

# Fungal communities on decaying leaves in streams: a comparison of two leaf species

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**Abstract** Decomposition of leaf litter is a microbial mediated process that helps to transfer energy and nutrients from leaves to higher trophic levels in woodland streams. Generally, aquatic hyphomycetes are viewed as the major fungal group responsible for leaf litter decomposition. In this study, traditional microscopic examination (based on identification of released conidia) and phylogenetic analysis of 18S rRNA genes from cultivated fungi were used to compare fungal community composition on decomposing leaves of two species (sugar maple and white oak) from a NE Ohio stream. No significant differences were found in sporulation rates between maple and oak leaves and both had similar species diversity. From the 18S rRNA gene sequence data, identification was achieved for 12 isolates and taxonomic affiliation of 12 of the remaining 14 isolates could be obtained. A neighbor-joining tree (with bootstrap values) was constructed to examine the taxonomic distribution of the isolates relative to sequences of known operational taxonomic units (OTUs). Surprisingly, only 2 of the isolates obtained were aquatic hyphomycetes based

on phylogenetic analysis. Overall, there were no differences between the two leaf types and a higher diversity was observed via culturing and subsequent 18S rRNA gene sequencing than by conidia staining. These differences resulted from the fact that traditional microscopy provides estimates of aquatic hyphomycete diversity while the other approach revealed the presence of both aquatic hyphomycete and non-aquatic hyphomycete taxa. The presence of this broad array of taxa suggests that the role of aquatic hyphomycetes relative to other fungi be re-evaluated. Even though the functional role of these non-aquatic hyphomycetes taxa is unknown, their presence and diversity demonstrates the need to delve further into fungal community structure on decomposing leaves.

**Keywords** Aquatic hyphomycetes · Microscopy · Phylogenetic analysis

## Introduction

In woodland streams, leaf litter is an important energy source (Minshall 1967; Fisher and Likens 1973) and, among the fungi, aquatic hyphomycetes play a pivotal role in the decomposition of dead leaves in these ecosystems (Bärlocher 1992a). Phylogenetically, the aquatic hyphomycetes constitute a heterogeneous group of anamorphic fungi belonging to ascomycetes and basidiomycetes that are characterized by mostly tetra- or multibrachial conidia with a few forming sigmoid conidia. (Bärlocher 1992a). They provide nutrition to higher trophic levels, mainly macroinvertebrates, by enzymatic degradation of leaf material (Sinsabaugh and Linkins 1990). Thus, by “conditioning” leaf litter for invertebrates (Kaushik and Hynes 1971; Bärlocher 1985; Suberkropp 1992a), aquatic

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hyphomycetes along with bacteria serve as important intermediaries in the energy flow of food webs in lotic ecosystems (Suberkropp and Klug 1976; Bärlocher and Kendrick 1981; Findlay and Arsuffi 1989; Bärlocher 1992b).

There are biologically important differences among leaves of different species that may affect microbial community structure, decomposition, and nutrient availability to higher trophic levels. Factors, such as N content, lignin concentration, cuticle toughness, and concentrations of secondary compounds, influence decomposition rates and microbial activity (e.g., Webster and Benfield 1986). Therefore, the types of organisms growing on leaves with different properties may differ. However, results from prior studies comparing fungal communities among different leaf types, based on conidia formation and molecular analyses of DNA extracts (Nikolcheva and Bärlocher 2005) or DNA fingerprinting, like DGGE, alone (Das et al. 2007), have yielded mixed results across the methods and analyses employed. Gulis (2001), in a comparison of leaves of several species as well as wood, concluded, based on conidia examination, that there are likely substrate preferences in some species of aquatic hyphomycetes, with distinct fungal assemblages being present in grass or wood compared to leaves.

Traditional methods for estimating fungal diversity on leaves rely on examination of sporulation structures on leaves (Bärlocher and Kendrick 1974; Suberkropp and Klug 1976), or on conidia (spores) released from leaves (Bärlocher 1982). However, the absence of conidia does not necessarily mean a given species is absent, as non-sporulating mycelia may also be present on decaying leaves (Nikolcheva et al. 2003). Moreover, traditional conidia staining is a method most suited for identification of the sigmoid and tetra-radiate conidia of aquatic hyphomycetes. Although aquatic hyphomycetes are generally believed to be the main group of fungi involved in leaf decay, relying solely on conidia staining might preclude the observation of other fungal groups involved in the decay process, such as those that produce ascomata (Nikolcheva and Bärlocher 2005).

