

# The molecular phylogeny of freshwater *Dothideomycetes*

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**Abstract:** The freshwater *Dothideomycetes* species are an ecological rather than taxonomic group and comprise approximately 178 meiosporic and mitosporic species. Due to convergent or parallel morphological adaptations to aquatic habitats, it is difficult to determine phylogenetic relationships among freshwater taxa and among freshwater, marine and terrestrial taxa based solely on morphology. We conducted molecular sequence-based phylogenetic analyses using nuclear ribosomal sequences (SSU and/or LSU) for 84 isolates of described and undescribed freshwater *Dothideomycetes* and 85 additional taxa representative of the major orders and families of *Dothideomycetes*. Results indicated that this ecological group is not monophyletic and all the freshwater taxa, except three aeroaquatic *Tubeufiaceae*, occur in *Pleosporomycetidae* as opposed to *Dothideomycetidae*. Four clades comprised of only freshwater taxa were recovered. The largest of these is the *Jahnulales* clade consisting of 13 species, two of which are the anamorphs *Brachiosphaera tropicalis* and *Xylomyces chlamydosporus*. The second most speciose clade is the *Lindgomycetaceae* clade consisting of nine taxa including the anamorph *Taeniolaella typhoides*. The *Lindgomycetaceae* clade consists of taxa formerly described in *Massarina*, *Lophiostoma*, and *Massariosphaeria* e.g., *Massarina ingoldiana*, *Lophiostoma brevipendiculatum*, and *Massariosphaeria typhicola* and several newly described and undescribed taxa. The aquatic family *Amniculicolaceae*, including three species of *Amniculicola*, *Semimassariosphaeria typhicola* and the anamorph, *Anguillospora longissima*, was well supported. A fourth clade of freshwater species consisting of *Tingoldiagio graminicola*, *Lentithecium aquaticum*, *L. arundinaceum* and undescribed taxon A-369-2b was not well supported with maximum likelihood bootstrap and Bayesian posterior probability. Eight freshwater taxa occurred along with terrestrial species in the *Lophiostoma* clades 1 and 2. Two taxa lacking statistical support for their placement with any taxa included in this study are considered singletons within *Pleosporomycetidae*. These singletons, *Ocala scalariformis*, and *Lepidopterella palustris*, are morphologically distinct from other taxa in *Pleosporomycetidae*. This study suggests that freshwater *Dothideomycetes* are related to terrestrial taxa and have adapted to freshwater habitats numerous times. In some cases (*Jahnulales* and *Lindgomycetaceae*), species radiation appears to have occurred. Additional collections and molecular study are required to further clarify the phylogeny of this interesting ecological group.

**Key words:** Ascomycetes, aquatic, evolution, *Jahnulales*, *Pleosporales*.

## INTRODUCTION

Freshwater ascomycetes comprise a diverse taxonomic assemblage of about 577 species (Shearer *et al.* 2009). These fungi are mostly saprobic on submerged woody and herbaceous debris and are important in aquatic food webs as decomposers and as a food source to invertebrates (see Gessner *et al.* 2007, Simonis *et al.* 2008). Although in the early ascomycete taxonomic literature some species were reported and/or described from plants in or near aquatic habitats, little was noted about whether the fungi were on aerial or submerged parts of their hosts/substrates. For the purpose of this study, we consider freshwater ascomycetes as only those species that occur on submerged substrates; ascomycetes on aerial parts of aquatic plants are considered terrestrial and not dealt with herein.

Ingold was the first to recognise that a distinctive freshwater ascomycota might exist and published a series of papers about fungi on submerged substrates in the Lake District, England (Ingold 1951, 1954, 1955, Ingold & Chapman 1952). Ingold was collecting from the submerged stems of aquatic macrophytes in the English Lake District when he discovered the magnificent freshwater *Dothideomycete*, *Macrospora scirpicola* on *Schoenoplectus lacustris*, the lakeshore bulrush (Ingold 1955). This fungus has ascospores equipped with a gelatinous sheath (Fig. 1A) that

elongates and becomes sticky after the ascospores are discharged into water (Fig. 1B), a feature thought to improve the probability that ascospores will attach to substrates in moving water (Hyde & Jones 1989, Shearer 1993, Jones 2006). This feature is found in numerous freshwater *Dothideomycetes* (see species monograph, Shearer *et al.* 2009). The ascospores also germinate immediately upon contact with a firm substrate (Fig. 1C), which may help them adhere to substrates in moving water. *Macrospora scirpicola* is one of the earliest known freshwater *Dothideomycete* species; DeCandolle originally described it in 1832 as *Sphaeria scirpicola*, and Pringsheim first reported it from freshwater in 1858.

The early literature dealing specifically with freshwater ascomycetes, including *Dothideomycetes*, has been reviewed by Dudka (1963, 1985) and Shearer (1993). Since the 1990's, interest in aquatic ascomycetes has grown and the number of species reported and/or described from freshwater habitats has increased by 370 to a total of 577 taxa (Shearer *et al.* 2009). For more recent reviews of the freshwater ascomycetes, see: Goh & Hyde (1996), Wong *et al.* (1998), Shearer (2001), Tsui & Hyde (2003), Shearer *et al.* (2007), and Raja *et al.* (2009b). Approximately 30 % of the 577 freshwater ascomycetes are *Dothideomycete* species, and based on morphology, belong primarily in *Pleosporales* or secondarily in *Jahnulales*. Exceptions include four species in *Capnodiales* (*Mycosphaerellaceae*) and four species in *Tubeufiaceae*.

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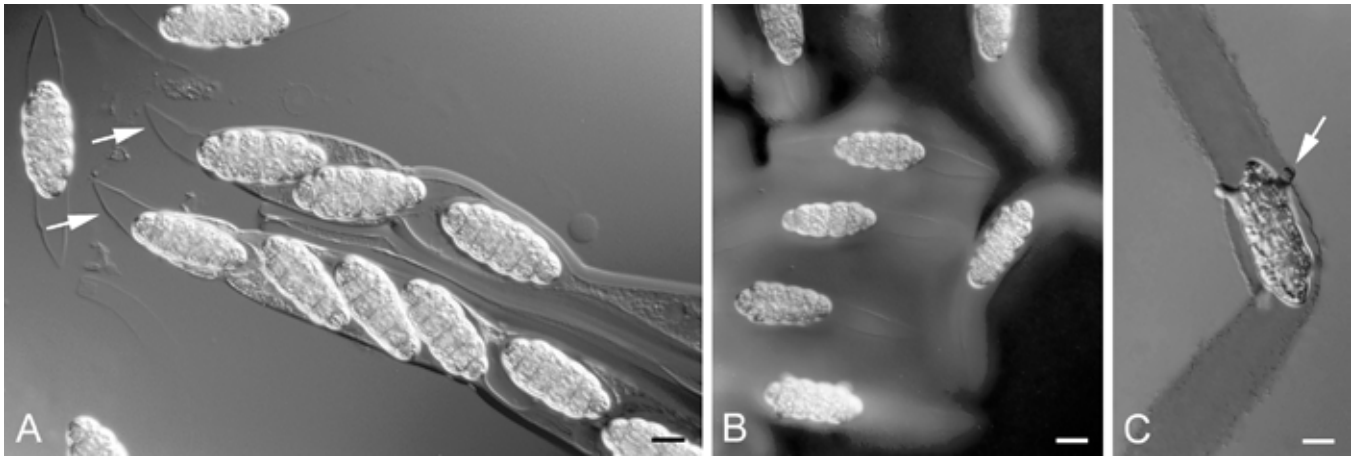
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**Fig. 1.** *Macrospora scirpicola* A27-1. A. Ascospores being discharged from bitunicate asci showing bipolar gelatinous appendages. B. Ascospores showing an outer and inner sheath when stained with India ink. C. Ascospore on a glass slide germinating within its gelatinous sheath stained with India ink. Scale Bars: = 20  $\mu$ m.

