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A Comparative Taxonomic and Diversity Study of Litter-Associated Fungi in Northwest Arkansas Forests

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A Comparative Taxonomic and Diversity Study of Litter-Associated Fungi in Northwest Arkansas Forests

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by

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Abstract

Fungi are taxonomically the most species-rich group of organisms on the earth, ecologically occupy distinctive niches and interact with diverse other organisms throughout their biogeographic distributions and functionally play key roles through their various lifestyles. Plant litter, in particular, is a keystone component in ecosystems and provides heterogeneous microhabitats for the often overlooked litter-decomposing fungi and other organisms on the floor of temperate deciduous forests. Litter fungi involve indirect interactions with the plant, soil and whole food web network. However, the community structure and functions of litter-associated fungi as well as patterns of species richness distributed across various litter microhabitats have been largely neglected and potentially threatened by forest management activities. This is because of the primary focus on fungi with direct interactions with plants or soils and methodological limitations. In this project, fruiting body inventories, incubation chambers and Sanger sequencing and DNA-metabarcoding techniques were applied to document and compare the hidden diversity and productivity of the fungi associated with litter microhabitats on the forest floor in the temperate deciduous forests of northwest Arkansas. Using traditional methods (inventories and incubation chambers), a total of 126 taxa, likely with many new species, were recorded, the majority of which were associated with dead leaves in the Pea Ridge National Military (PR) Park and Devil's Den State (DD) Park. Functionally, saprotrophs were dominant and appeared to shape the community structure of other overlapping guilds present. On each site, diverse macrofungal species inhabited multiple microhabitats of this heterogeneous substrate. The taxonomic and functional diversity of community assemblages were significantly distributed over the forest floor litter and clustered across the twenty plots on the investigated study sites. The rDNA- metabarcoding of the fungal ITS2 region generated a more meaningful data body

once direct sampling of leaf litter as the main component of litter forest floor in these deciduous forests as opposed to traditional methods. There were 415 OTUs including 349 macrofungal OTUs and 66 OTUs with new macrofungal sequences recorded for the first time across both investigated sites. The deep sequencing presented more diverse and overlap functional groups inhabiting leaf litter without significant differences in richness of the dominant saprotrophs, while the variations recorded for the richness of the ectomycorrhizas, parasites, and members of other uncertain functional groups in both sites. Molecular identification for the DNA macrofungi through direct samplings of leaf litter identified distinctive communities and indicator species. The results of both methods revealed that several abiotic and biotic factors were apparent, but other complex factors not considered in the present study may be important for characterizing the litter-associated macrofungi of forests in the Ozark Mountains. The effect of prescribed burning practices on the litter macrofungi was assessed and it was concluded that as a result of prescribed burning, neither total species richness nor total macrofungal productivity were substantially different between the compared sites. Nevertheless, prescribed burning shaped the composition of the fungal assemblage on various litter microhabitats, resulting in a decline in the species richness and numbers of fruiting bodies inhabiting the various microhabitats, and reduced the indicator species in PR sites. The data presented herein provide an unprecedented insight into the macrofungal diversity taxonomically, functionally and compositionally in the litter microhabitat of the Ozark forests aiding in future fungal investigation and forest management decisions.

Dedication

I wholeheartedly dedicated my dissertation to my dearest mother who passes away more than year and a half ago before my graduation, and my cherished father who passed away forty days before my defense.
My parents loved and supported me through every moment and challenge in my life and especially through my long education starting from the first day in elementary school until before taking my doctorate. Their words of engorgement still echo in my mind to this day and gave me the strength to keep going and striving to reach my goal even after losing them.

Table of Contents

Chapter 1. General Introduction……..…….……..……..1

1.1 Forest floor litter and associated fungi…….……...…...…1

1.2 Litter-associated fungi as microfungi and macrofungi……..…….....6

1.3 Functional diversity of litter-associated fungi: fungal lifestyle and their interconversions……...…...9

1.4 Techniques for taxonomic litter fungi: Traditional morphological and DNA sequencing methods versus modern next-generation sequencing approaches ……...13

1.5 Outline and objectives of the thesis……...…….19

1.6 References …….....….30

Chapter 2. Fungi Associated with Forest Floor Litter in Northwest Arkansas….....38

2.1 Abstract……....…….…….…….38

2.2 Introduction…….…….……...…….....38

2.3 Materials and Methods……..…….…….....……..…40

2.3.1 Study areas and fungal collection….....…….……...……...…….40

2.3.2 Processing and identification of fruiting bodies...……...……...……..…41

2.3.3 DNA extraction and amplification……...……...…….…….....….....42

2.3.4 Sanger sequencing and taxonomic determinations…….….....43

2.4 Results……..…….…….…….……44

2.4.1 Taxonomic diversity…….……..…….……..…44

2.4.2 Functional diversity……..……..……..44

2.5 Discussion…….....……...……53

2.6 References …….……56

Chapter 3. Taxonomic, Functional, and Compositional Variabilities of Macrofungi Associated with Various Litter Microhabitats in the Forests of the Ozark Mountains.....59

3.1 Abstract…….....…….....59

3.2 Introduction ……....……...…….……...….60

3.3 Materials and Methods …….…….....64

3.3.1 Sampled plots with macrofungal processing and identification……...…….64

3.3.2 DNA extraction, PCR amplification and Sanger sequencing……...……..…65

3.3.3 Taxonomic affiliations and functional assignments……...……...…...….....65

3.3.4 Statistical analyses of diverse macrofungal taxa and functional guilds on various litter macrohabitats ….....66

3.3.5 Comparation and correlation between environmental variables and compositional and functional variability on distinctive litter microhabitats…....67

3.4 Results ……...……...….....71

3.4.1 Taxonomical affiliations and community patterns…….…….71

3.4.2 Macrofungal species richness and productivity patterns based on taxonomic affiliations, litter microhabitats and functional guilds…..……...…….72

3.4.3 Macrofungal community assemblies across investigated sites …….…….73

3.5 Discussion.…….…….…….. 91

3.5.1 Community patterns of litter-associated fungi: overall taxonomic affiliations, microhabitat distributions and functional guilds …….……...…….…...…..91

3.5.2 Interactions and comparations of fungal community populations across investigated sites …….……..…..……..96

3.6 Conclusions ……..……...……..100

3.7 References…….……...……...…..…..102

Chapter 4. Metataxonomic Analyses of Assemblages of Leaf Litter Macrofungi: Assessing Taxonomic and Functional Diversity and Correlation Between the Community Assemblages……...……...……...……..…….……...109

4.1 Abstract……....…….……..……...……109

4.1 Introduction ……..…….…….……...……110

4.2 Materials and Methods…….…….....113

4.3.1 Study sites and sampling processes …….……..……113

4.3.2 DNA extraction, PCR amplification and NGS library sequencing....…...…114

4.3.3 Bioinformatic analyses.……...……..115

4.3.4 Taxonomic and functional assignments…….……...…….116

4.3.5 Statistical analyses……...….....118

4.4 Results… …….……...……120

4.4.1 Overview of sequence taxonomical identifications and community structure.……...……..120

4.4.2 Macrofungal taxonomic and functional diversity for leaf litter…..…….….122

4.4.3 Comparing taxonomic and functional community assemblages of macrofungi across sampling sites……..……....123

4.4.3.1 Correlation between site variables and compositional taxonomic variability……...……...…….123

4.4.3.2 Correlation between investigated sites and functional guilds ……...….124

4.5 Discussion ……...…145

4.5.1 Taxonomic diversities and structures of fungal OTU assemblages.....145

4.5.2 Functional structures and diversities of macrofungal assemblages on leaf litter……..…….147

4.5.3 Comparisons, correlations and distributions between different macrofungal assemblages and variables across plot sites …….....……..150

4.6 Conclusions …….……...…..…..…153

4.7 References…….……..…...……...…...154

4.8 Supplementary Materials…….……...…..161

Chapter 5. Effect of prescribed burning on the taxonomic and functional diversity of litter-associated macrofungi in the Pea Ridge National Military Park, Arkansas …...…..171

5.1 Abstract……...…….171

5.2 Introduction …….....……...…….……...172

5.3 Materials and Methods …….……...…….....175

5.3.1 Study sites and sampled plots……...….……175

5.3.2 Processing and identification of macrofungi…….....…….176

5.3.3 DNA extraction, PCR amplification, and Sanger sequencing……177

5.3.4 Taxonomic affiliations and functional assignments……..…....177

5.3.5 Statistical analyses……...…….…….177

5.4 Results ……...…….....179

5.4.1 Taxonomical identifications and community structures……...…..….179

5.4.2 Species richness and productivity patterns for litter microhabitats and ecological functions……...…...…181

5.4.3 Comparing macrofungal community assemblies across investigated sites..182

5.5 Discussion ……...……....192

5.5.1 Assemblages of litter-associated fungi: overall taxonomic diversity ……192

5.5.2 Distributions of litter macrofungal communities on their microhabitats……194

5.5.3 Comparison of functional diversities of litter-associated macrofungi……196

5.6 Conclusions ……...…….....198

5.7 References……...…..…200

5.8 Supplementary Materials……...…….……..206

Chapter 6. General Conclusions……...….210

List of Tables

Table 2.1. Identified fungi associated with the litter microhabitats in the forests of northwest Arkansas……..……..…….……46

Table 3.1. Correlation coefficients of the vectors fitted on the ordination axes using separate NMDS analyses for the variables of Pea Ridge (PR) and Devil's Den (DD) plots (n=20), fungal families (A) and fungal richness assemblages (B) ……..…..…..86

Table 3.2. Macrofungal taxa and their codes for species clustered on a matrix axis after using two-way cluster analysis for DD and PR plots……..…..…88

Table 3.3. Indicator species value (IV%) litter associated macrofungi for both investigated sites…….…….....90

Table 4.1. The results of charectarizing and comparing fungal variables of Pea Ridge (PR) and Devil's Den (DD) plots (n=20). ……..……...……...……..…….142

Table 4.2. The indicator values (IV%), standard deviation (SD), probabilities (*P*-value) and accession numbers of indicator taxa OTUs associated with the leaf litter in both phyla, Ascomycota (A) and Basidiomycota (B) with *P*-values ≤ 0.05 as identified from sequencing rDNA-ITS2 region for both Pea Ridge (PR) and Devil's Den (DD) sampled sites…….....144

Table 4.1S. Unique molecular identifier tags (MIDs) assignment for samples collected from Pea Ridge (PR) and Devil's Den (DD) sites…….…..…..…….……...161

Table 4.2S. Sequences of Ion Torrent adaptors and gene primers for sequencing the ITS2 region…….162

Table 4.3S. The summary of raw sequances and read length histograms of the next-generation sequancing for each sample from Devil's Den investigated plots (D1-D10) …...……163

Table 4.4S. The summary of raw sequances and read length histograms of the next-generation sequancing for each sample from Pea Ridge investigated plots (PN1-PN10)……..164

Table 4.5S. Macrofungal taxa and their codes for species clustered on a matrix axis after using two-way cluster analysis for DD and PR plots……165

Table 5.1. The indicator values (IV%), standard devotion (S.D) and probabilities of litter indicator species with *P*-values ≤ 0.05 for both investigated unburned (PN) and burned (PB) sites…….…….…….....191

Table 5.1S. The results of prescribed burning effects based on richness of fungal taxonomic group across study plots (n=20) as revealed by correlation coefficients between fungal families recorded (A) and community variables (B) with ordination axes using two separated nonmetric multidimensional scaling (NMDS) …….....206

Table 5.2S. Macrofungal taxa with their codes for two-way cluster analysis…….....207

List of Figures

Fig. 1.1. Mycelial growth and fruiting body development of *Sebacina* sp. on the mixed litter of the forest floor of Pea Ridge National Military Park……..……..18

Fig. 1.2. Geographic locations of the investigated forests in northwest Arkansas including Pea Ridge National Military and Devil‘s Den Parks……..…..…….….21

Fig. 1.3. Archetypal locations of the deciduous mixed forest in Pea Ridge National Military Park during two intervals. ……...…….…….……...……...…….22

Fig. 1.4. Representative locations in the deciduous mixed forest in Devil’s Den State Park during two intervals. …….……...……..…..23

Fig. 1.5. Forest floor components of the Ozark study sites……..……..……....……..24

Fig. 1.6. Incubation chamber experiment for forest floor litter materials with some isolated macrofungi. ……..……...……25

Fig. 1.7. Monthly air temperature (°C) and precipitation (cm) averages of Devil’s Den Park (blue) and Pea Ridge National Military Park (orange) for Northwest Arkansas region in 2016…….……...…….…26

Fig. 1.8. Map of fungal ribosomal genes and target regions of PCR primers for the current study for both Sanger sequencing and next-generation sequencing techniques….....27

Fig. 1.9. Geographic locations of the two investigated sites in the Pea Ridge National Military Park; unburned (PN) and burned (PB) sites…….……...…….28

Fig. 1.10. Represented forest floor components of burned plot site that yielded fruiting bodies in Pea Ridge National Military Park……...…….……...…….29

Fig. 2.1. Families represented by litter-associated fungi recorded in the present study……50

Fig. 2.2. The relative proportion of the most abundance of macrofungal functional guilds of the classified litter-associated fungi.……..…….…….…….…….51

Fig. 2.3. Selected species of litter-associated fungi recorded on the present study…..……..…52

Fig. 3.1. Overstory tree structure based on stem density, basal area and importance value of each tree species divided into large trees (A) with size class >10 DBH (cm) and small trees (B) with size class > 2.5 - 9.9 DBH (cm) for tree Devil’s Den plot site…….....69

Fig. 3.2. Overstory tree structure based on stem density, basal area and importance value of each tree species divided into large trees (A) with size class >10 DBH (cm) and small trees (B)

with size class > 2.5 - 9.9 DBH (cm) for tree Pea Ridge National Military plot site……..……...……...70

Fig. 3.3. Morphological diversity of macrofungal fruiting bodies on various floor litter microhabitats in the Pea Ridge site……..……....76

Fig. 3.4. Morphological diversity of macrofungal fruiting bodies on various litter microhabitats in the Devil's Den site…….…..……..……...……...……....77

Fig. 3.5. Taxonomic structure of litter-associated fungi for Devil's Den (A) and Pea Ridge (B) sites at the genus level based on macrofungal richness illustrated by Krona plots ..……78

Fig. 3.6. The means of species richness (A) and a number of fruiting bodies (B) of litter-associated macrofungi for the sets of the investigated sites…….……..…79

Fig. 3.7. The species richness (A-C) and number of fruiting bodies (D-F) of leafy, woody and mixed litter-associated macrofungal assemblages on various microhabitats across Devil's Den (DD) and Pea Ridge (PR) sites…….……..……..……...80

Fig. 3.8. The species richness (A-C) and productivity of fruiting bodies (D-F) of the putative ecological functions of saprotrophs, mycorrhizas, and *incertae sedis* guilds, of litter associated macrofungi across plots within Devil's Den (DD) and Pea Ridge (PR) forests....81

Fig. 3.9. Non-metric multidimensional scaling (NMDS) ordination plots of litter-associated fungi within sampling plots of the Devil's Den (DD) and Pea Ridge (PR) forests based on the binary data……...……..……...…82

Fig. 3.10. The cluster analysis of litter-associated fungi on various microhabitats across 20 sampling plots in the Devil's Den (DD) and Pea Ridge (PR) forests using Euclidean distance and Ward's linkage method (B) based on the binary data of macrofungal species ….....83

Fig. 3.11. Non-metric multidimensional scaling (NMDS) analysis of the forest plots and family structure variables overlaid as vectors on ordination axes based on a number of macrofungal species per taxonomic family of Devil's Den (DD) and Pea ridge (PR) datasets……....……...…84

Fig. 3.12. Non-metric multidimensional scaling (NMDS) analysis of the richness of taxonomic and functional groups and of litter microhabitat species, and the forest plot variables for the Devil's Den (DD) and Pea ridge (PR) sites fitted as vectors on NMDS axes…….…….……....85

Fig. 3.13. Distribution patterns of 73 species in various litter microhabitats clustered on the horizontal matrix axis and distributed across investigated plots for investigated Devil's Den (red color) and Pea Ridge (green color) sites, grouped on the vertical axis using two-way cluster analysis…….…….....87

Fig. 3.14. Diverse morphological patterns of macrofungal fruiting bodies for indicator species……..89

Fig. 4.1. Characterizing and comparing the phylum composition of leaf litter fungi from plots in Devil's Den and Pea Ridge forests based on fungal OTU read abundance……..…….…....126

Fig. 4.2. The number of OTU phyla of macrofungi from plots in Devil's Den and Pea Ridge forests……...……..…….…..…127

Fig. 4.3. Overview of leaf litter fungi in Devil's Den (blue bars) and Pea Ridge (orange bars) forests based on the number of OTU sequences (A) ascomycete orders, (B) basidiomycete orders, and (C) basidiomycete orders after excluded members of Tremellales…....…..…128

Fig. 4.4. Krona plot diagrams that characterize and compare taxonomically the classified community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of DD site (A) and PR site (B)……...…..…129

Fig. 4.5. Krona plot diagrams that characterize and compare taxonomically the ascomycete community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of DD plots (A) and PR plots (B). …….130

Fig. 4.6. Krona plot diagrams that characterize and compare taxonomically the basidiomycete community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of DD site (A) and PR site (B)……131

Fig. 4.7. Total fungal richness (A) and diversity analyses (B-D) of all taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites…….……...…132

Fig. 4.8. Total ascomycete richness (A) and diversity analyses (B-D) of taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites. ……...…..……133

Fig. 4.9. Total basidiomycete richness and diversity analyses of taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites…….…...…134

Fig. 4.10. Characterizing and comparing the OTU richness of saprotrophs on the different substrates and the total OTU richness of saprotrophs with other functional guilds of litter-associated fungi…….....……135

Fig. 4.11. Leaf litter macrofungal community assemblage of 97 genera clustered on the horizontal matrix axis and distributed across 20 investigated plots for Devil's Den (red color) and Pea Ridge (green color) sites, grouped on the vertical axis using two-way cluster analysis……..……..……...……136

Fig. 4.12. Cluster analysis of leaf litter-associated fungi across the sampling sites of both Devil's Den (DD) and Pea Ridge (PR) plots using Euclidean distance and Ward's linkage method based on number of operation taxonomic unites (OTUs) per a taxonomic group…….137

Fig. 4.13. Non-metric multidimensional scaling (NMDS) ordination plots of leaf litter-associated fungi across the sampling sites of the both Devil's Den (DD) and Pea Ridge (PR) forests based on the number of the operation taxonomic unites (OTUs) per taxonomic group..…138

Fig. 4.14. Non-metric multidimensional scaling (NMDS) ordination plots of the fungal and leaf chemical characteristic variables of leaf litter-associated fungi across the sampling sites for the both Devil's Den (DD) and Pea Ridge (PR) forests based on the binary datasets……..139

Fig. 4.15. Non-metric multidimensional scaling (NMDS) ordination plots of the plot site and order assemblage variables of the leaf litter-associated fungi overlaid as vectors on ordinate axes based on binary datasets of Devil's Den (DD) and Pea Ridge (PR) plots.……140

Fig. 4.16. Structuring functional variables of (A) all phylum functional guilds after gathering all saprotrophs with respecting to their substrates (B) all species guilds with respecting to their higher rank taxa groups and dividing saprotrophs into portions based on substrates based on macrofungal OTU richness……...…...…….……141

Fig. 4.1S. Representave samples of electrophoreses for triplicate PCR products with negative control (co.) for each sequenced sample…….…….……..167

Fig. 4.2S. Electrophoresis of pooled PCR products for 10 samples of each investigated site; Devil's Den (DD) and Pea Ridge (PR) sites……...……...168

Fig. 4.3S. The raw run summary of Ion Torrent sequancing……...……....169

Fig. 4.4S. Rarefaction curve of sampled leaf litter-associated fungi for DNA sequence reads of ITS2 region across 20 sampled plots of both Pea Ridge (PR) and Devil's Den (DD) sites.....170

Fig. 5.1. The estimated percentages of macrofungal genus productivity for burned (orange bars) and unburned (blue bars) sites based on a number of fruiting bodies produced by a particular species…….…….…….…….....184

Fig. 5.2. The taxonomic comparison of fungal community assemblage in the burned and unburned sites based on the number of species per taxon.....185

Fig. 5.3. The total species richness (A) and number of fruiting bodies (B) of litter-associated macrofungi for both investigated sites with no significant differences apparent between sites based on the pairwise Tukey's test at $P < 0.05$……..…….…….……..186

Fig. 5.4. The species richness (A) and number of fruiting bodies (B) of litter-associated macrofungal assemblages on the various microhabitats, leafy litter (LL), woody litter (WL) and mixed litter (ML), in burned and unburned sites. ……..…..……...187

Fig. 5.5. Comparison of the putative ecological functions of litter-associated macrofungi, ectomycorrhizas (EC), saprotrophs (SA) and *incertae sedis* guilds (IS) in relation to species richness (A), and productivity of fruiting bodies (B)……..……..……..188

Fig. 5.6. Non-metric multidimensional scaling (NMDS) ordinations for (A) family compositions and (B) fungal structure variables of environmental variables overlaid as vectors on investigated plots, based on richness data.……..….....…….189

Fig. 5.7. The species distribution of 91 macrofungi, clustered on the horizontal matrix axis, across 20 investigated plots for unburned (green color) and burned (red color) sites, grouped on the vertical axis using two-way cluster analysis……...190

Fig. 5.1S. The forest composition of the sampled sites after dividing trees into large tress with stems ≥ 10 cm diameter at breast height (DBH) and small trees with stems ≥ 2.5-9.9 cm DBH……...209

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Chapter 1. General Introduction

1.1 Forest Floor Litter and Associated Fungi

Plant litter materials are the most valuable renewable resource on the earth and an essential component in the terrestrial ecosystem. Globally, more than 100 gigatons of terrestrial plant biomass are produced each year. Up to 90% of this plant productions enters the dead organic matter pool to be decomposed and sequestered as organic carbon stored either in soils or sediments, both of which are central components of ecosystem functioning (Cebrian, 1999; Gessner et al., 2010). Ecologically, the term litter has two meanings, referring to the layer of dead plant materials present on the soil surface or those materials detached from a living plant. Whether dead fallen substrates or attached branches, there are no benchmarks for the beginnings of decomposition of plant litter in various forest types (Krishna and Mohan, 2017). In forests, most of the decomposition process takes place in and on the forest floor and involves a complex set of processes including chemical, physical and biological mediators acting upon various organic substrates that are constantly changing (Berg and McClaugherty, 2008). Leaf litter is estimated to be more than 70% of aboveground litterfall and the rest consists of stems, small twigs and propagative structures (Robertson and Paul, 1999). In temperate deciduous forests, for example, Ozark Mountains of northwest Arkansas, the primary forested community is dominated by species of oak (*Quercus*) and hickory (*Carya*) in addition to American beech (*Fagus grandifolia* Ehrh.) (Leis, 2018). These produce a mixture of diverse host litter and a large amount of detritus each year, and these are the driving force behind the establishment of the soil profile and turnover of nutrients to the soil forest ecosystem through litter decomposition. The quality of plant litter is usually determined on the basis of the chemical compositions of nitrogen, phosphorus, potassium and cell wall components, such as lignin, cellulose, and hemicelluloses

which differ in their relative proportions based on the plant species and parts (leaves, stems, roots, bark) involved (Berg and McClaugherty, 2008). The productivity of terrestrial ecosystems is directly connected with nutrient cycling between the various components of the plant and soil system (Vitousek, 1984; Terror et al., 2011).

Litter decomposition plays a vital part in the nutrient cycling of a forest ecosystem, where the vegetation is influenced most significantly by a breakdown of organic matter into CO2 and nutrients. The rate of litter decomposition is influenced by several connected factors including physical, biological and chemical pathways in a forest system. For example, climatic features, such as temperature, rainfall, and seasonal variations, can be considered as prime factors in determining the rates of litter decomposition and may influence the existence of microbes and other soil fauna. The dead leaves and other types of non-woody detritus provide microhabitats for the often-overlooked litter-decomposing fungi and their consumers (Berg and McClaugherty, 2008; Krishna and Mohan 2017). In this process, fungi have more than a 75% greater potential to decompose organic matter than bacteria (Kjoller and Struwe, 1992). This likely leads to an extraordinarily diverse community of fungi and soil organisms connected by highly complex interactions in forest ecosystems (Hattenschwiler et al., 2005).

The fungal kingdom is the second most diverse eukaryotic lineage on earth after the insects on earth, with an updated estimate of 12 million compared to 3.5–5.1 million species estimated previously, due to technical developments, particularly next-generation sequencing approaches (Blackwell, 2011; Wu et al., 2019). Hawksworth and Rossman (1997) suggested that most of the unidentified fungi inhabit understudied microhabitats and known habitats explored by using new techniques. Litter-associated fungi are an understudied assemblage and presumably support a high level of hidden diversity due to methods that have been used (Baldrian, 2017).

Litter-associated fungi mostly belonging to the 69 families of the Basidiomycetes, Ascomycetes and Mucoromycotina are the major contributors to decomposition, carbon sequestration, and nutrient immobilization as well serving as both soil stabilizers and biological remediators (Berg and McClaugherty 2008, Claridge et al. 2009; Osono, 2020). Additionally, macrofungal taxa of the Basidiomycetes and Ascomycetes also have an economic importance as food and medical resources (Miles and Chang, 2004). Diverse assemblages of tree species and litter traits could also lead to the greatest fungal species richness and fruiting body productions (Cline and Zak, 2015; Alanbagi et al., 2019). The reachable nutrients of leaf litter, for example, could trigger fungal spore germination and develop networks of mycelial growth and later influence the colonization success of ligninolytic fungi or other later arriving fungal species (Voříšková and Baldrian, 2013; Baldrian, 2017). These fungi are progressively colonized by several different fungal strategies and patterns of hyphal foraging with various potential enzymatic activities. This species dissimilarity in the use of resources in a complementary way forms a community that is more efficient in acquiring available resources (Goswami et al., 2017), eventually resulting in enhanced ecosystem functions (Mori et al., 2013).

Decomposing activity for recalcitrant compounds has been recorded in many macrofungal isolates with selective decaying ability for litter materials from tropical, temperate and subalpine forests. Saprotrophic fungi are major contributors as a result of their high enzymatic capacity during the decomposition processes. That is structurally altered from predominant members of the Ascomycota in the early decomposition of easily attainable carbon to increased members of Basidiomycota with the accumulation of recalcitrant compounds. Families such as the Rhytismataceae and Xylariaceae in the Ascomycota and the Marasmiacease, Mycenaceae in the Basidiomycota were specifically detected as fungal taxa with ligninolytic ability under pure

culture tests in the laboratory and during inventory methods used for the litter layer (Berg and McClaugherty 2008; Osono, 2020). The ligninolytic activity varied among individual fungal species and with the quality and type of substrate when being inoculated adjacent to sterilized leaves and other substrates. Litter communities of freshly fallen leaf litter are dominated primarily by species of *Marasmius*, *Marasmiellus*, and *Mycena*, and other litter materials are dominated by species of *Clitocybe*, *Collybia*, *Geastrum*, *Lepiota*, and *Leucocoprinus* in diverse forest types using inventory and culture dependent methods (Osono, 2007, 2019; Alanbagi et al., 2019). Some of these agarics have been recorded in the different wet forest floors of planted (Hedger,1985), tropical (Lodge 1993; Braga-Neto et al., 2008) and temperate forests (Bahnmann et al., 2018). They usually are rapidly trapping freshly fallen leaves and binding them to the litter mat (Lodge and Asbury, 1988) and some are found to translocate significant quantities of ^{32}P and other nutrients from partly decomposed litter into freshly fallen leaves (Lodge 1993; Berg and McClaugherty, 2008). Thus, litter-binding species would reduce the loss of litter and soil organic matter from the ecosystem through erosion and increase the competitive abilities of these decomposers by increasing the speed by which they can colonize newly fallen leaves. This seemingly results in accelerating the rate of litter decomposition and nutrient recycling due to efficient extracellular enzyme activity, mostly ligninolytic enzymes with a Fenton system, which is an alternative mechanism for depolymerization and biomodification of plant litter based on reduction of Fe^{3+} to Fe^{2+}, that might be accomplished by extracellular fungal metabolites or reductive enzymes (Lodge 1993; Berg and McClaugherty, 2008; Eastwood et al., 2011).

The factors driving the assemblage and productivity of macrofungal litter are also poorly studied and understood (Kauserud et al., 2010; Collado et al., 2018). Identification of species diversity and the factors influencing macrofungal assemblages is thus a crucial step to determine structure

and function invaders in decomposition. This knowledge is also important to conserve their diversity (Berg and McClaugherty, 2008; Krishna and Mohan, 2017; Chen et al., 2017), since in the last century their growth conditions have been dramatically influenced by human activities (Osono, 2007; Sun et al., 2015; Santín and Doerr, 2016). Macrofungal fruiting bodies face several natural challenges by climatic factors such as rainfall and temperature (Osono, 2007; Ágreda et al., 2016). Moreover, the emergence of fruiting bodies is likely accompanied by a drastic change in exposure to the limited availability of oxygen and high concentrations of carbon dioxide in the substrate microenvironment (Manachère, 1980). Diverse organisms associated with litter decomposition also impact mycelia and fruiting body developments including such interactions as other fungal and microorganism competitors, fungal metabolite feeders and invertebrate grazers (Hättenschwiler et al., 2005; Gessner et al., 2010). Other challenges for litter-associated fungi are artificial encounters made directly or indirectly by humans (Senn-Irlet et al., 2007; Sun et al., 2015; Arthur et al., 2017). Fires, for instance, the primary natural disturbance factor in forest ecosystems are predicted to increase in the incidence and severity of large global uncontrolled forest fires in the future due to the potential impact of global climate change (Pechony and Shindell, 2010; Liu et al., 2010, 2013). Prescribed burning as an additional example of the forest management has been applied for sustainable and restoration purposes with insufficient knowledge of fungal responses (Schuler and McClain, 2003; Oliver et al., 2015; Dove and Hart, 2017). For example, saprotrophic productivity has been relatively erratic across forest biomes (Kauserud et al., 2010; Collado et al., 2018). It seems that the litter fungal group is disappearing or threatened before being documented. Therefore, more diversity information is needed through the use and combining of the inventory methods with new research techniques. This might be useful to make considerable progress through offering a

means to sample the communities more extensively as well as to detect taxa that have low levels of abundance, resulting in a more comprehensive view of the litter microfungal and macrofungal diversity. These two groups of fungi are morphologically, ecologically and methodologically different.

1.2 Litter-Associated Fungi as Microfungi and Macrofungi

Fungi are essentially microorganisms and spend most of their life cycle in a microbial stage. They can range morphologically from microscopic single-celled yeasts to typical filamentous molds (systems of branching tubes known collectively as a mycelium and singly as hyphae) and as macroscopic multicellular mushrooms. The conquest of new, often patchy resources is significantly facilitated by the production of numerous spores, where a food source is colonized most efficiently by hyphal growth. The fungal hypha is about 5–10 μm in diameter, and their direct outcome on the environment is restricted to a few micrometers on each side of these hyphae (Oberle-Kilic et al., 2013). However, the growth nature of the fungal structures allows them to exploit large volumes of their environment, from intimate associations with other organisms, and influence at much larger spatial scales (Figs. 1.1 and 1.5). Spores can be actively and rapidly germinating on new resources. The germinating tubes and later branching tubes of hyphae radially extend and intensely colonize substrates with an exoenzymatic influence around individual hyphae (Oberle-Kilic et al., 2013). These effectively access the new resources after developing a mycelial network architecture under complex interactions with each other and other fungi and with other organisms (Figs. 1.5C and D; Osono, 2007; Webster and Weber, 2007). The importance of these interactions is little known under field conditions (Osono, 2007). As the first step in the sexual reproduction, the monokaryotic mycelium of previously developed mycelia for either the Ascomycota (self-incompatible) or Basidiomycota ultimately meet another comparable

monokaryotic mycelium of the same species but with a different mating type to form a dikaryotic mycelium. After the establishments of a dikaryotic mycelium through highly complex developments for the formation of fruiting bodies, these processes require special environmental conditions and are controlled by many developmentally regulated genes (Fincham, 1996; Boddy et al., 2008).

Macrofungi, Basidiomycota in particular, have a more persistent presence in the environments owing to their long-lived mycelial structures. Some aggregated mycelia produce structures that are easily visible to the naked eye and their distribution as mycelial cords or rhizomorphs in the litter layer until they unite around the resources. These hyphae and mycelial strands develop long-lived mycelial connections between the different components of litter floor and are relatively common in the Basidiomycota and some Ascomycota (Fig. 1.5; Webster and Weber, 2007; Boddy et al., 2008; Alanbagi, 2014). Bidirectional translocation of nutrients along vegetative or specific mycelium is an important structural feature to support energy needs and growth development for both saprotrophic and ectomycorrhizal fungi (Hattenschwiler et al., 2005). The macrofungal community populations depend on a number of different nutritional strategies and patterns of foraging mycelia and enzymatic activities in this highly competitive microenvironment (Boddy et al., 2008; Bodeker et al., 2016). These hyphal networks with nutrient movements result in reducing forest floor resource heterogeneity and communication channels between not only plants but also the food web as a whole (Peay et al., 2016). The septate or coenocytic mycelia with few exceptions cannot be observed directly with the naked eye or identified in the field but many of them can produce obviously sexual or asexual structures to ensure spore dispersal or other propagules to disperse to novel substrates at a certain stage of development. These interesting structures depend on the potential abilities of hyphal

characteristics and mycelial differentiation. Many of the identified fungi have been determined from the sexual form and their sexual structure although still others are not. The fruiting bodies differ very much in form, but all produce some sort of microscopically small spores which upon germination give rise to a new mycelium (Mueller et al., 2011; Alanbagi, 2014). These frequently could be perceived from their distribution on various substrates and divided into microscopic or macroscopic fruiting bodies based on fruiting body size. These present specific characteristic shapes for each species but only limited tissue differentiation are collectively known as macrofungi (Boddy et al., 2008).

There is no specific definition for macrofungi. However, the mycologist community usually morphologically refers to those fungi that are able to produce fruiting bodies visible to the naked eye or ≥ 1 mm in length or diameter in different habitable environments. The remaining fungi are called microfungi (e.g., Arnolds 1996; Alanbagi, 2014; Brunner et al., 2017; Chen et al., 2018). The limits between the macrofungi and microfungi are very haphazard with respect to taxonomic or ecological units. Within a single genus there can be species belonging to macrofungi and microfungi. For example, *Tremella encephala* Pers produces striking cushion-shaped fruiting bodies up to 40 mm broad, while *Tremella obscura* (Olive) M.P. Christ forms a completely reduced thallus and parasitizes within the small fruiting bodies of *Dacrymyces stillatus* Nees: Fr. (Winterhoff, 2012). Some groups of macrofungi are strongly underrepresented because of their hidden sporocarps, such as members of the Tuberales, Plectascales and Aphyllophorales growing underground or on the underside of wood (e.g. De Vries, 1990). A large number of macrofungi have been recorded around the world. The estimated number was between 53,000 and 110,000 macrofungal species derived using plant/macrofungal species ratio data. However, based on a meta-analysis for the combined data set of macrofungal names, the estimated totals for the world

were 56,679 species. These include a total of 21,679 names of “known” macrofungi; half of which were from North America and Western Europe and 16-41% of them have been described to date. An additional about 35,000 macrofungal taxa were estimated to be “unknown” by the contributing authors. These were compiled by Mueller et al. (2007). For ectomycorrhizas, for instance, the species richness would likely be 20,000-25,000 (Rinaldi et al., 2008). A final estimation could be more than these assessments when applied to areas of different sizes, with a distinctive diversity of the plant community, climate, and environmental factors (Mueller and Schmit, 2007).

Macrofungi associated with forest floor litter materials, that are of particular interest in this regard, are mainly known from their macroscopic reproductive sexual structures with diverse and noteworthy macro-microscopic features (Boddy et al., 2008; Gessner et al., 2010). A satisfactory understanding of this important part of biodiversity is still far from the most likely species-rich taxonomic group during a highly complex process of litter decomposition (e.g., Mueller et al. 2007).

1.3 Functional Diversity of Litter-Associated Fungi: Fungal Lifestyle and Their Interconversions

Diverse fungal assemblages can be ecologically associated with dead organic matter, various plant parts, and animals as a crucial component of forest diversity (Osono, 2007; Baldrian, 2017), while in most cases, the role of an individual fungus in nature is still unknown (Schmit and Mueller, 2007). A growing body of evidence highlights the importance of biodiversity for ecosystem stability and maintenance of the best system function especially with newly emerged concepts based on measures of diversity and richness for both taxonomic species and functional

traits (Mori et al., 2013). In the forest ecosystem, trees and shrubs create an array of microhabitats for those fungi associated with plants. Specifically, the forest floor consists of complex fungal assemblages with close and various interactions among fungi in different stratifications and mainly plant living parts and litter with other organisms. Within their varied natural habitats, the plant-fungal interactions have been extensively investigated and putatively divide into three main lifestyles in macrofungi. Those associated with dead organic matter of plants are considered as saprotrophs, those involved in a mutual symbiosis with plant roots as mycorrhizas, and those living as necrotrophic fungi on dead cells of woody plants, insects and fungi as parasites (Hättenschwiler et al., 2005; Boddy et al., 2008; Winterhoff, 2012). These functions are similar to microfungi, but their ecological main points are different. For example, most microfungi are involved in the degradation of the simplest carbon compounds, the breakdown of numerous cellulose and hemicellulose, but only a few are involved in lignin decay (Boddy et al., 2008). However, saprotrophic macrofungi contribute significantly to the chemical decomposition of cellulose, hemicellulose, and lignin, the latter material being almost entirely attacked by mainly macrofungal Basidiomycota (Winterhoff, 2012; Alanbagi et al., 2019; Osono, 2020). The majority of microfungal mutualists with living plant roots are vesicular-arbuscular mycorrhizas, while most macrofungi are participating as ectomycorrhizas. These ectomycorrhizas belong mostly to the Basidiomycota and, to a lesser extent, the Ascomycota (Tedersoo et al., 2010; Winterhoff, 2012). Relatively few macrofungal species can also function as basidiomycete necrotrophies on woody plants, such as is the case for *Armillaria mellea* (Yahl: Fr.) Kumm. ss. str. *Heterobasidion annosum* (Fr.) Bref. or as ascomycete parasites on insects and fungi such as members of the genus *Cordyceps* (Winterhoff, 2012). Macrofungal litter is also an important food source of insects and other fauna (Gessner et al., 2010). A number of edible and

medicinal species with other uses, in addition, are commercially important for improving human and ecosystem health (Miles, and Chang, 2004; Ferreira et al., 2016).