Other approaches to examining fungal community structure on leaves are based on DNA extraction and analysis (Smit et al. 1999; May et al. 2001; Nikolcheva and Bärlocher 2005) or on cultivation (Gamboa et al. 2002). Although cultivation-based approaches may not depict the community structure in its entirety, they provide living organisms that can be identified, characterized and used in experiments. Identification of these isolates can be done by traditional microscopic methods, which rely on formation of reproductive structures (Bärlocher 1992a). However, some isolates may remain sterile under laboratory conditions and identification can be time-consuming and

laborious (Pérez-Sierra and Henricot 2002). In such cases, DNA sequencing provides a means for taxonomic identification (Ward et al. 1990). Molecular techniques can also provide insight into the phylogenetic relationships of fungi that had previously been based largely on morphological traits (Winka 2000; Down 2002).

The objective of this study was to compare the fungal community on leaves of two species decaying in a stream using both traditional conidia staining methods and cultivation followed by 18S rRNA gene sequencing. We selected sugar maple and white oak because they are common in this watershed and differ in leaf chemistry and decay rates. For example, Ostrofsky (1997) reported white oak to have greater lignin, greater toughness, and a 5-fold slower decay rate than sugar maple. We hypothesized that there would be differences in fungal communities between the two leaf species and that cultivation and sequencing of isolates would reveal members of the fungal community that were not detected by conidia staining. Nevertheless, we expected aquatic hyphomycetes to represent the majority of the fungal community, as this group is believed to play a major role in leaf processing (e.g., Cummins 1974; Suberkropp and Klug 1980).

## Methods

### Study site

The study was conducted in a 3rd order reach of the West Branch of the Mahoning River (WBM) in Northeastern Ohio. The WBM lies within the Erie/Ontario Lake Plain Ecoregion (OHEPA 1996) and at the study site (20°41'N, 51°80'W) flows through a mature hardwood forest of primarily oaks (*Quercus* spp.), sugar maple (*Acer saccharum*), and sycamore (*Platanus occidentalis*). There are no point source pollution discharges to the stream, but non-point source inputs occur within the watershed from surrounding residences. The WBM is a hardwater stream with annual average dissolved inorganic nitrogen and soluble reactive phosphorus concentrations of 0.18 mg N/l and 0.15 mg P/l, respectively (Olapade and Leff 2005). The streambed consists mainly of gravel and small cobbles and the channel contains a typical pool-riffle sequence. During baseflow, the stream is 4–5 m wide and riffle-run habitats are less than 30 cm deep. The site has been used extensively for studies of aquatic microbial ecology and additional descriptions of the physical and chemical characteristics of the stream are available (e.g., Olapade and Leff 2004; McNamara and Leff 2004). During the present study, the temperature ranged from 0.7 to 21.4°C while conductivity values were as high as 1,777  $\mu$ S/cm to as low as 463  $\mu$ S/cm.

## Study design

During October 2003, senescent leaves of sugar maple (*A. saccharum*) and white oak (*Q. alba*) were collected by shaking several trees along the stream. Leaves were air-dried and leaf packs constructed by placing about 4.5 g of sugar maple or white oak leaves in nylon litter bags with a mesh size of 3 mm ( $n=33$  for each leaf type). On 31 October 2003, the litter bags were placed in a riffle-run habitat and secured to the streambed with plastic stakes. Three replicate litter bags of each leaf type were retrieved after 1, 6, 15, 30, 60, 90, 120, 150 and 181 days of incubation. At retrieval, each litter bag was rinsed gently, placed in individual plastic bags, and transported to the laboratory on ice. In the laboratory, leaves were removed from the litter bags, rinsed with distilled water, and ten leaf disks were cut from the leaves using a cork-borer (14 mm diameter) and used for sporulation and cultivation of fungi (described below). The wet, dry, and ash-free dry mass of the disks used for sporulation were also measured.

Water samples ( $n=3$ , but  $n=1$  for 4 November 2003) for examination of conidia were collected on 3 dates during November and December of 2003 from the riffle at the study site in sterile Whirlpaks. In the laboratory, 200 ml of the sample was immediately used for counting of conidia in the water column as described below.

## Conidia identification and sporulation rates

Leaf disks were incubated on a shaker in 200 ml of autoclaved, distilled water at room temperature for 7 days. From each flask, 100  $\mu$ l of the conidial suspension was used for cultivation and the remainder was filtered through a 5- $\mu$ m MF-mixed cellulose ester, hydrophilic filter (47 mm diameter) from Millipore (Bedford, MA), fixed in lactophenol and stained with 0.01% cotton blue in 60% lactic acid to examine spores of aquatic hyphomycetes. Filters were mounted and 20 fields observed at 400 $\times$  magnification. Spores were identified (Barron 1968; Ingold 1975; Hanlin 1990) and enumerated. Sporulation rates were expressed as number of conidia  $g^{-1}$  (dry mass)  $day^{-1}$ . For water samples, conidia were fixed, stained and identified as above after filtering samples through 5- $\mu$ m cellulose membrane filters (47 mm diameter; Millipore).

