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Fungal communities of submerged fine detritus from temperate peatland and stream habitats

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ABSTRACT: Fungi are essential in aquatic ecosystems, transforming organic matter into energy sources that support higher trophic levels. However, researchers do not yet know the extent of fungal diversity and species distribution within these important ecosystems. Therefore, we examined the detrital fungal communities from contrasting aquatic habitats (temperate peatlands and streams) to provide an in-depth inventory and greater understanding of how these communities differ. Fine submerged detritus or substrate on or beneath the stream bed were collected from 6 sites. Fungal cultures were isolated from samples collected in May, July/August, and November from 2 sites in 2014 and 4 sites in 2016. Culture-independent analyses were conducted on 42 environmental samples collected in November 2016. Results indicated that peatland and stream fungal communities were taxonomically diverse, phylogenetically distinct, and harbored many unknown taxa from the kingdom Fungi. Specifically, stream habitats were more species-rich, in both number of species and phylogenetic diversity, compared to peatland habitats. In addition, fungal species and phylogenetic distribution within most major fungal classes were distinct between peatland and stream fungal communities. In light of global climate change, habitat loss, and water pollution, it has become increasingly important to examine these understudied and essential fungal communities within these ecosystems.

KEY WORDS: Ascomycota · Basidiomycota · Bogs · Chytridiomycota · Mortierellomycota

1. INTRODUCTION

Freshwater fungi provide essential ecosystem functions including litter decay (Bärlocher 1992), nutrient cycling (Tant et al. 2015), and parasitism of algae and invertebrates (Barron 2004, Sime-Ngando 2012). However, considering their importance within aquatic ecosystems, interest in aquatic fungi did not advance until Ingold (1942) connected the conidia of aquatic hyphomycetes to mycelia colonizing submerged leaves. Even then, aquatic fungi were generally ignored by stream ecologists until Triska (1970) and Kauskih & Hynes (1971) demonstrated that fungal-conditioned leaves were a vital food source for stream invertebrates (Bärlocher 1992). Consequently, following these previous discoveries, the study of aquatic fungi remained limited to Ingoldian fungi, which typically inhabit submerged decaying leaves (Wong et al. 1998).

More recently, there has been a push to expand our understanding of fungal biodiversity found on submerged coarse-woody debris (minimum diameter of 2.5 cm) (Wong et al. 1998, Jones et al. 2019), as endophytes of aquatic plants (Sati & Belwal 2005, Sandberg et al. 2014), river, lake, and marine sediments (Liu et al. 2015, Rédou et al. 2015, Wahl et al. 2018), wetlands (Thormann et al. 2007), and within the hyporheic zone of streams (Bärlocher et al. 2006). However, there are still very few studies examining fungal biodiversity in detritus smaller than coarse-woody debris, which we define as fine detritus (organic matter particle size <2.5 cm in diameter).

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Fine detritus is non-living organic matter from dead microbes, feces, and dead tissues of animals and plants and can be characterized based on chemical composition and size (Moore et al. 2004). Detritus found within aquatic ecosystems is predominantly from autochthonous and allochthonous plant sources, and large amounts of detrital input are usually episodic (storms, die-off) or seasonally dependent (Benbow et al. 2020). In many ecosystems, including aquatic habitats, detritus is a key component to ecosystem function because it is the dominant energy base (O'Neill & Reichle 1980), central to energy flow (Lindeman 1942), and acts as a habitat modifier (Moore et al. 2004) and reservoir of stored carbon. Findlay et al. (2002) indicated that while fungi are present on very fine detritus, they tend to be the predominate microbes on detritus >1 mm in size. Consequently, a greater understanding of the fungal communities within submerged fine detritus will provide insight into which taxa are potentially utilizing this important resource.

Currently, the use of both culture-dependent and culture-independent techniques (environmental DNA sampling techniques) has allowed researchers to better characterize fungal community composition and diversity within samples rather than using one method alone. Romão et al. (2017) showed that each method was complementary as many of the cultured fungi were not found within the culture-independent data. In addition, the use of different genetic markers for determining species identity is not standardized. A review by Duarte et al. (2013) indicated that studies on aquatic fungi predominately used the internal transcribed spacer (ITS) ITS1-5.8S-ITS2 region (57%), small subunit (SSU) ribosomal DNA (16%), large subunit (LSU) ribosomal DNA (12%), and other methods (15%). They suggested that targeting several loci would be advantageous for accuracy and reproducibility. Due to primer biases and different levels of taxonomic resolution for each genetic marker, conducting environmental studies using multiple genetic markers has the potential to capture a greater proportion of the total fungal community.

Therefore, the aim of the present study was to provide an in-depth inventory and phylogenetic understanding of fine detritus inhabiting aquatic fungal communities from temperate peatland and stream habitats. Our goals were to (1) use culture-dependent methods to isolate fungi from each habitat, (2) isolate environmental DNA from fine detritus samples and use multiple genetic markers to characterize the fungal communities, (3) compare culture-dependent and culture-independent techniques, and (4) determine environmental factors that influence community composition. We expected to find phylogenetically distinct fungal communities due to abiotic habitat-specific selection pressures and to find unknown fungal species due to the few taxonomic studies on aquatic fine detritus inhabiting fungi. This research will provide an in-depth species list for future studies to build upon, provide methodological insights for future sample collection and community analyses, and provide a phylogenetic context to important fungal genera isolated from fine detritus.

2. MATERIALS AND METHODS

2.1. Sampling sites

Three sites from each of 2 different freshwater habitats (peatlands and streams) were sampled for a total of 6 sites. Four sites were sampled in Pennsylvania: Black Moshannon State Park, Nescopeck State Park (Pennsylvania Department of Conservation and Natural Resources Bureau of State Parks), Pepper Run (private property), and Tannersville Cranberry Bog Preserve (The Pennsylvania Nature Conservancy). Two sites were sampled in Wisconsin: Beulah bog (Wisconsin Department of Natural Resources) and Honey Creek State Natural Area (The Wisconsin Nature Conservancy). Sites were sampled in May, late July/early August, and late November, with 10 samples collected each month at each sampling location with permission from the landowners. Black Moshannon State Park and Pepper Run were sampled in 2014 for culture-dependent analyses while the remaining 4 sites were sampled in 2016 for culture-dependent analyses. All sites were sampled in 2016 for culture-independent analyses (Fig. 1). All 3 stream sites were shallow, first-order streams that run through dense forests, while all 3 peatland sites contained *Sphagnum* spp. moss, native carnivorous plants (*Drosera ratundifolia* L., *D. intermedia* Hayne, *Sarracenia purpurea* L.), and native cranberry *Vaccinium* spp. species. The dominant plant overstory in Tannersville Cranberry bog and Beulah bog consisted of tamarack *Larix laricina* (Du Roi) K. Koch, while Black Moshannon State Park contained leather leaf *Chamaedaphne calyculata* (L.) Moench and *Vaccinium* species. Water parameters were collected at the time of sample collection. Dissolved oxygen and temperature were recorded with a YSI 556 handheld multiparameter meter, pH was recorded with a Milwaukee model MW102 pH meter, and general hardness, carbonate hardness, NO_3, and NO_2 were determined using commercially available API test strips (Table 1).

