Long- and short-read metabarcoding technologies reveal similar spato-temporal structures in fungal communities

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# Abstract

Fungi form diverse communities and play essential roles in many terrestrial ecosystems, yet there are methodolical challenges in xxx. We investigated spatio-temporal structure of a fungal community using soil metabarcoding with four different sequencing strategies: short amplicon sequencing of the ITS2 region (300–400 bp) with Illumina MiSeq, Ion Torrent Ion S5, and PacBio RS II, as well as long amplicon sequencing of the full ITS and partial LSU regions (1200–1600 bp) with PacBio RS II. Resulting community structure and diversity depended more on statistical method than sequencing technology. The use of long-amplicon sequencing enables construction of a phylogenetic tree from metabarcoding reads, which facilitates taxonomic identification of sequences. However, long reads present issues for denoising algorithms in diverse communities. We present a solution that splits the reads into shorter homologous regions prior to denoising, and then reconstructs the full denoised reads. In the choice between short and long amplicons, we suggest a hybrid approach using short amplicons for sampling breadth and depth, and long amplicons to characterize the local species pool for improved identification and phylogenetic analyses.

# Introduction

Ectomycorrhizas (ECM) form a symbiosis between fungi and plants. The fungi provide nutrients and protection from pathogens in exchange for carbon from the plant (Smith & Read, 2010). Approximately 8% of described fungal species are believed to take part in ECM symbiosis (Ainsworth, 2008; Rinaldi, Comandini, & Kuyper, 2008). Although only about 2% of land plant species form ECM, these include ecologically and economically important stand-forming trees belonging to both temperate/boreal groups such as Pinaceae and Fagaceae, and tropical groups such as Dipterocarpaceae, and Fabaceae tr. Amherstieae (Brundrett, 2017).

Although many ECM fungi form well-known mushrooms (e.g., *Amanita*, *Cantharellus*, *Boletus*), some instead produce inconspicuous (e.g., *Tomentella*) or no (e.g., *Cenococcum*) fruit bodies. Even when fruitbodies are large, they are ephemeral, so study of ECM communities is facilitated by looking at vegetative structures (Horton & Bruns, 2001). Unlike many saprotrophic fungi which grow easily in axenic culture, ECM fungi are usually difficult to culture, so DNA barcoding is increasingly used to investigate vegetative structures. The advent of high-thoughput sequencing (HTS) has facilitated such studies by providing enough sequencing depth for metabarcoding of bulk environmental samples such as soils (Lindahl et al., 2013).

As additional techniques and methods are developed for HTS, there is an increasing array of choices for researchers investigating fungal communities. Traditionally, fungal metabarcoding studies have targetted the ITS1 or ITS2 regions, which provide sufficient resolution to distinguish fungal species in many groups, and which are usually short enough for HTS (Lindahl et al., 2013; Schoch et al., 2012). The resulting sequencing reads are clustered by sequence similarity to form operational taxonomic units (OTUs), which are then used as the units for further community analysis (Lindahl et al., 2013). If taxonomic identification is desired in order to put OTUs in a wider context and associate functional information, it has usually been performed by database searches using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990; Lindahl et al., 2013). However, this traditional approach comes with some potential weaknesses.

While ITS1 and ITS2 often have suitable variation to distinguish species, they cannot be reliably aligned over the fungal kingdom (Lindahl et al., 2013; Tedersoo et al. 2018 Fungal div). Additionally the wide range of length variation of these regions may introduce bias in recovery of different taxa (ref). Further bias is introduced by variation in the 5.8S region which separates the two ITS regions, which makes it difficult to design primers that are suitable for all fungi (Tedersoo et al., 2015). The recent long-read HTS technologies such as Pacific Biosciences Single Molecule Real Time sequencing (PacBio) enable sequencing longer amplicons, including both the ITS regions and the flanking, more conserved SSU and/or LSU regions (Tedersoo et al., 2018). This can potentially improve taxonomic placement of sequences that lack close database matches and the alignment of metabarcoding reads for further phylogenetic analysis. However, long-read technologies are currently more expensive per read, and so their use entails a trade-off with sequencing depth and/or sample number (Kennedy, Cline, & Song, 2018).

In addition xxx. Distance-based clustering conflates intra-species variation and sequencing error, and results are dataset-specific. In contrast, more recent denoising methods such as DADA2 (Callahan, McMurdie, & Holmes, 2017), Deblur (Amir et al., 2017), and UNOISE2 (Edgar, 2016b) utilize read quality information to control for sequencing error while leaving intra-species variation. The resulting units are known as amplicon sequence variants (ASVs) or exact sequence variants (ESVs), as they should represent true amplicon sequences from the sample. Unlike cluster-based OTUs, ASVs can capture variation of as little as one base pair, and are not as dataset specific (Callahan et al., 2017).

Assignment of taxonomic identities using BLAST requires *a priori* choice of thresholds for different taxonomic ranks. Several algorithms specifically designed for taxonomic assignment have been published which use information about variability within different taxa in the reference database to assign unknown sequences, along with confidence estimates for these assignments (e.g., Wang, Garrity, Tiedje, & Cole, 2007; Edgar, 2016a; Murali et al., 2018a). In addition, methods have been published which integrate predictions from multiple algorithms to increase the reliability of assignments (Gdanetz, Benucci, Vande Pol, & Bonito, 2017; Somervuo, Koskela, Pennanen, Henrik Nilsson, & Ovaskainen, 2016).

