Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream

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Traditional techniques for studying the fungal community composition in streams favour the detection and identification of aquatic hyphomycetes. Our objective was to use molecular techniques to determine the presence and contributions of other fungal groups. We designed primers specific for the ITS regions in Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Oomycota. The primers were used to amplify DNA from linden, maple, and beech leaves, and birch wood submerged in a stream for 4 weeks in summer, autumn, winter and spring. The amplification products were separated by denaturing gradient gel electrophoresis. Ascomycota were present in large phylotype numbers (up to 21) on all substrates and all dates and represented ≥ 75 % of the fungal biomass. Basidiomycota were the second most abundant group in summer and autumn (up to 13 % on wood) and were absent only on linden and maple in spring. There were consistently large numbers of phylotypes from Chytridiomycota and their relative contribution to the microbial community peaked in winter on all substrates. Oomycota were present in summer and abundant only on wood. Zygomycota were present in low numbers and their estimated contribution to fungal biomass was ≤ 1%. Using primers to target individual groups facilitates a more balanced approach to studying fungal diversity in freshwater ecosystems.

Leaves and woody debris derived from the riparian vegetation represent major energy sources in lotic ecosystems (ALLAN 1995). Bacterial biomass and productivity are low during the initial phases of decomposition, which are dominated by fungi (SUBERKROPP & KLUG 1976; SUBERKROPP & WEYERS 1996). Estimates of fungal biomass and production have been based on measuring ergosterol (GESSNER, BÄRLOCHER & CHAUVET 2003), a membrane-bound molecule largely restricted to the higher or true Fungi (Kingdom Fungi; ALEXOPOULOS, MIMS & BLACKWELL 1996). Chytridiomycota (Fungi), Oomycota (Stramenopila) and other fungus-like organisms do not contain ergosterol; their potential biomass and production are therefore not measured by this technique.

To identify members of the fungal community on decaying leaves, newly collected substrates have been screened under the microscope for reproductive structures. A large proportion of the fungal biomass on the leaf, however, consists of fungal hyphae that cannot be identified by direct observation. Indirect methods to study the fungal community rely on inducing reproduction, generally by submersing the substrate in water and increasing turbulence by aeration or shaking (for review, see GESSNER, BÄRLOCHER & CHAUVET 2003). This technique stimulates heavy sporulation by aquatic hyphomycetes (though a few primarily terrestrial genera, such as *Fusarium* or *Cylindrocarpon*, also sporulate under water). Aquatic hyphomycetes (Ingoldian fungi) are classified on the basis of asexually produced propagules (conidia), which are typically tetraradiate or sigmoid, both clearly adaptations to dispersal in running water (WEBSTER 1959; WEBSTER & DAVEY 1984). Thus far, more than 300 species have been described. Only 10 % of these anamorph species have been connected to teleomorphs (sexual states). Most of them belong to the phylum Ascomycota, and about 10 % to the Basidiomycota (WEBSTER 1992).

Even though aquatic hyphomycetes are thought to be the dominant decomposers of decaying vascular plant detritus in streams, other fungal taxa have been recorded (fungal or fungi is used here in a very broad sense and includes all major groups that have traditionally been studied by mycologists; ALEXOPOULOS, MIMS & BLACKWELL 1996). Terrestrial anamorphs are ubiquitous on senescent and newly immersed leaves (KAUSHIK & HYNES 1971; BÄRLOCHER & KENDRICK 1974). Ascomata commonly develop on woody debris incubated in damp chambers (GOH & HYDE 1996; SHEARER 1992, 1993). Oomycota and Zygomycota have been observed in the early stages of leaf (BÄRLOCHER & KENDRICK 1974) and wood decay (SHEARER & VON BODMAN 1983), and sporangia of Chytridiomycota occasionally appear on leaf-agar plates incubated with decaying leaves at close to 0 °C (Bärlocher, pers. comm.). However, the frequency of occurrence, seasonality, and relative

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abundances of these taxa during leaf decomposition in streams are essentially unknown.