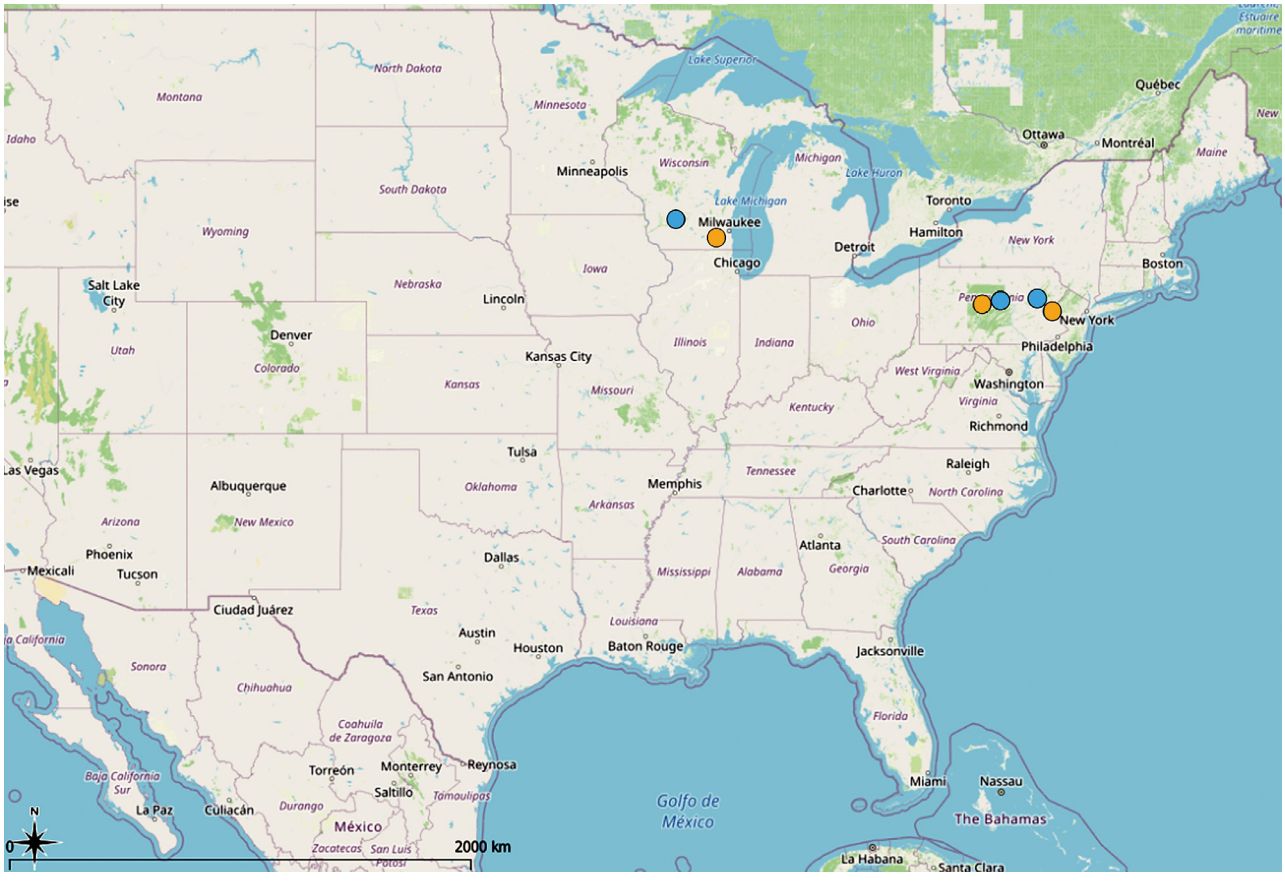

Fig. 1. Location of sampling sites in Pennsylvania and Wisconsin. Orange dots: peatland sites; blue dots: stream sites. Map generated using QGIS v.3.6.3

2.2. Culture-dependent sample collection and fungal isolation

Approximately 100 g of submerged fine detritus (<2.5 cm particle size; ca. 20 cm below the water level for peatland samples, or sediment/fine detritus on or beneath the stream bed for stream samples) were placed into sealable plastic storage bags and maintained at 4°C during transport to the laboratory. For environmental sequencing, a portion of each sample was stored in a 2 ml cryogenic vial and maintained at –20°C; 2 g of sample were added to 200 ml of distilled water in a blender, pulsed for 15 s, and 300 µl of the solution was lawn plated onto 2 pH-adjusted media: (1) malt extract agar (Difco) supplemented with 0.1 g l^{-1} chloramphenicol and (2) 0.05 g l^{-1} Rose Bengal (pH 3–5 and 8–10) and tea agar supplemented with 0.20 g l^{-1} chloramphenicol (Mehrotra et al. 1982), using sterilize glass rods and wrapped with Parafilm. May and November samples were incubated at 7°C for the first 2 wk, at 14°C for an additional 2 wk, and at 21°C thereafter. August samples were incubated at 14°C for the first 2 wk and at 21°C thereafter. The different incubation regimes were used to isolate fungi that would be preferentially growing at the current environmental temperature, and the later incubation temperatures were to isolate additional fungi within the sample that may prefer a higher temperature. Fungal colonies were transferred to malt extract agar and incubated at room temperature for 1 wk and then transferred to 7°C for storage. Negative control plates consisted of uninoculated and water-inoculated plates. Uninoculated control plates were wrapped with Parafilm and subjected to the same conditions as the inoculated plates above. Water-inoculated control plates consisted of lawn plating 300 µl of sterile water onto 2 plates of each pH value following the above process. Three representative substrate samples from each location were microscopically inspected for the presence of actively growing fungi, and subsequently baited with pollen (*Pinus* sp.) and examined for the presence of chytrid species. Chytrid species were baited with dried onion peel and pine

Table 1. Physical and chemical properties of sampling sites. Mean ± SD are shown for temperature. GH: general hardness; KH: carbonate hardness; LOD: limit of detection; NO_2: nitrite; NO_3: nitrate; DO: dissolved oxygen

Location	Designation	State	Latitude	Temp. (°C)		GH (ppm)	KH (ppm)	pH	NO_2 (ppm)	NO_3 (ppm)	DO (%)
				May	Jul/Aug	Nov					
Peatlands											
Black Moshannon State Park	Fen	Pennsylvania	40° 54.049′ N	12.2 ± 3.7	19.1 ± 1.4	1.0 ± 0.1	<LOD	<LOD	5.1–6.5	<LOD	<LOD	< 20
Tannersville Cranberry Bog Preserve	Acid-poor fen	Pennsylvania	41° 02.236′ N	18.2 ± 1.7	18.9 ± 0.9	8.6 ± 0.2	<LOD	<LOD	4.8–5.3	<LOD	<LOD	< 20
Streams											
Honey Creek State Natural Area	First-order	Wisconsin	43° 22.865′ N	12.8 ± 0.3	13.4 ± 0.3	11.4 ± 0.3	180	120	7–8	<LOD	0–20	> 80
Pepper Run	First-order	Pennsylvania	41° 05.569′ N	13.5 ± 0.5	16.7 ± 0.2	6.9 ± 0.2	0–30	<LOD	7–7.5	<0.5	0–20	> 80
Nescopeck State Park	First-order	Pennsylvania	41° 03.121′ N	13.1 ± 1.3	15.9 ± 1.8	5.0 ± 0.1	0–30	0–40	7–8.2	<LOD	0–20	> 80

pollen (Powell et al. 2019) and visually screened since they would not have been detected using lawn plate isolation techniques.