Molecular studies of freshwater *Dothideomycetes* have been of four basic types. The first type was to determine the overall taxonomic placement of one or more undescribed taxa (e.g., Inderbitzin *et al.* 2001, Cai & Hyde 2007, Kodsueb *et al.* 2007, Cai *et al.* 2008, Zhang *et al.* 2008a, b, 2009a, c, Raja *et al.* 2010). In these studies one or more nuclear genes were sequenced to place a newly described fungus in an order or family within the *Dothideomycetes* framework. In the second type, the goal was to use single or multi-gene phylogenies to elucidate the evolutionary relationships among a group of closely related taxa, and to evaluate which suite of morphological characters might be informative for predicting evolutionary relationships and which might be misleading or homoplasious (e.g., Liew *et al.* 2002, Pang *et al.* 2002, Campbell *et al.* 2006, 2007, Tsui & Berbee 2006, Zhang *et al.* 2009a, c, Hirayama *et al.* 2010). The third type of molecular study was used to identify relationships between aquatic anamorphic and teleomorphic *Dothideomycetes* (see Baschien 2003, Belliveau & Bärlocher 2005, Baschien *et al.* 2006, Campbell *et al.* 2006, Tsui *et al.* 2006, 2007). Here the goal was to use sequence data to place the aquatic anamorphs within the teleomorph phylogeny to better understand the phylogenetic affinities of freshwater anamorphs. The fourth type addressed the evolution of freshwater ascomycetes (Vijaykrishna *et al.* 2006).

*Dothideomycetes* possess freshwater hyphomycetous anamorphs rather rarely. Approximately only 10 % of 86 aquatic hyphomycete species, which are at least tentatively assigned to an ascomycete family, order or class, have affinity to *Dothideomycetes*. Four of them are connected to known teleomorphs via cultural studies: *Tumularia aquatica* to *Massarina aquatica* (Webster 1965), *Anguillospora longissima* to *Massarina* sp. (Willoughby & Archer 1973), *Clavariopsis aquatica* to *Massarina* sp. (Webster & Descals 1979), and *Aquaphila albicans* to *Tubeufia asiatica* (Tsui *et al.* 2007). Four connections are published on the basis of molecular phylogenetic rather than cultural studies, but some of these connections are controversial and require further molecular study using additional genes and/or cultural studies. These connections include: *Anguillospora rubescens* in *Dothideales* (Belliveau & Bärlocher 2005), *Lemonniera pseudofloscula* and *Goniopila monticola* in *Pleosporales* (Campbell *et al.* 2006), and *Mycocentrospora acerina* to *Mycosphaerellaceae* (Stewart *et al.* 1999). (Note: Data on affinity of *Mycocentrospora* is not explicitly given in the text, but is in the GenBank entry AY266155).

Most of the above-mentioned molecular studies have used limited taxon sampling of various orders and families currently in the *Dothideomycetes*, as well as a single gene (either nuc SSU rDNA or nuc LSU rDNA) to understand the phylogenetic affinities of the freshwater taxa. A review of past molecular phylogenetic studies of freshwater *Dothideomycetes* revealed that very few of the approximately 170 freshwater *Dothideomycete* species have been sequenced. In addition, different genes and different regions of the same genes have been sequenced for different taxa making any comprehensive molecular analysis impossible. Clearly more sequences are needed for taxa already studied and more taxa need to be sequenced if we are to understand the phylogeny of the freshwater *Dothideomycetes*.

The purpose of this study, therefore, was to obtain two gene sequences (nuc SSU rDNA & nuc LSU rDNA) for as many freshwater *Dothideomycetes* (teleomorphs and anamorphs) as possible to conduct molecular sequence analyses to place these taxa within a phylogenetic framework comprised of a broader taxonomic and ecological taxon sampling from major orders and families using the most current classification system proposed for the *Dothideomycetes* (Schoch *et al.* 2006, Hibbett *et al.* 2007).

## MATERIALS AND METHODS

### Taxon sampling

The species used in this study, their isolate numbers, sources and GenBank accession numbers are listed in Table 1 - see online Supplementary Information. The datasets contained 156 taxa for the SSU and 160 taxa for LSU, while the combined dataset consisted of 169 taxa with some missing data. Twenty-two aquatic taxa were newly sequenced for the SSU gene and/or the LSU gene, while sequences of several other aquatic taxa included in the analyses were obtained from very recently published or unpublished phylogenetic studies of freshwater fungi (Zhang *et al.* 2008a, b, 2009a, c, Hirayama *et al.* 2010, Raja *et al.* 2010). Sequences of a wide array of taxa representing various orders and families within the *Dothideomycetes* based on Schoch *et al.* (2006) were included in this study. In addition to taxa from the *Dothideomycetes*, members of *Arthoniomycetes*, *Lecanoromycetes*, *Sordariomycetes* and *Leotiomyces* were also included in the analyses. Members of the *Pezizomycetes* were used as outgroup taxa.

## DNA extraction and PCR amplification

For extraction of genomic DNA, mycelium from axenic cultures was scraped with a sterile scalpel from nutrient agar in plastic Petri dishes and ground to a fine powder in liquid nitrogen using a mortar and pestle. Approximately 400  $\mu\text{L}$  of AP1 buffer from the DNAeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) was added to the mycelial powder and DNA was extracted following the manufacturer's instructions. The DNA was finally eluted in 30  $\mu\text{L}$  distilled water. Fragments of SSU and LSU nrDNA were amplified by PCR using PuReTaq™ Ready-To-Go PCR beads (Amersham Biosciences Corp., Piscataway, New York) according to Promputtha & Miller (2010). Primers NS1 and NS4 for SSU (White *et al.* 1990) and LROR and LR6 for LSU (Vilgalys & Hester 1990, Rehner & Samuels 1995) were used for PCR reactions in addition to 2.5  $\mu\text{L}$  of BSA (bovine serum albumin, New England Biolabs, Ipswich, MA) and/or 2.5  $\mu\text{L}$  of DMSO (dimethyl sulfoxide, Fisher Scientific, Pittsburgh, PA). PCR products were purified to remove excess primers, dNTPs and nonspecific amplification products with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, California). Purified PCR products were used in 11  $\mu\text{L}$  sequencing reactions with BigDye Terminators v. 3.1 (Applied Biosystems, Foster City, California) in combination with the following SSU primers: NS1, NS2, NS3, NS4 (White *et al.* 1990), and LSU primers: LROR, LR3, LR3R, LR6 (Vilgalys & Hester 1999, Rehner & Samuels 1995). Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer at the UIUC Biotech facility. Sequences were also obtained using other methods outlined in Hirayama *et al.* (2010) and Zhang *et al.* (2009c).

