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Marine fungal lineages in the *Hypocreomycetidae*

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ABSTRACT

Phylogenetic analyses of DNA sequences from protein coding and ribosomal nuclear loci support the placement of a number of marine fungal species associated with a well-supported clade containing fungicolous species of *Melanospora* and wood inhabiting *Coronophorales*. Three subclades containing marine species were recovered including *Torpedospora radiata* plus *T. ambispinosa*, *Swampomyces* species plus *Juncigena adarca*, and two *Etheirophora* species plus additional *Swampomyces* species. The monophyly of these three subclades, as well as a subclade containing representatives of *Coronophorales* and *Melanospora*, is well supported statistically. However, relationships among the different subclades remain largely unresolved. A sister relationship for this group with *Hypocreales* is significantly supported by Bayesian and ML methods. In addition to the *Halosphaeriales* and *Lulworthiales*, this clade, which is characterized by considerable morphological and ecological diversity, represents a third major clade of marine *Sordariomycetes*.

Introduction

The marine genera *Torpedospora* and *Swampomyces* have been placed within *Sordariomycetes incertae sedis* in the most recent classification of *Ascomycota* (Eriksson 2006). *Torpedospora radiata*, the type species of the genus, was first described from yellow pine wood submersed in seawater (Meyers 1957). It produces dark coloured perithecia with elongated, early deliquescing asci and paraphyses growing irregularly through the venter of the ascoma. The ascospores are triseptate with radiating appendages at one end. The other species in the genus, *T. ambispinosa* is described with appendages at both ends (Kohlmeyer 1960). *Swampomyces armeniacus*, the type species of its genus, was first isolated as a saprobe from mangrove roots in Belize and described with perithecia forming a clypeus and septate paraphyses (Kohlmeyer & Volkmann-Kohlmeyer 1987a). The original description provisionally placed this genus in the order *Phyllachorales*, which contains several biotrophic parasites. Subsequent ultrastructural studies did not clarify its taxonomic position and Read *et al.* (1995) therefore recommended assigning *Swampomyces armeniacus* to *Ascomycota incertae sedis*. *Swampomyces* and *Torpedospora* were never postulated to be related based on morphology, but comparative analysis of LSU and SSU rDNA sequences (Sakayaroj *et al.* 2005) showed an unexpected close phylogenetic relationship of the two genera. The clade containing these species could be placed in the subclass *Hypocreomycetidae*, but its relationship with other orders could not be elucidated with good statistical support.

The Assembling the Fungal Tree of Life Project (AFTOL; http://aftol.org) has produced large-scale generation of sequence data from multiple loci (Lutzoni *et al.* 2004). As part

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of this process a focused study combining data generated by AFTOL and other researchers presented the most thoroughly sampled phylogenetic hypothesis for the *Sordariomycetes* to date (Zhang *et al.* 2006). This analysis found support for several subclasses and revealed some previously unexpected relationships, such as a clade including *Coronophorales* and *Melanospora* within the *Hypocreomycetidae*. However, none of the marine species discussed in this study was included in this analysis. Another paper (Spatafora *et al.* 2006) containing a wider selection of taxa in the *Pezizomycotina*, but without taxonomic representatives of the *Coronophorales* or *Melanospora*, also placed an isolate of *T. radiata* within the *Hypocreomycetidae*. The goal of this study is to verify the earlier claims of shared ancestry (Sakayaroj *et al.* 2005) and, as far as possible, combine the various data sets mentioned above. We will also include the genera *Etheirophora* and *Juncigena*, which were revealed as being possibly closely related to *Torpedospora* and *Swampomyces* as part of the AFTOL data collection. This study also aims to investigate the ordinal and familial relationships of these fungi with additional molecular characters obtained from the protein coding RNA polymerase II second largest subunit gene (*RPB2*).

Materials and methods

Isolates and reference material

Specimens were isolated by cutting the top of an ascoma with a sterilized razor blade, removing the contents of the centrum by making a spore suspension and then streaking the spores on antibiotic seawater agar (Kohlmeyer & Kohlmeyer 1979). Small blocks of agar with a single germinating spore were later transferred to plates with the same medium, but without antibiotics.

Material examined (all collected and isolated by *J. Kohlmeyer* and *B. Volkmann-Kohlmeyer*, all vouchers in IMS): *Etheirophora blepharospora*: **French Polynesia**: *Moorea*, 12 Oct. 1990, J.K. 5397A; **USA**: *Florida*, 9 Jun. 1989, J.K.5289C. *Etheirophora unijubata*: **USA**: *Maui*, 22 Jan. 1992, J.K.5543B. *Juncigena adarca*: **USA**: *North Carolina*, 9 May 1989 and 1 Jan. 1995, J.K.5235A (paratype), J.K.5548A (paratype). *Swampomyces armeniacus*: **Australia**: *Queensland*, 5 Nov. 1989, J.K.5325A); **U.S. Virgin Islands**: *St Croix*, 2 Oct. 1987, J.K.5059C. *Torpedospora radiata*: **Australia**: *Queensland*, 4 Mar. 1988, J.K.5154C; **Belize**: *Blue Ground Range*, 4 Jun. 1989, J.K.5252C; **USA**: *Kauai*, 10 Mar. 1987, J.K.5095A; *North Carolina*, 30 Mar.1989, J.K.5231A.