2.3. Culture-dependent identification of fungal isolates

Fungal isolates were identified using the nuclear ribosomal ITS region (Schoch et al. 2012). A simple NaOH DNA extraction method (Osmundson et al. 2013) was used to obtain fungal DNA. In short, fresh mycelium was ground in a 1.5 ml centrifuge tube containing 200 µl 0.5 M NaOH solution, centrifuged at 14 000 rpm (16 873 × *g*) for 2 min, and 5 µl of the resulting supernatant added to 495 µl 100 mM Tris-HCl buffered with NaOH to pH 8.5–8.9. PCR was conducted on a Bio-Rad PTC 200 thermal cycler with a total reaction volume of 25 µl (12.5 µl GoTaq® Green Master Mix, 1 µl of each 10 µM primer ITSIF and ITS4, 3 µl Tris-HCl-DNA extraction solution, and 7.5 µl DNA-free water). Gel electrophoresis (1% tris-borate-EDTA [TBE] agarose gel stained with ethidium bromide) was used to verify the presence of PCR product before purification using a Wizard® SV Gel and PCR Clean-Up System (Promega). A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems) was used to obtain a forward sequence for the ITS region in one direction using the ITS5 primer on an Applied Biosystems 3730XL high-throughput capillary sequencer. Sequence quality and bp calls were visually reviewed using Sequencher 5.1. Unique isolates were determined through the Bayesian implementation of the Poisson tree process model (bPTP; Zhang et al. 2013). In short, class level sequence alignments were conducted in PASTA (Mirarab et al. 2015) using MAFFT (Katoh et al. 2002), and a maximum likelihood tree was built using RAxML (Stamatakis 2014) in CIPRIS (Miller et al. 2010) followed by species delineation using 'bPTP.py' script (Zhang et al. 2013) with 500 000 iterations. A tree-based species delineation model was chosen to (1) reduce the influence of sequence read length on species determination, (2) remove anamorph/teleomorph nomenclatural redundancies, and (3) distinguish the number of cryptic taxa within genera that contain many species with ≥97% ITS sequence similarity. Final species identification was made through sequence match using BLASTn against the NCBI database using the following criteria: (1) genus level, ≥85% query coverage with ≥93% ID; and (2) species level, ≥85% query coverage with ≥97% ID (Wahl et al. 2018). All sequence data

and taxonomic assignments were deposited in the University of Illinois Databank (https://doi.org/10.13012/B2IDB-6862941_V2) and NCBI GenBank (MW764107–MW765222).

2.4. Culture-dependent statistical analyses

Confidence intervals using the modified Wald method (Agresti & Coull 1998) were completed to determine if the number of unique cultured isolates per class was different per habitat. Principal component analysis (PCA) using Hellinger-transformed presence–absence data (Ramette 2007) was completed at the order level to visualize the importance of time on isolation using the 'vegan' package (Oksanen et al. 2018) in R statistical software (R Core Team 2013). Statistical significance was assessed with 'adonis' and ANOSIM using the 'vegan' package in R.

2.5. Culture-independent samples and sequencing

Seven random samples per site were selected for environmental sequencing for a total of 42 environmental samples and one negative control. Environmental DNA was extracted from approximately 0.4 g of detritus using the MP FastDNA™ Spin Kit for Soil following the instruction protocol, with the following modifications: samples were stored at −20°C overnight, homogenized for 12 min using a Vortex Genie™, placed on ice for 5 min, homogenized for 8 additional minutes, followed by 2 protein precipitation steps per optional protocol. The negative control consisted of 400 µl Millipore water following the same procedure. We amplified 4 nuclear DNA regions (ITS1, ITS2, nuclear ribosomal LSU, beta-tubulin [TUB]) using the Fluidigm Access Array (Brown et al. 2016). Amplicons were sequenced on 2 separate Illumina MiSeq v2 platform runs using rapid 2 × 250 nt paired-end reads (Table 2). One Illumina run contained ITS1, ITS2, and TUB amplicons size-selected into <500 and >500 nt sub-pools, then remixed together <500:>500 nt by nM concentration in a 1×:3× proportion. A second Illumina run contained LSU Fluidigm amplicons that were 400–800 nt in length. All amplification and sequencing steps were performed at the Roy J. Carver Biotechnology Center at the University of Illinois Urbana-Champaign.

2.6. Culture-independent Illumina read processing and taxonomic assignment

Illumina forward reads were processed in QIIME 1.9 (Caporaso et al. 2010). Only forward reads were used instead of paired-end reads, because (1) forward reads were higher quality than reverse reads, (2) reads with large insertions would be removed due to lack of paired-end overlap, and (3) retaining as much of the overall depth of sequence data as possible provides a more complete fungal inventory (Wahl et al. 2018). Sequence reads with a quality score of less than 25 were removed using the 'split_libraries_fastq.py' script. Operational taxonomic units (OTUs) for ITS1 and ITS2 were binned at 97% similarity using the 'pick_open_reference_otus.py' script with the following parameters: 'otu_picking_method' set to 'uclust', 'reference_fp' set to the UNITE database 01.12.2017, and 'min_otu_size' set to 5. OTUs for LSU were binned at 97% similarity, to be conservative, using the SILVA database (Quast et al. 2013). Taxonomic assignment of representative ITS1 and ITS2 OTUs was completed using the UNITE database in CONSTAX (Gdanetz et al. 2017), whereas the online Ribosomal Database Project (RDP) classifier (Wang et al. 2007) was used for LSU-representative OTU taxonomy assignment. Beta-tubulin OTUs were binned as above using a QIIME-compatible TUB database containing 84 477 entries of 200–2500 bp reference sequences from the NCBI database (Benson et al. 2005) constructed on 27 June 2018 using

Table 2. Fluidigm amplification primers

Amplification target	Primer	Primer sequence	Reference
BT2	BT2AF	5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′	Glass & Donaldson (1995)
	BT2BR	5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′	
LSU	LROR	5′-CCG CTG AAC TTA AGC ATA TCA-3′	Vilgalys & Hester (1990)
	LR3	5′-CCG TGT TTC AAG ACG GG-3′	Vilgalys & Gonzalez (1990)
ITS1	ITS1F	5′-CTT GGT CAT TTA GAG GAA GTA A-3′	Gardes & Bruns (1993)
	ITS2	5′-GCT GCG TTC TTC ATC GAT GC-3′	White et al. (1990)
ITS2	fITS7	5′-GTG ART CAT CGA ATC TTT G-3′	Ihrmark et al. (2012)
	ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′	White et al. (1990)

the 'entrez_qiime.py' script (Baker 2016). Beta-tubulin-representative OTU taxonomic assignments were completed in QIIME using the above database, and taxonomic assignment was completed using 'assign_taxonomy.py' set to blast. Raw and processed data files are publicly available from the University of Illinois Databank (https://doi.org/10.13012/B2IDB-6862941_V2), and raw Illumina reads have been deposited into the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/PRJNA714435).

2.7. Culture-independent statistical analyses

Community analyses were completed using ITS1 and LSU environmental sampling data since these data sets captured the greatest proportion of the fungal community. Four LSU samples (BM6, BB23, TV21, and TV24) were removed due to low sequence depth, and count data was normalized using relative abundance per sample based on hierarchical cluster analyses. Relative abundance of OTUs within the ITS1 and LSU data sets were examined for congruency using correlation analysis. ViroBlast (Deng et al. 2007) was used to identify ITS1 OTUs with high similarity to culture sequences.

Alpha diversity, the diversity within a community, was assessed in R using the 'pez' (Pearse et al. 2015) and 'Picante' (Kembel et al. 2010) packages for species richness, phylogenetic richness, phylogenetic divergence, and phylogenetic regularity. Species richness at each site was estimated using the Chao 1 estimator. Phylogenetic richness (amount of evolutionary history at each site) was determined using Faith's phylogenetic distance, and phylogenetic regularity was determined using the entropic measure of phylogenetic diversity metric (H_{ed}; Cadotte et al. 2012). Phylogenetic regularity is a metric that indicates how regularly species are spread along the phylogenetic tree and how evenly distant species are apart (Tucker et al. 2017). High H_{ed} suggests that species are more closely related to each other, while low H_{ed} suggests a greater proportion of evolutionary distinct species (Cadotte et al. 2012).

Beta-diversity, the difference between communities, was assessed in R using (1) 'adonis' function (permutational distance-based multivariate analysis of variance, PERMANOVA) in the 'GUniFrac' package (Chen et al. 2012) using the generalized Unifrac distance matrix with alpha 0.5 and 1000 permutations, (2) 'anosim' function (analysis of similarity) within the 'vegan' package using a Bray-Curtis dissimilarity index matrix and 1000 permutations, (3) 'anosim' function using presence/absence matrix and 1000 permutations, and (4) 'adonis' function in the 'vegan' package with the Bray-Curtis dissimilarity index matrix (relative abundance). Bray-Curtis dissimilarity was used because it was found to be one of the least vulnerable distance matrix indices to several types of errors, and generalized Unifrac distance matrix with alpha 0.5 was found to be more robust than unweighted-Unifrac or weighted-Unifrac distance matrixes (Chen et al. 2012). For both ITS1 and LSU analyses, the removal of OTUs with ≤9 count data or presence–absence analysis ('anosim') did not significantly alter the results of the beta-diversity analyses. A circular cladogram was constructed in EvolView (He et al. 2016) from post-processed RAxML analysis using PASTA based on partial LSU Illumina sequence data.

