Reviews, Critiques and New Technologies

Fungal diversity during leaf decomposition in a stream assessed through clone libraries

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Maple, linden and oak leaf disks were exposed for 17 days in a first order, softwater stream in Nova Scotia, Canada. After aerating disks in water for 2 days, we recovered conidia of aquatic hyphomycetes belonging to 7 (oak) or 13 (maple, linden) species. We also extracted DNA from stream-exposed disks and amplified partial SSU rDNA sequences. The diversity of these sequences was evaluated by construction of clone libraries. The 151 sequenced clones were divided into 40 operational taxonomix units (OTUs). The majority of these were most similar to sequences of uncultured fungi, most of them with close affinities to various Ascomycetes. Other fungal categories included Basidiomycetes and Chytridiomycetes. Taxon accumulation curves and estimated diversity were considerably higher for sequenced clones than for species of aquatic hyphomycetes identified from their spores. We conclude that traditional, microscopy-based studies underestimate fungal diversity on leaves decaying in streams.

Key words: Aquatic hyphomycetes, clone libraries, diversity, OTU, SSU rDNA

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Introduction

Over 600 species of freshwater fungi (300 anamorphic fungi, 300 ascomycetes, a few chytrids and oomycetes) are known today (Wong *et al*., 1998; Vijaykrishna *et al*., 2006; Shearer *et al*., 2007). They play a pivotal role in the degradation of dead leaves and other plant detritus in lotic environments. Aquatic fungi secrete enzymes that degrade leaf polysaccharides and other polymers (Nilsson, 1964; Thornton, 1965; Suberkropp and Klug, 1980; Singh, 1982; Chandrashekar and Kaveriappa, 1988; Mansfield, 2005). These changes in substrate composition and the concurrent increase in fungal biomass "condition" plant detritus for invertebrate consumption by raising its nutritional value (Bärlocher, 1985; Suberkropp, 1992). On leaves, these functions are assumed to be performed primarily by aquatic hyphomycetes, a polyphyletic group of anamorphic fungi (Belliveau and Bärlocher, 2005). Their identification relies primarily on conidial shapes. The pressure to adapt to dispersal in flowing water has resulted in convergent evolution favouring two spore morphologies, tetraradiate or sigmoid. This can make identifications based on spore shapes ambiguous (Bärlocher, 2007). In addition, the presumed dominance of aquatic hyphomycetes is based on their prolific spore production and therefore at least in part based on circular reasoning. Mycelia may be actively involved in leaf decomposition without releasing the relatively large tetraradiate or sigmoid spores associated with aquatic hyphomycetes.

Molecular methods characterize nucleic acids that are present in all stages of the fungal

life cycle, and can circumvent some of the problems associated with microscopy-based techniques. In aquatic mycology, two methods have been applied successfully: terminal restriction fragment length polymorphism (T-RFLP) analysis and denaturing gradient gel electrophoresis (DGGE) (Nikolcheva *et al*., 2003, 2005). These two approaches allow estimates of fungal diversity independent of presence or absence of reproductive structures, but their ability to distinguish among major fungal groups is limited. As a refinement, Nikolcheva and Bärlocher (2004) designed primers specific for Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota (Kingdom Fungi) and Oomycota (Stramenopila), and investigated phylum-specific diversities and relative amounts of amplified sequences. Their study confirmed the dominance of Ascomycota (to which most aquatic hyphomycetes belong) but found substanital contributions by Basidiomycota, Chytrodiomycota and, to a lesser degree, by Oomycota and Zygomycota. Duong *et al*. (2006) have also extracted whole DNA from living leaves to reveal new endophyte phylotypes.

An alternative approach is based on the extraction of whole-community DNA, followed by amplification with fungal-specific primers and the establishment of ribosomal gene libraries (Vandenkoornhuyse *et al*., 2002; Schadt *et al*., 2003). Phylogenetic analyses of randomly selected cloned sequences allow estimating the frequencies of occurrence of various fungal groups based on their contribution to the community DNA pool. In theory, this approach allows much greater resolution and unequivocal assignment to a taxon. In both DGGE and T-RFLP different sequences may result in the same signal, whose homogeneity cannot be established without further analyses (e.g., by extracting and sequencing the DNA from an individual DGGE band). On the other hand, our knowledge of biases and errors in DNA extraction, followed by cloning and sequencing is incomplete. In addition, we lack sufficient data to confidently evaluate the equivalency of percentage sequence similarities and species, genera or even families.

Our objective was to apply this sequence-based analysis to the fungal community on leaves decaying in a stream. Maple, linden and oak leaves were exposed for 17 days. Diversity was then assessed through construction of clone libraries and complemented with traditional microscopic observation and documentation of aquatic hyphomycetes. We anticipated the whole-community DNA to be dominated by sequences with affinities to the Ascomycota, with smaller numbers belonging to Basidiomycota and Chytridiomycota.

