Mycobiome sequencing and analysis of the assemblages of fungi associated with leaf litter on the Fernow Experimental Forest in the Central Appalachian Mountains of West Virginia

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Abstract

High-throughput sequencing techniques have become widely used for identification, analyses of community composition, and functional significance of fungi in forest ecosystems. Because many fungi cannot be cultured, seldom produce visible sexual structures, and are often overlooked, the use of such techniques is especially appropriate. In the study reported herein, DNA-metabarcoding techniques were applied to samples of forest floor leaf litter collected from a temperate deciduous forest to explore the hidden diversity of fungi and to compare distinct assemblages of leaf litter-associated fungi. The samples were collected from the Fernow Experimental Forest (FEF) in the central Appalachian Mountains of eastern central West Virginia. The datasets of metataxonomic sequences were obtained from samples of leaf litter collected from 10 plots. These revealed high OTU abundance and richness estimates linked to five fungal phyla, at least 85 orders, and 24 classes in addition to numerous unidentified fungi. The majority of unknown fungi could be assigned only to the Ascomycota and Basidiomycota. Based on horizontal diversity indices, Shannon's diversity indices, and evenness values, the highest diversity and evenness values of the assemblages investigated appeared to be most closely related to species of ascomycetes. Morphologically, the fungi contributing to species richness were microscopic species of ascomycetes and macroscopic basidiomycetes. Functionally, the deep sequencing revealed varied and overlapping functional guilds for filamentous fungi in which saprotrophs independently were dominant and contributed mostly to bifunctional and multifunctional guilds. The presented data provides an insight into the diversity of fungi morphology, taxonomically, functionally, and compositionally for the leaf litter microhabitats, setting the stage for forest management decisions and future fungal research.

Key words – Bioinformatic analyses – fungal functional diversity – ITS – metataxonomic approach – metabarcoding

Introduction

Advances in high-throughput sequencing technologies and the availability of high-performance computers have largely replaced culture-independent methods of studying microbial diversity and ecology (Nilsson et al. 2019). Through the increasing use of direct DNA sequencing of environmental samples, mycobiome (fungal microbiota) studies have been widely used to characterize the community structure and explore the dynamic nature of the fungal community taxonomically and functionally in different fungal microhabitats (Yahr et al. 2016, Nelsen 2017, Al Anbagi 2020) and along environmental gradients (Semenova et al. 2016).

The highly divergent fungal "nuclear ribosomal internal transcribed spacer" (ITS) region has been commonly amplified and used as the standard fungal barcode due to the historically broad depth of sequencing and availability of this information in public databases. ITS sequences are considered sufficient to differentiate fungal taxa for the purposes of classification (Schoch et al. 2012). This sequencing approach provides a novel insight into the Kingdom Fungi – the second most species-rich group of organisms after insects (Wu et al. 2019). Previous efforts to estimate the number of fungi were limited by traditional culture-dependent methods, but data derived from molecular sequencing has provided an updated estimate of 12 million species of fungi worldwide (Blackwell 2011, Wu et al. 2019). Recent studies that have applied molecular technologies to forest ecosystems have revealed the hidden fungal diversity associated with previous understudied microhabitats and substrates (e.g., Nelsen 2017, Al Anbagi 2020).

The important roles of fungi in symbiotic and pathogenic relations with plants as well as in the transformation of plant litter and nutrient cycling have also been detected in previous studies (e.g., Lindahl et al. 2013). These studies provided a deeper insight into the structure and function of entire ecosystems and the primary ecological mechanisms responsible for interactions among species as well as between species and the surrounding environment.

The leaf litter mycobiome is one important yet understudied and underappreciated element of forest and other ecosystems, and the taxa involved are among the key players in the decomposition of leaf litter. These fungi establish the soil profile and determine the turnover of nutrients (Boddy et al. 2008, Al Anbagi 2020). This group of fungi inhabits litter – the most valuable renewable resource and key structural component of the forest floor; this litter is comprised of aboveground litterfall (ca. 70%) but also contains stems, small twigs, and propagative structures such as seeds and fruits (Robertson & Paul 1999, Al Anbagi 2020). For example, in deciduous forests, the heterogeneous litter microhabitat usually carpets most of the forest floor and changes during the annual cycle in response to a complex set of chemical, physical, and biological factors related to the decomposition process (Berg & McClaugherty 2008, Boddy et al. 2008). The admixture of material making up forest floor litter supports highly diverse taxonomic and functional groups of litter-associated fungi and the latter presumably interact competitively to exploit the litter resource, proliferate and develop various taxonomic and functional assemblages. The availability and heterogeneity of litter microhabitats result in several taxonomically and functionally discrete assemblages of fungi coexisting within the same microhabitat (Al Anbagi 2020). These assemblages include forms ranging from microscopic unicellular to complex, macroscopic and multicellular forms, with multiple transitions of cellular complexity as a key to their ecological success (Naranjo-Ortiz & Gabaldón 2020). Except for the microscopic unicellular form, the fungi present in litter produce bifurcating fungal hyphae which develop into mycelial networks that extend over large areas, maintaining their growth by bidirectional material movement of nutrients, growth-limiting elements, and cellular components to nutrient-poor patches and metabolic activity zones (Frey et al. 2000, Lin et al. 2015, Naranjo-Ortiz & Gabaldón 2020). For example, this mycelial architecture enables fungi to effectively translocate essential minerals from partially decomposed leaf litter to newly fallen leaves over considerable distances (Berg & McClaugherty 2008, Boddy et al. 2008). It also allows these fungi to optimize their behavior and make suitable responses to environmental signals, including reproduction, morphogenesis, and metabolism (Brand & Gow 2009). As a result of participating in a highly complicated process of litter decomposition, it has been suggested repeatedly (e.g., Gessner et al. 2010, Baldrian 2017) that more

information on patterns of diversity and community structure is needed for litter-associated fungi, to provide a more comprehensive overview of this overlooked group.

The historically important Fernow Experimental Forest (FEF) is located south of the town of Parsons in eastern central West Virginia in the United States. The FEF encompasses an area of 1,900 ha within the Allegheny Mountains subsection of the Central Appalachian Plateau. In this mixed deciduous forest, the few previous studies of fungi have investigated such things as the ectomycorrhizas associated with oak (*Quercus*) and beech (*Fagus*) based on the traditional inventory methods and Sanger sequencing of root tips (Bursick 2013). Only a single study has used a modern next-generation sequencing approach of root tips (Nelsen 2017). The purpose of this study was to characterize litter fungal diversity of FEF. Due to the FEF being understudied but relatively undisturbed for more than a century except for the selective removal of American chestnut (*Castanea dentata* [Marshall] Borkh.) during the 1940s, it was considered to be an especially appropriate place to characterize the leaf litter fungal community assemblages taxonomically, and infer their assemblages morphologically, and functionally based on sequence identification of taxa and previous references. Another research question addressed was whether forest floor leaf litter may capture and preserve fungal propagules from diverse sources and habitats and thus provide an insight into fungal diversity patterns of the entire FEF.