3. RESULTS

3.1. Observed fungi

A total of 1383 strains were isolated in culture, for which ITS sequence data were obtained for 1196 (86.5%), of which 1100 (92%) had a very high-quality match (E-value ≤ $1E^{-50}$) to a culture-independent ITS1 representative OTU and 1120 (93%) of the culture sequences had the same level match to a culture-independent ITS2-representative OTU. The most frequently isolated classes within Ascomycota were Sordariomycetes (36%), Dothideomycetes (20%), and Eurotiomycetes (19%), and the most frequently isolated classes within the Basidiomycota were Tremellomycetes (7%) and Microbotryomycetes (2%). Isolates within Mucoromycota (1.3%) were isolated more frequently than isolates within Mortierellomycota (0.7%) (Fig. 2). In addition, fungi frequently collected in polar regions, consisting of *Cryptococcus filicatus*, *C. gastricus*, *C. saitoi*, *C. macerans*, *Mrakia blollopis*, *Rhodotorula glacialis*, and *Recurvomycetes mirablis*, were isolated from both peatland and stream habitats. Across all fungal cultures, site location accounted for 30% of the variation in the data ($p = 0.0005$) and habitat explained 13% ($p = 0.035$). Collection time was not statistically significant ($r^2 = 0.16$, $p = 0.141$).

Illumina sequencing of extracted DNA produced 10 364 390 LSU forward reads, of which 59% passed quality resulting in 7025 fungal OTUs; 2 267 937 ITS1 forward reads, of which 67% passed quality resulting in 6578 fungal OTUs; 1 838 408 ITS2 forward

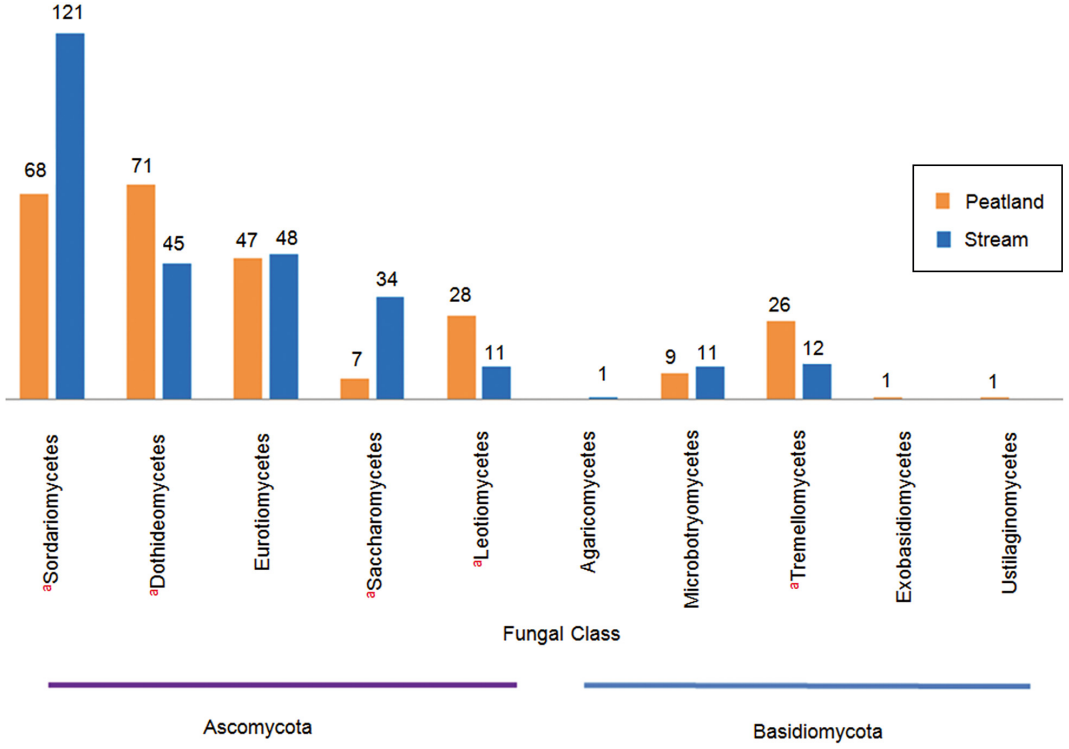

Fig. 2. Culture-dependent results for the Ascomycota and Basidiomycota fungal classes. ([a]) represents statistical significant habitat collection representation. Numbers above bars represent unique Bayesian Poisson tree process species per class. Statistically, there was a greater number of Sordariomycetes and Saccharomycetes species isolated from stream habitats compared to the peatland habitats, while Dothidiomycetes, Leotiomycetes, and Tremellomycetes species were isolated in a greater number from peatland habitats compared to the stream habitats

reads, of which 69% passed quality resulting in 4335 fungal OTUs; 2 654 311 TUB forward reads, of which 77% passed quality resulting in 1563 OTUs. Beta-tubulin OTU taxonomic assignments were found to be unreliable due to limited species representation, and average QIIME OTU over-inflation was higher for ITS2 (23%) compared to ITS1 (16%) forward reads. Sampling depth was adequate for most sites (Fig. 3A,B), and site sample clustering was not altered by the removal of rare taxa (Fig. 3C,D). The proportion of ITS1 and LSU OTUs assigned to taxonomic rank were correlated ($p < 0.001$) at the class ($r^2 = 0.97$), order ($r^2 = 0.90$), and family ($r^2 = 0.61$) levels. Within the ITS1 data set, there was a maximum of 3% difference in the OTU relative abundance of each major fungal class per habitat (Fig. 4A). In total, 1131 unique taxonomic determinations were assigned from across all 6 sites using ITS1, ITS2, TUB, and culture data (Table S1 in the Supplement at www.int-res.com/articles/suppl/a086p191_supp.pdf). No single gene region captured all of the fungal diversity. Phylogenetic beta-diversity analysis using ITS1, ITS2, and LSU data sets indicated that the peatland and stream fungal communities were distinct, with most of the variation being attributed to site location ($r^2 = 0.47$) followed by habitat ($r^2 = 0.22$).

3.2. Stream fungal communities

Among the cultured fungi, there was a greater number of Saccharomycetes and Sordariomycetes species isolated from streams compared to peatlands (χ^2, $p < 0.01$, n = 34:7; $p < 0.0001$, n = 121:68, respectively) (Fig. 2). No chytrids were isolated from stream samples. From environmental sequencing data, the most abundant OTUs in the stream habitats were assigned to the genera *Flagellospora*, *Ganoderma*, *Hannaella*, *Mortierella*, *Neobulgaria*, *Neonectria*, *Neopestalotiopsis*, *Paraconiothyrium*, *Penicillium*, *Plectosphaerella*, *Preussia*, *Pyrenochaetopsis*, *Ramularia*, *Sphaerulina*, and *Tolypocladium*, in addition to 10 unidentified fungal OTUs. Rarefraction indicated that streams had a greater number of species than peatlands and that each location was adequately sampled (Fig. 3A,B). Alpha-diversity metrics indicated that stream sites were richer in both number of species and phylogenetic diversity compared to peatland sites (Table 3).

