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Rivers may constitute an overlooked avenue of dispersal for terrestrial fungi

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ABSTRACT

While fungi are intimately associated with substrates in freshwater systems, the role of fungi in the open water column is less well defined. Using next generation sequencing of 0.2 μm–1 μm filtered water column samples, we detected abundant and diverse fungal sequences across 25 stream and river sites in the Ozark region of Oklahoma and Arkansas. Fungal communities were only weakly related to stream environmental metrics with the exception of total phosphorus (TP). We infer from our results that TP is acting as a proxy for unique catchment effects. We observed patterns of dominant community taxa at higher taxonomic groupings but lower taxonomic groupings were site specific. OTU functional assignment showed the majority of sequences to be related to plant and animal pathogens, and some saprotrophs. The likely allochthonous origin and strong site specificity of these fungi suggest overlooked dispersal via lotic waterways, which may have important biogeographic consequences for fungi.

1. Introduction

Fungi in aquatic ecosystems have been extensively studied, typically focusing on substrate surfaces such as allochthonous leaf litter in freshwater in the context of decomposition (Suberkropp and Klug, 1976; Nikolcheva and Bärlocher, 2005; Sridhar et al., 2008). One of the topics that has been largely neglected is fungi in the water column not associated with substrates or sediment. One study that assessed fungal biomass in the upper 1 m of the water column in 32 temperate streams in Poland found a significant correlation between fungal biomass and total nitrogen (N) and phosphorus (P) in water using regression and Pearson correlation analysis (Gorniak et al., 2013). Community structure and role were not directly investigated but direct fungal participation in water column nutrient cycling was hypothesized (Gorniak et al., 2013).

Another possible explanation for fungal presence in the water column is simply the deposition of hyphal fragments or other potential propagules from air, upstream water, detritus deposition, and root to stream contact. Water column dispersal is well studied for aquatic hyphomycetes (Ingold, 1942; Thomas et al., 1991; Suberkropp and Wallace, 1992; Sridhar and Bärlocher, 1994) but such inputs could provide an overlooked means of dispersal for terrestrial fungi as well. In fact, fungus-like oomycete plant pathogens of the genus *Phytophthora* are well known to disperse via river systems (Li, 2016). Recent studies clearly show that some true fungi are dispersal-limited (Peay et al., 2012; Cline and Zak, 2014; Peay and Bruns, 2014), leading to strong biogeographic distribution patterns (Taylor and Bruns, 1999; Peay et al., 2010).

Most work on dispersal in terrestrial fungi has focused on movement of aerial spores (Brown and Hovmøller, 2002; Pashley et al., 2012; Savage et al., 2012; Grinn-Gofroń and Bosiacka, 2015), including next generation sequencing studies of indoor air (Amend et al., 2010; Adams et al., 2013). While aquatic hyphomycetes have received attention with respect to diversity of decomposers and macroscopic life stages (Fabre, 1998a, 1998b, 1998c), there have been few studies of the roles of river systems in the dispersal of true fungi in general, particularly by very small propagules. Given the fact that streams and rivers serve as ecological aggregators of processes throughout their watershed catchments (Frissell et al., 1986; Allan, 2004; Bormann and Likens, 2012), and that riverine dispersal

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is important in numerous other taxa, e.g. fish, insects, reptiles, and plants (Maguire, 1963; Bermingham and Avise, 1986; Bunn and Hughes, 1997; Bernatchez and Wilson, 1998; Miller et al., 2002; Santamaría, 2002; Petersen et al., 2004; Pellegrino et al., 2005; Vanschoenwinkel et al., 2008), the lack of fungal studies represents a major gap.

A potential hurdle to investigating dispersal via the water column is determining whether or not fungi detected are active aquatic community members or are transient and inactive. There is a large body of evidence linking fungi to the phosphorus (P) cycle in soils (Bolan, 1991; Schachtman et al., 1998; Van Der Heijden et al., 2008). High total phosphorus (TP) is also an indicator of excessive nutrients from catchments feeding into streams and rivers (Schindler, 1977; Carpenter et al., 1998) and has a large impact on aquatic systems (Bennett et al., 2001; Anderson et al., 2002; Hart et al., 2004). Exploring fungi across a gradient of TP allows identification of relationships of free living fungal communities to P in the water column.

The bulk of water-column particulate matter consists of eroded soils and particulate organic matter (Schlesinger and Melack, 1981; Waters, 1995; Bilotta and Brazier, 2008). Fungi are both ubiquitous in soils and are directly involved in colonizing and decomposing organic matter in streams (Christensen, 1989; Gessner, 1997). To focus on dispersal via small fungal cells, fragments, and spores in the water column, it is desirable to exclude fungi associated with particles using a method like size filtering (APHA, 1998). Here, we analyze total fungal diversity in a microscopic fraction (0.2–1.0 μm) across a river system spanning a range of TP. While many fungal cells are larger than 1 μm, we anticipated good detection of fungi through small cells, spores, and cell fragments. The ecological gradient is representative of differences in multiple catchment properties such as vegetation and nutrient cycling across the varied watersheds enhancing the exploration of relationships between fungal communities and the environment in the system.

