available online at www.studiesinmycology.org
doi:10.3114/sim.2009.64.08

STUDIES IN MYCOLOGY 64: 145–153. 2009.

The molecular phylogeny of freshwater *Dothideomycetes*

C.A. Shearer[1*], H.A. Raja[1], A.N. Miller[2], P. Nelson[3], K. Tanaka[4], K. Hirayama[4], L. Marvanová[5], K.D. Hyde[6] and Y. Zhang[7]

[1]*Department of Plant Biology, University of Illinois, 505 S. Goodwin Ave., Urbana, IL 61801, U.S.A.;* [2]*Illinois Natural History Survey, University of Illinois, 1816 South Oak St., Champaign, IL, 61820, U.S.A.;* [3]*University of Minnesota, Ecology, Evolution, and Behavior, 100 Ecology Building, St. Paul, MN 55108, U.S.A.;* [4]*Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan;* [5]*Czech Collection of Mircroorganisms, Institute of Experimental Biology, Faculty of Science, Masaryk University, Tvrdého 14, Brno CZ-602 00, Czech Republic;* [6]*School of Science, Mae Fah Luang University, Tasud, Muang, Chiang Rai 57100, Thailand;* [7]*Division of Microbiology, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, P.R. China*

**Correspondence*: C.A. Shearer, carolshe@uiuc.edu

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 *Taeniolella typhoides*. The *Lindgomycetaceae* clade consists of taxa formerly described in *Massarina, Lophiostoma, and Massariosphaeria e.g., Massarina ingoldiana*, *Lophiostoma breviappendiculatum*, 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 *Tingoldiago 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.*

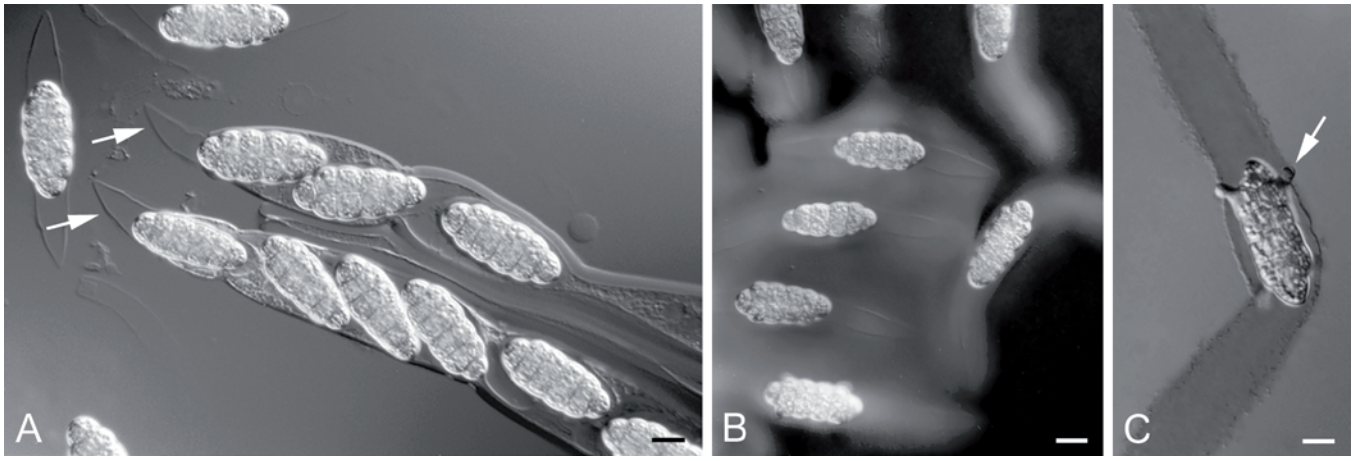

Fig. 1. *Macrospora scirpicola* A27-1. A. Ascospores being discharged from bitunicate asci showing bipolar gelatinous appendages. B. Ascospores showing an outer and inner sheath when stained with India ink. C. Ascospore on a glass slide germinating within its gelatinous sheath stained with India ink.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

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 asiana* (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 *Leotiomycetes* 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 µL 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 µL 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 µL of BSA (bovine serum albumin, New England Biolabs, Ipswich, MA) and/or 2.5 µL 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 µL 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<–>C = 0.9722, A<–>G = 2.7980, A<–>T = 1.1434, C<–>G = 0.6546, C<–>T = 5.1836, G<–>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 000th 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. *Leotiomycetes* 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