The species diversity, community structure and value of functional diversity measures on litter decomposition have been insufficiently investigated (Gessner et al., 2010; Baldrian, 2017). However, recently, litter associated fungi have been increasingly studied and determined functional groups with progressive development of more advanced molecular approaches and unexpectedly functional diversity associated with litter decomposition documented (Smith et al., 2017; Osting et al., 2018; Osono, 2020). During litter decompositions, fungi play a central role due to their capabilities to attack a wide range of chemical materials, specifically the lignocellulose matrix in litter largely as saprotrophs that other functional organisms are not able to assimilate efficiently (Boddy et al., 2008; Osono et al., 2019). The fungal overlap between the different functional traits also could be suggested, especially with the recent use of DNA barcoding approaches (Van der Wal et al., 2013; Smith et al., 2017). For example, it has been documented that the litter layer not only harbors saprotrophs but also other lifestyles such as mycorrhizas, parasites or plant pathogens for energy needs and fruiting body productions on litter microhabitats (Rinaldi et al., 2008; Bodeker et al., 2016; Bahnmann et al., 2018). Several studies have reported that ectomycorrhizas, in particular, compete on the same litter substrates with saprotrophs for nutritional and reproductive reasons (Bödeker et al., 2016; Smith et al., 2017 b) with the detection of peroxidase-encoding genes for degrading recalcitrant polyphenolic compounds in a wide range of ectomycorrhizal genera (Bödeker et al., 2009). The species diversity, the habit of hyphal growth and functioning of litter decomposers might be influenced by litter types and temperature, although only a few studies have been compared these factors across multiple sites of different climatic regions (Osono, 2014).

With distinctive litter microhabitats, many macrofungal species have been recorded inhabiting these nutritional rich resources with various trophic modes such as prevalent saprotrophs, various ectomycorrhizas and few parasites with many transitions between these general functions or their subdivided functions in the temperate deciduous forests (Bodeker et al., 2016; Alanbagi et al., 2019). That would suggest that all kinds of intermediates and transitions or even enigmatic in functional groups might occur in litter macrofungi as has been proposed for other macrofungi (e.g., Winterhoff, 2012; Halbwachs et al., 2013; Nguyen et al., 2016). For example, recently some saprotrophic species have been isolated from plant roots under field conditions (Nelsen, 2017) and evidently function as facultative biotrophs with plant roots under lab conditions (Smith et al., 2017a). Thus, it is suggested for living plant-fungi interaction that saprotrophs have the potential of switching to another life type mode comparable to altering endophytes to latent pathogens and most likely to be host-specific or host-recurrent based on the evolution history with the plant's evolutionary history (Photita et al., 2004). What triggers fungi to switch among lifestyles is still an important question that needs to be answered (Rai and Agarkar, 2016) with several interesting questions arising and considerations for recategorizations of functional guilds. If the functional redundancy and variation within a functional effect group are carefully characterized and understood, it would be expected that the assemblage of species respond differentially to environmental and disturbance changes. This is important for management decisions, for example, that can be made for its active maintenance (Mori et al., 2013). In conclusion, the litter layer constitutes from diverse macrofungal taxa include a considerable part of the saprotrophs, many ectomycorrhizas, and most enigmatic functions. These play a vital role in the lifecycle of the biosphere through mineralization, transformation and recycling soil nutrients from all plant debris generated overtime.

1.4 Techniques for Taxonomic Litter Fungi: Traditional Morphological and DNA Sequencing Methods Versus Modern Next-Generation Sequencing Approaches

Fungal taxonomy focuses on discovering, describing, and classifying all species of fungi. All taxonomic concepts are human-made and thus subject to a certain degree arbitrary. Researchers continuously try to provide and improve tools for fungal identification to detect species diversity on the earth (Webster and Weber, 2007). The major limitation of this goal is that traditionally the only fungi targeted are those that either easily cultured on artificial media or produced fruiting bodies that can be sampled in field, which is also a matter of human opinion for the description of a particular character (Webster and Weber, 2007; Boddy et al., 2008). Thus, several groups of fungi have been inadequately explored. Despite the important role of litter-inhabiting fungi in the decomposition process, our knowledge of the macrofungal diversity and factors shaping the dynamics of their species richness, productivity, functional diversity, and community composition is scarce (Kauserud et al., 2010; Collado et al., 2018). This is due primarily to methodological limitations regarding the classic inventory approaches, complemented by culture-dependent methods for characterization of litter fungal assemblages (Boody et al., 2008). Inventory methods rely on collecting visible sexual structures or fruiting bodies of fungi with *in situ* monitoring. Fruiting bodies usually persist for only a short duration, and the strong periodicity and fluctuations of fruiting are influenced by temperatures and precipitation within a geographic region and forest practices leading to changes in phenology and productivity of fruit bodies (Kauserud et al., 2010; Mueller et al., 2011). Isolation of fungi growing out from litter components placed on artificial media has also been used. Culture techniques largely have been applied for microfungi with unique macro or microscopic features due to the complete absence of larger basidiomycetes from incubated samples (Osono, 2020; Winterhoff, 2012). However,

challenges have been faced by the widespread occurrence of fungal hyphae that do not sporulate in culture, are capable of being cultured or ever reproduce sexually and those lost during sterilization methods of specimens, are slow-growing taxa, or are sterile, which are usually macrofungi (Boody et al., 2008). Later, classification systems of fungi were updated to more accurately be identified by molecular characters for previous challenges and morphological traits that are convergent, reduced, or missing among the taxa considered. These molecular methods are based on the polymerase chain reaction (PCR) amplification and sequencing of a small part of the fungal genome based on the internal transcribed spacer (ITS) region using fungal- specific primers (White et al., 1990) and DNA extracted from field samples as a template in the early 2000s. These methods began by the cloning approach for DNA identification of fruiting bodies or sporulating cultures from a single fungal taxon. Cloning has to be combined with community fingerprinting methods such as terminal restriction fragment length polymorphism (TRFLP) if large numbers of samples are analyzed (Lindahl et al., 2007; Boody et al., 2008). The DNA sequencing results clearly indicated that the total mycobiota far exceeds the diversity recognizable through fruiting bodies and cultivation (O'Brien et al., 2005).

With the continuous development in molecular techniques and primers, next-generation sequencing technologies and high-performance computers defined the culture-independent era of microbial ecology (Nilsson et al., 2019). DNA-barcoding approaches were first applied for taxonomic identification and detection of species diversity. Estimation of fungal diversity in various natural environments uncovered hidden fungal diversity based on specimen-based studies, mostly with soil and root-associated fungi (e.g., Clemmensen et al., 2015; Grau et al., 2017). These next-generation sequencing approaches are now extensively deployed for community characterization, detecting novel taxa, and exploring the dynamic nature of fungal

richness and composition along environmental gradients and different fungal substrates through directly DNA sequencing of environmental samples (Semenova et al., 2016; Yahr et al., 2016; Nelsen, 2017). High-throughput sequencing (HTS) has been applied through using slandered, short sequences with several fungal genomic regions that have been used as barcode markers, including the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) genes, as well as two noncoding internal transcribed spacers (ITS1 and ITS2) (Tedersoo et al., 2015; Grau et al., 2017). As a result of providing greater richness and taxonomic resolution as compared to other markers (Tedersoo et al., 2015), the formal acceptance of the fungal ITS region ultimately became the slandered practice (Schoch et al., 2012; Yahr et al., 2017), with an exponentially growing number of publications characterizing not only the fungal communities but also functional diversity and assemblages in various ecosystems (Nilsson et al., 2017; Li et al., 2020). The results of these techniques also indicated that many previously regarded "unculturable fungi" could be cultured on certain substrates under specific conditions and this represented a powerful means to detect novel taxa (Wu et al., 2019). However, HTS approaches come with a range of pitfalls and feasible biases, cautioning against unsuspecting application and interpretation of HTS technologies and results (Nilsson et al., 2019). Practically, the ITS region is too long to be fully sequenced by current second-generation sequencing. Thus, either the ITS1 or the ITS2 region are targeted (Li et al., 2020). Targeting the full ITS region and including a greater taxonomic resolution by means of the third-generation sequencing techniques may enable the resolving of some of the disadvantages of the earlier generations and increasingly improving the read quality of third-generation platforms could be enough to use in metabarcoding and community analysis (Nilsson et al., 2019). Several studies have companied both culture-dependent and independent methods for fungal investigation. However, the fungal species that

were detected by both approaches were not actually overlapping even for the dominant species (Fischer et al., 2012; Smith et al., 2017b) which suggested the intrinsic selectivity of each method.

Research efforts have been decided towards sequencing different ecosystems and substrates to discover at least the nearest previously estimated fungal diversity of 1.5–6 million species, with roughly over 100,000 fungal described species (Blackwell, 2011; Taylor et al., 2014). That is clearly still far below the estimated number of fungal species. That is because the slow progress in specimen collection and reference database improvement as well as technological limitations such as the biases and errors introduced during sampling, DNA extraction, PCR and sequencing (Halwachs et al., 2017; Lindahl et al., 2013; Nilsson et al., 2019; Taylor et al., 2016).

The identification and compiling of information data on forest fungal communities in the heterogeneous litter have been fragmentary and largely incomplete due to previous concerns. Recently, HTS approaches have been developed fungal identification and used extensively to analyze taxonomically the community structure of litter-associated fungi and the timing of colonization and decomposition processes to enhance understanding of the hidden species and functional diversity of this group (Voříšková and Baldrian, 2013; Osting et al., 2018; Bahnmann et al., 2018). The usual focus of these studies has been on the microfungal community assemblages with detecting few litter macrofungi and interesting mostly ectomycorrhizal fungi. With applying these methods, some studies have also highlighted the important of biotic and abiotic parameters such as litter mixtures and quality (Purahong et al., 2016; Osting et al., 2018), tree species diversity (Urbanová et al., 2015; Schimann et al., 2017) that shape the diversity and distribution of the fungal community in forest floor litter across tropical, boreal, and temperate ecosystems. These processes are important in influencing the distribution and structure of fungal

ecological guilds in litter and switching among lifestyles although these types of studies have received less attention and expected factor effects remain poorly understood (Rai and Agarkar, 2016; Bahnmann et al., 2018). Although next-generation sequencing approaches have several problematic issues with respect to meeting these challenges, the techniques do provide a powerful tool to characterize the distribution, structure and function of mycobiome assemblage in heterogenous litter and confirm the enormous diversity of fungi. With the exponentially growing volume of data gathered from high throughput sequencing technology, the DNA barcoding with metagenomic studies and metataxonomic tools used side by side with collection-based sequencing and newly described species are important tools and certainly a priority for the coming decades. These also required more knowledge of the key functional attributes of different fungal species to interact with other ecosystem components as well as the identification of the appropriate scale at which we can predict features of the mycobiome.

Fig. 1.1. Mycelial growth and fruiting body development of *Sebacina* sp. on the mixed litter of the forest floor of Pea Ridge National Military Park. Photograph by the author.

1.5 Outline and Objectives of the Thesis

The study reported here is the first effort to provide data for a more complete understanding of the species and functional diversity of litter-associated macrofungi, species richness and distributions on multiple floor litter microhabitats, community structures and functional assemblages in the Ozark Mountain forests based on different objectives and methodologies. Also, research described herein investigated some biotic-abiotic factors and prescribed burning management practice on macrofungal litter assemblages distributed in the investigated sites. To our knowledge, this is the first attempt to mainly describe the macrofungi based on litter microhabitats in detail and specific defining of macrofungi on very fine woody debris. The following project objectives and results were presented in chapters 2 to 5. The thesis objectives were:

(1) Documenting and characterizing of the overall taxonomic species richness, fruiting body productivity and functional guilds for the litter-associated macrofungi in a temperate deciduous forest located in the Ozark Mountains of northwest Arkansas (Figs. 1.2-1.4). The goal of this objective was based initially on the fruiting body inventories and the incubation chambers of the forest floor litter for categorizing litter microhabitats and macrofungal distribution across these microhabitats (Figs. 1.5-1.6).

(2) Describing and comparing litter macrofungal assemblages taxonomically, functionally and compositionally across sampling two sites in the Ozark forest, Pea Ridge National Military Park and Devil's Den State Park (Figs. 1.3-1.4). Additionally, species and functional richness with their productivities based on a number of fruiting bodies on leafy, woody and mixed litter microhabitats were also detected. The data obtained used general morphological characters and Sanger sequencing of taxon fruiting bodies, taxonomic and functional databases and non-metric

multidimensional scaling (NMDS) and cluster analyses supported by various statistical analyses (Fig. 1.8). This part also focused on effects of some site features on macrofungal community compositions such as temperatures and precipitation (Fig. 1.7).

(3) Providing novel insights into the macrofungal diversity and suggesting some drivers that shape the community structures of litter macrofungi through environmental samplings of the leaf litter microhabitats as the main component of a heterogenous litter layer in a temperate deciduous forest ecosystem on the Ozark Plateau (Figs.1.3-1.5). For this microhabitat, the representative OTUs were identified taxonomically and segregated compositionally for the leaf litter microhabitat of twenty plots in two Ozark forests. The deep sequencing presented also diverse and overlapping functional groups inhabiting leaf litter. The knowledge of the molecular data was constructed using next-generation sequencing through rDNA metabarcoding of the fungal ITS2 region, metataxonomic and functional tools and diversity analyses (Fig.1.8). High-throughput sequencing technologies using amplicon approaches were used as powerful tools to investigate the hidden diversity in the challenged substrate investigation.

(4) Determining the effect of prescribed burning management practices on the macrofungi associated with each litter microhabitat in the Pea Ridge National Military Park, a historic forest in northwest Arkansas using fruiting body inventories, morphological characters, and Sanger sequencing with various statistical analyses and visual methods (Figs. 1.9 and 1.10). These results are important for future forest management decisions to preserve fungal diversity, productivity, microhabitats, and ecological function as well as to maintain and restore planted forests.

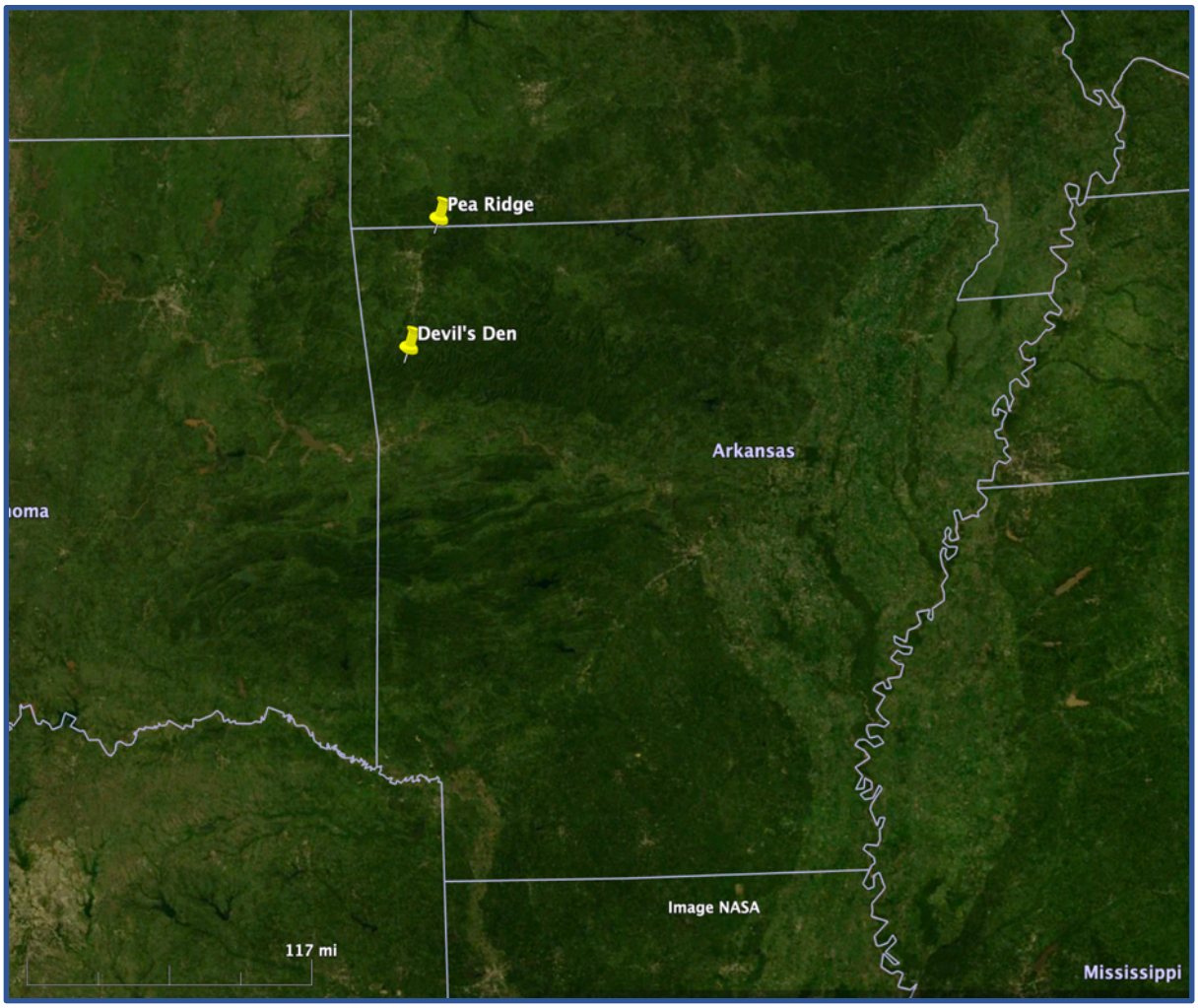

Fig.1.2. Geographic locations of the investigated forests in northwest Arkansas, including Pea Ridge National Military and Devil's Den Parks (Google Earth © 2020 Google).

Fig. 1.3. Archetypal locations of the deciduous mixed forest in Pea Ridge National Military Park during two intervals. (A) Winter season. (B) Spring season. Photograph by the author.

Fig. 1.4. Representative locations in the deciduous mixed forest in Devil's Den State Park during two intervals. (A) Winter season. (B) Spring season. Photograph by the author.

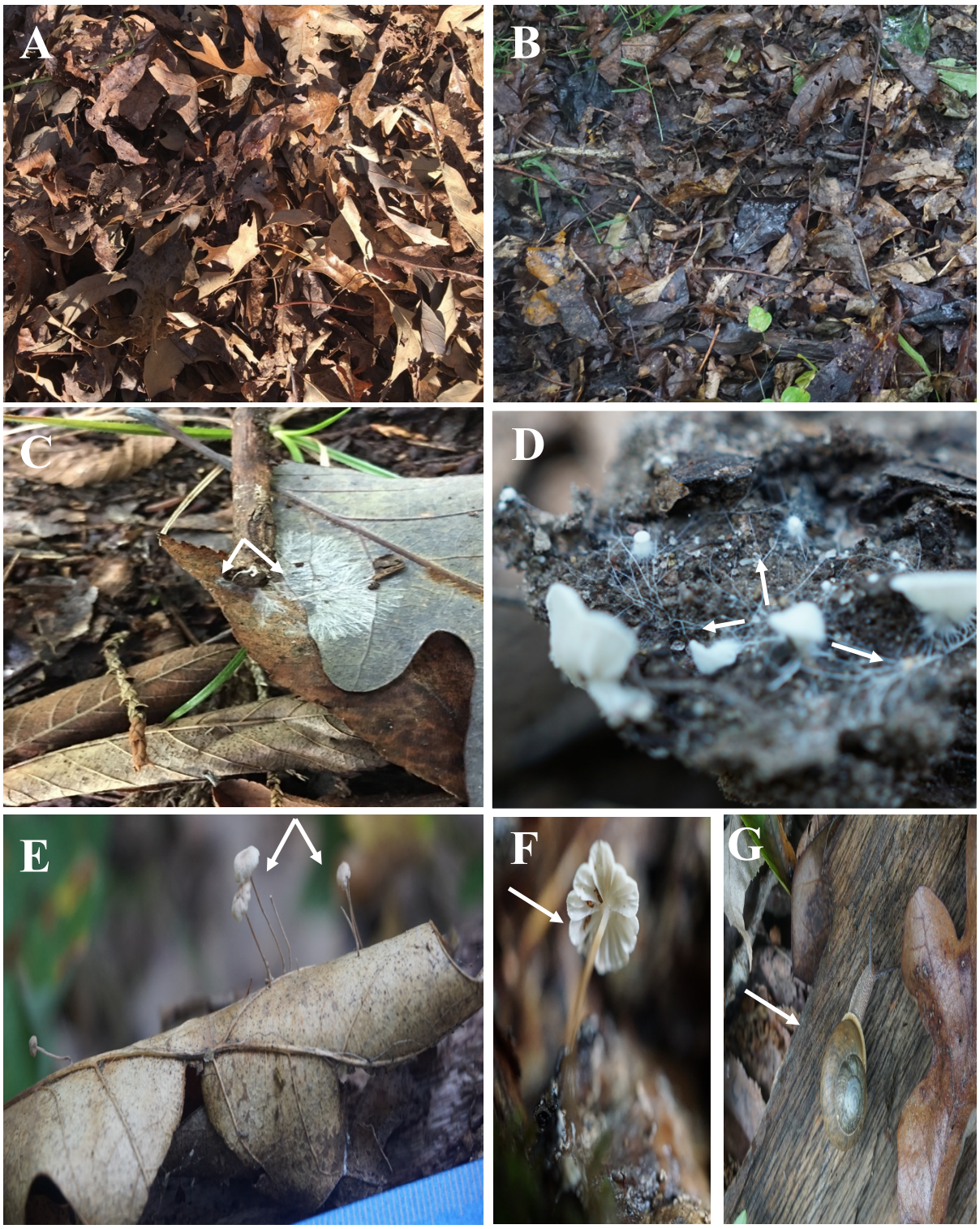

Fig. 1.5. Forest floor components of the Ozark study sites. (A) Represented leafy matters carpet the forest floor. (B) Litter forest after a rainy day. (C) Fungal propagules used woody litter to relocate and develop mycelial growth on leafy litter. (D) Architecture mycelial growth distributed on small litter patches to extend to new resources. (E) Fruiting bodies of *Mycetinis opacus* on leafy litter. (F) Tiny invertebrates feeding on *Gymnopus foliiphilus*. (G) Snail and other invertebrates inhabit, break down and feed on the leafy and woody litter as well as fungal mycelium. Photograph by the author.

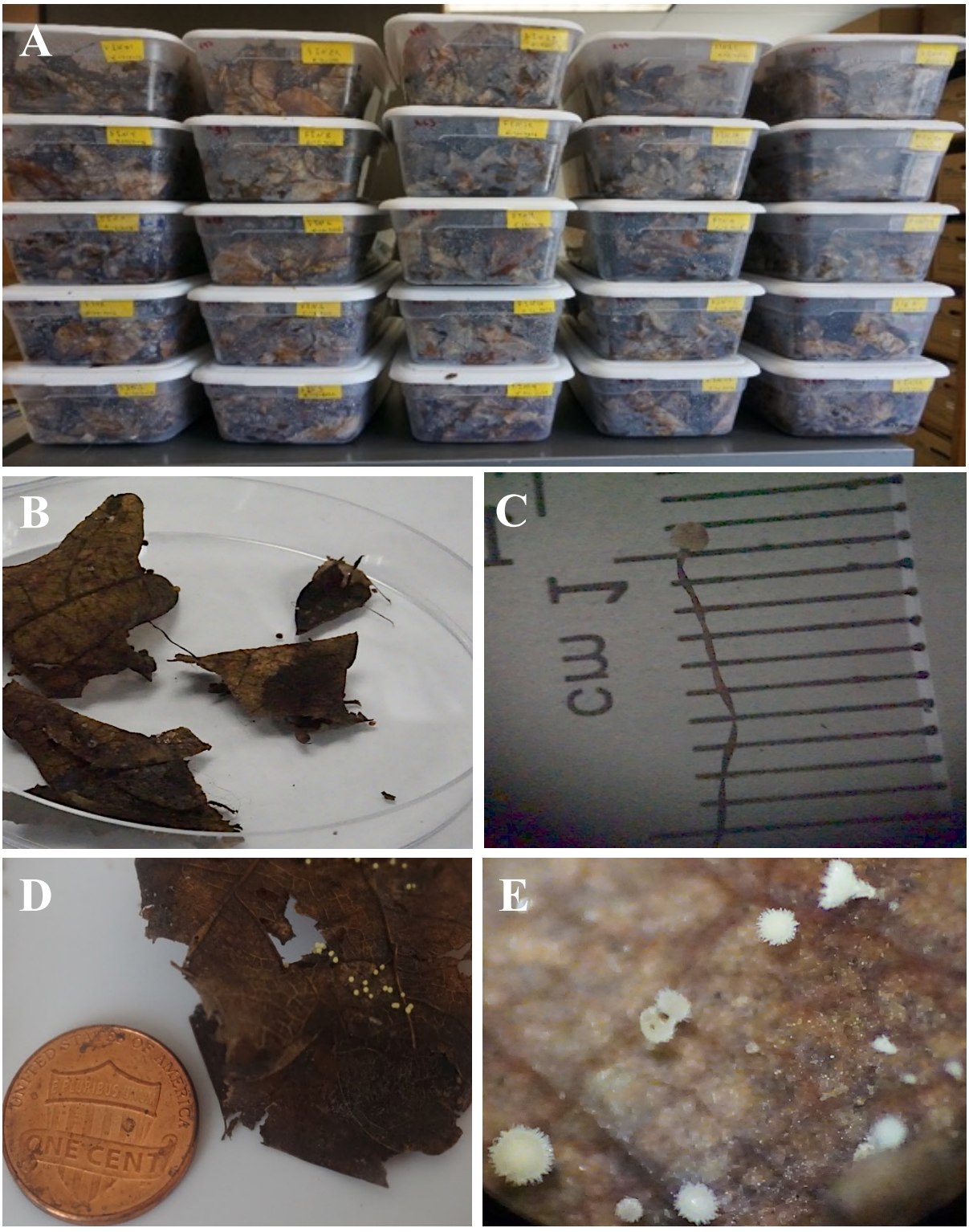

Fig. 1.6. Incubation chamber experiment for forest floor litter materials with some isolated macrofungi. (A) Incubation chambers under lab. conditions. (B & C) Fruiting bodies of the basidiomycetes *Mycena* sp. (D & E) Fruiting bodies of ascomycete *Incrucipulum ciliare*. Photograph by the author.

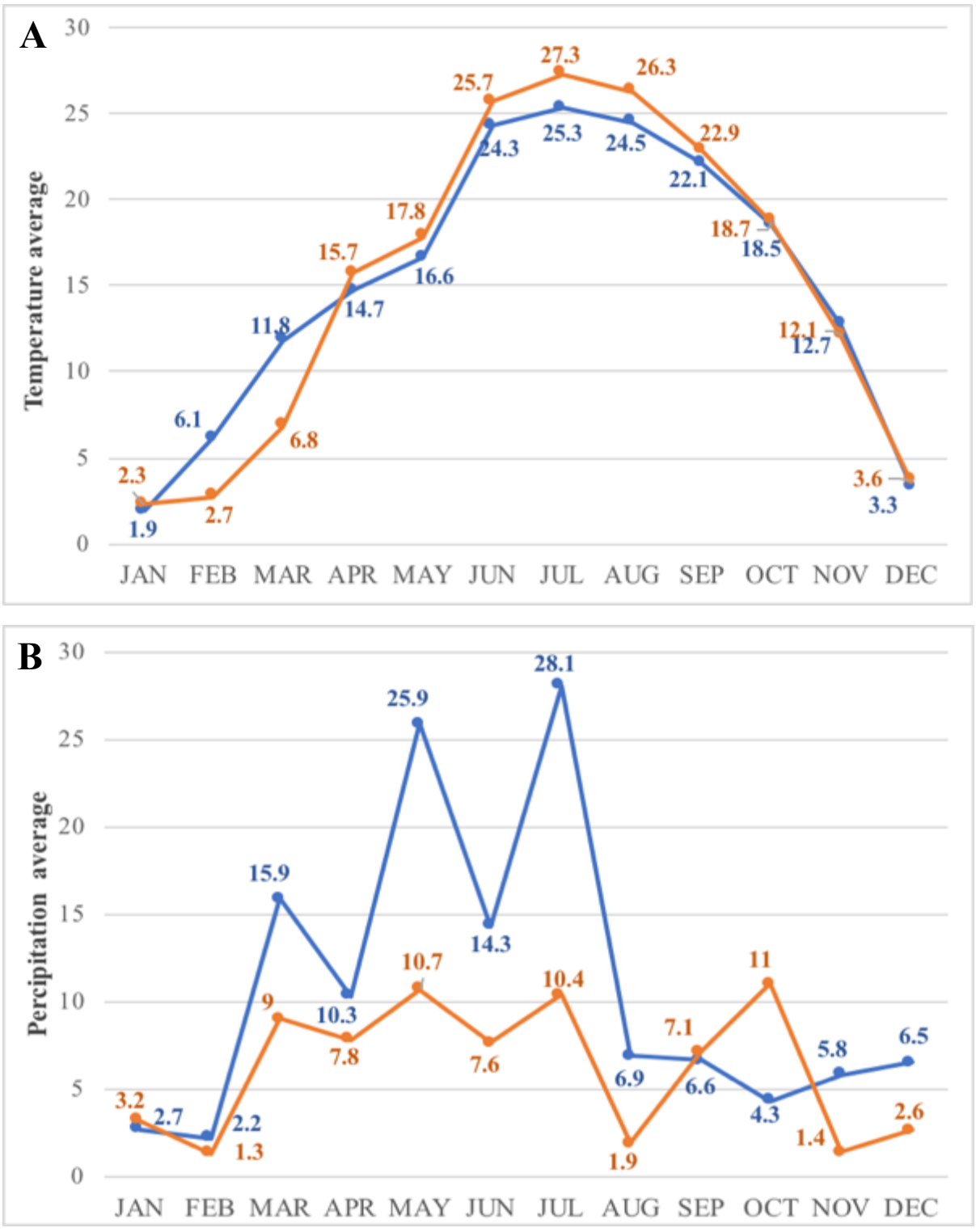

Fig. 1.7. Monthly air temperature (°C) and precipitation (cm) averages of Devil's Den Park (blue) and Pea Ridge National Military Park (orange) for northwest Arkansas region in 2016. Data from National Centers for Environmental Information © 2019.

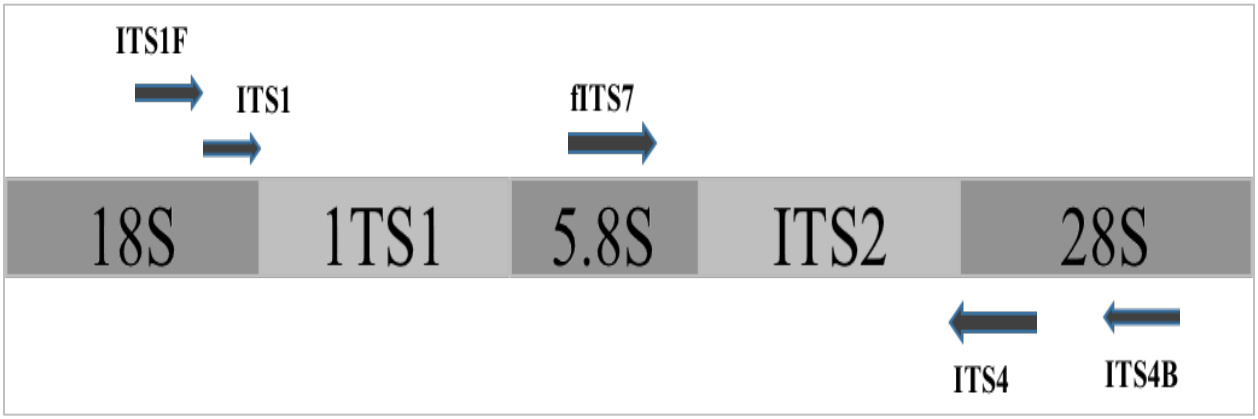

Fig. 1.8. Map of fungal nuclear ribosomal genes and target regions of PCR primers for the current study of both Sanger sequencing and next-generation sequencing techniques. Positions of forward (right-pointing arrow) and reverse (left-pointing arrow) primers are shown on the map of ITS regions and partial large subunit (LSU). The figure was exemplified by the author based on Ihrmark et al. (2012).

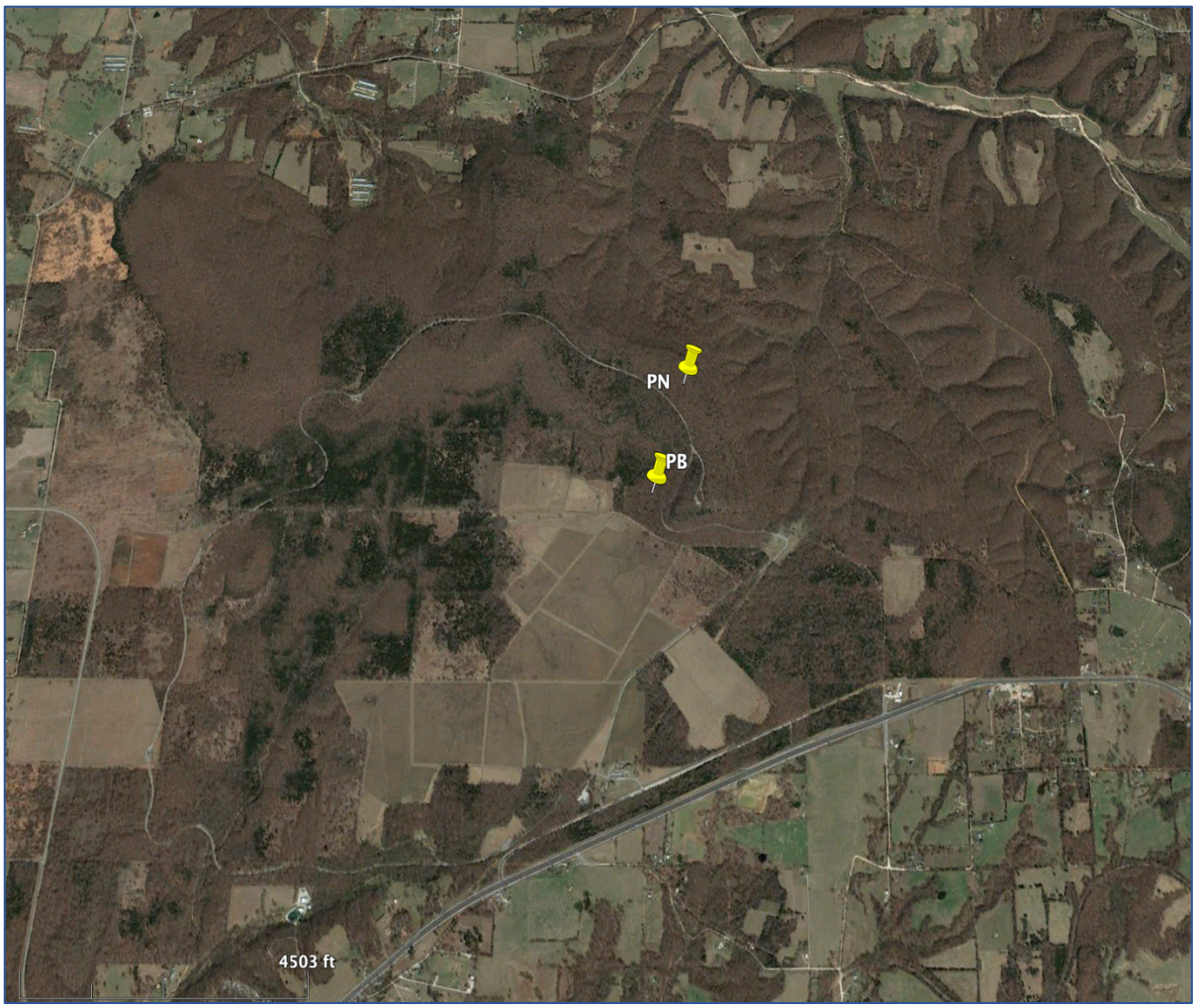

Fig. 1.9. Geographic locations of the two investigated sites in the Pea Ridge National Military Park- unburned (PN) and burned (PB) sites. (Google Earth © 2020 Google).

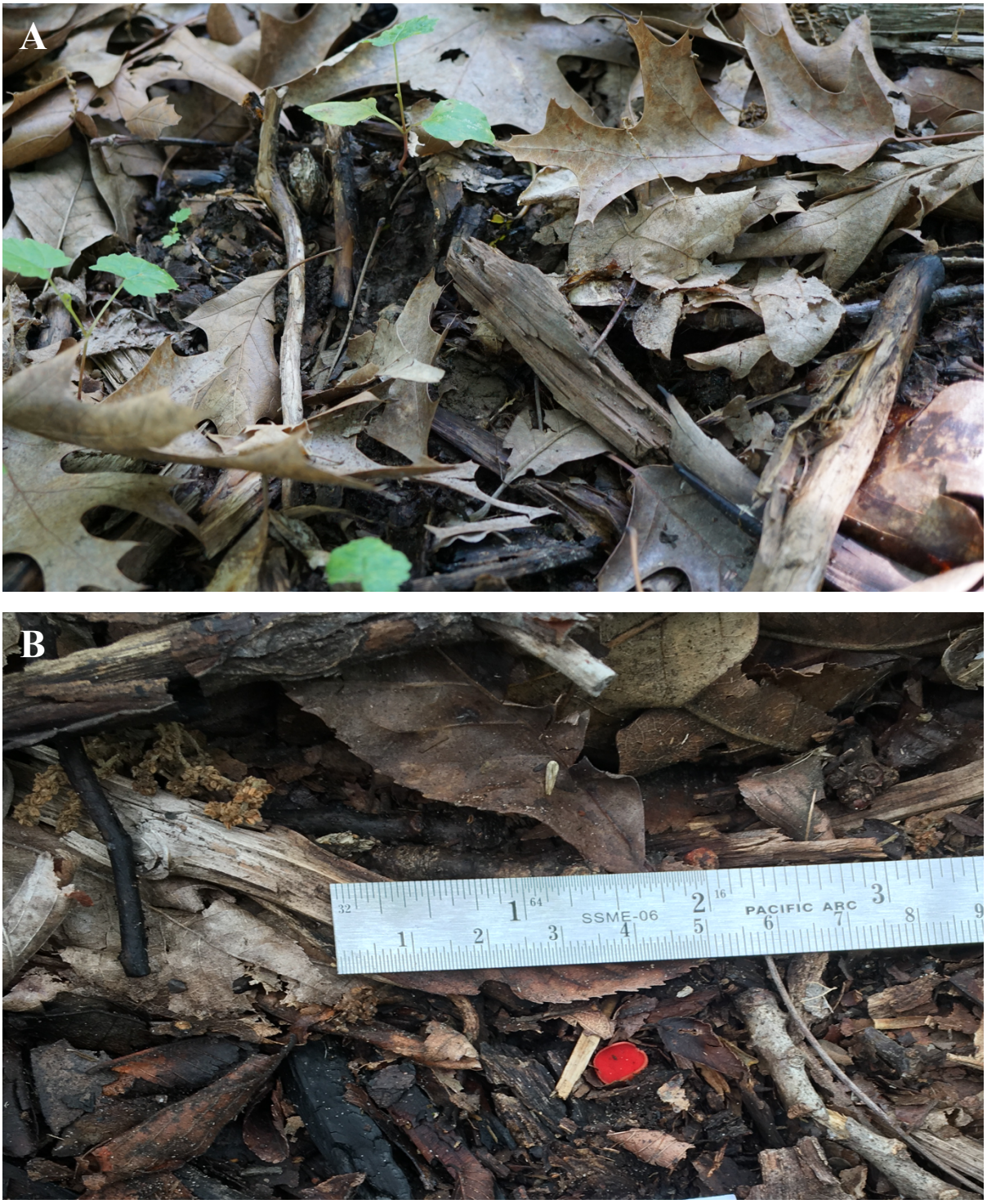

Fig. 1.10. Representative forest floor components of burned plot site that yielded fruiting bodies in Pea Ridge National Military Park. (A) Mixed leafy litter and burned pieces of woody debris on the forest floor. (B) Litter on the forest after a rainy day. Photograph by the author.