DNA extraction, amplification and sequencing

DNA extraction from 3–5-d-old cultures was performed using Q-biogene Fast DNA Kit (Q-biogene, Morgan Irvine, CA) according to the manufacturer's instruction. PCR for the following genes: nuSSU and nuLSU rDNA, and approximately 1000 bp of the second largest subunit of RNA polymerase gene (*RPB2*) spanning domains 5 to 7. Amplifications and sequencing were conducted with primers listed on the AFTOL website (http://aftol.biology.duke.edu/pub/primers/viewPrimers). The following primers were used: nuSSU: NS1, NS2, NS4, NS24; nuLSU: LR0R, LR5 LR7; RPB2 fRPB2-5F, fRPB2-7cR. RPB2 PCR reactions were done as follows. A single reaction of 30 μl contained 5 μl of a 4 M Betaine solution (Tokyo Kasei Kogyo, Tokyo, Japan) and was set up under manufacturer-recommended conditions using Eppendorf Hotmaster Taq Polymerase (Hamburg) solution and run on an iCycler from Biorad (Hercules, California). The following conditions were used: 94 °C for 2 min; five cycles of 94 °C for 40 s, 50 °C for 45 s lowering by 0.8 °C per cycle and 65 °C for 90 s; 30 cycles of 94 °C for 30 s, 46 °C for 45 s and 65 °C for 120 s and a final cycle for 10 min at 65 °C. Ribosomal PCR reactions and sequencing reactions were performed under conditions mentioned previously (Lutzoni *et al.* 2004).

Phylogenetic analysis

DNA sequences for the *Torpedospora/Bertia/Melanospora* (TBM) clade are listed in Table 1. The remaining sequences were listed as indicated in Zhang *et al.* (2006). New sequences obtained in this study were edited using Seqmerge program in GCG software (Accelrys, San Diego, CA). Sequences of each gene were then initially aligned with Clustal X (Thompson *et al.* 1997) and concatenated into a single, combined data set. The initial alignment was manually edited as

Table 1 – Sequences used in this study representing the *Torpedospora/Bertia/Melanospora* clade

Taxon[a]	nrSSU	nrLSU	*RPB2*
Bertia moriformis[b]	-	AU695260	AY780151
Chaetosphaerella phaeostroma[b]	-	AY346274	AY780172
Etheirophora blepharospora[c] (J.K.5397A)	EF027717	EF027723	EF027731
E. blepharospora[c] (J.K.5289C)	-	EF027724	EF027732
E. unijubata[c] (J.K.5443B)	EF027718	EF027725	EF027733
Euacanthe foveolata[b]	-	AY695267	-
Fracchiaea broomeana[b]	-	AY695268	-
Juncigena adarca[c] (J.K.5235A)	EF027719	EF027726	EF027734
J. adarca[c] (J.K.5548A)	EF027720	EF027727	EF027735
Melanospora singaporensis[d]	-	AY015629	-
M. tiffanii[d]	AY015619	AY015630	AY015637
M. zamiae[d]	AY046578	AY046579	AY046580
Nitschkia grevillei[b]	-	AY346294	-
Scortechinia conferta[b]	-	AY695272	-
Sphaerodes fimicola[d]	-	AY015628	-
Swampomyces aegyptiacus[e] (CY2973)	AY858943	AY858950	-
S. armeniacus[e] (CY2799)	AY858944	AY858951	-
S. armeniacus[c] (J.K.5059C)	EF027721	EF027728	-
S. armeniacus[c] (J.K.5325A)	-	EF027729	EF027736
S. clavatispora[e] (LP83)	AY858945	AY858952	-
S. triseptatus[e] (CY2802)	AY858942	AY858953	-
Torpedospora ambispinosa[e] (CY3386)	AY858941	AY858946	-
T. ambispinosa[e] (BCC16003)	AY858940	AY858949	-
T. radiata[c] (J.K.5252C)	EF027722	EF027730	EF027737
T. radiata[c] (J.K.5154C)	-	-	EF027738
T. radiata[c] (J.K.5231A)	-	-	EF027739
T. radiata[c] (J.K.5095A)	DQ470999	DQ470951	DQ470902
T. radiata[e] (PP7763)	AY858939	AY858947	-
T. radiata[e] (BCC11269)	AY858938	AY858948	-

a Strain numbers are listed in brackets where appropriate.
b References: Huhndorf *et al.* (2004).
c This paper.
d Zhang and Blackwell (2002).
e Sakayaroj *et al.* 2005.

necessary in MacClade 4.0 (Maddison & Maddison 2000) and ambiguously aligned regions were excluded from phylogenetic analyses. Alignments were deposited at TreeBASE (SN 3076).

