The molecular phylogeny of freshwater Dothideomycetes

C.A. Shearer1*, H.A. Raja1, A.N. Miller2, P. Nelson3, K. Tanaka4, K. Hirayama5, L. Marvanová6, K.D. Hyde6 and Y. Zhang7

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 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 Capnodiiales (Mycosphaerellaceae) and four species in Tubeufiaceae.
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), Anguilliospora 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: Anguilliospora 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 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 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 Prompputha & Miller (2010). Primers NS1 and NS4 for SSU (White et al. 1990) and LR0R 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: LR0R, 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 sequences 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” (Morrison 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-MCMC) 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 (Swoford 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-genus 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.
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 % MLB 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 Lentitheciaceae; A-25-1, F-60, and F-65 in Jahnulales; A369-2B in Pleosporales; and F80-1 as sister taxon to Lentitheciaceae. 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 A273-1 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.

Fig. 2. (Continued).
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. imersa 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 alternata* (Webster 1965) was placed with Lophiostoma giblatronicum, an aquatic fungus collected in mountain streams in France on submerged wood of *Alnus glutinosa*, *Fagus sylvatica* and *Salix* sp. (Zhang et al. 2009c). *Teenirolella typhoides* occurred in a well-supported group with members of *Lindgomycetaceae* in *Pleosporales*. *Lemoniera pseudoflorsula* 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 (*Amniculicolaeeae*) 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 *Amphiphaeria aquatica* by Piöttner 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 *Aliquandistipitum*, 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, *Jahnuliales*, 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, Pinrnan 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 *Lepidotopella 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 ascii; 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. *Lepidotopella palustris* has black, cleistothecial ascomata appearing as raised dome-shaped structures on the substrate; hamatheicum of hyaline, septate, narrow pseudoparaphyses not embedded in a gel matrix; thick-walled, globose to subglobose, broadly rounded, fissitunicate ascii; 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 & Bärlocher (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 & Bärlocher 2005, Campbell et al. 2006, 2007, Zhang et al. 2009c). These taxa are: Anguillospora longissima, Lemnoniera pseudofloscula, Taeniocella 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 aeroaerobic 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 for “similar to Taeniocella typhoides, Tumularia aquatica. Here it forms a well-supported sister clade with Dothideomycetes. This idea is supported by the presence of similar ascospore modifications such as ascomata and hamathecia in interpreting phylogenetic relationships among freshwater Dothideomycetes. 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.

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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. 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.