## Sequence alignment

Each sequence fragment obtained was subjected to an individual blast search to verify its identity. Individual fragments were edited and contigs were assembled using Sequencher v. 4.9 (Gene Codes Corp., Ann Arbor Michigan). Newly obtained sequences were aligned with sequences from GenBank using the multiple sequence alignment program, MUSCLE® (Edgar 2004) with default parameters in operation. MUSCLE® was implemented using the programs Seaview (Galtier *et al.* 1996) and Geneious Pro v. 4.7.6 (Biomatters) (Drummond *et al.* 2006). Sequences were aligned in MUSCLE using a previous (trusted) alignment made by eye in Sequencher v. 4.9, based on a method called "jump-starting alignment" (Morrisson 2006). The final alignment was again optimised by eye and manually corrected using Se-Al v. 2.0a8 (Rambaut 1996) and McClade v. 4.08 (Maddison & Maddison 2000).

## Phylogenetic analyses

Separate alignments were made for SSU and LSU sequences. The aligned SSU and LSU datasets were first analysed separately and then the individual datasets were concatenated into a combined dataset. Prior to combining the datasets, the possibility of clade conflict was explored. Independent maximum likelihood (ML) analyses were run with a GTR model including invariable sites and discrete gamma shape distribution and 100 bootstrap replicates were performed using the program Seaview (Galtier *et al.* 1996). The individual SSU and LSU phylogenies were then examined for conflict by comparing clades with bootstrap support (Wiens 1998). If clades were < 50 % they were considered weakly supported, whereas 70–100 % indicated a strong support. We combined

the datasets since there was no obvious clade conflict for 90 % of the taxa included in our study. Subsequent analyses were then performed on the combined SSU + LSU dataset. In the final combined dataset, 13 ambiguously aligned regions were delimited and excluded from all further analyses.

Modeltest v. 3.7 (Posada & Crandall 1998) was used to determine the best-fit model of evolution for the dataset. ML analyses were performed using RAxML v. 7.0.4 (Stamatakis 2006) with 100 successive searches and the best-fit model, which was the (GTR) model with unequal base frequencies (freqA = 0.2666, freqC = 0.2263, freqG = 0.2664, freqT = 0.2407), a substitution rate matrix (A $\leftrightarrow$ C = 0.9722, A $\leftrightarrow$ G = 2.7980, A $\leftrightarrow$ T = 1.1434, C $\leftrightarrow$ G = 0.6546, C $\leftrightarrow$ T = 5.1836, G $\leftrightarrow$ T = 1.0000), a proportion of invariable sites (– 0.2959) and a gamma distribution shape parameter (– 0.4649). For the ML analyses constant characters were included and again 13 ambiguously aligned regions were excluded. Each search was performed using a randomised starting tree with a rapid hill climbing option. One thousand fast bootstrap pseudoreplicates (Stamatakis *et al.* 2008) were run under the same conditions.

Bayesian Metropolis Coupled Markov Chain Monte Carlo (B-MCMCMC) analyses were performed with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) as an additional means of assessing branch support. Constant characters were included. A comparable model to the ML analyses was used to run 10 million generations with trees sampled every 1 000<sup>th</sup> generation resulting in 10 000 total trees. The first 1 000 trees which extended beyond the burn-in phase in each analysis were discarded and the remaining 9 000 trees were used to calculate posterior probabilities. The consensus of 9 000 trees was viewed in PAUP v. 4.0b10 (Swofford 2002). The analysis was repeated twice each with four Markov Chains for the dataset starting from different random trees.

## RESULTS

### Sequence alignment

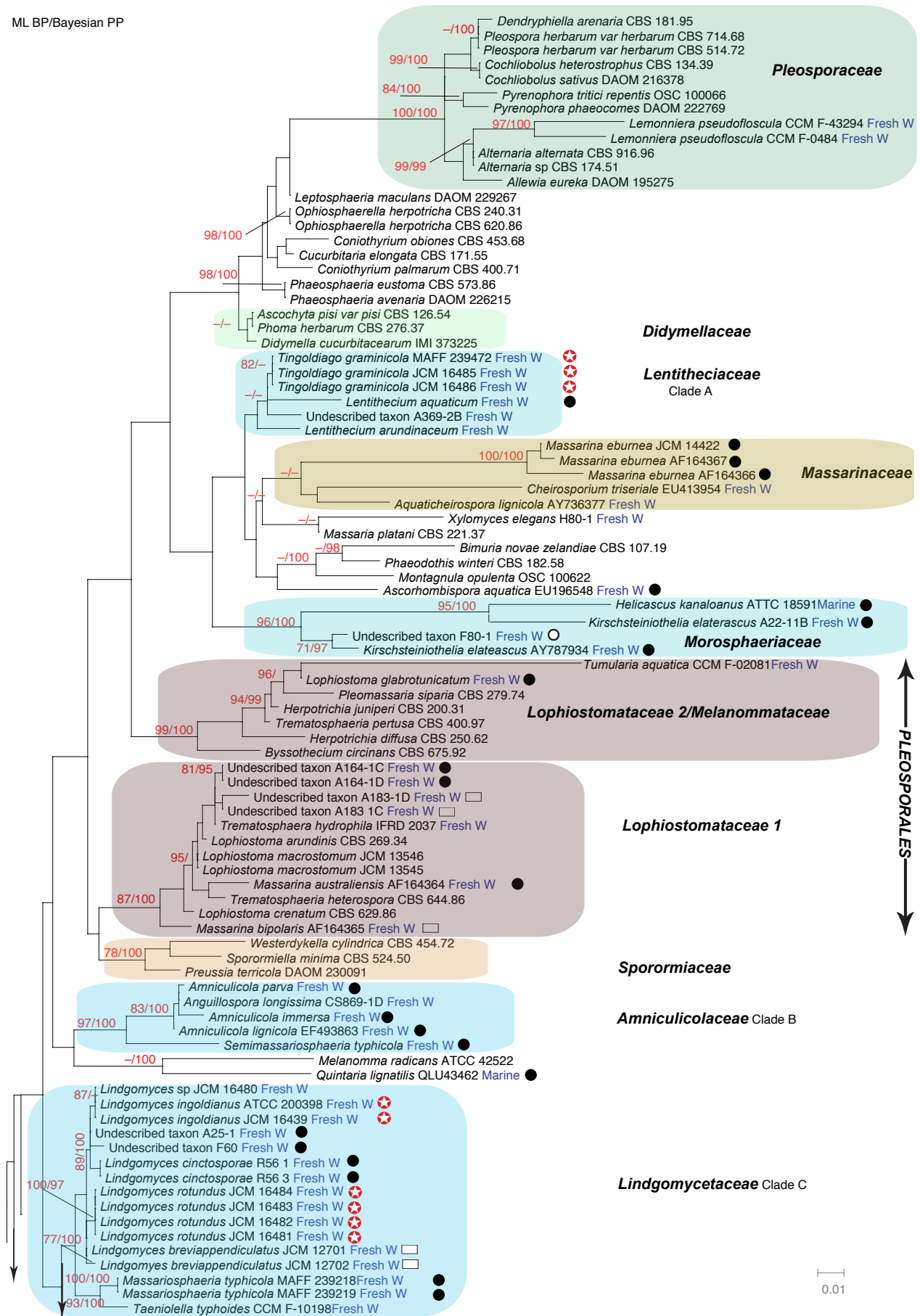
The complete dataset (combined SSU and LSU alignment) along with intron regions and ambiguous characters had 169 taxa and 7 264 characters. The dataset consisted of 169 taxa and 3 641 characters after removal of intron regions. We then delimited and removed 548 ambiguous characters from the final alignment along with characters from the 5' and 3' end regions due to missing information in most taxa included in the alignment. The final dataset after removal of all the intron regions and 13 ambiguous regions along with missing data from the 5' and 3' ends consisted of 1816 characters. There were no significant conflicts among the clades in the separate SSU and LSU analyses in either SSU or LSU datasets (data not shown) therefore we used all 169 taxa in the combined SSU and LSU analyses.