Material & Methods

Study area and sampling methodology

As noted above, the specific study site was in the Fernow Experimental Forest (FEF) (39° 03'21" N and 79° 40'06" W; elevation 770 m) in Tucker County, West Virginia. During 2016, average temperatures ranged from -3.0 C in January to 24.1 C in August, and the total precipitation ranged from 69 mm in March to 225 mm in May (Supplementary Fig. 1). Litter samples were collected in Watershed 4 of the FEF. The investigated site is a mixed temperate deciduous forest dominated by sugar maple (*Acer saccharum* Marshall), American beech (*Fagus grandifolia* Ehrh.), northern red oak (*Acer rubrum* L.), sweet birch (*Betula lenta* L.) and black cherry (*Prunus serotina* Ehrh.). The thickness of the litter layer ranged between 5–15 mm, and the mean pH was 5.5 (determined with a portable pH meter). The samples of leaf litter were collected from the forest floor in 10 plots (each 10 m x 10 m) in mid-May of 2016. The leaf litter consisted of partially decomposed but still largely intact leaves from the previous field season. These plots were located along a transect, approximately 5 m apart, and delimited in the same manner as described by Alanbagi et al. (2019). Within each plot, samples of leaf litter were collected from a representative area of the forest floor. A total of 10 collected samples were put in paper bags, brought to the laboratory, and homogenized with liquid nitrogen as described in Al Anbagi (2020). The homogenized pieces of leaf litter from each sampled plot were stored at -80°C until processed for DNA extraction, library preparation, and sequencing.

DNA extraction, amplicon amplification and NGS library sequencing

Genomic DNA was extracted from 80 mg of each homogenized leaf litter sample. To minimize DNA extraction bias, two replicates of each litter sample were extracted using the NucleoSpin® Plant II DNA extraction kit (Macherey-Nagel Gmbh & Co., Düren, Germany). The replicates of extracted DNA were then pooled together, purified, and concentrated using the Wizard SV Clean and Concentrated DNA kit (Promega, USA). The DNA quantity and purity were checked using a Fluorimeter (GloMax®-Multi Jr, Promega, USA) and a NanoDrop2000™ (Thermo Fisher Scientific, USA), respectively, and the extractions were then stored at -20°C. The fungal amplicon-based sequencing libraries were generated for Ion Torrent sequencing using the internal transcribed spacer 2 region (ITS 2) of the nuclear ribosomal rDNA region as described in previous studies (e.g., Geml et al. 2014, Al Anbagi 2020). The primers fITS7 with the trP1 adaptor (Ihrmark et al. 2012) and labeled ITS4 primer (White et al. 1990) with a unique sample Multiplex Identification DNA (MID) tag were used to amplify the targeted region. All information related to the primers, Ion

Torrent adaptors and MIDs are listed in the Supplementary Table 1. The PCR reactions were carried out in three independent replicates. The negative control consisted nuclease-free water instead of DNA, and was run for every barcode, as described in Al Anbagi (2020) and Geml et al. (2014). The amplified reactions with their negative controls were verified on 1% agarose gels. The triplicate PCR fragments were pooled for each sample, then purified with AmPure® beads (Beckman Coulter, Beverly, Massachusetts, USA), and quantified with Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). The amplicon libraries were sequenced using an Ion 318[TM] Chip on an Ion Torrent Personal Genome Machine (PGM) (Life Technologies, USA), by Macrogen Inc., Seoul, South Korea. The Ion Torrent dataset of raw data contained 1,294,763 sequence reads with mean read lengths of 232 (Supplementary Table 2).

Bioinformatic analyses

Raw sequence data were filtered by the PGM software to remove low quality and polyclonal sequences. The sequences were concurrently sorted according to samples, and the primers, adapters, and MID tags were removed. All PGM quality approved, trimmed, and filtered data were exported as FASTQ files (Supplementary Table 2). The low-quality ends of sequences were then trimmed based on 0.02 error probability limit sequences by Geneious 9.1.8 software (Biomatters, New Zealand). The sequences were filtered using USEARCH v.2.8.0 (Edgar 2010) with the following settings: truncating the sequences to 200 bp and discarding sequences with expected errors >1. Identical sequences were collapsed into unique sequence types while preserving corresponding read counts. After merging individual sample sequence files into a single file, the quality-filtered sequences were clustered into operational taxonomic units (OTUs) with a 97% sequence similarity using USEARCH, simultaneously detected and excluded putative chimeric sequences using the UPARSE-OUT algorithms as previously recommended in meta-barcoding studies (e.g., Geml et al. 2010, Semenova et al. 2016, Al Anbagi 2020). The remaining 614,427 sequences were collapsed into unique sequence types on a per-sample basis while preserving read counts. Global singletons were discarded and OTUs with less than four sequence reads were excluded as suggested by Lindahl et al. (2013) to minimize the false positives. OTUs with <80% similarity or <150 pairwise alignment length to a fungal sequence were excluded as suggested by Grau et al. (2017). The resulting 608,505 high-quality sequences remained and were grouped into 1233 OTUs. The representative sequences of fungal OTUs were submitted to GenBank under the GenBank accession numbers (MW337303 – MW338535). Good's coverage (complement of the ratio between local singleton OTUs and the total sequence reads) was performed to estimate the sequencing depth of the fungal OTUs coverage across the sampled plots (Brown et al. 2013).

Litter mycobiome: functional and Taxonomic assignments

The representative sequences were assigned to taxonomic fungal groups based on pairwise similarity searches using two methods. These were the Basic Local Alignment Search Tool (BLASTn) algorithm and Sequence Classifier with the curated UNITE and INSD (GenBank, EMBL, DDBJ) fungal ITS reference databases (version released on 22 August 2019), containing identified fungal sequences with assignments to species Hypothesis groups (Kõljalg et al. 2013). For an accurate taxonomic assignment, the search results of query sequences were manually checked for both methods, and the lowest taxonomic level at which both methods agreed was selected. The representative sequences were assigned to the taxonomic affiliation and named using the current online Species Fungorum database (www.indexfungorum.org) as implemented in UNITE, following standard reference criteria (Geml et al. 2010, Tedersoo et al. 2014, Agostinelli et al. 2018). For those OTUs that had the same percentage of similarity with several fungal genera or families, the assignment was made at the level of family or order, respectively. The morphotyping patterns of 2,002 OTU sequences represented 501 genera. These fungi were classified into (1) filamentous forms, including microscopic and macroscopic fungi, based on the size of fruiting bodies and (2) yeast forms. The fungal functional guilds were putatively and manually assigned for only those OTUs that were identified to the genus or species level. These functional assignments

were as follows: saprotrophs, ectomycorrhizas, mycoparasites, plant pathogens, and bi-multifunctional forms (for those being assigned to two or more functions such as saprotrophs and other functions based on available references). The *incertae sedis* category was also used for assigning fungi to functional guilds if no information was available (e.g., no phylogenetical assignments or methodological isolations from specified substrates known). The unknown term was used for fungi that could not be assigned to a functional guild based on available references. These ecological categories describe the fungal functional guilds and the transition between guilds in the investigated forest ecosystem. The morphological traits and functional guilds of identified fungi were assigned based on the relevant published literature (Tedersoo et al. 2014, Nguyen et al. 2016, Agostinelli et al. 2018).