MP analyses were performed using PAUP 4.0 (Swofford 2002) on a combined data set of nrLSU, nrSSU and *RPB2* sequences and a focused data set containing only nrLSU. We included three species from the *Leotiomycetes* as outgroups. These were obtained from GenBank (listed in the order nrSSU, nrLSU, *RPB2*): *Botryotinia fuckeliana*: AY544695, AY544651, DQ470876; *Leotia lubrica*: AY544687, AY544644, DQ247786; *Microglossum rufum*: DQ471033, DQ470981, DQ470933 (not shown in Fig 1). The second data set included an expanded set of taxa for the *Coronophorales* and *Melanospora* for which only nrLSU sequences were available, plus three hypocrealean species as outgroups (*Hypocrea lutea*, *Nectria cinnabarina* and *Cordyceps cardinalis*). The inclusion of the *Halosphaeriales*/*Microascales* in Fig 2 without indicating them as outgroup was done in order to have the highest diversity of closely related marine sequences present in this single gene data set. The intention was not to test a relationship, or force a monophyly with the members of the *Hypocreales* and the species relevant to this study (clade TBM) but to confirm whether their monophyly could be verified in the presence of a closely related group of marine fungi. Similar MP analyses were performed for both data sets, using only parsimony informative characters with the following settings: 100 replicates of random sequence addition, tree bisection–reconnection (TBR) branch swapping, and multrees in effect. We repeated the same process without third base position in the *RPB2* gene in the combined data set, excluding 314 characters. In order to verify the influence of missing data and single genes on our complete analyses we used the same parsimony search options and ran a set of focused analyses under the following conditions. We only used taxa from subclades I–IV (Fig 1) with three hypocrealean taxa as outgroup. In each case we used complete taxon sets that had nucleotide data for the one of the three genes used in this study. This resulted in three single gene datasets—nrSSU, nrLSU and *RPB2*—with different taxon samplings but with the same outgroup. We also analysed the *RPB2* nucleotide data set without the third base codon positions and ran another combined three gene analysis as mentioned above. The BS trees from the three single gene analyses plus one from an analysis of RPB2 without third codon positions were compared in order to test for different resolutions and nodal support by different partitions of the total combined data set.

ML analysis was performed with RAxML-VI-HPC v2.0 using a GTRCAT model of evolution with 25 rate categories on combined and nrLSU data sets (Stamatakis *et al.* 2005). Nodal support was verified by nonparametric bootstrapping using 500 replicates. Bayesian analyses were performed on both abovementioned data sets with a parallelized version of MRBAYES v3.1.2 across four processors (Altekar *et al.* 2004; Huelsenbeck & Ronquist 2001). The data set was partitioned according to codon position and gene, yielding five partitions. The GTR + I + Γ model was applied to each partition and 5M generations was run in four chains with sampling every 100 generations, yielding 50K trees of which the first 5K were discarded as 'burn in'. Three separate Bayesian runs were completed for both data sets. For both ML and Bayesian analysis we chose the most complex evolutionary models possible, based on arguments that the parameter-rich models perform better in estimating likelihoods and PPs (Lemmon & Moriarty 2004).

Results

The combined three gene data set consisted of 4040 characters and 90 taxa. A total of 357 ambiguously aligned characters were excluded, yielding a final character set of 3683 with 1488 parsimony informative characters. The breakdown of characters per gene was as follows: nrSSU: 1451 total with 374 characters informative and 41 excluded; nrLSU: 1421 total with 498 characters informative and 118 excluded; and *RPB2*: 970 total with 670 characters informative and 198 excluded. An MP search yielded nine most parsimonious trees of 12,958 steps (CI = 0.244 RI = 0.552 RC = 0.135). A second MP analysis excluding 323 third base positions in the *RPB2* gene yielded a total data set of 3360, with 1174 characters being parsimony informative. This resulted in 352 trees consisting of 6943 steps (CI = 297 RI = 0.653 RC = 0.194). The Bayesian analyses converged on the plateau of the log-likelihood with a harmonic mean value of −95,262. A 50 % majority rule tree from Bayesian analyses is shown in Fig 1 with MP and ML BS proportions, as well as the Bayesian PPs. Internodes were considered strongly supported if they received all of BS proportions ≥70 % and PPs≥95 % (Lutzoni *et al.* 2004). For the nrLSU data set we analysed 43 taxa with 1289 characters, after removing 138 ambiguously aligned there were 411 parsimony informative characters. MP analyses resulted in 76 most parsimonious trees (CI = 0.244 RI = 0.552 RC = 0.135). Bayesian analyses of the nrLSU converged on the plateau of the log-likelihood with a harmonic mean value of −17,806.92. A 50 % majority rule tree from Bayesian analyses is shown in Fig 2 with all of the BS proportions mentioned above.

The phylogeny presented in Fig 1 covers representatives of the majority of the ordinal lineages in the *Sordariomycetes*. In order to complete a comparison with the marine species in the study we specifically included taxa contained in orders of perithecial marine species, the *Lulworthiales* and *Halosphaeriales*. As in previous studies (Spatafora *et al.* 1998; Kohlmeyer *et al.* 2000), these analyses did not confidently resolve the placement of the *Lulworthiales* in relation to the other *Sordariomycetes*. The MP and ML BS supported placement of *Lulworthiales* as a sister group to the remaining *Sordariomycetes* in contrast to the placement presented in Fig 1. Generally we found agreement for placement of the other major orders with the phylogeny of Spatafora *et al.* (1998) and Zhang *et al.* (2006), notably with the *Microascales* being paraphyletic due to the inclusion of the *Halosphaeriales*. A number of taxa were included that only had two of three genes present, including a set of taxa with only nrLSU and nrSSU sequences of relevant taxa from a previous study (Sakayaroj *et al.* 2005). According to Wiens (2006) 50 % of data can be missing for phylogenetic accuracy and adding additional taxa is desirable in order to break up long-branch attraction and other artefacts (Wiens 2005).