Most aquatic hyphomycetes can occur on a wide variety of leaf species, but different substrates affect their diversity and the relative frequencies of occurrence of individual species (Gulis, 2001; Bärlocher and Graça, 2002; Nikolcheva and Bärlocher, 2005). Analyses of clone libraries may allow us to determine how leaf species influences fungal diversity at a more fundamental level, such as the relative contributions by various phyla or subphyla.

Materials and methods

Isolates and morphology

The field experiment was conducted in Boss Brook, a first order softwater stream in Fenwick, Nova Scotia, Canada (45°43.000′ N; 64°09.567′ W). The stream runs through a mixed forest dominated by white birch (*Betula papyrifera* Marsh), several species of maple (*Acer rubrum* L., *A. saccharum* Marsh, *A. spicatum* Lam) and white spruce [*Picea glauca* (Moench) Voss].

Freshly shed leaves of oak (*Quercus alba* L.), maple (*Acer rubrum* L.) and linden (*Tilia cordata* Mill.) were collected from three trees. The leaves were leached for 24 hours in running tap water, 2-cm disks were punched out with a cork borer and autoclaved.

Litterbags (12 cm × 12 cm) were prepared with nylon mesh (2 mm × 2 mm) to hold 15 leaf discs of a single type of leaf. Five bags of each tree species (total of 75 leaf discs) were submerged in Boss Brook on June 1, 2005 and retrieved after 17 days. Ten discs were chosen randomly from each bag and rinsed in distilled water to remove debris. Six of the ten discs were used for DNA extraction, and four were used for sporulation measurements.

Sporulation and morphological identification

Four leaf disks were placed in a 250 mL Erlenmeyer flask with 150 mL sterile distilled

water (5 replicate flasks per leaf species). To induce sporulation, the suspensions were aerated through a glass pipette (dark, 15°C). After 48 hours, the entire 150 mL was sucked through a 8 µm membrane filter. The spores retained on the filter were stained with lactophenol cotton blue (50 mg L^{-1}), counted and identified under a light microscope (Gulis *et al*., 2005). Percentage contributions of the dominant species were based on their frequencies of occurrence in the first 500 spores encountered. The remainders of the filters were scanned to detect rare species. The leaf disks were air-dried to constant weight to calculate the number of spores released per unit mass.

DNA isolation and amplification

DNA was extracted from freeze-dried discs of maple, linden and oak leaves with the Soil DNA Extraction kit (MoBio Laboratories, Solana Beach, CA; Bärlocher *et al*., 2008). It was amplified with the fungal specific primers AU2 and AU4, which flank the SSU region with (Vandenkoornhuyse *et al*., 2002). PCR was performed in a T Gradient Biometra thermocycler (Whatman) with Taq Ready-To-Go PCR Beads (Amersham Biosciences, Oakville, Canada), using the following protocol: (i) Initial denaturation 94°C, 2 min; (ii) Denaturation, 94°C, 30 sec; (iii) Annealing, 51°C, 30 sec; (iv) Extension, 70°C, 90 sec: (v) Repeat (ii)-(iv) for 35 cycles; (v) Final extension, 70°C, 5 min; (vi) Pause at 4°C until retrieved. The amplified products were cleaned (GFX™ PCR DNA and Gel Band Purification kit, Amersham Biosciences), and run on a agarose gel (1.3%) to ascertain the presence of the desired band.

Generation of SSU r DNA clone library and sequencing

A TOPO TA Cloning® Kit For Sequencing (Invitrogen) was used to clone the approximately 1330 bp SSU rDNA insert. Selective Luria-Bertani (LB) plates were prepared with 70 µg mL^{-1} Ampicillin (Sigma Aldrich) and spread with 70 µL of Ultra Pure X-GAL (20 mg mL^{-1} in DMF, Invitrogen). *E. coli* cells were thawed on ice, and transformed according to the One Shot® Chemical Transformation Protocol. Volumes of 15 µL, 30 µL and 50 µL were spread onto LB plates and incubated overnight at 37°C. Sterilized wooden toothpicks were used to transfer clones from a single white colony onto a labelled grid plate of LB media containing Ampicillin, and then into a 3 mL LB broth tube. Tubes of inoculated LB broth were shaken at overnight 37°C (12-14 hours, 35º angle, ~150 rpm) with caps loosened slightly to allow aeration of the *E. coli* cells. Plasmids were extracted with the Wizard® Plus SV MiniPreps DNA Purification System (Promega, Nepean, Canada). Plasmid DNA was stored at 4°C until a restriction digest could be performed to verify the presence of insert. Restriction digests were carried out on the plasmid extracts using ECOR1 (New England Biolabs, 20,000 units mL^{-1}, kept on ice at all times) and 10X "Unique buffer" (New England Biolabs). A 1.3% agarose gel was run at 90 V to verify the presence of inserts in the extracted plasmids (presence of a band between 1250 and 1500 bp on the 250-kb ladder (Invitrogen). The 1.5 mL Eppendorf tubes containing plasmid extract that tested positive for insert were sequenced. In total, digests from 151 individual clones were sequenced at the Genome Quebec Innovation Centre at McGill University (Montreal, Quebec) using the primers T3 and T7. The expected length of the insert was 1380 bp. Sequences were submitted to Genbank, with the following accession numbers: Linden1-Linden50: EU200001-EU200050; Maple1-Maple53: EU200051-EU200103; Oak1-Oak48: EU200104-EU200151.

