British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/fbr

Review

Molecular approaches applied to aquatic hyphomycetes

Felix BÄRLOCHER*

Department of Biology, Mount Allison University, Sackville, New Brunswick, E4L 1G7, Canada

Keywords:
Aquatic hyphomycetes
Ecology
Molecular methods
Phylogeny

ABSTRACT

Since aquatic hyphomycetes were discovered in 1942, much has been learned about their taxonomy and biology, their seasonal and geographic distribution, responses to pollutants and potential connections between diversity and ecological functions. Aquatic hyphomycetes are now recognized as essential intermediaries in food webs of streams. Despite these advances, the inability of identifying the metabolically active phase, mycelium, continues to impede progress. Molecular methods do not rely on the presence of reproductive stages to identify taxa. Their application has modified or refined our understanding of many aspects of the taxonomy and ecology of aquatic hyphomycetes, and makes accessible entirely new avenues of research.

© 2007 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Research on aquatic hyphomycetes was initiated by Ingold's (1942) discovery of multiradiate (often tetraradiate) and sigmoid conidia in foam which forms naturally in many running waters. Ingold showed that the preferred substrates of the fungi releasing these conidia were deciduous leaves that had fallen into the stream. In the first few decades following his discovery, aquatic hyphomycetes (or Ingoldian fungi) were isolated and described from all over the world, resulting in the early recognition that many (morpho)species are cosmopolitan, though some appear to be restricted to certain latitudes. Ingold postulated that the similarity of conidial shapes in many species was due to convergent evolution and therefore phylogenetically non-informative. The selective advantage was shown to be increased probability of settling on smooth surfaces such as leaves in turbulent water (Webster 1959). The first teleomorph of an aquatic hyphomycete was found by Ranzoni (1956); today, roughly 10 % of described species have a known sexual stage (Webster 1992; Sivichai & Jones 2003). Meiospores are typically more robust than the delicate conidia, and their long-distance dispersal may explain the apparently world-wide distribution of species.

By the late 1960s, a few biologists had become fascinated by aquatic hyphomycetes, but overall, little research was done on these fungi by mycologists or by limnologists – Ainsworth (1976) labeled it "a minor mycological industry". This changed when ecologists recognized the importance of deciduous leaves in stream food webs. Two studies demonstrated that invertebrates prefer to eat leaves colonized by fungi (Triska 1970; Kaushik & Hynes 1971; Bärlocher 1992a). This pointed to a crucial fungal role as intermediaries between a dominant food source (leaves) in streams and its consumers (shredders), and stimulated research into methods to measure fungal biomass and growth. Today, ergosterol is widely considered a suitable indicator of living fungal biomass (Gessner *et al.* 2003), and fungal production is estimated by incorporation of ^{14}C acetate into ergosterol (Newell & Fallon 1991). These techniques allowed quantitative estimates of the fungal involvement in stream food webs: up to 17 % of decaying leaf biomass has been attributed to fungal biomass (Gessner 1997; Gessner *et al.* 2003), and fungal productivity approaches or exceeds that of bacteria or invertebrates (Suberkropp 1997; Carter & Suberkropp 2004). Numbers such as these raised interest in

* Tel.: +1 506 364 3501; fax: +1 506 364 2505.
E-mail address: fbaerlocher@mta.ca

1749-4613/$ – see front matter © 2007 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.fbr.2007.02.003

aquatic hyphomycetes, and there is a growing literature on their geographic distribution, seasonal succession, substrate preferences, enzyme production, reaction to pollution, and potential connections between fungal diversity and ecological functions (for reviews, see Bärlocher 1992b, c, 2005; Bärlocher & Corkum 2003; Gessner & Van Ryckegem 2002; Gessner *et al.* 1997; Krauss *et al.* 2003).