### Phylogenetic analyses

The combined matrix analysed in this study produced 852 distinct alignment patterns and the most likely tree (Fig. 2) had a log likelihood of -17187.0385 compared to the average (100 trees) of -17191.7927. Several major clades presented in the multi-gene phylogeny of Schoch *et al.* (2006) were recovered in our combined SSU and LSU phylogeny. *Leotiomyces* was not monophyletic in our analyses, but this relationship was not supported.

Eighty-four Dothideomycete isolates from freshwater habitats, including meiosporic and mitosporic representatives, were included

ML BP/Bayesian PP



**Fig. 2.** Freshwater *Dothideomycetes* phylogeny. The most likely tree (Ln L = -17187.0385) after 100 replicates of a RAxML analysis of combined SSU and LSU data. Orders, classes, and families are indicated on the tree. ML bootstrap support values greater than 70 % are indicated along with Bayesian posterior probabilities  $\geq 95$  % for nodes. Members of *Pezizomycetes* are used as outgroup taxa. Freshwater lineages are labeled as Clades A–D and taxa isolated and described from freshwater habitats are indicated with Fresh W. Ascospore modifications are indicated by: ☆ = greatly elongating sheath; ● = thin to thick non-elongating sheath; □ = apical appendages; ○ = no sheath; ▲ = gelatinous pads. Scale bar indicates nucleotide substitutions per site.

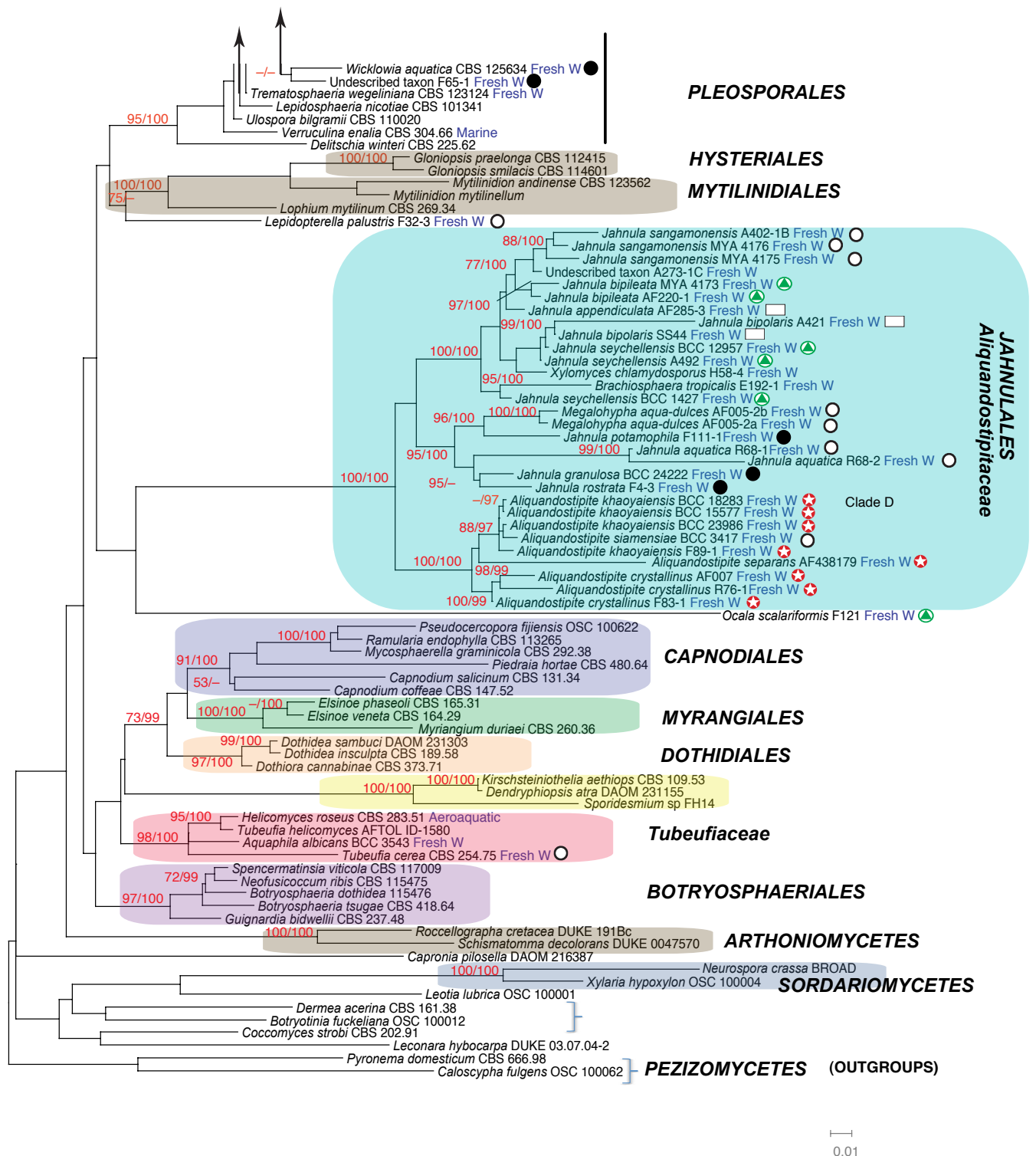


Fig. 2. (Continued).

in this study. The majority of freshwater *Dothideomycetes* had phylogenetic affinities to taxa in *Pleosporales* (Fig. 2). Four major clades (A–D) of freshwater fungi were recovered, of which three clades received  $\geq 70\%$  Maximum Likelihood Bootstrap (MLB) support and  $\geq 90\%$  Bayesian Posterior Probability (BPP) (Fig. 2). *Lentitheciaceae* (Clade A) included six taxa, together with undescribed taxon A369-2B but was not supported by either MLB or BPP. *Amniculicolaceae* (Clade B) was well supported with 97% ML bootstrap support and 100% BPP. *Lindgomycetaceae* (Clade C) was also supported with 77% MLB and 100% BPP values.