Diversity and statistical analyses

The relative OTU number of sequence abundance and richness based at the taxonomic level of phylum were determined and visualized for providing an overview of community structure. The alpha taxonomic diversity for all OTU datasets including both identified and unidentified fungi was investigated for observed richness (S), diversity Shannon-Weaver (H) index and Evenness (H/In S), all calculated using the Vegan package (Oksanen et al. 2013) in R software for statistical computing (R Core team 2017) for the ten sampling plots. Additional richness analyses were performed and compared independently for dominant OTU datasets first for all ascomycete and basidiomycete members and then to identified taxa only at the level of genus or species. Species richness values of OTU morphotypes for dominant phyla were also analyzed and compared across all litter samples. Richness values were determined for all fungal functional groups for various assemblages in all investigated plots. The results were statistically evaluated and compared using analysis of variance (ANOVA) and Tukey's test for all result pairs among the ten plots. The cut-off value was 0.05. The results were visualized using Excel and JMP software (SAS Institute Inc., Cary, North Carolina, USA). The relative richness levels of identified OTUs also were generated to determine, compare, and visualize the distribution of fungal phyla to orders using Krona plot diagrams. The mean per-plot OTU richness of fungal genera was calculated and visualized as the distribution of the highest mean OTU-richness genera values across all plots.

Results

Overview of litter mycobiome taxonomic affinities, community structures and diversity analyses

The datasets of metataxonomic sequences for retrieving fungal genomes directly from litter samples revealed a large OTU abundance and richness. Fungi identified belonged to five fungal phyla. In addition, numerous fungi were identified as unknown members of the Ascomycota or Basidiomycota (Fig. 1). The 890 OTUs belonged to the Ascomycota and 234 OTUs belonged to the Basidiomycota, with relatively few belongings to other phyla – Chytridiomycota (3 OTUs), Glomeromycota (3 OTUs) and Mortierellomycota (2 OTUs) (Figs 1, 2). On the level of taxonomic class, 25 taxa were identified. The Dothideomycetes (30%), Leotiomycetes (14%), and Sordariomycetes (14%) were the most prominent of these among the Ascomycota, while the Agaricomycetes (9%) and Tremellomycetes (5%) were prominent among the Basidiomycota (Fig. 3). Overall, the fungal OTUs represented at least 85 orders and 210 families. A group of 95 OTUs could not be identified further than Kingdom Fungi, suggesting either novel taxa or relatively rare taxa yet to be sequenced (Figs 2, 3). Of the 291 genera, there were 54 ascomycetous genera that met the average of at least one OTU per plot, while only 16 basidiomycetous genera presented the average of at least one OTU per plot. The genera *Cylindrium* (Hypocreales, family *incertae sedis*) and *Sarcinomyces* (Chaetohyriales, family *incertae sedis*) which belong to the Ascomycetes, along with *Hannaella* (Tremellales, Bulleribasidiaceae) and *Mycena* (Agaricales, Mycenaceae), which belong to Basidiomycetes, had the highest OTUs richness per plot among all fungal genera (Fig. 4).
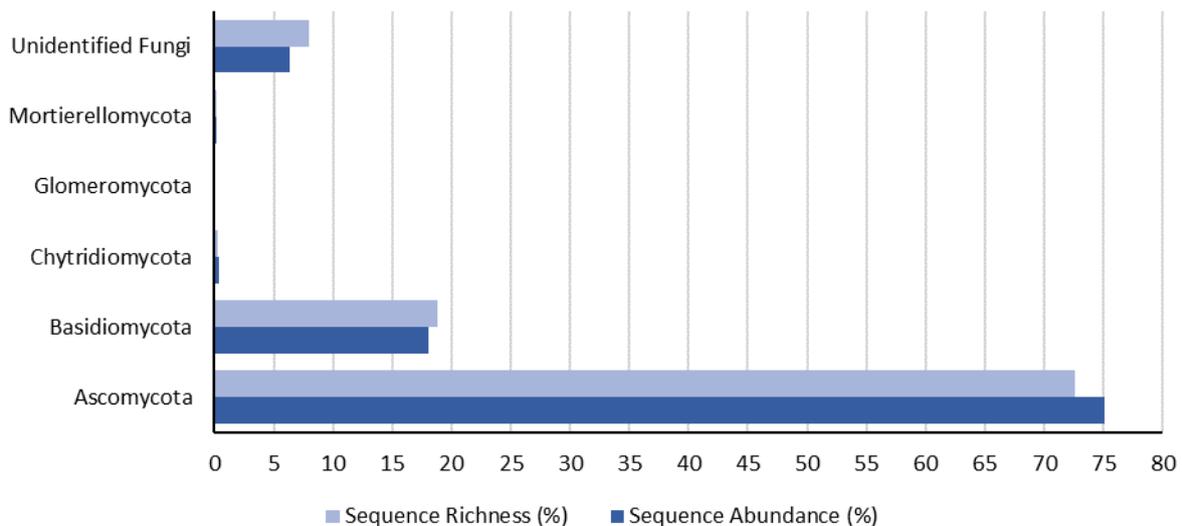
Fig. 1 – Mycobiome overview of sequence taxonomic abundance (Navy blue bars) and richness (Light blue bars) for phylum assemblages of leaf litter fungi detected in ten sampling plots on the Fernow Experimental Forest.
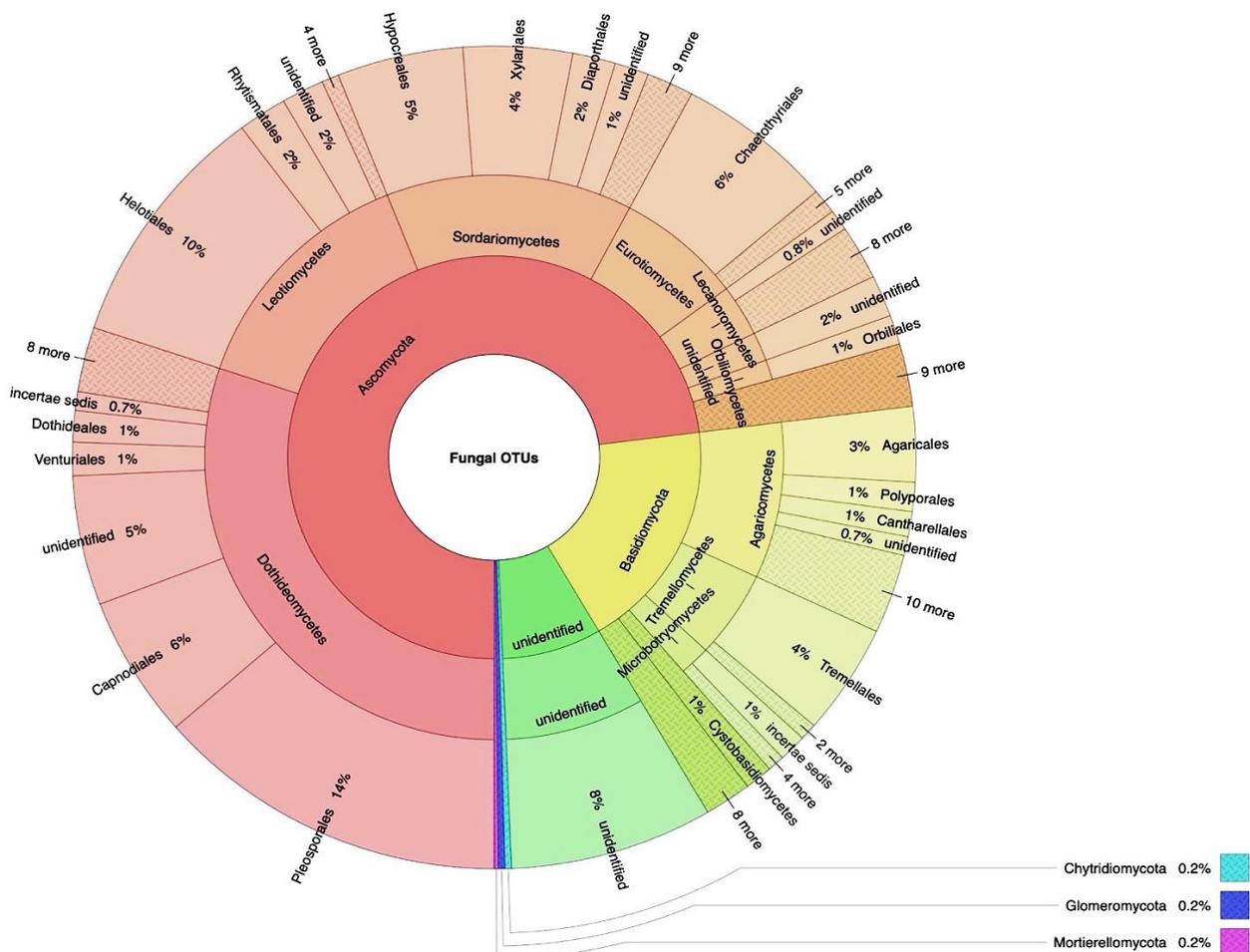