In Fig 1 we found good support with all three methods used (Bayesian inference, ML and MP) for a single clade (referred to hereafter as the TBM clade) encompassing a number of

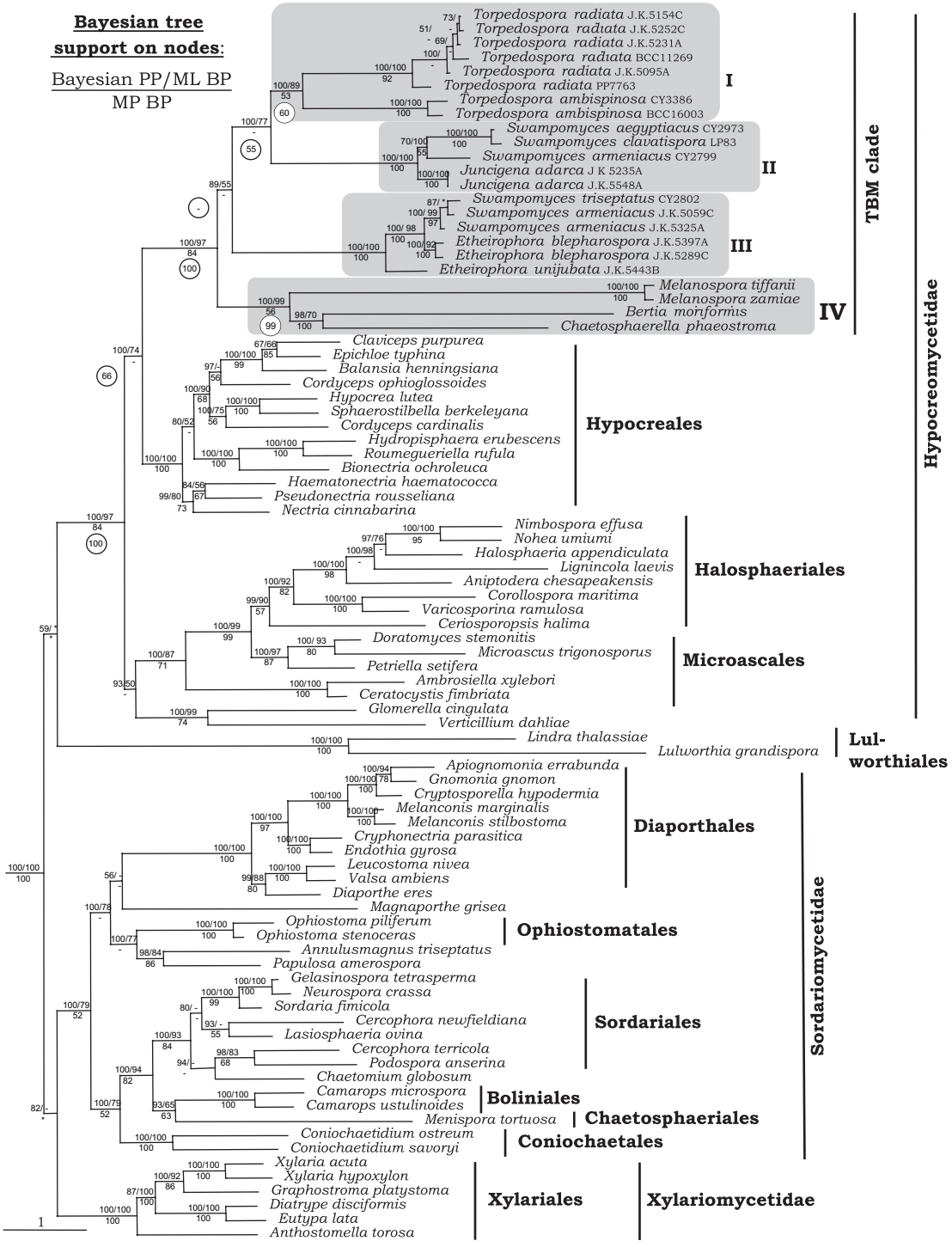

Fig 1 – Combined ribosomal and protein phylogeny (nrSSU, nrLSU, RPB2) of the *Sordariomycetes* emphasizing the TBM clade. The tree is a 50 % majority rule consensus tree of 45K trees obtained by Bayesian MCMCMC under GTR + I + Γ. Nodes of interest are labelled with support values shown above and below as indicated in the legend. Gaps (-) are shown for nodes with no support and an asterisk (*) indicates nodes that are differently resolved under the specific statistical sampling method used. Circles show selected MP BS results without third codon position in *RPB2*.

marine species and members of *Coronophorales* and *Melanospora*. This was confirmed in the presence of an expanded data set of taxa, including several marine species in *Halosphaeriales* and *Microascales* using nrLSU sequences (Fig 2). In Fig 1 BS support for this node increased with the removal of third base positions in the *RPB2* gene suggesting saturation may play a role in the contribution of these characters. The same pattern is seen in other nodes not supported by BS,