*Jahnulales* (Clade D) received 100% MLB and 100% BPP support and formed a strong monophyletic group.

Eight undescribed freshwater *Dothideomycetes* were dispersed throughout the *Pleosporomycetidae* as follows: A369-2B in *Lentitheciaceae*; F80-1 as sister taxon to *K. elaterascus*; A164 and A183 in *Lophiostomataceae* 1; A-25-1, F-60, and F-65 in *Lindgomycetaceae*; and A273-1c in *Jahnulales*. A few singletons such as *Lepidopterella palustris* and *Ocala scalariformis* are on single lineages without any relationships to known groups included in the analyses.

The anamorph genus *Xylomyces* was polyphyletic, with one species, *X. elegans*, placed with *Massarina* species in the *Pleosporales*, and the other, *X. chlamyosporus*, placed within *Jahnulales* (Fig. 2). The affinity of *Anguillospora longissima* (CS869-1D, Shearer isolate) to *Amniculicola lignicola*, *A. immersa* and *A. parva* (Fig. 2) confirms this relationship reported previously for a different isolate of *A. longissima* (Zhang *et al.* 2009a). *Tumularia aquatica*, originally assigned to *Massarina aquatica* (Webster 1965) was placed with *Lophiostoma glabrotunicatum*, an aquatic fungus collected in mountain streams in France on submerged wood of *Alnus glutinosa*, *Fagus sylvatica* and *Salix sp.* (Zhang *et al.* 2009c). *Taeniolella typhoides* occurred in a well-supported group with members of *Lindgomycetaceae* in *Pleosporales*. *Lemmoniera pseudofloscula* isolates occurred among terrestrial taxa as a highly supported sister taxon to a clade of *Alternaria alternata*, *Alternaria sp.* and *Allewia eureka*. This placement is somewhat controversial and a more detailed study with additional isolates and more gene regions should be carried out.

## DISCUSSION

Within *Dothideomycetes*, the freshwater species occur in *Pleosporomycetidae* but not *Dothideomycetidae*. It is interesting to speculate on possible reasons for this pattern. First, overall there are more taxa in the *Pleosporomycetidae* than *Dothideomycetidae* resulting in a numerical imbalance between subclasses in most ecological and taxonomic groups. Second, many of the orders in *Dothideomycetidae* contain specialised plant pathogens, e.g., *Capnodiales*, *Myriangiiales*, and *Botryosphaeriales*, many of which grow on leaves. It is possible that such specialised fungi have lost the genetic potential to adapt to a submerged, saprobic lifestyle. Third, the absence of pseudoparaphyses in *Dothideomycetidae* taxa may limit survival in aquatic habitats with fluctuating water levels. Pseudoparaphyses of aquatic species in *Pleosporomycetidae* are often abundant and surrounded by gel, which may protect the asci from desiccation during dry conditions. There is currently no experimental evidence, however, to support this idea.

Freshwater *Dothideomycete* species are distributed throughout the *Pleosporomycetidae* (Fig. 2). Several clades, however, contain numerous freshwater species and merit discussion. Clade A (*Lentitheciaceae*), which consists entirely of freshwater taxa, is not well supported in this study (Fig. 2). Reasons for this lack of support are not clear at this time. For a discussion of this clade, see Zhang *et al.* (2009b; this volume). The well-supported Clade B (*Amniculicolaceae*) consists of four freshwater teleomorph species and one aquatic hyphomycete anamorph species. This family is established and described in detail by Zhang *et al.* (2009b; this volume).

A third exclusively freshwater lineage is Clade C (*Lindgomycetaceae*) (Fig. 2). This well supported clade was first revealed during a recent molecular sequence-based study of *Massarina ingoldiana* Shearer & Hyde s. l. (Hirayama *et al.* 2010). A number of dothideomycetous aquatic species that have 1-septate, hyaline ascospores surrounded by a prominent gelatinous sheath that elongates greatly in water were included in this study. Analyses of a combined dataset of SSU and LSU sequences for a number of aquatic isolates of *M. ingoldiana* and other morphologically similar fungi along with the type specimens of *Massarina* and *Lophiostoma* were conducted. Their results showed that none of the aquatic taxa belonged in *Massarina* or *Lophiostoma* and that convergent evolution in ascospore morphology had occurred, confounding

systematic placement based on ascospore morphology. Our results support the study by Hirayama *et al.* (2010) which found that taxa with 1-septate, hyaline ascospores with a large, elongating gelatinous sheath have evolved independently in several lineages within *Dothideomycetes* (*Lentitheciaceae*, *Lindgomycetaceae*, and *Aliquandostipitaceae*) (Fig. 2). Thus in freshwater *Dothideomycetes*, this form of the gelatinous sheath is not taxonomically informative at the family or genus level.

Clade D (*Jahnulales*) contains the greatest number of freshwater species (Fig. 2). The type species of *Jahnula*, *J. aquatica*, was described as *Amphisphaeria aquatica* by Plöttner and Kirschstein in 1906 from *Salix* wood in a wet ditch in Germany. Kirschstein (1936) subsequently changed the name of this fungus to *Jahnula*. The genus remained monotypic until 1999, when Hyde & Wong (1999) described five new tropical species based on morphological data. Currently, *Jahnula* and *Aliquandostipite*, a genus morphologically similar to *Jahnula* that was established by Inderbitzen *et al.* (2001), represent a well-supported lineage in *Dothideomycetidae* based on molecular and morphological data (Inderbitzen *et al.* 2001, Pang *et al.* 2002, Campbell *et al.* 2007, Suetrong *et al.* 2009, 2010). Pang *et al.* (2002) established a new order, *Jahnulales*, for this group. *Jahnulales* now contains numerous species representing four meiosporic genera and two mitosporic genera from freshwater habitats (Hyde 1992, Hyde & Wong 1999, Pang *et al.* 2002, Pinruan *et al.* 2002, Raja *et al.* 2005, 2008, Ferrer *et al.* 2007, Raja & Shearer 2006, 2007). *Manglicola guatemalensis*, collected from mangroves, was recently confirmed to belong in *Jahnulales* (Suetrong *et al.* 2010). There appear to be four, possibly five, separate lineages within *Jahnulales*, but further molecular work is needed to confirm these lineages. Species in this clade are well adapted for aquatic habitats with large-celled pseudothecia and ascospores filled with lipid guttules and equipped with a variety of gelatinous appendages, pads and sheaths (Fig. 2). Thus far, all members in the order have broad vegetative hyphae (10–40 µm) that attach the fungi to softened, submerged wood.

Clade *Lophiostomataceae* 1 was well supported as a whole in this study and studies by Tanaka & Hosoya (2008) and Zhang *et al.* (2009c), but relationships within this clade were not well resolved. Several taxa within this clade are undescribed and additional morphological and molecular data are needed to further resolve relationships within this group.