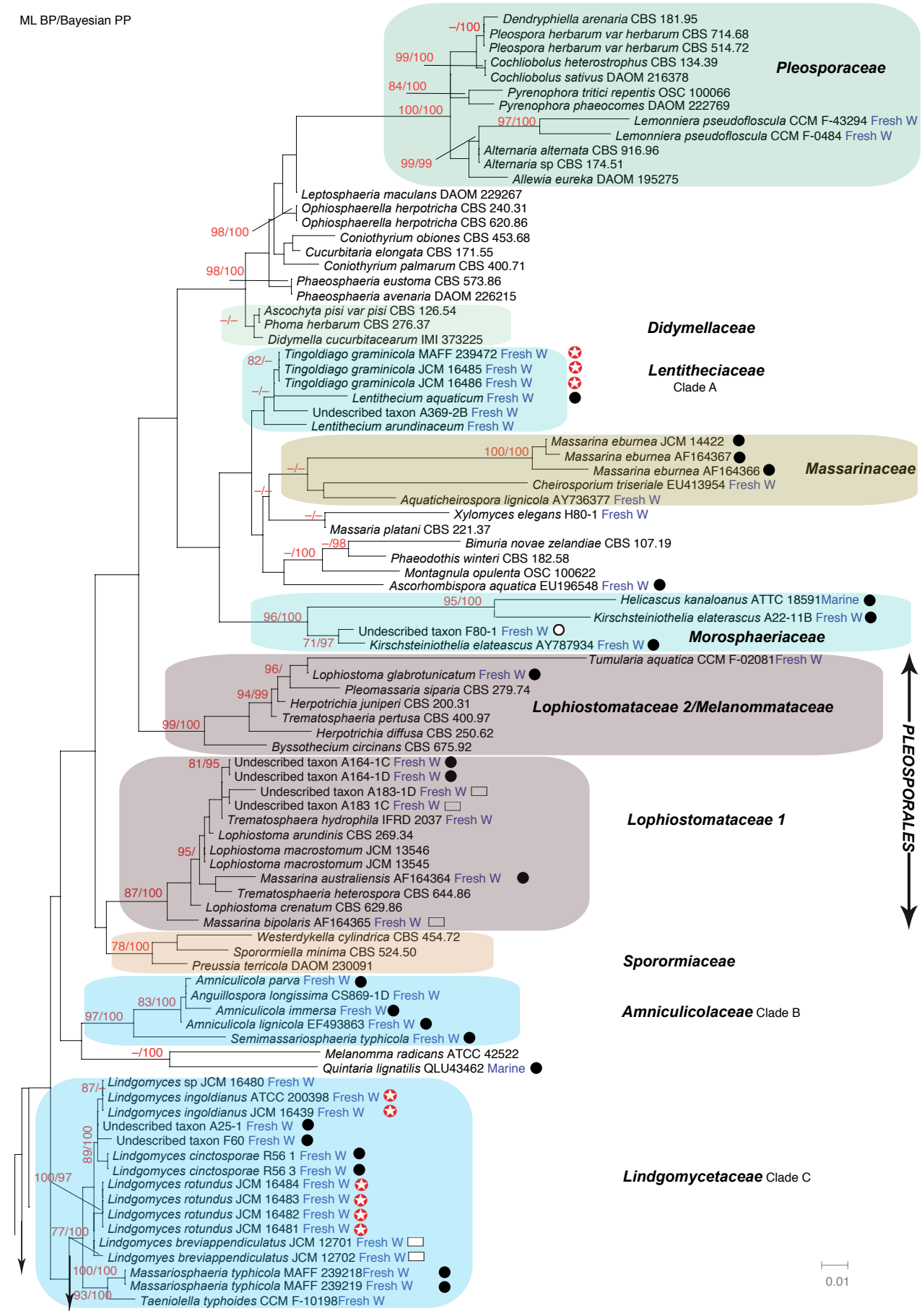

Fig. 2. Freshwater *Dothideomycetes* phylogeny. The most likely tree (Ln L = -17187.0385) after 100 replicates of a RAxML analysis of combined SSU and LSU data. Orders, classes, and families are indicated on the tree. ML bootstrap support values greater than 70 % are indicated along with Bayesian posterior probabilities ≥ 95 % for nodes. Members of *Pezizomycetes* are used as outgroup taxa. Freshwater lineages are labeled as Clades A–D and are shaded in blue 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.