We have recently applied molecular techniques to reassess fungal diversity and identities during the early stages of plant litter decay in streams (NIKOLCHEVA, COCKSHUTT & BÄRLOCHER 2003). Polymerase chain reactions (PCR) with fungal-specific primers, followed by separation of phylogenetically distinct fungal strains (phylotypes) by denaturing gradient gel electrophoresis (DGGE), have significantly modified our interpretation of potential contributions of aquatic and terrestrial anamorphs. Our objectives in the current study were to design and test PCR primers that target specific fungal groups but exclude all other organisms. These primers were used to amplify DNA extracted from leaves and wood incubated for four weeks in a stream on four separate occasions. Our primary goal was to determine seasonal variations in the presence of five groups: Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota (Kingdom Fungi) and Oomycota (Kingdom Stramenopila). Our secondary objectives were to estimate the relative contributions of these five groups to total fungal biomass, and the number of genetically different strains in each group. We expected the fungal community to be dominated by Ascomycota in all seasons (most aquatic and terrestrial anamorphs belong to the Ascomycota), but particularly in autumn, when leaf fall results in a huge increase in the number of aquatic hyphomycete conidia carried in the water column of temperate streams (BÄRLOCHER 1992a). We also expected a lower but consistent presence of Basidiomycota. Various Oomycota are early transient colonizers of dead plant material; this suggests that the state of decay may be more important than season for the occurrence of Oomycota (BÄRLOCHER 1992b). The abundance of Mucorales (Zygomycota) often appears to be directly related to the amount and frequency of sewage effluents (COOKE 1976), while the number of Chytridiales (Chytridiomycota) species may peak in winter (WATERHOUSE 1942; BÄRLOCHER 1992b).

Material and methods

Study site

The field experiment was conducted in Boss Brook, a first order stream in Fenwick, Nova Scotia, Canada (45°43.000' N; 064° 09.567' W; BÄRLOCHER 1987). This stream runs through a mixed forest dominated by white birch (*Betula papyrifera* Marsh.), several maple species (*Acer rubrum* L., *A. saccharum* Marsh., *A. spicatum* Lam.) and white spruce (*Picea glauca* (Moench) Voss). The stream bed consists of stones and gravel. At the sampling site, the stream was 2–3 m wide and 20–50 cm deep.

Sample preparation

Leaves from red maple (*Acer rubrum)*, linden (*Tilia cordata* Mill.) and beech (*Fagus sylvatica* L.) were collected from single trees on the university campus in the fall of 2001. They were leached in tap water for 2 hours, cut into 18-mm diameter discs with a corkborer and dried at room temperature. Wooden popsicle sticks (*Betula papyrifera*) were cut into 1 x 6 cm sections. Sets of 10 preweighed leaf discs or 4 preweighed wood sticks were placed in fiberglass mesh bags (20 x 15 cm, 2 mm mesh size), and submerged in the stream for four weeks in the summer (introduced on July 22, 2002), autumn (October 11, 2002), winter (January 6, 2003) and spring (April 7, 2003). After recovery, the substrates were aseptically transported to the laboratory, where they were rinsed to remove any adherent soil material and freeze-dried. The samples were stored at –80 °C.

DNA sources

Cultures of *Anguillospora filiformis* Greathead (CCM F-20687), *Anguillospora longissima* (Sacc. et Syd.) Ingold (CCM F-00980), *Articulospora tetracladia* Ingold (CCM F-12499), *Clavariopsis aquatica* De Wild. (CCM F-10791), and *Flagellospora curvula* Ingold (CCM F-20799) were provided by L. Marvanová from the Czech Collection of Microorganisms, Brno, Czech Republic. A culture of *Heliscus lugdunensis* Sacc. et Thérry (CS-950) was provided by G. Krauss from UFZ – Centre for Environmental Research, Halle, Germany. A culture of *Tetrachaetum elegans* Ingold (30-426) was provided by Dr. E. Chauvet from CNRS – Université Paul Sabatier, Toulouse, France. *Aspergillus niger* van Tiegh. (85 W 4100), *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 (85 W 0400), *Chlamydomonas reinhardtii* Dangeard (86 W 0102), and *Rhizopus stolonifer* (Ehrenb) Vuill. (85 W 4900) were obtained from Ward's Supply Company (St. Catharines, ON, Canada). *Allomyces arbuscula* Butler (DN 15-5910), *Hyphochytridium catenoides* Karling (DN 25-6055), and *Pythium irregulare* Buisman (DN 15-6214) were obtained from Carolina Biological Supplies (Burlington, NC). *Thalassiosira pseudonana* Hasle et Heindal CCMP 1007 was obtained from the Centre for Culture of Marine Phytoplankton (West Woothbay Harbour, ME). *Agaricus bisporus* (Lange) Pilát was obtained from a local grocery store; DNA was extracted as described below.

DNA extraction

A small agar plug (3 mm x 3mm) from the agar cultures was used for DNA extraction. DNA extraction from submerged leaves (approx. 25 mg) or pure cultures (approx. 20 mg) was performed with MoBio UltraClean Soil DNA extraction kit (MoBio, Solana Beach, California) according to manufacturer's instructions. It is based on homogenizing the material with glass beads; the extractant contains a PCR inhibitor removal solution.