Fig. 2 – Krona plot diagram of OTU richness (1219) for taxonomically describing and comparing assemblages of litter associated fungi at the level of order. The largest groups (Ascomycota accounts for 73% of the total OTUs) are shown in the wheel with varieties of red color levels and the second largest groups in light green color levels, including unidentified taxa in both groups. (Basidiomycota encompasses 19% of the total OTUs).

Fig. 3 – Krona plot diagrams of OTU richness for taxonomically characterizing and comparing assemblages of litter fungi at the level of order for Ascomycota (A) and Basidiomycota (B). Wheel

varieties of the distinct color levels are shown where the largest groups are in varieties of the red color levels with other groups in distinct color levels.
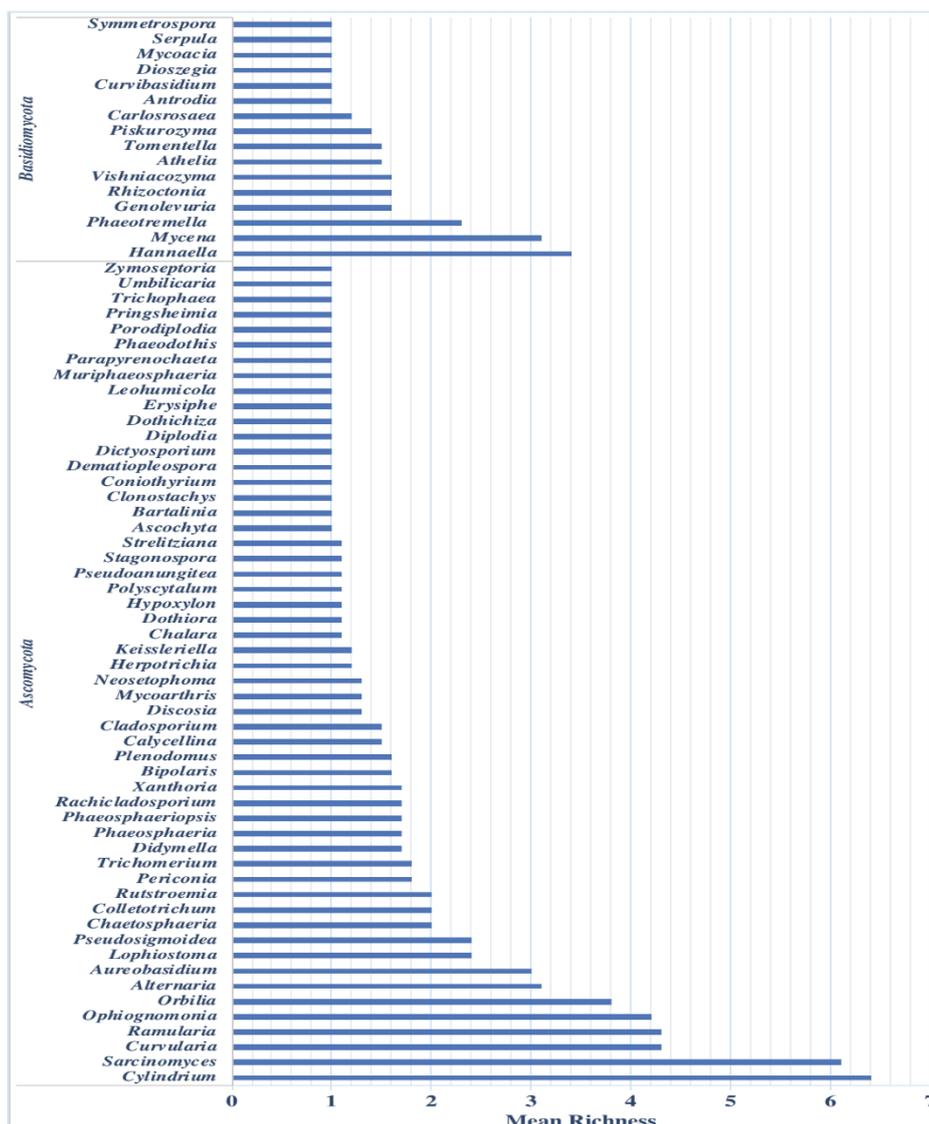

Fig. 4 – The mean richness of the most dominant genera for the major phyla Ascomycota and Basidiomycota. The data represent the mean value of each genus isolated from samples of deciduous leaf litter collected from ten forest plots.

The High Good's coverage estimator (99.15 ± 0.16%) indicated equally deep OTU recovery across the sampled plots (Supplementary Fig. 2). The diversity indices (Shannon's and the evenness values) of the overall community structure did not differ from the one for ascomycete community structure but were higher than the corresponding values for basidiomycete community structure (Fig. 5). High diversity within the plot correlated with the relative abundance of ascomycete taxa (Fig. 5B, C).