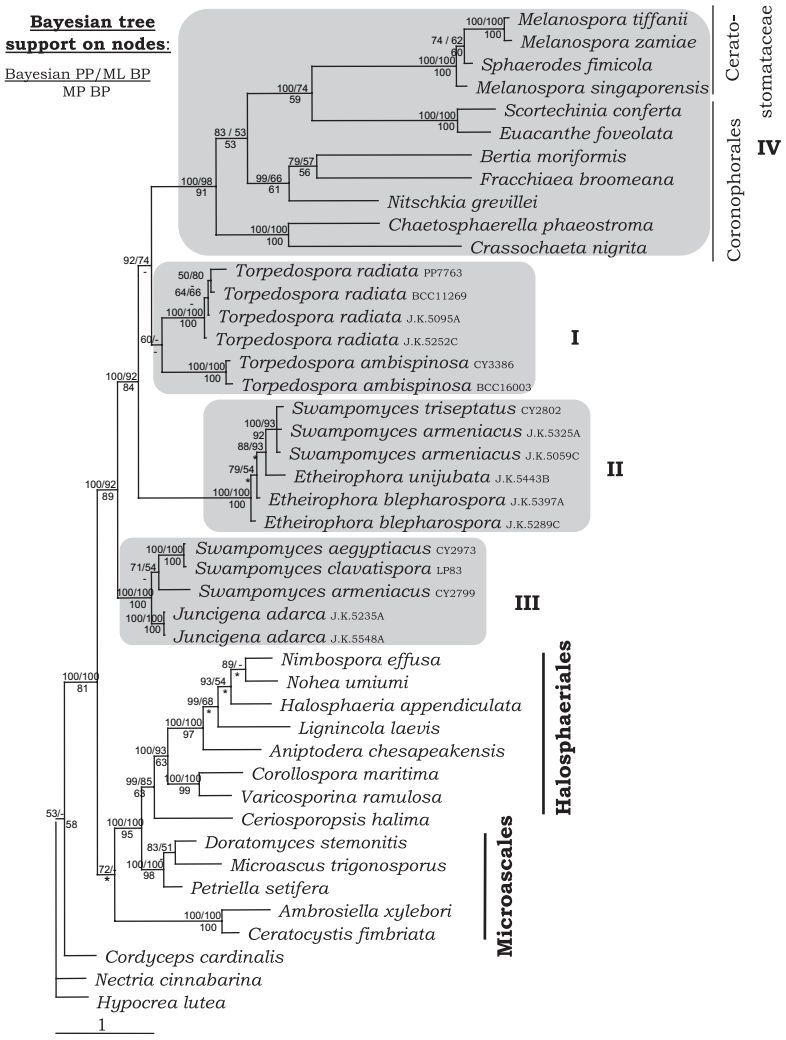

Fig 2 – Placement of *Coronophorales/Melanospora* together with marine lineages using an expanded taxon set data from the nrLSU. The tree is a 50 % majority rule consensus tree of 45K trees obtained by Bayesian MCMCMC under GTR + I + Γ applied to nrLSU sequence data. Nodes of interest are labelled as in Fig 1.

albeit still with low BS values. The marine species of the TBM clade could be distinguished into three subclades (I, II, III), while the taxa representing the *Coronophorales* and *Melanospora* formed a single lineage (IV) but only supported with Bayesian and ML BS and with MP BS without third position bases in *RPB2*.

The placement of the marine taxa in Fig 1 generally agreed with those taxa used in an earlier phylogenetic analysis of *Swampomyces* and *Torpedospora* spp. using nrLSU and nrSSU ribosomal sequences (Sakayaroj *et al.* 2005). In that study strains of *T. radiata* and *T. ambispinosa* grouped together with significant BS support under MP. Likewise, a clade consisting of *S. aegyptiacus*, *S. clavatispora* and *S. armeniacus* had good support, but only marginal support for an association with *S. triseptatus*. Importantly, all of these aforementioned taxa were supported by a single node, but with varying support depending on the gene sequence used. MP comparisons of nrSSU and nrLSU yielded 63 and 87 % BS support, respectively, and a combined weighted parsimony analysis had lowered support of 57 %. The TBM clade placed within the subclass *Hypocreomycetidae* encompassing the *Hypocreales*, *Microascales* and *Halosphaeriales*, but without strong statistical support for deep nodes of the subclass. Our current study expands the Sakayaroj *et al.* (2005) data set with additional sequences and taxa within a broader taxonomic and phylogenetic sampling of *Sordariomycetes*.

A subclade consisting of *S. aegyptiacus*, *S. clavatispora* and *S. armeniacus* sequences from GenBank is strongly supported as being monophyletic with *Juncigena adarca* sequences obtained in this study, but differences were noted with the earlier work (Sakayaroj *et al.* 2005). The sequences obtained from the various *Swampomyces* species did not form a monophyletic group. The two *S. armeniacus* isolates in this study (J.K. 5059C and J.K. 5325A) showed an affinity with isolate CY2802 collected in the Bahamas and possibly misidentified as *S. triseptatus*. This subclade (11) also contained the two *Etheirophora* taxa, *E. unijubata* and *E. blepharospora* with good statistical support. The monophyly of *Etheirophora* was not supported due to the

placement of the three *Swampomyces* isolates. It is also clear from the molecular data that the *Swampomyces* sequences used in this study and the previous ones from GenBank are not congeneric.