Primer design

Our goal was to design primers that differed between but not within the target group and enclosed a sequence sufficiently

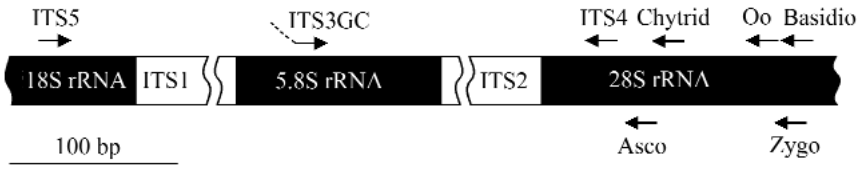

Fig. 1: Diagram of the binding sites of primers ITS4Asco (Ascomycete-specific), ITS4Basidio (Basidiomycete-specific), ITS4Chytridio (Chytridiomycete-specific), ITS4Oo (Oomycete-specific), ITS4Zygo (Zygomycete-specific) on the 28S rRNA gene. The binding sites of ITS4 and ITS5 (White *et al*. 1990) are also shown. The ITS region is not drawn to scale

variable to allow species-specific differentiation by DGGE or similar techniques (NIKOLCHEVA 2003; NIKOLCHEVA, COCKSHUTT & BÄRLOCHER 2003). We chose the region extending from within 18S rDNA to 28S rDNA (WHITE et al. 1990; Fig. 1). Primers specific to Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota (Kingdom Fungi) and Oomycota (Kingdom Stramenopila) were designed. Sequences from the 5' end of the nuclear large subunit of the ribosome (28S rDNA) of each the targeted groups were downloaded from GenBank (National Center for Biotechnology Information). Origins and accession numbers of the sequences are listed in Table 1. In the case of Ascomycota and Basidiomycota, sequences from representative species of all available orders were obtained. Large subunit nuclear rDNA sequences from bacteria, vascular plants, algae (including diatoms) and animals were used as negative controls. The sequences were aligned with Multalin (CORPET 1988). The binding site of primer ITS4 (WHITE et al. 1990) was located on the aligned sequences and all designed primers were positioned downstream from ITS4 on the 28S rRNA gene (Fig. 1). The sequences were scanned for regions that were conserved among all members of a targeted group (e.g. Basidiomycota), but different in all other groups (Ascomycota, Zygomycota, Chytridiomycota, Oomycota, algae (including diatoms), bacteria, vascular plants and animals). The primers had the following sequences:

- ITS4Asco (Ascomycota-specific):
  5' CGTTACTRRGGCAATCCCTGTTG3';
- ITS4Basidio (Basidiomycota-specific):
  5' GCRCGGAARACGCTTCTC3';
- ITS4Chytrid (Chytridiomycota-specific):
  5' TTTTCCCGTTTCATTCGCCA 3';
- ITS4Oo (Oomycota-specific):
  5' ATAGACTACAATTCGCC 3';
- ITS4Zygo (Zygomycota-specific):
  5' AAAACGTWTCTTCAAA 3'.

They were used in combination with the forward primer ITS5 (WHITE et al. 1990; Fig. 1).

Primer optimization

The primers were synthesized by MWG-Biotech. Amplification reactions were performed with Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) with 10 pmol of forward primer (ITS5), 10 pmol of reverse primer (one of the newly designed primers) and 1 µl of DNA template. Initially, the annealing temperature of each primer pair was optimized by running amplification reactions at 10 °C below to 5 °C above the calculated annealing temperature of the new primer at 1 °C increments. The primer specificity to the template was tested with DNA isolated from Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota, Oomycota, algae (including diatoms), vascular plants and bacteria in separate amplification reactions at the optimal annealing temperature. The Mg^{2+} concentration provided in the Ready-To-Go PCR beads did not require optimization. The amplification products were stained with 100X GelStar and visualized on a 1% agarose gel in 0.5 X TBE (Tris-borate-EDTA).

Amplification and DGGE

Amplification was performed as described above, at the optimal annealing temperature of each primer pair, with 1 µl of DNA template isolated from the decaying leaves. The amplification products were visualized on a 1 % agarose gel. All successful amplification products were used as templates (1 µl) for PCR with the primers ITS4 and ITS3GC (modified with a 50 base GC tail, MUYZER, DE WAAL & UITTERLINDEN 1993), suitable for separation by DGGE. These two primers amplify a subsequence within the original amplification product. The new amplification products were separated using the DCode mutation detection system (BioRad). Samples (10 µl) were loaded on a 8 % (w/v) polyacrylamide gels in 1X TAE with denaturing gradient from 30 to 80 % (100 % denaturant corresponds to 40 % formamide and 7 M urea). The gels were run in 1X TAE at 60 V for 15 hours and stained with 1X GelStar (BioWhittaker Molecular Applications, Rockland, Maine). The gel images were captured under UV light with a Sony digital videocamera and analyzed with NIH Image (National Institutes of Health, Bethesda, Maryland). The amplification products with the specific primers from a substrate/date combination were run next to the amplification product with ITS4 and ITS3GC. The migration distances of bands in all lanes were compared and the members of Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota, and Oomycota were