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Chapter 2. Fungi associated with forest floor litter in northwest Arkansas

2.1 Abstract

A diverse assemblage of fungi is associated with the litter layer found on the forest floor. Many of these are litter-decomposing fungi, which play a major role as functional decomposers, carbon sequesters and nutrient immobilizers while also serving as both soil stabilizers and biological remediators. Each year, temperate deciduous forests such as those of northwest Arkansas produce a considerable amount of dead leaves and other types of plant detritus which accumulates on the forest floor. Although it has long been recognized that there is an assemblage of fungi associated with this microhabitat, few studies have been carried out to document the taxa present. The study reported herein represented the first effort of which we are aware to document the fungi associated with forest floor litter in northwest Arkansas. Specimens of fungi collected during the 2016 field season were identified through sequencing of the nuclear ribosomal internal transcribed spacer region (nrDNA–ITS). A total of 126 taxa were recorded, the majority of which were clearly associated with dead leaves. Overall, saprophytes were the dominant ecological group, but the total assemblage also included some taxa with other biological roles (e.g., mycorrhizal fungi).

2.2 Introduction

The dead leaves and other types of plant detritus which accumulate on the forest floor represent the most valuable renewable resource on the earth. It has been estimated that 90% of the more than 100 gigatons of terrestrial plant biomass entering the dead organic matter (detritus) pool consists mostly of leaves and other types of plant-derived detritus, including coarse woody debris (Cebrian, 1999), and this detritus is the driving force behind the establishment of the soil profile

(Berg and McClaugherty, 2003). The dead leaves and other types of non-woody detritus provide microhabitats for the often overlooked litter-decomposing fungi and their consumers in forest ecosystems. These fungi play a major role as functional decomposers, carbon sequesterers and nutrient immobilizers as well serving as both soil stabilizers and biological remediators (Berg and McClaugherty, 2003; Claridge et al., 2009). Moreover, fungi are a major energy input for heterotrophic organisms and are directly involved in nutrient cycling as a result of the decomposition processes in which they are involved (Bödeker et al., 2016; Purahong et al., 2016; Jacobsen et al., 2018).

Within forest ecosystems, litter-decomposing fungi significantly colonize, enzymatically degrade and efficaciously transform carbon litter materials and immobilize sufficient quantities of detrital nutrients to support their growth and reproduction (Berg and McClaugherty, 2003; Baldrian, 2017). Saprotrophic fungi are almost exclusively responsible for lignocellulose decomposition, with mycorrhizal fungi also playing some role when they revert to a saprophytic lifestyle (Tedersoo et al., 2014; Bahnmann et al., 2018). These functions connect debris breakdown with overall complex ecosystem processes, and the presence of the fungi allows complex ecological networks involving other organisms, especially invertebrates, to be established (Stephenson, 2010; Baldrian, 2017; Jacobsen et al., 2018). There is little question that these networks have a considerable impact upon the overall forest ecosystem.

Although litter-decomposing fungi are keystone organisms in the decomposition processes that take place in forest ecosystems, they have rarely been studied to the extent that is true for other groups such as wood-decay fungi and mycorrhizal fungi (Hättenschwiler et al., 2005; Prakash et al., 2015). The study reported herein represented the first effort of which we are aware to document the fungi associated with forest floor litter in the Ozark Mountains of northwest

Arkansas. Community composition, microhabitat distribution and putative ecological functions were assessed for the litter-associated fungi in two study areas. These were Pea Ridge National Military Park and Devil's Den State Park. Pea Ridge National Military Park, which covers an area of approximately 1740 ha, is the site of a Civil War battle which occurred in 1862, while Devil's Den State Park consists of about 890 ha and encompasses the largest sandstone crevice area and one of the best preserved Civilian Conservation Corps (CCC) projects in the United States.

2.3 Materials and Methods

2.3.1 Study areas and fungal collection

As noted above, the study areas in the forests of northwest Arkansas were Pea Ridge National Military Park (36°27'28" N, 94°01'18" W, elevation 484 m) and Devil's Den State Park (35°46'32" N, 94°14'46" W, elevation 454 m) in the Ozark Mountains of northwest Arkansas (Fig. 1.2). The forests present in both areas are dominated by an admixture of several species of oak (*Quercus alba* L., *Q. velutina* Lam., and *Q. marilandica* Muenchh.), hickory (*Carya* spp.), winged elm (*Ulmus alata* Michx), and red cedar (*Juniperus virginiana* L.). In addition, red maple (*Acer rubrum* L.) and sugar maple (*Acer saccharum* Marshall) occur at Devil's Den, which is the more mesic of the two study areas (Figs 1.3 and 1.4; Stephenson et al., 2007; Leis, 2018).

At Pea Ridge, fruiting bodies of fungi were collected from thirty 10 × 10 m study plots in the manner described in Lodge et al. (2004). The plots were located along a transect and placed 35–50 m apart in order to minimize sampling the same genet frequently and also to obtain fruiting bodies from a wide variety of microsites on the forest floor. The corners of the plots were delimited with stake wire flags. At Devil's Den, areas of forest floor litter were examined in an

opportunistic manner as described by Cannon and Sutton (2004), and any fruiting bodies of fungi associated with this microhabitat were collected.

2.3.2 Processing and identification of fruiting bodies

Fruiting bodies were collected directly from the litter layer on the forest floor in these plots on a series of visits that took place at approximately two or three week intervals during the period of May to September 2016. After recording substrate data, fruiting bodies were photographed in their natural habitats, placed in the compartments of a plastic fishing tackle box and transferred to the laboratory. The substrates upon which fruiting bodies occurred were segregated into three microhabitat categories. These were leafy litter (LL), consisting of dead leaves present on the forest floor; woody litter (WL), consisting of small twigs (<2 cm in diameter), small pieces of woody debris, and tiny fragments of bark (no more than 0.5–2.5 cm in diameter); and mixed litter (ML) representing instances in which the mycelium associated with a fruiting body proliferated across both leafy and woody substrates or the fruiting bodies were collected from a combination of LL and WL.

Fruiting bodies were dried on a food dehydrator at a temperature 45 °C for at least 24 h, placed in small pasteboard boxes and then deposited in the mycological herbarium of the University of Arkansas (UARK) for future study. The morphological characteristics of fruiting bodies were recorded in either the field or laboratory for use in identification, and the latter was accomplished with the use of appropriate keys and various other publications (e.g., Breitenbach and Kränzlin, 1984, 1995; Binion et al., 2008; Elliott and Stephenson, 2018). Later, macrofungal identifications were confirmed by amplifying the partial sequences of the internal transcribed spacer 1 and 2 with the complete sequence of the 5.8S ribosomal RNA gene (Fig.1.8).

In another component of the project described herein, samples of forest floor litter consisting

mostly of fallen leaves were collected from the four corners and the center of each investigated plot in May 2016, pooled together and placed in plastic incubation chambers (31.5 cm long × 17.5 cm wide × 10.5 cm tall). These incubation chambers were maintained with adequate moisture via spraying the surface of the sample material with water in order to induce the growth and development of fungi. At regular intervals, the incubation chambers were checked for the presence of fruiting bodies, and these were collected, conserved and processed in the same manner as fruiting bodies collected in the field.

2.3.3 DNA extraction and amplification

Genomic DNA was extracted from small fragments (usually taken from the pileus or stipe) of selected fruiting bodies and processed with the use of the Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin), following the manufacturer's protocol. The extracted DNA was amplified and sequenced using the fungal specific primer pairs ITS1F–ITS4 or ITS4B (Gardes and Bruns, 1993) along with the universal primers ITS1 and ITS4 (White et al., 1990). Polymerase chain reactions were carried out with 2X Go Taq Green master mix and amplification was carried out with a BioRed T100 ™ thermal cycler. The PCR protocol of specific primer pairs involved a hot start at 95 °C for 5 min, followed by 37 cycles of 30 s denaturation at 95 °C, 30 s annealing at 56 °C, 1 min amplification at 72 °C, and a final 5 min elongations at 72 °C. Similar preheated and final elongated conditions were applied for the primers ITS1-ITS4 in the thermal cycler program with performing 35 cycles of 45 s denaturation at 96 °C, 45 s annealing at 52 °C, 1.30 min amplification at 72°C.

The size and purity of PCR products were assessed via electrophoresis and resolved on 1% agarose gel in 0.5X TAE buffer, stained by SYBR safe, and sized using the DNA Ladder (Quick-Load®, New England BioLabs, Ipswich, Massachusetts). Afterwards, amplified bands were

visualized and photographed with an ultraviolet transilluminator using a Gel-Doc image analysis system.

2.3.4 Sanger sequencing and taxonomic determinations

The successfully amplified products were sent for direct Sanger sequencings by GENEWIZ (Boston, Massachusetts) after enzymatical clean-up of the PCR sequencing templates. The resulting sequences were edited and aligned with the Geneious program version 9.1.8 (Biomatters Ltd., Newark, New Jersey) to obtain a consensus sequence after manually correcting the alignment errors. For a verified identity, each sequence fragment was subjected to an individual Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) GenBank (www.ncbi.nlm.nih.gov/genbank/). Determining the region of similarity and then comparing and calculating the statistical significances between fungal nucleotide sequences was completed using query coverage of $\geqslant$ 80 % and $\geqslant$ 97–100% sequence similarity based on consideration of the results from the GenBank BLAST search. The taxonomic level and current taxonomic names for the taxa of fungi listed herein follow those given on Index Fungorum (www.indexfungorum.org). After being processed and named the sequences, sequences were deposited in GenBank under the accession number MK213351-MK213368, MK217417-MK217467, MK234170- MK234227, MN148653-MN148660. Each identified taxon was putatively assigned to a particular ecological functional group based on information available in the relevant literature (e.g., Rinaldi et al., 2008; Tedersoo et al., 2014).

2.4 Results

2.4.1 Taxonomic diversity

A total of 126 taxa of litter-associated fungi were recorded from the 2585 fruiting bodies collected and/or recorded from the two study areas and the incubation chambers (Table 2.1). As noted, these taxa were taxonomically identified, first from morphological characters and then this identification was confirmed genetically from ITS sequence data. These 126 taxa represented 62 genera, at least 36 families and 14 orders. The majority of the identified taxa belong to the families Marasmiaceae (15 taxa), Inocybaceae (14 taxa), Omphalotaceae (14 taxa), Entolomataceae (10 taxa) and Mycenaceae (10 taxa) in the phylum Basidiomycota. However, several families such as the Lachnaceae, Rutstroemiaceae, Sarcoscyphaceae, Orbiliaceae and Xylariaceae in the phylum Ascomycota also were identified (Table 2.1, Fig. 2.1). The members of a particular family tend to be associated with a different type of litter material (e.g., small twigs versus dead leaves), and those examples collected from dead leaves and mixed litter were the most common (Table 2. 1). As can be noted in Table 2.1, there were an appreciable number of specimens which yielded sequences that could not be matched beyond genus with anything in GenBank. It seems likely that the majority of these are either relatively rare taxa yet to be sequenced or possibly taxa new to science.

2.4.2 Functional diversity

Saprotrophs made up the single most dominant ecological functional group on litter materials in the temperate deciduous forests considered in the present study, although an ecological function could not be assigned to a number of taxa (Fig. 2.2). Most of the saprotrophic taxa belong to the Omphalotaceae, Marasmiaceae, Agaricaceae and Entolomataceae, all of which were collected predominantly from leafy and woody litter materials. The majority of these taxa produce

relatively small (albeit visible to the naked eye and thus possible to collect in the field) fruiting bodies (Fig. 2.3).

Table 2.1 Identified fungi associated with the litter microhabitats in the forests of northwest Arkansas. Note: MH = microhabitat (LL= leafy litter, WL = woody litter and ML = mixed litter), and ID = percent (%) sequence identity for the current sequences to homologous sequences in the GenBank.

Family	Taxon	MH	Accession number	ID
	Phylum Ascomycota			
Hyaloscyphaceae	*Incrucipulum ciliare* (Schrad.) Baral*	LL	KT876985	99
Lachnaceae	*Lachnum brevipilosum* Baral	WL	AB267643	99
Lachnaceae	*Lachnum virgineum* (Batsch) P. Karst.	WL	AB481268	99
Orbiliaceae	*Orbilia luteorubella* (Nyl.) P. Karst.	WL	U72607	99
Parmeliaceae	*Punctelia hypoleucites* (Nyl.) Krog	WL	HQ650685	99
Pezizaceae	*Peziza succosa* Berk.	WL	MG663289	99
Pyronemataceae	Pyronemataceae (unknown species)	ML	KJ209693	82
Pyronemataceae	*Humaria* sp.	ML	MG019762	99
Rutstroemiaceae	*Rutstroemia* sp. 1	LL	AB926083	91
Rutstroemiaceae	*Rutstroemia* sp. 2	LL	LT158447	93
Sarcosomataceae	*Galiella rufa* (Schwein.) Nannf. & Korf	WL	AF485073	99
Sclerotiniaceae	*Moellerodiscus* sp.	LL	AB926070	95
Sarcoscyphaceae	*Sarcoscypha occidentalis* Schwein.) Sacc.	WL	MF992165	99
Xylariaceae	*Xylaria cornu-damae* (Schwein.) Berk.	WL	MH859400	99
Xylariaceae	*Xylaria hypoxylon* (L.) Grev.	ML	AY327477	99
Xylariaceae	*Xylaria* sp. 1	ML	JQ761773	99
Xylariaceae	*Xylaria* sp. 2	LL	GU300082	99
	Phylum Basidiomycota			
Agaricaceae	*Crucibulum laeve* (Huds.) Kambly	WL	DQ071701	98
Agaricaceae	*Cyathus* sp.	ML	NR_119589	96
Agaricaceae	*Cystolepiota seminuda* (Lasch) Bon	ML	AY176350	97
Agaricaceae	*Lepiota* sp. 1	LL	HQ647294	90
Agaricaceae	*Lepiota* sp. 2	LL	MH211803	100
Agaricaceae	*Leucoagaricus* sp. 1	LL	MG050099	99
Agaricaceae	*Lycoperdon perlatum* Pers.	ML	KP340193	99
Amanitaceae	*Amanita bisporigera* G.F. Atk.	LL	EU819411	99
Amanitaceae	*Amanita* sp.	LL	KP711841	97
Bolbitiaceae	*Conocybe pubescens* (Gillet) Kühner	ML	MH855752	99
Bolbitiaceae	*Conocybe* sp.	WL	JF907826	95
Bolbitiaceae	*Conocybe nigrescens* Hauskn. & Gubitz	WL	JX968234	98
Bolbitiaceae	*Galerella nigeriensis* Tkalčec, Mešić & Čerkez	ML	JX968251	98
Cortinariaceae	*Cortinarius hinnuleoarmillatus* Reumaux	LL	DQ499460	97
Entolomataceae	*Clitocella popinalis* (Fr.) Kluting, T.J. Baroni & Bergemann	LL	FJ770397	98
Entolomataceae	*Clitopilus* sp. 1	WL	KC176282	94
Entolomataceae	*Clitopilus* sp. 2	WL	NR_137867	96
Entolomataceae	*Clitopilus* sp. 3	WL	NR_137867	94
Entolomataceae	*Clitopilus* sp. 4	ML	KU862859	88
Entolomataceae	*Entoloma rhodocylix* (Lasch) M.M. Moser	LL	KJ001414	99
Entolomataceae	*Entoloma* sp. 1	LL	KX387621	90

Table 2.1. Cont.

Family	Taxon	MH	Accession number	ID
Entolomataceae	*Entoloma* sp. 2	WL	JF908003	89
Entolomataceae	*Entoloma* sp. 3	WL	JF908007	92
Entolomataceae	*Entoloma* sp. 4*	LL	KX387621	96
Hydnangiaceae	*Laccaria bicolor* (Maire) P.D. Orton	LL	KM067816	99
Hydnangiaceae	*Laccaria laccata* (Scop.) Cooke	LL	KY744187	99
Hymenochaetaceae	*Coltricia confluens* P.-J. Keizer	WL	NR_137867	99
Incertae sedis	***Resinicium pinicola*** (J. Erikss.) J. Eriksss & Hjortstam	WL	KJ668463	98
Inocybaceae	*Crepidotus* sp. 1	WL	MF461325	87
Inocybaceae	*Crepidotus* sp. 2	LL	KF830099	86
Inocybaceae	*Inocybe grammata* Quél.	LL	GQ166896	99
Inocybaceae	*Inocybe radiata* Peck	LL	GU819490	99
Inocybaceae	*Inocybe subfulva* Peck	ML	KP641623	99
Inocybaceae	*Inocybe subradiata* Murrill	ML	MF992157	99
Inocybaceae	*Inocybe* sp. 1	ML	KJ432283	89
Inocybaceae	*Inocybe* sp. 2	LL	KY990545	90
Inocybaceae	*Inocybe* sp. 3	ML	MF992163	99
Inocybaceae	*Inocybe* sp. 4	ML	HQ604204	90
Inocybaceae	*Inocybe* sp. 5	LL	NR_153163	81
Inocybaceae	*Inocybe* sp. 6	LL	JF908124	94
Inocybaceae	*Phaeomarasmius* sp.	WL	MH856667	94
Inocybaceae	*Simocybe sumptuosa* (P.D. Orton) Sanger	WL	KT715798	98
Lyophyllaceae	*Lyophyllum* sp. 1	LL	MF773643	99
Marasmiaceae	***Crinipellis nigricaulis*** Har.Takah	LL	FJ573197	97
Marasmiaceae	***Crinipellis setipes*** (Peck) Sanger	LL	JF930641	99
Marasmiaceae	*Crinipellis zonata* (Peck) Sacc.	WL	MH979260	99
Marasmiaceae	*Gerronema subclavatum* (Peck) Sanger ex Redhead	WL	U66434	99
Marasmiaceae	*Marasmius capillaris* Morgan*	LL	MH979289	99
Marasmiaceae	*Marasmius conchiformis* J.S. Oliveira & Capelari	LL	KF741996	99
Marasmiaceae	*Marasmius pulcherripes* Peck	LL	MF161270	99
Marasmiaceae	*Marasmius rotula* (Scop.) Fr.	WL	DQ182506	99
Marasmiaceae	*Marasmius siccus* (Schwein.) Fr.	LL	HQ607384	99
Marasmiaceae	*Marasmius* sp. 1	LL	KY366495	91
Marasmiaceae	*Marasmius* sp. 2	LL	KF774157	94
Marasmiaceae	*Marasmius* sp. 3*	LL	KP826788	87
Marasmiaceae	*Marasmius* sp. 4	WL	KP013041	96
Marasmiaceae	*Trogia* sp. 1	LL	MF100962	86
Marasmiaceae	*Trogia* sp. 2	ML	AY329595	87
Meruliaceae	*Gloeoporus dichrous* (Fr.) Bres.	WL	MG748583	99
Meruliaceae	*Steccherinum* sp.	WL	KP814318	99
Mycenaceae	*Hemimycena* sp. 1	LL	MH856249	93
Mycenaceae	*Hemimycena* sp. 2	LL	MK169368	91
Mycenaceae	*Mycena albiceps* (Peck) Gilliam*	LL	KY744172	99
Mycenaceae	*Mycena citrinomarginata* Gillet*	LL	GU234150	99
Mycenaceae	*Mycena pearsoniana* Dennis	ML	JN182200	99
Mycenaceae	*Mycena pura* (Pers.) P. Kumm.	ML	MF955190	98

Table 2.1. Cont.

Family	Taxon	MH	Accession number	ID
Mycenaceae	*Mycena stylobates* (Pers.) P. Kumm. *	LL	JF908439	99
Mycenaceae	*Mycena* sp. 1	LL	MH136827	86
Mycenaceae	*Mycena* sp. 2	LL	JF908384	90
Mycenaceae	*Mycena* sp. 3	ML	LT671449	87
Mycenaceae	*Mycena* sp. 4	WL	DQ490645	86
Omphalotaceae	***Gymnopus androsaceus*** (L.) Della Magg. & Trassin	LL	MH857176	97
Omphalotaceae	*Gymnopus cremeostipitatus* Antonín, Ryoo & Ka	LL	NR_152898	98
Omphalotaceae	*Gymnopus dichrous* (Berk. & M.A. Curtis) Halling	LL	KY026696	97
Omphalotaceae	***Gymnopus disjunctus*** R.H. Petersen & K.W. Hughes	LL	NR_137865	99
Omphalotaceae	*Gymnopus foliiphilus* R.H. Petersen	LL	KY026633	100
Omphalotaceae	*Gymnopus junquilleus* R.H. Petersen & J. L. Mata	LL	AY256693	99
Omphalotaceae	*Gymnopus spongiosus* (Berk. & M. A. Curtis) Halling	WL	AF505784	99
Omphalotaceae	*Gymnopus subnudus* (Ellis ex Peck) Halling	ML	KY777383	99
Omphalotaceae	*Gymnopus* sp. 1	LL	KY026619	99
Omphalotaceae	*Marasmiellus rhizomorphigenus* Antonín, Ryoo & H.D. Shin	LL	GU319116	99
Omphalotaceae	*Marasmiellus* sp. 1	WL	MF161165	98
Omphalotaceae	*Mycetinis copelandii* (Peck) A.W. Wilson & Desjardin*	LL	KY696751	98
Omphalotaceae	***Mycetinis opacus*** (Berk. & M.A. Curtis) A.W. Wilson & Desjardin	ML	MG663278	99
Omphalotaceae	*Mycetinis scorodonius* (Fr.) A.W. Wilson & Desjardin	WL	JQ272364	99
Physalacriaceae	*Strobilurus* sp.	LL	HQ604789	91
Pluteaceae	*Pluteus leoninus* sensu Rea, Cooke	WL	HM562190	100
Polyporaceae	*Trametes conchifer* (Schwein.) Pilát	WL	JN164939	99
Psathyrellaceae	*Coprinellus* sp. 1	LL	MH856811	96
Psathyrellaceae	*Coprinellus xanthothrix* (Romagn.) Vilgalys, Hopple & Jacq. Johnson	WL	KJ028784	99
Psathyrellaceae	*Coprinus sclerocystidiosus* (M. Lange & A.H. Sm.) Vilgalys, Hopple & Jacq. Johnson	WL	KC992942	99
Psathyrellaceae	*Psathyrella candolleana* (Fr.) Maire	WL	HQ436117	98
Russulaceae	*Lactarius camphoratus* (Bull.) Fr.	ML	EU819480	97
Russulaceae	*Lactarius pterosporus* Romagn.	LL	KF432963	98
Russulaceae	*Lactarius subserifluus* Longyear	WL	EU819486	98
Schizophyllaceae	*Schizophyllum commune* Fr.	WL	MH307932	99
Sclerodermataceae	*Scleroderma areolatum* Ehrenb.	WL	EU819438	99
Sebacinaceae	*Sebacina candida* L.S. Olive	ML	KF061277	99
Sebacinaceae	*Tremellodendron schweinitzii* (Peck) G.F. Atk.	LL	KY744167	97
Strophariaceae	*Deconica sp. 1*	WL	KM270756	95
Thelephoraceae	*Thelephora anthocephala* (Bull.) Fr.	WL	AF272927	98
Thelephoraceae	*Thelephora sp.*	WL	KP783471	93
Tremellaceae	*Tremella yokohamensis* (Alshahni, Satoh & Makimura) Yurkov	WL	KP986529	97
Tricholomataceae	*Clitocybe subditopoda* Peck	ML	KM453734	99
Tricholomataceae	*Clitocybe* sp. 1	ML	KJ680984	86
Tricholomataceae	*Infundibulicybe gibba* (Pers.) Harmaja	LL	MG663274	99

Table 2.1. Cont.

Family	Taxon	MH	Accession number	ID
Tricholomataceae	*Resupinatus alboniger* (Pat.) Sanger	WL	KP026234	99
Tubariaceae	*Tubaria* sp. 1	ML	MF039263	97
Tubariaceae	*Tubaria* sp. 2	LL	MF039263	87

Note: Names in bold represent taxa recorded both in the field and from incubation chambers, whereas "*" indicates taxa recorded only from incubation chambers

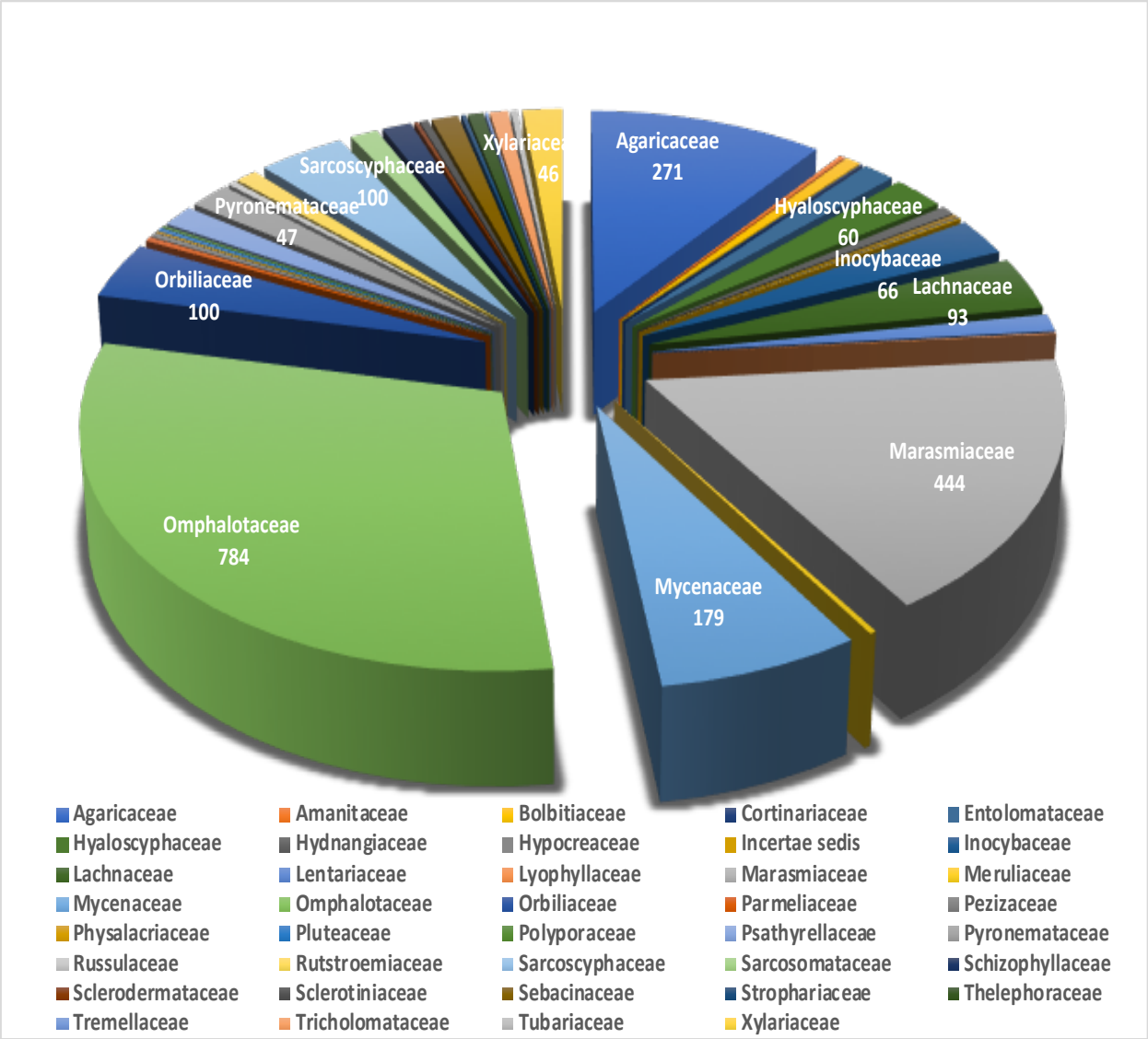

Fig. 2.1. Families represented by litter-associated fungi recorded in the present study. The numbers reflect their relative abundance in the litter microhabitat.

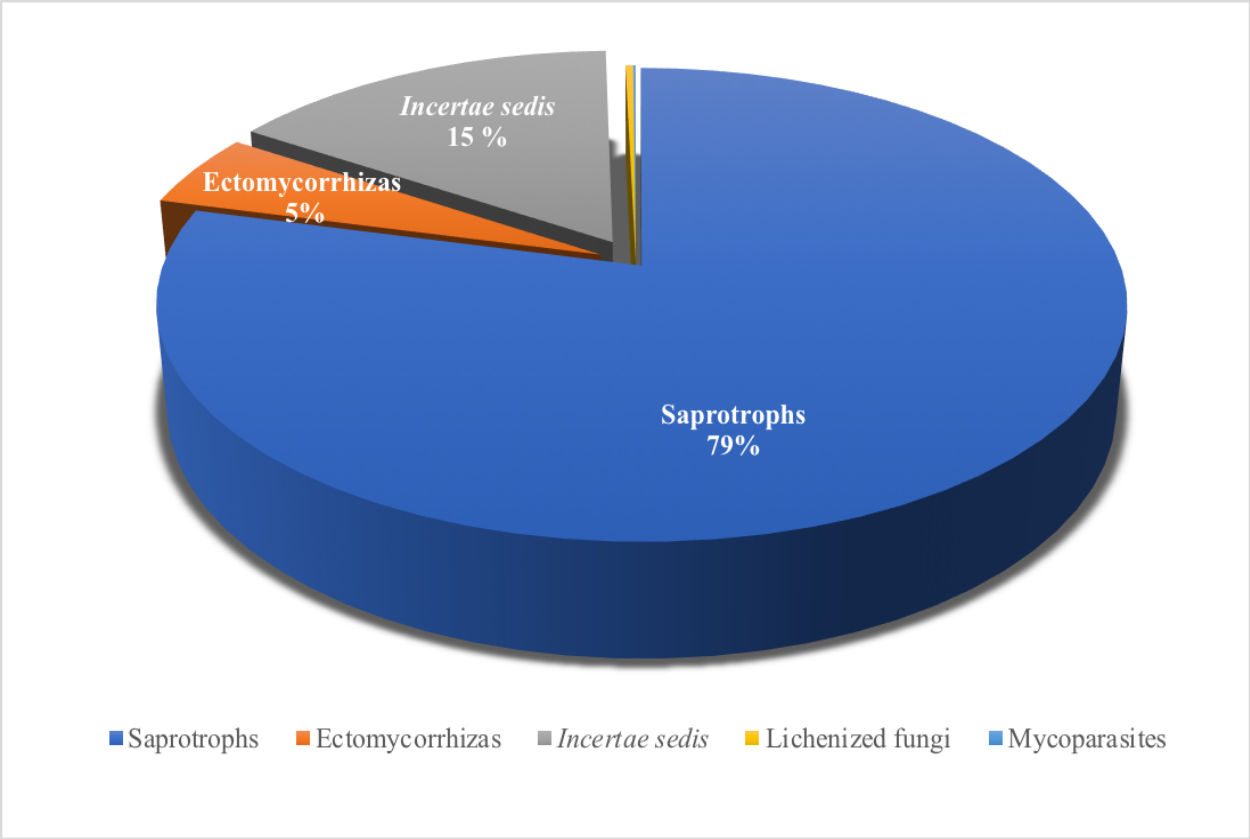

Fig. 2.2. The relative proportions of the most abundance of macrofungal functional guilds of the classified litter-associated fungi.

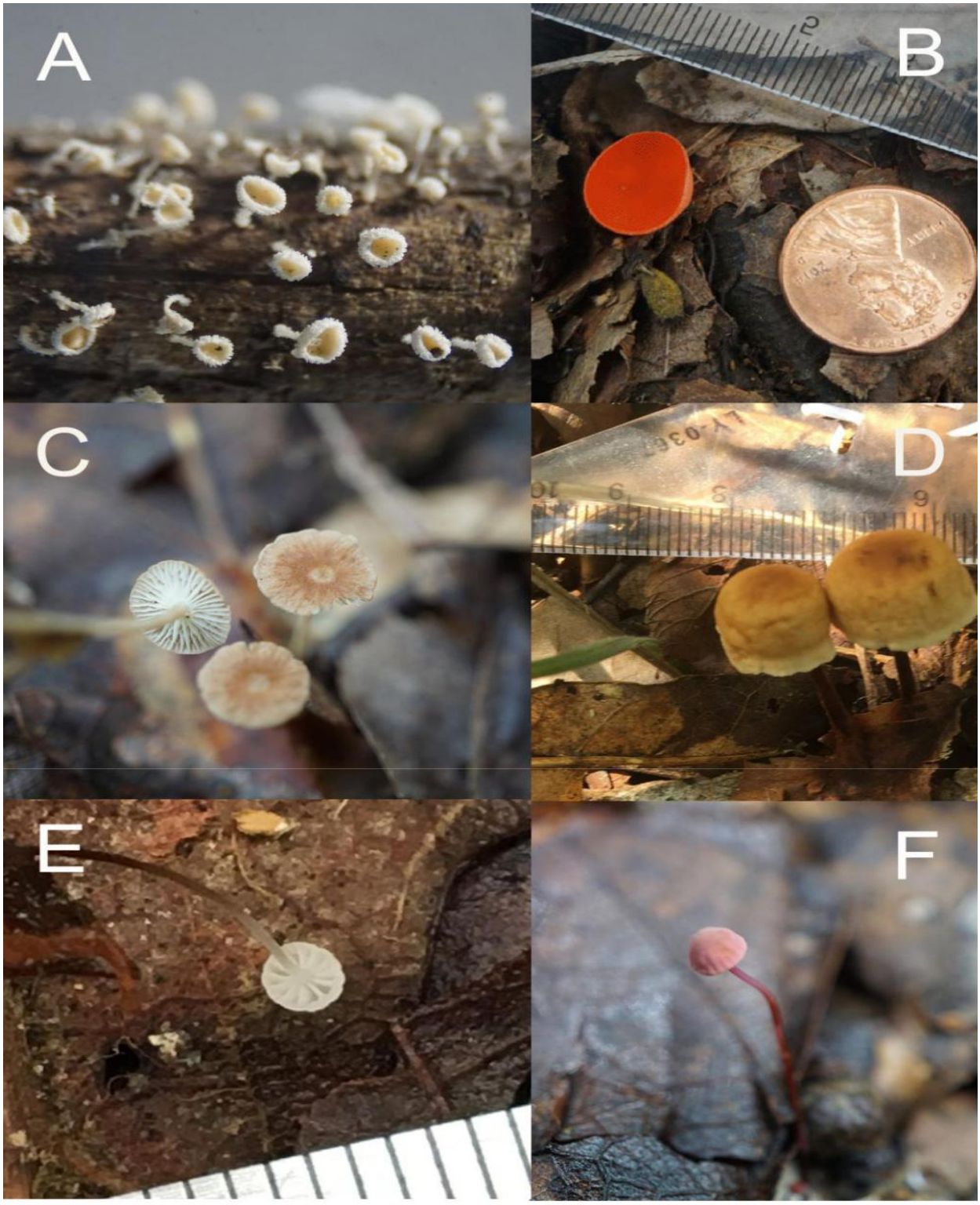

Fig. 2.3. Selected species of litter-associated fungi recorded on heterogeneous microhabitats in the present study. (A) *Lachnum virgineum* (B) *Sarcoscypha occidentalis*. (C) *Gymnopus disjunctus*. (D) *Gymnopus spongiocus* (E) *Mycena albiceps*. (F) *Marasmius pulcherripes*. Photograph by the author.

2.5 Discussion

The research described herein produced a comprehensive body of data on what is an understudied assemblage of fungi-those species associated with forest floor litter. The total of 126 taxa, all of which produce macroscopic fruiting bodies, clearly exhibited a high level of taxonomic diversity, since they were assigned to 63 genera, at least 36 families and 14 orders (Table 2.1). This was not surprising, since a high level of biodiversity has been reported in a number of other studies (e.g., Kubartová et al., 2009, Bahnmann et al., 2018). Forest floor litter is a heterogenous microhabitat consisting of a mixture of several different types of detritus (e.g., dead leaves, small twigs, and tiny pieces of bark) which provide substrates with various chemical and physical properties that presumably represent different niches for fungi to exploit (Berg and McClaugherty, 2003, Elliott and Stephenson, 2018). Some fungi are likely to display a preference for detritus from particular kinds of trees, so the overall biodiversity of the forest almost surely represents a factor accounting for the number of taxa present (Hattenschwiler et al., 2005).

Fungi that decompose dead leaves were the group characterized by the greatest abundance and richness (Table 2.1) in the litter microhabitat. Newly fallen leaves provide relatively nutrient-rich and thus favorable substrates for fungi, and once established, their mycelia grow and develop. The presence of the mycelia provides a setting for ecological interactions with bacteria and invertebrates (Boddy et al., 2008; Hardoim et al., 2015; Prakash et al., 2015). To what extent these interactions influence the structure and composition of the assemblages of fungi associated with litter is not yet known. Presumably, the stage of litter decomposition also represents a factor in determining just what taxa of fungi are present on a particular substrate. It has been suggested that as fungi colonize and enzymatically degrade and assimilate the preferred or available

biopolymers, the differences in the metabolic abilities which exist from taxon to taxon contribute to the overall fungal biodiversity (Purahong et al., 2016). All of these factors underscore the complexity of the fungus-substrate relationships associated with forest floor litter, including the manner in which the fungal mycelium, once established, is able to colonize new substrates.

In the present study, as already noted, the taxa recorded from the litter microhabitat were assigned to ecological functional groups (Fig. 2.2). Saprotrophs, with a total of 1986 fruiting bodies (roughly 79% of the total) were clearly the most abundant fungi present. These fungi undoubtedly possess the various functional characters that allow them to exploit the types of plant detritus that make up litter (Goswami et al., 2017). However, fungi representing other functional groups also were recorded, thus providing evidence of the functional overlap that exists between fungal saprophytes and fungi with different ecological roles. For example, 137 of the fruiting bodies (roughly 5% of the total) collected were those of ectomycorrhizal fungi, which not only use forest floor litter as a substrate for fruiting, but also have a role as organic decomposers to meet some of their energy needs (Rinaldi et al. 2008; Bödeker et al., 2016; Bahnmann et al., 2018).