The strains of *T. radiata* and *T. ambispinosa* grouped together to form subclade I with marginal MP BS but with good PP and ML support (Fig 1). The single gene tree in Fig 2 resulted in no statistical support for subclade I as measured by PP and bootstrapping. None of the single gene analyses where these taxa were present (nrSSU and nrLSU) supported the monophyly of this node. It is clear that several differences between these taxa exist in the sequences analysed, although we cannot exclude the influence of missing data. Strong support for the monophyly of the genus *Torpedospora*, as currently defined, awaits further testing with a more complete data set.

In addition to the marine species in this study, the fungicolous *Melanospora* species and the wood inhabiting *Coronophorales* forming subclade IV (Fig 1) were unexpectedly placed together in a recent study (Zhang *et al.* 2006), but this relationship was only well supported when third base positions of the protein coding *RPB2* gene was removed. Previous studies containing taxa from these two groups have placed them independently in the *Hypocreomycetidae* with good support (Zhang & Blackwell 2002; Huhndorf *et al.* 2004; Castlebury *et al.* 2004). However, this is the first data set that places all these groups together with several marine species in a single analysis. In order to expand the taxa of *Melanospora* and *Coronophorales* used in this study with taxa for which there are only nrLSU sequences available, we ran a single gene tree with sequence data obtained from GenBank for the nrLSU using *Nectria cinnabarina* as an outgroup. We also included representatives of the *Halosphaeriales*, an exclusively marine order in the *Hypocreomycetidae* (Fig 2). Similar to results from the multi-gene analyses (Fig 1), three subclades of marine taxa are well supported by the various approaches used.

A number of separate gene analyses were performed to verify the impact of missing data and the contribution of specific genes. In order to assess the relationship between missing data and long branch attraction (Bergsten 2005) we used MP, putatively the process most sensitive to this artefact, and compared BS analyses supporting nodes within subclades I–IV in Figs 1 and 3. Outgroups were the same in all cases but ingroup taxon sets varied among the different nucleotide gene sequences as follows: 17 taxa for the nuSSU, 21 taxa for nrLSU, and 14 taxa for *RPB2* (Fig 3). Each taxon set had at least two representatives of the subclades I–IV used in Fig 1. One notable difference was the placement of the two *Melanospora* species sister to *Juncigena* and distant from the *Coronophorales* in the *RPB2* only data set. The removal of third base nucleotides within the *RPB2* gene had the effect of placing these two species sister to *Bertia* with 66 % BS consistent with saturation at this position. Additionally, a complete analysis with all available characters for this taxon set that also included missing data for the other genes changed its position as monophyletic within subclade IV with 72 % BS (data not shown). Another notable observation is the relationships among the four subclades within this study. As can be seen from a comparison of Figs 1–3, subclade IV's position in the phylogeny is unstable, depending on the set of nucleotide characters used and taxon sampling.

Discussion

This study confirmed the existence of a novel marine lineage in the *Hypocreomycetidae* overlapping with a previous report by Sakayaroj *et al.* (2005). We find additional support for a phylogenetic relationship between *Swampomyces* and *Torpedospora* as mentioned by these authors, but important conflicts are noted. Their *S. triseptatus* (CY2802) from the Bahamas is resolved as a close relative to the two *S. armeniacus* isolates sequenced for this study (subclade I; Fig 1), while their *S. armeniacus* (CY2799) appears to be closely related to *S. aegyptiacus* and *S. clavatispora* (subclade II; Fig 1). Given the fact that only single isolates were used to represent their respective species it seems possible that the *S. triseptatus* and *S. armeniacus* isolates were inadvertently switched. Taking this into account, the present molecular results would indicate that *S. aegyptiacus*, *S. clavatispora* and possibly also *S. triseptatus* belong to a different genus. In support of this assertion these three species differ from *S. armeniacus* by the presence of a pseudostroma (instead of a clypeus) and three-septate ascospores (instead of one-septate ascospores). Additionally, we find support for a close relationship of these species with *Juncigena*. *Juncigena adarca* was identified from a salt-marsh on the US east coast, occurring on the black needle rush, *Juncus roemerianus* (Kohlmeyer *et al.* 1997) and is currently classified as a member of the *Magnaporthaceae* (Eriksson 2006). This species was also linked with a *Cirrenalia* anamorph. Its phylogenetic affinity with the three-septate *Swampomyces* species [assuming that *S. armeniacus* (CY2799) in reality is *S. triseptatus* (CY2802)] is unexpected. However, there are a number of morphological similarities that would support this finding, i.e. the ascomata are immersed, periphysate, ostiolate and similar in shape (although without pseudostroma) and paraphyses are unbranched. Asci are unitunicate with an apical ring (Kohlmeyer *et al.* 1997), while the *Swampomyces* species have an apical thickening (Sakayaroj *et al.* 2005). Ascospores for *Juncigena* are also three-septate without appendages and look very similar to *S. triseptatus* and *S. clavatispora* ascospores.