Morphotyping and functional assignments for fungal leaf litter OTUs
The result of sorting fungal community members on the basis of taxa that form filaments, the size of sexual structures, and yeast forms revealed that the filamentous community of deciduous leaf litter was characterized by significantly higher species richness (436 taxa) compared to the yeast community (51 taxa; Fig. 6). The community structures formed by filamentous fungi were mainly microscopic ascomycetes, which had higher species richness than predominantly

macroscopic basidiomycetes. However, the vast majority of yeasts were species that belonged to the Basidiomycetes (20.1, Figs 6A, B).
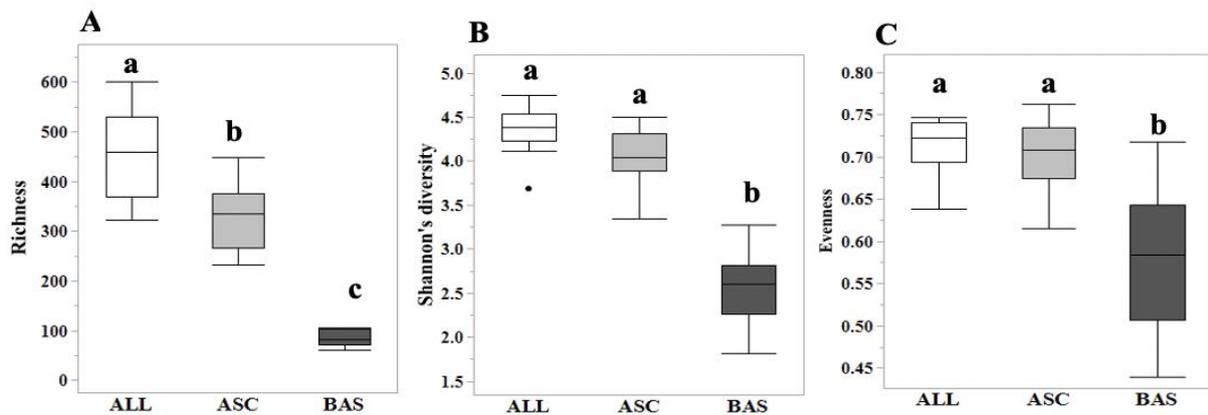

Fig. 5 – Means of taxonomic richness (A) and diversity analyses (B and C) of all identified and unidentified fungal assemblages (ALL) with identified fungi at the major phylum levels for Ascomycota (ASC) and Basidiomycota (BAS). The different letters indicate significant differences between the compared groups based on the pairwise using Tukey's test.
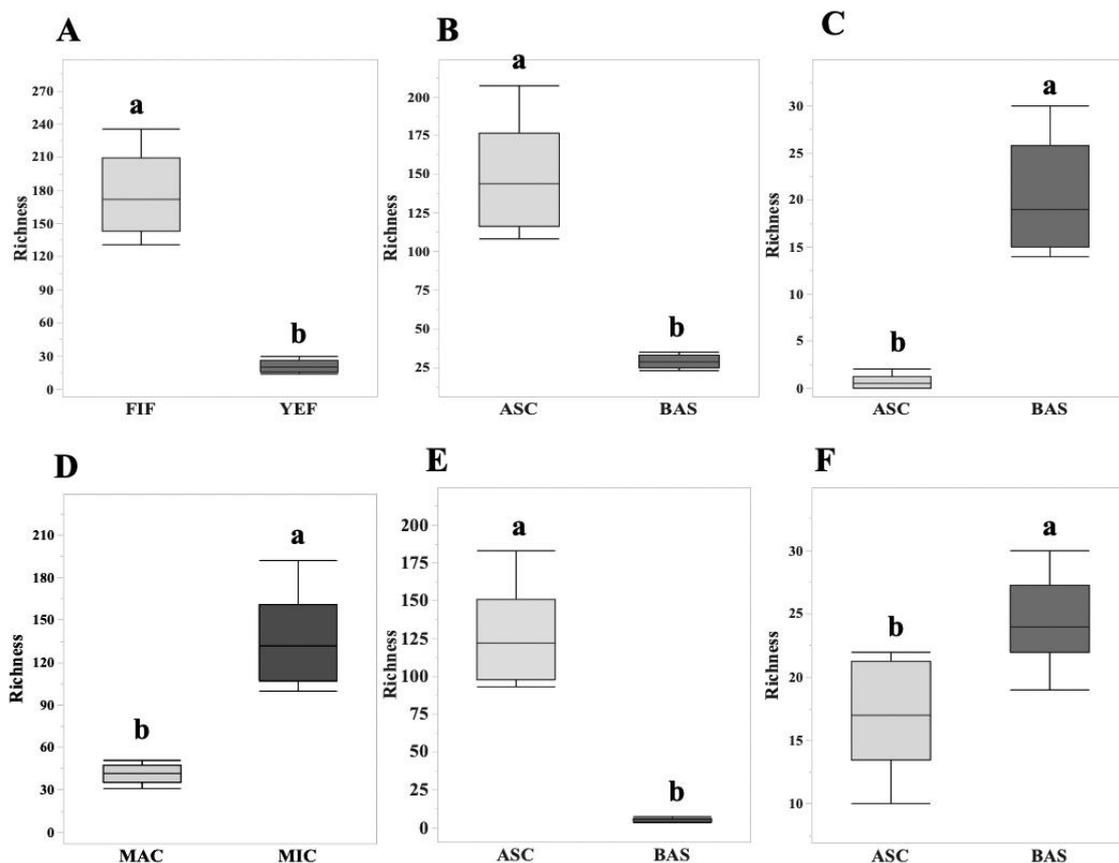

Fig. 6 – Typifying and comparing the OTU richness of the dominant fungal morphotypes, filamentous (FIF) and yeast (YEF) fungi (A) with categorized them (B and C) into the dominant phyla (Ascomycota (ASC) and Basidiomycota (BAS)). The prevailing filamentous fungi are also characterized based on the size of reproductive structures into the microscopic (MIC) and macroscopic assemblages (MAC, D–F). The different letters indicate significant differences between the compared groups based on the pairwise using Tukey's test.

Functionally, the leaf litter layer of the FEF was inhabited by various filamentous species that represented various functional guilds based on currently available knowledge. These involve about 40% of the total classified sequences (Fig. 7). The functional diversity patterns were distinct in their species richness at the level of the dominant phyla and are apparently driven by the members of Ascomycota. For that microhabitat, leaf litter saprotrophs had higher OTU richness than for other detected functional groups with significant differences between both dominant phyla (Fig. 7). The litter species richness values of multifunctional and difunctional guilds were most characteristic after saprotrophs and were clearly different between the Ascomycota and Basidiomycota. In addition, the population assemblage of fungal pathogens contributed little to functional diversity. However, no significant differences were detected for mycorrhizas between both dominant phyla (Fig. 7).
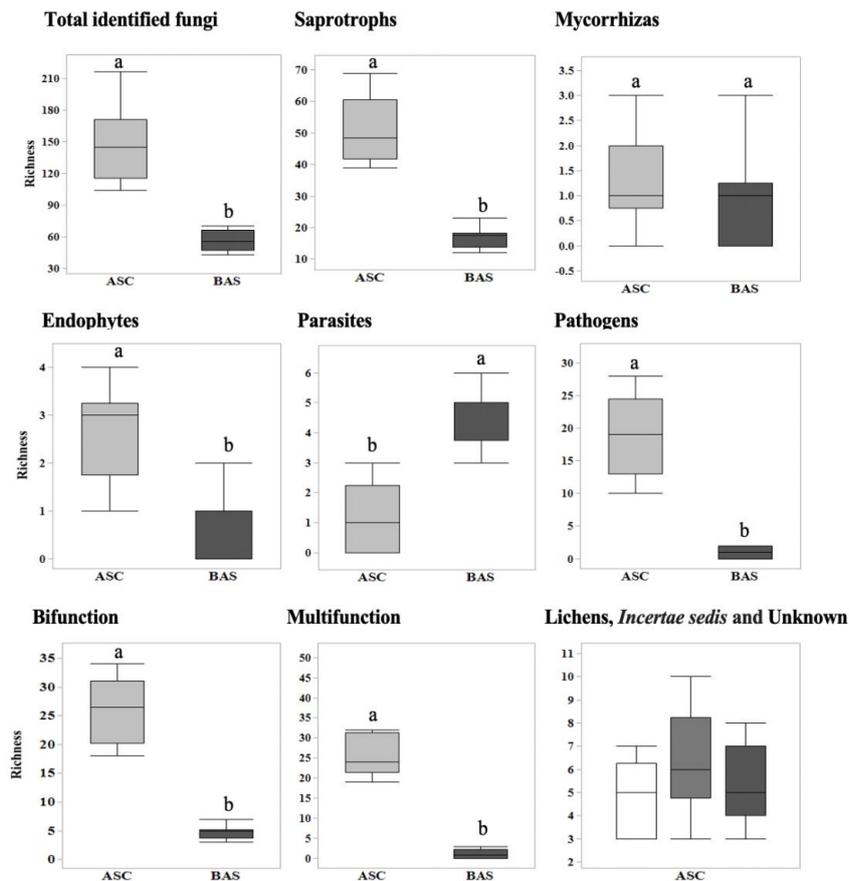