Long-amplicons allow phylogenetic analysis xxx. The resulting information has the potential to both improve taxonomic assignment and provide alternative measures of community alpha and beta diversity. Because OTU clustering may both “clump” different species into a single OTU, and “split” a single species into multiple OTUs (Ryberg, 2015), diversity measures based on counting species within a community or shared species between two communities may give different results depending on the clustering threshold. In contrast, phylogenetic community distance measures like UNIFRAC (Wong, Wu, & Gloor, 2016) are relatively insensitive to species/OTU delimitation, but require a phylogenetic tree. Phylogenetic placement algorithms have been developed to place short amplicon reads onto a reference tree (Berger, Krompass, & Stamatakis, 2011; Matsen, Kodner, & Armbrust, 2010), but are not easy to apply to ITS sequences because they require that the query sequences be aligned to a reference alignment. Additionally, methods exist to place OTUs on a simplified tree based on taxonomic assignments (Tedersoo, Sánchez-Ramírez, et al., 2018). However, long amplicon sequencing allows the inclusion of alignable regions for construction of more fully resolved phylogenetic trees directly from metabarcoding reads.

Here we investigated the effects of different sequencing strategies and post-analysis on biological conclusions using measurement of the spatial community turnover rate along a transect in an ECM Soudanian woodland in Benin. We investigated turnover range in both the total fungal community and ECM community by metabarcoding of bulk soil, sampled at narrow intervals, over two years. We compare three different sequencing platforms (PacBio RS II, Illumina MiSeq, Ion Torrent Ion S5), long and short amplicons, three different taxonomic assignment algorithms (RPP classifier, SINTAX, IDTAXA) and reference databases (Unite, Warcup, RDP), and two different community distance measures (Bray-Curtis vs. weighted UNIFRAC). We also present new algorithms for dividing the rDNA into regions, combining denoising results from multiple regions, and incorporating phylogenetic information into taxonomic assignments.

Turnover scale is the distance at which two communities can be considered to be independent samples of the local species pool. Knowledge of turnover scale is important when planning studies of local diversity and its environmental correlates. It varies between different ecosystems and taxonomic groups. Turnover scale is often measured by the range at which a Mantel correlogram indicates significant autocorrelation, or by fitting a function to an empirical distance-decay curve community dissimilarity vs. distance (Legendre & Legendre, 2012).

Several studies have investigated the turnover scale in ECM forests, but so far they have been based on analysis of ECM root tips, rather than bulk soil. Lilleskov, Bruns, Horton, Taylor, & Grogan (2004) found autocorrelation only at ranges <2.6 m at most sites in temperate forests, based on Sanger sequencing. Similarly, Pickles, Genney, Anderson, & Alexander (2012) found autocorrelation at distances <3.4 m, also in temperate forests, based on T-RFLP. Two studies have included data from tropical African woodlands and wooded savannahs. Tedersoo et al. (2011) found autocorrelation at ranges <10 m in Miombo woodland based on Sanger sequencing. However, this study was based on irregular sampling with a minimum spacing of 8 m, so this as the smallest distance class. Bahram et al. (2013) performed a meta-analysis of turnover scale in ECM communities, including data from Benin, where they found turnover range of 65 m based on distance-decay curves. They concluded that the range of distance decay is greater in tropical forests than temperate forests.

We investigated turnover range in both the total fungal community and ECM community by metabarcoding of bulk soil, sampled at narrow intervals, over two years. We compare three different sequencing platforms (PacBio RS II, Illumina MiSeq, Ion Torrent Ion S5), long and short amplicons, three different taxonomic assignment algorithms (RPP classifier, SINTAX, IDTAXA) and reference databases (Unite, Warcup, RDP), and two different community distance measures (Bray-Curtis vs. weighted UNIFRAC). We also present new algorithms for dividing the rDNA into regions, combining denoising results from multiple regions, and incorporating phylogenetic information into taxonomic assignments.

# Methods

## Sampling

Sampling was conducted at two sites (Ang: N 9.75456° W 2.14064°; Gan: N 9.75678° W 2.31058 °) approximately 30 km apart in the *Forêt Reservée de l’Ouémé Supérieur* (Upper Ouémé Forest Reserve) in central Benin. Both locations were located in woodlands dominated by the ECM host tree *Isoberlinia doka* (Caesalpinioideae). At each site, 25 soil samples were collected along a linear transect at intervals of 1 m in May 2015. One third of the sample locations (3 m spacing) were resampled one year later in June 2016. For each sample, any coarse organic debris was removed from the soil surface and a sample of approximately 5cm×5cm×5cm was extracted with a knife blade. Each sample was sealed in a plastic zipper bag and homogenized by shaking and manually breaking apart soil aggregations. Approximately 50 mg total of soil was collected from two locations in the homogenized soil sample and placed into a separate 2.0 mL microtube containing 750 mL of lysis buffer and lysis beads (XpeditionTM Soil/Fecal DNA miniprep, Zymo Research Corporation, Irvine, California, USA) and lysed in the field using a handheld bead-beater (TerraLyserTM; Zymo Research Corporation).