## Cultivation of isolates

Cultivation and isolation of fungi from the leaf disks was accomplished by plating 100  $\mu$ l of suspension from the sporulation flasks on 0.1% malt extract agar (MEA) containing 100  $\mu$ g/ml chloramphenicol to inhibit bacterial growth. Plates were incubated at room temperature for 7–14 days. When colonies appeared, the growing edge was

transferred to 2% MEA plates with 100  $\mu$ g/ml chloramphenicol. As soon as a colony appeared on this second plate, the growing edge was transferred to 2% MEA slants containing 100  $\mu$ g/ml chloramphenicol. After 14 days, the slants were stored at 12°C. The isolates were transferred to fresh media every 3–4 months.

## DNA extraction and gene amplification

In preparation for DNA extraction, fungal isolates were grown on 2% MEA plates and the growing edge transferred to 2% malt extract broth containing 100  $\mu$ g/ml chloramphenicol. These cultures were incubated on a shaker at room temperature for 7–14 days. When growth was observed, the mycelia were filtered through a 5- $\mu$ m cellulose membrane filter and ground in liquid nitrogen. DNA was isolated from 30 mg of ground mycelia using the Ultraclean Microbial DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's instructions.

Amplification of the 5' end of the 18S rRNA gene was done with the universal primer NS1 (White et al. 1990) and the fungus-specific primer GCFung (May et al. 2001) according to Nikolcheva et al. (2003) with slight modification. PCR was performed using Ready-To-Go-PCR beads (Amersham Biosciences, Piscataway, NJ), 0.8  $\mu$ M concentration of each of the primers, and 50 ng of DNA. The conditions for the reaction were: 2 min of initial denaturation at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C, and extension for 1 min at 72°C. Any partial polymerization that might have occurred was completed by doing a final extension step for 5 min at 72°C. The 370-bp PCR products were checked via 1% (w/v) agarose gels containing ethidium bromide (4  $\mu$ l, 10 mg/ml; Bio-Rad) against a 100-bp molecular ladder (Promega, Madison, WI). *Tetrachaetum elegans* (provided by V. Gulis at University of Alabama) was used as a control for the PCR reactions.

## PCR product purification

PCR products were purified using low melting NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) and Wizard PCR Preps DNA Purification System (Promega). The 1.5% gel was made by adding 0.75 g NuSieve agarose to 50 ml of 0.5  $\times$  TBE to which 5  $\mu$ l of 10 mg  $ml^{-1}$  ethidium bromide was added. The gel was placed on a transilluminator under the lowest intensity to minimize formation of pyrimidine dimers and segments of gel containing PCR products were excised, and gel slices were incubated at 70°C until the agarose melted. The Wizard PCR Preps DNA purification system (Promega) was then used following manufacturer's directions. The purified

products were eluted in 50 µl of autoclaved, distilled water and again checked on a 1% agarose gel. The amount of DNA obtained was determined in a GeneQuant Pro spectrophotometer (Amersham Biosciences).

#### Sequencing and phylogenetic tree construction

Purified amplification products of fungal isolates were cycle-sequenced in both directions using an automated 3730 DNA analyzer (Applied Biosystems) and BigDye Terminator Cycle Sequencing chemistry at the Plant-Microbe Genomics Facility at the Ohio State University, Columbus, OH. Sequences were edited in the Sequencher program (GeneCodes Corporation, Ann Arbor, MI), aligned automatically with Sequencher and then manually with the Se-Al program (Dept. of Zoology, University of Oxford, Oxford, UK). Consensus sequences were then matched against the National Center for Biotechnology Information's (NCBI) nucleotide database using Basic Local Alignment Search Tool (BLAST) to identify the isolates. A  $\geq 98\%$  similarity match of an isolate with a sequence in GenBank was used as the criterion for identification of a particular isolate as a given operational taxonomic unit (OTU). A neighbor-joining bootstrap mid-point rooted tree was constructed using PAUP\* software (Swofford 2001) and *Saccharomyces cerevisiae* as an outgroup. The neighbor-joining method of tree construction is a distance-based method that starts with a star-like tree and then clusters the sequences or operational taxonomic units by minimizing the sum of the branch lengths (Saitou and Nei 1987), based on the distance or dissimilarity between pairs of OTUs. The Jukes–Cantor model for nucleotide substitution was used in the tree construction (Jukes and Cantor 1969). The phylogenetic tree was constructed using sequences of our isolates and reference sequences from GenBank (NCBI) that shared a  $\geq 95\%$  sequence homology with the isolates. Bootstrapping was done in order to place confidence levels on the topologies of the phylogenetic tree (Felsenstein 1985). With this method, a bootstrap proportion of  $\geq 70\%$  usually reflects a 95% probability that a particular clade in the tree is correct (Hillis and Bull 1993).

## Results

#### Conidia identification and sporulation rates

In total, 13 species of aquatic hyphomycetes were identified from leaf samples based on examination of conidia (Table 1). Six other distinct but unidentified types of spores were also observed, but 5 of the 6 were rare and each occurred on a single sampling date. The maximum number of taxa recorded on sugar maple leaves was 7, compared to a maximum of 12 on white oak leaves.