Despite these advances, progress continues to be hampered by an inherent and long-recognized conundrum in fungal ecology (e.g., Garrett 1952). Fungi may occur as mycelia, fructifications or spores. Only metabolically active mycelia decompose substrates, but they cannot be identified. Fructifications and spores can often be identified but do not participate in decomposition. With aquatic hyphomycetes, ecologists have relied on types and numbers of conidia in the water column to describe large-scale seasonal changes (Iqbal & Webster 1973); community changes on substrates have been characterised by stimulating spore production in turbulent solutions, followed by collecting, counting and identifying the released conidia (Bärlocher 1982). Even though maximum fungal biomass (estimated by ergosterol) and spore production (total number of released spores) observed during leaf decay correlate (e.g., Gessner & Chauvet 1994), this correlation breaks down when applied to individual species, which react differently to nutrient enrichment or temperature changes. The reliance on spores is based on the assumption that aquatic hyphomycetes are the dominant fungal group, which seems a case of circular reasoning – these fungi produce abundant spores on leaves in streams. Methods are used that favour production of such spores; not surprisingly, many spores are produced from decaying leaves, which is used as evidence that these fungi dominate leaf decomposition. To my knowledge, there has been only one attempt to evaluate the potential occurrence of other fungal groups using traditional methods (Bärlocher & Kendrick 1974).

Molecular methods do not rely on the presence of reproductive stages to identify taxa. Their application has modified or refined our understanding of the ecology of aquatic hyphomycetes. It allowed tackling problems that had been largely inaccessible through traditional methods. It continues to carry the promise of opening entirely new avenues of research.

1. Fungal diversity on substrates

Molecular methods typically evaluate nucleic acids that are present in all stages of the life cycle. These stages include structures not identifiable with microscopy, such as fungal mycelia without associated spores. The basic procedure begins with extracting DNA from substrates colonized by fungi. The DNA is then amplified with fungal-specific primers (e.g., White *et al.* 1990), which typically creates a mixture of sequences from several species. If a suitable gene has been amplified, all products will be of comparable length but will show slight species- or strain-specific differences in their sequence. These differences can be evaluated by various techniques (Kennedy & Clipson 2003; Osborn & Smith 2005). Both DGGE (denaturing gradient gel electrophoresis) and T-RFLP (terminal restriction fragment length polymorphism) have been applied to aquatic hyphomycetes. With a section of 18S rRNA, 21 fungal species yielded 10 distinct T-RFLP fragments and 11 bands with DGGE (Nikolcheva *et al.* 2003). With an ITS2 section, these numbers increased to 17 and 18, respectively (Nikolcheva & Bärlocher 2004), which still does not represent complete resolution. In addition, if the abundance of a species in a mixture dropped below 3 % of total relative abundance, it could no longer be detected. Despite these shortcomings, molecular diversity exceeded spore diversity in early successional stages, suggesting the presence of non-sporulating mycelia (Nikolcheva *et al.* 2003). Based on molecular methods, Nikolcheva *et al.* (2005) concluded that fungal diversity peaked 2–3 d after leaf immersion. This included terrestrial taxa, present in the phyllosphere or as endophytes, and newly arrived conidia of aquatic hyphomycetes that had settled on the leaf surface. At later stages, molecular diversity declined. This was attributed to the gradual disappearance of terrestrial taxa, and increasing dominance by a few aquatic hyphomycetes, who tended to drown out the signals by rare taxa. Overall, the data supported the view of fungal succession suggested by a combination of microscopy and culture techniques (Bärlocher & Kendrick 1974), with the important difference that spore identification allowed detection of aquatic hyphomycetes at much lower relative densities. On the other hand, by using phylum-specific primers, Nikolcheva and Bärlocher (2004) demonstrated the presence of many fungal taxa that largely escaped detection by the microscope because they did not release identifiable spores. Both traditional and molecular techniques suggested that strict exclusion of fungi by leaf type is rare, and that presence of different species is governed by season (Nikolcheva & Bärlocher 2005).