Sequence analyses

The SSU rDNA gene sequences of clones were edited manually for consensus with Se-Al v2.0 (Sequence alignment editor; Rambaut, 1995). Discrepancies were resolved by verifying the chromatogram for both forward and reverse sequences. The sequences were aligned with ClustalX (Thompson *et al*., 1997) with 10.00 and 0.10 as gap opening and extension penalities, respectively, in pairwise and multiple alignments and the divergent sequences were delayed 25%. The aligned sequences

were edited to restrict the phylogenetic analysis to regions of nucleotides that were unambiguously alignable in all sequences using MacClade 3.07 (Maddison and Maddison, 1992). The sequences were submitted to a BLAST search at the NCBI sever (Altschul *et al*., 1997), using the search option "short, exact nucleotide matches". Pair-wise genetic distances between the aligned clone sequences were calculated using uncorrected 'p' and lower triangle parameter settings of PAUP*4.0b10 (Swofford, 2000). The sequences that differed by ≤ 1% were assigned to the same Operational Taxonomic Unit (OTU; Vandenkoornhuyse *et al*., 2002).

Diversity analyses

Estimated species richness based on spore and OTU counts was calculated with EstimateS (Colwell, 2005). Since the estimated CV for abundance distribution was > 0.5, we used the classic option to calculate the Chao1 indicator. It was consistently lower than ACE (abundance-based coverage indicator). We therefore report ACE and ICE (incidence-based coverage indicator) values, as recommended by Colwell (2005).

Choa's abundance-based Jaccard index and the Bray-Curtis index were calculated separately for spore and OTU data to estimate pair-wise similarities among the 3 substrates (Colwell, 2005).

Species accumulation curves based on OTUs were calculated with EcoSim 7 with 1000 iterations (Gotelli and Entsminger, 2004).

Results

Sporulation analyses

After 17 days in Boss Brook, maple, linden and oak leaf discs were recovered and aerated in the laboratory. The number of released conidia over 2 days was highest on linden (1520 ± 380 conidia mg^{-1} day^{-1}), followed by maple (390 ± 170 conidia mg^{-1} day^{-1}) and oak (130 ± 7 conidia mg^{-1} day^{-1}). Differences were significant for all pair-wise comparisons ($p < 0.001$) except between maple and oak ($p > 0.05$; ANOVA, followed by Tukey-Kramer's Multiple Comparisons Test).

In total, approx. 40,000, 152,000 and 13,000 conidia released from maple, linden and oak leaf disks, respectively, were scanned to evaluate diversity. Two species, *Anguillospora filiformis* and *Articulospora tetracladia*, together accounted for over 84% of all released conidia (Table 1).

Table 1. Percentage contributions of aquatic hyphomycete species to conidium production from maple oak and linden leaf disks exposed in Boss Brook for 17 days. + : contribution < 0.1%.

	Maple	Linden	Oak
Anguillospora filiformis	33.7	51.4	54.5
Anguillospora longissima	3.0	1.9	
Articulospora tetracladia	58.2	32.8	40.0
Clavariopsis aquatica	1.2	0.5	2.3
Culicidospora aquatica	2.0	0.2	
Clavatospora longibrachiata	0.4	10.4	0.5
Geniculospora inflata	0.2	0.9	0.9
Heliscella stellata		0.3	
Heliscus lugdunensis	+		
Lemonniera aquatica	+	+	
Lemonniera pseudofloscula	0.6	1.4	1.8
Mycocentrospora acerina	0.6	0.2	
Tetracladium marchalianum	+	+	+
Tricladium angulatum	+	+	

Molecular analyses

Table 2 lists the 151 DNA sequences recovered from the three leaf species. Three sequences originating from linden disks (Linden2, 41, 47) were ≥ 99 % similar to published sequences of two aquatic hyphomycete species, *Alatospora acuminata* and *Anguillospora rosea*. The majority of closest hits (75) were classified as uncultured fungi. The taxonomic distribution of closest hits for the three leaf species is summarized in Table 3. Since several counts were low (e.g, 0 for aquatic hyphomycetes on maple and oak), a χ^2 test was not appropriate, and we used a randomization test with the sum of squared deviations from expected values as test statistic (Bärlocher, 2005). Distribution of the taxonomic units among the leaf species differed significantly ($p = 0.007$).

Clone sequences that differed by ≤ 1% were assigned to the same Operational Taxonomic Unit (OTU; Vandenkoornhuyse *et al*., 2002). This resulted in 40 OTUs. Their distribution among the three leaf species is listed in Table 4. Of the 40 OTUs, 32 occurred only on one of the three substrates: 12 were restricted to maple, 9 to linden, and 11 to oak.