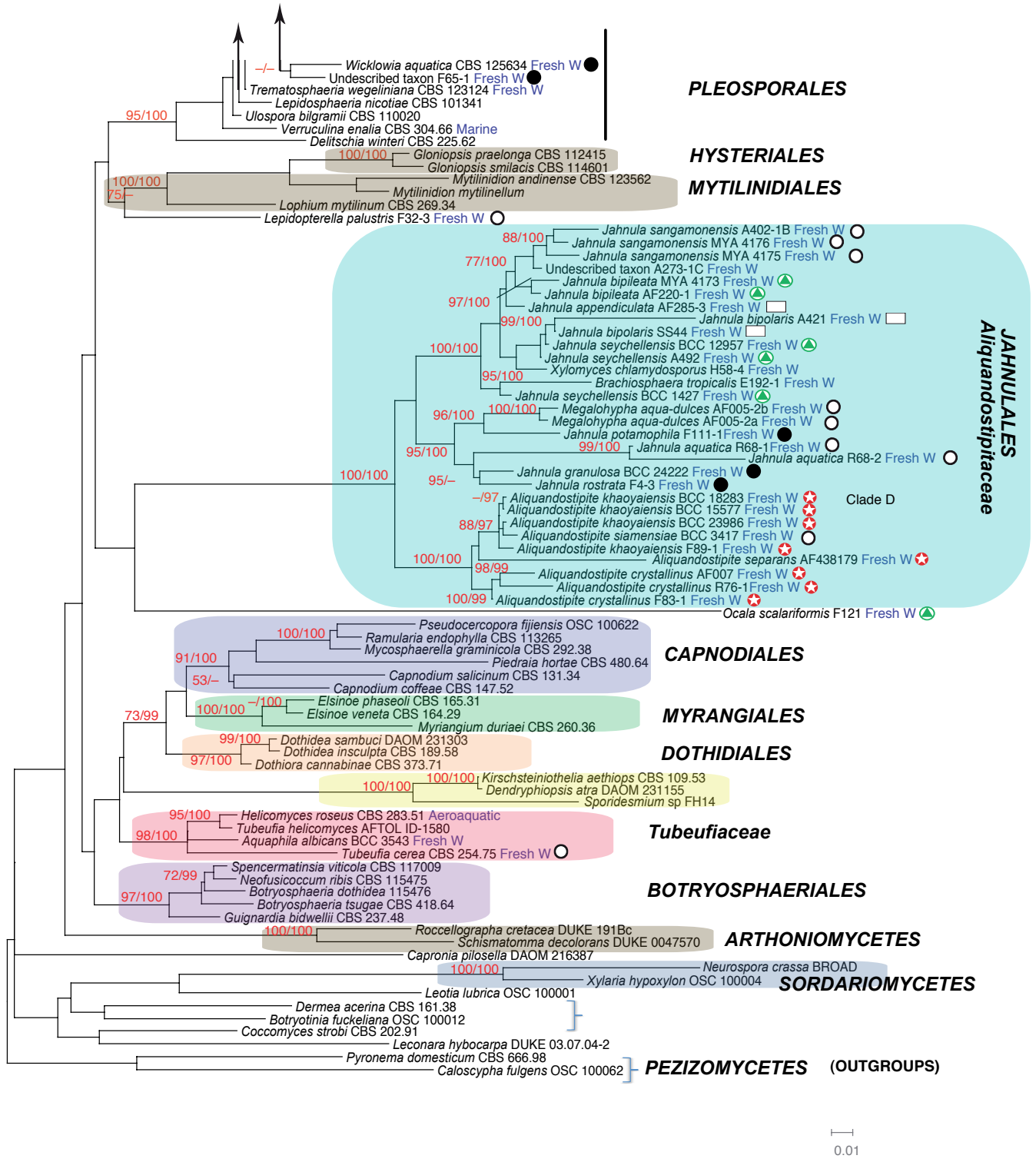

Fig. 2. (Continued).

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 ≥ 70 % Maximum Likelihood Bootstrap (MLB) support and ≥ 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. chlamydosporus*, 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. Lemonniera 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, Myriangiales, 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 phyologenetic affinities to the *Dothideomycetes* based on previous studies (Belliveau & Bärlocher 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 & Bärlocher 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, Mytilinidiales* (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 tetraradiate 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 *Leotiomycetes* 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 hamathecia 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 tetraradiate 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.

ACKNOWLEDGEMENTS

We thank Conrad Schoch for his helpful comments on the taxon sampling as well as for his assistance with the RAxML analysis. This work was partially supported by a foundation from the Institute for Fermentation, Osaka (IFO). Participation of L. Marvanová on this study was partly supported by the project (MSM00216222416) of the Czech Ministry of Education Youth and Sports. Financial support from the National Science Foundation and National Institutes of Health (NSF No. DEB 03-16496, NSF DEB 05-15558, NSF DEB 08-44722 and NIH No. R01GM-60600) helped make this research possible. We are grateful to the curator of IMI for the loan of a specimen. Any opinions, findings and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Foundation and National Institutes of Health.

REFERENCES

Baschien C (2003). *Development and evaluation of rDNA targeted in situ probes and phylogenetic relationships of freshwater fungi.* PhD Thesis. Technische Universität Berlin, Berlin.

Baschien C, Marvanová L, Szewzyk U (2006). Phylogeny of selected aquatic hyphomycetes based on morphological and molecular data. *Nova Hedwigia* **83**: 311–352.

Belliveau MJ-R, Bärlocher F (2005). Molecular evidence confirms multiple origins of aquatic hyphomycetes. *Mycological Research* **109**: 1407–1417.

Cai L, Guo XY, Hyde KD (2008). Morphological and molecular characterisation of a new anamorphic genus *Cheirosporium,* from freshwater in China. *Persoonia* **20**: 53–58.

Cai L, Hyde KD (2007). *Ascorhombispora aquatica* gen. et sp. nov. from a freshwater habitat in China, and its phylogenetic placement based on molecular sequence data. *Cryptogamie Mycologie* **28**: 291–300.

Campbell J, Ferrer A, Raja HA, Sivichai S, Shearer CA (2007). Phylogenetic relationships among taxa in the *Jahnulales* inferred from 18S and 28S nuclear ribosomal DNA sequences. *Canadian Journal of Botany* **85**: 873–882.

Campbell J, Shearer CA, Marvanová (2006). Evolutionary relationships among aquatic anamorphs and teleomorphs: *Lemonniera, Margaritispora,* and *Goniopila*. *Mycological Research* **110**: 1025–1033.

Descals EC, Nawawi A, Webster J (1976). Developmental studies in *Actinospora* and three similar aquatic hyphomycetes. *Transactions of the British Mycological Society* **67**: 207–222.

Descals EC, Webster J (1978). *Miladina lechithina (Pezizales),* the ascigerous state of *Actinospora megalospora. Transactions of the British Mycological Society* **70**: 466–472.

Drummond AJ, Kearse M, Heled J, Moir R, Thierer T, Ashton B, Wilson A, Stones-Harvas S (2006). Geneious v. 2.5, Available from htt://www.geneious.com/.