Tab. 1: Details of sequences used for primer design. In the GenBank database, *Saagaromyces* is misspelled as *Sagaaromyces*. *Apiotrichum porosum* is also listed as *Trichosporon porosum* in GenBank (same accession number)

Phylum	Genus/Species	GenBank no.
Ascomycota	*Acremonium strictum*	AY138482
	Acremonium strictum	AY138485
	Aureobasidium sp.	AY167611
	Bartalinia laurina	AF382369
	Beauveria bassiana	AF280637
	Botryosphaeria ribis	AY004336
	Candida sp.	AF389527
	Ceratocystis moniliformis	AF275499
	Chaetosphaeria fusiformis	AF178554
	Clavispora intechensis	AF538871
	Cordyceps subsessilis	AF373285
	Corollospora filiformis	AF491256
	Curvularia cymbopogonis	AF163996
	Curvularia oryzae	AF163991
	Discula destructiva	AF277136
	Fusarium falciforme	AY097326
	Fusarium lichenicola	AY097321
	Fusarium solani	AY097316
	Geotrichum sp.	AY225313
	Glomerella cingulata	AF543786
	Hypocrea jecorina	AF510497
	Hypoxylon fragiforme	AY083829
	Lignicola tropica	AF539474
	Lignincola longirostris	AF534473
	Mycosphaerella cryptica	AF309585
	Nectria ventricosa	AF228361
	Ophiostoma montium	AY194948
	Penicillium boreae	AF481122
	Penicillium crustosum	AF484409
	Peziza sp.	AF335171
	Phialocephala compacta	AF326083
	Phoma herbarum	AY293790
	Pichia farinosa	AF335974
	Pichia sydowiorum	AJ508573
	Polycephalomyces ramosus	AY259503
	Pseudallescheria boydii	AY228123
	Pseudocyphellaria coriacea	AF351149
	Saagaromyces abonnis (see *Sagaaromyces abonnis*)	AF539469
	Terfezia claveryi	AF435823
	Trichoderma viride	AY291123
	Truncatella angustata	AF382383
	Tuber californicum	AF127120
	Verticillium sp.	AY089746
Basidiomycota	*Amylostereum chailletii*	AF518599
	Apiotrichum porosum	AF189833
	Boletus satanas	AF336242
	Bullera coprosmaensis	AF363660
	Clavulina cinerea	AF335456
	Corticium roseum	U80647
	Duportella tristicula	U80649
	Fellomyces distylii	AF363652
	Gloeocystidiellum porosum	AF310095
	Gloeocystidiellum porosum	AF310091
	Gymnopaxillus nudus	AY177266
	Gyroporus castaneus	AF336253
	Hydnellum aurantiacum	AF347113
	Hydnellum gracilipes	AY012676
	Hygrophoropsis aurantiaca	AF352816
	Hysterangium stoloniferum	AF336259
	Lactarius vellereus	AF325294
	Lentinellus auricula	AF506415
	Peniophora cinerea	AF506424
	Peniophora proxima	U80660
Basidiomycota	*Phellinus bicuspidatus*	AY059022
	Phellinus contiguus	AF311029
	Phellodon melaleucus	AY228355
	Phlebia radiata	AY089740
	Phlebia serialis	AF141629
	Phlebopus portentosus	AF336260
	Ramaria pinicola	AF213112
	Ramaria stricta	AF287887
	Rhizopogon pumilionus	AY177252
	Rhodotorula sonckii	AY213009
	Russula aurantiaca	AF506427
	Scleroderma sp.	AF336271
	Serpula incrassata	AF098401
	Sistotrema brinkmannii	AF506473
	Trechispora sp.	AF347088
	Trichosporon pullulans	AJ507665
	Tubulicium vermiculare	AJ406424
	Vararia insolita	AF518665
	Vararia investiens	AF506484
	Veligaster columnaris	AF336273
	Vuilleminia comedens	AF518666
	Xerocomus rubellus	AF514829
Chytridiomycota	*Blastocladiella emersonii*	X90411
Oomycota	*Achlya ambisexualis*	AF218026
	Achlya bisexualis	AF218203
	Albugo blitii	AY035543
	Albugo candida	AY035540
	Albugo tragopogonis	AY035542
	Aphanomyces laevis	AF218198
	Apodachlya brachynema	AF218199
	Basidiophora entospora	AY035513
	Bremia lactucae	AY035507
	Bremia lactucae	AY035512
	Bremiella megasperma	AY035516
	Dictyucus sterilis	AF218193
	Paraperonospora leptosperma	AY035515
	Peronophytophthora litchii	AY035531
	Peronospora alta	AY035493
	Peronospora camelinae	AY035506
	Peronospora dentariae	AY035505
	Phytophthora cambivora	AY035533
	Phytophthora capsici	AY035532
	Plasmopara densa	AY035525
	Plasmopara geranii	AY035520
	Plasmopara halstedii	AY035523
	Plasmopara obducens	AY035522
	Plasmopara viticola	AY035524
	Pseudoperonospora cubensis	AY035496
	Pseudoperonospora urticae	AY035495
	Pythiopsis cymosa	AF218172
	Pythium aquatile	AF218200
	Pythium sp.	AY035537
	Sclerospora graminicola	AY035514
	Sclerospora graminicola	AY035513
	Thraustotheca clavata	AF218181
Zygomycota	*Glomus caledonium*	AF396794
	Glomus coronatum	AF141739
	Glomus fragilistratum	AF145747
	Glomus geosporum	AF145745
	Glomus geosporum	AJ510241
	Glomus mosseae	AF396798
	Glomus mosseae	Y07565
	Scutellospora calospora	AJ510231
	Scutellospora pellucida	AF396784