Two interesting freshwater taxa in *Dothideomycetidae* included in this study, *Ocala scalariformis* and *Lepidopterella palustris*, did not show strong phylogenetic affinities with any of the major families and orders included in the *Dothideomycetes* (Fig. 2). These so called singletons each has a distinctive combination of morphological characteristics that perhaps make them unique among other *Dothideomycetes* taxa included in the phylogeny. *Ocala scalariformis* possesses morphological characters that include superficial to erumpent, globose to subglobose, hyaline perithecial ascomata with an ostiole; cellular pseudoparaphyses; fissitunicate asci; and hyaline, 1-septate, thick-walled ascospores with appendages (Raja *et al.* 2009a). However, based on the combined SSU and LSU phylogeny, *Ocala scalariformis* is placed as basal to the *Jahnulales*, without any statistical support. *Lepidopterella palustris* has black, cleistothecial ascomata appearing as raised dome-shaped structures on the substrate; hamathecium of hyaline, septate, narrow pseudoparaphyses not embedded in a gel matrix; thick-walled, globose to subglobose, broadly rounded, fissitunicate asci; and brown butterfly shaped ascospores (Shearer & Crane 1980, Raja & Shearer 2008). Based on our phylogeny it forms a single branch by itself, basal to the

*Mytilindiales* with moderate bootstrap support (Fig. 2). It is possible that these singletons represent new lineages currently unknown in the *Dothideomycetes*.

Belliveau & Baerlocher (2005) showed that aquatic hyphomycetes have multiple origins within the ascomycetes. In this study, we included some hyphomycete taxa that had phylogenetic affinities to the *Dothideomycetes* based on previous studies (Belliveau & Bärlöcher 2005, Campbell *et al.* 2006, 2007, Zhang *et al.* 2009c). These taxa are: *Anguillospora longissima*, *Lemonniera pseudofloscula*, *Taeniolella typhoides*, *Tumularia aquatica*, and *Brachiosphaera tropicalis*. Previous studies showed that *Anguillospora longissima* had a strong affinity to *Pleosporales* and was a sister species to *Kirschsteiniothelia maritima* (Baschien 2003, Belliveau & Bärlöcher 2005). In contrast, Voglmayr (2004) reported a close relationship between an aeroaquatic fungus, *Spirosphaera cupreorufescens*, and *A. longissima*. Baschien *et al.* (2006) confirmed the close relationships of the five isolates of *A. longissima* to *Spirosphaera cupreorufescens*. Zhang *et al.* (2009c) in a maximum parsimony tree generated from partial 28S rDNA gene sequences showed a 91 % bootstrap support for a clade formed by *A. longissima*, *Spirosphaera cupreorufescens*, *Repetophragma ontariense* and three species of *Amniculicola*. In our analyses, *A. longissima* is placed in the new aquatic family *Amniculicolaceae* (Clade B) Fig. 2 (See Zhang *et al.* 2009b; this volume).

*Taeniolella typhoides* was described without a teleomorph. Here it forms a well-supported sister clade with *Massariosphaeria typhicola*. The epithet of *T. typhoides* may indicate some relationship to *Typha*, but this is a casual coincidence only as “*typhoides*” is for “similar to *Typha*”. The teleomorph of *Taeniolella* is *Glyphium*, *Mytilindiales* (Kirk *et al.* 2008).

*Tumularia aquatica* is the type species of *Tumularia* and was connected by Webster (1965) to the teleomorph, *Massarina aquatica*. *Massarina aquatica* was later recombined on the basis of morphology in *Lophiostoma* as *L. aquatica* (Hyde *et al.* 2002). In this study, *T. aquatica* is placed with *Lophiostoma glabrotunicatum* in the *Lophiostomataceae* 2/*Melannomataceae* Clade, but lacks significant bootstrap support (Fig. 2).

*Brachiosphaera tropicalis* has conidia very similar to those of *Actinosporella megalospora* and the two species are sometimes confused with each other. On the basis of pure culture studies Descals *et al.* (1976) pointed out the essentially different conidiogenesis (blastic sympodial in *Brachiosphaera* vs. retrogressive thallic in *Actinosporella*) and also subtle differences in conidial morphology (constricted appendage insertion in *Brachiosphaera* vs. unconstricted in *Actinosporella*). The placement of *Brachiosphaera* within *Jahnulales* (Campbell *et al.* 2007) confirms its unrelatedness to *Actinosporella*, which has been connected to the *Pezizales* by Descals and Webster (1978).

The genus *Lemonniera* is characterised by tetradiate conidia with long arms, phialidic conidiogenesis, and formation of minute dark sclerotia in culture. Previously, it has been shown to be polyphyletic and different species of *Lemonniera* are placed in two distinct clades, namely the *Leotiomyces* and the *Dothideomycetes* (Campbell *et al.* 2006). In our study we used two isolates of *L. pseudofloscula* previously sequenced by Campbell *et al.* (2006). These isolates form a strongly supported monophyletic group within the *Pleosporaceae*.

More recently, Prihatini *et al.* (2008) have shown that *Speiropsis pedatospora* (Tubaki 1958) has phylogenetic affinities within the *Jahnulales* based on ITS rDNA data. Also, in another recent study by Jones *et al.* (2009), *Sigmoidea prolifera* and *Pseudosigmoidea cranei*, two aquatic hyphomycetes were shown to have phylogenetic

affinities with the *Phaeotrichaceae*, *Pleosporales* based on SSU data. Sequencing of additional aquatic hyphomycete taxa in the future will continue to shed light on the evolutionary relationships of freshwater aquatic hyphomycetes to different lineages within the *Dothideomycetes*.

## CONCLUSIONS

The freshwater *Dothideomycetes* occur primarily in the *Pleosporomycetidae* as opposed to the *Dothideomycetidae* and appear to have adapted to freshwater habitats numerous times, often through ascospore adaptations, and sometimes, through anamorph conidial adaptations. Ascospores and conidiospores of freshwater fungi are under strong selective pressure to disperse and attach to substrates in freshwater habitats in order for the fungi to complete their life cycles. Thus ascospore features that facilitate dispersal and attachment may not be as reliable as other morphological features such as ascomata and hamothecia in interpreting phylogenetic relationships among freshwater *Dothideomycetes*. This idea is supported by the presence of similar ascospore modifications such as the presence of gelatinous ascospore sheaths in phylogenetically distant taxa. Further support is the presence of tetradiate conidia present in widely separated clades.

The presence of morphologically unique singletons within the molecular-based phylogenetic tree of *Dothideomycetes* suggests that we need to further sample the freshwater ascomycetes to identify close relatives of these taxa.

We expect that future collections from freshwater habitats will modify the phylogeny presented in this paper by increasing the size and support values of existing clades containing freshwater species and in increasing the number of exclusively freshwater clades.

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## SUPPLEMENTARY INFORMATION

Table 1. Species used in this study.