Table 2. List of the SSU rDNA sequences recovered from maple, linden and oak disks with closest BLAST hits, their accession numbers, and % similarity to cloned sequence. Genbank accession numbers of cloned sequences: linden1-linden50 = EU200001-EU200050; maple1-maple53 = EU200051- EU200103; oak1-oak48 = EU200104-EU200151.

Clone	closest BLAST hit	Accession No.	% Similarity
maple1	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple2	Chytridiales sp. JEL207	AF164261.1	96
maple3	*Eimeriidae* environmental sample clone Amb_18S_749	EF023410.1	99
maple4	Uncultured fungus clone CCW48	AY180024.1	94
maple5	Uncultured cercozoan clone	AY620339.1	100
maple6	Leucosporidium scottii isolate AFTOL-ID	AY707092.1	97
maple7	Uncultured cercozoan	AY620339.1	100
maple8	*Nuclearia* sp.	AY496008.1	99
maple9	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple10	Uncultured fungus	AM114816.1	100
maple11	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple12	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
maple13	Uncultured cercozoan clone	AY620339.1	100
maple14	*Nuclearia* sp.	AY496008.1	96
maple15	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple16	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple17	Uncultured fungus	AM114816.1	100
maple18	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple19	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple20	Uncultured fungal contaminant	EF053584.1	100
maple21	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	99
maple22	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple23	*Nowakowskiella* hemisphaerospora	AF164283.1	90
maple24	Uncultured fungal contaminant	EF053584.1	100
maple25	Uncultured eukaryote clone Zeuk2	AY916571.1	99
maple26	*Leucosporidium scottii* isolate AFTOL-ID 718	AY707092.1	96
maple27	Uncultured fungus clone B13	AF504784.1	96
maple28	Eimeriidae environmental sample clone Amb_18S_	EF023410.1	99
maple29	*Cercomonas* sp. HFCC89	DQ211598.1	100
maple30	*Cordierites sprucei*	AF292089.1	99
maple31	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple32	*Camptobasidium hydrophilum*	U75449.1	100
maple33	*Cercomonas* sp. HFCC89	DQ211598.1	100
maple34	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
maple35	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple36	Uncultured cercozoan clone	AY620339.1	99
maple37	Uncultured fungal contaminant	EF053584.1	99
maple38	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
maple39	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
maple40	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple41	*Nowakowskiella* sp. JEL127 isolate AFTOL	AY635835.1	97
maple42	Uncultured fungus	AM114816.1	100

Table 2 (continued). List of the SSU rDNA sequences recovered from maple, linden and oak disks with closest BLAST hits, their accession numbers, and % similarity to cloned sequence. Genbank accession numbers of cloned sequences: linden1-linden50 = EU200001-EU200050; maple1- maple53 = EU200051- EU200103; oak1-oak48 = EU200104-EU200151.

Clone	closest BLAST hit	Accession No.	% Similarity
maple43	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
maple44	*Camptobasidium hydrophilum*	U75449.1	100
maple45	Uncultured fungus	AM114816.1	99
maple46	Uncultured eukaryote clone IAFDv110	AY835696.2	97
maple47	*Sporobolomyces roseus* AFTOL-ID 1549	DQ832235.1	100
maple48	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
maple49	*Nuclearia* sp.	AY496008.1	96
maple50	Uncultured fungus	AM114816.1	99
maple51	Uncultured fungus	AM114816.1	100
maple52	Uncultured fungal contaminant	EF053584.1	99
maple53	Uncultured fungus clone PFB1AU2004	DQ244005.1	99
linden1	Uncultured fungus	AM114816.1	100
linden2	*Alatospora acuminata* strain 102-280	AY357261.1	100
linden3	Uncultured fungus	AM114816.1	100
linden4	Uncultured fungus	AM114816.1	100
linden5	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
linden6	Uncultured cercozoan clone	AY620339.1	100
linden7	*Mollisia cinerea* isolate AFTOL-ID 76	DQ470990.1	98
linden8	Uncultured fungal contaminant	EF053584.1	100
linden9	*Athalamea* environmental sample clone Amb_18S_1145	EF023543.1	88
linden10	Eimeriidae environmental sample clone Amb_18S_	EF023410.1	99
linden11	Eimeriidae environmental sample clone Amb_18S_749	EF023410.1	100
linden12	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
linden13	Eimeriidae environmental sample clone Amb_18S_749	EF023410.1	90
linden14	Uncultured soil fungus clone FChW6m75 18S	DQ994544.1	97
linden15	Uncultured fungus	AM114816.1	99
linden16	*Rhizosphaera pini* isolate rhpisr	EF114733.1	100
linden17	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94
linden18	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94
linden19	Uncultured fungus	AM114816.1	100
linden20	*Filobasidium capsuligenum*	AB075544.1	98
linden21	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
linden22	*Mollisia cinerea*	DQ470990.1	99
linden23	*Nuclearia* sp.	AY496008.1	96
linden24	Nowakowskiella sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94
linden25	*Ascozonus woolhopensis*	AF010590.1	100
linden26	Uncultured cercozoan clone	AY620339.1	100
linden27	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94
linden28	*Mollisia cinerea*	DQ470990.1	99
linden29	*Cordierites sprucei*	AF292089.1	99
linden30	*Cordierites sprucei*	AF292089.1	99
linden31	Chytridiales sp. JEL187 isolate AFTOL-ID 39	AY635825.1	97
linden32	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94
linden33	Uncultured fungus	AM114816.1	100

Table 2 (continued). List of the SSU rDNA sequences recovered from maple, linden and oak disks with closest BLAST hits, their accession numbers, and % similarity to cloned sequence. Genbank accession numbers of cloned sequences: linden1-linden50 = EU200001-EU200050; maple1- maple53 = EU200051- EU200103; oak1-oak48 = EU200104-EU200151.