Fig. 7 – Characterizing and comparing the total OTU richness of functional guilds of identified filamentous fungi. The different letters indicate significant differences based on the pairwise Tukey's test. Bifunctions and multifunction refer to species with two or more different functional guilds. *Incertae sedis* refers to species and genera for which the common function has yet to be determined.

Discussion

The metagenomic results for the 10 plots sampled in the present study showed that the putative mycobiome of the leaf litter layer is taxonomically, morphologically, and functionally diverse but also dominated by a few key groups. Obtaining fungal genome data directly from leaf litter samples revealed high OTU abundance and richness linked to five fungal phyla – Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Mortierellomycota (Fig. 1), indicating that litter decomposition is a highly complex process mediated by numerous fungal taxa characterized by various catabolic capabilities (Osono et al. 2006, Voříšková & Baldrian 2013). The data obtained on community structure and distributions of these phyla that make it up were

confirmed statistically via fungal diversity analyses for the overall community and separately for both dominant phyla – the Ascomycota and the Basidiomycota (Figs 2, 3, 5).

These assemblages were apparently driven by the members of the Ascomycota and Basidiomycota (Fig. 2), which are the largest fungal phyla, the most species-rich, and the best-studied groups (Naranjo-Ortiz & Gabaldón 2020). In these two phyla, the most abundant ascomycetous classes were the Dothideomycetes, Leotiomycetes, and Sordariomycetes; the basidiomycetous classes Agaricomycetes and Tremellomycetes were represented by certain orders on leaf litter (Fig. 3) Most of these orders have been documented previously in other fungal successional and diversity studies involving leaf litter in different types of forests (e.g., Voříšková & Baldrian 2013, Wang et al. 2019, Osono 2020). Representatives of the Pleosporales and Capnodiales (Dothideomycetes) were detected in all stages of succession in the decomposition of leaf litter in a temperate forest, but the latter order predominated in the early stages. Fungi related to the Helotiales (Leotiomycetes) and Atheliales (Agaricomycetes) also were also documented and present during the entire decomposition process, while the Tremellales (Tremellomycetes) dominated in the late stages (Voříšková & Baldrian 2013). The previous studies demonstrated substantial changes for the fungal assemblages and pointed to specific cellulolytic fungi as well as taxa associated with a complex set of extracellular enzymes during the different stages of litter decomposition. Members of the orders such as the Pazizales, Xylariales, and Sebacinales also have been documented as associated with the various litter microhabitats, including leaf litter in the temperate deciduous forests of northwest Arkansas (Al Anbagi 2020).

The high level of fungal diversity on leaf litter in the current study reflects the influence of many interconnected site factors, including the forest vegetation type and the dominant plant species contributing to the build-up of a nutrient-rich layer of decaying organic matter (Berg & McClaugherty 2008, Al Anbagi 2020). On the forest floor, the physiochemical characters of the heterogenous litter fall undoubtedly provide different and novel niches for current fungi to exploit and thus potentially increase fungal diversity during organic matter transformation from easily utilizable compounds to recalcitrant ones (Berg & McClaugherty 2008). Multi-kingdom interactions such those involving bacteria and detritivores also may play a role in shaping the litter assemblages in forest litter (Gessner et al. 2010, Štursová et al. 2012). However, the actual development and composition of decomposer communities on decaying litter are still seriously understudied (Voříšková & Baldrian 2013, Baldrian 2017).

The leaf litter assemblages were primarily related to diverse species differing in abundance and richness and primarily belong to a few dominant genera (Fig. 4). Most of the highlighted genera in this study have been documented for fungal community composition in the forest leaf litter using traditional and DNA metabarcoding techniques (Mašínová et al. 2017, Alanbagi et al. 2019, Osono 2020). Most of these genera are known for their specific enzyme capabilities to decompose cellulose, hemicellulose and lignin in leaf litter, and their mineralization of lignin and other compounds due to comprising a sat of related genes such as cellobiohydrolase I and exoglucanase, and β-glucosidase (Voříšková & Baldrian 2013, Osono 2020, Wang et al. 2019). Similar to other observations (Voříšková & Baldrian 2013, Mašínová et al. 2017), the organic materials in this study were also support yeast growth where the basidiomycetous yeasts predominated over ascomycetous ones. In this study, the prevailing genera related to the Ascomycota are morphologically classified as microfungi, including such examples as *Cylindrium*, *Sarcinomyces*, *Curvularia*, *Ramularia*, and *Ophiognomonia* with functions as endophytes, saprotrophs, and pathogens. Other macrofungi such as *Orbillia* and *Rutstroemia* likely act as saprotrophs. Basidiomycetous genera commonly inhabiting leaf litter included saprotrophic yeasts such as *Hannaella* and filamentous genera with macroscopic fruiting bodies such as *Mycena* and *Tomentella.* Other basidiomycetes belonged to the *incertae sedis* guilds or had multiple functions (e.g., saprotrophs or symbiotrophs). Some have very specific functions, as is the case for the genus *Phaeotremella*, which is a mycoparasite. The antagonistic activity of current genera might also contribute to their dominance as being reported in some yeasts against the fungal mycelial growth (Into et al. 2020). The dominant genera potentially relate to diverse interactions in the same fungal

kingdom or even cross other kingdoms such as bacteria that are considered driving forces for determining the structure of litter assemblages (Bödeker et al. 2016, Velez et al. 2018).