Seasonal changes in stream fungal communities are typically assessed by filtering water and identifying trapped conidia. Taken over an entire year, molecular diversity of trapped fungal material did not differ from conidial diversity (Raviraja *et al.* 2005). However, when conidial density on filters was low, it was often surpassed by molecular diversity, suggesting incomplete resolution by visual inspection or the presence of DNA in nonconidial structures. Further refinement of this approach may be useful for checking the accuracy of morphology-based taxonomy. It is now possible to extract and amplify DNA from a single spore, though the success rate is low (Nikolcheva, pers. comm.). If performed on a routine basis with informative DNA sequences, it could greatly simplify evaluating and comparing fungal communities in different regions.

2. Spatial arrangement of fungal colonies

Extracting and amplifying DNA informs us about the number of different sequences, typically called phylotypes, in a substrate, irrespective of the fungal life stage. It may also provide information about the relative quantities of the various phylotypes (e.g., combined with real time PCR), though there might be distortions due to sequence-specific biases in extractability and amplification (Röling & Head 2005). However, at least as currently practiced, we lose information on spatial arrangements by homogenizing relatively large amounts of substrates. Classic studies have demonstrated close intermingling of several species by plating out leaf squares of 1 mm^2 (Bärlocher & Kendrick 1974), or by identifying conidia on squares of 4 mm^2

(Chamier *et al.* 1984), or 36 mm^2 (Shearer & Lane 1983). With the increasing sensitivity of molecular methods, it is feasible to extract and amplify DNA from similar sized or even smaller squares.

Two alternative approaches to investigate spatial arrangement of individual species have been suggested. Fluorescence *In Situ* Hybridization (FISH) includes fixation of target cells, hybridization with fluorescently labelled oligonucleotide probes (typically targeting SSU rRNA), removal of nonhybridized probes and visualization and quantification by epifluorescence or confocal laser scanning microscopy (Bidartando & Gardes 2005). Since rRNAs have regions of variable sequence conservation, probes can be designed to be kingdom, genus, or species-specific (''phylogenetic stains''). However, it has been difficult to develop consistently useful probes for fungi because their cell walls often impede probe entry and because of autofluorescence of hyphae and/or substrates. Some success with aquatic hyphomycetes has been reported (Baschien *et al.* 2001; McArthur *et al.* 2001). A modification uses *in situ* PCR (fixed substrates or cells) followed by hybridization. This has not been attempted with aquatic fungi, but its feasibility has been shown with mycorrhizas (Bago *et al.* 1998).

Monoclonal antibodies allow both quantification of mycelia belonging to specified taxa through enzyme-linked immunosorbent assays (ELISA) and their localization in substrates through immunofluorescence. They have successfully been raised against four species of aquatic hyphomycetes (Bermingham *et al.* 1995, 1996, 1997, 2001).

3. Phylogeny

Ingold (1942) recognized that the characteristic shapes of aquatic hyphomycete conidia evolved in response to identical selection pressures. Their value as indicators of phylogenetic relationships is therefore limited, and closely related teleomorphs can indeed produce very different conidial shapes, and very similar conidia are produced by distantly related teleomorphs (Webster 1992; Sivichai & Jones 2003). These studies relied on observing both reproductive stages on the same mycelium. Today, an alternative approach is based on comparing nucleotide sequences of selected genes. This allows comparing groups at all taxonomic levels and, if chosen with care, is independent of an organism's stage in the life cycle or its reproductive phase. Ribosomal genes have been an important source of phylogenetic information for many taxa, including fungi. Sequence analysis can potentially place an anamorph taxon within an order or even a teleomorph genus without having to observe the latter (e.g., Berbee & Taylor 2001). An analysis of 18S rDNA sequences of 7 strains (5 species) of *Tetracladium* placed them in the vicinity of the Ascomycete orders Onygenales, Erysiphales and Leotiales (Nikolcheva & Bärlocher 2002). Additional gene sequences (ITS, 28S) still did not allow unequivocal allocation to a defined Ascomycete order, but the genus clearly belonged to the (paraphyletic) Leotiomycetes (Baschien *et al.* 2006). More strains and sequences from additional loci (e.g., β-tubulin, or α-1-elongation factor) may resolve this issue.