identified in the amplification product of ITS4 and ITS3GC. The total band (phylotype) intensity from each lane was calculated in NIH Image. To calculate the relative intensity of each phylotype, its intensity was divided by total lane intensity. Since this method estimates relative contributions to total phylotype intensity within each lane, calibration within and between gels is inappropriate.

Results

Optimal annealing temperatures of the designed primers, using ITS5 as forward primer were 45 °C (ITS4Zygo), 49 °C (ITS4Oo), 53 °C (ITS4Chytrid), 55 °C (ITS4Asco), and 58 °C (ITS4Basidio). The primers amplified DNA extracted from the members of the group that they were designed to target and did not amplify other fungal templates Fig. 2 shows the results of one complete cross with primers and targets. Similarly, each specific primer, in combination with ITS5, was tested on DNA templates isolated from algae (including diatoms) and vascular plants, and yielded no amplification products (data not shown).

The DNA from stream-exposed leaves was extracted and checked for presence of fungal DNA with the fungal-specific primer pair ITS3 and ITS4 (WHITE et al. 1990). There were detectable levels of fungal DNA from the four substrates on all dates. The DNA extract was amplified with the primers specific for the individual fungal groups. All substrates dates had amplifiable levels of Ascomycota (Table 2). Basidiomycota and Zygomycota were absent from some substrates in April; Chytridiomycota were absent from linden and maple in April; Oomycota were only present in August.

The amplification products with the group-specific primers were successfully reamplified with ITS4 and ITS3GC. These products were then separated on DGGE. When the same DNA template was amplified with the five different primers, there were no overlapping bands (phylotypes) among the primers (Fig. 3). The number of phylotypes found on each substrate and date with each primer is summarized in Table 2. The total numbers of phylotypes on birch wood and beech were almost always higher than on linden and maple.

We amplified the initial extract of fungal DNA with the less specific primer pair ITS4 and ITS3GC which targets all fungi and oomycetes. The resulting product was run on the same gel as the amplification reactions with the specific primers designed in this study. The position and intensity of the bands from the amplification with specific primers was compared to that of ITS4 and ITS3GC. Assuming that all primers had the same extension efficiency during PCR, we could calculate what percentage of the entire fungal community (Fungi and Oomycota, amplified with the ITS4 and ITS3GC primers) was represented by members of the individual fungal groups. Based on the analysis of band intensities, we found that Ascomycota dominated (≥ 75 % of the phylotype intensity) the fungal community on all substrates and all dates (Table 3).

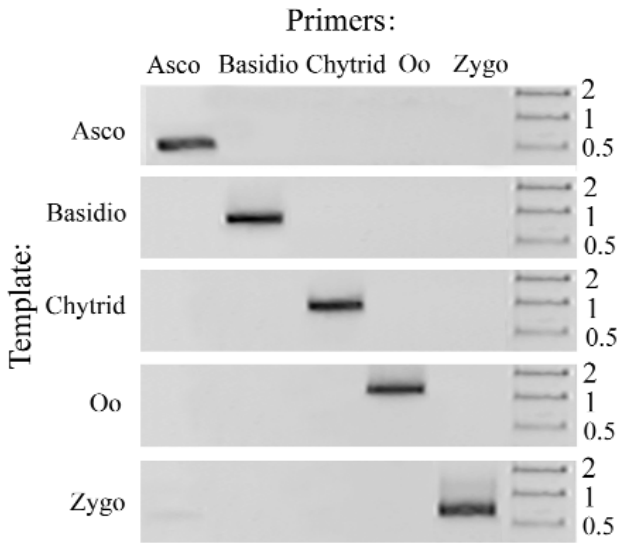

Fig. 2: Gel electrophoresis of the PCR products of template DNA extracted from *Aspergillus niger* (Ascomycota), *Agaricus bisporus* (Basidiomycota), *Allomyces arbuscula* (Chytridiomycota), *Pythium irregulare* (Oomycota), and *Rhizopus stolonifer* (Zygomycota) amplified with the primers designed in this study. Each row represents amplification reactions containing the same template. Each lane contains amplification reactions with the same primer pair. ITS5 was used as a forward primer in all reactions. The numbers on the right indicate sizes of DNA standards (in kilobases)

Basidiomycota contributed up to 13 % of the phylotype intensity on wood and beech; Chytridiomycota were fairly common on all substrates in winter (up to 21 % on wood). Oomycota were only present in the summer and their contribution was lower than that of Basidiomycota. Zygomycota contributed less than 1 % to total band intensity.