Morphologically, the deciduous leaf litter examined in this study harbored a variety of cellular morphologies consisting mostly of highly polarized multicellular hyphae and secondarily of unicellular yeast cells (Fig. 6). The mycelial fungi seem more likely to facilitate adaptation and allow greater distribution for colonization of new environmental niches (Boyce & Andrianopoulos 2015). The long-lived and distance-exploration mycelia are also preferred for habitat connection, substrate mineralization, immobilizing N and P (Boddy et al. 2008, Bödeker et al. 2016), and translocation of nutrients or water (Baldrian 2017). The vast majority of the filamentous fungi identified in the present study were ascomycetes, mainly microfungi, while the yeast populations were mostly basidiomycetous yeasts (Fig. 6). These morphotypic differences at the phylum level agreed with observations of overall community composition in the litter of a European temperate forest (Mašínová et al. 2017) and of decomposing litter of a Czech Republic oak forest (Voříšková & Baldrian 2013), with significant variations in substrate preference based on the dominated trees (Mašínová et al. 2017). Generally, the species-richness of the Ascomycota has been suggested to be related to a faster evolutionary rate in this phylum as opposed to the Basidiomycota, and the former phylum includes two-thirds of all described species (Wang et al. 2010). In addition, most ascomycete taxa identified in this study were microfungi. Ascomycete microfungi may have become established in the litter layer earlier than basidiomycetes, thus having an advantage of (1) gaining immediate access to readily available nutrients (Koide et al. 2005) and (2) in later stages of decomposition, feeding on available cellulose (Šnajdr et al. 2011). Macroscopic basidiomycetes produce diverse fruiting bodies and have lifestyles associated with a wide array of enzymes that allow them to degrade plant lignin (Wang et al. 2010). As a result of various adaptations, litter-associated yeasts represent typical transient species that are introduced to the soil habitat with newly falling leaves or other litter materials, as suggested by Yurkov et al. (2016), and are considered to be the common occupants of leaf surfaces and have a saprotrophic function (Buck 2002, Sláviková et al. 2007). Most of the orders of fungi (Fig. 3) recorded in the present study include taxa known to produce distinct patterns of morphogenesis in cells of hyphal and yeast morphotypes such as members of the Capnodiales, Saccharomycetales and Tremellales that are often induced by extracellular conditions and executed by fungal genetic factors to develop and adapt to their niches (Lin et al. 2015). Differences in the metabolic abilities of the assemblages of filamentous fungi (Hättenschwiler et al. 2005, Osono 2020), for example, could contribute to the overall fungal diversity, community structure, and the dominant group associated with FEF litter (Fig. 6C, D). As an assemblage of populations in nature, these activities are the main modes of actions using different strategies in the competition for nutrients and space that could be either intra-specific or inter-specific as has been reported and suggested for diverse morphotypes (Spadaro & Droby 2016, Di Francesco et al. 2017, Al Anbagi 2020).

Based on the currently available information on species richness and fungal ecology, the leaf litter of FEF was inhabited by various filamentous species that represented various overlapping functional guilds (Fig. 7). These assemblages may share genes and/or metabolic paths for decomposing litter (Hättenschwiler et al. 2005, Osono 2020). A considerable degree of fungal overlap between functional groups has been recently recorded for macrofungi using an inventory method for the various floor litter microhabitats (Alanbagi et al. 2019) and metabarcoding approaches for the leaf litter of deciduous forests (Al Anbagi 2020). In agreement with other findings, both previous used techniques found that saprotrophs were the primary decomposers of the litter layer in general and the dead leaf microhabitat specifically (Voříšková & Baldrian 2013, Bahnmann et al. 2018, Al Anbagi 2020). The diverse functional guilds examined in the present study suggest that some taxa may switch between different lifestyles (e.g., endophytism versus saprotrophism) to fulfill their need to acquire nutrients (van der Wal et al. 2013, Bödeker et al. 2016). The filamentous nature of the dominant saprotrophic fungi may give them the capacity to enter into facultative biotrophic relationships with plant roots as has been reported for basidiomycetes (Smith et al. 2017). Although yeast functions were not detected in this study,

several previous studies have revealed that saprotrophic yeasts are common occupants of leaf litter and soil due to active production of a wide spectrum of carbon sources as well as hydrolytic enzymes that break down lignocellulose-related sugars (Voříšková & Baldrian 2013, Mašínová et al. 2017). Some of identified yeast species also may have other ecological functions. However, the exact functions of the *Incertae sedis* fungal guild remain unclear.

In summary, the metabarcoding data obtained for leaf litter DNA presented in this study provides a higher quantitative resolution of distinct fungal assemblages than was previously documented for the deciduous mixed leaf litter microhabitat of FEF in the Allegheny Mountains of the eastern United States. Compared to traditional methods, next-generation sequencing yields a more complete body of knowledge to describe and explore the diverse morphotypes of fungal assemblages in nature without being biased towards easily cultured and rapidly growing taxa of filamentous microfungi. Although metabarcoding approaches also provide clues about fungal biodiversity and where potentially new species can be found, there are still various issues that need to be considered through using a short DNA fragment from environmental samples for taxon naming with needing to develop classification systems for huge environmental DNA sequences (Hongsanan et al. 2018, Wu et al. 2019). In the present study, after considering various considerations and recommendations mentioned in previous studies, there remain certain macrofungal and plant pathogenic fungal genera that found to need specific attention when naming species. This is because (1) many genera are already known to contain a considerable number of cryptic species and (2) the difficulty in species segregation when using the ITS region as the standard fungal barcode, specifically ITS1 or ITS2 as presently being applied, with an increasingly urgent need for specific primers for certain taxa and fungal barcoding databases (Yahr et al. 2016, Wu et al. 2019). This is time consuming, due to the many synonyms that exist for some species, incomplete taxonomic points, and dynamic changes in the phylogenic placement of particular species – all of which were challenges in the present study. Previous taxonomic studies using metabarcoding techniques have confirmed the power of sequencing approaches for scaling up the discovery of the hidden diversity of litter-associated fungi and their ecological roles (Voříšková & Baldrian 2013, Yahr et al. 2016). The results presented herein suggest that the heterogeneity of the FEF leaf litter supports diverse fungal functional guilds, with overlapping roles during the decomposition process. Studies such as this one has increased our understanding of fungal diversity and community structure on substrates in the forest other than soil. These data are essential for assessing fungal community diversity and may help in making future decisions in aspects of forest management such as prescribed burning, to preserve fungal diversity and support to forest productivity through sustaining the network of relationships between fungi and other organisms. Additional research is needed to develop an even more complete understanding of community structures and the roles played by the individual fungal taxa by using metaproteomic techniques during entire litter decomposition process to link fungal diversity with functional roles. For example, what role does seasonality play on fungal assemblages during the entire year. Among other things, these data may help forest managers determine the best time for prescribed burning.

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Supplementary Materials

Supplementary Table 1 Sequences of (A) Ion Torrent adaptors and gene primers for sequencing the ITS2 region and (B)Unique molecular identifier tags (MIDs) for samples collected from the Fernow Experimental Forest (FEF).

A. Sequences of Ion Torrent adaptors and gene primers for sequencing the ITS2 region	
Sequence Name	Sequence
Ion Torrent Adaptor	
Sequencing adaptor P1	5`-CCTCTCTATGGGCAGTCGGTGAT-3`
Sequencing adaptor A	5`-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3`
Gene Primer	
fITS7-trP1	5`-GTGARTCATCGAATCTTTG-3`
ITS4	5`-TCCTCCGCTTATTGATATGC-3`

Supplementary Table 1 Continued.

B. Unique molecular identifier tags (MIDs)		
Sample Name	MID Tag	MID Sequence
FEF1	IonXpress_021	TGAGGCTCCGAC
FEF2	IonXpress_022	CGAAGGCCACAC
FEF3	IonXpress_023	TCTGCCTGTC
FEF4	IonXpress_024	CGATCGGTTC
FEF5	IonXpress_025	TCAGGAATAC
FEF6	IonXpress_026	CGGAAGAACCTC
FEF7	IonXpress_027	CGAAGCGATTC
FEF8	IonXpress_028	CAGCCAATTCTC
FEF9	IonXpress_029	CCTGGTTGTC
FEF10	IonXpress_030	TCGAAGGCAGGC

Supplementary Table 2 Summary of raw sequences and read length histograms of the next generation sequencing for each sample from investigated plots in the Fernow Experimental Forest (F1-F10).