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
<i>Aliquandostipite crystallinus</i> *	F83-1	Raja & Shearer	GU266221	GU266239
	AF007	–	EF175631	EF175652
	R76-1	–	EF175630	EF175651
<i>Aliquandostipite khaoyaiensis</i>	F89-1	Raja & Shearer	EF175625	EF175647
	SS2961	BCC 15577	EF175626	EF175648
	SS3028	BCC 23986	EF175627	EF175649
	SS3321	BCC 18283	EF175628	EF175650
<i>Aliquandostipite separans</i>		–	AF438179	–
<i>Aliquandostipite siamensis</i>	SS81.02	BCC 3417	EF175645	EF175666
<i>Allewia eureka</i>		DAOM 195275	DQ677994	DQ678044
<i>Alternaria alternata</i>		CBS 916.96	DQ678031	DQ678082
<i>Alternaria</i> sp. (as <i>Clathrospora diplospora</i> )		CBS 174.51	DQ678016	DQ678068
<i>Amniculicola immersa</i>	–	KD Hyde	GU456295	FJ795498
<i>Amniculicola lignicola</i>	–	KD Hyde	EF493863	EF493861
<i>Amniculicola parva</i>		KD Hyde	GU296134	FJ795497
<i>Anguillospora longissima</i> *	CS869-1D	Shearer	GU266222	GU266240
<i>Aquaticheirospora lignicola</i>		–	AY736377	AY736378
<i>Aquaphila albicans</i>		BCC 3543	DQ341093	DQ341101
<i>Ascochyta pisi</i> var. <i>pisi</i>		CBS 126.54	DQ678018	DQ678070
<i>Ascorhombispora aquatica</i>		–	–	EU196548
<i>Bimuria novae-zelandiae</i>		CBS 107.19	AY016338	AY016356
<i>Botryosphaeria dothidea</i>		CBS 115476	DQ677998	DQ678051
" <i>Botryosphaeria</i> " <i>tsugae</i>		CBS 418.64	AF271127	DQ767655
<i>Botryotinia fuckeliana</i>		OSC 100012	AY544695	AY544651
<i>Brachiosphaera tropicalis</i>	E192-1	Shearer	GU266223	EF175653
<i>Byssothecium circinans</i>		CBS 675.92	AY016339	AY016357
<i>Caloscypha fulgens</i>		OSC 100062	DQ247807	DQ247799
<i>Capnodium coffeae</i>		CBS 147.52	DQ247808	DQ247800
<i>Capnodium salicinum</i>		CBS 131.34	DQ677997	DQ678050
<i>Capronia pilosella</i>		DAOM 216387	DQ823106	DQ823099
<i>Coccomyces strobil</i>		CBS 202.91	DQ471027	DQ470975
<i>Cheirosporium triseriale</i>		–	–	EU413954
<i>Cochliobolus heterostrophus</i>		CBS 134.39	AY544727	AY544645
<i>Cochliobolus sativus</i>		DAOM 216378	DQ677995	DQ678045
<i>Coniothyrium obiones</i>		CBS 453.68	DQ678001	DQ678054
<i>Coniothyrium palmarum</i>		CBS 400.71	DQ678008	DQ767653
<i>Cucurbitaria elongata</i>		CBS 171.55	DQ678009	DQ678061
<i>Delitschia winteri</i>		CBS 225.62	DQ678026	DQ678077
<i>Dendryphiella arenaria</i>		CBS 181.85	DQ471022	DQ470971
<i>Dendryphiopsis atra</i>		DAOM 231155	DQ677996	DQ678046
<i>Dermea acerina</i>		CBS 161.38	DQ247809	DQ247801
<i>Didymella cucurbitacearum</i>		IMI 373225	AY293779	AY293792
<i>Dothidea insculpta</i>		CBS 189.58	DQ247810	DQ247802
<i>Dothidea sambuci</i>		DAOM 231303	AY544722	AY544681

**Table 1.** (Continued).

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
<i>Dothiora cannabinae</i>		CBS 373.71	DQ479933	DQ470984
<i>Elsinoë phaseoli</i>		CBS 165.31	DQ678042	DQ678095
<i>Elsinoë veneta</i>		CBS 164.29	DQ678007	DQ678060
<i>Gloniopsis praelonga</i>		CBS 112415	FJ161134	FJ161173
<i>Gloniopsis smilacis</i>		CBS 114601	FJ161135	FJ161174
<i>Guignardia bidwelli</i>		CBS 237.48	DQ678034	DQ678085
<i>Helicascus kanaloanus</i>		ATCC 18591	AF053729	–
<i>Helicomycetes roseus</i>		CBS 283.51	DQ678032	DQ678083
<i>Herpotrichia diffusa</i>		CBS 250.62	DQ678019	DQ678071
<i>Herpotrichia juniperi</i>		CBS 200.31	DQ678029	DQ678080
<i>Jahnula appendiculata*</i>	AF285-3	Shearer	GU266224	GU266241
<i>Jahnula aquatica</i>	R68-1	Raja & Shearer	EF175633	EF175655
	R68-2	Raja & Shearer	EF175632	NA
<i>Jahnula bipileata</i>	F49-1	MYA 4173	EF175635	EF175657
	AF220-1	Shearer	EF175634	EF175656
<i>Jahnula bipolaris</i>	SS44	BCC 3390	EF175637	EF175658
	A421	Shearer	EF175636	–
<i>Jahnula granulosa</i>	SS1562	BCC24222	EF175638	EF175659
<i>Jahnula potamophila*</i>	F111-1	Raja & Shearer	GU266225	GU266242
<i>Jahnula rostrata</i>	F4-3	MYA4176	GU266226	EF175660
<i>Jahnula sangamonensis</i>	A482-1B	MYA 4174	EF175640	EF175662
	A402-1B	Shearer	EF175639	EF175661
	F81	MYA 4175	EF175641	EF175663
<i>Jahnula seychellensis</i>	SS2133.1	BCC 14207	EF175644	EF175665
	SS2113.2	BCC 12957	EF175643	EF175664
	A492	Shearer	EF175642	GU266243
<i>Kirschsteiniothelia aethiops</i>		CBS 109.53	AY016344	AY016361
<i>Kirschsteiniothelia elaterascus</i>	A22-11B-/	–	AF053728	–
			–	AY787934
<i>Lecanora hybocarpa</i>		DUKE 03.07.04-2	DQ782883	DQ782910
<i>Lentithecium aquaticum</i>		CBS 123099	FJ795477	FJ795434
<i>Lentithecium arundinaceum</i>		CBS 619.86	DQ813513	DQ813509
<i>Lemonniera pseudofloscula</i>		CCM F-0484	–	DQ267631
		CCM F-43294	–	DQ267632
<i>Leotia lubrica</i>		OSC100001	AY544687	AY544644
<i>Lepidopterella palustris*</i>	F32-3	Raja & Shearer	GU266227	GU266244
<i>Leptosphaeria maculans</i>		DAOM 229267	DQ470993	DQ470946
<i>Lepidosphaeria nicotiae</i>		CBS 101341	–	DQ678067
<i>Lindgomyces cinctosporae</i>	R56-1		AB522430	AB522431
	R56-3	Raja & Shearer	GU266238	GU266245
<i>Lindgomyces breviappendiculatus</i>	KT 215	JCM 12702/MAFF 239291	AB521733	AB521748
	KT 1399	JCM 12701/MAFF 239292	AB521734	AB521749
<i>Lindgomyces ingoldianus</i>	A39-1	ATCC200398	AB521719	AB521736
	KH 100	JCM 16479	AB521720	AB521737
<i>Lindgomyces</i> sp.	KH 241	JCM16480	AB521721	AB521738
<i>Lindgomyces rotundatus</i>	KT 966	JCM 16481/MAFF 239473	AB521722	AB521739
	KT 1096	JCM 16482	AB521723	AB521740

Table 1. (Continued).