Another genus included in this study as a close relation of *Swampomyces* is *Etheirophora*. *E. blepharospora* was originally described in the genus *Keissleriella* as a possibly bitunicate species, isolated from bark of mangrove prop roots (Kohlmeyer & Kohlmeyer 1965). However, further examination showed that it is not functionally bitunicate and this species was later placed in *Etheirophora* (Kohlmeyer & Volkmann-Kohlmeyer 1989a) together with the two newly described species, *E. unijubata* and the type species of the genus, *E. bijubata*. The inclusion of *E. blepharospora* and *E. unijubata* in the multi-gene nucleotide sequencing data demonstrates a close phylogenetic affinity with *S. armeniacus* (subclade I; Fig 1) that is well supported by a number of morphological characters. Both are periphysate, have a clypeus, rarely branched paraphyses in a gel matrix, unitunicate, persistent asci, which do not turn blue in potassium hydroxide solution, and are morphologically very similar, as well as one-septate ascospores. The only difference is that all *Etheirophora* species have appendaged ascospores. Interestingly, however, *E. unijubata* has appendages on only one end of the ascospore and does not form a monophyletic genus with *E. blepharospora*. This indicates that the genus concepts employed

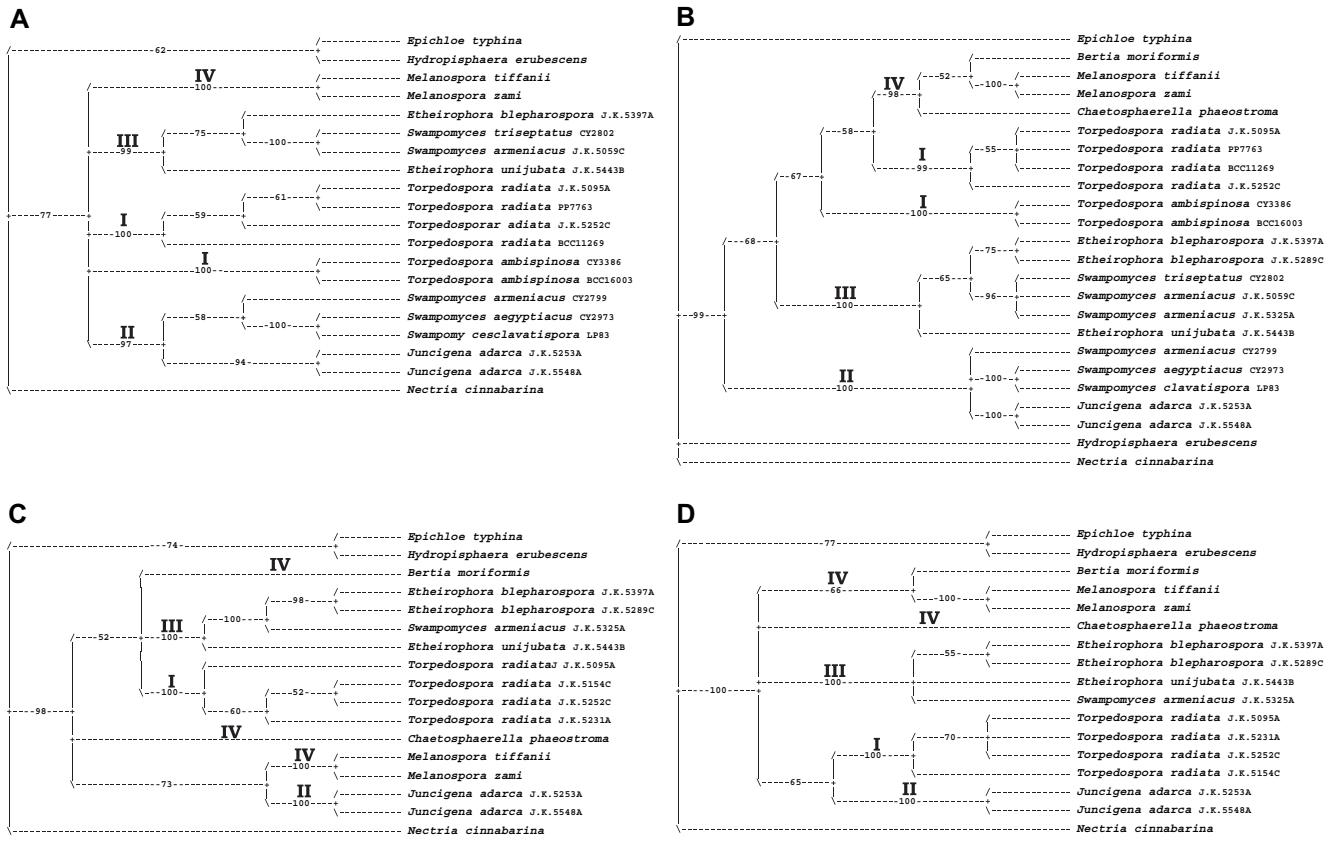

Fig 3 – Comparison of taxon sets having complete nucleotide sequence data for a single gene using MP BS. Branches containing members of relevant subclades are labelled. Nucleotide character sets used were A: nrSSU, B: nrLSU, C: *RPB2*, D: *RPB2* only, without third base nucleotides.

for *Swampomyces* and *Etheirophora* in this subclade need to be reassessed with more intensive sampling.