An additional sample was collected at every sampling location (1-m spacing) in 2016 using LifeGuardTM Soil Preservation Solution (MO BIO, Carlsbad, CA; USA) for preservation, without field lysis. Sequencing results for these samples significantly? differed from results preserved using the XpeditionTM lysis buffer (Supplementary Figures S11 and S12); as such these samples were excluded from our spatial analyses. However, reads from these sequences were included in the full bioinformatics workflow, including ASV calling, OTU clustering, and phylogenetic trees.

## DNA extraction, amplification, and sequencing

After field lysis, DNA was extracted using the XpeditionTM Soil/Fecal Prep kit (see above). Samples preserved using LifeGuard were first centrifuged at 10000×g for 1 minute, after which the supernatant was removed and DNA was extracted from the remaining soil using the Soil/Fecal Prep kit as for the other samples. DNA was quantified using fluorometrically using Quant-iTTM PicoGreenTM dsDNA (Thermo Fisher Scientific, Waltham, MA, USA) fluorescent indicator dye on a Infinite F200 plate spectrofluorometer (Tecan Trading AG, Männedorf, Switzerland) according to the manufacturer’s protocol.

Two different fragments of the nuclear rDNA were amplified (Figure 1). The short amplicon (approximately 300 bp) targetted the full ITS2 region as well as parts of the flanking 5.8S and large subunit (LSU) rDNA, using gITS7 (Ihrmark et al., 2012) as the forward primer and a mix of ITS4 (White, Bruns, Lee, & Taylor, 1990) and ITS4a (Urbina, Scofield, Cafaro, & Rosling, 2016) as the reverse primer (hereafter, ITS4m). The long amplicon (approximately 1500 bp) targetted the full ITS region including the 5.8S rDNA and approximately 950 bp at the 5’ end of the LSU, including the first three variable regions (Figure 1), using ITS1 (White et al., 1990) as the forward primer and LR5 (Vilgalys & Hester, 1990) as the reverse primer.

The gITS7 primers for the short amplicon were indexed for multiplexing (**Supplementary File**). Amplification was performed by polymerase chain reaction (PCR) in 20µl reactions containing 200 µM dNTP mix, 250 µM indexed gITS7 primer, 150µM ITS4m, 2mM MgCl2, 0.1 U *Taq* polymerase (Dream *Taq*, Thermo Fisher Scientific, Waltham, MA, USA) and 3–7 ng purified DNA in Dream *Taq* buffer. The reaction conditions were 10 min at 95°, followed by 35 cycles of 60 s at 95°, 45 s at 56°, and 50 s at 72°, and finally 3 min at 72°. Each reaction was conducted in three technical replicates to reduce the effect of PCR stochasticity, which were pooled after amplification.

Both primers for the long amplicon were indexed for multiplexing (**Supplementary File**). PCR was performed as for the short amplicons, but with 500 µM of each of the two primers. Reaction conditions were 10 min at 95°, 30 cycles of 45 s at 95°, 45 s at 59°, and 90 s at 72°, and finally 10 min at 72°. Each reaction was performed in three technical replicates as for short amplicons.

Each PCR run also included a blank sample and a positive control consisting of freshly extracted DNA from a commercially purchased fruitbody of *Agaricus bisporus*.

Amplicons were purified using SPRI beads (Vesterinen et al., 2016) and quantified fluorometrically as above. An aliquot of 100 ng of DNA from each sample (or the total PCR product if less than 100 ng) was pooled into two libraries each for long and short amplicons. Each library was sequenced using Single Molecule Real Time (SMRT) sequencing on a Pacific Biosciences (PacBio) RS II sequencer at the Uppsala Genome Center (UGC; Uppsala Genome Center, Science for Life Laboratory, Dept. of Immunology, Genetics and Pathology, Uppsala University, BMC, Box 815, SE-752 37 UPPSALA, Sweden). Short amplicon libraries were sequenced on two SMRT cells each, while long amplicon libraries were sequenced on four SMRT cells each.

Additionally the short amplicon libraries were combined and sequenced using an Ion S5 (Ion Torrent) sequencer using one 520 chip at UGC, and a MiSeq (Illumina Inc.) sequencer using v3 chemistry with a paired-end read length of 300 bp at the SNP&SEQ Technology Platform (Dept. of Medical Sciences, Uppsala University, BMC, Box 1432, SE-751 44 UPPSALA, Sweden). The Illumina library was pooled with samples for another project, with one half of the reads from one lane devoted to this project.

## Bioinformatics

Circular consensus sequence (CCS) basecalls for PacBio sequences were made using ccs version 3.4 (Pacific Biosciences, 2016/2019) using the default settings. The resulting sequences, as well as the paired-end Illumina sequences, were demultiplexed and sequencing primers were removed using cutadapt version 2.8 (Martin, 2011). Sequencing primers were similarly removed from the Ion Torrent sequences, but interference between the tagged gITS7 primers and the Ion XPress tags used in library prep made full demultiplexing of the Ion Torrent sequences impossible. For Ion Torrent and PacBio, reads were discarded if they did not have the appropriate primers on both ends. Reads were searched in both directions, and reads where the primers were found in the reverse direction were reverse complemented before further analysis. For Illumina sequences, primers were also searched for and removed on the 3’ ends of the reads, in case of readthrough with short amplicons. Read pairs were retained when PCR primers were detected at the 5’ ends of both the forward and reverse read. Read pairs where the primers were found in reverse orientation were kept in seperate files, but were retained in their original orientation until after denoising.