Dudka IO (1963). Data on the flora of aquatic fungi of the Ukrainian SSR. II. Aquatic hyphomycetes of Kiev Polessye. *Ukraine Botanical Journal* **20**: 86–93.

Dudka IO (1985). Ascomycetes, components of freshwater biocoenosis. *Ukraine Botanical Journal* **42**: 86–95.

Edgar R (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Ferrer A, Sivichai S, Shearer CA (2007). *Megalohypha*, a new genus in the *Jahnulales* from aquatic habitats in the tropics. *Mycologia* **99**: 456–460.

Galtier N, Gouy M, Gautier C (1996). SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Computer Applications in Bioscience* **12**: 543–548.

Gessner MO, Gulis V, Kuehn KA, Chauvet E, Suberkropp K (2007). *Fungal decomposers of plant litter in aquatic ecosystems.* In: *The Mycota IV. Environmental and microbial relationships. 2nd ed.* (Kubicek CP, Druzhinina IS, eds). Berlin: Springer-Verlag: 301–324.

Goh TK, Hyde KD (1996). Biodiversity of freshwater fungi. *Journal of Industrial Microbiology* **17**: 328–345.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, *et al.* (2007). A higher-level phylogenetic classification of the Fungi. *Mycological Research* **111**: 509–547.

Hirayama K, Tanaka K, Raja HA, Miller AN, Shearer CA (2010). A molecular phylogenetic assessment of *Massarina ingoldiana* sensu lato. *Mycologia*: In press.

Hyde KD (1992). Australian Freshwater Fungi. V. *Bombardia* sp., *Jahnula australiensis* sp. nov., *Savoryella aquatica* sp. nov. and *S. lignicola. Australian Systematic Botany* **6**: 161–167.

Hyde KD, Jones EBG (1989). Observations on ascospore morphology in marine fungi and their attachment to surfaces. *Botanica Marina* **32**: 205–218.

Hyde KD, Wong SW (1999). Tropical Australian freshwater fungi. XV. The ascomycete genus *Jahnula,* with five new species and one new combination. *Nova Hedwigia* **68**: 489–509.

Hyde KD, Wong SW, Aptroot A (2002). Marine and estuarine species of *Lophiostoma* and *Massarina*. In: *Fungi in marine environments*. (Hyde KD, ed.). *Fungal Diversity Research Series* **7**: 93–109.

Inderbitzin P, Landvik S, Abdel-Wahab MA, Berbee ML (2001). *Aliquandostipitaceae,* A new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. *American Journal of Botany* **88**: 52–61.

Ingold CT (1951). Aquatic ascomycetes: *Ceriospora caudae-suis* n.sp. and *Ophiobolus typhae. Transactions of the British Mycological Society* **34**: 210–215.

Ingold CT (1954). Aquatic ascomycetes: Discomycetes from lakes. *Transactions of the British Mycological Society* **37**: 1–18.

Ingold CT (1955). Aquatic ascomycetes: further species from the English Lake District. *Transactions of the British Mycological Society* **38**: 157–168.

Ingold CT, Chapman B (1952). Aquatic ascomycetes. *Loramyces juncicola* Weston and *L. macrospora* n. sp. *Transactions of the British Mycological Society* **35**: 268–272.

Jones EBG (2006). Form and function of fungal spore appendages. *Mycoscience* **47**: 167–183.

Jones EBG, Zuccaro A, Mitchell J, Nakagiri A, Chatmala I, Pang K-L (2009). Phylogenetic position of freshwater and marine *Sigmoidea* species: introducing a marine hyphomycete *Halosigmoidea* gen. nov. *(Halosphaeriales). Botanica Marina* **52**: 349–359.

Kirk PM, Cannon PF, David JC, Stalpers JA (2008). *Ainsworth and Bisby's Dictionary of the Fungi.* 10th ed. CAB International, Wallingford, U.K.

Kirschstein V W (1936). Beiträge zur Kenntnis der Ascomyceten und ihrer Nebenformen besonders aus der Mark Brandenburg und dem Bayerischen Walde. *Annales Mycologici* **34**: 180–210.

Kodsueb R, Lumyong S, Ho WI, Hyde KD, McKenzie E, Jeewon R (2007). Morphological and molecular characterization of *Aquaticheirospora* and phylogenetics of *Massarinaceae (Pleosporales). Botanical Journal of the Linnean Society* **155**: 283–296.

Liew ECY, Aptroot A, Hyde KD (2002). An evaluation of the monophyly of *Massarina* based on ribosomal DNA sequences. *Mycologia* **94**: 803–813.

Maddison WP, Maddison DR (2000). *MacClade: analysis of phylogeny and character evolution*. Sinauer, Sunderland, Mass., U.S.A.

Morrison DA (2006). Multiple sequence alignment for phylogenetic purposes. *Australian Systematic Botany* **19**: 479–539.

Pang KL, Abdel-Wahab MA, Sivichai S, El-Sharouney HM, Jones EBG (2002). *Jahnulales (Dothideomycetes, Ascomycota):* a new order of lignicolous freshwater ascomycetes. *Mycological Research* **106**: 1031–1042.

Pinruan U, Jones EBG, Hyde KD (2002). Aquatic fungi from peat swamp palms: *Jahnula appendiculata* sp. nov. *Sydowia* **54**: 242–247.