As a general observation, saprotrophic activity by fungi undoubtedly extends throughout the litter layer, but some degree of vertical stratification also exists, with the mycelia of ectomycorrhizal fungi likely to be confined to the lower humus/soil layer (O'Brien et al., 2005, Bahnmann et al., 2018). One aspect of the ecology of the fungi associated with forest floor litter could not be addressed during the present study. This related to the temporal variation that occurs over the course of the fruiting season, as noted by Rudolph et al. (2018). Voříšková et al. (2014), who used a pyrosequencing technique, reported maxima in saprophytic genera on oak litter in the autumn, with ectomycorrhizal taxa more abundant in the summer. Just what seasonal

differences exist in northwest Arkansas remains unknown. An appreciable number of fungi identified in the present study (a total of 342 specimens) could not be assigned to a functional group. It seems likely that many of these "*incertaine sedis*" fungi decompose litter, but other functional roles are possible, as evidenced by the presence of some taxa known to be mycoparasites.

The presence of lichenized fungi in forest floor litter (Fig. 2.1) is probably the result of fragments derived from the thallus of one of these organisms on some other substrate (e.g., the bark of trees, where they are often quite common).

In conclusion, the results presented herein indicate that the fungi associated with forest floor litter are a diverse and dynamic assemblage (Figs. 2.1 and 2.3) whose contributions to the overall ecology of forest ecosystems should not be ignored, since they contribute to the structural stability, nutrient availability, productivity and other critical aspects of ecosystem functioning. The present study documented this assemblage of fungi for the first time in northwest Arkansas, using current molecular methods to identify the various taxa present. Many of these undoubtedly represent new records for both northwest Arkansas and the Ozark region of the central United States.

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Chapter 3. Taxonomic, functional, and compositional variabilities of macrofungi associated with various litter microhabitats in the forests of the Ozark Mountains

3.1 Abstract

Diverse assemblages of macrofungi are associated with the heterogeneous litter microhabitats that occur on the forest floor. Functionally, these organisms are referred to as litter-decomposing fungi, and they represent a primary component of ecosystem functioning. However, the taxonomic diversity, functional diversity and community composition of litter-associated macrofungi are seriously understudied. The objectives of the present study were first to document and then to compare the diversity, function and distribution of litter-associated macrofungi associated with multiple microhabitats on the forest floor at two sites in the Ozark Mountains of northwest Arkansas. Taxonomically, neither total species richness nor macrofungal productivity was substantially different between the two sites, but the composition of the two assemblages varied. The community patterns comprised of 47 and 42 macrofungal species documented and related to 27 and 24 genera, at least 21 and 17 families for the Devil's Den Park (DD) and Pea Ridge (PR) Park, respectively. These include a group of 16 species belonging to 11 genera that were recorded in the datasets at both sites. Diverse functions overlapped various litter microhabitats at both sites, but based on species richness and fruiting productivity, saprotrophs were dominant and appeared to shape the community structure of the guilds present. The leafy litter species formed approximately 62.7% and 60.9% of the reproductive structures with distinct reproductive traits for PR and DD plots, respectively. Four species seemingly represent indicator taxa in PR plots, whereas only two taxa could be characterized as indicator taxa in DD plots. Several abiotic and biotic factors were apparent, but other complex factors not

considered in the present study may be important for characterizing the litter-associated macrofungi of forests in the Ozark Mountains. The present results documented species that sexually succeed to reproduce, develop, and form fruiting bodies under highly competitive environments between inter and intra populations in fungal diverse community assemblages and other organisms.

3.2 Introduction

Fungi represent one of the most species-rich groups of organisms with various growth forms. With the production of numerous spores and a growth system consisting mostly of mycelial networks that comprise an interconnecting series of branched hyphae, they conquer new and often patchy resources and colonize different food resources. This network architecture characterizes an optimal adaptation to different lifestyles or ecological roles (Boddy et al., 2008). In forest ecosystems, fungi grow saprotrophically, parasitically, and mutualistically on a wide range of substrates. Owing to their recognizable enzymatic abilities, fungi adapt to shift taxonomically and functionally in composition and abundance across various investigated sites, reflecting aspect-driven environmental structuring, vegetation types and dynamically nutrient changes of fungal microhabitats at smaller scales in the forest landscape (Puraphong et al., 2016; Geml, 2019). Macrofungi are a major ecological grouping of fungi forming extensive mycelial networks, long-lived and constantly renewed vegetative mycelia, functioning mainly as saprotrophs or mycorrhizas but also sometimes as pathogens. These mycelial networks are restricted to the forest soil-litter interface or distributed into various organic resources (Muller et al., 2007; Boddy et al., 2008). They are categorized by the production of more compact tissues, short-lived and spore-producing fruiting bodies, communally known as mushrooms, which are

the result of the sexual combination of dikaryotic hyphae (Boddy et al., 2008). Fruiting bodies are broadly diverse in their size and shape and macrofungi are visible to the naked eye. Macrofungi exhibit patterns of diversity that are related mainly to substrate and host availability, and their fruiting is climate-driven (Lodge et al., 2004). These macrofungi primarily belong to the Ascomycota and Basidiomycota, the largest fungal phyla. Those are the most conspicuous groups of fungi recorded and are associated with different plant parts and decomposing materials in forest ecosystems and are characterized by a wide range ecological functions (Prakash et al., 2015; Baldrian, 2017). Macrofungai are also considered the most difficult taxonomic group to study and monitor, due to the fact that they often require a high field sampling effort, the hidden and ephemeral nature of their fruiting bodies and the difficulty of determining an appropriate approach (Ambrosio et al., 2017). As a result, the composition of litter decomposer communities, in particular, remain largely unknown (Baldrian, 2017).

Litter-associated fungi, especially macrofungi, are specifically overlooked but need to be investigated because of their high enzymatic ability and variability to efficiently attack the recalcitrant materials and rapidly succeed assemblages on their microhabitats. Based on their ecological role, for example, these fungi substantially facilitate carbon and nutrient cycling and potentially translocate nutrients and moisture to the whole forest ecosystem through their extensive hyphal networks and varied enzymatic activities in the detritusphere (Boddy et al., 2008; Bahnmann et al., 2018). For life strategies to continuously exist, litter-associated macrofungi are characterized by developing fleshly small-sized or fragile fruiting bodies with an investment of a large number of resources (Boddy et al., 2008). These reproductive structures tend to be easily dehydrated and quickly decompose a portion of the litter microhabitat upon which they are present and abundant in nature. Many factors can affect their diversity and

productivity, such as forest structure, site characteristics, and management (Collado et al., 2019). However, climate is the key factor driving fruiting body emergence and yield and also acts as a limiting factor depending on local conditions (Büntgen et al., 2012; Collado et al., 2019). Litter fungi are the among the most species-rich genera, with a high diversity of macrofungal species (Boddy et al., 2008; Alanbagi et al., 2019). However, the patterns of variation in macrofungal litter distribution and productivity on their microhabitats and possible drivers of these patterns are not especially well known.

Although how ecological niche breadth evolves is essential for adaptation and speciation (Sexton et al., 2017), few studies have focused on fungal litter microhabitats based on fruiting body distribution. Moreover, until recently, studies of fungal ecology in the forest ecosystem have been focused mainly on specific trophic guilds such as mycorrhizas in the soil or decomposers in wood while essentially ignoring the rest of the co-occurring members of the fungal community in the litter habitat (Nguyen et al., 2015), or even on neglected litter microhabitats.

Within forest ecosystems, defining the species niche of some populations of litter fungi has occasionally been done. For example, species of *Marasmius*, *Marasmillus*, *Mycena*, and *Collybia* have been intensively sampled on the litter layer and considered to be the dominant litter decomposers (Murphy and Miller, 1993; 1997). Some of them are even known to inhabit a specific part of their microhabitat to support minimum fruiting bodies with only a small amount of resources (Boddy et al., 2008), but there are more investigations to be done for other macrofungal litter residents. Species occurrence data and productivity trends have been used to model macrofungal distributional or functional, recently detailed. The macrofungal diversity and corresponding sporocarp production have been typically studied for characterizing macrofungal assemblages in specific ecosystems (Trudell and Edmonds, 2004; Dejene et al., 2017), assessing

potential impacts of environmental changes (Sexton et al., 2017; Collado et al., 2019) and/or of ecosystem managements (Dooley and Treseder, 2012; Köster et al., 2015).

Functionally, in the litter layer specifically, saprotrophs are typically responsible for litter decomposition, but fungi in other guilds such as mycorrhizas may revert to a saprophytic lifestyle (Tedersoo et al., 2014; Bahnmann et al., 2018; Alanbagi et al., 2019). Through inhabiting various microhabitats with multiple ecological guilds such as litter decompositions, fungi usually are an important component in the soil biosphere and one of the driving forces behind the establishment of the soil profile in forest ecosystems (Puraphong et al., 2016; Alanbagi et al., 2019). During the microbial decay activities of lignin, lignocellulosic and other components of leafy and or woody microhabitats, nutrients are usually released to plants or stored in the stable humus or in fungal mycelium or consumers (Berg and McClaugherty, 2008; Baldrian, 2017; Zotti et al., 2013). Decayed woody debris also aids in the establishment of mycorrhizal associations with seedlings and the maintenance of mycorrhizal fungi in seasonally dry forests (Huhndorf et al., 2004). Fungi participate in multiple ecological roles as organic decomposers, nutrient immobilizers and releasers, soil stabilizers, biological remediators (Berg and McClaugherty, 2008; Claridge et al., 2009), and attractive dietary resources and/or habitat for heterotrophic organisms (Bödeker et al., 2016; Purahong et al., 2016; Jacobsen et al., 2018).

In the present study, the main goal was to taxonomically characterize the species richness and fruiting body productivity of litter-associated macrofungi on various litter microhabitats using a plot-based survey for the two sites within the Ozark Mountains of northwest Arkansas. The species ecological guilds were defined, and numbers of fruiting bodies were determined. All previous community assemblages were compared within and between the two sites.

3.3 Materials and Methods

3.3.1 Sampled plots with macrofungal processing and identification

Two study sites in the Ozark Mountains of northwest Arkansas in the United States of America were selected for sampling of fruiting bodies of litter-associated fungi in various litter microhabitats on the forest floor. These sites were Devil's Den State Park (35°46'32" N, 94°14'46" W) and Pea Ridge National Military Park (36°27'37" N 94°01'21" W). At both sites the forest present had a relatively closed canopy and was dominated by several tree species (Stephenson et al., 2007; Diamond et al., 2013). The tree species were divided into two categories using the diameter at breast height (DBH), large trees with size class >10 DBH (cm) and small trees with size class > 2.5 - 9.9 DBH (cm). The relative density and basal area with the importance value of each forest tree species for the vegetation composition of the investigated sites were calculated, gathered and illustrated in Figs. 3.1 and 3.2. Prior to the fruiting body collecting, the depth of the carpeting litter layer was measured at the four corners and the center of each plot using a small centimeter ruler. The mean depths of the Devil's Den (DD) and Pea Ridge (PR) litter layers were 2.2 and 3.1 cm, respectively, and did not differ significantly. Fruiting bodies were collected directly from the litter layer on the forest floor within twenty plots located along the study site boundaries. In the same manner described in detail in Alanbagi et al. (2019), each explored site was divided into ten plots, each of which was 10 m × 10 m and located 35-50 m apart from the neighbored plot. The total area sampled was approximately 0.2 ha for each study area, while fungal datasets were collected within 10 plots located along the site boundaries. The surveys of macrofungi were limited to the fruiting bodies visible to the naked eye, including those with a cap diameter ⩾ 1 mm and 1 cm for Ascomycota and Basidiomycota, respectively.

Due to specific features of litter-associated fungi as characterized by the development of small sized fruiting bodies, easily dehydrated, on their microhabitats, a series of visits after rainy days took place at approximately two or three week intervals during the period of May to September 2016. The number of fruiting bodies of litter-associated fungi were recorded and photographed in their natural microhabitats and tallied within each plot. The samples of macrofungi and their litter microhabitats upon which fruiting bodies occurred were then processed and segregated as described in Chapter 2. For accuracy of species identifications and classification, the morphological features of the litter-associated fungi and the molecular amplifications of the internal transcribed spacer (ITS) region of the fungal ribosomal DNA (rDNA) were used, and then confirmed, based on the available references.

3.3.2 DNA extraction, PCR amplification and Sanger sequencing

DNA extraction, protocol amplification, PCR product visualization, and Sanger sequencing were described in detail elsewhere (Alanbagi et al., 2019). The genomic DNA was extracted mostly from small portions of fruiting bodies using the Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin), according to the manufacturer's instructions. Later, the extracted DNA was amplified by targeting the ITS regions using the universal primers ITS1 and ITS4 (White et al., 1990) and fungal-specific primer pairs ITS1F–ITS4 or ITS4B (Gardes and Bruns, 1993) and sequenced thru Sanger sequencing technique (Fig.1.8). The sampled sequences of the successful sequencing were obtained after editing and alignment of the sequencing results using the Geneious program version 9.1.8 (Biomatters Ltd., Newark, New Jersey).

3.3.3 Taxonomic affiliations and functional assignments

Representative sequences of each fragment were used to verify individually the sequence identity using The Basic Local Alignment Search Tool (BLAST) available on the website (www.ncbi.

nlm.nih.gov/genbank/) of the National Center for Biotechnology Information (NCBI) GenBank. Defining taxa of fungi was completed using a sequence similarity of ⩾97% with a query coverage of ⩾80% depending on the result consideration of from the GenBank BLAST search. Fungal name and taxonomy follow those given on the Index Fungorum database (www.indexfungorum). Based on its taxonomic affiliates, the identified taxon was putatively assigned to a functional guild according to the relevant published literature (e.g., Rinaldi et al., 2008, Tedersoo et al., 2014; Morgado et al., 2016).

3.3.4 Statistical analyses of diverse macrofungal taxa and functional guilds on various litter macrohabitats

The distribution of main taxon rank starting from phyla until the genus level was displayed in a web-browser based on the richness data using Krona Plots (Ondov et al., 2011) to describe the general taxonomic patterns between both sets of study plots. The total number of the identified taxa (species richness) was assessed taxonomically and functionally by characterizing and comparing datasets on the taxonomic boundaries inhabiting an admixture of litter microhabitats for both PR and DD sites. For these analyses, all species of macrofungi in the different litter categories were included in the general category ‘litter’. The total productivity of fruiting bodies was quantified generally based on the fungal taxa and specifically for each taxon functional guild in both compared sites. Values of the species richness and total productivity of fruiting bodies on each litter microhabitat; leafy litter (LL), woody litter (WL), mixed litter (ML) were also calculated separately to assess and compare their prevalence between the two sites. The previous outcomes were statically evaluated using analysis of variance (ANOVA). Tukey’s test was performed to compare means of these results, selecting 0.05 as the significant value for all statistical tests.

3.3.5 Comparation and correlation between environmental variables and compositional and functional variability of macrofungi on distinctive litter microhabitats

The characterized assemblages of litter-associated macrofungi across sampling plots of the DD and PR forests were first compared by performing one-way cluster using Euclidean distance and Ward's linkage method based on the binary data in PC-Ord v.6.1.2. Then, these assemblages, were compared taxonomically and functionally through the dissimilarity in datasets and a visual estimate based on multivariate analyses in PC-Ord as well (McCune and Grace, 2002). The non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance was conducted. In brief, the primary species matrix based on binary data was generated. The secondary matrix consisted of categorical values for the two sites with the number of species per taxonomic family. The total species richness and the number of species per taxonomic phylum, functional groups and for the distinctive litter microhabitats for both compared plot sets were also included in additional NMDS analyses. Variables with $| r | \geq 0.5$ values for either NMDS axis were considered essential for characterizing changes in fungal assemblage structure and overlaid on the ordinations as strength vectors and directions (Roger et al., 2009; Geml et al., 2014). The NMDS ordination plots were orthogonally rotated by the treatment to visualize correlations between forest sites and fungal community assemblages. Multi-response Permutation Procedure (MRPP), providing a *P* - value and an *A*- statistic (effect size) and Permutation based MANOVA (PerMANOVA), providing *P*-value and *F*-statistic were performed based on Bray-Curtis distances for detecting differences in the multivariate data based on treatment type (Anderson, 2001; McCune and Grace, 2002).

To compare patterns of rarity (number of species occurrence) and distributions of litter associated species across the investigated plots, two-way cluster analysis was performed and

visualized, using Bray-Curtis distances as the linkage method in PC-Ord (McCune and Grace, 2002). Finally, Indicator Species Analysis (Dufrêne and Legendre, 1997) was tested to detect any preferences of individual species for a particular forest site and a significance of observed indicator values through Monte Carlo test with permutations (4999) using previous software with $P < 0.05$.

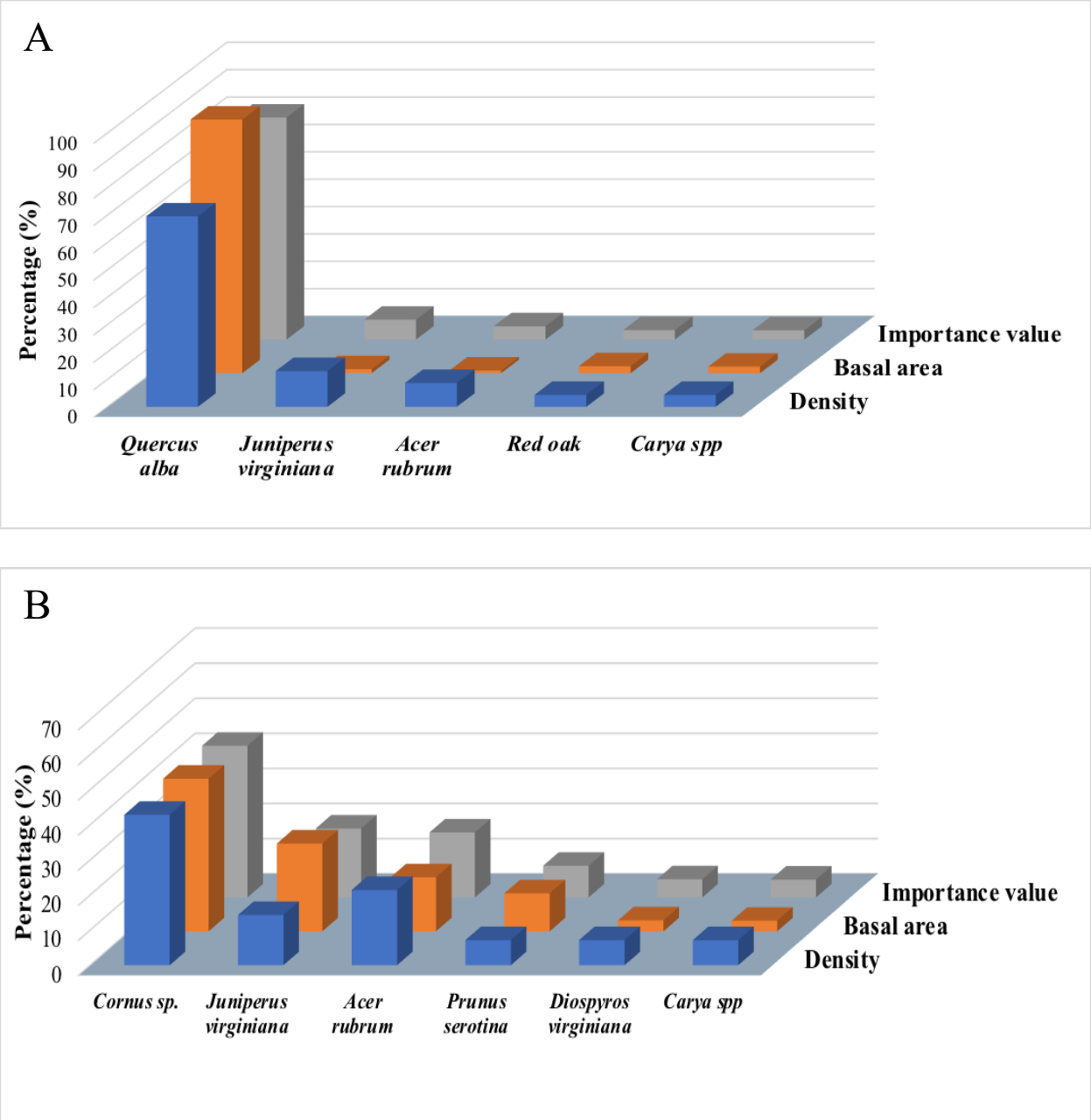

Fig. 3.1. Overstory tree structures based on stem density, basal area and importance value of each tree species divided into large trees (A) with size class >10 cm DBH and small trees (B) with size class > 2.5 - 9.9 cm DBH for tree Devil's Den plot site.

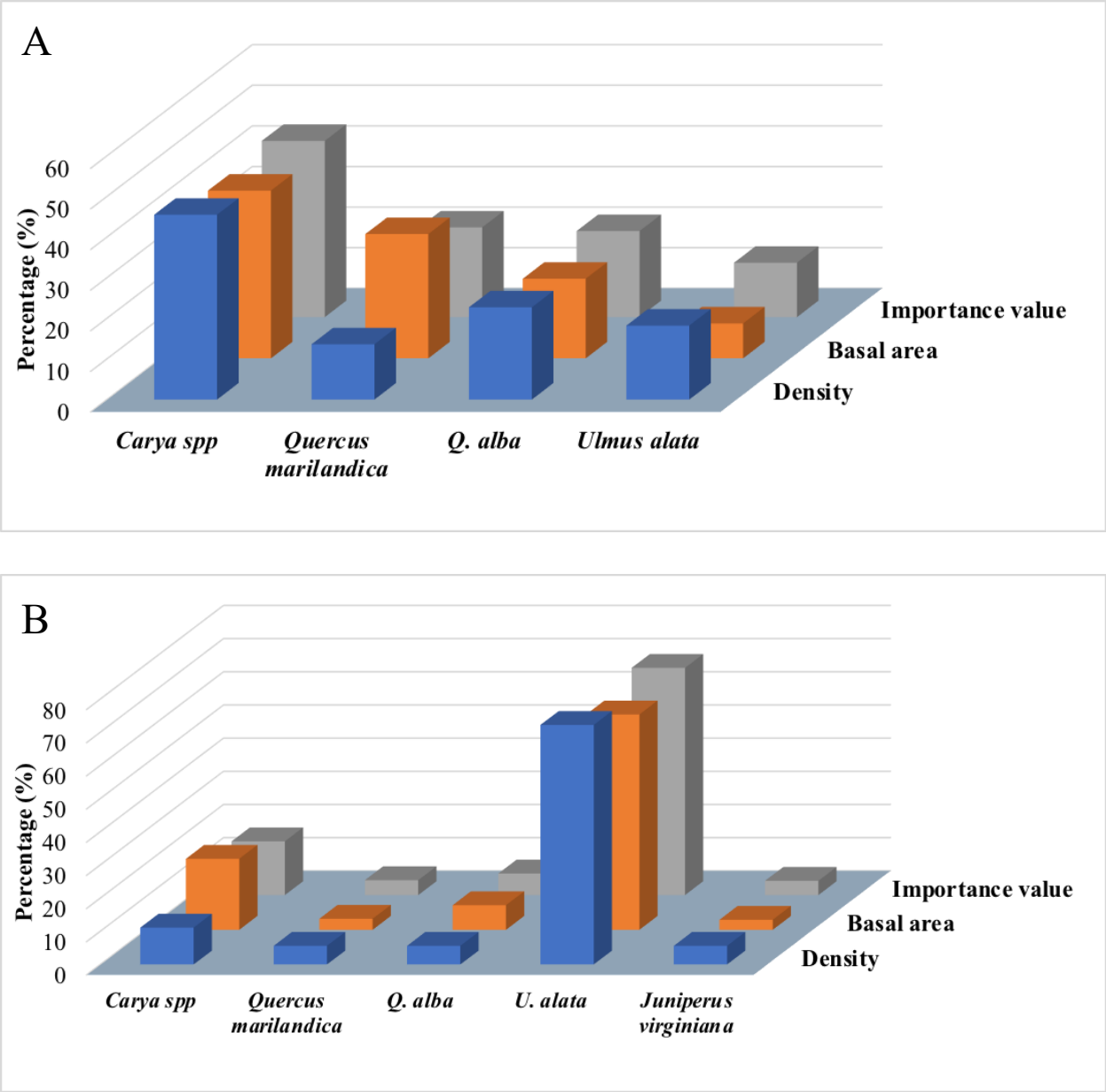

Fig. 3.2. Overstory tree structures based on stem density, basal area and importance value of each tree species divided into large trees (A) with size class >10 cm DBH and small trees (B) with size class > 2.5 - 9.9 cm DBH for tree Pea Ridge National Military plot site.

3.4 Results

3.4.1 Taxonomical affiliations and community patterns

A diverse assemblage of species was recorded, and these were dispersed across the plot sites based on available resources (Figs. 3.3 and 3.4). Many of them tend to be rather inconspicuous or sporadic in their incidence and thus not always easy to detect in the forest sites. These small and fragile species persist on their microhabitats in nature for one or few days. A total of 73 macrofungal species were morphologically identified and genetically confirmed, with a total of 1,685 fruiting bodies counted on the various litter microhabitats after a series of visits to both DD and PR areas. Taxonomically, the community patterns comprised of 47 and 42 macrofungal species documented and these belonged to 27 and 24 genera. At least 21 and 17 families belonged to 8 and 7 orders for DD and PR sites, respectively. These included a group of 16 species belonging to 11 genera that fruited and were recorded in the datasets of both plot sites (Fig. 3.5). Also, these taxa included an appreciable number of specimens that could not be resolved to a species level with anything in the GenBank. It seems likely that the majority of these are either relatively rare taxa yet to be sequenced or taxa new to science.

The taxonomic patterns of the taxa recorded in the present study were seemingly driven by the fruiting bodies of members of the Ascomycetes (4 orders) and Basidiomycetes (6 orders) on the various litter microhabitats. Members of the Basidiomycota comprised 93% and 83% of macrofungal species of the PR and DD sites, respectively, while the Ascomycota encompassed 7% and 17% of PR and DD species assemblages, respectively. The order Agaricales (87% and 90% of Basidiomycota for DD and PR plots, respectively) and Sebacinales (5% for both sites) were predominant. Several orders in the phylum Ascomycota also were documented as litter-inhibiting fungi, including examples in the Pezizales and Helotiales with higher percentages for

DD litter plots. The Ozark Mountains of Northwest Arkansas forests were dominated by genera with the highest species richness such as *Marasmius* (8 species), *Mycena* (7) and *Gymnopus* (6), in addition to various other genera (Fig. 3.5). These species-rich genera typically occupied leafy and mixed litter and function as saprotrophs in both forest sites. Although some of these species were distributed in both forest sites, others were unique to a specific site.

3.4.2 Macrofungal species richness and productivity patterns based on taxonomic affiliations, litter microhabitats and functional guilds

The litter layers for both sets of study sites were apparently inhabited by diverse hidden foraging mycelia that obviously succeed in sexually reproducing and form fruiting bodies on various litter microhabitats under suitable environmental conditions. The average species richness (10.8 species and 10.2 species for PR and DD plots, respectively) and productivity responses (941 and 744 fruiting bodies for PR and DD plots, respectively) of litter-associated macrofungi did not differ remarkably for the two fungal assemblages of investigated sites with respect to the unique species for each site (Fig. 3.6). At microhabitat scales, these species tended to be distributed differentially on heterogeneous plant detritus, and 68.5% and 56.9 % of species mostly populated leafy litter (LL) of PR and DD plot areas, respectively. The LL species formed approximately 62.7% and 60.9% of the reproductive structures with distinct reproductive traits for PR and DD plots, respectively. However, the means of richness and productivity of LL species did not vary substantially between the two sites (Figs. 3.7A and D). The mean richness of woody litter (WL) and mixed litter (ML) species showed no statistical differences among the forest sites (Figs. 3.7B and C). The number of fruiting bodies of ML species were significantly higher in PR site but did not differ between sites on WL (Figs. 3.7E and F).

With respect to functionality of unassigned guilds (*incertae sedis*) for a number of taxa, the saprotrophic species assemblages may functionally shape the community structure of the presented guilds as these taxa were numerically greater in both species richness and fruiting body productivity than the other detected functional groups during sampling of the various litter microhabitats without any statically difference between both compared sites (Fig. 3.8). Most of these species belong to the order Agaricales in the Basidomycota in both sites. However, the ascomycete saprotrophic species *Sarcoscypha occidentalis* belongs to the order Pezizales, and three species of *Xylaria* (order Xylariales) were mainly prevalent in PR and DD plots, respectively. The majority of these were collected predominantly from leafy and woody litter in both sets of study sites.

The species richness of ectomycorrhizal fungi was significantly smaller in DD plots than in PR plots, while the mean productivity of DD ectomycorrhizas was obviously less than PP ectomycorrhizas. However, the productivity differences were not significant (Figs. 3.8B and E). Species representing other functional groups in mostly unassigned guilds were also recorded in both investigated areas, with significantly greater numbers of fruiting bodies for the PR litter compared to the DD litter (Figs. 3.8C and F).

3.4.3 Macrofungal community assemblies across investigated sites

The NMDS-ordination depicted an obvious structuring of the macrofungal litter assemblages across NMDS plots of both forest sites with a four-dimensional solution. The analysis resulted in a final stress of 9.74371, with a final instability < 0.02803 (Fig. 3.9). This was also evident by one-way clustering (Fig. 3.10). At the taxonomic family level, with the significant variation in species composition expressed mostly on the 1^{st} and 2^{nd} axes, the family Hydnangiaceae (r = 0.589) and the family Omphalotaceae (r = 0.514) were correlated positively with axis 1, while

the family Xylariaceae (r = - 0.518) was negatively correlated with this axis. The Marasmiaceae (r = -0.674), Sichizophyllaceae (r = -0.635), Sarcoscyphaceae (r = -0.558) and Lachnaceae (r = -0.410) were significantly correlated with axis 2 (Fig. 3.11 and Table 3.1). The Mycenaceae (r = 0.590) displayed also strong correlation with the third ordination axis.

The richness of functional groups, fungal microhabitats and taxonomic phylum composition of species assemblages for the datasets related to the compared sites also were clearly displayed on the NMDS diagram, with a final stress of 10.42367 and a final instability < 0.02839, respectively. In the NMDS ordination plots, the values for ectomycorrhizas (r = 0.556), leafy species (r = 0. 507), and Basidiomycota (r = 0.403) correlated positively with the axis 1, while the richness of the woody litter assemblages (r = - 0.701) and Ascomycota (r = -0.604) correlated negatively. The saprotrophs (r = -0.652) and total richness (r = 0. 583) displayed a significant relationship, with somewhat greater richness in PR plots (Fig. 3.12 and Table 3.1). The previous results were statistically confirmed by MRPP conclusions (A = 0.0713, P < 0.0000137) and PerMANOVA (F= 3.880, P = 0.00020).

The two-way cluster analysis also confirmed the NMDS-ordinations and previous statistical results in which the macrofungal assemblages strongly clustered, based on the datasets of two investigated sites. Species that gathered closely on the x-axis dendrogram were distributed in similar plots, and plots that assembled tightly on the y-axis dendrogram had similar species assemblages. Some species were distributed in both sites, but unique species assemblages were also present on a specific site (Fig. 3.13 and Table 3.2). The Indicator Species Analysis of the Ozark Plateau litter showed that the northwest Arkansas area was characterized by a diverse assemblage of species (Table 3.3 and Fig. 3.14). These dominant species were mostly members of the families Omphalotaceae, Marasmiaceae, and Tricholomataceae and mainly inhabited leafy

and mixed litter. *Mycetinis opacus* (Peck) Gilliam had the highest important value index, with a significant difference of the indication value when compared with *Gymnopus disjunctus* R.H. Petersen & K.W. Hughes, *G. spongiosus* (Berk. & M.A. Curtis) Halling, and *Infundibulicybe gibba* (Pers.) Harmaja in PR plots. Conversely, only the two species *Crinipellis setipes* (Peck) Sanger and *Gymnopus foliiphilus* R.H. Petersen were significantly reliable indicators for the DD plots (Table 3.3 and Fig. 3.14).

Fig. 3.3. Morphological diversity of macrofungal fruiting bodies on various floor litter microhabitats in Pea Ridge site; *Clitocella popinalis* (Fr.) Kluting, T.J. Baroni, Entolomataceae on leafy litter (LL) (A), *Crinipellis zonata* (Peck) Sacc., Marasmiaceae, on woody litter (WL)(B), *Crucibulum leave* (Huds.) Kambly, Agaricaceae, WL (C), *Gymnopus subnudus,* (Ellis ex Peck) Halling, Omphalotaceae, on mixed litter (ML) (D), *Lycoperdon perlatum* Pers., Agaricaceae, on ML (E), *Marasmius rotula* (Scop.) Fr. Marasmiaceae, on WL (F), *Mycena* sp. Mycenaceae, on LL (G), *Punctelia hypoleucites* (Nyl.) Krog Parmeliaceae on WL (H), *Tremellodendron schweinitzii* (Peck) G.F. Atk. Sebacinaceae, on LL (I). Photograph by the author.

Fig. 3.4. Morphological diversity of macrofungal fruiting bodies on various litter microhabitats in the Devil's Den site; *Collybia subnuda* Ellis ex Peck, Tricholomataceae on leafy litter (LL) (A), *Crinipellis nigricauli* Har.Takah, Marasmiaceae, on LL (B), *Gerronema subclavatum* (Peck) Sanger ex, Marasmiaceae, on woody litter (WL) (C), *Gymnopus androsaceus* (L.) Della Magg. & Trassin, Omphalotaceae on LL (D), *Marasmius* sp., Marasmiaceae, on LL (E), *Marasmiellus rhizomorphigenus* Antonín, Ryoo & H.D. Shin, Omphalotaceae on LL (F), *Resupinatus alboniger* (Pat.) Sanger, Tricholomataceae, on WL (G), *Sebacina candida* L.S. Olive, Sebacinaceae, on LL (H). *Xylaria cornu-damae* (L.) Grev Xylariaceae, on WL (I) Photograph by the author.

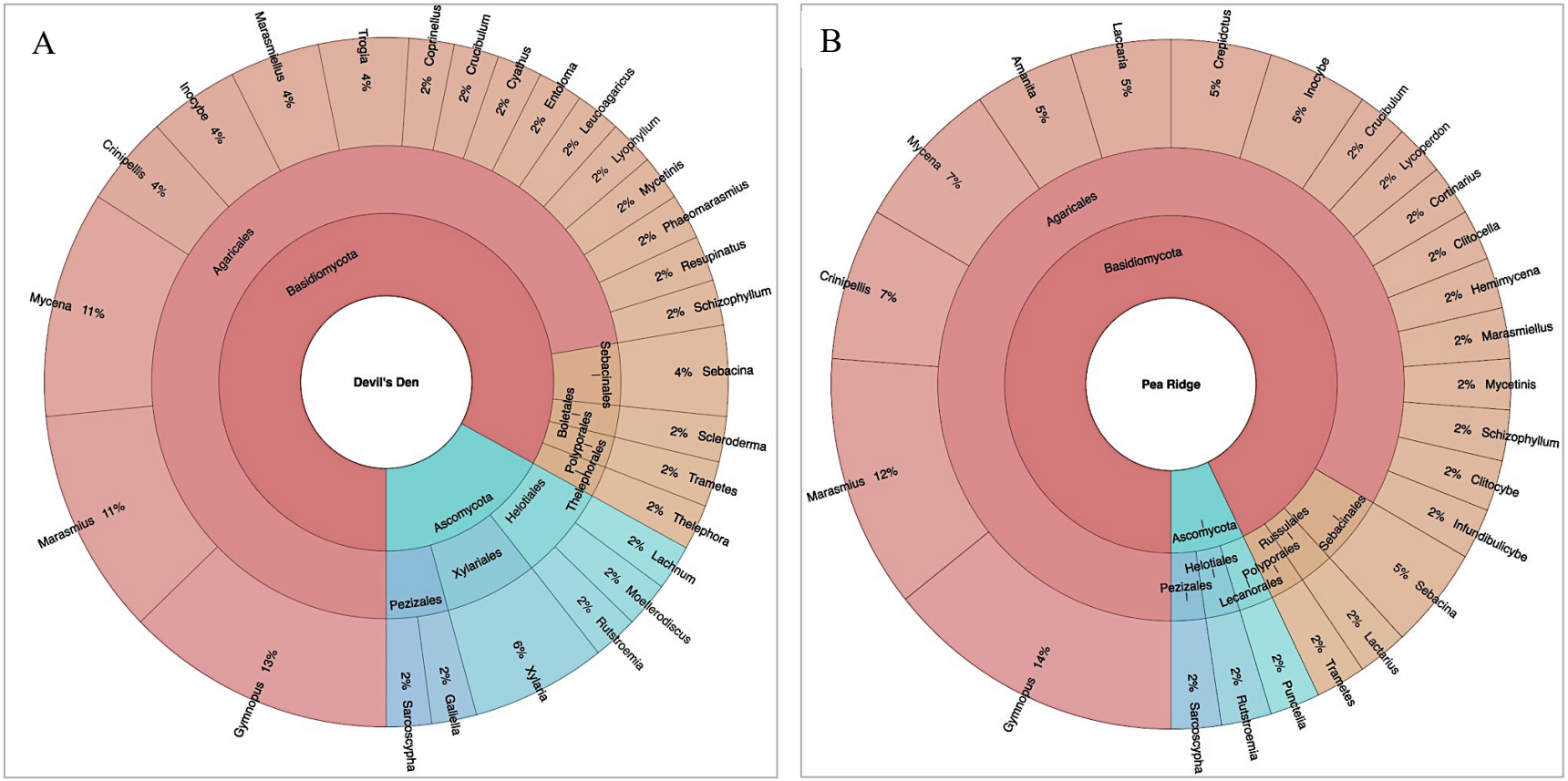

Fig. 3.5. Taxonomic structure of litter-associated fungi for Devil's Den (A) and Pea Ridge (B) sites at the genus level based on macrofungal richness illustrated by Krona plots. Wheel varieties of the distinct color levels are shown where the largest groups are in varieties of the red color levels, while the smallest proportions are in distinct turquoise color levels. The OTU members of the Basidiomycota comprise 93% and 83% of macrofungal species of the PR and DD sites, respectively while the Ascomycota encompasses 7% and 17% of PR and DD species assemblages, respectively.