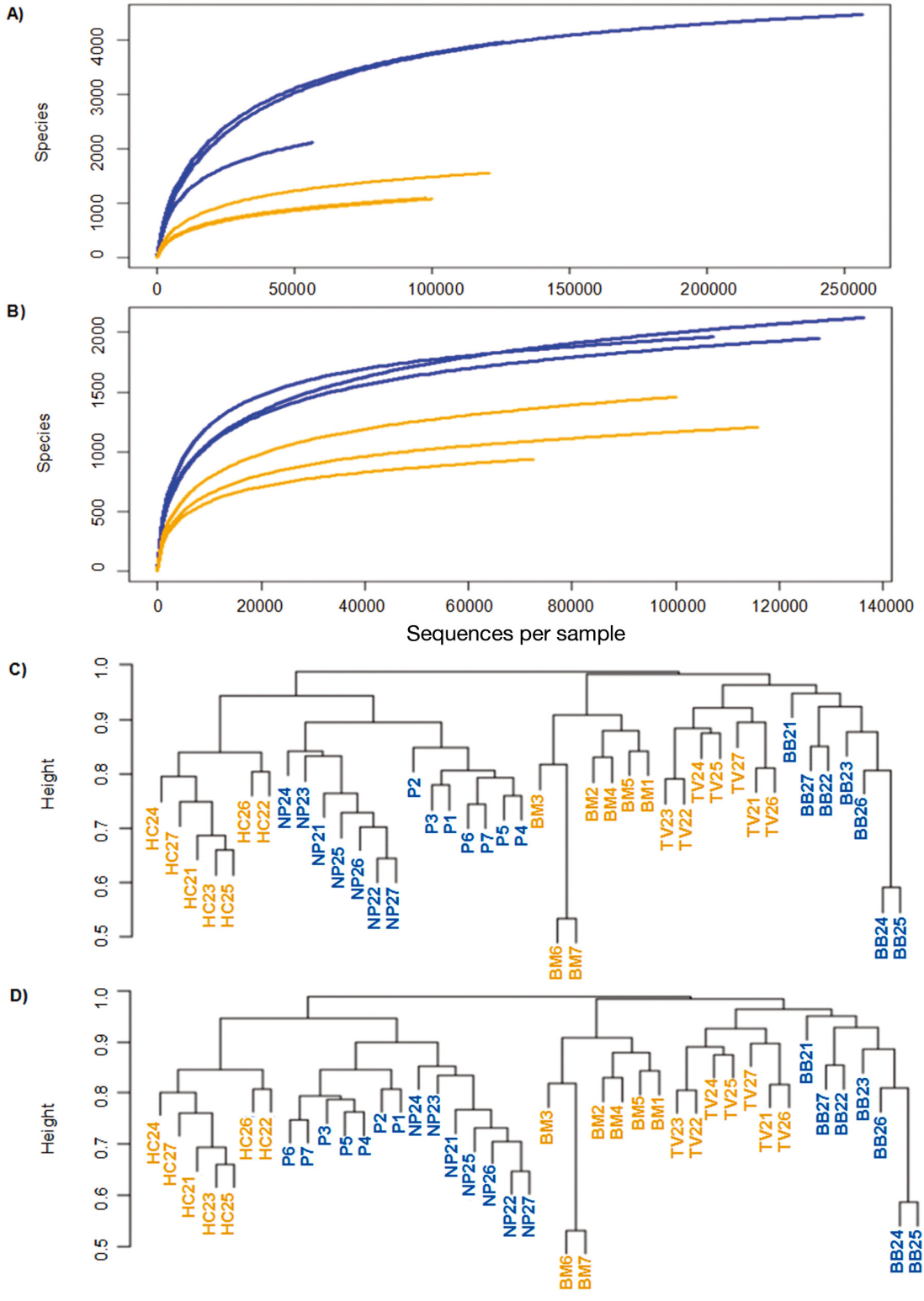

Fig. 3. Internal transcribed spacer (ITS1) and large subunit (LSU) sequencing metrics. (A,B) LSU and ITS1 rarefraction analyses by site. (C,D) ITS1 hierarchical cluster analyses using average linkage on full data set and reduced data set (rare species with read abundance <9 removed), respectively. Rarefraction indicated that streams had a greater number of species compared to peatlands and that each location was adequately sampled. Cluster analyses indicated that all replicate samples for each location were more similar to one another regardless of the inclusion of low abundant reads, indicating that low abundant reads would not influence downstream analyses. Orange represents peatland sites, blue represents stream sites

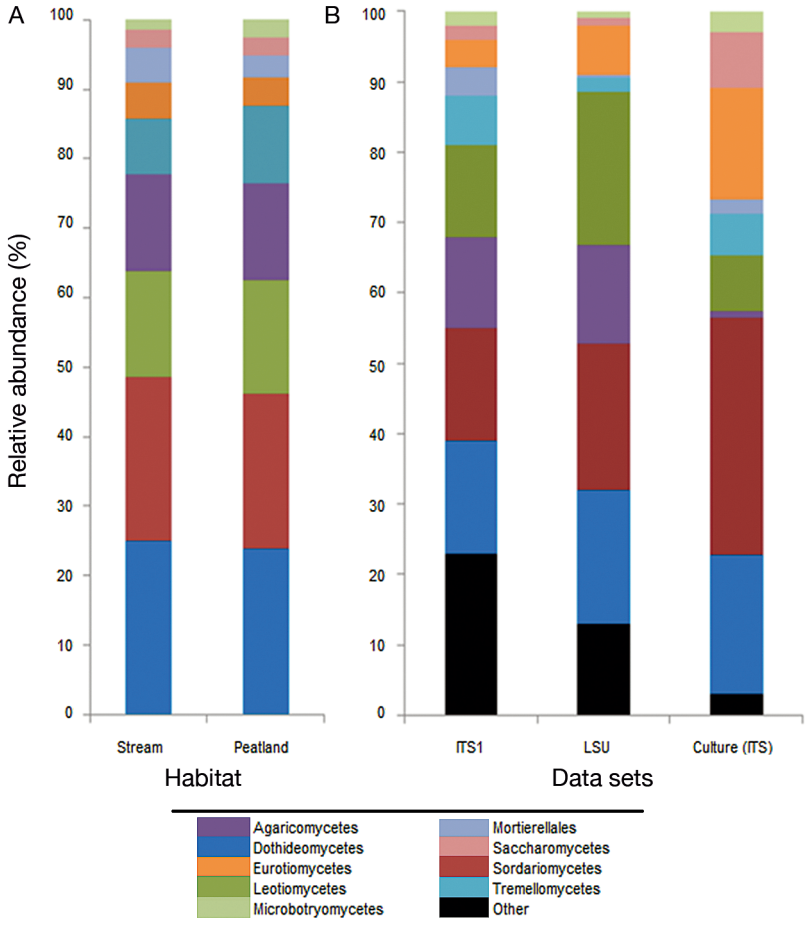

Fig. 4. Taxonomic distribution of the 9 most abundant fungal classes within the environmental sequencing ITS1, NGS LSU, and culture ITS data sets. Relative abundance at the (A) class level and (B) class level based on habitat using the NGS ITS1 data set

Table 3. Site alpha-diversity metrics. Faith's phylogenetic distance (PD) and the entropic measure of phylogenetic diversity (H_{ed}) were based on the ribosomal large subunit (LSU) data set

Location	Richness			Regularity
	Chao 1 (LSU)	Chao 1 (ITS1)	Faith's PD	Mean H_{ed}
Streams				
Pepper Run	4633	2558	201	29
Nescopeck	4995	2436	218	24
Honey Creek	2619	2480	127	46
Peatlands				
Tannersville	1945	1877	85	57
Beulah Bog	1487	1322	83	77
Black Moshannon	1596	1794	99	56

3.3. Peatland fungal communities

Among the fungal cultures, there was a greater number of Dothideomycetes, Leotiomycetes, and Tremellomycetes species isolated from peatland habitats compared to stream habitats (χ^2, $p < 0.001$, n = 71:45; $p < 0.0001$, n = 28:11; $p < 0.02$, n = 26:12, respectively) (Fig. 2). Environmental sequencing data revealed the most abundant OTUs in the peatland habitats were assigned to Dothideomycetes, Sordariomycetes, Leotiomycetes, Agaricomycetes, and Tremellomycetes (Fig. 4B). At the genus level, the genera *Amanita*, *Lactarius*, *Lipomyces*, *Russula*, *Sugiyamaella*, and *Tomentella* were most abundant in addition to 12 unidentified fungal OTUs. Although not cultured, several saprotrophic and parasitic chytrids were observed within peatland samples (Fig. 5).