**Table 1** Aquatic hyphomycete taxa found on sugar maple (*Acer saccharum*) and white oak (*Quercus alba*) on different days (1, 6, 15, 30, 60, 90, 120, 150 & 181) based on conidia staining method

| Hyphomycete taxa                 | Leaf species (days) |            |
|----------------------------------|---------------------|------------|
|                                  | Maple               | Oak        |
| <i>Alatospora</i> sp.            | 6–120, 181          | 1–181      |
| <i>Anguillospora crassa</i>      | 1, 6, 90, 120       | 15–150     |
| <i>Anguillospora longissima</i>  | 6–181               | 30–181     |
| <i>Articulospora tetracladia</i> | 30, 90              | 30         |
| <i>Clavariopsis aquatica</i>     | 30–181              | 15–181     |
| <i>Dactylella aquatica</i>       |                     | 90         |
| <i>Flagellospora curvula</i>     | 1, 6, 150, 181      | 6–60       |
| <i>Lemnoneira aquatica</i>       | 181                 | 120, 181   |
| <i>Lunulospora curvula</i>       | 60, 90, 181         | 15, 30, 90 |
| <i>Tetrachaetum elegans</i>      | 1–181               | 1–181      |
| <i>Tetracladium marchalianum</i> | 1–30                | 1–120      |
| <i>Tetrasporium asterinearum</i> | 1                   |            |
| <i>Tricladium angulatum</i>      |                     | 1–15, 90   |
| Unknown # 1                      | 6–60                | 15–60      |
| Unknown # 2                      |                     | 30         |
| Unknown # 3                      |                     | 30         |
| Unknown # 4                      | 6                   |            |
| Unknown # 5                      |                     | 6          |
| Unknown # 6                      |                     | 1          |

The most common aquatic hyphomycetes from the decaying leaves were *Alatospora* sp., *Anguillospora longissima* (Sacc. & Syd.) Ingold, *Clavariopsis aquatica* de Wildeman, *Tetrachaetum elegans* Ingold, and *Tetracladium marchalianum* de Wildeman. Conidia from these taxa were also found in the water column (Table 2). In general, sampling leaves revealed greater diversity than sampling the water column (9 fungal types) and all aquatic hyphomycetes observed in the water column were also identified from leaves.

Conidia counts in the stream water column ranged between  $3.48 \times 10^5 \text{ l}^{-1}$  to  $6.48 \times 10^5 \text{ l}^{-1}$ . Sporulation rates of aquatic hyphomycetes followed a similar pattern on both leaf species, reaching a peak on day 15 (Fig. 1). A two-way ANOVA (with days and species as factors) revealed no statistically significant differences between sporulation rates on sugar maple and white oak leaves ( $F=9.91$ ,  $p=0.93$ ). Maximum sporulation rates of approximately  $4.4 \times 10^5$  and  $4.7 \times 10^5$  conidia  $\text{g dry mass}^{-1} \text{ day}^{-1}$  were measured on sugar maple and white oak leaves, respectively. After 120 days of incubation, sporulation on both leaf types declined by about an order of magnitude from the day 15 peak. By day 15 of the study, *T. elegans* represented  $> 80\%$  of the total conidia production on both sugar maple and white oak leaves (Fig. 2). This taxon continued to dominate conidium production for the remainder of the study with the next common species rarely exceeding 20% of total conidium production.

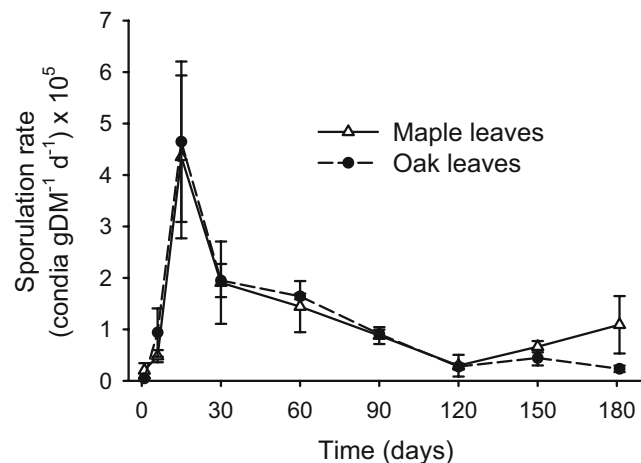
**Table 2** Hyphomycete taxa found in water samples based on conidia staining

| Hyphomycete taxa                 | Dates      |            |            |
|----------------------------------|------------|------------|------------|
|                                  | 4 Nov 2003 | 6 Nov 2003 | 6 Dec 2003 |
| <i>Alatospora</i> sp.            | *          | *          | *          |
| <i>Anguillospora crassa</i>      | *          | *          | *          |
| <i>Anguillospora longissima</i>  | *          | *          | *          |
| <i>Articulospora tetracladia</i> |            |            | *          |
| <i>Clavariopsis aquatica</i>     |            | *          | *          |
| <i>Flagellospora curvula</i>     | *          | *          | *          |
| <i>Tetrachaetum elegans</i>      | *          | *          | *          |
| <i>Tetracladium marchalianum</i> |            | *          |            |
| Unknown # 1                      | *          |            |            |