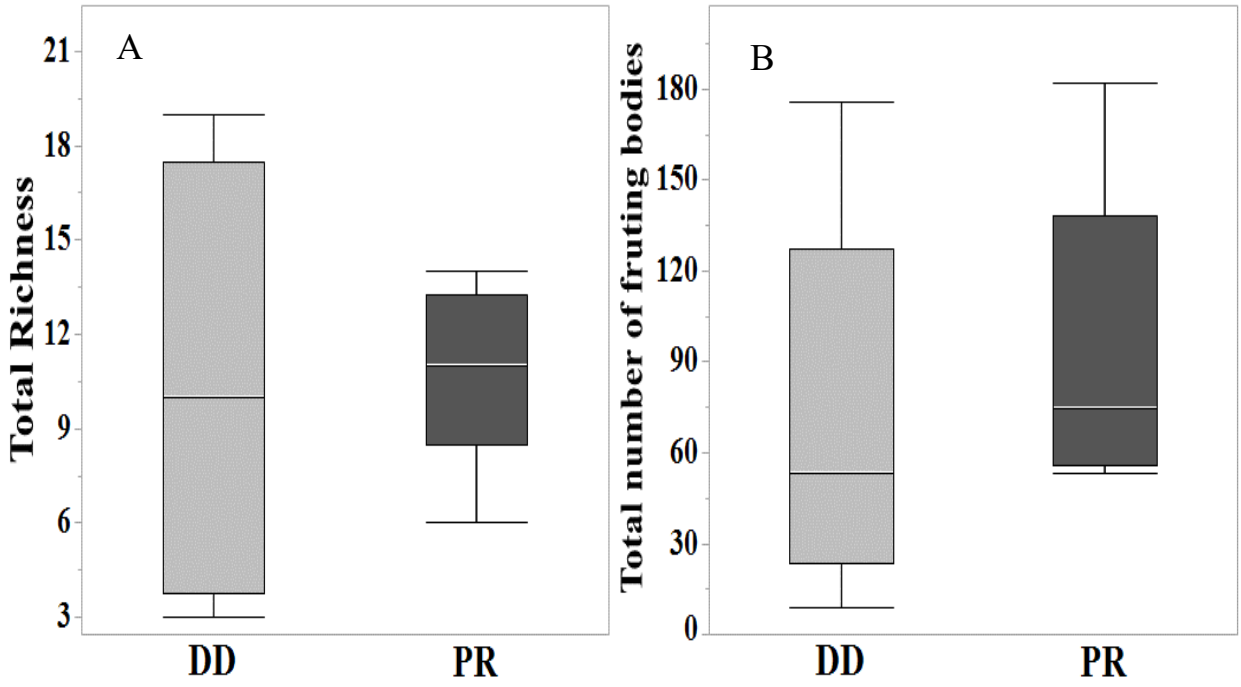

Fig. 3.6. The means of species richness (A) and number of fruiting bodies (B) of litter-associated macrofungi for the sets of the investigated sites. Means were compared using ANOVA and the Tukey's tests without showing significant differences at $P < 0.05$.

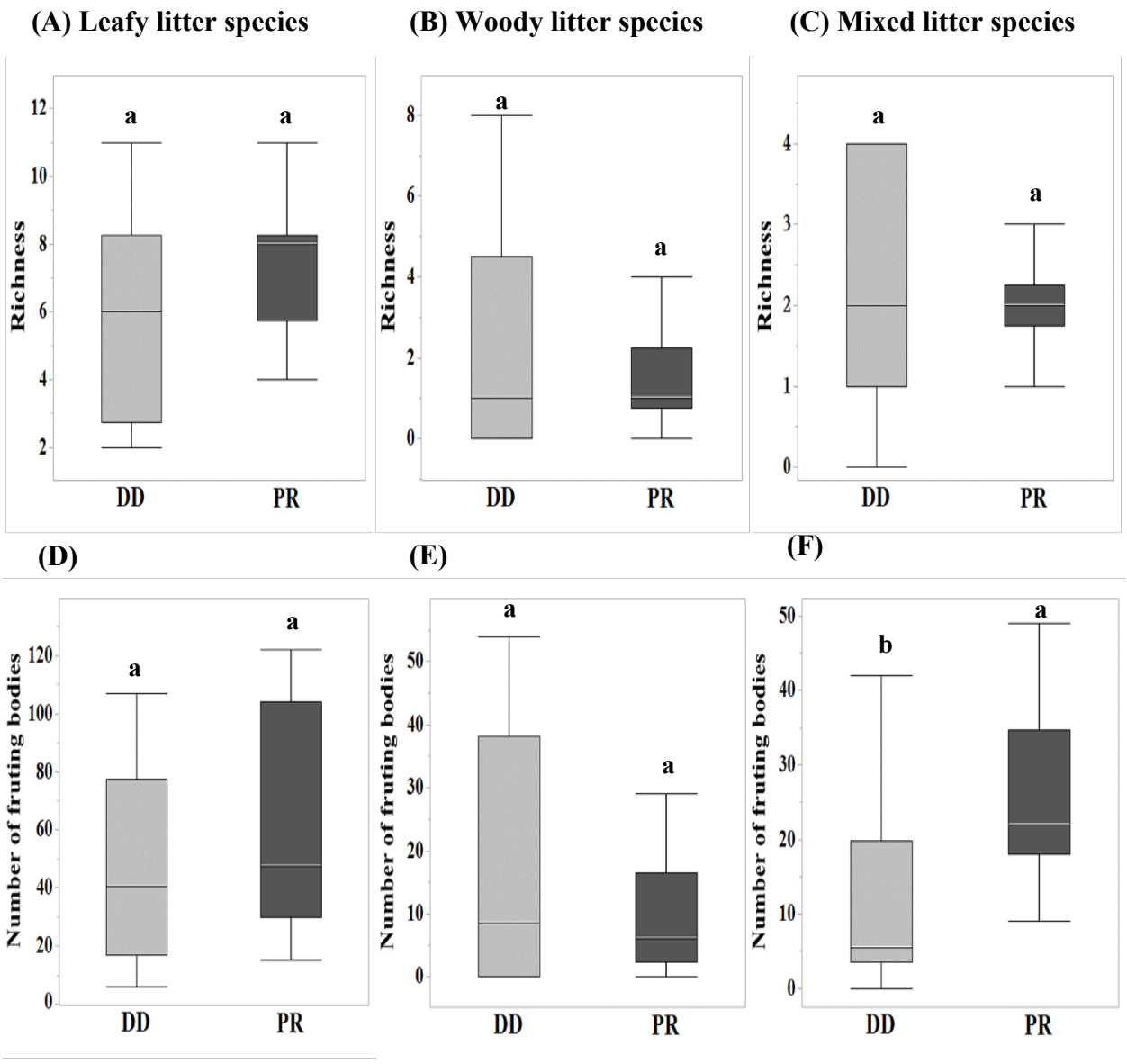

Fig. 3.7. The species richness (A-C) and number of fruiting bodies (D-F) of leafy, woody and mixed litter-associated macrofungal assemblages on various microhabitats across Devil's Den (DD) and Pea Ridge (PR) plot sites. Means were compared using ANOVA and the Tukey's tests. Figures connected by same letters are not significant different, while only F figure connected by different letters indicate significant differences at $P < 0.05$.

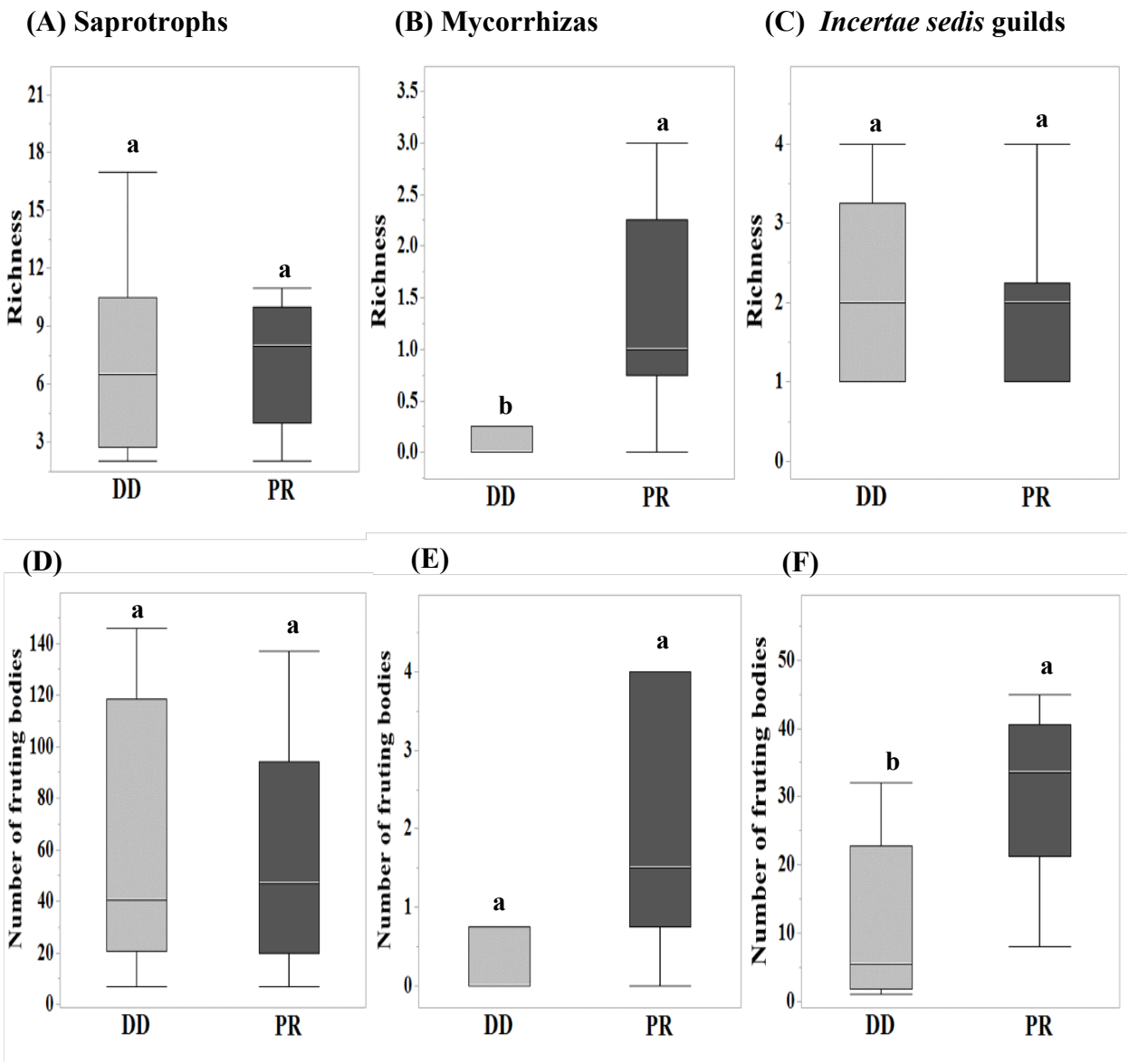

Fig. 3.8. The species richness (A-C) and productivity of fruiting bodies (D-F) of the putative ecological functions of saprotrophs, mycorrhizas, and *incertae sedis* guilds, of litter associated macrofungi across 20 plots within Devil's Den (DD) and Pea Ridge (PR) forests. Means were compared using ANOVA and the Tukey's tests. Figures connected by same letters are not significant different, while B and F figures connected by different letters indicate the significant differences at $P < 0.05$. Lichens and mycoparasites were excluded from the functional illustrations due to their smallest richness.

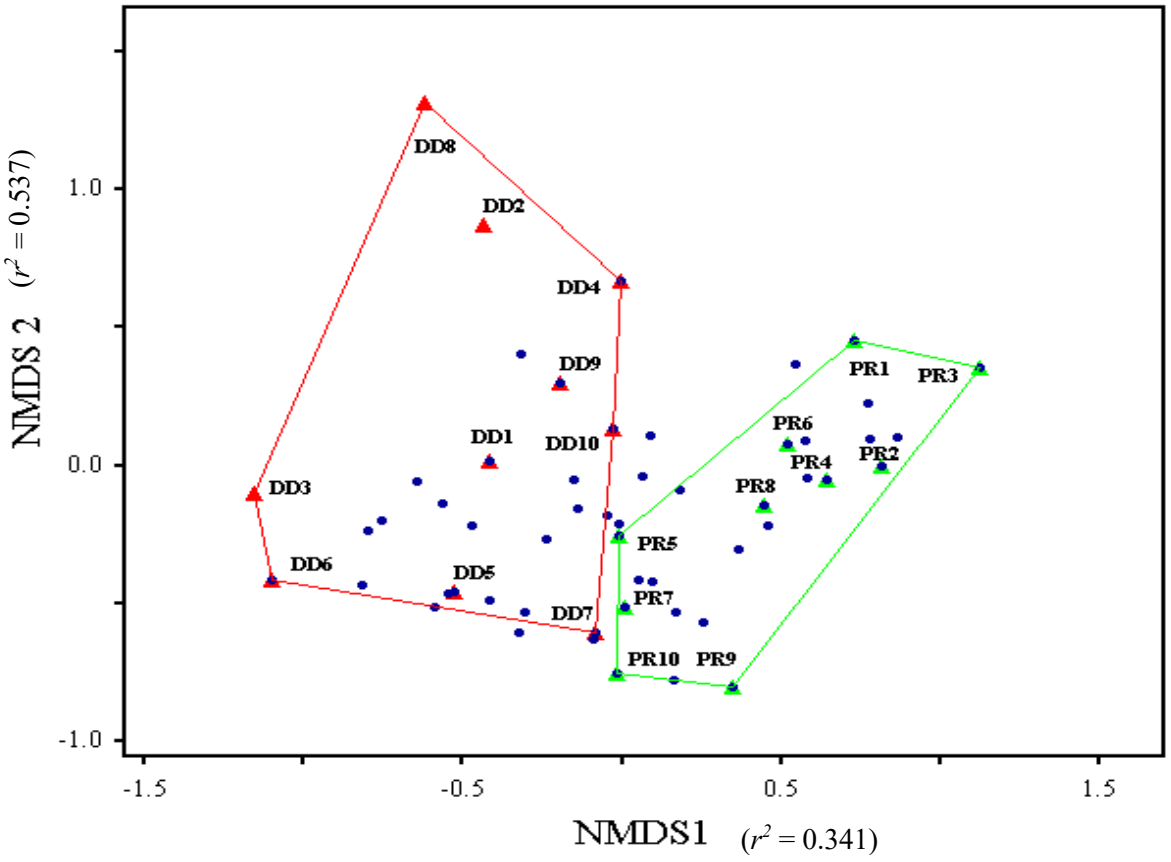

Fig. 3.9. Non-metric multidimensional scaling (NMDS) ordination plots of litter-associated fungi within sampling plots of the Devil's Den (DD) and Pea Ridge (PR) forests based on the binary data. The placements of blue variables presented the fungal assemblages distributed within and between both investigated sites.

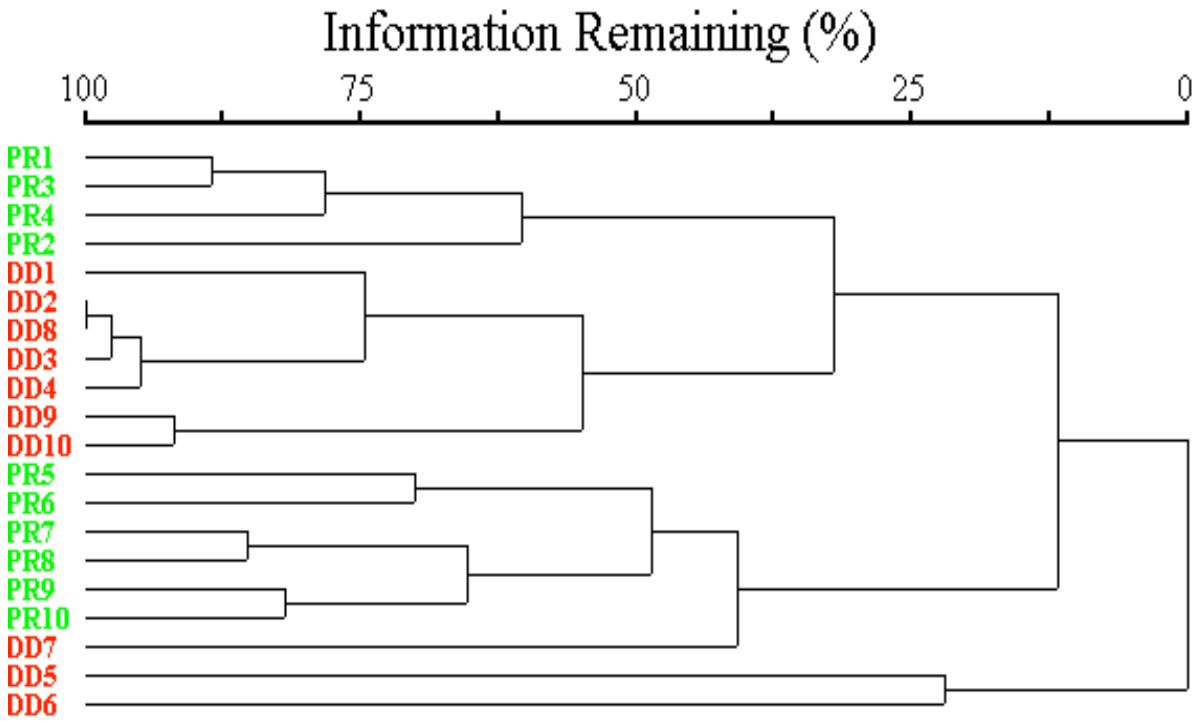

Fig. 3.10. Cluster analysis of litter-associated fungi on various microhabitats across 20 sampling plots in the Devil's Den (DD) and Pea Ridge (PR) forests using Euclidean distance and Ward's linkage method (B) based on the binary data of macrofungal species.

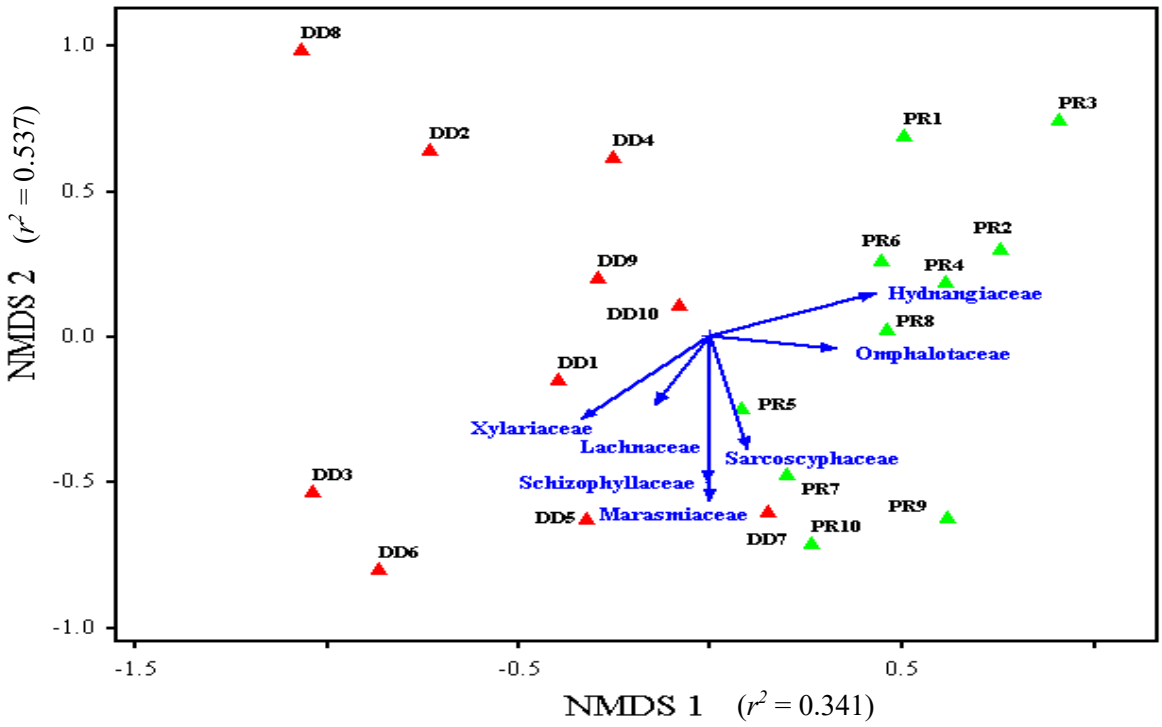

Fig. 3.11. Non-metric multidimensional scaling (NMDS) analysis of the forest plots and family structure variables overlaid as vectors on ordination axes based on a number of macrofungal species per taxonomic family of Devil's Den (DD) and Pea ridge (PR) datasets. Vectors directions and lengths designate strengths of correlations within the ordination axes. Variables with weakly correlations were not illustrated on the NMDS axes.

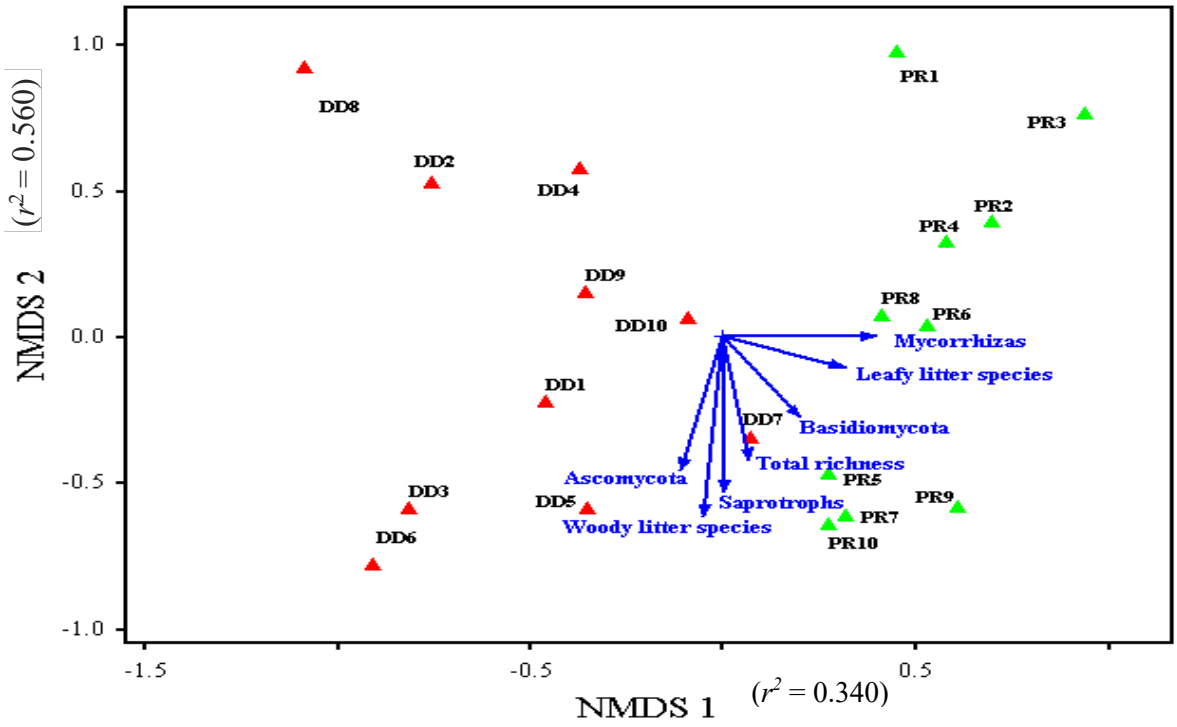

Fig. 3.12. Non-metric multidimensional scaling (NMDS) analysis of the richness of taxonomic and functional groups and of litter microhabitat species, and the forest plot variables for the Devil's Den (DD) and Pea ridge (PR) sites fitted as vectors on NMDS axes. All variables in this figure were strongly correlated with at least one of the two ordination axes.

Table 3.1. Correlation coefficients of the vectors fitted on the ordination axes using separate NMDS analyses for the variables of Pea Ridge (PR) and Devil's Den (DD) plots (n=20), fungal families (A) and fungal richness assemblages (B). Negative and positive values indicate strong correlations with ordination axes at $|r| \geq 0.5$ and are plotted in the NMDS figures.

Variables	1st axis correlation	2nd axis correlation	Variables	1st axis correlation	2nd axis correlation
A- Fungal family			**B- Fungal richness assemblages**		
Hydnangiaceae	0.589	0. 345	Ascomycota	-0.294	-0.604
Lachnaceae	-0.037	-0.437	Basidiomycota	0.403	-0.470
Marasmiaceae	-0.034	-0.674	Woody litter species	-0.200	-0.701
Sarcoscyphaceae	0.28	-0.558	Leafy litter species	0.507	-0.296
Schizophyllaceae	-0.063	-0.635	Mycorrhizas	0.566	-0.019
Xylariaceae	-0.518	-0.477	Saprotrophs	0.050	-0.652
Omphalotaceae	0.514	-0.184	Total species richness	0.232	-0.583

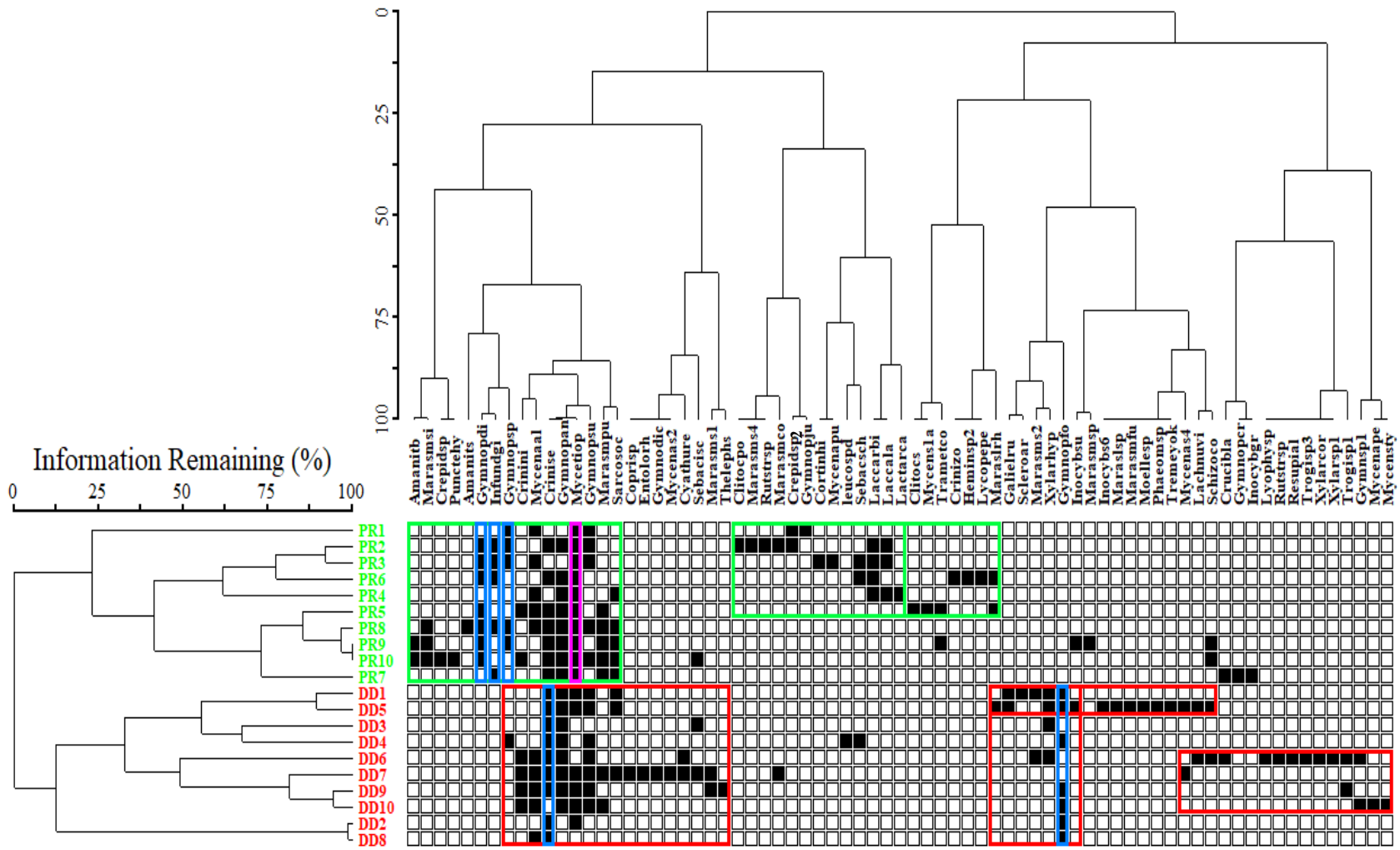

Fig. 3.13. Distribution pattern of 73 species in various litter microhabitats clustered on the horizontal matrix axis and distributed across plots investigated for Devil's Den (red color) and Pea Ridge (green color) sites, grouped on the vertical axis using two-way cluster analysis. The blue color represents indicator species for both sites, with *P*-values ≤ 0.05, and rose indicates the largest indicator species with a *P*-value = 0.0002. See Table 3.2 for complete species names.

Table 3.2. Macrofungal taxa and their codes for species clustered on a matrix axis after using two-way cluster analysis for DD and PR plots.

Macrofungal Taxa	**Codes**	**Macrofungal Taxa**	**Codes**
Amanita bisporigera	Amanitb	*Marasmius conchiformis*	Marasmco
Amanita sp.	Amanits	*Marasmius fulvoferrugineus*	Marasmfu
Clitocybe subditopoda	Clitocs	*Marasmius* sp. 1	Marasms1
Clitocella popinalis	Clitocpo	*Marasmius* sp. 2	Marasms2
Coltricia confluens	Coltrico	*Marasmius* sp. 4	Marasms4
Coprinellus sp.	Coprisp	*Marasmius pulcherripes*	Marasmpu
Cortinarius hinnuleoarmillatus	Cortinhi	*Marasmius* sp.	Marsmsp
Crepidotus sp. 1	Crepidsp	*Mycena albiceps*	Mycenaal
Crepidotus sp. 2	Crepidsp2	*Mycena pura*	Mycenapu
Crinipellis nigricaulis	Crinini	*Mycena* sp. 1	Mycens1a
Crinipellis setipes	Crinise	*Mycena* sp. 2	Mycenas2
Crinipellis zonata	Crinizo	*Mycena* sp. 4	Mycenas4
Crucibulum laeve	Crucibla	*Mycena pearsoniana*	Mycenape
Cyathus sp.	Cyathure	*Mycena stylobates*	Mycensty
Entoloma rhodocylix	Entolorh	*Mycetinis opacus*	Mycetiop
Galiella rufa	Galielru	*Moellerodiscus* sp.	Moellesp
Gymnopus dichrous	Gymnodic	*Phaeomarasmius* sp.	Phaeomsp
Gymnopus androsaceus	Gymnopan	*Punctelia hypoleucites*	Punctehy
Gymnopus cremeostipitatus	Gymnopcr	*Resupinatus alboniger*	Resupial
Gymnopus disjunctus	Gymnopdi	*Rutstroemia* sp126	Rutstrsp126
Gymnopus junquilleus	Gymnopju	*Rutstroemia* sp.	Rutstrsp
Gymnopus spongiosus	Gymnopsp	*Sarcoscypha occidentalis*	Sarcosoc
Gymnopus subnuda	Gymnopsu	*Schizophyllum commune*	Schizoco
Gymnopus sp.1	Gymnopsp1	*Scleroderma areolatum*	Scleroar
Gymnopus foliiphilus	Gymnopfo	*Sebacina schweinitzii*	Sebacsch
Hemimycena sp. 2	Hemimysp2	*Sebacina cadida*	Sebacisc
Infundibulicybe gibba	Infundgi	*Thelephora anthocephala*	Thelepan
Inocybe grammata	Inocybgr	*Trametes conchifer*	Trametco
Inocybe sp. 5	Inocybs5	*Trametes yokohamensis*	Tremeyok
Inocybe subfulva	Inocybsu	*Trogia* sp.1	Trogisp1
Laccaria bicolor	Laccarbi	*Trogia* sp.2	Trogisp2
Laccaria laccata	Laccala	*Thelephora* sp.	Thelephs
Lachnum virgineum	Lachnuvi	*Xylaria cornu-damae*	Xylarcor
Lactarius camphoratus	Lactarca	*Xylaria hypoxylon*	Xylarhyp
leucoagaricus sp.	leucosp	*Xylaria* sp.	Xylarsp1
Lycoperdon perlatum	Lycopepe		
Lyophyllum sp.	Lyophysp		
Marasmiellus rhizomorphigenus	Maraslrh		
Marasmiellus sp.	Maraslsp		

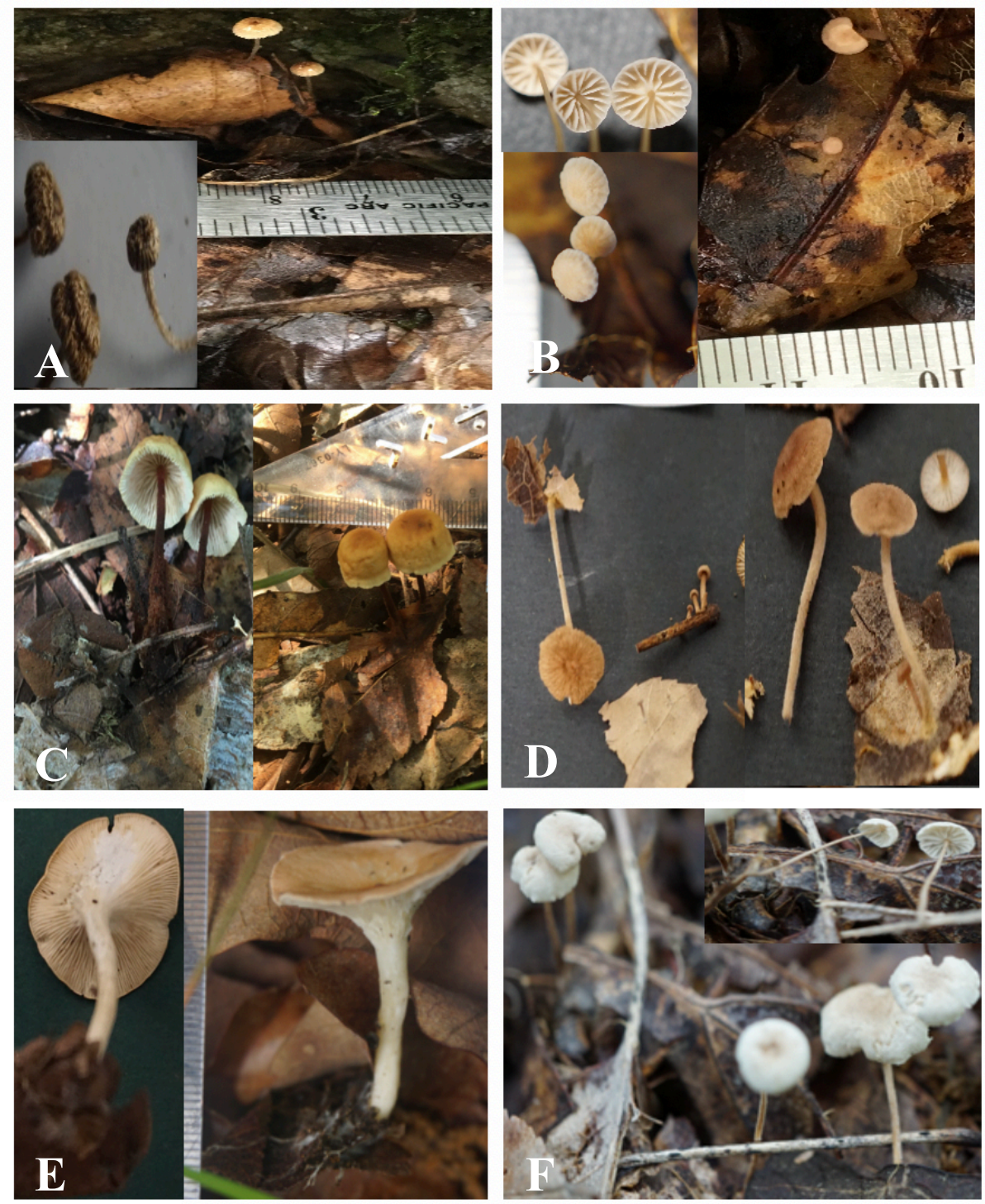

Fig. 3.14. Diverse morphological patterns of macrofungal fruiting bodies for indicator species; *Crinipellis setipes*, Marasmiaceae, (A), *Gymnopus foliiphilus*, Omphalotaceae, (B), *Gymnopus spongiocus* (C), *Gymnopus disjunctus* (D), *Infundibulicybe gibba* (E) and *Mycetinis opacus*, Tricholomataceae, (F) on various litter microhabitats for investigated sites at Ozark Mountains of northwest Arkansas forests. Photograph by the author.

Table 3.3. Indicator species value (IV%) litter associated macrofungi for both investigated sites.

Indicator Species	**Taxonomic Family**	**Microhabitat**	**Site**	**IV**	**SD**	***P*-value**
Crinipellis setipes	Marasmiaceae	Leafy litter	DD	73	9.47	0.0374
Gymnopus disjunctus	Omphalotaceae	Leafy litter	PR	70	9.42	0.0034
Gymnopus foliiphilus	Omphalotaceae	Leafy litter	DD	70	9.7	0.0036
Gymnopus spongiosus	Omphalotaceae	Mixed litter	PR	48	9.97	0.0562
Infundibulicybe gibba	Tricholomataceae	Leafy litter	PR	50	8.63	0.031
Mycetinis opacus	Omphalotaceae	Mixed litter	PR	92	8.37	0.0002

Note: Identifier: DR= Devil's Den area, PR = Pea Ridge area, Observed Indicator Value (IV%), Standard Devotion (S.D)

3.5 Discussion

3.5.1 Community patterns of litter-associated fungi: overall taxonomic affiliations, microhabitat distributions and functional guilds

The field data presented herein represented the first effort to characterize and compare the species richness, composition, and function of litter-associated fungi on the various litter microhabitats of the forest floor at the local level in two temperate deciduous mixed forests of the Ozark Mountains of northwest Arkansas. The small-sized and fragile fruiting bodies of these fungal species usually exist in nature for one or few days. However, the data for the PR and DD sites clearly highlighted the greatest taxonomic diversity and prevalent fruiting bodies of basidiomycetes species in both investigated sites, along with some ascomycete taxa. These results have been noted by using traditional methods or next-generation sequencing of sampling litter fungi generally (Boody et al., 2008; Bahnmann et al., 2018). Nevertheless, few studies have focused on the litter decomposer community (Baldrian, 2017) and the distribution of litter macrofungi on various microhabitats (Alanbagi et al., 2019) because of the difficulty of studying macrofungi related to needing a high field sampling effort along with the cryptic and nature of reproductive structures (Ambrosio et al., 2018).

Taxonomically, there were 73 species with a total of 1685 fruiting bodies in both investigated sites. Most of the species recorded in both the Ascomycota and Basidiomycota are known to efficiently decompose lignin and other recalcitrant materials and play decisive roles in litter decomposition processes (Boody et al., 2008; Van der Wal et al., 2013) while contributing distinctively for producing a unique fungal assemblage for each site. Statistically, these assemblages showed no differences in mean values for species richness and fruiting bodies for both DD and PR sites (Fig. 3.6). Also, within structure of these communities, the Ascomycota

was characterized by more productivity and higher species percentages for DD litter plots than for PR plots, mainly of the presence of species of *Xylaria* (Osono, 2020) along with species of *Lachnum* and *Rutstroemia*, which are known to secrete ligninolytic peroxidases and oxidoreductases (Liers et al., 2011; Nghi et al., 2012). However, it has been assumed that members of the Basidiomycota may utilize recalcitrant compounds more easily than members of the Ascomycota (Voříšková and Baldrian, 2013).