Discussion

Design of primers specific for groups of organisms requires the presence of DNA sequences conserved among members of the targeted group but different in all other groups. If the amplified region of DNA is required for identification of phylotypes or organisms (such as separation on DGGE or T-RFLP, or cloning and sequencing), then the sequence of the region bounded by the primers should be sufficiently variable to allow differentiation of distinct strains or species. The nuclear small subunit rRNA gene (18S rRNA), which has been the most extensively used gene in phylogenetics and community analysis of fungi, is a functional gene with low interspecific variability. Since separation by DGGE requires the amplification of a short region (350–450 bp) for optimal separation (MUYZER, DE WAAL & UITTERLINDEN 1993), the variability per nucleotide of the 18S rRNA gene may be too low to allow

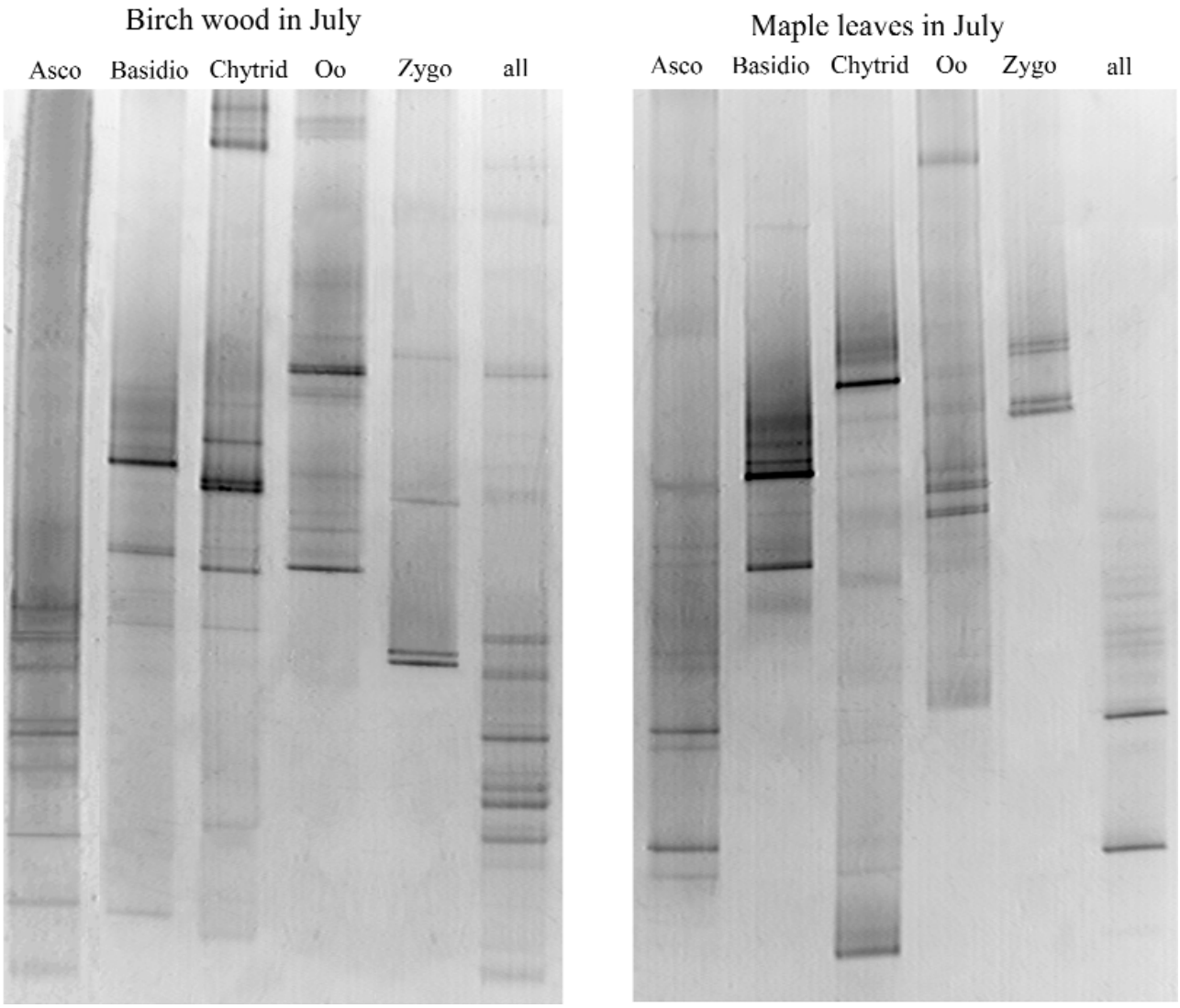

Fig. 3: DGGE of DNA isolated from maple leaves and birch wood submerged in a stream in July and amplified with the specific primers. The lane labeled "all" represents an amplification of the same template with ITS4 and ITS5