Based on LSU sequencing, most Basidiomycota, Chytridiomycota, and Zygomycota fungal OTUs formed well-defined clusters, whereas the Ascomycota classes did not (Fig. 6). Most of the unknown assigned taxa were within the Ascomycota, with the majority placed within a Dothideomycetes or Leotiomycetes cluster (Fig. 6). Sordariomycetes (16–21%), Dothideomycetes (16–19%), and Leotiomycetes (13–22%) were the most represented classes in the Ascomycota. The Agaricomycetes (13–14%), Tremellomycetes (2–7%), and Microbotryomycetes (1–2%) were the most represented classes in the Basidiomycota, and the most represented class in the zygomycotan fungi was Mortierellomycetes (0.4–4%) (Fig. 6).

4. DISCUSSION

We evaluated detrital fungal communities from stream and peatland habitats using culture-dependent and environmental DNA sampling approaches. We found some strong differences in the community characterization between the 2 methods and some consistent ecological signals between stream and peatland habitats. Using these approaches together deepened our understanding of the fungal communities within these habitats and appreciation of biases presented by both laboratory techniques.

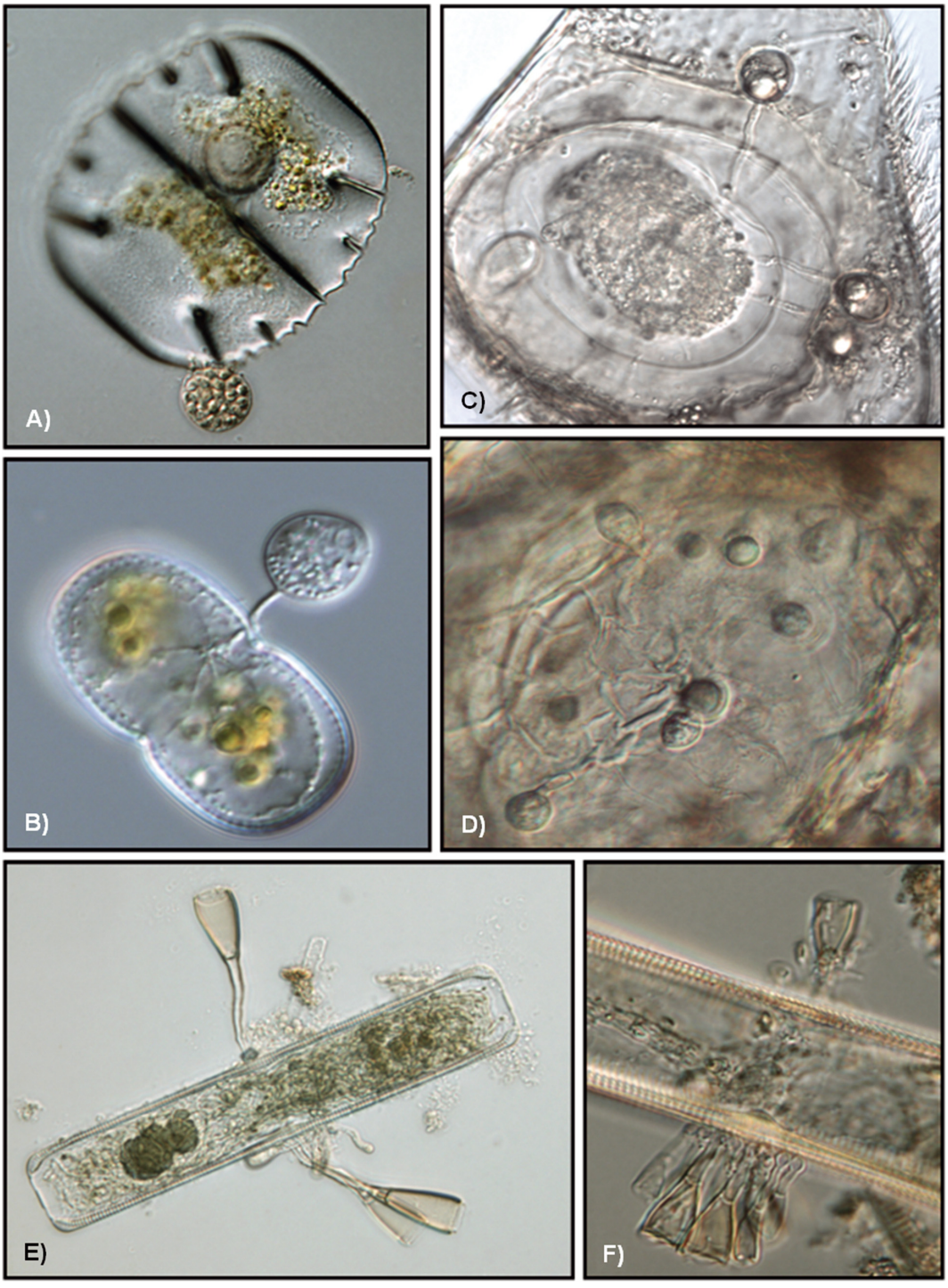

Fig. 5. Chytrids found in peatlands. (A,B) eucarpic chytrids on *Micrasterias truncata*; (C) eucarpic chytrid infecting an arthropod egg; (D) unknown *Polychytrium* chytrid infecting pollen; (E,F) unidentified *Podochytrium* chytrid infecting a *Pinnularia* sp.

The results met the 4 goals for the study: (1) use culture-dependent methods to isolate fungi from each habitat, (2) isolate environmental DNA from fine detritus samples and use multiple genetic markers to characterize the fungal communities, (3) compare culture-dependent and culture-independent techniques, and (4) determine environmental factors that influence community composition.