### Denoising

We attempted to denoise both long and short PacBio amplicons using DADA2 according to the steps outlined in the supplementary information in Callahan et al. (2019). However, only 38 amplicon sequence variants (ASVs) were obtained for the long amplicons, representing 12% of the trimmed reads. We believe that this poor performance was due to a combination of long read length and low sequencing depth relative to community diversity. The DADA2 algorithm requires that the seed sequence of each ASV be represented by at least two error-free reads (Callahan et al., 2016). If sequencing errors are uniformly distributed, then the probability that a given read will be error-free is , where is the sequencing error rate and is the read length in base pairs. Then the number of reads of a given sequence that would be required to obtain two error-free reads in expectation is . For the combination of long reads (median after trimming) and moderate error rate (mean based on ccs quality scores) for the long amplicon in this study, the expected number of reads required to achieve two error-free reads is 126 659. Given the high diversity relative to sequencing depth in this study (501 ASVs based on PacBio short amplicons, 1.08598^{5} trimmed long amplicon reads), this requirement could not have been met for the long amplicons except by the most abundant sequences. In comparison, the equivalent requirement for the short amplicon (, ) is only 3.8 reads. We therefore developed a new workflow to assemble ASVs from the long amplicons, as follows:

Raw reads were divided into shorter regions by matching to covariance models (CM), which are similar to stochastic hidden markov models (HMM), but account for both nucleotide sequence and RNA secondary structure (Eddy & Durbin, 1994). First, the 5.8S rDNA was located in each read by searching for Rfam model RF0002 (Kalvari et al., 2018) using cmsearch from Infernal 1.1.2 (Nawrocki & Eddy, 2013), and all bases before the 5.8S were assigned to ITS1. No attempt was made to remove the approximately 12 bp fragment of the SSU from the 5’ end of ITS1 in the long amplicons; it was too short to be reliably detected by a CM or the HMMs employed by ITSx (Bengtsson‐Palme et al., 2013). A reference alignment including conserved RNA base pairing between and within the 5.8S and relevant portions of LSU was generated from the fungal 28S RNA seed alignment from the Ribosomal Data Project (RDP) release 11.5 (Cole et al., 2014; Glöckner et al., 2017) by truncating after the LR5 primer site and using the reference line to annotate the variable regions *sensu* Michot et al. (1984) and Raué, Klootwijk, & Musters (1988). A CM was generated from the alignment using cmbuild from Infernal. The fragment of each read beginning with the 5.8S rDNA was then aligned to the CM using cmalign from Infernal. The annotation line in the CM alignment for each read was then used to split the reads into alternating more-conserved and less-conserved regions as shown in Figure 1, where LSU1-4 represent the conserved regions of LSU flanking the variable D1-3 regions (Michot et al., 1984). For short amplicons, only (partial) 5.8S, ITS2, and (partial) LSU1 were extracted. Code to extract the regions, including annotated seed alignments and CMs, is available in the new R package LSUx, available on github (<https://github.com/brendanf/LSUx>).

Each of the extracted regions was independently filtered for length (Supplementary Table 3) and a maximum of three expected errors. Sequences were then dereplicated and denoised into amplicon sequencing variants (ASVs) using DADA2 version 1.12.1 (Callahan et al., 2016, 2019). The error model for DADA2 denoising was fit using the 5.8S region for long amplicons, and using the entire read for short amplicons. Independent error models were fit for each sequencing run (i.e., long *vs.* short amplicons, different sequencing technologies). For PacBio libraries, DADA2 was run with complete pooling and a band size of 16. For Ion Torrent libraries, pseudo-pooling and a band size of 32 were used, and the homopolymer gap penalty was set to -1, as recommended by the DADA2 FAQ (<https://benjjneb.github.io/dada2/faq.html>). Chimeras within each region were removed using removeBimeraDenovoTable from DADA2.

For each ITS2 ASV from the long amplicon data set, the denoised sequences for the other regions corresponding to the same sequencing reads were concatenated to form a set of full-length reads. For reads which were not assigned a denoised sequence for each region, the raw read for the region was used instead. Because ITS2 is the most variable of the amplified regions (Supplementary Figure S3), reads with identical ITS2 regions are expected have highly similar sequences in the other regions, unless the amplicon was chimeric. The concatenated ASVs representing each long read were aligned in R using the DECIPHER package (Wright, 2015). Outlier sequences, as determined by mean pairwise distance from the rest of the alignment, were removed from each alignment using the odseq package (Jehl, Sievers, & Higgins, 2015), using the default threshold of 0.025. The consensus of the remaining aligned sequences was assigned as the full-length ASV sequence. Full-length ASV sequences with more than three ambiguous bases (i.e., no nucleotide >50% at a given position) were removed. The count and sample distribution of reads assigned to each ASV were calculated in order to form a sample × ASV community matrix. A similar process was used to generate a consensus ITS (ITS1–5.8S–ITS2) and LSU (LSU1–D1–LSU2–D2–LSU3–D3–LSU4) sequence for each ASV. The process of assigning consensus full-length ASVs was carried out using the new tzara package for R, available on github (<https://github.com/brendanf/tzara>).

Because the Illumina dataset consisted of paired-end reads, regions were not extracted prior to denoising. ASVs were instead generated according to a standard workflow for DADA2. Demultiplexed reads were truncated after the first base with quality score <= 10, and then reads with more than 3 expected errors in either read were discarded. Forward and reverse reads were denoised using DADA2 version 1.12.1 (Callahan et al., 2016) using seperate error models and pseudo-pooling, and then forward and reverse reads were merged. The ITS2 region was extracted from the ASVs using LSUx for comparison to the other technologies.