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
	KH 114	JCM 16484	AB521725	AB521742
	KT1107	JCM 16483	AB521724	AB521741
<i>Lophiostoma arundinis</i>		CBS 269.34	DQ782383	DQ782384
<i>Lophiostoma crenatum</i>		CBS 629.86	DQ678017	DQ678069
<i>Lophiostoma glabrotunicatum</i>		IFRD 2012	FJ795481	FJ795438
<i>Lophiostoma macrostomum</i>	KT 635	JCM 13545	AB521731	AB433273
	KT 709	JCM 13546 MAFF 239447	AB521732	AB433274
			SSU	LSU
<i>Lophium mytilinum</i>		CBS 269.34	DQ678030	DQ678081
<i>Massaria platani</i>		CBS 221.37	DQ678013	DQ678065
<i>Massarina australiensis</i>		–	AF164364	–
<i>Massarina bipolaris</i>		–	AF164365	–
<i>Massarina eburnea</i>	H 3953	JCM 14422	AB521718	AB521735
		–	AF164366	–
		–	AF164367	–
<i>Massariosphaeria typhicola</i>	KT 667	MAFF 239218	AB521729	AB521746
	KT 797	MAFF 239219	AB521730	AB521747
<i>Megalohypha aqua-dulces*</i>	AF005-2a	–	GU266228	EF175667
	AF005-2b	–	–	EF175668
<i>Melanomma radicans</i>		ATCC 42522	U43461	U43479
<i>Montagnula opulenta</i>		CBS 168.34	AF164370	DQ678086
<i>Mycosphaerella graminicola</i>		CBS 292.38	DQ678033	DQ678084
<i>Myriangium duriaei</i>		CBS 260.36	AY016347	DQ678059
<i>Mytilinidion andinense</i>		EB 0330 (CBS 123562)	FJ161159	FJ161199
<i>Mytilinidion mytilinellum</i>		CBS 303.34	FJ161144	FJ161184
<i>Neofusicoccum ribis</i>		CBS 115475	DQ678000	DQ678053
<i>Neurospora crassa</i>		BROAD	X04971	AF286411
<i>Ocala scalariformis*</i>	F121-1	Raja & Shearer	GU266229	–
<i>Ophiosphaerella herpotricha</i>		CBS 620.86	DQ678010	DQ678062
		CBS 240.31	DQ767650	DQ767656
<i>Phaeodothis winteri</i>		CBS 182.58	DQ678021	DQ678073
<i>Phaeosphaeria avenaria</i>		DAOM 226215	AY544725	AY544684
<i>Phaeosphaeria eustoma</i>		CBS 573.86	DQ678011	DQ678063
<i>Phoma herbarum</i>		CBS 276.37	DQ678014	DQ678066
<i>Piedraia hortae</i>		CBS 480.64	AY016349	AY016366
<i>Pleomassaria siparia</i>		CBS 279.74	DQ678027	DQ678078
<i>Pleospora herbarum</i> var. <i>herbarum</i>		CBS 714.68	DQ767648	DQ678049
		CBS 514.72	DQ247812	DQ247804
<i>Preussia terricola</i>		DAOM 230091	AY544726	AY544686
<i>Pseudocercospora fijiensis</i>		OSC 100622	DQ767652	DQ678098
<i>Pyrenophora phaeocomes</i>		DAOM 222769	DQ499595	DQ499596
<i>Pyrenophora tritici-repentis</i>		OSC 100066	AY544716	AY544672
<i>Pyronema domesticum</i>		CBS 666.98	DQ247813	DQ247805
<i>Quintaria lignatilis</i>		–	QLU43462	–
<i>Ramularia endophylla</i>		CBS 113265	DQ471017	DQ470920
<i>Roccellographa cretacea</i>		DUKE 191Bc	DQ883705	DQ883696
<i>Schismatomma decolorans</i>		DUKE 0047570	AY548809	AY548815

**Table 1.** (Continued).

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
<i>Semimassariosphaeria typhicola</i> **			GU296174	FJ795504
<i>Spencermartinsia viticola</i>		CBS 117009	DQ678036	DQ678087
<i>Sporormiella minima</i>		CBS 524.50	DQ678003	DQ678056
<i>Sporidesmium</i> sp.	FH14	–	GU266230	–
<i>Taeniolella typhoides</i>		CCM F-10198/extype	GU266231	–
<i>Tingoldiagio graminicola</i>	KH 68	JCM 16485	AB521726	AB521743
	KT 891/	MAFF 239472	AB521727	AB521744
	KH 155/	JCM 16486	AB521728	AB521745
<i>Trematosphaeria hydrophila</i>		IFRD 2037	GU261721	–
<i>Trematosphaeria heterospora</i>		CBS 644.86	AY016354	AY016369
<i>Trematosphaeria pertusa</i>		CBS 400.97	DQ678020	DQ678072
<i>Trematosphaeria wegeliniana</i>		CBS 123124	GU261720	GU261722
			SSU	LSU
<i>Tubeufia cerea</i>		CBS 254.75	DQ471034	DQ470982
<i>Tubeufia helicomyces</i>		–	DQ767649	DQ767654
<i>Tumularia aquatica</i>		CCM F-02081	AY357287	–
<i>Ulospora bilgramii</i>		CBS 110020	DQ678025	DQ678076
<i>Verruculina enalia</i>		CBS 304.66	DQ678028	DQ678079
<i>Westerdykella cylindrica</i>		CBS 454.72	AY016355	AY004343
<i>Wicklowlia aquatica</i> *	F76-2	CBS 125634	GU266232	GU045445
<i>Xylaria hypoxylon</i>		OSC 100004	AY544719	AY544676
<i>Xylomyces chlamydosporus</i> *	H58-4		GU266233	EF175669
<i>Xylomyces elegans</i> *	H80-1		GU266234	–
Undescribed taxon A25-1*		Shearer	–	GU266246
Undescribed taxon R60-1*		Raja & Shearer	GU266235	GU266247
Undescribed taxon F65-1		Shearer	GU266236	GU266248
Undescribed taxon A369-1*		Raja & Shearer	–	GU266249
Undescribed taxon F80-1*		Shearer	GU266237	GU266250
Undescribed taxon A164-1C*		Shearer	–	GU266251
Undescribed taxon A164-1D*		Shearer	–	GU266252
Undescribed taxon A183-1C*		Shearer	–	GU266253
Undescribed taxon A183-1D*		Shearer	–	GU266254
Undescribed taxon A273-1C*		Shearer		GU266255