separation of species from the same genus. We therefore focused on the internal transcribed spacer region positioned between the 18S rRNA gene and the nuclear large subunit of the ribosome (28S rRNA). The internal transcribed spacer is a non-coding region. It has high interspecific variability allowing differentiating species within a genus, but low intraspecific variability preventing separation of individuals or strains within the same species (LEE & TAYLOR 1992; GARDES & BRUNS 1993; BRUNS 2001).

In the primer design, we considered the convenience of the already designed and tested primers of the ITS series (WHITE et al. 1990), specifically ITS5, which amplifies all Fungi as well as Oomycota. ITS5 can be used as a forward primer and combined with a more specific reverse primer to target specific microbial phyla. Primers specific for Ascomycota (ITS4A, LARENA et al. 1999) and Basidiomycota (ITS4B, GARDES & BRUNS 1993) have already been described for this region. However, if used in combination with ITS5, ITS4A does not exclude Oomycetes (LARENA et al. 1999), and ITS4B weakly amplifies vascular plant DNA (GARDES & BRUNS 1993). To find suitable reverse primers, we were searching for sequences downstream from ITS4, since we already had optimized conditions for DGGE for the section between it and ITS3GC (NIKOLCHEVA, COCKSHUTT & BÄRLOCHER 2003). The major problem we encounted was the scarcity of published sequences within that region, particularly for Chytridiomycota and Zygomycota. Primers designed from an insufficient number of sequences may not amplify all members of a group, resulting in underestimates of within-group diversity. Alternatively, the primers may not be specific enough and amplify some unsequenced members of another group, overestimating diversity. In our study, the former scenario is more likely since we found no overlap of the newly designed ITSChytridio, ITSOo and ITSZygo with the much larger database from

Tab. 2: Seasonal variation of number of phylotypes on DGGE belonging to different fungal groups, on linden (L), maple (M), beech leaves (B) and birch wood (W) decaying in a stream

		Ascomycota	Basidiomycota	Zygomycota	Chytridiomycota	Oomycota
Jul	L	21	7	4	3	5
	M	11	6	3	12	5
	B	14	8	4	10	12
	W	13	10	4	13	8
Oct	L	8	4	4	8	
	M	10	6	5	11	
	B	9	6	3	9	
	W	12	9	4	11	
Jan	L	10	8		11	
	M	9	10		13	
	B	10	7		14	
	W	9	9	6	13	
Apr	L	13				
	M	11				
	B	10	8			
	W	6	8			

Tab. 3: Seasonal variation of percentage contributions by fungal groups to total band intensity on DGGE. Zygomycota contributions were always ≤ 1 %, Symbols same as in Tab. 2

		Ascomycota	Basidiomycota	Chytridiomycota	Oomycota
Jul	L	93	6		1
	M	96	1		3
	B	95	2		3
	W	77	13		8
Oct	L	89	11	3	
	M	91	9		
	B	90	4	4	
	W	92	3	7	
Jan	L	86		14	
	M	85		15	
	B	75	3	17	
	W	80	4	21	
Apr	L	100			
	M	100			
	B	91	8		
	W	96	8		

which we designed ITSAsco and ITSBasidio. Nevertheless, both sources of error are real and can only be mitigated by access to more sequences. To some extent, results based on 'specific' primers always have to be considered as preliminary. This is particularly true for ITS4Chytrid, which is based on DNA from three species – on the other hand, it clearly discriminated against the other fungal groups (no matches in database, no amplification of test organisms).

Once DNA from a circumscribed fungal group has been amplified, the products can be reamplified in nested PCR with less specific primers from the ITS series (WHITE et al. 1990) and analyzed by terminal restriction fragment length polymorphism or DGGE. This determines the minimum number of fungal strains from each group in the sample (NIKOLCHEVA, COCKSHUTT & BÄRLOCHER 2003). Alternatively, the PCR product can be cloned and sequenced to determine the phylogenetic composition of a fungal community (BUCHAN et al. 2002, 2003).