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**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     |
| **Alleuria eureka**                     |                | DAOM 195275    | DQ677994     | DQ678044     |
| **Alternaria alternata**                | CBS 916.96     |                | DQ678031     | DQ678082     |
| **Alternaria sp. (as Clathrospora diplospora)** |          |                |             |             |
| **Amniculicola immera**                 | –              | 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**         | –              | –              | AY36377      | AY36377      |
| **Aquaphila albicans**                  |                | BCC 3543       | DQ341093     | DQ341101     |
| **Ascochyta pisi var. pisi**            |                | CBS 126.54     | DQ678018     | DQ678070     |
| **Asconombispora aquatica**             | –              | –              | –            | EU196548     |
| **Bimuria novae-zelandiae**             |                | CBS 107.19     | AY016338     | AY016356     |
| **Botryosphaeria dothidea**             |                | CBS 115476     | DQ677998     | DQ678051     |
| **“Botryosphaeria” tsugae**             |                | CBS 418.64     | AF271127     | DG706765     |
| **Botryotinia fuckeliana**              |                | OSC 100012     | AY544695     | AY544651     |
| **Brachiosphaera tropicalis**           | E192-1         | Shearer        | GU266223     | EF175653     |
| **Byssothecium cincinans**              | 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     |
| **Cheirosporum trisenale**              | –              | –              | EU196548     |             |
| **Cochliobolus heterostrophus**         | CBS 134.39     |                | AY544727     | AY544645     |
| **Cochliobolus sativus**                | DAOM 21637     |                | DQ677995     | DQ678045     |
| **Coniothyrium obiones**                | CBS 433.68     |                | DQ678001     | DQ678054     |
| **Coniothyrium palmarum**               | CBS 400.71     |                | DQ678008     | DQ676753     |
| **Cucurbitania 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     |                | DQ471031     | DQ470971     |
| **Didymella cucurbitacearum**           | IMI 373225     |                | AY293779     | AY293792     |
| **Dothidea insculpta**                  | CBS 189.58     |                | DQ247810     | DQ247802     |
| **Dothidea sambuci**                    | DAOM 231303    |                | AY544722     | AY544681     |
| 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 praetectora        | CBS 112415     |                | FJ161134    | FJ161173     |
| Gloniopsis squalaccis         | CBS 114601     |                | FJ161135    | FJ161174     |
| Guignardia bidwellii          | CBS 237.48     |                | DQ678034    | DQ678085     |
| Helicascus kanaloianus        | ATCC 18591     |                | AF053729    | –            |
| Helicomyces roseus            | CBS 283.51     |                | DQ678032    | DQ678083     |
| Herpotrichia diffusa          | CBS 250.62     |                | DQ678019    | DQ678071     |
| Herpotrichia juniperi         | CBS 200.31     |                | DQ678020    | DQ678080     |
| Jahnula appendiculata*        | AF225-3        | Shearer        | GU266224    | GU266241     |
| Jahnula aquatica              | R68-1          | Raja & Shearer | EF175633    | EF175655     |
| Jahnula bipileata             | F49-1          | MYA 4173       | EF175635    | EF175657     |
| Jahnula bipolaris             | SS44           | BCC 3390       | EF175637    | EF175658     |
| Jahnula granulosa             | A421           | Shearer        | EF175636    | –            |
| Jahnula potamophila*          | F111-1         | Raja & Shearer | GU266225    | GU266242     |
| Jahnula rostrata              | F4-3           | MYA4176        | GU266226    | EF175660     |
| Jahnula sangamonensis         | A462-1B        | MYA 4174       | EF175640    | EF175662     |
| Jahnula seychellensis         | SS2133.1       | BCC 14207      | EF175644    | EF175665     |
| Kirschsteiniothelia aethiops  | A22-11B-/-     | –              | AF053728    | –            |
| 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-4084     |                | –           | DQ267631     |
| Leotia lubrica                | OSC100001      |                | AY544687    | AY544644     |
| Lepidopterella palustris*     | F32-3          | Raja & Shearer | GU266227    | GU266244     |
| Leptosphaeria maculans        | DAOM 229267    |                | DQ470993    | DQ470946     |
| Lepidosphaeria nicolai        | CBS 101341     |                | –           | DQ678067     |
| Lindgomyces cinctosporeae     | R56-1          |                | AB522430    | AB522431     |
| Lindgomyces breviappendiculatus| R56-3          | Raja & Shearer | GU266238    | GU266245     |
| Lindgomyces ingoldianus       | A39-1          | ATCC200398     | AB521719    | AB521736     |
| Lindgomyces sp.               | KH 100         | JCM 16479      | AB521720    | AB521737     |
| Lindgomyces rotundatus        | KT 966         | JCM 16481/MAFF 238473 | AB521722 | AB521739 |
|                             | KT 1096        | JCM 16482      | AB521723    | AB521740     |
Table 1. (Continued).