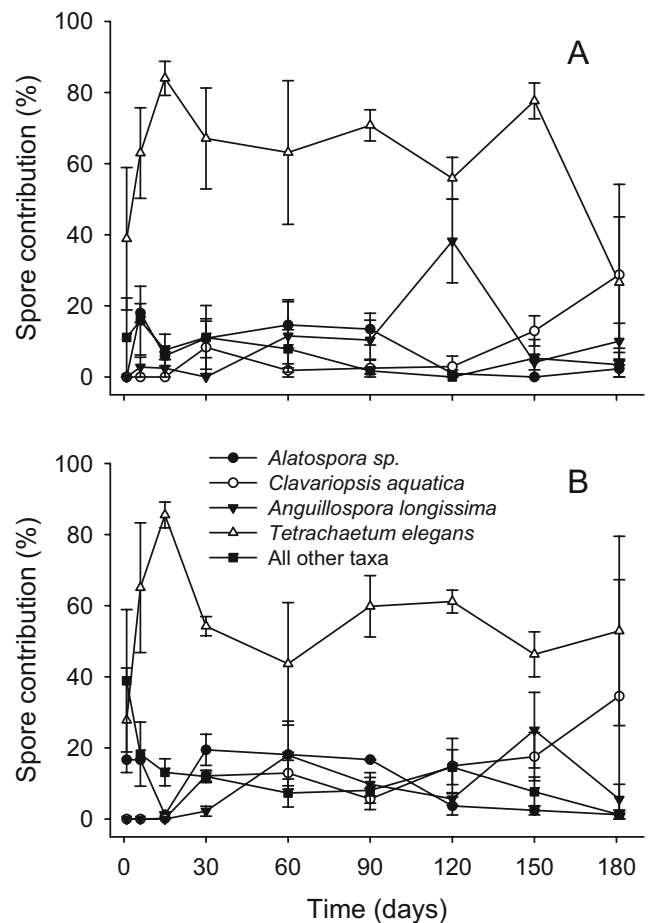
Fungal isolate analysis

Thirty-two fungal isolates were obtained by culturing, of which 26 were analyzed by 18S rRNA gene sequencing. The remaining 6 exhibited the same colony morphology as another of the isolates that were sequenced and hence were not sequenced. Out of 26 isolates, 12 isolates were identified ( $\geq 98\%$  identity) as a particular OTU by comparing the obtained sequences to the NCBI database (Table 3). The remaining isolates could not be matched to any known sequence based on the resolution of the sequences we obtained (Table 4).

The majority of the isolates were ascomycetes (the taxonomic group to which most aquatic hyphomycetes belong) and most belonged to the subphylum Pezizomycotina, class Sordariomycetes, and some belonged to the class



**Fig. 1** Sporulation rate of aquatic hyphomycetes on decomposing sugar maple (*Acer saccharum*) and white oak (*Quercus alba*) leaves. Values are means ( $\pm$ SE) of triplicate samples



**Fig. 2** Relative contribution of different hyphomycete taxa to spore production on decomposing leaves of (a) sugar maple and (b) white oak. Values are means ( $\pm$ SE) of triplicate samples

Dothideomycetes. Although isolate H30 is apparently a mitosporic ascomycete, no information on its class/order affinity is listed in the public databases and thus it is listed as ‘incertae sedis’. Two isolates (H21 and H32) matched partial sequences of 18S rRNA genes in the NCBI database from uncultured fungi obtained from environmental samples.

Isolates with similar colony morphology usually also yielded the same sequence data (e.g., H27 and H28, H17 and H20). However, despite having identical colony morphology to H17 and H20 (which were *Colletotrichum gloeosporioides*), H4 was designated as *Phyllachorales* sp. H4 because of its lower value of identity (95%) to *Colletotrichum gloeosporioides*. Yet, this was not true for H1 and H18 which differed morphologically but shared sequence identity with *Pestalotiopsis jesteri*. Interestingly, although H21 and H32 were obtained via cultivation, they were homologous to sequences obtained from environmental DNA, with H21 matching an uncultured fungus clone (GenBank Accession No. AY321700), and H32 an uncultured endophytic fungus (GenBank Accession No. AM161480).