### Taxonomy assignment

Taxonomic annotations of the Ribosomal Data Project’s LSU fungal training set (RDP) version 11.5 (Cole et al., 2014) and Warcup ITS training set (Wang et al., 2007) were mapped to the taxonomic classification system used in the Unite database version 8 (Nilsson et al., 2019). In particular, the classification for fungi was according to Tedersoo, Sánchez-Ramírez, et al. (2018), and for non-fungal eukaryotes was according to the proposed system of Tedersoo (2017). Although the latter system is not formally published, it is consistent with the annotations for non-fungal eukaryotes in the Unite database. Additionally, it is a system with both purportedly monophyletic taxa and a uniform set of taxon ranks, which make it more appropriate for sequence-based taxonomic assignment algorithms than more accepted classification systems such as that of the International Society of Protistologists (Adl et al., 2019), which utilizes hierarchical nameless ranks. FASTA format files of the re-annotated RDP and Warcup training sets are available at (*somewhere*).

Taxonomic assignment was performed to genus level separately on the ITS and LSU regions using Unite/Warcup and RDP, respectively, as taxonomic references. For each region/reference combination, taxonomy was assigned using three algorithms: the RDP Naïve Bayesian Classifier (RDPC) as implemented in DADA2; SINTAX (Edgar, 2016a) as implemented in VSEARCH v2.9.1 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016); and IDTAXA (Murali et al., 2018b). Each ASV was thus given up to nine preliminary taxonomic assignments (three references three algorithms).

Sequences were assigned as ECM based on taxonomic assignments using the FUNGuild database (Nguyen et al., 2016) via the R package FUNGuildR (<https://github.com/brendanf/FUNGuildR>). All taxa which included “Ectomycorrhiza” in the guild assignment at any level of confidence were included.

### Clustering

For comparison with clustering-based methods, ASVs were clustered into operational taxonomic units (OTUs) at 97% similarity using VSEARCH v2.9.1 (Rognes et al., 2016).

### Alignment and phylogenetic inference

Full length long amplicon ASVs were aligned using DECIPHER (Wright, 2015) with up to 10 iterations of progressive alignment and conserved RNA secondary structure calculation and 10 refinement iterations. This alignment was truncated at a position after the D3 region corresponding to base 907 of the *Saccharomyces cerevisiae* S288C reference sequence for LSU, because several sequences had introns after this position, as also observed in several fungal species by Holst-Jensen, Vaage, Schumacher, & Johansen (1999).

ML trees were produced using RAxML version 8.2.12 (Stamatakis, 2014) using the GTR+GAMMA model and rapid bootstrapping with the MRE\_IGN stopping criterion. The LSU tree was not constrained topologically, but the long amplicon tree was constrained by the result for the LSU tree. *Trees and alignments are available in Treebase. (not yet)*

The tree was rooted outside the Fungi by using the most abundant ASV which was confidently assigned to a non-fungal kingdom by all 6 applicable taxonomic assignment methods. Assignments based on Warcup were not used at this step because non-Fungi are not included in the dataset. The kingdom Fungi was identified as the minimal clade containing all ASVs which were confidently identified (consensus of at least 6 of 9 assignments) to a fungal phylum. ASVs falling outside this clade were not included in the fungal community analysis.

Taxonomic assignments of ASVs from the long amplicon dataset were refined using the phylogenetic tree (Supplementary Figure S1). A taxon at a particular rank was assigned to a node and all its descendents if that taxon was consistent with the reference-based taxonomic assignments for each of the descendents. A taxon assignment was considered to be consistent if at least one algorithm assigned that taxon at greater than 50% confidence, or if no algorithm successfully classified the sequence at greater than 50% confidence. The result of this process was to give a taxonomic assignment to ASVs which were previously unassigned if they were nested within a clade where which was consistently given an assignment, as well as clarify the assignment of ASVs where different algorithms had resulted in different assignments, but only one of these was consistent with the assignments of other ASVs in the same clade. This refinement algorithm is referred to as “PHYLOTAX”.

ASVs from the short amplicon datasets were refined using only the final strict consensus step, i.e., an assignment at a given rank was accepted if there was no conflict between the different assignment algorithms at greater than 50% confidence. This refinement method is referred to as “Consensus”. Additionally, a hybrid method, was applied to the short amplicon datasets, in which assignments from PHYLOTAX were used for ASVs which could be linked by an identical ITS2 region to a long amplicon, and assignments from Consensus were used for the remaining ASVs.

## Spatial analysis

Ecological community dissimilarity matrices were calculated using the ASV/OTU based Bray-Curtis metric (both long and short amplicons) and the phylogenetically based weighted UNIFRAC method (only long amplicons) in phyloseq version 1.26.0. Each of these distance matrices was used to calculate a Mantel correlogram for distances of 0–12 m. Separate correlograms were drawn for samples taken during the same year, and samples separated in time by one year, in order to assess the degree to which the soil community changes over the course of one year.