Posada D, Crandall KA (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **49**: 817–818.

Pringsheim N (1858). Ueber das Austreten der Sporen von *Sphaeria scripi* aus ihren Schläuchen. *Jahrbuch für Wissenschaftlichen Botanik* **1**: 189–921.

Prihatini R, Nattawut B, Sivichai S (2008). Phylogenetic evidence that two submerged-habitat fungal species, *Speiropsis pedatospora* and *Xylomyces chlamydosporus* belong to the order *Jahnulales incertae sedis Dothideomycetes. Microbiology Indonesia* **2**: 136–140.

Promputtha I, Miller AN (2010). Three new species of *Acanthostigma (Tubeufiaceae, Pleosporales)* from the Great Smoky Mountains National Park. *Mycologia*: In press.

Raja HA, Carter A, Platt HW, Shearer CA (2008). Freshwater ascomycetes: *Jahnula apiospora* (*Jahnulales, Dothideomycetes*), a new species from Prince Edward Island, Canada. *Mycoscience* **49**: 326–328.

Raja HA, Ferrer A, Miller AN, Shearer CA (2010). Freshwater Ascomycetes: *Wicklowia aquatica,* a new genus and species in the *Pleosporales* from Florida and Costa Rica. *Mycoscience*: In press.

Raja HA, Ferrer A, Shearer CA (2005). *Aliquandostipite crystallinus,* a new ascomycete species from submerged wood in freshwater habitats. *Mycotaxon* **91**: 207–215.

Raja HA, Ferrer A, Shearer CA (2009a). Freshwater ascomycetes: A new genus, *Ocala scalariformis* gen. et sp. nov, and two new species, *Ayria nubispora* sp. nov., and *Rivulicola cygnea* sp. nov. *Fungal Diversity* **34**: 79–86.

Raja HA, Schmit JP, Shearer CA (2009b). Latitudinal, habitat and substrate distribution patterns of freshwater ascomycetes in the Florida Peninsula. *Biodiversity and Conservation* **18**: 419–455.

Raja HA, Shearer CA (2006). *Jahnula* species from North and Central America, including three new species. *Mycologia* **98**: 312–332.

Raja HA, Shearer CA (2007). Freshwater ascomycetes: *Aliquandostipite minuta* (*Jahnulales, Dothideomycetes*), a new species from Florida. *Mycoscience* **48**: 395–398.

Raja HA, Shearer CA (2008). Freshwater ascomycetes: new and noteworthy species from aquatic habitats in Florida. *Mycologia* **100**: 467–489.

Rambaut A (1996). *Sequence Alignment Editor.* Version 2.0. Department of Zoology, University of Oxford, Oxford. Available from http://evolve.zoo..ox.ac.uk/Se-Al/Se-Al.html.

Rehner SA, Samuels GJ (1995). Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73** (Suppl 1): S816–S823.

Ronquist F, Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Schoch CL, Crous PW, Groenewald JZ, Barrés, Boehm EWA, *et al*. (2009). A class-wide phylogenetic assessment of *Dothideomycetes. Studies in Mycology* **64**: 1–15.

Schoch CL, Shoemaker RA, Seifert KA, Hambleton S, Spatafora JW, Crous PW (2006). A multigene phylogeny of the *Dothideomycetes* using four nuclear loci. *Mycologia* **98**: 1041–1052.

Shearer CA (1993). The freshwater ascomycetes. *Nova Hedwigia* **56**: 1–33.

Shearer CA (2001). The distribution of freshwater filamentous Ascomycetes. In: *Trichomycetes and other fungal groups*. (Misra JK, Horn BW, eds). Science Publishers, Plymouth, U.K.: 225–292.

Shearer CA, Crane JL (1980). Taxonomy of two cleistothecial ascomycetes with papilionaceous ascospores. *Transactions of the British Mycological Society* **75**: 193–200.

Shearer CA, Descals E, Volkmann-Kohlmeyer B, Kohlmeyer J, Marvanova L, *et al.* (2007). Fungal biodiversity in aquatic habitats. *Biodiversity and Conservation* **16**: 49–67.

Shearer CA, Fallah PM, Ferrer A, Raja HA, Schmit JP (2009). Freshwater ascomycetes and their anamorphs. URL: www.fungi.life.uiuc.edu (accessed Nov 2009).

Simonis JL, Raja HA, Shearer CA (2008). Extracellular enzymes and soft rot decay: Are ascomycetes important degraders in fresh water? *Fungal Diversity* **31**: 135–146.

Stamatakis A (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Stamatakis A, Hoover P, Rougemont J (2008). A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology* **57**: 758–771.

Stewart EL, Liu Z, Crous PW, Szabo LJ (1999). Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. *Mycological Research* **103**: 1491–1499.

Suetrong S, Sakayaroj J, Phongpaichit S, Jones EBG (2010). Morphological and molecular characteristics of a poorly known marine ascomycete, *Manglicola guatemalensis* (*Jahnulales: Pezizomycotina; Dothideomycetes*, Incertae sedis): new lineage of marine ascomycetes. *Mycologia*: In press.

Suetrong S, Schoch CL, Spatafora JW, Kohlmeyer J, Volkmann-Kohlmeyer B, *et al.* (2009). Molecular systematics of the marine *Dothideomycetes*. *Studies in Mycology* **64**: 155–173.