Clone	closest BLAST hit	Accession No.	% Similarity
linden34	Uncultured eukaryote clone 1871	AY494502.1	97
linden35	*Cordierites sprucei*	AF292089.1	99
linden36	Uncultured cercozoan clone	AY620339.1	100
linden37	Uncultured fungal contaminant	EF053584.1	99
linden38	Uncultured fungal contaminant	EF053584.1	99
linden39	Uncultured fungal contaminant	EF053584.1	100
linden40	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
linden41	*Alatospora acuminata* strain 102-280	AY357261.1	99
linden42	*Cordierites sprucei*	AF292089.1	99
linden43	Uncultured fungus	AM114816.1	99
linden44	Uncultured ascomycete isolate dfmo4345.073	AY969282.1	97
linden45	Uncultured fungal contaminant	EF053584.1	100
linden46	Uncultured fungal contaminant	EF053584.1	99
linden47	*Anguillospora rosea* strain CCM F-08983	AY357265.1	100
linden48	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	93
linden49	Uncultured fungus	AM114816.1	100
linden50	Eimeriidae environmental sample clone Amb_18S_749	EF023410.1	90
oak1	*Helicogloea* sp. TUB FO42773	DQ198793.1	93
oak 2	*Cordierites sprucei*	AF292089.1	99
oak 3	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
oak 4	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	97
oak 5	Uncultured fungal contaminant	EF053584.1	100
oak 6	*Colacogloea peniophorae* isolate AFTOL-ID 709	DQ234564.1	90
oak 7	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	88
oak 8	Uncultured fungal contaminant	EF053584.1	100
oak 9	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
oak 10	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
oak 11	Uncultured fungal contaminant	EF053584.1	99
oak 12	Uncultured fungal contaminant	EF053584.1	99
oak 13	Uncultured eukaryote clone 1871	AY494502.1	97
oak 14	*Filobasidium capsuligenum*	AB075544.1	97
oak 15	Uncultured fungus	AM114816.1	100
oak 16	Uncultured fungus	AM114816.1	100
oak 17	Uncultured fungus	AM114816.1	100
oak 18	Uncultured fungus	AM114816.1	100
oak 19	Uncultured Closteriaceae clone Amb_18S_970	EF023700.1	95
oak 20	*Cordierites sprucei*	AF292089.1	99
oak 21	Uncultured fungal contaminant	EF053584.1	99
oak22	*Mollisia cinerea*	DQ470990.1	99
oake23	Uncultured fungal contaminant	EF053584.1	99
oak24	Uncultured eukaryote clone D4P08E02	EF100330.1	94
oak 25	*Nowakowskiella* sp. JEL127 isolate AFTOL-ID 146	AY635835.1	94

Table 2 (continued). List of the SSU rDNA sequences recovered from maple, linden and oak disks with closest BLAST hits, their accession numbers, and % similarity to cloned sequence. Genbank accession numbers of cloned sequences: linden1-linden50 = EU200001-EU200050; maple1- maple53 = EU200051- EU200103; oak1-oak48 = EU200104-EU200151.

Clone	closest BLAST hit	Accession No.	% Similarity
oak 26	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
oak 27	*Cordierites sprucei*	AF292089.1	99
oak 28	Uncultured fungal contaminant	EF053584.1	99
oak 29	*Mollisia cinerea* isolate AFTOL-ID 76	DQ470990.1	99
oak 30	Uncultured fungal contaminant	EF053584.1	99
oak 31	Uncultured fungus	AM114816.1	99
oak 32	Uncultured fungal contaminant	EF053584.1	100
oak 33	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
oak 34	Fungal endophyte isolate 3395	DQ979481.1	99
oak 35	Uncultured fungal contaminant	EF053584.1	98
oak 36	Uncultured soil fungus clone FChW6m75	DQ994544.1	99
oak 37	Uncultured fungal contaminant	EF053584.1	97
oak 38	Uncultured soil fungus clone FChW6m75	DQ994544.1	100
oak 39	Uncultured fungus	AM114816.1	99
oak 40	Uncultured fungus	AM114816.1	100
oak 41	*Mollisia cinerea* isolate AFTOL-ID 76	DQ470990.1	97
oak 42	Uncultured fungus	AM114816.1	100
oak 43	Uncultured Tremellaceae clone Amb_18S_1097	EF023503.1	100
oak 44	Uncultured fungus	AM114816.1	99
oak 45	*Cordierites sprucei*	AF292089.1	99
oak 46	*Cordierites sprucei*	AF292089.1	99
oak 47	Uncultured fungus	AM114816.1	100
oak 48	Uncultured marine eukaryote clone M1_18C07	DQ103822.1	87

Table 3. Taxonomic distribution of closest hits to cloned SSU rDNA sequences from maple, linden and oak disks.