Additionally, empirical distance-decay curves were generated by plotting mean community dissimilarity as a function of spatial distance, and fit to an exponential model of the form given by Legendre & Legendre (2012) using the nls function in R. Points in the empirical distance-decay curve were weighted by the number of comparisons within the distance class and the inverse of the distance for the purposes of model fitting. For datasets where the Mantel correlogram indicated spatial correlation between samples taken in separate years, the model was re-fit with an additional term to represent temporal correlation:

where , , and represent the community dissimilarity, spatial distance, and time lag between samples, respectively, and the parameters are , the community dissimilarity from replicate samples (“nugget”); , the community dissimilarity at long distances (“sill”); the spatial range at which the community dissimilarity has moved 95% of the way from “nugget” to “sill”, and the equivalent temporal range. 95% confidence intervals were calculated for the spatial and temporal range parameters by profiling using the MASS package in R.

# Results

DNA concentrations after extraction and PCR, as well as sequencing reads for PacBio and Illumina, are shown per sample in Supplementary Figure S10. Samples from Ang in 2015 yielded low quantities of DNA, poor PCR performance, and ultimately very few sequencing reads, especially in the long amplicon library, where only one sample produced more than 100 reads. Consequently, Ang samples were excluded from spatial analysis, although they were retained for denoising, phylogenetic reconstruction, and taxonomic assignment.

The number of sequencing reads and ASVs at each stage in the bioinformatics pipeline are shown in Table 1. Long (short) amplicon sequencing with PacBio yielded 1.25034^{5} (4.9511^{4}) circular consensus reads, of which 1.08598^{5} (4.3272^{4}) were successfully trimmed and demultiplexed. Illumina sequencing yielded 1.0756939^{7} read pairs, of which 9.513433^{6} were successfully trimmed and demultiplexed, and 7.674712^{6} passed quality filtering. Ion Torrent sequencing yielded 1.5208677^{7} demultiplexed reads. 4.5133^{4} (41.6%) of long amplicon PacBio reads passed length and quality filtering stages, compared with 4.0615^{4} (93.9%) of short amplicon PacBio reads, 7.674712^{6} (80.7%) of Illumina read pairs, and 1.2177705^{7} (80.1%) of Ion Torrent reads.

## Region extraction

The 5.8S rDNA was located in 1.04298^{5} long amplicon PacBio reads (96.0% of demultiplexed total), 4.1987^{4} short amplicon PacBio reads (97.0%), and 1.4946121^{7} Ion Torrent reads (98.3%). Length and quality filtering resulted in 9.0041^{4} (82.9% of demultiplexed total) ITS2 sequences from the long amplicon PacBio reads, 3.9994^{4} (92.4%) ITS2 sequences from the short amplicon PacBio reads, and 1.3474669^{7} (88.6%) ITS2 sequences from the Ion torrent reads. The length distribution of different regions extracted from the long amplicon are shown in Supplementary Figure S4.

## Reproducibility of read counts from different technologies

The number of ASVs and OTUs shared between the different sequencing strategies are shown in Supplementary Tables 4 and 5, respectively, and graphically in Figure 2. ASVs shared between all datasets represented 65%, 79%, 67%, and 56% of the reads for the long and short PacBio datasets, Illumina dataset, and Ion Torrent dataset, respectively. These fractions increased to 73%, 88%, 83%, and 80% when differences at the intra-species scale were removed by clustering the ASVs into 97% OTUs. In particular, 99%, 92%, and 89% of reads in the PacBio, Illumina, and Ion Torrent short-amplicon datasets belonged to OTUs shared between all three datasets. In contrast, 21% of reads in the long PacBio dataset belonged to ASVs which were unique to that dataset, and the fraction only reduced to 20% after OTU clustering.

Figure 3 shows the correspondence between the read count for different ASVs/OTUs in the different technologies, where shared ASVs/OTUs are plotted as circles, and unshared OTUs are plotted on the margins. In all cases, the read counts for shared ASVs/OTUs were correlated , with a minimum value of 0.46. Correlations between read counts for the three technologies using the short amplicon library were increased by OTU clustering (0.68 to 0.75, 0.49 to 0.78, and 0.73 to 0.87, for PacBio vs. Illumina, PacBio vs. Ion Torrent, and Illumina vs. Ion Torrent, respectively); but not between the long amplicon library and short amplicon library (0.65 to 0.61, 0.59 to 0.58, and 0.46 to 0.49, for PacBio long amplicon reads vs PacBio, Illumina, and Ion Torrent short reads, respectively).

Almost all of the short amplicons from all three technologies were between 240 and 375 bp long (Supplementary Figure S2a). the length profile of the three sequencing runs were not significantly different (p>0.05), Illumina MiSeq had the largest fraction of reads near the top of the range, followed by Ion Torrent Ion S5 and PacBio RS II (Supplementary Figure S2b). In contrast, the length of the long amplicon reads varied widely, from 696 to 1638 bp, with a median of 1477 bp.

## Taxonomic assignment

The fraction of reads and ASVs assigned to each taxonomic rank is shown in Figure 4. For all sequencing datasets and taxonomic assignment protocols, a higher proportion of reads was assigned than of ASVs, indicating that common ASVs were more likely to be identified than rare ASVs. A greater fraction of ITS reads and ASVs were assigned using the Unite database than the Warcup database across sequencing technologies, amplicons, algorithms, and taxonomic ranks. Across sequencing technologies, amplicons, and reference databases, the RDPC algorithm assigned the greatest fraction of reads and ASVs, followed by SINTAX, and then IDTAXA, with the exception of lower taxonomic ranks (order for the Long amplicon, family and genus for both amplicons) using the warcup reference database, where SINTAX assigned the greatest fraction of reads, but not ASVs.