Studies of 31 species placed the majority (74 %) within the Leotiomycetes (Belliveau & Bärlocher 2005; Campbell *et al.* 2006; Baschien *et al.* 2006). They also supported the assumption that several anamorph genera are highly heterogeneous; Campbell *et al.* (2006) concluded that ''no morphological character was entirely congruent with the molecular derived phylogeny''. In addition, several teleomorph genera (e.g., *Nectria*, *Massarina*) associated with aquatic anamorphs are no longer considered to be monophyletic (Belliveau & Bärlocher 2005; Campbell *et al.* 2006). These data suggest that a considerable amount of diversity and heterogeneity remains to be uncovered among aquatic hyphomycete taxa, both in their anamorph and teleomorph phases. This will require analysing multiple sequences in multiple isolates from many locations. For comparison, a recent study on the morphospecies *Neurospora discreta* investigated three nuclear loci in 73 strains and revealed at least eight phylogenetic species (Dettman *et al.* 2006).

Shearer (1993) suggested that when plants invaded freshwater habitats, they brought with them fungal pathogens, endophytes, and saprobes. Alternatively, dead branches and leaves of terrestrial plants may have fallen into streams, precolonized by fungal endophytes and saprobes. Some of these fungal taxa may have adapted to dispersal and reproduction in water. It is known that several aquatic hyphomycetes occur as endophytes in terrestrial and submerged roots (reviewed in Bärlocher 2006), and one species has been found in needles of black spruce (Sokolski *et al.* 2006). Molecular studies of aquatic and terrestrial species may shed additional light on these connections.

4. Genetic diversity

Biodiversity is most commonly evaluated at the species level. But is it becoming increasingly clear that there is considerable intraspecific diversity. Initially, this was evaluated with morphological differences. Later, variability at the level of gene products (isozymes) was investigated. Today, it is possible to evaluate diversity at the level of genes themselves. This has not been done with aquatic hyphomycetes, but Lyons *et al.* (2003) amplified laccase sequences from salt marsh fungi. The products were inserted into plasmids, cloned and sequenced. Several fungal species yielded multiple laccase gene sequences. Investigating the diversity of functional genes is more advanced in bacteriology, where prototype gene arrays are available (e.g., Wu *et al.* 2001).

Intraspecific diversity of aquatic hyphomycetes was first studied by Peláez *et al.* (1996) with the RAPD technique (random amplified polymorphic DNA) applied to the 5.8S rRNA of strains of *Heliscus lugdunensis* and *Articulospora tetracladia*, both with known teleomorphs. They found 7 and 5 RAPD types in the two species, randomly distributed in foam patches along a 1 km long stream reach. Charcosset and Gardes (1999) investigated *Tetrachaetum elegans* (no known teleomorph) with RAPD. They found 13 types in 96 strains. Single leaves were often colonized by multiple genotypes, but frequencies of genotypes differed among leaf species, suggesting substrate preferences among strains. However, the reliability of RAPD markers for population genetic inferences is increasingly being questioned and other methods are now recommended (Rademaker *et al.* 2005). One of these, AFLP (amplified

fragment length polymorphism) was applied to 97 isolates of *Tetrachaetum elegans*, isolated from leaves in 9 streams (Laitung *et al.* 2004). There was a limited degree of polymorphism among the studied markers, and 20 % of the genetic variation was due to differences between streams. A few multilocus genotypes occurred in several locations, suggesting gene flow through animal or aerial dispersal. The results also pointed to a large degree of linkage disequilibrium, indicating a limited recombination (which is expected in clonally reproducing species).

An interesting application was suggested by Seymour *et al.* (2004): genetically more diverse (RAPD and rDNA-ITS sequencing) strains of *Cylindrocarpon destructans* and *Heliscus lugdunensis* produced a greater diversity of unique metabolites.