| Species                  | Isolate number | Source             | GenBank No.   |
|--------------------------|----------------|--------------------|---------------|
|                          |                |                    | **SSU**       | **LSU**       |
| **Lophiostoma arundinis**| KH 114         | JCM 16484          | AB521725      | AB521742      |
|                          | KT 1107        | JCM 16483          | AB521724      | AB521741      |
| Lophiostoma crenatum     | CBS 269.34     |                    | DG782383      | DG782384      |
| Lophiostoma glabrotrunicatum | IFRD 2012 |                    | FJ95481       | FJ95438       |
| Lophiostoma macrostomum  | KT 635         | JCM 13545          | AB521731      | AB433273      |
|                          | KT 709         | JCM 13546 MAFF 239447 | AB521732 | AB433274      |
|                          |                |                    | SSU           | LSU           |
| Lophium mytilinum        | CBS 269.34     |                    | DG782030      | DG782081      |
| Massaria platani         | CBS 221.37     |                    | DG782013      | DG782065      |
| Massarina australiensis  | –              |                    | AF164364      | –             |
| Massarina bipolaris      | –              |                    | AF164365      | –             |
| Massarina eburnea        | H 2953         | JCM 14422          | AB521718      | AB521735      |
|                          | –              |                    | AF164366      | –             |
| Massariopsis typhicola   | KT 667         | MAFF 239218        | AB521729      | AB521746      |
|                          | KT 797         | MAFF 239219        | AB521730      | AB521747      |
| Megalothypha aqua-dulces*| AF005-2a       | –                  | GU266228      | EF175667      |
|                          | AF005-2b       | –                  | –             | EF175668      |
| Melanomma radicans       | ATCC 42522     |                    | U43461        | U43479        |
| Montagnula opulenta      | CBS 168.34     |                    | AF164370      | DG782086      |
| Mycosphaerella graminicola | CBS 292.38   |                    | DG783033      | DG782084      |
| Myriangium diniae         | CBS 260.36     |                    | AO16347       | DG782059      |
| Mytilidion andinense     | EB 0330 (CBS 123562) |                | FJ161159      | FJ161199      |
| Mytilidion mytilinellum  | CBS 303.34     |                    | FJ161144      | FJ161184      |
| Neofusisoccum riberi     | CBS 115475     |                    | DG782000      | DG782053      |
| Neurospora crassa        | BROAD          |                    | X04971        | AF286411      |
| Ocala scalariforms*      | F21-1          | Raja & Shearer     | GU266229      | –             |
| Ophiophaerella herpotricha| CBS 260.86     |                    | DG782010      | DG782062      |
|                          | CBS 240.31     |                    | DG767650      | DG767656      |
| Phaeodothis winteri      | CBS 182.58     |                    | DG782021      | DG782073      |
| Phaeosphaeria avenaria    | DAOM 226215    |                    | AY544725      | AY544684      |
| Phaeosphaeria eustoma    | CBS 573.86     |                    | DG782011      | DG782063      |
| Phoma herbarum           | CBS 276.37     |                    | DG782014      | DG782066      |
| Piedraia hortae          | CBS 480.64     |                    | AO16349       | AO16366       |
| Pleomassaria siparia     | CBS 279.74     |                    | DG782027      | DG782078      |
| Pleospora herbarum var. herbarum | CBS 714.68 |                    | DG767648      | DG782049      |
|                          | CBS 514.72     |                    | DG247812      | DG247804      |
| Preussia terricola       | DAOM 230091    |                    | AY544726      | AY544686      |
| Pseudocercospora fijiensis| OSC 100622    |                    | DG767652      | DG767698      |
| Pyrenophora phaeocomes   | DAOM 222769    |                    | DG499595      | DG499596      |
| Pyrenophora tritic-repentis| OSC 100066  |                    | AY544716      | AY544672      |
| Pyronema domesticum      | CBS 666.98     |                    | DG247813      | DG247805      |
| Quintaria lignatis       | –              |                    | QLU43462      | –             |
| Ramularia endophylla     | CBS 113265     |                    | DG471017      | DG470920      |
| Rocciolegrapha cretacea  | DUKE 191Bc     |                    | DG83705       | DG83696       |
| Schismatomata decolorans | DUKE 0047570  |                    | AY548809      | AY548815      |
| Species                              | Isolate number | Source               | GenBank No.          |
|-------------------------------------|----------------|----------------------|---------------------|
| **Semimassariosphaeria typhicola**  |                |                      | GU296174 FJ795504   |
| Spencermartinia viticola            | CBS 117009     |                      | DG678036 DG678087   |
| Sporormiella minima                 | CBS 524.50     |                      | DG678003 DG678056   |
| *Sporidesmium sp.*                  | FH14           | –                    | GU266230 –          |
| *Taenicella typhoides*              |                | CCM F-10198/extype   | GU266231 –          |
| Tingoldiago graminicola             | KH 68          | JCM 16485            | AB521276 AB521743   |
|                                     | KT 891/        | MAFF 239472          | AB521277 AB521744   |
|                                     | KH 155/        | JCM 16486            | AB521278 AB521745   |
| *Trematosphaeria hydrophila*        |                | IFRD 2037            | GU261721 –          |
| *Trematosphaeria heterospora*       | CBS 644.86     |                      | AY016354 AY016369   |
| *Trematosphaeria pertusa*           | CBS 400.97     |                      | DG678020 DG678072   |
| *Trematosphaeria wegeliniana*       | CBS 123124     |                      | GU261720 GU261722   |
|                                     |                | SSU                  | GU261725 LSU        |
| Tubeufia cerea                      | CBS 254.75     |                      | DJ471034 DJ470982   |
| Tubeufia helicomyces                | –              |                      | DJ767649 DJ767664   |
| Tumularia aquatica                  | CCM F-20081    |                      | AY357287 –          |
| Ulospora bigramii                   | CBS 110020     |                      | DG678025 DG678076   |
| Verruculina enalia                  | CBS 304.66     |                      | DG678028 DG678079   |
| Westerdykella cylindrica            | CBS 454.72     |                      | AY016355 AY004343   |
| Wicklowia aquatica*                 | F78-2          | 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            |