Taxonomic composition of the sequenced soil community is summarized in Figure 5. According to the PHYLOTAX assignments, Fungi represented 90.1% of the reads and 75.6% of the ASVs in the long amplicon library, compared to 98.4%–98.9% of the reads and 89.7%–94.5% of the ASVs in the short amplicon library. Fungi categorized as ECM made up 39.0% of reads and 8.4% of ASVs in the long amplicon library, and 36.2%–46.3% of the reads and 6.2%–14.0% of the ASVs in the short amplicon library (Supplementary Figure S8).

### Positive control

*Agaricus bisporus*, the positive control, was represented by a single ASV in the positive control samples for both long- and short-amplicon PacBio datasets, and in the Ion Torrent dataset. Additional ASVs belonging to the genus *Agaricus* were also detected in the same wells as the positive control in the Ion Torrent dataset, but these were sufficiently different that they probably represent additional *Agaricus* species from the field samples, and were found in the same wells due to incomplete demultiplexing. *A. bisporus* was represented by two ASVs in the Illumina dataset, which differed at one base pair. The abundance of the second ASV was 1.06% and 0.96% that of the primary ASV in the two Illumina positive controls. The consistency of this ratio across PCR replicates suggests that it represents true inter-copy variation within the specimen, rather than sequencing or PCR error.

The primary *A. bisporus* ASV was the most abundant ASV in the long-amplicon PacBio dataset and the Ion Torrent dataset, and the fifth most abundant ASV in the short-amplicon PacBio dataset, and the third most abundant ASV in the Illumina dataset. This ASV was also found in 8.9% , 9.3% , 59.3%, and 97.0% of the non-control samples, representing 0.11%, 0.08%, 0.09%, and 0.09% of non-control reads, in the PacBio long, PacBio short, Illumina, and Ion Torrent datasets, respectively.

## Spatial analysis

Results of spatial analysis were qualitatively similar between the two amplicon libraries and between PacBio and Illumina sequencing, with significant autocorrelation at ranges of up to 3 m for the total fungal community, and 1–2 m for the ECM community (Figure 6). In both cases, the greatest correlation magnitudes and distances are associated with Illumina sequencing of the short amplicon library, followed by PacBio sequencing of the long amplicon library, with PacBio sequencing of the short amplicon library detecting the least spatial structure.

In contrast, the weighted UNIFRAC distance, which was only calculated for the long amplicon library, shows very little spatial structure, with only the total fungal community in the 1 m distance class showing a significant correlation at .

The Bray-Curtis metric showed significant () positive correlation at a time lag of 1 year and 0 m separation for both the total fungal and ECM communities in the long amplicon library. For the short amplicon library, although the general profile of the correlograms was similar, correlation at 0m and 1 year was not significant, but there was a negative correlation at time lag of 1 year and a distance of 1m for both sequencing technologies. This negative correlation was significant in all short amplicon correlograms.

Although a distance decay model was fit for the weighted UNIFRAC distance applied to the total fungal community, the result was very poorly constrained, and a range of 0 m was included in the 95% confidence interval (Figure 7). Although the best fit spatial ranges vary between the different datasets by a factor of about 3, there is overlap of the 95% confidence intervals for all of the Bray-Curtis variograms across communities, amplicon libraries, and sequencing technologies.

# Discussion

## Comparison of sequencing strategies

The three sequencing technologies gave similar results for the short amplicon library, the major difference being in sequencing depth. Although a greater fraction of PacBio raw reads were ultimately mapped to ASVs (76.0%) compared to Illumina (62.9%) or Ion Torrent (64.8%), the latter two technologies provided much greater sequencing depth for a similar cost, allowing a greater diversity of rare ASVs to be recovered.

DADA2 denoising may perform differently on different technologies (or perhaps sequencing runs), indicated by the fact that clustering ASVs at 97% led to substantially higher correspondence between both the set of sequences recovered from the same library by different technologies (Figure 2) and the read counts for each sequence (Figure 3). The large number of ASVs unique to Ion Torrent, while only the Illumina dataset recovered an apparent intragenomic variant in the positive control sample, suggests that DADA2 may not control sequencing error as effectively in Ion Torrent sequences as in Illumina, for which it was developed (Callahan et al., 2016).

Although the longer read length capabilities of PacBio would allow recovery of longer ITS2 sequences than the other two technologies, no such amplicons were sequenced by any of the technologies, and Illumina showed a slightly higher fraction of longer sequences than Ion Torrent, which in turn showed slightly longer sequences than PacBio (Supplementary Figures S2 and S5).

21% of long amplicon reads belonged to ASVs which occurred only on long amplicon dataset, and clustering at 97% similarity only reduced this fraction to 20%. Additionally, ITS2 sequences extracted from the long amplicon dataset included some sequences that were much shorter than those recovered from the short amplicon datasets 10. Taxonomic assignments revealed that many of these non-shared sequences fall outside kingdom Fungi (Supplementary Figure S7), and that in particular the short ITS2 sequences are mostly Alveolates (Supplementary Figure S6). Within Fungi, the short amplicon datasets recovered more Sordariomycetes, possibly because this group was represented by diverse but rare taxa which remained undetected in the PacBio datasets due to the lower sequencing depth. Additionally, several groups showed anomalous detection in either the long or short datasets, such as *Tulasnella* in the long amplicon dataset (Supplementary Figure S9), and *Xylaria* and *Myerozyma* in the short amplicon datasets (Supplementary Figure S7). These differences may be due to primer mismatches in these groups. However, neither amplicon recovered any sequences identifiable to *Cantharellus*, an ECM genus which is known from the area from fruitbodies.