5. Outlook

Molecular methods have refined or changed our understanding of aquatic hyphomycete biology, evolution and ecology, and have opened entirely new avenues of research. Nevertheless we have barely scratched the surface. It is becoming feasible to document the presence of fungi in smaller and smaller samples, such as single spores, or tiny collections from previously unexplored habitats such as the hyporheos (Bärlocher *et al.* 2006) or (potentially) animal guts (Foltan *et al.* 2005). Provided we connect our fungal sample to a unique sequence (Fell *et al.* 2000; Summerbell *et al.* 2005), we can achieve a much more detailed documentation of fungal distributions. A single sequence, however, is insufficient as a basis for fungal biogeography. In the morphospecies *Neurospora discreta*, multilocus sequence data of 73 strains revealed at least 8 separate phylogenetic species (Taylor *et al.* 2000; Dettman *et al.* 2006), several with distinct geographic distributions. Similar conclusions were reached with other fungal taxa (Taylor *et al.* 2006). This suggests that fungi do have a biogeography, i.e., most (phylogenetic) species do not occur ''everywhere'', as has been suggested for other eukaryotic microbial (morphological) species (Fenchel & Finlay 2004). One exception is *Aspergillus fumigatus*, which has never been observed to reproduce sexually but nevertheless seems capable of genetic recombination. Its ubiquitous distribution and lack of local adaptation have been tenatively attributed to its occurrence in composting vegetation, whose geographic distribution has been greatly expanded by human activity (Taylor *et al.* 2006).

Comparable analyses of aquatic hyphomycetes may become feasible once sufficient DNA can be extracted and analyzed from single spores (currently, analyses are based on pure cultures grown from single spores, which requires considerable skill and effort). They will reveal whether the apparently world-wide distribution of many aquatic hyphomycete species (Wood-Eggenschwiler & Bärlocher 1985) is an illusion due to poor resolution of morphology-based taxonomy, and to what extent there is genetic exchange among predominantly clonally reproducing lineages.

All previously mentioned methods rely on minute amounts of a DNA sequence that may not have originated from a metabolically active or even viable cell. To detect functioning genes, environmental RNA is extracted and reverse transcribed into DNA, which is then amplified by PCR (reverse transcriptase PCR, or RT-PCR; Bridge 2002). This allows identifying currently expressed genes. Microarrays can evaluate identities and/or activities of many microbial populations in complex environments (Gentry *et al.* 2006). This allows describing communities at previously unimagineable levels of resolution. Potential difficulties include biases in extraction, amplification, and statistical evaluations. In addition, it is questionable that this wealth of molecular information will easily translate into a deeper understanding of mechanisms of microbial coexistence and succession. In particular, investigating fungal properties at higher levels of integration (spore shape, population survival during resource scarcity, etc.) will continue to rely on more conventional techniques.

REFERENCES

Ainsworth GC, 1976. *Introduction to the History of Mycology*. University of Cambridge Press, Cambridge.

Bago B, Piche Y, Simon L, 1998. Fluorescently-primed *in situ* PCR in arbuscular mycorrhizas. *Mycological Research* **102**: 1540–1544.

Bärlocher F, 1982. Conidium production from leaves and needles in four streams. *Canadian Journal of Botany* **60**: 1487–1494.

Bärlocher F, 1992a. Historical background and overview. In: Bärlocher F (ed), *The Ecology of Aquatic Hyphomycetes*. Springer-Verlag, Berlin & New York, pp. 1–15.

Bärlocher F, 1992b. Community organization. In: Bärlocher F (ed), *The Ecology of Aquatic Hyphomycetes*. Springer-Verlag, Berlin & New York, pp. 28–76.

Bärlocher F (ed), 1992c. *The Ecology of Aquatic Hyphomycetes*. Springer, Heidelberg & New York.

Bärlocher F, 2005. Freshwater fungal communities. In: Dighton J, Oudemans P, White J (eds), *The Fungal Community*, third ed. CRC Press, Boca Raton, pp. 39–59.