Swofford DL (2002). PAUP 4.0b10: *Phylogenetic analysis using parsimony*. Sinauer Associates, Sunderland, MA, U.S.A.

Tanaka K, Hatakeyama S, Harada Y (2005). Three new freshwater ascomycetes from rivers in Akkeshi, Hokkaido, northern Japan. *Mycoscience* **46**: 287–293.

Tanaka K, Hosoya T (2008). *Lophiostoma sagittiforme* sp. nov., a new ascomycete *(Pleosporales, Dothideomycetes)* from Island Yakushima in Japan. *Sydowia* **60**: 131–145.

Tsui CKM, Berbee ML (2006). Phylogenetic relationships and convergence of helicosporous fungi inferred from ribosomal DNA sequences. *Molecular Phylogenetics and Evolution* **39**: 587–597.

Tsui CKM, Hyde KD (2003). *Freshwater mycology. Fungal Diversity Research Series* **10**: 1–350.

Tsui CKM, Sivichai S, Berbee ML (2006). Molecular systematics of *Helicoma, Helicomyces* and *Helicosporium* and their teleomorphs inferred from rDNA sequences. *Mycologia* **98**: 94–104.

Tsui CKM, Sivichai S, Rossman AY, Berbee ML (2007). *Tubeufia asiana,* the teleomorph of *Aquaphila albicans* in the *Tubeufiaceae, Pleosporales,* based on cultural and molecular data. *Mycologia* **99**: 884–894.

Tubaki K (1959). Studies on the Japenese Hyphomycetes V. leaf and stem group with discussion of the classification of Hyphomycetes and their perfect stages. *Journal of Hattori Botanical Laboratory* **20**: 142–244.

Vijaykrishna D, Jeewon R, Hyde KD (2006). Molecular taxonomy, origins and evolution of freshwater ascomycetes. *Fungal Diversity* **23**: 367–406.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Voglmayr H (2004). *Spirosphaera cupreorufescens* sp. nov., a rare aeroaquatic fungus. *Studies in Mycology* **50**: 221–228.

Webster J (1965). The perfect state of *Pyricularia aquatica. Transactions of the British Mycological Society* **48**: 449–452.

Webster J, Descals E (1979). The teleomorphs of waterborne hyphomycetes from fresh water. In: *The Whole Fungus*. (Kendrick B, ed.). National Museums of Canada and Kananaskis Foundation, Ottawa, **2**: 419–451.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, San Diego, California, U.S.A.: 315–322.

Wiens JJ (1998). Combining data sets with different phylogenetic histories. *Systematic Biology* **47**: 568–581.

Willoughby LG, Archer JF (1973). The fungal spora of a freshwater stream and its colonization pattern on wood. *Freshwater Biology* **3**: 219–239.

Wong MKM, Goh T-K, Hodgkiss IJ, Hyde KD, Ranghoo VM, Tsui CKM, Ho W-H, Wong WSW, Yuen T-K (1998). Role of fungi in freshwater ecosystems. *Biodiversity and Conservation* **7**: 1187–1206.

Zhang Y, Fournier J, Crous W, Pointing SB, Hyde KD (2009a). Phylogenetic and morphological assessment of two new species of *Amniculicola* and their allies *(Pleosporales)*. *Persoonia* **23**: 48–54.

Zhang Y, Fournier J, Pointing SB, Hyde KD (2008a). Are *Melanomma pulvis-pyrius* and *Trematosphaeria pertusa* congeneric? *Fungal Diversity* **33**: 47–60.

Zhang Y, Jeewon R, Fournier J, Hyde KD (2008b). Multi-gene phylogeny and morphotaxonomy of *Amniculicola lignicola:* a novel freshwater fungus from France and its relationships to the *Pleosporales. Mycological Research* **112**: 1186–1194.

Zhang Y, Schoch CL, Fournier J, Crous PW, Gruyter J de, *et al*. (2009b). Multi-locus phylogeny of the *Pleosporales*: a taxonomic, ecological and evolutionary re-evaluation. *Studies in Mycology* **64**: 85–102.

Zhang Y, Wang HK, Fournier J, Crous PW, Jeewon R, Pointing SB, Hyde KD (2009c). Towards a phylogenetic clarification of *Lophiostoma/Massarina* and morphological similar genera in the *Pleosporales. Fungal Diversity* **38**: 225–251.

SUPPLEMENTARY INFORMATION

Table 1. Species used in this study.