The results noted above are likely related to many interconnected site factors that include the differences from place to place in the dominant plant species contributing the material making up the litter layer of the deciduous mixed forests (Figs. 3.1 and 3.2) in northwest Arkansas (Schimann et al., 2017) or the soil and root-associated fungi (Dai et al., 2019; Nelsen, 2017) found beneath the litter layer. Devil's Den State Park had a more precipitation and a lower average temperature than Pea Ridge Park during the sampling year (Fig. 1.7), and this presumably presented another set of important environmental factors due to potential differences in climatic responses at the species level. This may have resulted in changes in species turnover and in the relative contributions of diverse species, perhaps leading to shaping the overall fungal community structure and to impacts on community functional traits (Ágreda et al., 2016; Boddy et al., 2014). For example, providing sufficient moisture possibly would proliferate mycelial growth of the ascomycete members in terms of species richness and fruiting body productivity, as in recording species of *Xylaria* only in DD plots, which is in agreement with observations of overall habitat preferences for an undistributed rainforest, as reported by Van der Gucht et al. (1996) and Geethanjali and Jayashankar (2016). However, temperature appears to be the main driver of mushroom production for temperate forest ecosystems, where soil humidity is maintained throughout the year (Büntgen et al., 2012; Sato et al., 2012). That may explain the

total number of fruiting bodies for drier PR plots due to the high productivity of species recorded in both areas such as *Mycetinis opacus, Gymnopus androsaceus*, *G. spongiosus*, *Mycena albiceps*, and *Schizophyllum commune* Fr. Because less research effort has been devoted to the response of individual taxa, and this has been restricted almost entirely to edible species, most studies have been carried out at a community level (Ágreda et al., 2016). Relatively few studies have depended upon plot-based methods for determining or comparing the macrofungal communities from forests in North America (Trudell and Edmonds, 2004), so it is difficult to clarify the differences in the species richness and productivity under climatic conditions in the current study. Nevertheless, other complex dynamic factors such as tree growth, fungal site history, competition, succession or antagonistic interactions among fungi may contribute to the final species litter diversity and productivity as well as the decay rate and nutrient allocation and cycling (Bodeker et al., 2016; Baldrian, 2017).

At microhabitat scales in the current study, diverse taxa distributed particularly based on the microhabitat prevalence, mostly leafy litter, and in a similar trend in both investigated sites (Fig. 3.7). The numbers of species and fruiting bodies of leafy litter (LL) species were higher in both sites compared to other microhabitats. That is because the leaf litter forest carpet provides easily accessible carbon and mineral nutrients that later follow a sequential pattern with different classes of organic compounds during the decay process, resulting in rapid successional and competitive changes of the fungal assemblages associated with these microhabitats based on their extracellular enzymatic abilities (Berg and McClaugherty, 2008). Thus, the accumulation of multispecies leaves with freshly fallen leaves support a microenvironment that is suitable for proliferated mycelial growth and development of fruiting bodies after a rainy day with appropriate temperatures. The richness of macrofungi on woody and mixed litter microhabitats

did not differ significantly when comparing them separately for both investigated areas, but the number of fruiting bodies of mixed species did vary for the two sites. The higher macrofungal productivities of PR site plots are mostly related to the ecological and biological features of the dominant *Mycetinis opacus* (Berk. & M.A. Curtis.) A.W. Wilson & Desjardin. This species was recorded with a high number of fruiting bodies (200 fruiting bodies) and found in gregarious to clustered groups inhabiting both fallen twigs and leaves of mostly species of *Quercus* in PR plots (Petersen and Hughes, 2017). Other species such as *Marasmius pulcherripes*, *Mycena albiceps*, *Gymnopus disjunctus, G. androsaceus* were also contributed to high macrofungal productivity in PR sites, colonizing leafy litter with gregarious fruiting bodies. Also, the substrate-level microenvironment conditions such as moisture and temperature both inside and surrounding mixed litter may influence the development of fruiting bodies of this species specifically and the community assembly generally. However, these notes are only occasionally recorded with observational fungal data (Ágreda et al., 2016; Dawson et al., 2019).

In the present results, various degrees of specificity for particular microhabitats among macrofungal species within the genus were observed. For example, species in the genera *Marasmius*, *Gymnopus*, *Mycena*, *Xylaria*, and *Crinipillus* colonized either leafy litter or woody litter. This observation could be an evolutionary strategy to avoid competition between closely related species similar to evaluated results of 68 species of basidiomycetes under lab conditions (Tanesaka et al., 1993).

Although the effect of litter microhabitat diversity on functional groups in forest ecosystems remain largely unknown (Otsing et al., 2018; Alanbagi et al., 2019), diverse functional guilds were associated with particular litter microhabitats in both investigated plots (Fig. 3.7). The present results suggested hypothetically occupied and utilized most of the litter microhabitats by

the specific species within community assemblages (Berg and McClaugherty, 2008; Baldrian, 2017) and closely linked by the same genes and metabolic functional pathways (Hättenschwiler et al., 2005; Osono, 2020). For those assemblages, saprotrophic populations, reflected by species richness and productivity (Fig. 3.8A and D), were the primary decomposers among the heterogeneous microhabitats as being expected in agreement with results from other studies (Voříšková et al., 2013; Alanbagi et al., 2019). However, there is also increasing evidence that some free-living saprotrophs can switch to facultative biotrophs with plant roots, without causing disease symptoms, this specialty is still not well studied (Smith et al., 2017). The possession of distinctive functional traits certainly allows saprotrophs to exploit the plant detritus that makes up litter (Goswami et al., 2017). Furthermore, documented ectomycorrhizal species in both datasets of the study sites provide evidence of overlapping litter microhabitats with saprotrophs and the switching lifestyle of ectomycorrhizas from symbiotic to saprotrophic interactions to access organically bounded nutrients (Bödeker et al., 2009; 2016; Bahnmann et al., 2018). Nevertheless, the ectomycorrhizal richness and productivity were significantly greater in PR plots than in DD plots (Fig. 3.8B and E), indicating that the capacity of unique ectomycorrhizal species in DD plots might contribute remarkably to inhabiting litter microhabitats, accessing nutrients, selectively absorbing nutrients to be later introduced into plants, and strategically to compete for intra- and inter-species interactions and with others ecosystem members (Baldrian, 2017; Bödeker et al., 2016; Tláskal et al., 2016).

Furthermore, variation in climatic conditions (Yang et al., 2012; Yuste et al., 2011), plant community composition (Laganière et al., 2010; Wang et al., 2019), nutrient availability (Boody et al., 2008) and forest management regimes (Köster et al., 2015) would probably affect fungal functional guilds differentially at the same site and between both investigated sites. That results

in changes in competitive balance, that could be observed as differences between PR and DD ectomycorrhizas.

Other identified species with uncertain functional guilds "*incertae sedis"* also were assigned greatest fruiting productivity in PR plots (Fig. 3.8C and F). Most are likely to be litter decomposers, but perhaps also switching from symbiosis to saprotrophism (Bodeker et al., 2016; Osting et al., 2018). This possibility was evidenced by the presence of some taxa known to be mycoparasites and lichens.

Since species richness and functional richness are inherently linked (Tilman, 1999; Goswami et al., 2017), it could be expected that the diverse macrofungal community might contain varied species with functional traits suited for exploitation of the heterogeneous litter nutrients. However, many biotic and abiotic factors influence the species and functional richness with proneness to compositional shifts and increased species dominance depending on these factors.

3.5.2 Interactions and comparations of fungal community populations across investigated sites

Community composition of litter-associated fungi significantly differed between both investigated sites (Figs. 3.9 and 3.10), but community structure was similar in the total taxon richness, various functional-guilds and overlapping microhabitats across site plots (Figs. 3.6-3.8). This pattern was presumably influenced by the complex relations driven by the dominant tree species and climate variation (Boddy et al., 2014) in addition to many other microenvironmental factors surrounding mycelial foraging for new or across litter resources, mostly biotic variables (Wang et al., 2019). At the macrofungal community level, diverse limiting factors, species-specific plasticity, and adaptative potential may trigger heterogeneous fungal responses (Malcolm et al., 2008) that determine community changes leading to shifts in species relative abundance and species turnover (Deslippe et al., 2011).

In the present study, members of the families Cortinariaceae and Hydnangiaceae mainly occupying leafy litter were unique to the PR basidiomycetes taxa and function as ectomycorrhizas, comprising 57% of mycorrhizal species mostly inhabited leafy litter and 62% of mycorrhizal productivity (Figs. 3.11 and 3.12). The current ectomycorrhizal species in genera such as *Laccaria, Lactarius and Cortinarius* with other ectomycorrhizal species on PR litter, may possess the genetic potential to produce extracellular enzymes such as Class II peroxidase that are efficient in lignin decomposition at the drier and lower N availability under field conditions (Trudell and Edmonds, 2004; Bödeker et al., 2009). These ectomycorrhizal community differences between both sites are most likely to develop over a long time and largely related to differences in ecosystem moisture, nutrients abundance such as nitrogen, (Trudell and Edmonds, 2004) and based on the evolutionary history with the host plants, as being suggested with other functional guilds (Photita et al., 2004). It seems that the mycorrhizal PR assemblage competed directly or indirectly for space or resources in the leaf litter with the dominant saprotrophic species in the Omphalotaceae and other families. The present species of ectomycorrhizal genera are known to produce abundant extraradical mycelium as rhizomorphs, cords or mat-like aggregations (Trudell and Edmonds, 2004). That may lead to a decrease the saprotrophic productivity of PR plots compared with DD plots when taking into account that lower soil moisture leads to lower wet deposition of nitrogen, as well as lower accumulation of CWD, and lower activity of free-living saprotrophs (Fig. 3.8A-D; Trudell and Edmonds, 2004). Hypothetically, all of these might expect to decrease in abundance of mineral nitrogen in the soil and would lead to greater allocation of carbon to growth and reproduction by the fungi and thus a competitive advantage for species with high carbon demand such as ectomycorrhizas (Wallander, 1995; Trudell and Edmonds, 2004). Accordingly, ectomycorrhizas could be

indirectly regulate the litter decomposition rate by restraining activities of more efficient litter decomposers, saprotrophs through competitive interactions (Bödeker et al., 2016). When comparing the total richness and dominant function" saprotrophs", they obviously clustered near PR NMDS based on previous community features. That implied large amounts of litter resources invested (Bässler et al., 2015), and the availability of resources could be limiting factor in the fungal productivity (Ágreda et al., 2016) in addition to previously mentioned factors such as environmental correlations.

Conversely, the distinctive community compositions of DD NMDS were exclusively clustered by the ascomycete members of the families Lachnaceae and Xylariaceae and significantly pointed to the basidiomycete taxa in the families Marasmiaceae and Schizophyllaceae (Fig.3.11 and Table 3.1). These distributed on various litter microhabitats but significantly characterized by prevailing woody species when comparing this group with PR NMDS (Fig. 3.12 and Table. 3.1). That suggested an important role of these assemblages inhabiting only very fine woody microhabitats or mixed with leafy litter not only in macrofungal diversity and productivity under field conditions, but also in understanding the adaptation and speciation of these species (Baldrian, 2017; Sexton et al., 2017) and prediction of fungal community assembly under environmental changes or forest management. However, few studies have focused on the very fine wood assemblages (Küffer and Senn-Irlet, 2005) or ecological niche breadth evolution of individuals or populations within a species (Sexton et al., 2017). Members of those families with ligninolytic species potentially capable of degrading a wide range of organic materials are largely unknown in the ascomycetes, except for Xylariaceous and Rhytismataceous fungi (Boddy et al., 2008; Liers et al., 2011; Osono, 2020). Most of woody litter taxa are saprotrophs which could help to provide a habitat for species that do not generally decay wood, such as

ectomycorrhizas (Fischer et al., 2012) or uncertain functional guilds "*incertae sedis"*. Microhabitat specificity of the litter decomposer community for each explored site could be related to several complex factors, such as a particular dominant tree species and overall forest tree diversity, as well as differences in nutrient availability or specific litter chemistry, has been revealed important dynamic influences (Table. 4.2S; Berg and McClaugherty, 2008; Bray et al., 2012).

The PR plots harbored the most indicator species with a saprotrophic function and inhabitants of leafy and mixed litter, including *Gymnopus disjunctus*, *G. spongiosus*, *Infundibulicybe gibba*, and *Mycetinis opacus,* while the indicator species of DD plots were *Crinipellis setipes* and *Gymnopus disjunctus* and both colonized leafy litter and function as saprotrophs (Figs. 3.13 and 3.14 and Table 3.2). The physiological and biological features of these species could contribute to their survival, reproductive potential and distribution on the relatively homogeneous continuous leaf litter layer and complemented other nutrients needs through utilizing the litter microhabitats (Boddy et al., 2008; Osono et al., 2020). For example, the indicator species in the current study are aggressive competitors with cord-forming mycelia (Boddy et al., 2008; Peay et al., 2013).

The previous results provide additional evidence that nutrient-rich microhabitats of plant litter matter are not only an important nutrition resource for thriving fungal growth, resulting in litter decomposition, humus formation, carbon sequestration (Berg and McClaugherty, 2008), but also a substrate for sexual reproduction and the formation of reproductive structures from intensive growth of mycelial foraging, leading to genetic variability and thus allowing the fungus to adapt to new environments surrounding their microhabitats.

3.6 Conclusions

This project characterized the assemblage of taxa, microhabitat distribution, and functional guilds of litter-associated macrofungi based on morphological and molecular genetic data for two investigated sites within the temperate mixed deciduous forests of northwest Arkansas. A high level of fungal taxonomic diversity characterizes these fungi found on the accumulation of heterogenous litter. The nutrient-rich microhabitats of the latter probably support specific populations that play essential roles in litter decomposition and sexual reproduction. Although total species richness and fruiting body numbers did not differ significantly between the two study sites, more than 60% of all of the species recorded were unique to one of the sites. Each litter microhabitat was characterized by a unique fungal community structure. Most of these microhabitats were colonized by leafy litter species. However, mixed and woody species contributed remarkably in terms of species richness and productivity, especially mixed litter in the PR plots. This specificity could provide important propagule sources after forest disturbances or management applications.

Members of distinctive functional guilds formed fruiting bodies in different microhabitats, but this had no apparent impact on the dominant saprotrophs. Documented diverse lifestyle patterns mean that litter-associated fungi have achieved a high degree of ecological flexibility to grow and reproduce in a wide range of litter microhabitats. Ectomycorrhizal fungi were associated mostly with leafy and mixed litter-microhabitats in the PR plots and with leafy and woody litter in the DD plots. The latter would contribute to their support of the growth of plant saplings and forest trees. An appreciable number of species in unassigned guilds appeared and produced a significant number of fruiting bodies, and these may provide another insight into the ecology of fungi in the current study. The previous results have the potential to be an important indicator for

efficient ecosystem management. Overall, knowledge regarding macrofungal community structure, litter microhabitats and functional assemblages in the heterogenous litter layer provide essential information for diverse applications and will contribute towards developing an effective plan for ongoing forest management in these forests.

3.7 References

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Chapter 4. Metataxonomic analyses of assemblages of leaf litter macrofungi: assessing taxonomic and functional diversity and correlation between the community assemblages

4.1 Abstract

Recently developed next-generation sequencing approaches provide novel insights into the fungal diversity associated with various substrates in terrestrial ecosystems. Leaf litter is main component of the heterogenous litter layer on the forest floor and the most valuable renewable resource in temperate deciduous forests. It is an important microhabitat for organic decomposition, nutrient cycling and food web interactions. However, knowledge about the fungal species diversity, community structure and functional guilds of this dynamic habitat is incomplete because of previous methodological limitations. This study examined and compared the macrofungal taxonomy and functional diversity of leaf litter and the community assemblages associated with10 plots from each of two temperate deciduous mixed forests on the Ozark Plateau using rDNA metabarcoding of the fungal ITS2 region. The representative OTUs were manually identified to the taxonomic levels and assigned for functional guilds. The study also highlighted the drivers that are presumably responsible for determining structure of the macrofungal assemblages on leaf litter in the study sites examined. The results obtained revealed that leaf litter has a hidden macrofungal component in both study sites. The Ascomycota and Basidiomycota with their various orders dominated in both sites, but their species taxonomic structures and abundances were different. There were 349 macrofungal OTUs in both investigated study sites, and these belonged to 97 genera and 94 families with many new macrofungal sequences recorded the first time across both study sites. These were clustered obviously across 20 plots based on a two-way cluster analysis of site genera. The indicator

species and their functions for both sites were also detected. The deep sequencing presented diverse and overlapping functional groups inhabiting leaf litter without significant differences in richness of the dominant saprotrophs. Nevertheless, the richness of ectomycorrhizas, parasites, and members of other uncertain functional guilds were statistically distinctive. Several investigated abiotic-biotic factors may synergistically shape the macrofungal taxonomic and functional assemblages. The data presented herein provide an unprecedented insight into the macrofungal diversity taxonomically, functionally and compositionally in leaf litter of Ozark forests. The results are important for future investigations of the sustainable diversity of macrofungi and forest managements such as for prescribed burning for plant community maintenance and restoration.

4.2 Introduction

Next-generation sequencing (NGS) provides a powerful tool for investigating the microbial communities. Microbiome research has grown rapidly over the past decade (Breitwieser et al., 2017) with the development of new sequencing technologies (Hilton et al., 2016) and the proliferation of new computational methods that can assist in the analysis of large and complex metagenomics data sets (Breitwieser et al., 2017). Knowledge was previously limited by culture-dependent methods and inventories of fruiting bodies. Metagenomics and metataxonomic tools can make wide fungal taxonomic classifications and provide essential information on the species diversity, composition and function of the microbiome in various environments (Breitwieser et al., 2017; Smith et al., 2017). These tools have successfully included fungi in studies of biological diversity, mostly by using fungal ITS barcoding. In particular, ITS sequencing has been used in unexplored habitats and localities (Yahr et al., 2017) or to uncover unexpected

fungal diversity, especially with databases growing in size and diversity (Hilton et al., 2016). Metagenomic studies are believed to provide more accurate estimates of a global fungal diversity and to determine taxonomic and functional groups of overlooked substrates with more in-depth an examination for fungi in a meaningful way (Yahr et al., 2017). This technology, with increased development, is expected to provide essential data about the uncertainty of the estimates of the global fungal species richness and an understanding of the importance of stochastic vs. deterministic forces in the assembly of fungal communities in general (Taylor et al., 2014). For example, substrate availability and preference are important drivers of macrofungal diversity, although few studies have examined this relationship (Ye et al., 2019). Leaf litter, for example, is an important microhabitat with a strong expectation to have a high level of hidden diversity due to fungal succession during litter decay, and it is still unclear which biological features of fungi and their sources of nutrition give rise to this pattern (Baldrian, 2017). A better understanding of key taxonomic features of litter-associated fungi, it should provide a more complete insight into the biology behind one of the largest fluxes of carbon and energy through the biosphere (Vivelo and Bhatnagar, 2019). However, culture-independent approaches highlight several challenges in fungal taxonomy for future investigations.

Litter-associated fungi are an important structural and functional component in any ecosystem through their role in the decomposition processes specifically, biogeochemical cycles in general (Hättenschwiler et al., 2005; Bisht et al., 2014.), and complex interactions involved with other microorganisms and other elements of the fauna (Hättenschwiler et al., 2005; Boddy et al., 2008). Macrofungi constitute an important part of this assemblage and exert profound biological and economic impacts (Miles and Chang, 2004). For example, they are able to rapidly migrate and colonize new or disturbed areas faster than some vascular plants (Claridge et al., 2009;

Alday et al., 2017). Within forest ecosystems, our knowledge of macrofungal taxa or community structures on litter materials has been limited by methodological aspects, or fungal morphological features largely related to their hidden nature and often short-lived fruiting bodies (Boddy et al., 2008, Ambrosio et al., 2017). The macrofungal propagules could not only include those from fungi restricted to the leaf carpet or other litter microhabitats, but those from diverse taxa with various functions that typically inhabit other forest substrates due to the rich nutrients of fallen leaves (Alanbagi et al., 2019; Boddy et al., 2008; Bodeker et al., 2016). However, the taxonomic structure of fungal assemblages and the functional diversity of fungal litter or the overlap between saprotrophs and other functional guilds on various litter microhabitats, on leaf litter specifically, are still not well studied (Gessner et al., 2010; Smith et al., 2017). Several factors that drive the structure and composition of community assemblage of macrofungal litter are also poorly studied and understood under field conditions. This group could face several natural challenges by climatic factors such as rainfall and temperature (Ágreda et al., 2016), and artificial encounters made directly or indirectly by humans (Senn-Irlet et al., 2007). The type of forest and the tree diversity of an investigated forest with different litter quality also have been detected as a major driver of fungal propagule richness and fruiting body productions (Cline and Zak, 2015; Nelsen, 2017; Alanbagi et al., 2019). Thus, identification of these drivers and filling in information gaps are a crucial step to conserve the diversity of litter-associated fungi (Chen et al., 2017).

Although leaf litter is an essential component in the forest ecosystems and major concern during management strategies, the macrofungal taxonomic and functional diversity and community structures associated with leaf litter have been underrepresented by traditional approaches or even recently by next-generation sequencing. The mycobiome results of the current study using

Ion Torrent PGM herein represented the first effort of which I am aware to document the fruiting bodies of macrofungal species associated with the multispecies leaf litter in two sites at the Ozark Mountains of northwest Arkansas. These were Pea Ridge National Military Park and Devil's Den State Park. Twenty litter samples were collected under more or less complete canopy cover from ten plots at each study site. The purpose of this study was to answer the following questions: (1) What is the taxonomic macrofungal diversity on leaf litter? (2) Are there varied functional guilds on that microhabitat? (3) What is the community assemblages of macrofungal species and functional guilds? (4) Are there different patterns in species and or functional community structures between both geographical forest sites, if so, what factors are the drivers of this variation?

4.3 Material and Methods

4.3.1 Study sites and Sampling Processes

Leaf litter samples were collected in the middle of May 2016 from forest floor of two sites in the Ozark Mountains of northwest Arkansas. The forests were characterized as mixed deciduous woodland (Stephenson et al., 2007; Diamond et al., 2013). These were Pea Ridge National Military Park (36°27'37" N 94°01'21" W, elevation 484 m) and Devil's Den State Park (35°46'32" N, 94°14'46" W, elevation 454 m), with both consisting mainly of diverse species of oaks with an admixture of several other species. The monthly mean temperatures and rainfall, and forest tree characteristics of the study sites are shown in Figs. 1.7, 3.1 and 3.2.

Ten replicate study plots were assigned at each site to collect multispecies litter samples consisting of fallen leaves (Fig. 1.2). Each site was divided into ten 10 m x10 m plots with the distance between them 35-50 m in the manner described in Alanbagi et al. (2019). Within each

plot, five leaf litter samples were collected from underlying forest floor to represent the substrates of litter-associated fungi. These replicate materials were pooled together, resulting in one sample per plot. A total of 20 collected samples from both sites were put in paper bags and transferred to the laboratory and processed. In the laboratory, leaves were air dried at room temperature. Then, each sampled plot of leaf litter was cut into the pieces approximately 1-2 cm pieces, homogenized and divided into two groups. The first group was stored at -80 °C until processed for DNA extractions, library preparation, and sequencing. Another group of leaf litter was sent for chemical analysis to Brookside Laboratories (Ohio, USA).

4.3.2 DNA extraction, PCR amplification and NGS library sequencing

For independent DNA extractions of each plot sample, leaf litter samples were ground using liquid nitrogen. Genomic DNA was extracted from 80 mg of the homogenized ground material with two replicates for each sample using the NucleoSpin® Plant II DNA extraction kit (Macherey-Nagel Gmbh & Co., Düren, Germany) according to the manufacturer's protocol. The two replicates of extracted DNA were then pooled together, purified and concentrated using the Wizard SV Clean and Concentrated DNA kit **(**Promega, USA) following the manufacturer's protocol. The DNA quantity and purity were checked using a fluorimeter (GloMax®-Multi Jr, Promega, USA) and a nanodrop apparatus (ThermoFisher Scientific, USA), respectively. The DNA extracts were afterward stored at -20 °C for further analysis.

The fungal amplicon libraries were obtained by Ion Torrent sequencing using internal transcribed spacer (ITS2) region based on the results of previous studies (Geml et al., 2014; Nelsen, 2017). 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 (MIDs) tag were used to amplify the ITS2 region of the nuclear ribosomal rDNA region. All information related to the Ion Torrent

adaptors, and MIDs are listed in Tables 4.1S and 4.2S. The components of the 40 µl PCR reactions were 1 µl (10-16 ng) DNA templates, 20 µl GoTaq® G2 Green Master Mix (Promiga, USA), 1µl (25 mM $MgCl_2$), 14.4 µl of nuclease-free water (QIAGEN, Valencia, California, USA) and 1.5 µl (10 mM) each forward fITS7 and reverse ITS4. The PCR reactions were carried out with three independent reactions from each DNA sample in addition to a negative control consisting of nuclease-free water instead of DNA for every barcode. The PCR thermocycler profiles were 1 initial denaturation cycle at 95ºC for 5 min, followed by 25 denaturation cycles at 95ºC for 20 sec., annealing at 56ºC for 30 sec., and with an extension at 72ºC for 1.30 min, with a final extension cycle at 72ºC for 7 min (Geml et al., 2014). Amplicons with their negative controls were verified on 1% agarose gels. The triplicate PCR products were pooled for each sample (Figs. 4.1S and 4.2S). Each pool was purified with AmPure® beads (Beckman Coulter, Beverly, Massachusetts, USA), and quantified with Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). The library was loaded onto and sequenced using an Ion 318^{TM} Chip on an Ion Torrent Personal Genome Machine (PGM)(Life Technologies, USA), and other related instrumentation by the Macrogen Inc., USA (Fig.4.3S). The total raw reads of Ion Torrent sequencing were 2,502,187 with approximately mean and median read lengths of 233 and 236 bp, respectively (Tables 4.3S and 4.4S).

4.3.3 Bioinformatic analyses

After sequencing, the raw individual sequence reads were filtered by the PGM software for low quality and polyclonal sequences removal. The sequences concurrently sorted according to samples, primers, adapter and MIDs were removed. All PGM quality approved, trimmed and filtered data were exported as FASTQ files (Tables 4.3S and 4.4S; Fig. 4.3S).

The low-quality ends of sequences, based on 0.02 error probability limit sequences were

trimmed in Geneious 9.1.8 software (Biomatters, New Zealand). Sequences were considered for further analyses when passing the quality filtered using USEARCH v.2.8.0 (Edgar, 2010) with default parameters based on the following criteria: truncating the sequences to 200 bp, removing reads with 100 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, all final, 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 previous recommended in previous meta-barcoding studies (e.g. Geml et al., 2010; Tedersoo et al. 2014; Semenova et al. 2016). Global singletons were discarded and OTUs with less than four sequence reads were excluded. A total of 2 080 high quality fungal OTUs were detected, truncated to the length of 200 bp and later used for taxonomic assignments to assesses all macrofungal sequences in the samples. The representative sequences with less than four sequences on a per sample were excluded from future analyses basis following the suggestion of Lindahl et al., 2013 and other researchers to minimize the false positives.

4.3.4 Taxonomic and functional assignments

Classification of sequences was performed for the representative sequences 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 internal transcribed spacer (ITS) reference databases (version released on 22 August 2019), containing identified fungal sequences with assignments to species Hypothesis groups (Kõljalg et al., 2013) All represented sequences were included for the goal of manually assessing macrofungal OTUs and diversity in the samples (e.g., Arnolds, 1981).

The representative sequences were then assigned as to the taxonomic affiliation and named using the current Index Fungorum classification (www.indexfungorum.org) as implemented in UNITE, following the reference criteria (e.g. Geml et al., 2010; Tedersoo et al., 2014; Agostinelli et al., 2018). For accurate taxonomic assignment, the search results of query sequences were manually checked in both methods and the lowest taxonomic level at which both methods agreed was selected. Additional manual blasts were run through the nucleotide blast (BLASTn) against the National Center for Biotechnology Information (NCBI) GenBank sequence database for those that had no blast hit in previous ITS sequence databases. Based on a megablast search using the ITS sequences, the closest hits of the matching sequences in the NCBI nucleotide database were then manually checked to improve phylum- and class-level classification. After excluding OTUs with < 80% similarity and microfungal sequences, a total of 155,091 fungal high-quality sequences were retained. The rarefied OTU accumulation curve were quantified to estimate the sequencing depth of the fungal OTUs per sample using "rarefy" function implemented in the Vegan package (Oksanen et al. 2013) in R software for statistical computing (R Core team, 2017).

The macrofungal functional guilds were putatively and manually assigned for only OTUs that were classified into the genus or species level based on the relevant published literature (e.g., Tedersoo et al., 2014; Morgado et al., 2016; Nguyen et al., 2016; Grau et al., 2017). These functional assignments were saprotrophs (leaf litter, wood substrates and multisubstrate on leaf and woody habitats), ectomycorrhizas, mycoparasites, plant pathogens, bifunction (for those being assigned to saprotrophs and another function based on available references). The *incertate sides* term was also used for assigning fungi to functional guilds when there was insufficient information to point out the dominant lifestyles or genera known to comprise species with

diverse functions based on phylogenetical assignments or methodological isolations from different substrates to track continuous changing in microhabitats or environments. These ecological categories describe the fungal functional guilds and the transition between guilds in the investigated forest systems.

4.3.5 Statistical analyses

Because sequencing reads have been considered as abundance data or semi-quantitative data when used in different fungal community analyses such as diversity analyses or NMDS ordinations (e.g. Amend et al., 2010; Nelsen 2017; Smith et al., 2017), the total number of sequences was determined and visualized for providing an overview of sequence taxonomical identifications and community structures. Alpha taxonomic diversity for all OTUs datasets included macrofungi and unidentified fungi was investigated with observed richness (S), diversity indexes via Shannon-Weaver (H) and Simpson (1-D), and Evenness (H/In S) using the Vegan package (Oksanen et al., 2013) in R software for statistical computing (R Core team, 2017) for both the Pea Ridge and Devil Den sites. Additional analyses were performed for OTU datasets of macrofungal belonging to the Ascomycota and Basidiomycota separately. Richness values of the macrofungal functions were also characterized in both sites by splitting saprotrophs into three groups, leafy, woody and mixed substrates. The previous results were statistically evaluated using analysis of variance (ANOVA) and Tukey's test for comparisons of all result pairs of both investigated study areas. The value of 0.05 was selected as the significant level for all statistical tests. These results were visualized using JMP software (SAS Institute Inc., Cary, North Carolina, USA). Differences in fungal taxonomic diversity between two distinctive approaches, traditional (inventory method) and mycobiome techniques were also performed. The non-metric multidimensional scaling (NMDS) analyses were assessed to compare the

dissimilarity in the datasets and to visualize the ordination patterns of the fungal taxonomic and functional diversities across the twenty investigated plots. These multivariate analyses were conducted separately based on Bray-Curtis dissimilarly index in PC-Ord™ software version 6.1.2 (McCune and Grace 2002). In brief, the primarily binary matrixes of OTUs were generated first with all represented OTUs, including macrofungi and fungi and then with only macrofungal taxa. The second matrix comprised the chemical data of leaf litter with macrofungal phyla and unidentified fungi for both compared plot sets as well as the categorial values of the two sites per taxonomic orders. Additional second matrices were prepared to run other NMDS analyses containing, first, the richness of fungal functional guilds divided based on their taxonomic phyla groups (e.g., ascomycete saprotrophs, basidiomycete mycorrhizas, basidiomycetes parasites. etc.). Then, NMDS analysis was performed for all functional subunits participating in the general macrofungal functions on leaf litter (e.g. litter or wood saprotrophs, plant pathogens, ectomycorrhizas). Variables with $| r | \geq 0.5$ values for either NMDS axis were considered essential for characterizing changes in fungal assemblage structure and overlaid on the ordinations as strength vectors and directions (Roger et al., 2009; Geml et al., 2014). The significance of the relationships between previous variables and the ordination axes were verified based on the Pearson's correlation coefficients. The multi-response permutation procedures (MRPP) were tested with PC-Ord software based on Bray-Curtis distances for statistically detected differences in the multivariate data across sampled plots in two forests according to *P*-values and an *A*- statistics (effect size) (McCune and Grace, 2002). Subsequently, Mantel tests were performed using PC-Ord to determine whether community structure is correlated with geographic distance and/or with the other variables.

Comparisons of the number of OTUs per taxonomic species and visualizations of the

distributions of all identified taxa of litter associated macrofungi across the investigated sites were carried out first by one-way cluster analysis, using Euclidean distance and Ward's linkage method. Later, two-way cluster analysis was conducted for the OTU-richness values of macrofungal genera across site plots using Bray-Curtis distances and flexible beta (ß= -0.25) as the linkage method in PC-Ord (McCune and Grace, 2002). Finally, Indicator Species Analysis (Dufrêne and Legendre, 1997) was conducted to detect any preferences of individual OTUs for a particular forest site. The significant differences of the observed indicator values were statistically compared through Monte Carlo test with permutations (4999) using PC-Ord as well ($P < 0.05$).

4.4. Results

4.4.1 Overview of sequence taxonomical identifications and community structures

The rarefaction curves of all plot samples reached a plateau, suggesting an equally deep OTU recovery and that almost all the fungal species in both sites were sequenced (Fig. 4.3S). Leaf litter samples hid a high diversity of associated fungi in both sampling sites based on the metagenomic sequences. After removal of low quality, singletons, chimeres, nonfungal sequences, and those with less than four reads, a total of 155,091 sequence reads were retained and classified into a total of 415 OTUs belonging to three macrofungal phyla with many of unidentified fungi in both investigated sites. Of these, 136,801 sequence reads were taxonomically classified into 349 macrofungal OTUs, mostly to ascomycete and basidiomycete phyla and minorly to the phylum Glomeromycota phylum in both datasets of study sites (Figs. 4.1 and 4.2). These sequences were deposited in GenBank under the Accession Number MT162736-MT162974 and MT228837-MT228844.

The Devil's Den (DD) site had the highest number of sequences for the identified taxa compared with the Pea Ridge (PR) site. The total number of the identified macrofungal sequences were mainly assigned to 130 OTUs of Ascomycota and 118 OTUs of Basidiomycota for the DD plots compared to 107 OTUs of Ascomycota and 109 OTUs of Basidiomycota for the PR plots (Fig. 4.2) with substantial differences in OTU sequence reads and numbers related to different orders (Figs. 4.3 and 4.4). These macrofungal OTUs represented 15 classes (Fig. 4.4). Of these, the higher macrofungal proportions were noticeably assigned to the Leotiomycetes with 37% and the 31% and the Agaricomycetes with 41% and 43% of the total OTU classes for DD and PR plots respectively (Figs. 4.5 and 4.6). The macrofungal OTUs represented at least 31 orders, 94 families referred to 97 identified genera distributed differentially across both site plots. Many other unidentified sequences that could not be matched beyond the level of genus with databases were also detected. It seems likely that the majority of these are either relatively rare taxa yet to be sequenced or taxa new to science.

At the ascomycete order level, there were 13 macrofungal orders in addition to *incertae sedis* and unidentified taxa in both investigated sites with substantial differences in their proportional richness contributions to the overall macrofungal community assemblages. The majority of the OTU richness was made up by the order Helotiales for 62% and 56% of the OTU Ascomycota in DD and PR plots respectively (Fig. 4.5). Conversely, there were 17 basidiomycete orders with unassigned, and unidentified orders were detected in both compared sites. Among them, the order Agaricales had the highest OTU richness for 35% and 39% of the OTU Basidiomycota in DD and PR plots respectively (Fig. 4.6), whereas members of the Tremellales had the highest sequence abundance, but with lower OTUs compared to previous orders (Figs 4.3B, C, and 4.6). There were only 16 % genus and 1.2% identified species overlaps between the compared

macrofungal results from the next-generation sequencing and inventory methods. These colonized various microhabitats (LL, WD and ML) with diverse ecological functions as genera and mostly saprotrophs as species.

4.4.2 Macrofungal taxonomic and functional diversity for leaf litter fungi

There were no significant differences between total richness, diversity indices (Shannon and Simpson), and evenness values of the overall species assemblages of the DD and PR sites (Fig. 4.7). The taxonomic diversity patterns described above were apparently driven by the members of the Ascomycota and Basidiomycota in both dataset sites, although no statistically significant variations in all four previous diversity analyses were detected for macrofungal species in both phyla when being measured separately (Figs. 4.8 and 4.9). The visual illustration of the 97 clustered genera indicated that the ascomycete genera of *Orbilia*, *Hymenoscyphus*, *Rutstroemia*, *Leptosphaeria*, and *Schizoxylon* and the basidiomycete genera of *Mycena*, *Tremella*, *Trametes Sebacina*, *Laccaria*, and *Athelia* had a wide distribution across the 20 plots (Fig. 4.11). Functionally, diverse functional guilds exemplified by the various macrofungal species inhabited leaf litter. For those identified to the genus or species level, 55,106 sequence reads (176 OTUs), 47.55% of the total classified sequences were functionally resolved based on the currently available knowledge. On that microhabitat, the saprotrophs of leaf litter, woody materials and different dead plant materials from multisubstrates in forest systems had higher OTU richness than for other detected functional groups without significant differences between them in both study sites (Fig. 4.10). The fungal richness of mycorrhizas and species with *incetae sedis* functions were most characteristic for DD plots and statically distinctive between both compared sites. In addition, the population assemblage of mycoparasites was obviously lesser in DD plots

while plant pathogens and taxa with bifunctions did not differ appreciably between the two types of study sites.

4.4.3 Comparing taxonomic and functional community assemblages of macrofungi across sampling sites

4.4.3.1 Correlation between site variables and compositional taxonomic variability

The one-way cluster analysis confirmed the NMDS-ordinations and statistical results in which the macrofungal assemblages for each site strongly clustered (Fig. 4.12). The NMDS-ordinations of macrofungal binary data also revealed a strong structuring of the fungal litter assemblages across sites apparently driven by forest location and other factors with a two-dimensional solution and the final stress of 12, 67695 resulted and final instability of 0, with coefficient correlations between axes: axis 1: $r^2 = 0.786$, axis 2: $r^2 = 0.094$. The fungal assemblages of the DD site cluster were more scattered than those of the PR site cluster without overlapping (Fig. 4.13). This was statistically confirmed by MRPP conclusions ($A = 0.1347$, $P = 0.0000041$). Taxonomically, the differences in the composition of all fungal assemblages (macrofungal taxa and unidentified fungi) amongst investigated plots were strongly and significantly correlated with both the investigated forest locations and available nutrient concentrations of leaf litter. The most of leaf litter chemical contents were highest significantly in the DD plots and obviously characterized and plotted on NMDS axes, except of Co. ($r = 0.470$) which seemed to relate with the first axis (PR plots) based on a joint biplot of OTU data. The litter macronutrients of Ca ($r = -0.895$), S ($r = -804$), P ($r = -717$), and N ($r = -0.705$) correlated strongly with the second axis. These chemical concentrations were influenced or correlated positively with the total Basidiomycete richness but negatively with the PR community structure when fitting all fungal community assemblages on NMDS axes with the final stress 12, 2303 and correlation

coefficients: axis 1: $r^2 = 0.822$, axis 2: $r^2 = 0.068$ (Fig 4.14 and Table 4.1). MARPP analyses indicated that compositional turnover was significant amongst investigated sites ($A = 0.1388$, $P = 0.0000041$). Mantel tests showed that chemical data of the leaf litter variables were weakly correlated with the fungal community structure, though still significant ($r = 0.2266$, $P = 0.005296$).