**Table 3** Identity of 12 out of 26 fungal isolates obtained during the study (along with their GenBank accession numbers) based on 18S rRNA gene sequencing

| Isolate | Name                                 | Leaf  | % identity | GenBank accession no. | Detected by conidia |
|---------|--------------------------------------|-------|------------|-----------------------|---------------------|
| H1      | <i>Pestalotiopsis jesteri</i>        | Maple | 98         | DQ310763              | No                  |
| H3      | <i>Sirococcus conigenus</i>          | Oak   | 98         | DQ310765              | No                  |
| H4      | Phyllachorales sp. H4                | Oak   | 95         | DQ310766              | No                  |
| H5      | <i>Hypocrea</i> sp. H5               | Maple | 98         | DQ310767              | No                  |
| H17     | <i>Colletotrichum gloeosporoides</i> | Oak   | 100        | DQ310773              | No                  |
| H18     | <i>Pestalotiopsis jesteri</i>        | Maple | 98         | DQ310774              | No                  |
| H20     | <i>Colletotrichum gloeosporoides</i> | Maple | 99         | DQ310776              | No                  |
| H21     | Pleosporales sp. H21                 | Oak   | 98         | DQ310777              | No                  |
| H27     | <i>Anguillospora longissima</i>      | Oak   | 97         | DQ310783              | Yes                 |
| H28     | <i>Anguillospora longissima</i>      | Maple | 99         | DQ310784              | Yes                 |
| H30     | <i>Tetrachaetum elegans</i>          | Oak   | 98         | DQ310786              | Yes                 |
| H32     | Fungal sp. H32                       | Maple | 98         | DQ310788              | No                  |

### Phylogenetic analyses

In the tree, the isolates form distinct clades (Fig. 3) with bootstrap values ranging from 63 to 100%. According to Hillis and Bull (1993), a topology has a 95% probability of being correct when it has  $\geq 70\%$  bootstrap support. Thus, the majority of the clades in Fig. 3 reflect a precise phylogeny.

The clade consisting of H23, H19, H32, H26 and H25 are interspersed with mostly dothideomycete sequences, suggesting that these isolates may taxonomically be more homologous to class Dothideomycetes. Isolates H16 and H22 cluster strongly with one dothideomycete and one uncultured soil ascomycete which may indicate their phylogenetic proximity towards dothideomycetes. Isolate H29 forms a strongly supported clade with *Tumularia aquatica* and *Clavariopsis aquatica*, which themselves

have been associated with the *Massarina* teleomorph (Belliveau and Barlocher 2005). Hence H29 probably is a dothideomycete, too. The other large clade, consisting of H1, H2, H12, H13, H15, H18 and H31, clustered strongly (92% bootstrap support) with reference sequences from the order Xylariales. The remaining isolate H24 clustered with an uncultured fungus clone and therefore no definitive conclusion concerning its phylogenetic affiliation can be drawn.

On comparing the fungal taxa that were observed through conidia staining and those obtained via culturing, only *Tetrachaetum elegans* and *Anguillospora longissima* were obtained by both methods. Another important aspect is that these two were also the only aquatic hyphomycetes that were obtained via cultivation in this study. The rest of the isolates are either mitosporic or teleomorphs and may not be aquatic hyphomycetes because repeated attempts to sporulate these isolates under laboratory conditions conventionally used for aquatic hyphomycetes failed. Also, it is difficult to say if the unresolved isolates are aquatic hyphomycetes based only on their broad taxonomic affinities to reference sequences.