Taxonomic group	Maple	Linden	Oak
Aquatic hyphomycetes	0	3	0
Uncultured Fungi	26	20	29
Ascomycetes	1	10	8
Basidiomycetes	5	1	4
Chytridiomycetes	8	8	3
Other Eukarya	13	8	4

Diversity estimates

Species and OTU accumulation curves for the three substrates, calculated with Ecosim (Gotelli and Entsminger, 2004), are shown in Fig. 1. The number of distinct taxa increased more rapidly with sample size when sequences rather than spores were evaluated.

Estimated species or OTU richness was calculated with EstimateS (Colwell, 2005). Values for ACE (abundance-based coverage indicator) and ICE (incidence-based coverage indicator) are listed in Table 5. Without exception, numbers of taxa were considerably higher when based on sequences rather than on conidia.

Pairwise comparisons of spore and OTU communities are listed in Table 6. Chao's Jaccard abundance-based estimator gave consistently higher similarity values than the complement to the classical Bray-Curtis dissimilarity index, and both gave higher values for spore data.

Discussion

Conidium production after 17 days of stream exposure varied with leaf species, and ranged from 128 conidia mg^{-1} d^{-1} on oak to 1519 conidia mg^{-1} d^{-1} on linden. These numbers

Table 4. List of SSU rDNA clones that differed by ≤ 1% and therefore assigned to the same operational taxonomic unit (OTU).

OUT	Maple	Linden	Oak
1	1, 9, 11, 15, 16, 18-20, 22, 24, 30, 31, 35, 39, 40, 43	5, 8, 12, 21, 25, 29, 30, 35, 39, 42	2, 3, 5, 8-12, 20, 24, 26, 27, 33, 36, 38, 45, 46
2	10, 17, 37, 42, 45, 50-52	1-4, 15, 19, 33, 37, 38, 41, 43, 45, 46, 49	15-18, 21, 23, 28, 30-32, 39, 40, 42, 47
3	21	17, 18, 24, 27, 32, 48	25
4	12, 23, 34, 38, 41, 48	40	4
5	5, 7, 13, 36	6, 26, 36	
6	3, 28	10,11	
7		7, 22, 28	22, 29, 34
8		20	14
9	32, 44		
10	29, 33		
11	6, 26		
12	2		
13	4		
14	8, 14		
15	25		
16	27		
17	46		
18	47		
19	49		
20	53		
21		13, 50	
22		9	
23		14	
24		16	
25		23	
26		31	
27		34	
28		44	
29		47	
30			1
31			6
32			7
33			13
34			19
35			35
36			37
37			41
38			43
39			44
40			48

fall within the range of earlier studies in the same stream (e.g., Maharning and Bärlocher, 1996; Nikolcheva and Bärlocher, 2005), and are at the lower end of values reported in the literature (Gessner, 1997). Similarly, the number of species identified from spores was relatively low, but not unprecedented at this location. The timing (stream exposure in June) was not ideal to maximize colonization by aquatic hyphomycetes. When comparing fungal community composition on the three leaf species, the choice of a similarity indicator was crucial. Chao's abundance-based Jaccard index is "based on the probability that two randomly chosen individuals, one from each of two samples, both belong to species shared by both samples (not necessarily to the same shared species)" (Colwell, 2005). It takes into account the contribution made by species present at both sites but not detected in one or both