The identical patterns of species assemblages were displayed on the NMDS ordinations at the taxonomic order level in the plots being compared (Fig. 4.15). However, there were substantial differences among the taxonomic orders of macrofungi in both sites. The order value associations with NMDS axis 1 of the Polyporales ($r = 0.714$), Auriculariales ($r = 0.671$), and Xylariales ($r = 0.560$) were negative while that of unidentified Leotiomycetes ($r = 0.797$), Orbiliales ($r = 0.638$), Hymenochaetales ($r = 0.561$) were positive. In addition, Exobasidiales ($r = 0.690$) and Diaporthales ($r = 0.616$) showed a negative and positive correlation, respectively, with the NMDS axis 2 whereas Taphrinales, Agaricales and Atheliales correlated negatively on both NMDS axes (Fig. 4.15 and Table 4.1). These results were statically confirmed by MRPP results ($A = 0.1346$, $P = 0.0000041$).

4.4.3.2 Correlation between investigated sites and functional guilds

The NMDS plots of datasets corresponding to the general and specific functional groups of macrofungi were strongly structured on the NMDS axes and returned a two-dimensional ordination with the final stress of 12,4845 (Fig. 4.16). The OTUs of Ascomycete functions with *incertae sedis*, undefined functional guilds, ($r = 0.670$), basidiomycete mycorrhizas ($r = 0.656$), basidiomycete saprotrophs ($r = 0.558$) had higher richness in the DD plots than in PR plots and negative correlations with the first axis for the NMDS ordinations of the functional groups based on taxa. In contrast, the OTUs richness of Ascomycete bifunctions ($r = 0.594$), and

basidiomycete parasites (r = 0. 497) were positive correlated with the first axis (Fig. 4.16A). In addition to basidiomycetes richness (r = 0. 582) and total richness (r = 0. 465), the ectomycorrhizas (r = 0.643) and *incertae sedis* (r = 0. 671) were negatively associated with the PR sites when splitting functional groups based on their substrates such as leaf litter and wood saprotrophs (Fig. 4.16B). The MARPP analyses confirmed the significant differences among sties in shaping functional structures of fungal assemblies (A = 0.1351, P = 0.0000041).

The two-way cluster analysis of genera showed that the previous clustered evidently driven by the site-specific genera when fitting the genera from both sites on the NMDS-ordinations and confirmed by the statistical results (Fig. 4.11). Indicator species analyses of the assigned OTUs revealed that *Marchandiobasidium* sp., *Trametes* spp., *Orbilia* sp, *Trichophaea flavobrunnea*, *Lycoperdon pratense*, *Hymenoscyphus immutabilis* were significantly reliable for PR plots with *Schizoxylon berkeleyanum* as the perfect indication value. In contrast, *Pestalotiopsis* sp., *Sirobasidium* sp.*, Uncobasidium* sp., *Russula vinacea*, *Exidia* spp., *Calvatia cyathiformis* were considerably species indicators for DD plots. Other unidentified taxa in both sites were also recognized as the OTU species indicators ($P \leq 0.05$, Table 4.2 and Fig. 4.11).

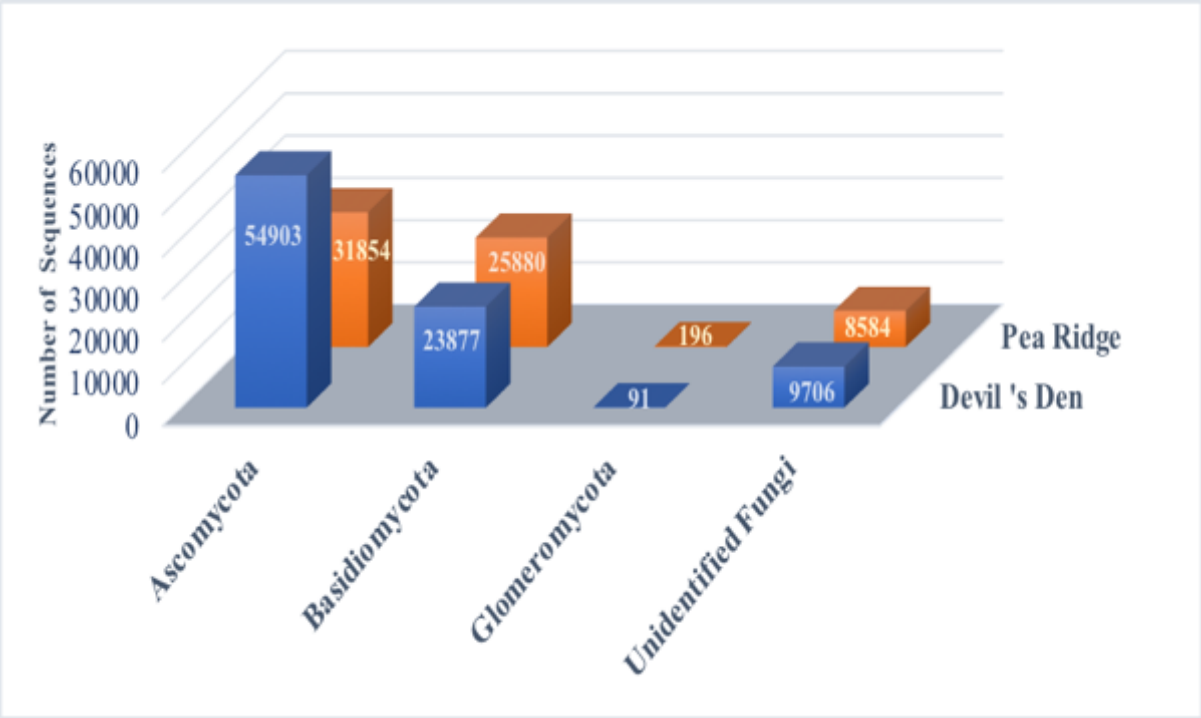

Fig. 4.1. Characterizing and comparing the phylum compositions of leaf litter fungi from plots in Devil's Den and Pea Ridge forests based on fungal OTU read abundance. Glomeromycota was included in this figure due to being identified the sequences into family (Glomeraceae) that are known to have genera that can produce macroscopic fruiting bodies.

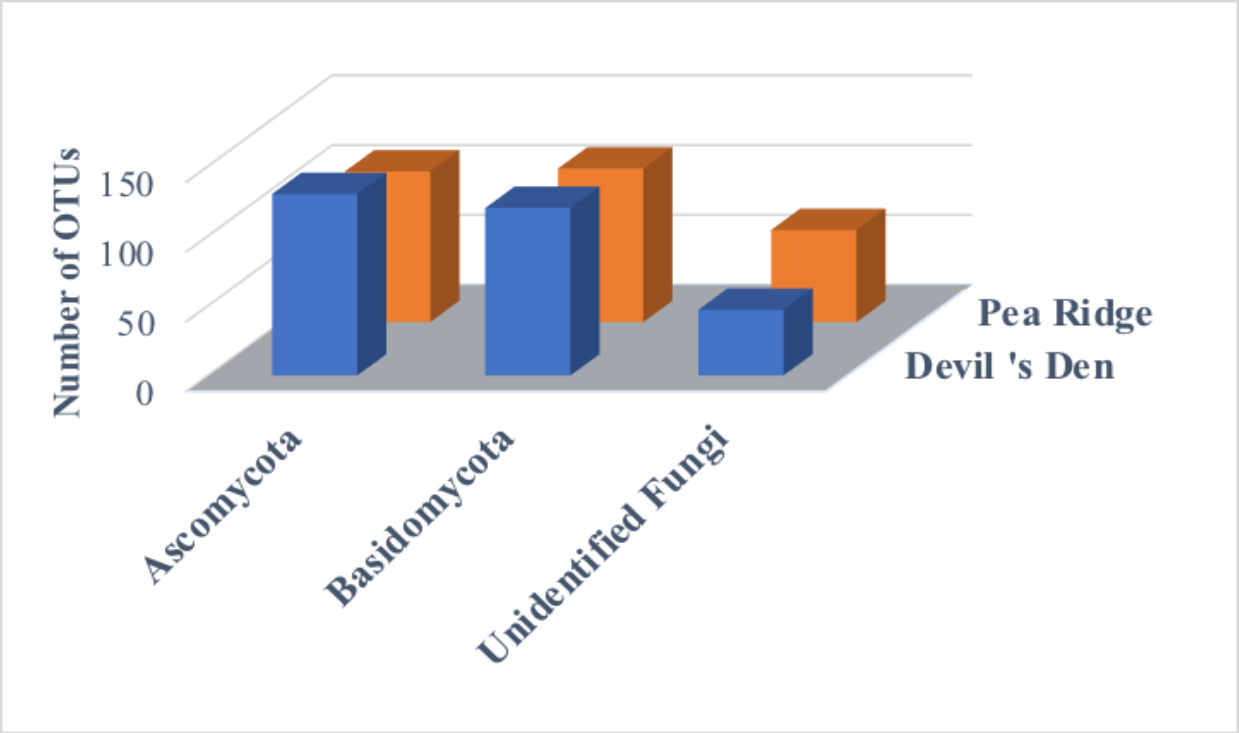

Fig. 4.2. The number of OTU phyla of macrofungi from plots in Devil's Den and Pea Ridge forests.

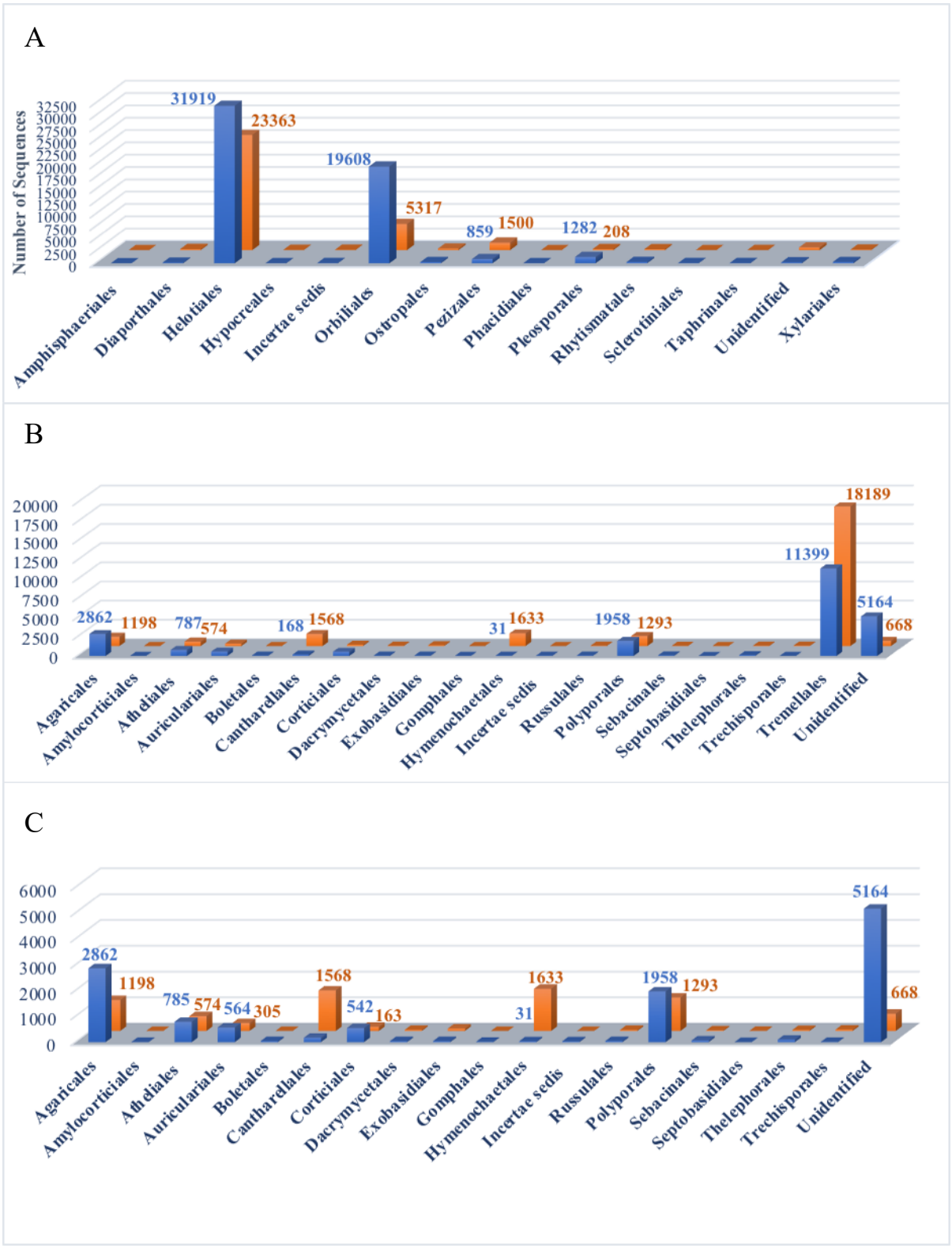

Fig.4.3. Overview of leaf litter fungi in Devil's Den (blue bars) and Pea Ridge (orange bars) forests based on the number of OTU sequences (A) ascomycete orders (B) basidiomycete orders and (C) basidiomycete orders after excluded members of Tremellales.

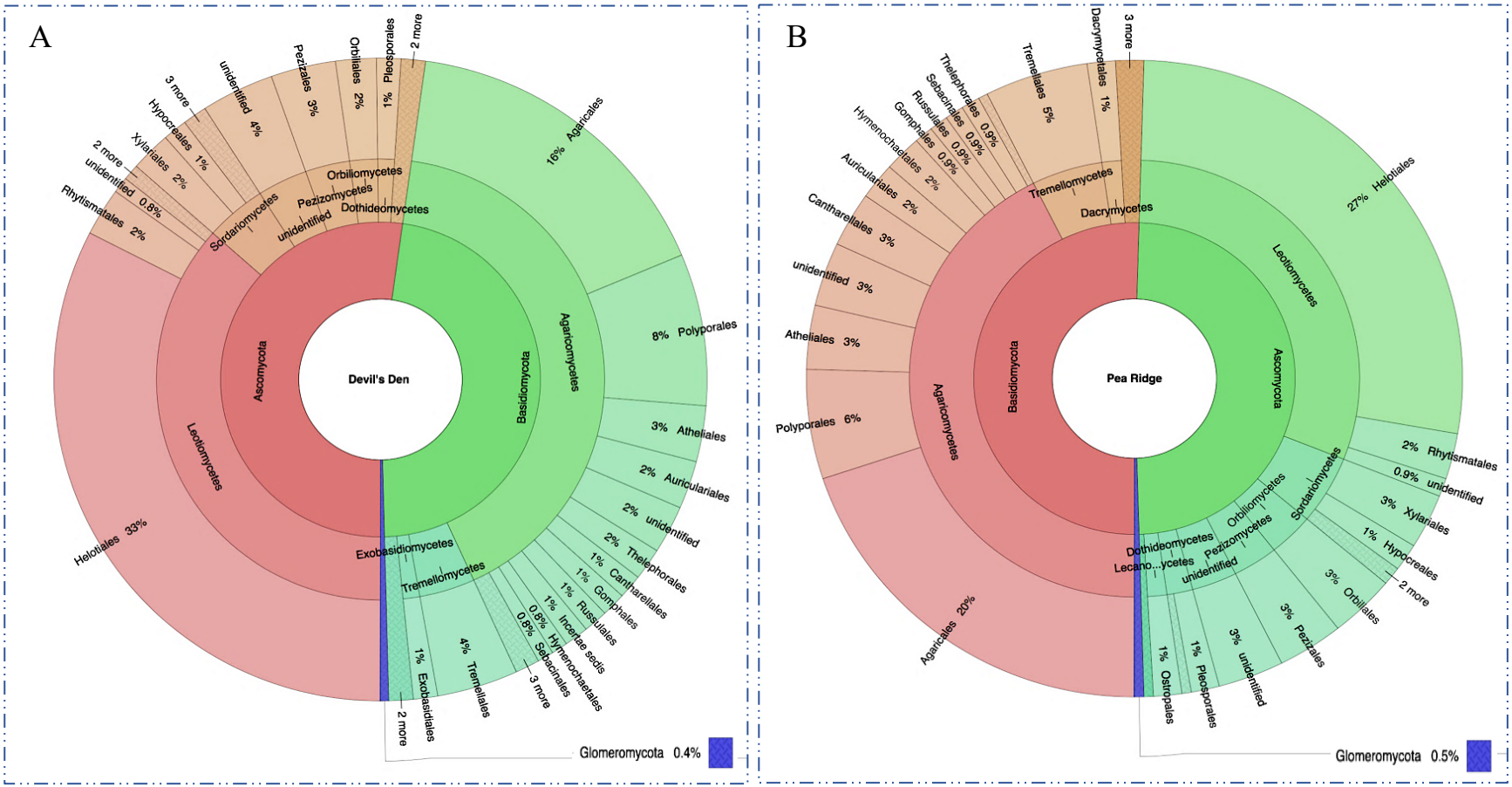

Fig. 4.4. Krona plot diagrams that characterize and compare taxonomically the community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of the DD site (A) and PR site (B). The largest groups are shown in wheel varieties of red color levels and second largest groups in light green color levels, including unidentified taxa in both. In the Devil's Den site, Basidomycota encompasses 47% of the total macrofungal OTU richness, while Ascomycota accounts for 52% of the total macrofungal OTU richness. In the Pea Ridge site, Basidiomycota comprises 50% of the total OTU richness, while Ascomycota accounts for 49% of the total OTU richness.

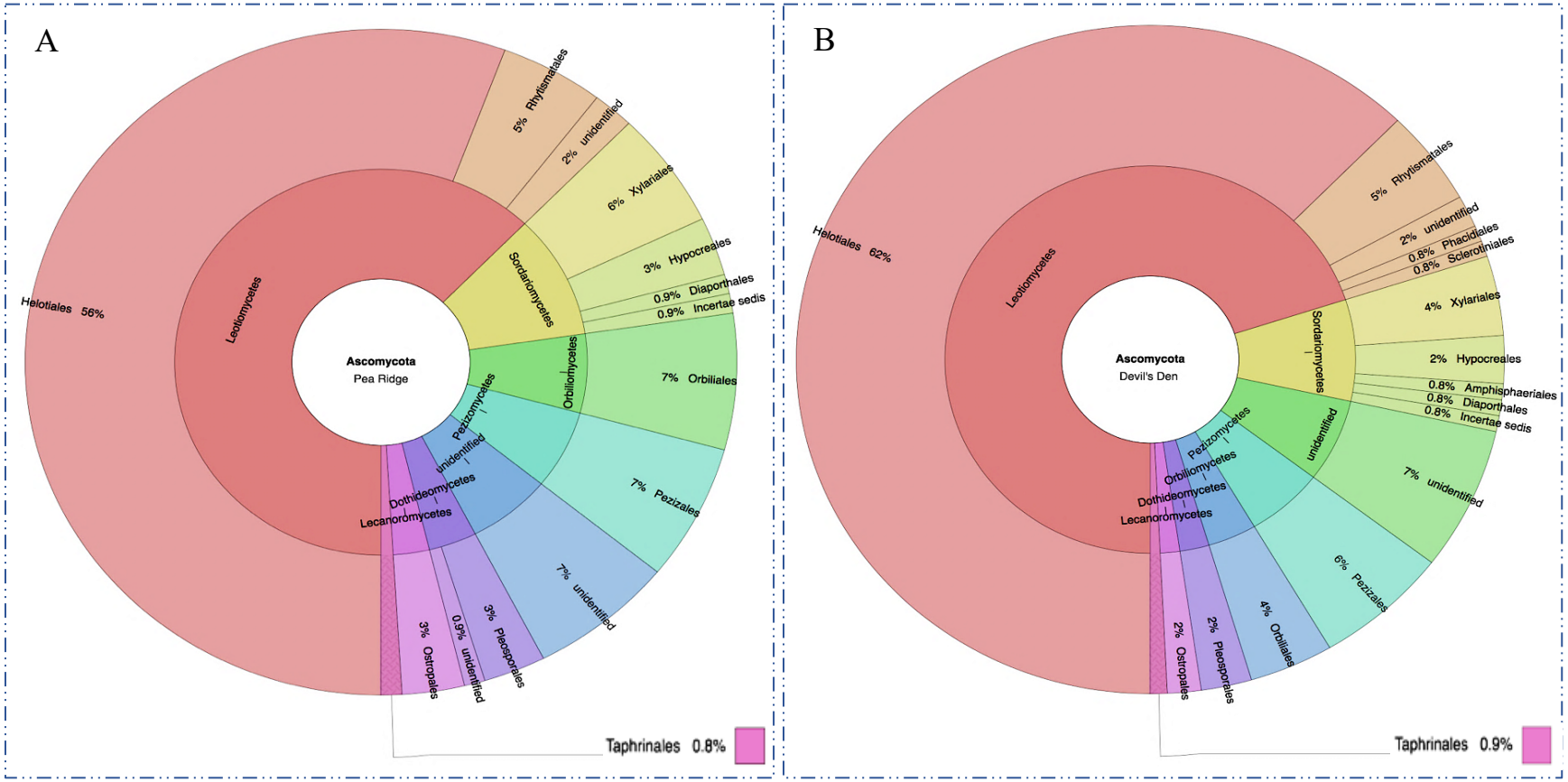

Fig. 4. 5. Krona plot diagrams that characterize and compare taxonomically the ascomycete community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of DD plots (A) and PR plots (B). Wheel varieties of the distinct color levels are shown where the largest groups are in varieties of the red color levels with other groups in distinct color levels. The majority of the OTU richness is made up by the order Helotiales for 62% and 56% of the ascomycete OTU in DD and PR plots, respectively. There is 5% and 6% of the Agaricales OTUs unidentified in DD and PR sites, respectively.

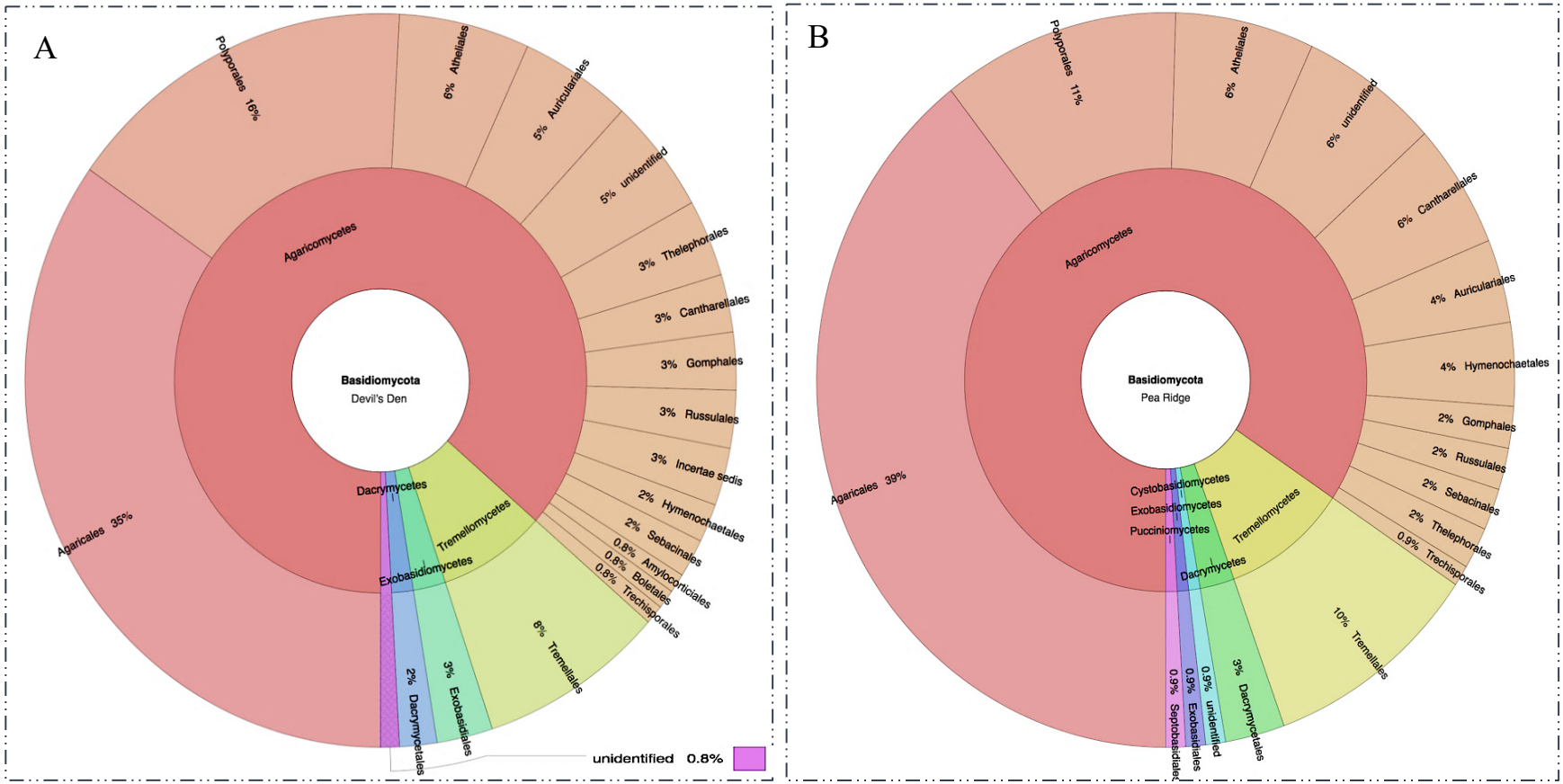

Fig. 4.6. Krona plot diagrams that characterize and compare taxonomically the basidiomycete community assemblages at the level of macrofungal orders of OTU richness in/on the leaf litter samples of DD plots (A) and PR plots (B). Wheel varieties of the distinct color levels are shown where the largest groups are in varieties of the red color levels with other groups in distinct color levels. The majority of the OTU richness is made up by the order Helotiales for 62% and 56% of the ascomycete OTUs in DD and PR sites, respectively. There is 7% of the OTU Ascomycota are unidentified to an order in both investigated sites.

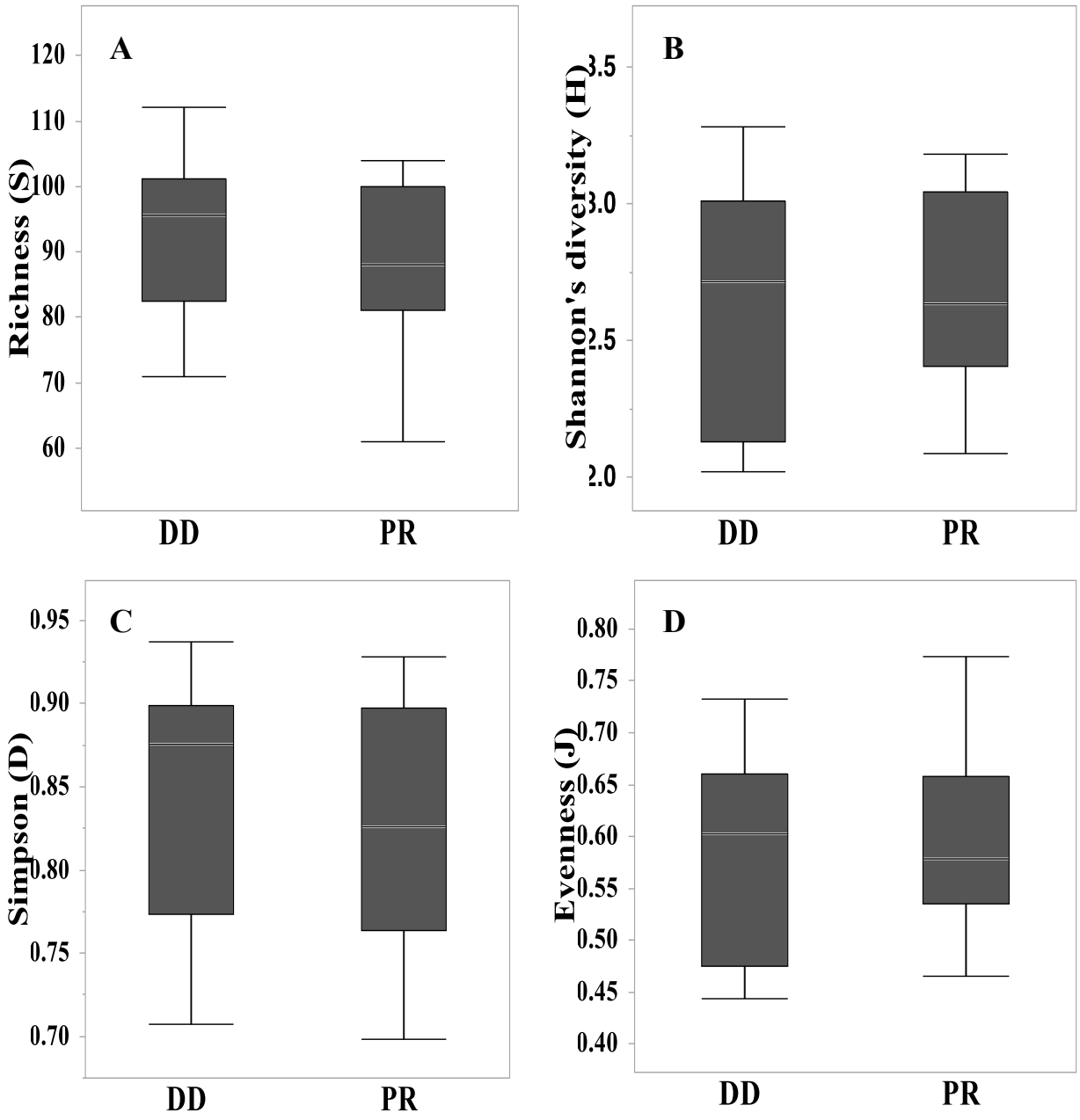

Fig. 4.7. Total fungal richness (A) and diversity analyses (B-D) of all taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites. There are not significant differences between the compared taxa based on the pairwise Tukey's test.

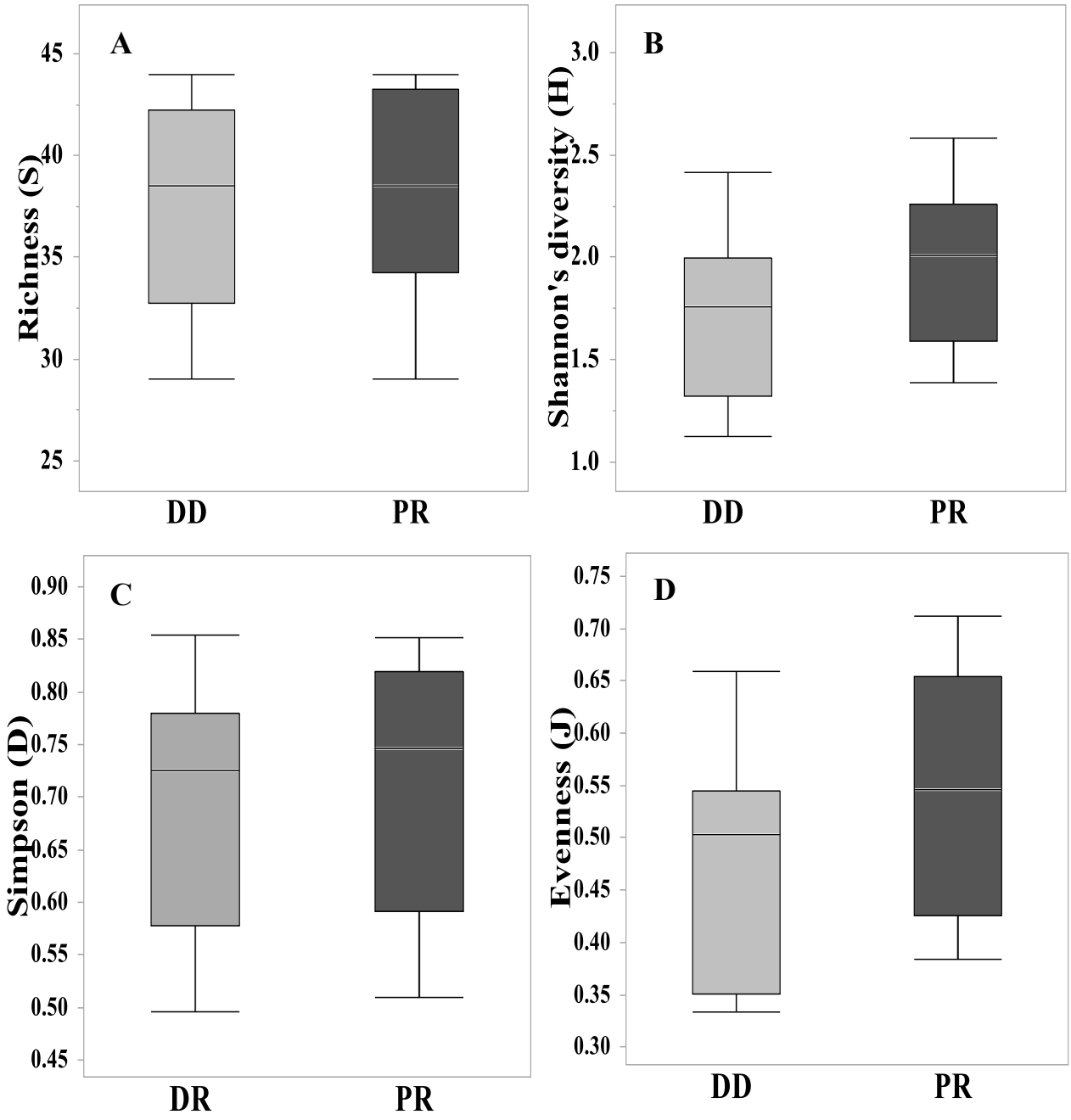

Fig. 4.8. Total ascomycete richness (A) and diversity analyses (B-D) of taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites. Means of analyses shown no significant differences between the compared taxa based on use of the pairwise using Tukey's test.

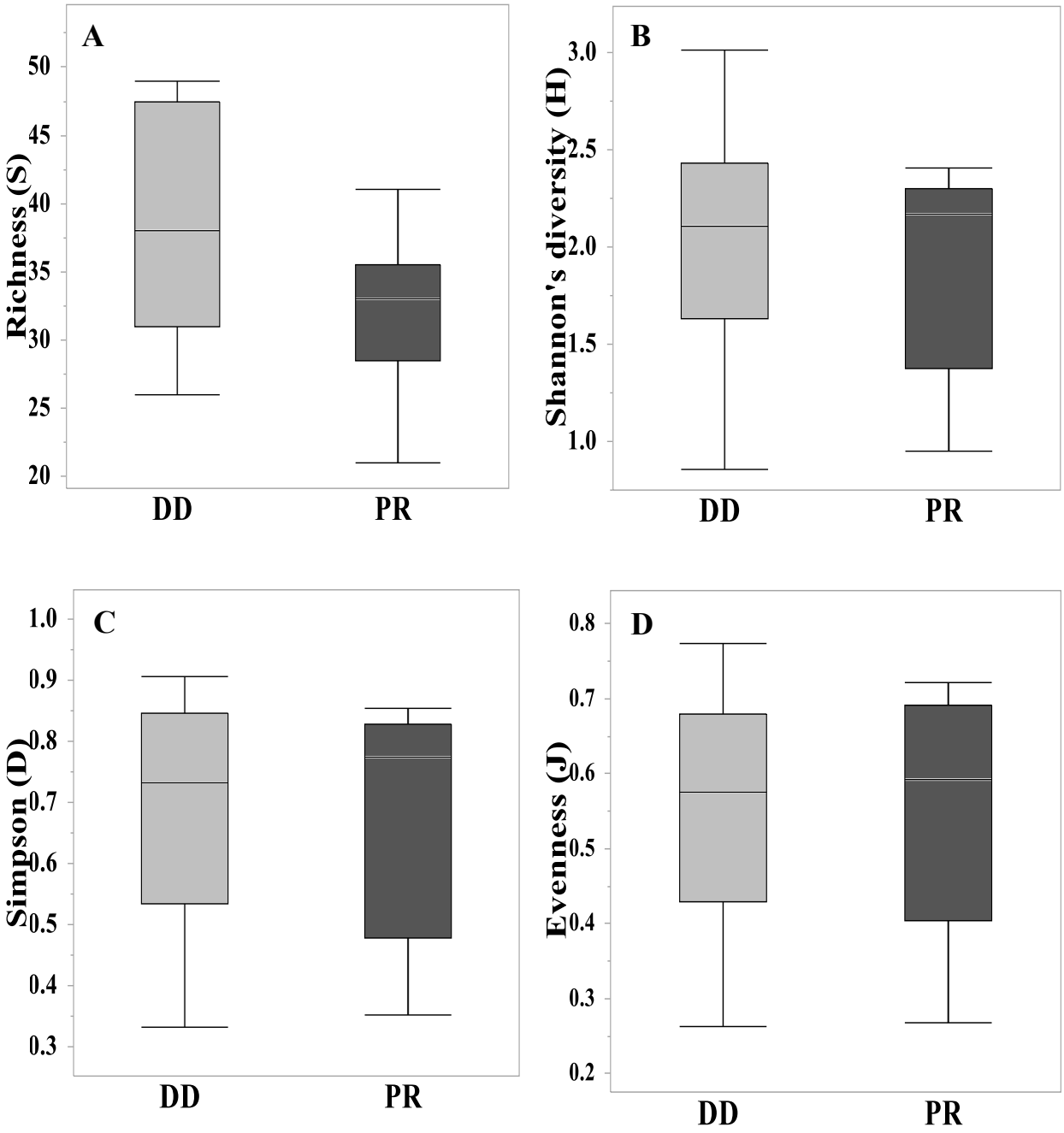

Fig. 4.9. Total basidiomycete richness and diversity analyses of taxonomic community assemblages for Devil's Den (DD) and Pea Ridge (PR) sites. Means of analyses shown no significant differences between the compared taxa based on the pairwise using Tukey's test.