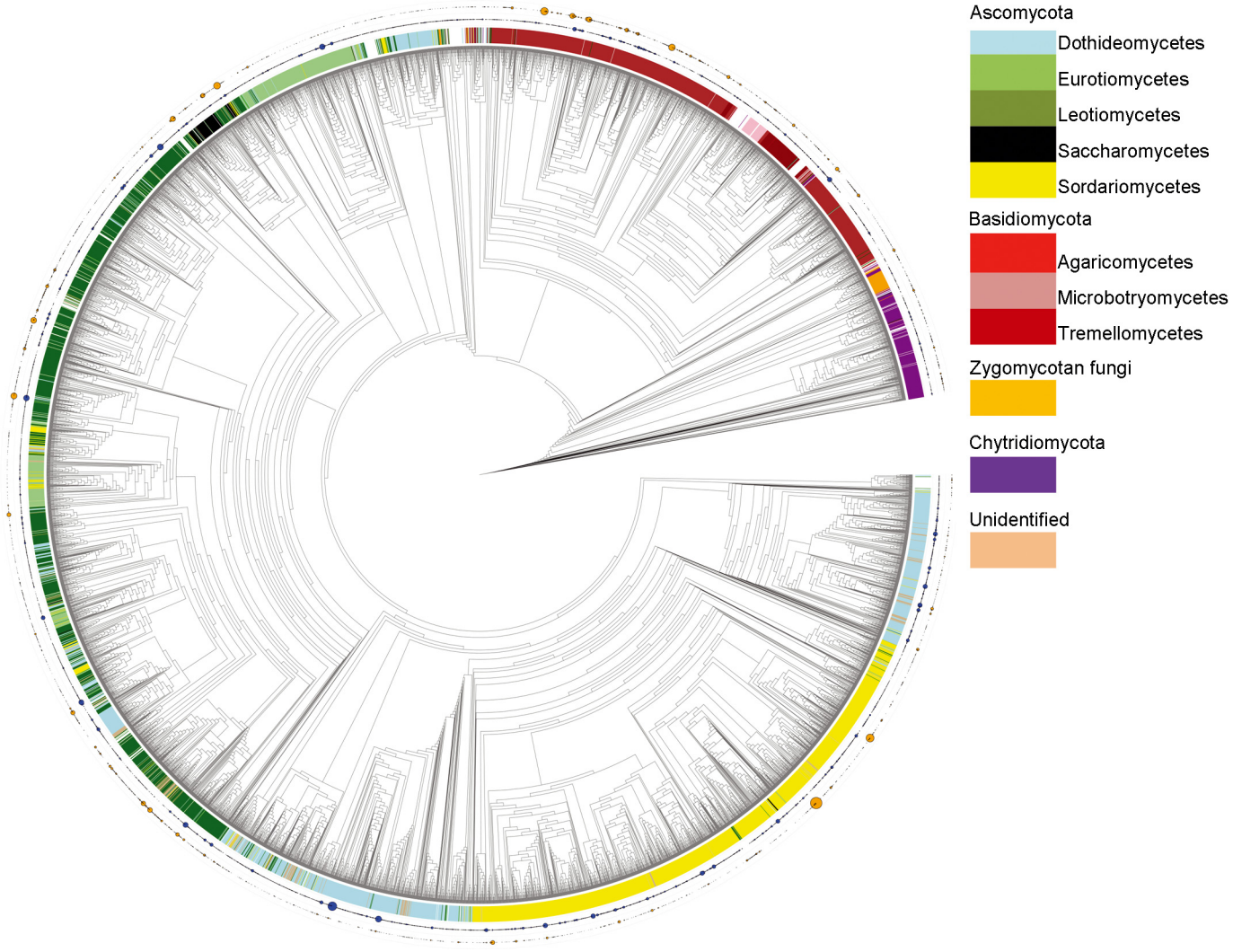

Fig. 6. Circular cladogram of fungal operational taxonomic units (OTUs) constructed in EvolView from post-processed RAxML analysis using PASTA based on partial LSU Illumina sequence data. Inner bar color indicates fungal group for each OTU. Outer circles represent relative OTU abundance of each OTU per habitat with blue indicating stream and orange indicating peatlands. Numerous unidentified OTUs are located throughout the Dothideomycetes and Leotiomycetes with an unknown Leotiomycetes cluster within the peatland sites and an unknown Dothideomycetes cluster within the stream sites. Cladogram suggests incorrect taxonomic assignment for some OTUs throughout all Ascomycota classes

4.1. Cultured fine detritus fungal communities

Phylum Ascomycota contained the largest portion of cultures, representing 5 classes from streams and bogs: Sordariomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Saccharomycetes. Sordariomycetes dominated cultured isolates as well as stream samples, with twice as many Sordariomycetes isolates than peatland samples (Fig. 2), consistent with Sordariomycetes being one of the 3 dominant classes within these ecosystems (Kujala et al. 2018, Wurzbacker et al. 2016). Dothideomycetes species made up the second largest Ascomycota class within both stream and peatland habitats (Fig. 4B). Dothideomycetes were the largest, most diverse class within the Ascomycota, with the subclass Pleosporomycetidae containing the majority of the aquatic fungal lineages (McLaughlin & Spatafora 2015). Eurotiomycetes had similar levels of presence when cultured from stream and peatland habitats.

Saccharomycetes species have a global distribution and are found in many habitats including freshwater and marine environments (McLaughlin & Spatafora 2015). Similarly, we found Saccharomycetes across habitats, but they were not as common as the other Ascomycota classes. Within Saccharomycetes,

Candida and *Pichia* were the most common genera found in peatlands (Thormann et al. 2007). In streams, *Candida* species were the most common species found on decaying leaves (Hagler et al. 2017). Golovchenko et al. (2013) indicated that *Candida* species were considered rare, occurring in 10–30% of collected samples. Wurzbacher et al. (2016) found Saccharomycetes species with similar abundance to Golovchenko et al. (2013) in coarse and fine particulate organic matter within streams, whereas Ahearn et al. (1968), during a survey of aquatic yeasts in Florida, USA, found that *Candida* species were widespread. Although Leotiomycetes was the dominant Ascomycetes class within the peatland sites and the third largest class within the stream sites, further class-level analyses would be needed to support a habitat preference because the phylogeny within Leotiomycetes needs further refinement (McLaughlin & Spatafora 2015, Johnston et al. 2019).

Basidiomycota were less common in both habitats, but the most frequently cultured classes were Microbotryomycetes and Tremellomycetes. Within Basidiomycota, peatland habitats had a greater presence of Tremellomycetes than streams (Fig. 2). Cultured Microbotryomycetes were similar between streams and peatlands. Several Mortierellomycetes cultures were obtained from stream samples. Mortierellomycetes currently contain the genera *Aquamortierella*, *Dissophora*, *Gamsiella*, *Mortierella*, *Modicella*, and *Lobosporangium* (Wijayawardene et al. 2018) and are frequently found in aquatic habitats (Kinsey et al. 2003, Thormann et al. 2003). Mortierellomycetes are saprotrophs but have been isolated from plant roots from many habitats (Watanabe 2010). Ectomycorrhizal Agaricomycetes lineages were not expected to be within our stream detritus. However, small tree root tips were visualized upon microscopic examination of several stream detrital samples, presumably from trees near the stream, providing a reasonable explanation for their presence within the environmental sequencing data.

Chytridiomycota are microscopic fungi found in terrestrial and aquatic habitats that are important decomposers of particulate organic matter and occur as plant and animal parasites (Gleason et al. 2008, Kagami et al. 2014). Although no chytrid cultures were obtained, microscopic evaluation of habitat samples revealed chytrids in peatland, but not stream, samples. Chytrids found in peatland samples included eucarpic chytrids on *Micrasterias truncata* Brébisson ex Ralfs, eucarpic chytrids infecting an arthropod egg, an unidentified *Podochytrium* chytrid infecting a *Pinnularia* sp., and a *Polychytrium* chytrid infecting pollen (Fig. 5). These findings are consistent with previous research indicating the importance of chytrids as pathogens and decomposers within peatland habitats. Ittner et al. (2018) indicated that chytrids are unique in their ability to degrade the outer pollen wall to obtain nutrients, and James et al. (2006) indicated the ubiquitous nature of chytrids in aquatic ecosystems and their importance as algal pathogens and saprobes.

4.2. Sequencing detrital fungal communities from environmental DNA

The majority of OTUs determined from environmental sequencing were assigned to the phylum Ascomycota. However, Ascomycota assignments also contained the most perceived sub-phylum-level error based on OTU LSU taxonomic assignment and subsequent phylogenetic analyses (Fig. 5). Recent research by Vu et al. (2019) reviewed the taxonomic resolution of LSU as it relates to a broad range of filamentous fungi and indicated that LSU could not discriminate 18% of the fungal species they examined. Therefore, because there was only a 3% difference in OTU relative abundance in this study among the largest Ascomycota classes (Dothideomycetes, Leotiomycetes, and Sordariomycetes) between the 2 habitats (Fig. 4B), we caution that this can only be interpreted as a minimum estimate of the diversity of Ascomycota in each habitat.

Within the environmental sequencing data, Dothideomycetes was the most abundant Ascomycota class. Within subclass Dothideomycetes, it is likely that at least one unknown peatland OTU cluster represented the genus *Meristemomyces* based on NCBI BLAST results. Interestingly, only 2 *Meristemomyces* species have been isolated. *M. frigidus* was isolated from within rocks (endolithic) in the Himalayas (Egidi et al. 2014), while *M. arctostaphyli* (Crous et al. 2019) was isolated from *Arcotostaphylos patula* leaves, an ericaceous plant, in Utah, USA. Peatlands would represent a new habitat for *Meristemomyces*, which seems plausible due to the presence of other cold-adapted fungal species and numerous ericaceous plant species within these peatlands. Sordariomycetes was the second most abundant Ascomycota class. Within Sordariomycetes, Hypocreales, Sordariales, and Xylariales contained the most species (Table S1). In addition, both ITS2 and TUB data sets were important in capturing more of the Sordariomycetes species because several genera (e.g. *Biscogniauxia*, *Diapor-*

thales, *Whalleya*) were not captured using ITS1. Ectomycorrhizal Agaricomycetes OTUs were identified, matching microscope observations.