Bärlocher F, 2006. Fungal endophytes in submerged roots. In: Schulz B, Boyle C, Sieber T (eds), *Microbial Root Endophytes*. Springer, Berlin & New York, pp. 179–190.

Bärlocher F, Corkum M, 2003. Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos* **101**: 247–252.

Bärlocher F, Kendrick B, 1974. Dynamics of the fungal population on leaves in a stream. *Journal of Ecology* **62**: 761–791.

Bärlocher F, Nikolcheva LG, Wilson KP, Williams DD, 2006. Fungi in the hyporheic zone of a springbrook. *Microbial Ecology*10.1007/s00248-006-9102-4.

Baschien C, Manz W, Neu TR, Szewzyk U, 2001. Fluorescence *in situ* hybridization of freshwater fungi. *International Review of Hydrobiology* **86**: 371–381.

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

Belliveau M, Bärlocher F, 2005. Molecular evidence confirms multiple origin of aquatic hyphomycetes. *Mycological Research* **109**: 1407–1417.

Berbee ML, Taylor JW, 2001. Fungal molecular evolution: gene trees and geologic time. In: Reynolds DJ, Taylor JW (eds), *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*. CAB International, Wallingford, UK, pp. 67–78.

Bermingham SL, Dewey FM, Maltby L, 1995. Development of a monoclonal antibody-based immunoassay for the detection and quantification of *Anguillospora longissima* colonizing leaf material. *Applied and Environmental Microbiology* **61**: 2606–2613.

Bermingham SL, Maltby L, Dewey FM, 1996. Monoclonal antibodies as tools to quantify mycelium of aquatic hyphomycetes. *New Phytologist* **132**: 593–601.