Sample ▲	Bases	>=Q20 Bases	Reads	Mean Read Length	Read Length Histogram
F1	25,638,636	22,945,619	105,886	242 bp	
F2	28,603,032	25,873,376	124,419	230 bp	
F3	35,464,699	31,995,457	151,660	234 bp	
F4	31,711,755	28,368,803	132,159	240 bp	
F5	31,743,010	28,197,710	143,491	221 bp	
F6	29,474,456	26,781,503	127,184	232 bp	
F7	29,694,660	26,693,627	144,012	206 bp	
F8	27,883,008	24,859,052	127,242	219 bp	
F9	31,196,833	28,674,829	122,266	255 bp	
F10	28,168,760	25,521,864	116,444	242 bp	
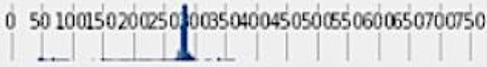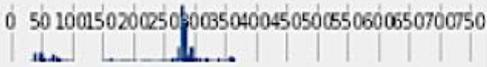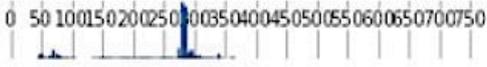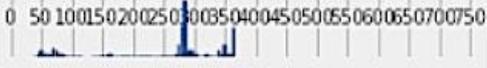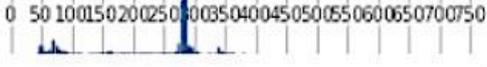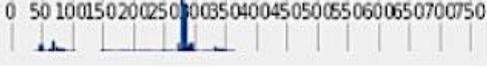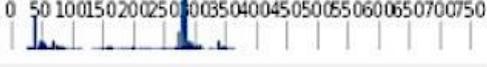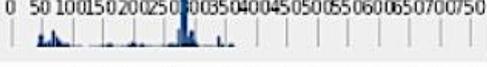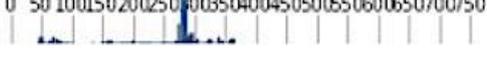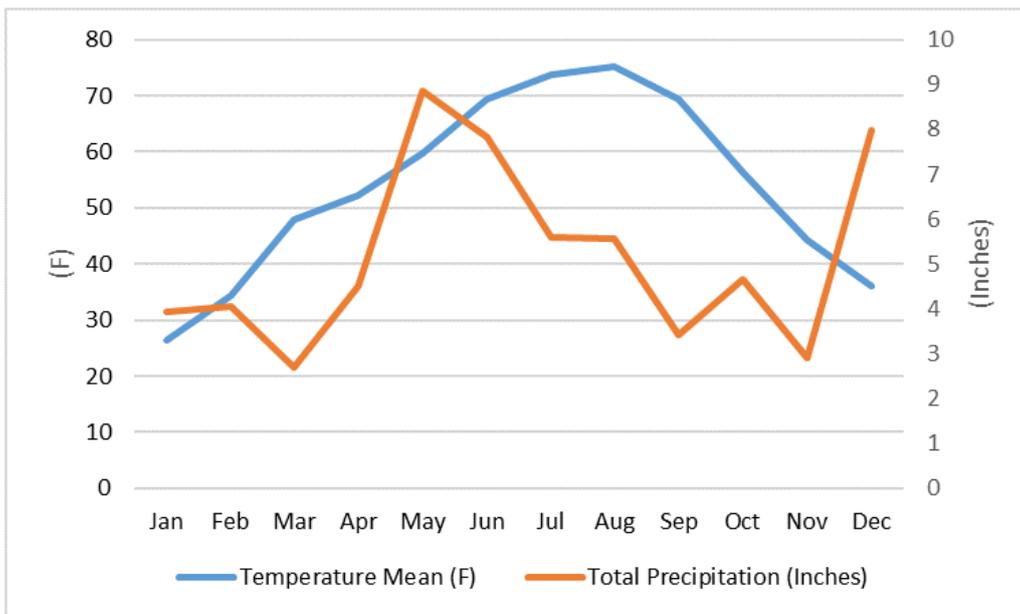
Supplementary Fig. 1 – Summary of the total precipitation and temperature means on the Fernow Experimental Forest according to the weather station Parsons 3 SE, WV US USC00466867 in 2016 (National Centre for Environmental Information, Asheville, North Carolina).
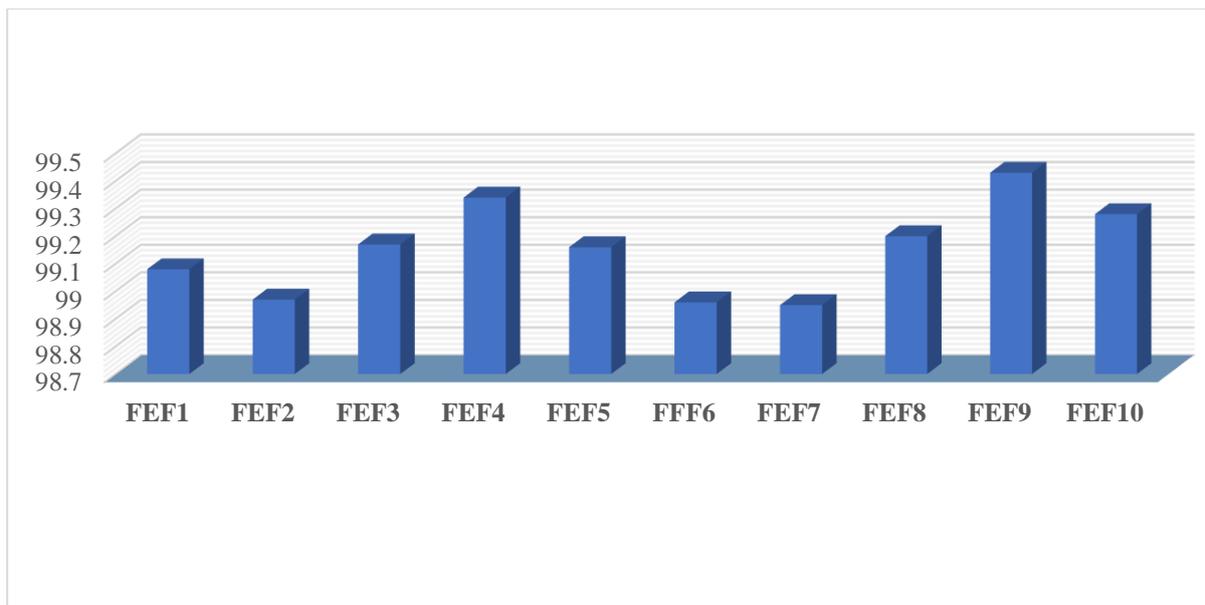
Supplementary Fig. 2 – The Good's coverage estimator across the FEF investigated plots. The high Good's coverage estimator (99.15 ± 0.16%) indicated equally deep OUT recovery across the sampled plots.