Fewer Basidiomycota taxa were present compared to Ascomycota, but peatland systems supported more Basidiomycota than streams. Environmental sequencing data indicated Agaricomycetes was the largest Basidiomycota class, although only one Agaricomycetes taxa was cultured (*Tyromyces chioneus*). The low representation of cultured Agaricomycetes species was expected due to known culture biases (Collado et al. 2007). The environmental sequencing results are consistent with previous research (Zhang et al. 2017, Kujala et al. 2018) on the fungal communities of peatlands in China and Finland. In this study, Tremellomycetes was the second largest Basidiomycota class, which agrees with Wurzbacher et al. (2016). They are found in both terrestrial and aquatic habitats and are typically isolated in a yeast stage (McLaughlin & Spatafora 2014). The genus *Cryptococcus* contained numerous OTUs and has been frequently isolated from peatland habitats (Golovchenko et al. 2013) and is also frequently encountered in environmental sampling of both peatland and stream habitats (Wurzbacher et al. 2016, Zhang et al. 2017, Kujala et al. 2018). Microbotryomycetes represents the smallest of the 9 most abundant classes in this study with about 2% of the total OTUs. Sporidiobolales, found in both peatlands and streams, was the most represented order with 8 species. Cultured Microbotryomycetes were similar between streams and peatlands, but environmental sequencing showed greater abundance of Microbotryomycetes in peatlands.

Phylum Mucoromycota was represented with 5 genera: *Bifiguratus*, *Endogone*, *Mucor*, *Rhizomucor*, and *Umbelopsis*. *Endogone* species were only found within peatland habitats, which is consistent with the fact that these species are generally found in humus-rich soils, leaf mold, or associated with mosses (Williams & Finney 1964). *Bifiguratus adelaidae* T. J. Torres-Cruz & A. Porras-Alfaro has been previously isolated from soils and as a moss endophyte (U'ren et al. 2010) and was identified in peatland and stream habitats. *Mucor* species have been previously reported in peatland and stream habitats as saprobes (Golovchenko et al. 2013, Seena et al. 2019), although it should be noted that Seena et al. (2019) found a large percentage of Mucoromycota on decaying leaves. Importantly, all *Mucor* OTUs were identified using the ITS2 data set, demonstrating a potential issue with the ITS1 primer pairs for this genus. *Rhizomucor* was only identified from culturing techniques and *Umbelopsis* was identified within the ITS1, ITS2, and culture data sets. The phylum Monoblepharomycota are zoosporic fungi with oogamous sexual reproduction and are only known as saprobes (McLaughlin & Spatafora 2014). Representative OTUs were found in all peatland sites and one stream site.

In this study, the class Mortierellomycetes within phylum Mortierellomycota represented 3% of the ITS1 OTUs, with most OTUs found within the stream habitats. Several Mortierellomycetes cultures were obtained but neither of the 2 most abundant stream OTUs were cultured. Mortierellomycetes currently contain the genera *Aquamortierella*, *Dissophora*, *Gamsiella*, *Mortierella*, *Modicella*, and *Lobosporangium* (Wijayawardene et al. 2018) and are frequently found in aquatic habitats (Kinsey et al. 2003, Thormann et al. 2003). Mortierellomycetes are saprotrophs but have been isolated from plant roots from many habitats (Watanabe 2010). This study also found Chytridiomycota representative OTUs in 4 classes (Chytridiomycetes, Lobulomycetes, Rhizophydiomycetes, and Spizellomycetes) with the most represented genera consisting of *Lobulomyces* (peatland), *Odontochytrium* (peatland and stream), and *Rhizophydium* (peatland and stream), matching microscope observations.

4.3. Culture-dependent and culture-independent comparison

Our study found that it is possible to obtain a high overlap between the culture-dependent and culture-independent components of the same study: 93% of our isolates were found within the culture-independent ITS2 representative OTUs, although this represents only a small percentage of the ITS2 representative OTUs with taxonomic assignment (25%). This finding is not surprising, as many fungi are still considered unculturable and are only known from environmental sequences.

Culture-independent techniques have certain issues: (1) no single gene target is perfect, (2) there are database limitations with taxonomic assignment, and (3) any DNA within the sample can be sequenced without regard to the state of the organism (inactive, actively growing, or dead) (Tedersoo et al. 2015). Recently, Tedersoo et al. (2015) determined that both LSU and SSU had lower species-level discrimination power compared to the ITS region, likely because they are more conserved. In

addition, long introns within the ITS region can prevent amplification and sequencing, and it has been noted that next-generation sequencing technology preferentially amplifies shorter sequences (Tedersoo et al. 2015).

The aim of this study was to determine fungal community composition within a phylogenetic framework requiring the use of multiple regions, including ITS1, ITS2, LSU, and TUB. Although ITS2 was preferred by Tedersoo et al. (2015), our study found that ITS2 had greater OTU inflation and did not capture as much of the community as the ITS1 region. In addition, the ITS region cannot be aligned across the entire fungal kingdom, so the LSU region was beneficial for determining the phylogenetic placement of unidentified fungal OTUs. Our study highlights the utility of the Fluidigm Amplification platform as a tool to characterize fungal communities using multiple gene targets permitting a greater number of community analyses, in addition to providing data sets that can be integrated into different single gene target specific studies (i.e. those that used only ITS1, ITS2, or LSU).

This study also highlights that current methods/databases used for taxonomic assignment of OTUs have limitations. Current sequence databases do not contain all of the known fungi and are not equally represented among the various taxa (Bruns 2001). Numerous incorrect class-level taxonomic assignments can be seen within the kingdom level LSU cladogram (Fig. 6), and these incorrect assignments make downstream taxonomic assignment-based analyses prone to misinterpretation, particularly in conjunction with OTU inflation. We believe that placing OTUs within a phylogenetic framework for analyses provides a valuable method to interpret community sequence data, allows visualization of misidentifications, and mitigates issues associated with taxonomic assignment-based analyses.

4.4. Environmental factors that influence community composition

We demonstrated that peatland and stream fungal communities are different and that individual sampling site was the most important factor, followed by habitat. In addition, phylogenetic analyses using the generalized Unifrac 0.5 distance matrix indicated that there is a phylogenetic component to species distribution in peatland and stream communities. Our results are not consistent with Duarte et al. (2016), who found high similarity between geographically distant fungal communities in similar climatic zones. In our study, the measured physical and chemical properties (Table 1) showed similar abiotic conditions (peatlands: low NO_3, pH 4.8–6.5, and <20% dissolved oxygen; streams: 0–20 ppm NO_3, pH 7–8.2, and >80% dissolved oxygen) for each habitat. Therefore, more rigorous physical and chemical analyses of the detritus will be needed to elucidate the differences between these microhabitats.

5. CONCLUSIONS

This research represents the first in-depth study using multiple genetic markers to provide a more complete and refined fungal inventory of fine detritus within peatlands and streams. We identified several limitations when using a single genetic marker and recommend using multiple genetic markers to capture more of the fungal community. We also provided additional evidence that current environmental sequencing taxonomic determinations, particularly within the Ascomycota, need further refinement.

All sites in this study contained numerous unidentified, abundant taxa but stream habitats contained more species, had greater phylogenetic diversity, and possessed greater phylogenetic distinctiveness compared to peatland habitats. Site variation had the greatest impact on community structure, followed by habitat and geographical distribution. We note that it can be difficult to determine which fungal species are utilizing fine detritus for habitat, nutrition, or are simply a part of the detritus. However, by utilizing a phylogenetic framework with environmental DNA, we can visualize habitat-specific OTU clusters that may provide new perspectives in elucidating their roles in these important ecosystems.

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