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
*Aliquandostipite crystallinus**	F83-1	Raja & Shearer	GU266221	GU266239
	AF007	–	EF175631	EF175652
	R76-1	–	EF175630	EF175651
Aliquandostipite khaoyaiensis	F89-1	Raja & Shearer	EF175625	EF175647
	SS2961	BCC 15577	EF175626	EF175648
	SS3028	BCC 23986	EF175627	EF175649
	SS3321	BCC 18283	EF175628	EF175650
Aliquandostipite separans		–	AF438179	–
Aliquandostipite siamensiae	SS81.02	BCC 3417	EF175645	EF175666
Allewia eureka		DAOM 195275	DQ677994	DQ678044
Alternaria alternata		CBS 916.96	DQ678031	DQ678082
Alternaria sp. (as *Clathrospora diplospora*)		CBS 174.51	DQ678016	DQ678068
Amniculicola immersa	–	KD Hyde	GU456295	FJ795498
Amniculicola lignicola	–	KD Hyde	EF493863	EF493861
Amniculicola parva		KD Hyde	GU296134	FJ795497
*Anguillospora longissima**	CS869-1D	Shearer	GU266222	GU266240
Aquaticheirospora lignicola		–	AY736377	AY736378
Aquaphila albicans		BCC 3543	DQ341093	DQ341101
Ascochyta pisi var. *pisi*		CBS 126.54	DQ678018	DQ678070
Ascorhombispora aquatica		–	–	EU196548
Bimuria novae-zelandiae		CBS 107.19	AY016338	AY016356
Botryosphaeria dothidea		CBS 115476	DQ677998	DQ678051
"Botryosphaeria" tsugae		CBS 418.64	AF271127	DQ767655
Botryotinia fuckeliana		OSC 100012	AY544695	AY544651
Brachiosphaera tropicalis	E192-1	Shearer	GU266223	EF175653
Byssothecium circinans		CBS 675.92	AY016339	AY016357
Caloscypha fulgens		OSC 100062	DQ247807	DQ247799
Capnodium coffeae		CBS 147.52	DQ247808	DQ247800
Capnodium salicinum		CBS 131.34	DQ6779977	DQ678050
Capronia pilosella		DAOM 216387	DQ823106	DQ823099
Coccomyces strobi		CBS 202.91	DQ471027	DQ470975
Cheirosporium triseriale		–	–	EU413954
Cochliobolus heterostrophus		CBS 134.39	AY544727	AY544645
Cochliobolus sativus		DAOM 216378	DQ677995	DQ678045
Coniothyrium obiones		CBS 453.68	DQ678001	DQ678054
Coniothyrium palmarum		CBS 400.71	DQ678008	DQ767653
Cucurbitaria elongata		CBS 171.55	DQ678009	DQ678061
Delitschia winteri		CBS 225.62	DQ678026	DQ678077
Dendryphiella arenaria		CBS 181.85	DQ471022	DQ470971
Dendyphiopsis atra		DAOM 231155	DQ677996	DQ678046
Dermea acerina		CBS 161.38	DQ247809	DQ247801
Didymella cucurbitacearum		IMI 373225	AY293779	AY293792
Dothidea insculpta		CBS 189.58	DQ247810	DQ247802
Dothidea sambuci		DAOM 231303	AY544722	AY544681

Table 1. (Continued).