**Table 4** GenBank accession numbers of remaining 14 isolates

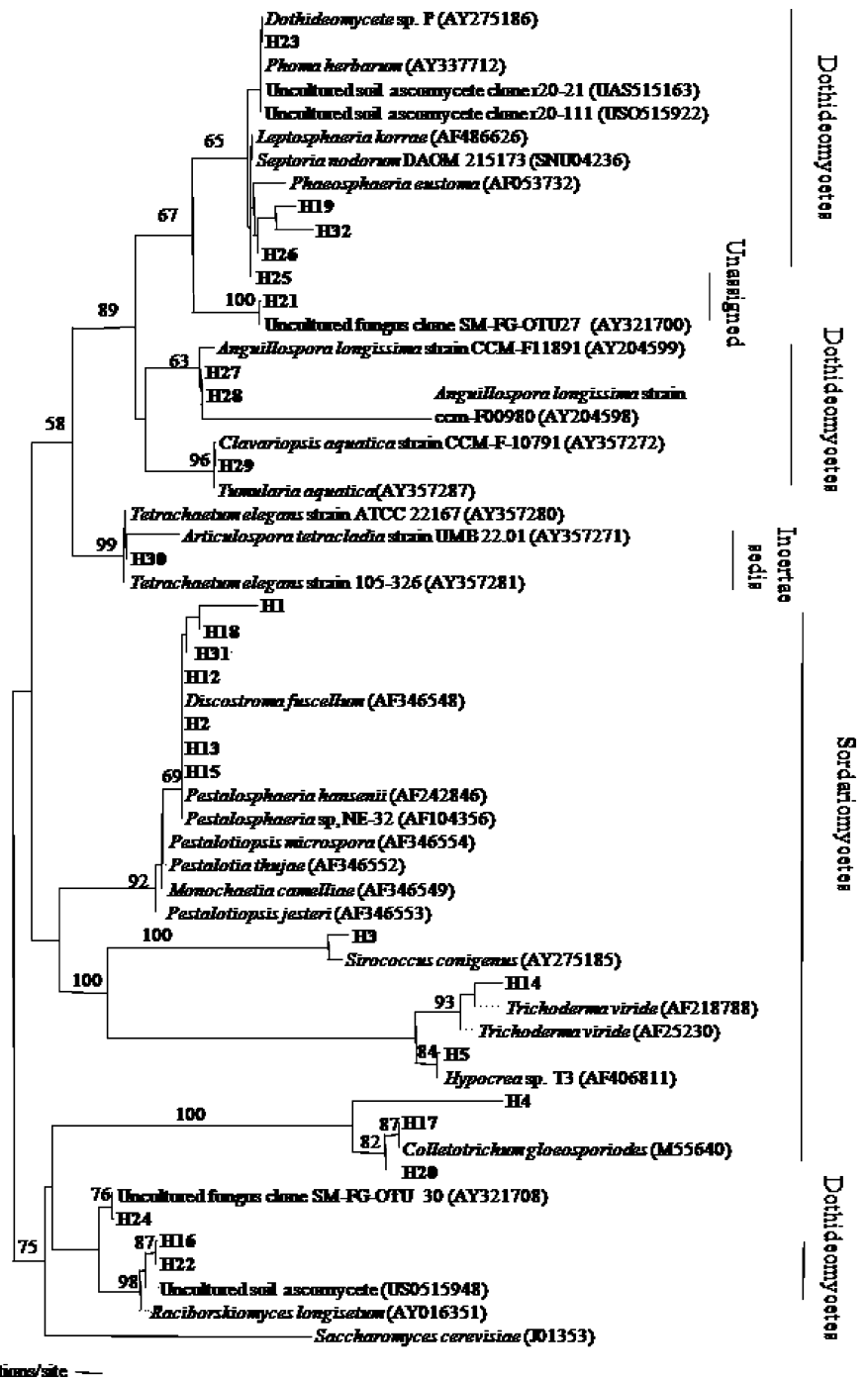
| Isolate | Name                 | Leaf  | GenBank Accession No. |
|---------|----------------------|-------|-----------------------|
| H2      | Xylariales sp. H2    | Maple | DQ310764              |
| H12     | Xylariales sp. H12   | Maple | DQ310768              |
| H13     | Xylariales sp. H13   | Maple | DQ310769              |
| H14     | Hypocreales sp. H14  | Oak   | DQ310770              |
| H15     | Xylariales sp. H15   | Maple | DQ310771              |
| H16     | Ascomycete sp. H16   | Maple | DQ310772              |
| H19     | Fungal sp. H19       | Maple | DQ310775              |
| H22     | Ascomycete sp. H22   | Oak   | DQ310778              |
| H23     | Fungal sp. H23       | Maple | DQ310779              |
| H24     | Fungal sp. H24       | Maple | DQ310780              |
| H25     | Pleosporales sp. H25 | Maple | DQ310781              |
| H26     | Pleosporales sp. H26 | Maple | DQ310782              |
| H29     | Ascomycete sp. H29   | Oak   | DQ310785              |
| H31     | Xylariales sp. H31   | Maple | DQ310787              |

Names reflect the taxonomic positions (order, family and so on) that could be corroborated by the available sequence information, followed by isolate codes (as used in the study)

### Discussion

Overall, there were fewer differences between the two leaf species than might be expected based on differences in leaf properties. Based on cultivation, a greater diversity of taxa was observed on maple leaves than on oak leaves. However, diversity observed via conidia examination and sporulation rates of fungi on the two leaf types were not different. The two leaf species have different decomposition rates as white oak has higher lignin content than sugar maple (Ostrofsky 1997), which constrains the availability of carbon to decomposers (Gessner and Chauvet 1994). This difference in resource quality could contribute to

**Fig. 3** Neighbor-joining (bootstrap) tree depicting phylogenetic relationships of the isolates to each other and to other known taxa whose sequences were obtained from GenBank. Reference taxa have their GenBank accession numbers listed in *parentheses*. The isolates are listed in *bold* and numbered H1 to H32. Bootstrap values are presented at each node in the tree. Class affinity of the different clades are also listed. “Unassigned” is listed for the isolate for which no phylogenetic information could be obtained



differences in community structure and diversity between the leaf types. However, the nature of the interaction between resource quality and fungal diversity on decaying leaves remains unclear, particularly for non-aquatic hyphomycetes.

