clear phylogenetic relationships can be advanced for *P. rhizophorae* and further species and DNA regions need to be sequenced.