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
Dothiora cannabinae		CBS 373.71	DQ479933	DQ470984
Elsinoë phaseoli		CBS 165.31	DQ678042	DQ678095
Elsinoë veneta		CBS 164.29	DQ678007	DQ678060
Gloniopsis praelonga		CBS 112415	FJ161134	FJ161173
Gloniopsis smilacis		CBS 114601	FJ161135	FJ161174
Guignardia bidwelli		CBS 237.48	DQ678034	DQ678085
Helicascus kanaloanus		ATCC 18591	AF053729	–
Helicomyces roseus		CBS 283.51	DQ678032	DQ678083
Herpotrichia diffusa		CBS 250.62	DQ678019	DQ678071
Herpotrichia juniperi		CBS 200.31	DQ678029	DQ678080
*Jahnula appendiculata**	AF285-3	Shearer	GU266224	GU266241
Jahnula aquatica	R68-1	Raja & Shearer	EF175633	EF175655
	R68-2	Raja & Shearer	EF175632	NA
Jahnula bipileata	F49-1	MYA 4173	EF175635	EF175657
	AF220-1	Shearer	EF175634	EF175656
Jahnula bipolaris	SS44	BCC 3390	EF175637	EF175658
	A421	Shearer	EF175636	–
Jahnula granulosa	SS1562	BCC24222	EF175638	EF175659
*Jahnula potamophila**	F111-1	Raja & Shearer	GU266225	GU266242
Jahnula rostrata	F4-3	MYA4176	GU266226	EF175660
Jahnula sangamonensis	A482-1B	MYA 4174	EF175640	EF175662
	A402-1B	Shearer	EF175639	EF175661
	F81	MYA 4175	EF175641	EF175663
Jahnula seychellensis	SS2133.1	BCC 14207	EF175644	EF175665
	SS2113.2	BCC 12957	EF175643	EF175664
	A492	Shearer	EF175642	GU266243
Kirschsteiniothelia aethiops		CBS 109.53	AY016344	AY016361
Kirschsteiniothelia elaterascus	A22-11B-/	–	AF053728	–
			–	AY787934
Lecanora hybocarpa		DUKE 03.07.04-2	DQ782883	DQ782910
Lentithecium aquaticum		CBS 123099	FJ795477	FJ795434
Lentithecium arundinaceum		CBS 619.86	DQ813513	DQ813509
Lemonniera pseudofloscula		CCM F-0484	–	DQ267631
		CCM F-43294	–	DQ267632
Leotia lubrica		OSC100001	AY544687	AY544644
*Lepidopterella palustris**	F32-3	Raja & Shearer	GU266227	GU266244
Leptosphaeria maculans		DAOM 229267	DQ470993	DQ470946
Lepidosphaeria nicotiae		CBS 101341	–	DQ678067
Lindgomyces cinctosporae	R56-1		AB522430	AB522431
	R56-3	Raja & Shearer	GU266238	GU266245
Lindgomyces breviappendiculatus	KT 215	JCM 12702/MAFF 239291	AB521733	AB521748
	KT 1399	JCM 12701/MAFF 239292	AB521734	AB521749
Lindgomyces ingoldianus	A39-1	ATCC200398	AB521719	AB521736
	KH 100	JCM 16479	AB521720	AB521737
Lindgomyces sp.	KH 241	JCM16480	AB521721	AB521738
Lindgomyces rotundatus	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
Lophiostoma arundinis		CBS 269.34	DQ782383	DQ782384
Lophiostoma crenatum		CBS 629.86	DQ678017	DQ678069
Lophiostoma glabrotunicatum		IFRD 2012	FJ795481	FJ795438
Lophiostoma macrostomum	KT 635	JCM 13545	AB521731	AB433273
	KT 709	JCM 13546 MAFF 239447	AB521732	AB433274
			SSU	LSU
Lophium mytilinum		CBS 269.34	DQ678030	DQ678081
Massaria platani		CBS 221.37	DQ678013	DQ678065
Massarina australiensis		–	AF164364	–
Massarina bipolaris		–	AF164365	–
Massarina eburnea	H 3953	JCM 14422	AB521718	AB521735
		–	AF164366	–
		–	AF164367	
Massariosphaeria typhicola	KT 667	MAFF 239218	AB521729	AB521746
	KT 797	MAFF 239219	AB521730	AB521747
*Megalohypha aqua-dulces**	AF005-2a	–	GU266228	EF175667
	AF005-2b	–	–	EF175668
Melanomma radicans		ATCC 42522	U43461	U43479
Montagnula opulenta		CBS 168.34	AF164370	DQ678086
Mycosphaerella graminicola		CBS 292.38	DQ678033	DQ678084
Myriangium duriaei		CBS 260.36	AY016347	DQ678059
Mytilinidion andinense		EB 0330 (CBS 123562	FJ161159	FJ161199
Mytilinidion mytilinellum		CBS 303.34	FJ161144	FJ161184
Neofusicoccum ribis		CBS 115475	DQ678000	DQ678053
Neurospora crassa		BROAD	X04971	AF286411
*Ocala scalariformis**	F121-1	Raja & Shearer	GU266229	–
Ophiosphaerella herpotricha		CBS 620.86	DQ678010	DQ678062
		CBS 240.31	DQ767650	DQ767656
Phaeodothis winteri		CBS 182.58	DQ678021	DQ678073
Phaeosphaeria avenaria		DAOM 226215	AY544725	AY544684
Phaeosphaeria eustoma		CBS 573.86	DQ678011	DQ678063
Phoma herbarum		CBS 276.37	DQ678014	DQ678066
Piedraia hortae		CBS 480.64	AY016349	AY016366
Pleomassaria siparia		CBS 279.74	DQ678027	DQ678078
Pleospora herbarum var. *herbarum*		CBS 714.68	DQ767648	DQ678049
		CBS 514.72	DQ247812	DQ247804
Preussia terricola		DAOM 230091	AY544726	AY544686
Pseudocercospora fijiensis		OSC 100622	DQ767652	DQ678098
Pyrenophora phaeocomes		DAOM 222769	DQ499595	DQ499596
Pyrenophora tritici-repentis		OSC 100066	AY544716	AY544672
Pyronema domesticum		CBS 666.98	DQ247813	DQ247805
Quintaria lignatilis		–	QLU43462	–
Ramularia endophylla		CBS 113265	DQ471017	DQ470920
Roccellographa cretacea		DUKE 191Bc	DQ883705	DQ883696
Schismatomma decolorans		DUKE 0047570	AY548809	AY548815

Table 1. (Continued).

Species	Isolate number	Source	GenBank No.	
			SSU	LSU
Semimassariosphaeria typhicola **			GU296174	FJ795504
Spencermartinsia viticola		CBS 117009	DQ678036	DQ678087
Sporormiella minima		CBS 524.50	DQ678003	DQ678056
Sporidesmium sp.	FH14	–	GU266230	–
Taeniolella typhoides		CCM F-10198/extype	GU266231	–
Tingoldiago graminicola	KH 68	JCM 16485	AB521726	AB521743
	KT 891/	MAFF 239472	AB521727	AB521744
	KH 155/	JCM 16486	AB521728	AB521745
Trematosphaeria hydrophila		IFRD 2037	GU261721	–
Trematosphaeria heterospora		CBS 644.86	AY016354	AY016369
Trematosphaeria pertusa		CBS 400.97	DQ678020	DQ678072
Trematosphaeria wegeliniana		CBS 123124	GU261720	GU261722
			SSU	LSU
Tubeufia cerea		CBS 254.75	DQ471034	DQ470982
Tubeufia helicomyces		–	DQ767649	DQ767654
Tumularia aquatica		CCM F-02081	AY357287	–
Ulospora bilgramii		CBS 110020	DQ678025	DQ678076
Verruculina enalia		CBS 304.66	DQ678028	DQ678079
Westerdykella cylindrica		CBS 454.72	AY016355	AY004343
*Wicklowia aquatica**	F76-2	CBS 125634	GU266232	GU045445
Xylaria hypoxylon		OSC 100004	AY544719	AY544676
*Xylomyces chlamydosporus**	H58-4		GU266233	EF175669
*Xylomyces elegans**	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