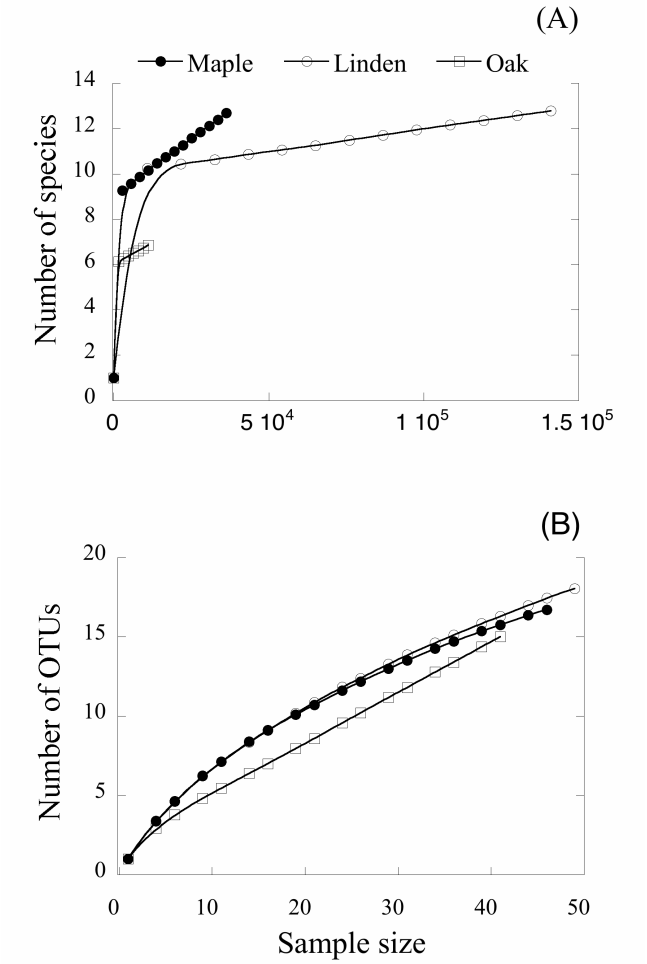

Fig. 1. Species accumulation curves: **(A).** based on spore identifications; **(B).** based on OTUs of sequenced clones.

Table 5. Species richness indicators ACE and ICE based on spore and OTU data from maple, linden and oak disks.

	Spores			OTU		
	Maple	**Linden**	**Oak**	**Maple**	**Linden**	**Oak**
ACE	11.6	15.9	15.8	69	66	114
ICE	41.4	18.6	15.9	113	159	174

Table 6. Pairwise similarity comparisons of spore and OTU communities from maple (M), linden (L) and oak (O) disks. Chao: Chao's Jaccard abundance based estimator of similarity; 1-(BC): 1-Bray-Curtis dissimilarity index.

	Spores		OTU	
	Chao	**(1-BC)**	**Chao**	**(1-BC)**
ML	0.99	0.60	0.62	0.51
MO	0.94	0.51	0.55	0.49
LO	0.97	0.84	0.77	0.39

samples, and reduces the negative bias in traditional similarity indices. The consistently high values (≥ 94% in pairwise comparisons, Table 6) suggest very similar aquatic hyphomycete communities on the three leaves.

Two species, *Anguillospora filiformis* and *Articulospora tetracladia*, together accounted for ≥ 84 % of all released spores (Table 1). Comparable or even greater dominance by conidia belonging to one or two species is common. For example, in samples taken a year later from the same stream, *Anguillospora filiformis* contributed 89% of all conidia, followed by *Articulospora tetracladia* with 8% (Kearns and Bärlocher, 2008). Not surprisingly, documenting additional, rare species, requires a considerable effort. This is reflected in the species accumulation curve (Fig. 1A), where the discovery of an additional species may require scanning as many as 100,000 additional conidia.

Diversity measures relying exclusively on conidia ignore aquatic hyphomycete mycelia in a non-reproductive state and other fungi that do not release easily identifiable conidia when submerged (some predominantly terrestrial taxa, such as *Fusarium* or *Cylindrocarpon*, do sporulate under water). The presence of some of these taxa was documented by incubating stream-exposed leaves on a range of media (Bärlocher and Kendrick, 1974). More recently, Nikolcheva and Bärlocher (2004) used PCR with phylum-specific primers, followed by analysis of the amplified sequences by DGGE. Taxa with close affinities to Ascomycota dominated, but there was a consistent presence of taxa assigned to Basidiomycota (with combined biomass of up to 13, as estimated by gel band intensities) and to Chytridiomycota (estimated biomass of up to 21%), with lower numbers assigned to Zygomycota and Oomycota.

Here, we evaluated diversity with a DNA sequence-based method, i.e., whole-community DNA was extracted and ribosomal gene libraries were constructed (Vandenkoornhuyse *et al*., 2002; Schadt *et al*., 2003). If an informative sequence is chosen, this approach allows identification at the genus or even species level. In the barcoding initiative, part of the conserved mitochondrial gene COI has successfully been used to discriminate between animal species (Hebert *et al.*, 2003; 2004). In

true Fungi, this sequence seems less suitable because the regions used for PCR priming evolve rapidly, which is a fatal flaw when investigating fungal diversity in the environment – different CO1 primers might be required for each genus. At a recent workshop on fungal barcoding, the recommendation was made that the entire ITS region should serve as the DNA barcoding locus for the true Fungi (Rossman, 2007). The sequence chosen here, SSU rRNA, is less suitable for discriminating among individual species; it typically aims at resolving differences at higher than genus level (Vandenkoornhuyse *et al*., 2002; Anderson and Cairney, 2004). Considering this relatively coarse resolution, the apparent diversity is even more astounding, but comparable to an earlier study in the hyporheic habitat of a stream with the same primers (Bärlocher *et al*., 2008). Again with the same primers, Vandenkoornhuyse *et al*. (2002) documented 49 distinct sequences from within the roots of a single plant species at a single location. Only 7 of their phylotypes were closely similar to known sequences. In the present study, between 40 and 60% of the phylotypes showed highest similarity to various uncultured fungi (Table 3). A majority of these probably belong to the Ascomycota (see Stoeck and Epstein, 2003; Moon-van der Staay *et al*., 2006; Hofstetter *et al*., 2007). In broad terms, this study confirms the conclusion by Nikolcheva and Bärlocher (2004): during leaf decomposition in streams, representatives of the Ascomycota are most common, followed by taxa with close affinities to Basidiomycota and Chytridiomycota. We did not, however, find any sequences related to Oomycota or Zygomycota. It would be interesting to apply this method to assess the biodiversity of fungi on submerged decaying wood. The present methodology relies on detecting fruting bodies that appear on the wood following incubation (see Pinnoi *et al*., 2006; Vijaykrishna and Hyde, 2006; Pinruan *et al*., 2007), which again discriminates against metabolically active or dormant but nonreproductive stages. We predict that the cloning method would also show a higher diversity of organisms in submerged decaying wood.