Bermingham SL, Maltby L, Dewey FM, 1997. Use of immunoassays for the study of natural assemblages of aquatic hyphomycetes. *Microbial Ecology* **33**: 223–229.
Bermingham SL, Dewey FM, Fisher PJ, Maltby L, 2001. Use of a monoclonal antibody-based immunoassay for the detection and quantification of *Heliscus lugdunensis* colonizing alder leaves and roots. *Microbial Ecology* **42**: 506–512.
Bidartando MI, Gardes M, 2005. Fungal diversity in molecular terms: profiling, identification, and quantification in the environment. In: Deighton J, White Jr JF, Oudemans P (eds), *The Fungal Community: its Organization and Role in the Ecosystem*. Taylor & Francis, Boca Raton, pp. 215–239.
Bridge P, 2002. The history and application of molecular mycology. *Mycologist* **16**: 90–99.
Campbell J, Shearer C, Marvanová L, 2006. Evolutionary relationships among aquatic anamorphs and teleomorphs: *Lemonniera*, *Margaritispora*, and *Goniopila*. *Mycological Research* **110**: 1025–1033.
Carter MD, Suberkropp K, 2004. Respiration and annual fungal production associated with decomposing leaf litter in two streams. *Freshwater Biology* **49**: 1112–1122.
Chamier A-C, Dixon PA, Archer SA, 1984. The spatial distribution of fungi on decomposing alder leaves in a freshwater stream. *Oecologia* **64**: 92–103.
Charcosset J-Y, Gardes M, 1999. Infraspecific genetic diversity and substrate preference in the aquatic hyphomycete *Tetrachaetum elegans*. *Mycological Research* **103**: 736–742.
Dettman JR, Jacobson DJ, Taylor JW, 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex. *Mycologia* **98**: 436–446.
Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A, 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Journal of Systematic and Evolutionary Microbiology* **50**: 1351–1371.
Fenchel T, Finlay BJ, 2004. The ubiquity of small species: patterns of local and global diversity. *Bioscience* **54**: 777–784.
Foltan P, Sheppard S, Konvicka M, Symondson WOC, 2005. The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular Ecology* **14**: 4147–4158.
Garrett SD, 1952. The soil fungi as a microcosm for ecologists. *Scientific Progress* **40**: 436–450.
Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J, 2006. Microarray applications in microbial ecology research. *Microbioal Ecology* **52**: 159–175.
Gessner MO, 1997. Fungal biomass, production and sporulation associated with particulate organic matter in streams. *Limnética* **13**: 33–44.
Gessner MO, Chauvet E, 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* **75**: 1807–1817.
Gessner MO, Van Ryckegem G, 2002. Water fungi as decomposers in freshwater ecosystems. In: Bitton G (ed), *Encyclopedia of Environmental Microbiology*. Wiley & Sons, New York, pp. 3353–3364.
Gessner MO, Suberkropp K, Chauvet E, 1997. Decomposition of plant litter by fungi in marine and freshwater ecosystems. In: Wicklow DT, Söderström B (eds), *The Mycota, Vol. IV: Environmental and Microbial Relationships*. Springer, Berlin, pp. 303–322.
Gessner MO, Bärlocher F, Chauvet E, 2003. Qualitative and quantitative analyses of aquatic hyphomycetes in streams. In: Tsui CKM, Hyde KD, Ho WH (eds), *Freshwater Mycology. A Practical Approach*. University of Hong Kong Press, Hong Kong, pp. 127–157.
Ingold CT, 1942. Aquatic hyphomycetes of decaying alder leaves. *Transactions of the British Mycological Society* **25**: 339–417.
Iqbal SH, Webster J, 1973. Aquatic hyphomycete spora of the River Exe and its tributaries. *Transactions of the British Mycological Society* **61**: 331–346.
Kaushik NK, Hynes HBN, 1971. The fate of the dead leaves that fall into streams. *Archiv für Hydrobiologie* **68**: 465–515.
Kennedy N, Clipson N, 2003. Fingerprinting the fungal community. *Mycologist* **17**: 158–164.
Krauss G, Bärlocher F, Krauss G-J, 2003. Effects of pollution on aquatic hyphomycetes. In: Tsui CKM, Hyde KD, Ho WH (eds), *Freshwater Mycology. A Practical Approach*. University of Hong Kong Press, Hong Kong, pp. 211–230.
Laitung B, Chauvet E, Feau N, Feve K, Chikhi L, Gardes M, 2004. Genetic diversity in *Tetrachaetum elegans*, a mitosporic aquatic fungus. *Molecular Ecology* **13**: 1679–1692.
Lyons JI, Newell SY, Buchan A, Moran MA, 2003. Diversity of ascomycete laccase gene sequences in a southeastern salt marsh. *Microbial Ecology* **45**: 270–281.
McArthur FA, Baerlocher MO, MacLean NAB, Hiltz MD, Bärlocher F, 2001. Asking probing questions: can fluorescent in situ hybridization identify and localise aquatic hyphomycetes on leaf litter? *International Review of Hydrobiology* **86**: 429–438.
Newell SY, Fallon RD, 1991. Toward a method for measuring instantaneous fungal growth rates in field samples. *Ecology* **72**: 1547–1559.
Nikolcheva LG, Bärlocher F, 2002. Phylogeny of *Tetracladium* based on 18S rDNA. *Czech Mycology* **53**: 285–295.
Nikolcheva LG, Bärlocher F, 2004. Taxon-specific primers reveal unexpectedly high diversity during leaf decomposition in a stream. *Mycological Progress* **3**: 41–50.
Nikolcheva LG, Bärlocher F, 2005. Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional vs. molecular evidence. *Environmental Microbiology* **7**: 270–280.
Nikolcheva LG, Cockshutt AM, Bärlocher F, 2003. Diversity of freshwater fungi on decaying leaves – comparing traditional and molecular approaches. *Applied and Environmental Microbiology* **69**: 2548–2554.
Nikolcheva LG, Bourque T, Bärlocher F, 2005. Fungal diversity during initial stages of leaf decomposition in a stream. *Mycological Research* **109**: 246–253.
Osborn AM, Smith CJ (eds), 2005. *Molecular Microbial Ecology*. Taylor & Francis, New York.
Peláez F, Platas G, Colldo J, Diez MT, 1996. Infraspecific variation in two species of aquatic hyphomycetes assessed by RAPD analysis. *Mycological Research* **100**: 831–837.
Rademaker JLW, Aarts HJM, Vinuesa P, 2005. Molecular typing of environmental isolates. In: Osborn AM, Smith CJ (eds), *Molecular Microbial Ecology*. Taylor & Francis, New York, pp. 96–134.
Ranzoni FV, 1956. The perfect stage of *Flagellospora penicillioides*. *American Journal of Botany* **68**: 31–35.
Raviraja NS, Nikolcheva LG, Bärlocher F, 2005. Diversity of aquatic hyphomycete conidia assessed by microscopy and by DGGE. *Microbial Ecology* **49**: 1–7.
Röling WFM, Head IM, 2005. Prokaryotic systematics: PCR and sequence analysis of amplified 16S rRNA genes. In: Osborn AM, Smith CJ (eds), *Molecular Microbial Ecology*. Taylor & Francis, New York, pp. 25–63.
Seymour FA, Cresswell JE, Fisher PJ, Lappin-Scott HM, Haag H, Talbot NJ, 2004. The influence of genotypic variation on metabolite diversity in populations of two endophytic fungal species. *Fungal Genetics and Biology* **41**: 721–734.
Shearer CA, 1993. The freshwater ascomycetes. *Nova Hedwigia* **56**: 1–33.
Shearer CA, Lane L, 1983. Comparison of three techniques for the study of aquatic hyphomycete communities. *Mycologia* **75**: 498–508.
Sivichai S, Jones EBG, 2003. Teleomorphic-anamorphic connections of freshwater fungi. In: Tsui CKM, Hyde KD, Ho WH (eds),