Low species diversity on the leaves based on conidium staining may be attributed to the occurrence of a couple of high discharge events during the study period. This may have caused scouring of the leaf bags and the streambed, resulting in reorganization of the fungal community. Hence

the number of species encountered was probably lower than might be expected when stream flow is not under the influence of such perturbations.

As was observed in our study, different methods may yield varying depictions of the leaf fungal communities. Decomposition studies in streams have primarily relied on evaluation of the diversity of the aquatic fungal community by staining conidia (Bärlocher 1982). However, it is possible that, at the time of collection, all the fungi present may not have been capable of sporulation (Nikolcheva et al.

2003). For example, high conidium production by a dominant aquatic hyphomycete species might mask spore production of other species, which thus would escape detection if diversity assays are solely based on conidia formation. Moreover, miscellaneous mitosporic ascomycetes that do not form typical aquatic hyphomycete conidia may be present in the leaf biofilm community (Nikolcheva and Bärlocher 2004). Although the role of such fungi in ecosystem-level processes like organic matter processing is largely unknown (Nikolcheva and Bärlocher 2004), several studies have documented the presence of other fungal taxa such as Basidiomycota, Chytridiomycota (Nikolcheva and Bärlocher 2004), Oomycota and Zygomycota (Bärlocher and Kendrick 1974; Nikolcheva and Bärlocher 2004) on decomposing plant litter. Therefore, in this project, fungal communities were assessed using the traditional conidia staining method as well as culturing and phylogenetic analysis.

Leaf decomposition studies report that terrestrial fungi are normally supplanted by aquatic hyphomycetes within 24 h of submersion of the leaves in the stream (Bärlocher and Kendrick 1974; Chergui and Pattee 1988). Adaptability to very low stream temperatures, a distinctive spore shape for attachment to solid substrata, and, most importantly, the ability to sporulate under water confers to the aquatic hyphomycetes a competitive advantage over the terrestrial fungi. Thus, to succeed on submerged leaves in a stream, terrestrial fungi would need to depend on a constant supply of hyphal fragments or exogenous propagules (Bärlocher and Kendrick 1974) which seem unlikely to be readily available. However, in our study, based on culturing, only two aquatic hyphomycete isolates were obtained, and non-aquatic hyphomycete fungi were recovered even from the latter stages of decomposition. This suggests that the latter were present in the community, perhaps as sterile mycelia not actively engaged in sporulation during the time of sampling. As a result, their spores could not be observed by microscopy. Also, the conventional technique of inducing sporulation by agitating water favors aquatic hyphomycetes, as they are considered to play the most important role in decomposition of leaf litter in streams (Bärlocher 1992a; Suberkropp 1992b). Consequently, fungi that may be present in a dormant or vegetative state will go undetected by this technique. Lastly, spores produced by taxa other than aquatic hyphomycetes may go undetected or remain unidentified because their morphology does not resemble the typical appearance of aquatic hyphomycete conidia.

As with the traditional approach, there are also limitations of the cultivation-based method. First, cultivation does not recover all fungi present as some fraction of natural microbial communities may not be readily cultivated using conventional means. Second, disparities were observed between the BLAST matches and phylogenetic

tree that was constructed. For example, although BLAST shows H1 and H18 to be *Pestalotiopsis jesteri*, they do not align adjacent to the reference sequence (GenBank Accession No. AF346553) of the same clade. This may be due to inter-strain differences between our isolates and the Genbank sequence. Also, different studies employ primers that target different portions of the 18S rRNA gene. Consequently, sequences generated by our primers may not align precisely with sequences in the database if they were generated from different primers. Due to the unavailability of complete 18S rRNA gene sequences from various fungi, the sequence matches are thus incomplete. In such cases, even though the level of sequence similarity obtained is adequate for matching to a known OTU/sequence present in the database, there may still be insufficient phylogenetic signal for correct clustering of isolates (like H1 and H18) to their reference GenBank sequences.

Although definitive identification could not be obtained for 14 of the 26 isolates that were sequenced, clues about their putative taxonomic positions could be observed for some of the isolates based on their clustering with known GenBank sequences; however, even this was not possible for four isolates. Further analyses targeting highly variable nucleotide sequences of shared genes, such as ITS regions of ribosomal genes (Nikolcheva and Bärlocher 2004), should facilitate their definitive identification.

Overall, greater diversity of fungi was observed via culturing compared to conidia staining; differences between the two leaf species were relatively limited and varied between the methods employed. This further emphasizes the need for assessment of fungal communities using a variety of techniques, especially given that conidia staining alone may not reveal the structure of the entire fungal community. In our study, the cultivation approach reflected the prevalence of mostly non-aquatic hyphomycete taxa while microscopy revealed the aquatic hyphomycete-component of the leaf fungal community. Our findings suggest that the non-aquatic hyphomycete taxa need to be explored in more detail to evaluate their role in leaf decomposition.

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