## Reconstruction of long amplicons from denoised subregions

ASV recovery for long amplicons was dramatically improved (12% to 75%) by denoising homologous subregions independently. Although newer sequencing platforms from PacBio (Sequel and Sequel II) feature increased sequencing depth and lower error rate compared to the RS II, long sequences inherently require much more sampling depth to identify ASVs; thus, this approach may increase ASV recovery from these platforms as well. It may also be adaptable to Oxford Nanopore sequencing, which has hitherto posed difficulties for application to complex community metabarcoding (Loit et al., 2019).

## Taxonomic identification

The RDP fungal training set and Unite performed comparably at placing long amplicon sequences. The Warcup database placed notably fewer sequences at all taxonomic levels for all datasets, probably in part due to the fact that only fungal sequences are included. However, even with this considered, IDTAXA performed very poorly with the Warcup database, placing < 25% of ASVs to kingdom in all datasets. IDTAXA placed fewer sequences than RDPC or SINTAX even with the other databases, but this is expected given its more conservative assignment of confidence scores (Murali et al., 2018a).

Gdanetz et al. (2017) showed that a majority-rule consensus of three assignment algorithms can improve the fraction of sequences assigned as well as decrease the false assignment rate. Here we find that strict consensus, which should provide more conservative taxonomic assignments, also usually increases the number of assigned sequences relative to any single method, except at family and genus level identifications, in line with what Gdanetz et al. (2017) showed xxx. This suggests that different assignment algorithms and databases bring mostly complementary, non-contradictory information at higher taxonomic levels. However, contradictory assignments between different methods is more common at lower taxonomic levels, which can be problematic because accurate assignment at the family or genus level is generally required for ecological guild assignment using FUNGuild.

For ASVs where a long amplicon sequence is available, PHYLOTAX uses phylogenetic relationships to resolve these disagreements in a principled manner. For instance, 52% and 79% of Illumina reads were assigned to genus and family, respectively, by the strict consensus of methods, however PHYLOTAX increased this fraction to 71% and 89%. This led to a corresponding increase in the fraction of reads assigned to a functional guild (Supplementary Figure S8).

## Turnover rate

Weighted UNIFRAC did not reliably detect spatial structure within this relatively homogeneous community. Although the Mantel test did show a small but significant positive autocorrelation in the fungal community at the smallest size category (1 m) (Figure 6), the distance-decay plot in Figure 7 does not show any clear relationship, and the functional fit showed poor convergence, with a 95% confidence interval for spatial range of 0-5700 m. This is probably due to the majority of weighted branch length in the community being between the Pezizomycotina and Agaricomycetes (Figure 5), which are both well represented in the majority of samples. UNIFRAC would be more suited at larger spatial scales and/or larger ecological gradients.

Fungal communities showed comparable spatial autocorrelation ranges in different platfomrs and amplicon datasets (Figure 6). These ranges were similar to the values reported for ECM root tips in temperate forests by Lilleskov et al. (2004) and Pickles et al. (2012), but substantially smaller than those observed by Tedersoo et al. (2011). Distance-decay plots (Figure 7, Table 2) gave substantially longer autocorrelation distances. There was little variation in the results between the Illumina and long-amplicon PacBio datasets for both the total fungal community and the ECM community, with best fit estimates ranging from 17–19 m. The 95% confidence interval was substantially wider than this variation, generally covering a range of 9–35 m. All of these values are smaller than the 65 m reported by Bahram et al. (2013), also from an ECM woodland habitat in Benin. The PacBio short amplicon dataset shows a longer spatial range, of 30 m for the total fungal community and 42–45 m for the ECM community, in both cases with wide confidence intervals spanning approximately 15–60 m. It is possible that the weaker fit for this dataset, which also showed weaker autocorrelation in the Mantel correlogram, is due to low sequencing depth.

The Bray-Curtis Mantel correlogram for both the total fungal and ECM communities from the long amplicon dataset show a significant positive correlation at 0 m and 1 year. The spatiotemporal distance-decay fit estimated the temporal turnover range as 3.1 years for the total fungal community and 5.5 years for the ECM community, but with overlapping confidence intervals. Both datasets from the short amplicon library showed a puzzling pattern with no autocorrelation at 0 m and 1 year, but a weak negative correlation at 1 m and 1 year. The general shape of the correlograms were similar for long and short amplicon datasets. We hypothesize that two different processes may be at work with differing spatiotemporal scales, whose superposition result in this pattern.

# Conclusion

The choice of amplicon and sequencing technology did not seem to affect the results of the spatial analysis, provided sufficient sequencing depth. However, the addition of long amplicon reads did allow the construction of a phylogenetic tree from the metabarcoding reads, which allowed refinement of taxonomic assignments. DADA2 ASV yield was initially poor for long reads, but this was improved through extraction of subregions, separate denoising, and then reconstruction of full-length sequences. This suggests a hybrid approach using long-read sequencing to acquire long amplicon sequences for the local species pool, and cost-effective short-read sequencing to provide high sampling depth and sample number.

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