In the current study, we used the primers as tools to study the diversity of fungi on plant litter decomposing in a stream. We successfully reamplified the initial PCR products with primers suitable for DGGE. When template DNA from the same substrate and date was amplified with the five primer pairs, there were no overlapping bands on DGGE. This indicates

that primers were very specific for the group they target and that the DNA amplified with each primer belonged to a phylogenetically distinct group (though it does not prove that the groups encompassed the entire targeted phylum, in particular the primers for the Chytridiomycota, which were based on very limited data). Keeping these limitations in mind, the results suggest that diversity was lowest in April – Ascomycota were found on all samples, Basidiomycota were restricted to linden and wood, and all other fungal groups were absent.

The largest numbers of fungal phylotypes belonged to the Ascomycota (Table 2). Based on conidial counts, the most common species of aquatic hyphomycetes from the four substrates throughout the year were *Anguillospora filiformis*, *Flagellospora curvula*, *Articulospora tetracladia* and *Clavariopsis aquatica*, whose 18S sequences place them within the Ascomycota (BELLIVEAU 2003). Terrestrial fungi, primarily hyphomycetes, dominate the fungal population on leaves before they are submerged in a stream (BÄRLOCHER & KENDRICK 1974), and may have contributed to the high diversity of Ascomycota. Finally, ascomycetous teleomorphs are commonly found on slowly decaying substrates, such as wood (SHEARER 1992). The approach used here, based on phylum-specific primers, cannot distinguish terrestrial from aquatic anamorphs or teleomorphs within the Ascomycota.

Diversity of Basidiomycota and Chytridiomycota, as characterized by DGGE, was surprisingly high. Freshwater Basidiomycetes are relatively rare, and their teleomorphs are generally most common on wood (GOH & HYDE 1996). Some aquatic hyphomycete anamorphs have been connected to basidiomycetous teleomorphs (WEBSTER 1992), but we did not encounter any of these in spore counts. It is therefore unlikely that all basidiomycete phylotypes detected with DGGE belong to aquatic hyphomycetes.

Chytridiomycota have generally been ignored by biologists (ALEXOPOULOS, MIMS & BLACKWELL 1996) but may play important roles in many ecosystems (POWELL 1993). We are not aware of any study that addressed the potential participation of this group in leaf decomposition, but casual observations (Bärlocher, pers. comm.) indicate that they do occur and may be more common in the colder seasons. Since we had only one sequence available for primer design (which was tested against two other species), it seems likely that Chytridiomycota were both more diverse and more plentiful than our results suggest.

Oomycota were restricted to the summer sample. Again, this is difficult to correlate with any potential role in leaf decomposition, since few studies have addressed this topic. There are reports, however, of rapid colonization by *Pythium* spp. of various cellulose substrates (WILLOUGHBY & REDHEAD 1973) and leaves (ROSSI et al. 1983) in summer.

The diversity of Zygomycota was generally low, supporting the assumption that they are essentially transients – their propagules may be trapped on exposed litter (PARK 1972), but their participation in the breakdown of cellulosic substrates is doubtful (COOKE 1976).

The PCR reaction can be extremely sensitive, especially when highly specific primers are used. By calculating relative intensities of DGGE bands belonging to different taxa, their contributions to total biomass can be estimated (MUYZER, DE WALL & UITTERLINDEN 1993). This analysis assumes that 1) the primer extension efficiency of the ITS3GC and ITS4 is identical for all templates in a mixture of extracted DNA and 2) the number of ribosomal operons is correlated to cell biomass. We have shown that in DNA mixtures of aquatic hyphomycete species, the DGGE band intensities are strongly correlated to fungal biomass (NIKOLCHEVA, COCKSHUTT & BÄRLOCHER 2003). While it is unlikely that all templates in the sample have identical extension efficiencies (HEAD, SANDERS & PICKUP 1998), the intensity of DGGE bands nevertheless provides a preliminary, semi-quantitative estimate for the abundances of the various fungal groups in an environmental sample. If we accept it as such, our data suggest that members of the Ascomycota (aquatic and terrestrial anamorphs and teleomorphs) clearly dominated the fungal community during leaf decomposition in Boss Brook. They were supplemented by a small but consistent basidiomycetous component. Chytridiomycota may have made a substantial contribution in winter, while Oomycota may have played a minor role in summer. There is no evidence that Zygomycota biomass was more than marginal in any sample.

The results presented here raise the possibility that during certain seasons, or during specific phases in leaf decomposition, fungi other than aquatic hyphomycetes may contribute substantially to leaf decomposition in streams. For a more complete and balanced understanding, a combination of traditional methods (direct observations, baiting techniques; BÄRLOCHER 1992b) and modern approaches, including fluorescent in situ hybridizations (FISH; BASCHIEN et al. 2001; MC ARTHUR et al. 2001), in situ PCR (BAGO, PICHE & SIMON 1998), and the use of genus or species-specific monoclonal antibodies (BERMINGHAM, MALTBY & DEWEY 1996) will be essential.

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