2. Methods

2.1. Sampling

Extraction of genomic DNA (gDNA) from water-column filter samples was described in detail in LeBrun et al., (2017). The study area was a collection of mid-order (3^{rd}-5^{th}) streams and rivers along the Oklahoma-Arkansas border, an area with known P enrichment problems (Fig. S1) (Green and Haggard, 2001; Haggard and Soerens, 2006; Haggard, 2010). Sampling was performed in October of 2014. Additional site characteristics are also available through a study by Cook et al., 2017 (*in press*) where data on these sites was collected at regular intervals over 2 y. The sampling sites represented a gradient of TP levels from 7 to 173 μg/L. Sample processing involved a stacked filtration of 50 mL of water from ~10 cm below the surface in the water column through a 1 μm glass fiber filter and then a 0.2-μm filter. Only components collected on the 0.2-μm filters were used in extracting the gDNA for this study (i.e. the 0.2–1.0 μm size fraction).

2.2. Environmental data

Environmental data including dissolved organic carbon (DOC), total phosphorus (TP), total N (TN), C:N ratio, C:P ratio, sestonic chlorophyll-a, total suspended solids (TSS), turbidity, pH, dissolved oxygen (DO), temperature, and specific conductance in stream/river water along with catchment size and catchment and land use factors including percentage pasture land, percentage impervious cover, percentage developed land were collected as reported in LeBrun et al., (2017). In brief, water chemistry was measured using YSI EXO2 multiparameter data sonde (Yellow Springs, OH) and standardized water testing (APHA, 1998). Catchments were delineated using ArcGIS and land usage was estimated from the National Land Cover Data (NLCD) raster (ESRI, 2011. *ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute.*, n. d.).

2.3. Library preparation

Library preparation for this study started with the gDNA collected in LeBrun et al. (2017). An initial PCR amplification of the ITS2 region was conducted using 5.8S_Fun and ITS4_Fun primers (Taylor et al., 2016) modified to include adapters for future indexing. PCR was performed using 2× Platinum™ Green Master Mix from Invitrogen. PCR specifications were 1 cycle for HotStart step at 94 °C for 2 min followed by 30 cycles of 94 °C denaturation step for 45 s, 50 °C annealing step for 1 min, and 72 °C elongation step for 1.5 min. Successful PCR was identified through 1% agarose gel electrophoresis run at 70 V for 40 min. PCR cleanup was conducted using an Agencourt AMPure XP kit (Beckman Coulter Life Sciences) following the manufacturer's protocol. Final PCR product quantification was conducted using a Qubit 3.0 system.

A second round of PCR amplification was run to add unique indices to each sample as well as Illumina sequencing adapters. PCR was again performed using 2× Platinum™ Green Master Mix. PCR specifications for the second round of PCR were 1 cycle for HotStart step at 94 °C for 2 min followed by 8 cycles of 94 °C denaturation step for 45 s, 59 °C annealing step for 1 min, and 72 °C elongation step for 1.5 min. Cleanup and quantification were performed in the same manner as the first round of PCR. Samples were then pooled so that 10 ng of DNA from each sample was present in final library.

Sequencing was performed on an Illumina MiSeq system using a MiSeq Reagent Kit v3 2 × 300 with paired-end reads. Libraries were spiked with 20% PhiX control.

2.4. Sequence processing

Demultiplexing was conducted through Illumina BaseSpace. Paired-end read FASTQ files for each sample were extracted for downstream processing. Additional sequence processing was carried out using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010). Paired-end reads were combined using the fastq-join algorithm from ea-utils (Aronesty, 2013). Un-paired reads were discarded at this time. The resulting sequences were then filtered with a maximum unacceptable Phred quality score of 20. Chimeric sequences were identified and removed using the UCHIME algorithm within USEARCH (Edgar, 2010). Operational taxonomic unit (OTU) picking was performed via open reference with a 0.50 pre-filter using UCLUST against the dynamic UNITE database version 7 with a 0.94 similarity cutoff. Singleton sequences were removed during OTU picking and taxonomy was assigned with the UNITE database as reference. Reads identified as Plantae or Protista were then manually removed from the resulting OTU table via filtering.

Functional information in the form of guild assignment to OTUs was performed using the online version of FUNGuild (Nguyen et al., 2016). FUNGuild parses OTUs into “guilds” or “functional groupings” based on their taxonomic assignments and ecological data extracted from the literature (Nguyen et al., 2016). Guilds are representative of species, whether related or unrelated, that exploit the same class of environmental resources in a similar way.

2.5. Statistical analysis software

All analyses were performed in the R software package v.3.2.3 (R Core Team, 2015) using various packages and scripts as identified. OTU table BIOM files from QIIME were either exported to tab delimited format directly from QIIME or imported for use in R using