Freshwater Mycology. A Practical Approach. University of Hong Kong Press, Hong Kong, pp. 259–274.
Sokolski S, Piché Y, Chauvet E, Bérubé JA, 2006. A fungal endophyte of black spruce (*Picea mariana*) needles is also an aquatic hyphomycete. *Molecular Ecology* **15**: 1955–1962.
Suberkropp K, 1997. Annual production of leaf-decaying fungi in a woodland stream. *Freshwater Biology* **37**: 169–178.
Summerbell RC, Lévesque CA, Seifert KA, Bovers M, Fell JW, Diaz MR, Boekhout T, de Hoog GS, Stalpers J, Crous PW, 2005. Microcoding: the second step in DNA barcoding. *Philosophical Transactions of the Royal Society B* **360**: 1897–1903.
Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.
Taylor JW, Turner E, Townsend JP, Dettman JR, Jacobson D, 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philosophical Transactions of the Royal Society B* **361**: 1947–1963.
Triska FJ, 1970. Seasonal distribution of aquatic hyphomycetes in relation to the disappearance of leaf litter from a woodland stream. Ph.D. Thesis, University of Pittsburgh, Pittsburgh.
Webster J, 1959. Experiments with spores of aquatic hyphomycetes. I Sedimentation, and impaction on smooth surfaces. *Annals of Botany* **23**: 595–611.
Webster J, 1992. Anamorph-teleomorph relationships. In: Bärlocher F (ed), *The Ecology of Aquatic Hyphomycetes*. Springer, Berlin & New York, pp. 99–117.
White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MS, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, pp. 315–322.
Wood-Eggenschwiler S, Bärlocher F, 1985. Geographical distribution of Ingoldian fungi. *Verhandlungen der internationalen Vereinigung für Limnologie* **22**: 2780–2785.
Wu L, Thompson DK, Li G, Hurt JM, Tiedje RA, Zhou J, 2001. Development and evaluation of functional gene arrays for detection of selected genes in the environment. *Applied and Environmental Microbiology* **67**: 5780–5790.