An outcome mentioned in other studies (Zhang *et al.* 2006; Sakayaroj *et al.* 2005) and highlighted here is the observation that members of the TBM clade share few morphological characters. A unique character of both *Torpedospora* species distinguishing them from all other genera with paraphyses is the composition of the hamathecium. Paraphyses are not arranged in parallel, but grow irregularly throughout the ascomatal venter. It is plausible that either higher rates of evolution have allowed for increased morphological divergence seen in these groups or that the TBM clade represents a clade of greater phylogenetic diversity than currently realized. Regardless, the TBM clade forms another example of an independent lineage of fungi adapting to marine environments from terrestrial lineages (Sakayaroj *et al.* 2005). This is in addition to the other main lineages of marine fungi in the *Sordariomycetes*—the *Lulworthiales* and *Halosphaeriales*.

The long branches of the *Coronophorales* and *Melanospora* lineages potentially complicate a clear assessment of their placement within the TBM clade and within *Hypocreomycetidae*. Members of these taxa were individually placed in the *Hypocreomycetidae* by previous studies (Huhndorf *et al.* 2004; Zhang & Blackwell 2002; Castlebury *et al.* 2004). The possible influence of long branch attraction was mentioned and Huhndorf *et al.* (2004) suggested that based on branch lengths the whole set of *Coronophorales* in their study evolved at a faster rate than the *Hypocreales*. The topology of the phylogenies in this study certainly indicates that all these taxa are on long branches with different phylogenetic resolutions between single genes and a complete dataset with missing characters (Figs 1–3). Nevertheless, in all cases the single node supporting the monophyly of the TBM clade, including *Coronophorales*, *Melanospora* and the three marine lineages, received significant support. It should be noted that none of the attempts made to verify whether support under conditions less sensitive to long-branch attraction (i.e. removing faster evolving third bases in the protein gene, comparing likelihood and parsimony methods) had the effect of collapsing or removing this node, and support remained significant in all cases.

Coronophorales is known for producing small pores surrounded by a ring (Munk pores) in the cell walls of their ascomata. Another unique feature of these fungi is the presence of the quellkörper in the apical region of the ascoma, 'a mass of small cells in a gel matrix that imbibes water and expands, finally rupturing the peridium' (Barr 1987). Structures similar or even identical to Munk pores (named pit connections in the subsequent papers) are formed in the central parenchyma of ascomata in a number of marine genera, i.e. *Antennospora* (Kohlmeyer 1980, as *Halosphaeria salina*), *Arenariomyces* (Kohlmeyer & Volkmann-Kohlmeyer 1989a) and *Corollospora* (Kohlmeyer & Volkmann-Kohlmeyer 1987b, 1989b, 1997), all belonging to the *Halosphaeriales*, as well as in *Kohlmeyeriella* (Kohlmeyer 1968, as *Corollospora tubulata*) in the *Lulworthiales*.

It is equally interesting that in the ostiolar canal of the genus *Corollospora* thick-walled cells can be found that appear to be homologous to the quellkörper (Kohlmeyer & Volkmann-Kohlmeyer 1987b, 1989b, 1997). However, it is clear from these analyses that these taxa do not collectively form a monophyletic clade and that these structures are homoplasious. Moreover, the majority of characteristics, including Munk pores and quellkörper, that define the *Coronophorales* are not present in subclades I, II, and III.

The case for synapomorphies linking the marine species of the TBM clade with the *Melanospora* spp. is even more confounding. The placement of *Melanospora* has been a long-standing taxonomic problem and it is currently classified under the *Ceratostomataceae* in the *Hypocreales* (Eriksson 2006). However, Zhang *et al.* (2006) revealed that it has a sister-group relationship with the *Coronophorales* and that it is not a member of the *Hypocreales*. It contains species with translucent perithecial ascomata with pseudoparenchymatous centra and clavate deliquescent asci with dark coloured ascospores containing germ pores. These species tend to be parasites of a wide range of other fungi spanning the *Basidiomycota* and *Ascomycota*. It shares very few features with the other species in this study except perhaps for clavate, deliquescent asci (shared with the *Torpedospora* species and members of the *Coronophorales*) (Zhang *et al.* 2006).

The TBM clade, therefore, contains order and family level relationships that remain unresolved and require additional character sampling from unlinked protein coding loci (e.g. RPB1, EF-1α). Furthermore, future taxon sampling will likely reveal additional phylogenetic and taxonomic diversity for this clade. Due to the numerous taxonomic uncertainties, we refrain from proposing any major taxonomic changes here and continue to treat all these lineages as *Hypocreomycetidae incertae sedis*. It also appears that current genus concepts of *Swampomyces* and to a lesser extent *Etheirophora* and *Torpedospora* should be re-assessed and the importance of morphological characters re-evaluated. The biological and morphological diversity of the TBM clade revealed in our analysis, however, highlights another example of a fungal clade that is well supported by data, but for which few if any shared morphological characters are known. Deliquescent asci are present throughout the clade, but not universally, and they are one of the more homoplastic traits of the *Ascomycota*. The majority of species are associated with wood in terrestrial or marine environments (except *Melanospora*) indicating that shared biochemical characters may be present. These results beg the question as to whether unequivocal synapomorphies for all clades will be found among morphological characters routinely used in fungal taxonomy. Rather, understanding the distinct evolutionary shifts supported by these phylogenetic analyses will likely require additional investigations at subcellular, enzymatic and genomic levels.

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