The estimated total OTU numbers on the three substrates varies are high (66-174; Table 5), especially in view of the fact that they are based on one sample date after 17 days of stream immersion. They exceed earlier estimates by DGGE and T-RLFP, also on leaves decaying in streams (Nikolcheva and Bärlocher, 2004, 2005), and raise the issue of potential overestimates. Two main sources of PCR-based errors are recognized: 1) PCR biases and artifacts, and 2) data interpretation (Bidartando and Gardes, 2005; Wintzingerode *et al*., 1997). Potential biases include preferential extraction and amplification of DNA from diverse communities (Anderson *et al*., 2003; Anderson and Cairney, 2004). The primers used in this study were designed to be fungus-specific (Vandenkoornhuyse *et al*., 2002), but 16.5% of the cloned sequences showed highest affinity to other eukaryotic taxa. Further progress is currently hampered by the relative scarcity of fungal and related eukaryotic sequences in databases.

PCR-produced heteroduplexes and chimeras may inflate diversity estimates. In a mixture of four proteobacteria, they accounted for between 9 and 28% of generated 16S rDNA sequences (Qiu *et al*., 2001). We are not aware of comparable controlled studies with fungal communities from streams or soils, where a known mixture of different sources of fungal DNA was analyzed with a PCR-based cloning approach. Here, we followed the protocol by Vandenkoornhuyse *et al.* (2002), designed to minimize such artifacts. Furthermore, 76% of our unknown fungal sequences showed close similarities to those reported by three other groups (Stoeck and Epstein, 2003; Moon-van der Staay *et al*., 2006; Hofstetter *et al*., 2007), who amplified DNA from other habitats using different primers. It seems unlikely that nearly identical artifacts would occur repeatedly under these conditions.

Estimating taxon richness based on a limited sample makes certain assumptions concerning community composition (Colwell, 2005). Again, these are best tested by comparing actual richness with sampling effort (e.g., Gönczöl *et al*., 2001).

Despite these uncertainties, our study confirms an earlier study that fungal diversity on leaves decomposing in streams extends beyond aquatic hyphomyetes (Nikolcheva and

Bärlocher, 2004), and that it may greatly exceed estimates based on cultureable colonies or on identification of released spores. Molecular methods, combined with computational improvements, have also resulted in much greater estimates of bacterial diversity. Gans *et al*. (2005) claim that 1 g of soil may contain as many as 1 mio distinct species. While the equivalency between genome and species is controversial and other studies have reported lower numbers, there is no doubt that bacterial diversity is much greater than estimated by conventional studies (Dykhuizen, 1998). Our data suggest that the same applies to fungi on leaves decaying in streams. This conclusion may even be extended to samples designed to collect conidia (e.g., Gönczöl and Révay, 2006): a comparison of DGGE and visual inspection of conidia revealed greater diversity of phylotypes (DGGE) than conventionally identified species (Raviraja *et al*., 2005).

The traditional assumption for microorganisms has been that "*everything is everywhere*, but, *the environment selects*" (formulated by Baas Becking, but often falsely attributed to Martinus Beijerinck; de Wit and Bouvier, 2006). This implies that global diversity is essentially identical with local diversity, and that there is no true microbial biogeography. Recent studies indicate that dispersal ability is probably not high for all bacteria, and while overall diversity in a given type of habitat may be similar independent of geography, the genotypes contributing to these communities may vary among locations (Dolan, 2006; Pommier *et al*., 2005; Ramette and Tiedje, 2007). There is no doubt that some microfungi have a true biogeography (Taylor *et al*., 2006). For example, multilocus sequence data of 73 strains of the morphospecies *Neurospora discreta* revealed at least 8 separate phylogenetic species (Dettman *et al*., 2006).

Documenting the presence of a fungal taxon, whether by traditional or molecular methods, does not by itself prove its participation in ecological processes. This would require, for example, analyzing the pool of mRNA, which allows differentiating members of the community that are active from those that are present but dormant (Anderson and Cairney, 2004; Bärlocher, 2007). Nevertheless, this newly appreciated taxonomic diversity, even if based on locally distinct assemblages, may also incorporate more redundancies of ecological functions than generally assumed. This has important implications for extrapolating results from microbial diversity/function studies in the laboratory (Bärlocher and Corkum, 2003; Dang *et al*., 2005, 2006) to conditions in the field.

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