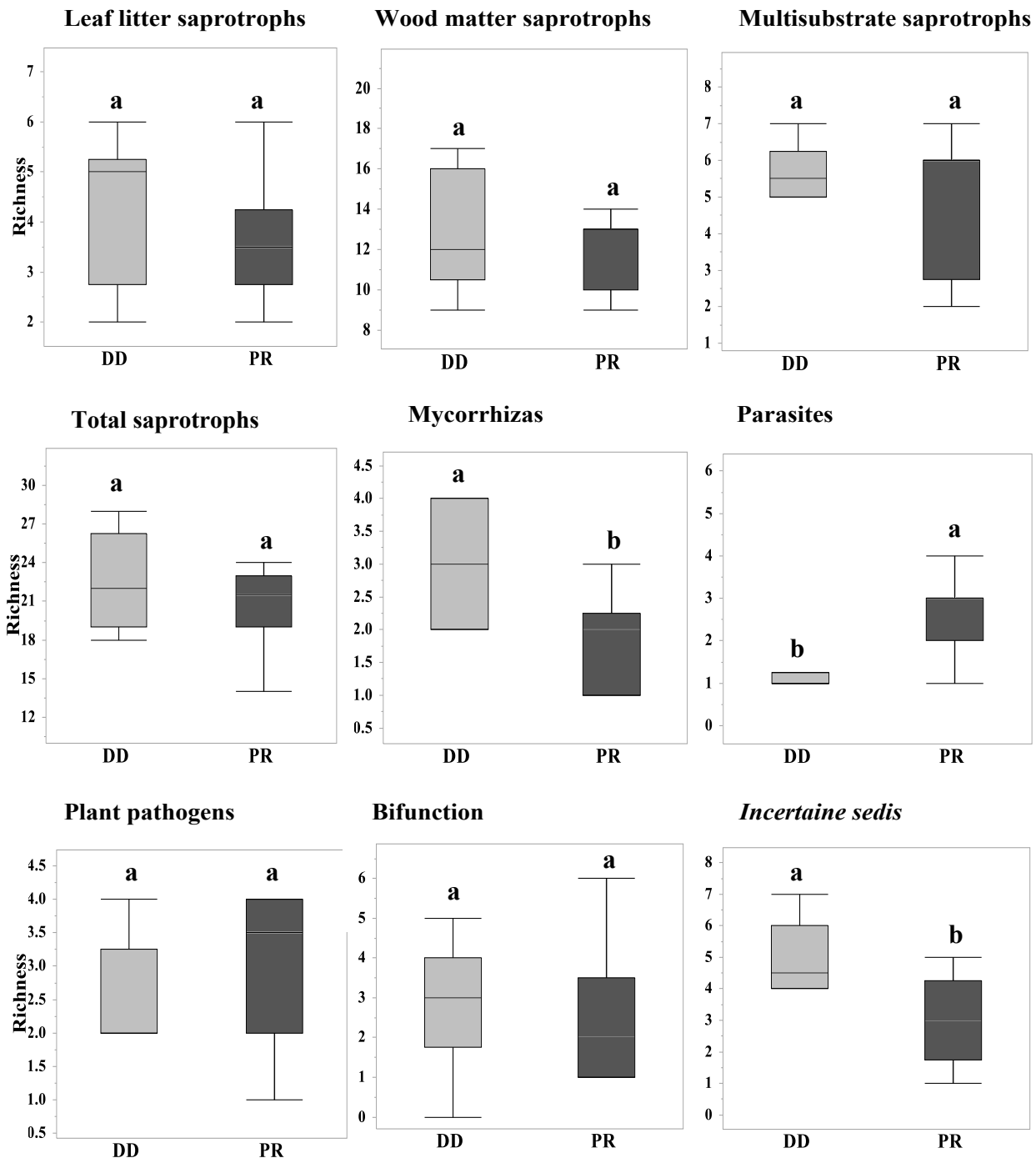

Fig. 4.10. Characterizing and comparing the OTU richness of saprotrophs on the different substrates and the total OTU richness of saprotrophs with other functional guilds of litter-associated fungi. The different letters indicate significant differences in the pairwise Tukey's test. Bifunctions refer to species with saprotrophs and/ or ectomycorrhizas, plant pathogens, parasites. *Incertaine sedis* refer to a species or genera for which their common function has yet to be determined.

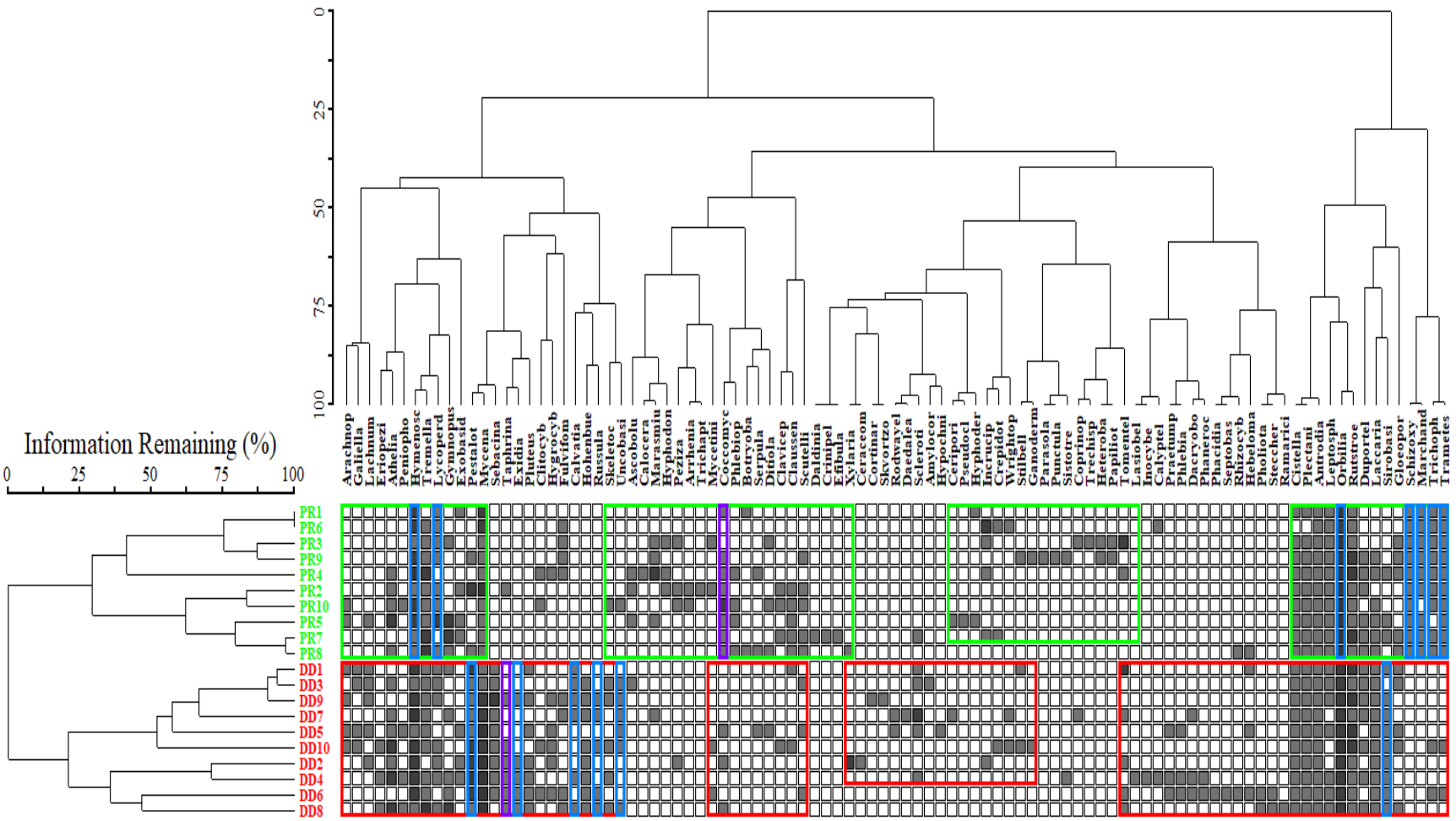

Fig. 4.11. Leaf litter macrofungal community assemblage of 97 genera clustered on the horizontal matrix axis and distributed across 20 investigated plots for Devil's Den (red color) and Pea Ridge (green color) sites, grouped on the vertical axis using two-way cluster analysis. The blue color represents genera with indicator species for both sites, with *P*-values ≤ 0.05, while purple indicates the genera of indicator species with a *P*-value = 0.066. See Table 4 S for complete genus names.

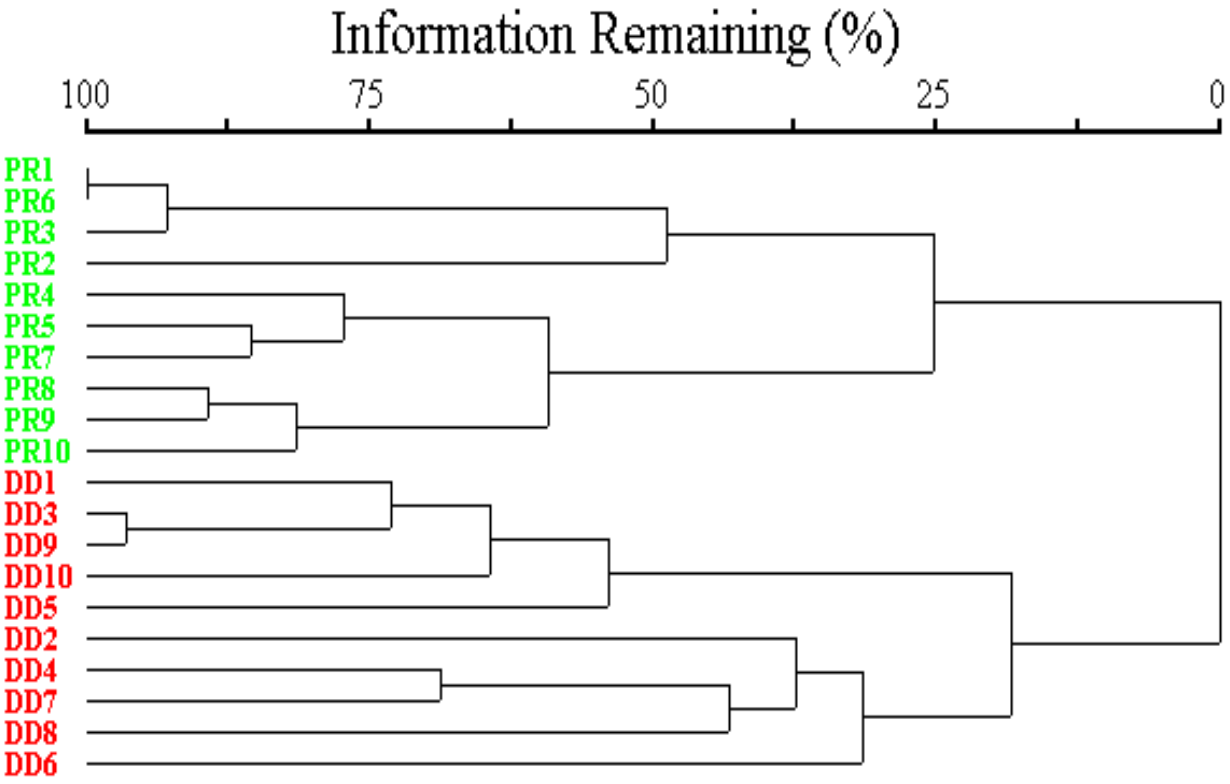

Fig. 4. 12. Cluster analysis of leaf litter-associated fungi across the sampling sites of both Devil's Den (DD) and Pea Ridge (PR) plots using Euclidean distance and Ward's linkage method based on number of operation taxonomic unites (OTUs) per a taxonomic group.

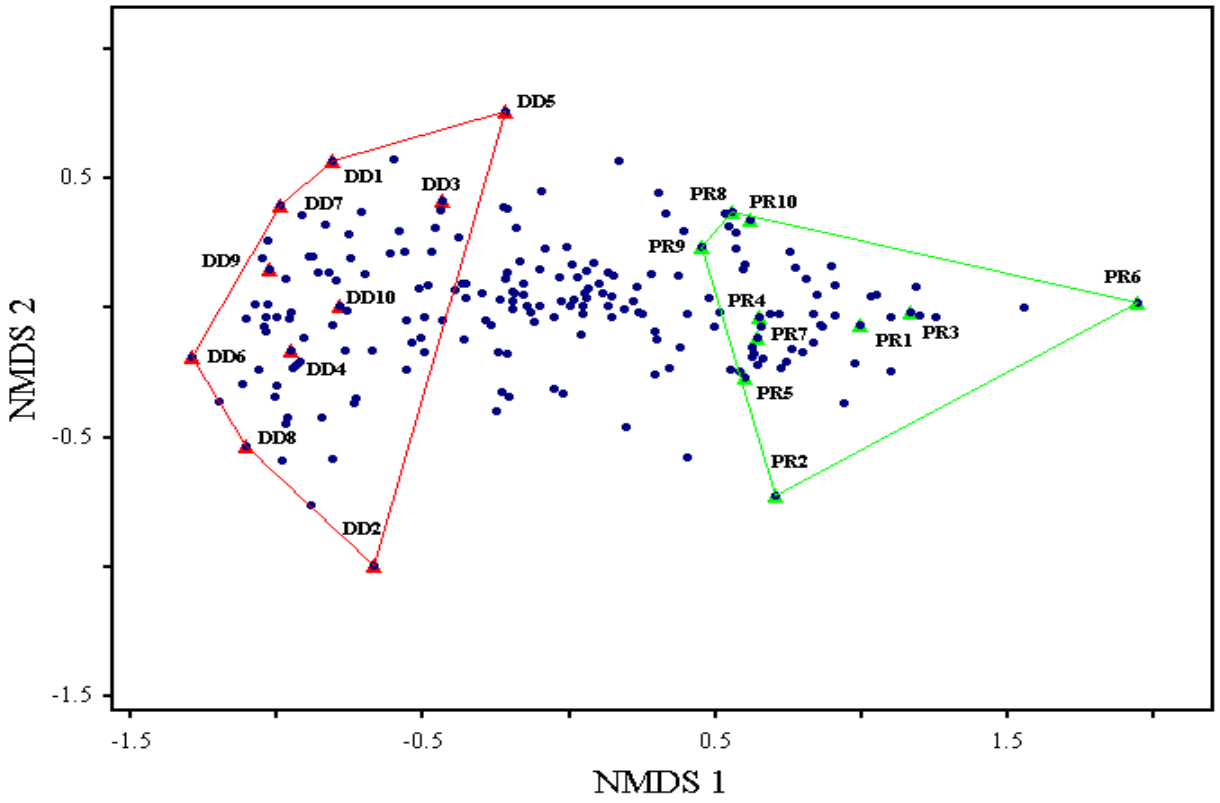

Fig. 4.13. Non-metric multidimensional scaling (NMDS) ordination plots of leaf litter-associated fungi across the sampling sites of the both Devil's Den (DD) and Pea Ridge (PR) forests based on the number of the operation taxonomic units (OTUs) per taxonomic group. The placements of blue variables presented the fungal assemblages distributed within and between both investigated sites.

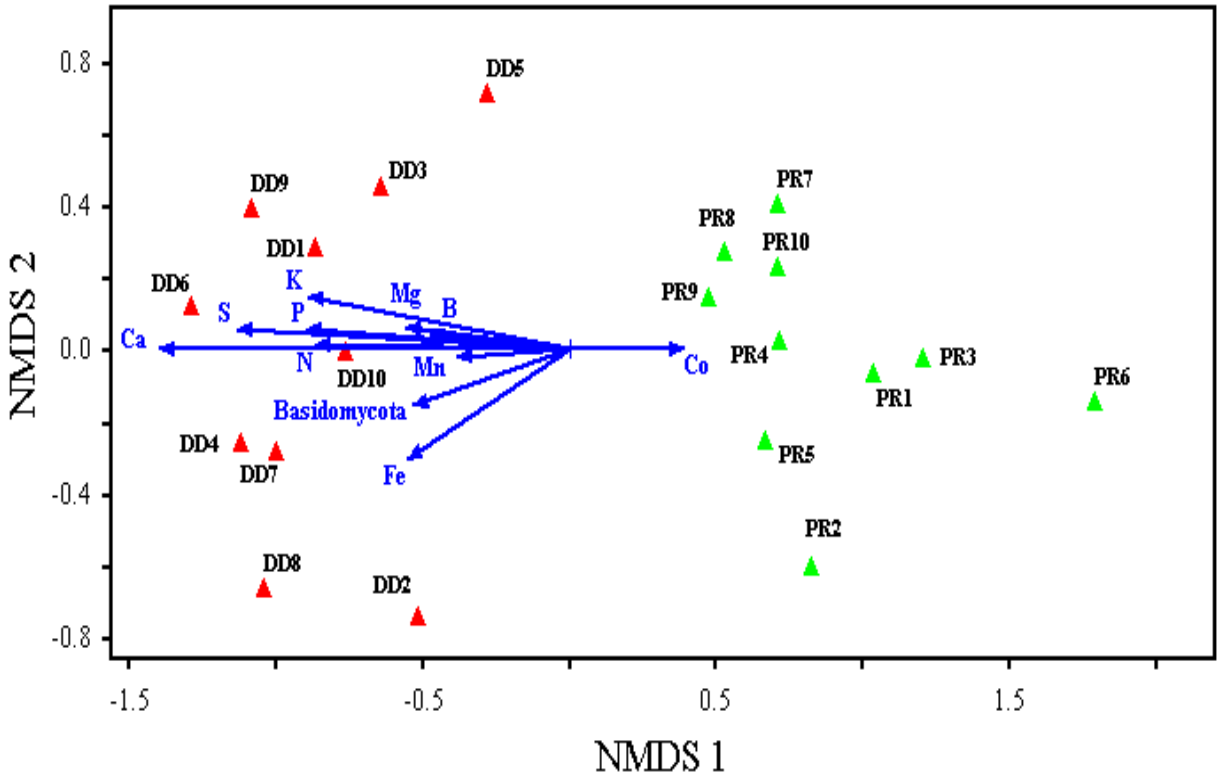

Fig. 4.14. Non-metric multidimensional scaling (NMDS) ordination plots of the fungal and leaf chemical characteristic variables of leaf litter-associated fungi across the sampling sites for the both Devil's Den (DD) and Pea Ridge (PR) forests based on the binary datasets. All variables in this figure were strongly related, either positively or negatively, on at least one of the two axes. Variables with weakly corelated were not illustrated on the NMDS axes.

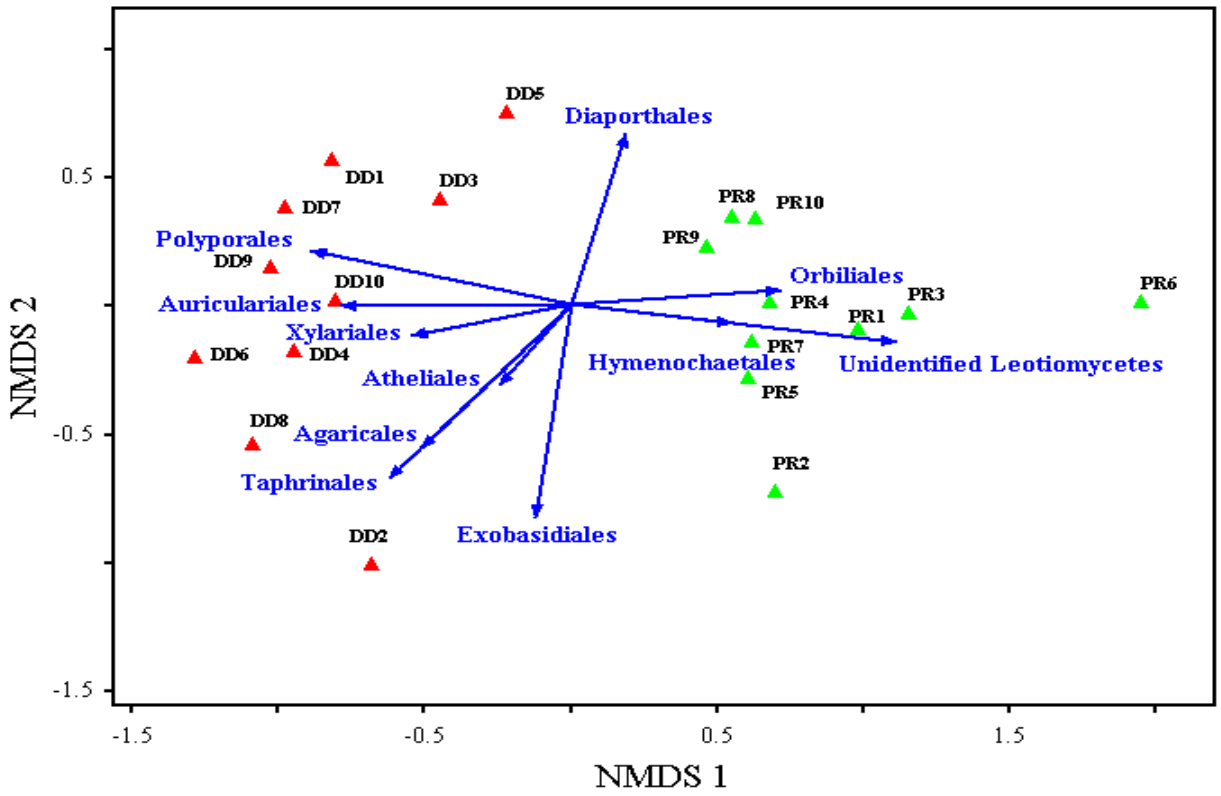

Fig. 4.15. Non-metric multidimensional scaling (NMDS) ordination plots of the plot site and order assemblage variables of the leaf litter-associated fungi overlaid as vectors on ordinate axes based on the binary datasets of Devil's Den (DD) and Pea Ridge (PR) plots. Vector direction and length designate strengths of correlations within the ordination. Variables with weak correlations were not illustrated on the NMDS axes.

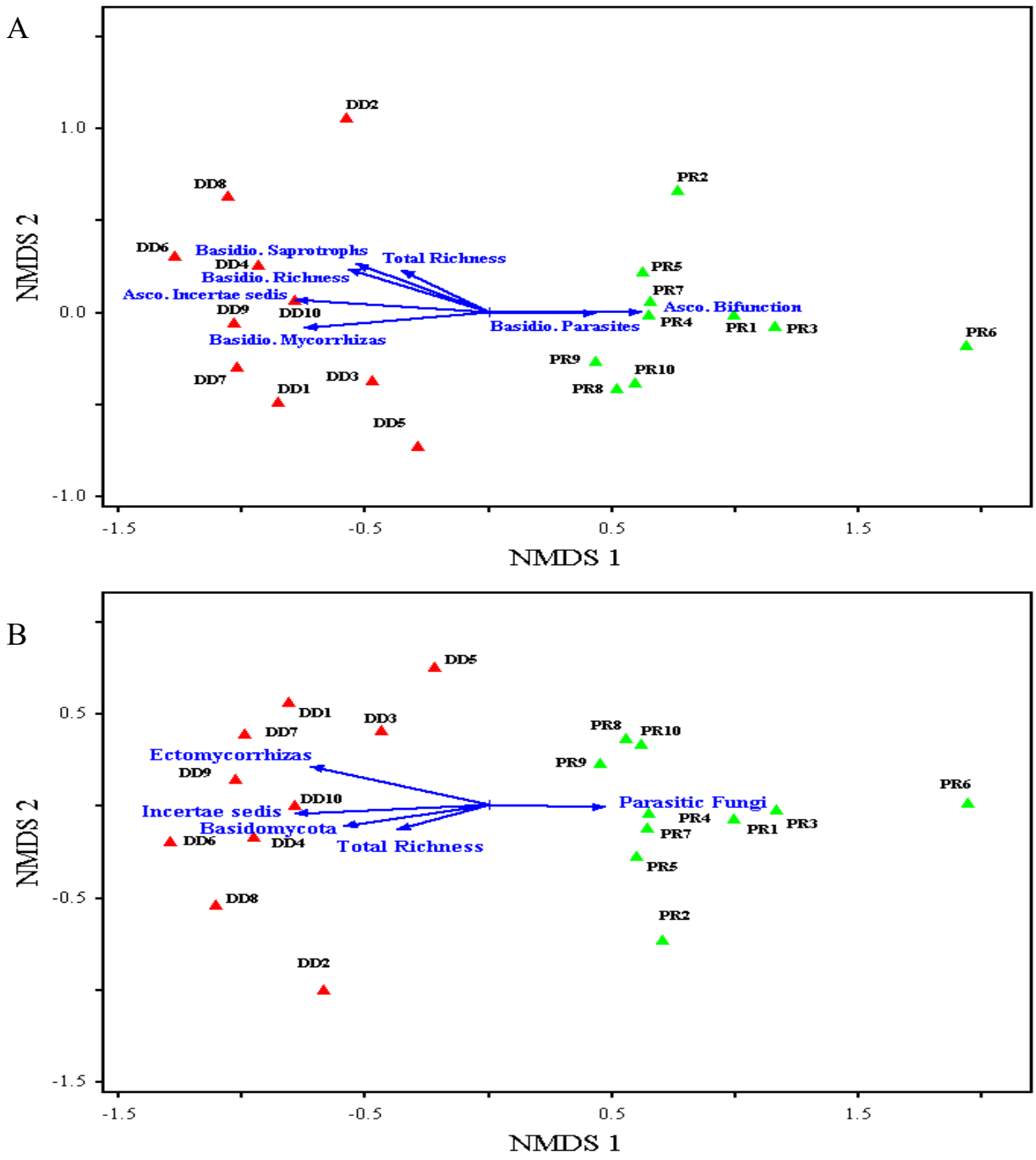

Fig. 4.16. Structuring functional variables of (A) all phylum functional guilds after gathering all saprotrophs with respecting to their substrates (B) all species guilds with respecting to their higher rank taxa groups and dividing saprotrophs into portions based on substrates of macrofungal OTU richness. These variables overlaid as vectors on NMDS ordinations with environmental variables of investigated plots of Devil's Den (DD) and Pea Ridge (PR) sites. Vector directions and length designate strengths of correlations within the ordinations. Vector directions and length designate strengths of correlations within the ordinations.

Table 4.1. The results of characterizing and comparing fungal variables of Pea Ridge (PR) and Devil's Den (DD) plots (n=20). These values revealed by correlation coefficients between fungal orders recorded (A) and functional guild variables (B) with considering general guild phyla first and without considering guild taxa, but considering their substrates using several separated nonmetric multidimensional scalings (NMDS)s. Negative and positive values indicate strong correlations with ordination axes at $|r| \geq 0.5$ and are plotted in the NMDS figures. Weak correlations with NMDS axes are not included in the table.

Variables	1st axis correlation	2nd axis correlation	Variables	1st axis correlation	2nd axis correlation
A- Leaf litter contents and fungal phyla			**B- macrofungal orders**		
B (ppm)	-0.567	0.181	Agaricales	-0.541	-0.566
Ca (%)	-0.895	-0.002	Atheliales	-0.373	-0.422
Co (ppm)	0.470	-0.011	Auriculariales	-0.671	-0.063
Fe (ppm)	-0.562	-0.419	Exobasidiales	-0.264	-0.690
K (%)	-0.713	0.286	Diaporthales	0.326	0.616
Mg (%)	-0.522	0.155	Hymenochaetales	0.561	-0.206
Mn (ppm)	-0.468	-0.11	Orbiliales	0.638	0.182
N (%)	-0.705	0.083	Polyporales	-0.714	0.344
S (%)	-0.804	0.173	Taphrinales	-0.595	-0.622
P (%)	-0.717	0.174	Xylariales	-0.560	-0.266
Total Basidiomycota richness	-0.555	-0.301	Unidentified Leotiomycetes	0.797	-0.288

Table 4.1 (Cont.)

Variables	1st axis correlation	2nd axis correlation	Variables	1st axis correlation	2nd axis correlation
C- Functional guild richness assemblages					
1- Phylum functional guilds			**2- Species functional guilds**		
Ascomycete bifunction	0.594	-0.035	Ectomycorrhizas	-0.643	0.344
Ascomycete *incertae sedis guilds*	-0.670	0.197	*Incertae sedis* guilds	-0.671	-0.171
Basidiomycete mycorrhizas	-0.656	-0.218	Parasites	-0.407	0.076
Basidiomycete parasites	0.497	-0.060	Total basidiomycete richness	-0.582	-0.26
Basidiomycete plant pathogens	0.361	0.076	Total macrofungal richness	-0.465	-0.278
Basidiomycete saprotrophs	-0.558	0.387			
Total basidiomycete richness	-0.571	0.364			
Total species richness	-0.453	0.36			

Table 4.2. The indicator values (IV%), standard deviation (SD), probabilities (*P*-value) and accession numbers of indicator taxa OTUs associated with the leaf litter in both Ascomycota(A) and Basidiomycota (B) phyla with *P*-values ≤ 0.05 as identified from sequencing rDNA-ITS2 region for both Pea Ridge (PR) and Devil's Den (DD) sampled sites.

Indicator taxa OTU	Site	IV	SD	*P*-value	Accession number
Ascomycota sp. OTU 876	DD	61.2	9.54	0.0194	KJ996070
Atheliaceae sp. OTU 8346	DD	50	7.98	0.0338	FM997948
Auriculariales sp. OTU 8279	DD	60	9.54	0.0148	FJ237097
Bolbitiaceae sp. OTU 9361	DD	66.7	6.14	0.0306	UDB038062
Calvatia cyathiformis OTU 8052	DD	50	7.93	0.0332	MF686508
Dacrymycetaceae OTU 884	DD	90	8.35	0.0002	KY271862
Exidia sp. OTU 9380	DD	50	8.1	0.036	KY801889&KY801871
Helotiaceae sp. OTU 9428	DD	60	9.44	0.0134	KY633581
Helotiales sp. OTU 9615	DD	71.1	8.66	0.0072	EF596821
Hyaloscyphaceae sp. OTU 8477	DD	50	7.88	0.0314	JN033412
Hyaloscyphaceae sp. OTU 9744	DD	67.5	8.76	0.0204	MH558296
Mycena robusta OTU 8312	DD	50	7.79	0.029	KJ705179
Pestalotiopsis sp. OTU 8864	DD	81	9.24	0.0018	KJ542337
Polyporaceae sp. OTU 8417	DD	81	9.04	0.0004	KY245961
Russula vinacea OTU 5913	DD	50	7.89	0.0316	MG680187
Sirobasidium sp. OTU 9526	DD	67.5	8.75	0.0176	AF444330
Uncobasidium sp. OTU 8582	DD	51.4	8.33	0.057	AB596025
Ascomycota sp. OTU 8419	PR	50	7.88	0.0312	UDB027460
Helotiales sp. OTU 9469	PR	90	8.48	0.0002	KX610428
Hymenochaetaceae sp. OTU 9868	PR	90.9	8.0	0.0002	KF438170
Hymenoscyphus immutabilis OTU 9428	PR	71.1	8.12	0.0046	KY744162
Leotiomycetes sp. OTU 9311	PR	90.9	8.0	0.0002	AB926064
Lycoperdon pratense OTU 9136	PR	70	8.31	0.0028	UDB031787
Marchandiobasidium sp. OTU 9000	PR	90	8.25	0.0002	HQ168397
Orbilia sp. OTU 9979	PR	76.9	7.3	0.0026	DQ656643
Schizoxylon berkeleyanum OTU 9190	PR	100	9.19	0.0002	MG281966
Trametes sp. OTU 9719	PR	90.9	7.88	0.0002	JN164970 &GQ280373
Trichophaea flavobrunnea OTU 989	PR	73.6	8.0	0.006	KY024696
Vibrisseaceae sp. OTU 9954	PR	64	9.13	0.0218	JX415337
Vibrisseaceae sp. OTU 9208	PR	90	8.24	0.0002	KY924872

4.5 Discussion

4.5.1 Taxonomic diversities and structures of fungal OTU assemblages

The results of the deeply sequenced samples examined using next-generation sequencing presented herein show that the mycobiome of the forest leaf litter is taxonomically and functionally diverse and that this microhabitat hides prime fungal species diversity in the leaf litter in both forest plots. These results confirmed the powerful sequencing technologies that have been extensively contributed to revealing an unexpected fungal diversity (Yahr et al., 2017) and have provided insight into fungal life in the leaf litter in the investigated locations of the Ozark forest. The species and functional diversity of the current sequencing results for temperate mixed deciduous forests are in agreement with pyrosequencing of a temperate beech forest in Central Germany that provided evidence of dynamic changes in species composition and abundances due to a dynamic functional succession during decomposing leaf litter that led to large fungal diversity (Purahong et al., 2016). In contrast to the previous inventory approach (Chapters 2 and 3), the metagenomic datasets in the current study detected a large number of macrofungal sequence reads with OTUs. Also, it revealed an even greater hidden diversity, from three fungal phyla beside numerous unidentified fungi than the inventory method (Figs. 4.1- 4.3). The high richness in the current metagenomic data may be related to the ability to detect most of the macrofungi present based on their propagules, somethings that could not be determined from their fruiting bodies through multiple visiting sites. That is because of difficulty to visualize the tiny size and short-lived fruiting bodies of litter-associated macrofungi (Boddy et al., 2008; Bässler et al., 2015) or those rarely producing fruiting bodies as dikaryotic fungi during their sexual reproduction (Fischer et al., 2012). When gathering the results of both and rDNA metabarcoding sequencings of leaf litter, there was an overlap of only 18.56% genus and 2.84% for species in the present study known as lignocellulose decomposers, supporting the idea that

fruiting bodies do not necessarily reflect the actually fungal proliferation (Taylor et al., 2010; Fischer et al., 2012; Smith et al., 2017). This disconnect between the two sets of results also suggests that many taxa may prefer to form long-lived and distance exploration mycelia than short-lived fruiting bodies with specific energy needs (Agerer, 2001).

In the last decade, the results obtained from high-throughput sequencing techniques have been revealed a number of novel taxa involving new divisions, classes, orders and new families. With recently generated data from traditional and molecular survey involving the same samples, the number of fungal species was estimated to be 12 million compared to 2.2-3.8 million species recently estimated based on various estimation techniques. It has been speculated that the current use of these techniques would reveal an even greater diversity than the current estimation (Wu et al., 2019). By combining the inventory method and DNA-metabarcoding approach of litter samples, this would enhance the knowledge about the successful sexual reproductions correlated with diverse ecological functions during sexual development within highly competitive assemblages in/on litter microhabitats. However, many unidentified species still need to be identified. These sequences are generally problematic for high-throughput sequencing approaches and the connection between fungal species and OTUs is challengeable and needs to be addressed (Wu et al., 2019).

In this study, the phyla Ascomycota and Basidiomycota were dominant in leaf litter of both sites, which is similar to the results obtained for the inventory approach utilized in the previous chapter although the OTU abundances and richness with species composition appeared to be different (Figs. 4.1-4.4). That confirms other suggestions that ascomycete fungi proliferate initially or earlier in plant litter decay and are replaced increasingly by basidiomycete species because of different enzymatic capacity for breakdown of litter polymers as decomposition progresses

(Osono, 2007; Purahong et al., 2016) and differences in abundances related to the competitive ability of individual species. Nevertheless, the actual development of fungal community on decaying litter is so far largely unknown under natural conditions (Voříšková and Baldrian, 2013.), so it is highly probably macrofungal mycelia of Ascomycota are present during all phases of leaf decomposition. This hypothesis is supported by detecting a considerable quantity of cellulose during the entire 24-month following a leaf litter deposition in a temperate forest dominated by *Quercus petraea* (Šnajdr et al., 2011) and reporting inventory results of presence of smaller fruiting bodies of the Ascomycota on leaf litter (Alanbagi et al., 2019). These dominant phyla were mostly driven by many species propagules belonging to the orders of Helotiales, Orbiliales, Pezizales, Agaricales, and Polyporales followed by others in both deciduous mixed forests of the present study (Figs 4.5 and 4.6), results were supported by DNA-metabarcoding results that revealed the high species richness and sequence reads of these orders from various habitat samples (Eduardo et al., 2018; Nelsen, 2017). The species with the most members, longer lifespans and inhabiting multiple substrates (Wang et al., 2006; Hibbett and Thorn, 2001; Baral et al., 2015) or varied microhabitats (Alanbagi et al., 2019), or in other words exhibited the most morphological plasticity, may be may demonstrate greater distributions, generally an essential characteristic in the kingdom fungi (Savile, 1969) and essential during fruiting body productions under preferable conditions (Boddy et al., 2008).

4.5.2 Functional structure and diversities of macrofungal assemblages on leaf litter

The high-throughput sequencing results comprehensively characterized the functional diversity and attributes and overlapping fundamental microhabitat of leaf litter macrofungi in Ozark forests and clearly were site-specific species related (Figs. 4.10 and 4.16). The total species, phylum, and dominant saprotroph richness of macrofungi did not differ significantly between

both sites, suggesting that the fungal communities shared similar genic profiles although they differed in taxonomic compositions (Sun et al., 2015). These communities also represented taxa that could transition between functional guilds and acquire bifunction or even multifunction with *"incertaine sedis"* on leafy litter; results that were in agreement with the inventory method of macrofungal litter for the same sites (Alanbagi et al., 2019) and molecular sequencings of root-associated fungi in the eastern North American (Nelsen, 2017). Results across studies signify that unique sets of genes are being retained during fungal evolution that probably allow some functional guilds to decompose lignocellulose (Sun et al., 2015). The basidiomycete mycorrhizas and saprotrophs were clearly richer in DD plots while basidiomycete parasites and ascomycete bifunctions were more prevalent in PR plots although the functions of *"incertaine sedis"* could be suggested as offering another view (Figs. 4.10 and 4.16). However, based on fruiting body production (Chapter 3), the ectomycorrhizas were more dominant in PR plots compared to DD plots, indicating that the inventory method may not reflect the actual functional diversity due to presumably highly competitive interactions between macrofungal assemblages at the microenvironment level. Also, that suggests that many ectomycorrhizal taxa may prefer to form long-lived and distance-exploration mycelia than short-lived fruiting bodies (Agerer, 2001), especially during mineralization and by immobilizing N and P in fungal mycelium (Boody et al., 2008; Näsholm et al., 2013; Bödeker et al., 2016). Taxa with saprotrophic activity have been assigned as a general observation to the litter layer (Bahnmann et al., 2018) or to litter microhabitats (Alanbagi et al., 2019) on the forest floor litter due to the great dependence on mineralizable C substrates with hyphal characters and invasive growth habits supported by a wide range of enzymatic capability (Wessels, 1999; Boddy et al., 2008; Fig.1.1). In the current study, the basidiomycete taxa belonging to the Omphalotaceae, Marasmiaceae, and Mycenaceae

presumably preserved the saprotrophic stability for the primary decomposers and known to be ligninolytic species that efficiently colonize distinct litter microhabitats mostly leaf and woody litter (Alanbagi et al., 2019) and decompose the individual components of the relatively homogeneous continuous leaf litter layer (Osono, 2007; Boddy et al., 2008). Other saprotrophic Basidiomycota members of the Orbiliaceae, Xylariaceae, Agaricaceae were also documented in agreement with other observations on different forest stratifications (Baldrian et al., 2012; Geml et al., 2017; Alanbagi et al., 2019). According to the current results, the prevalence of saprotrophic redundancy would probably determine ecosystem process rates (Nielsen et al., 2011) through influencing the persistence, mineralization and adjustment of soil organic matter (Boddy et al., 2008). The results also presumably provide primary knowledge about the forest health when taking into the account the macrofungal parasites, pathogens and capacity of some saprotrophs to enter into facultative biotrophic relationships with plant roots although this issue is not yet well investigated (Smith et al., 2017).

The results also determined the indicator species and their function for the investigated sites and may illustrate the unbalanced competition between dominant species or functional groups investigated in the present study. Interestingly, most of the indicator species of saprotrophs were known to inhabit mixed microhabitat litter or multi-substrates including *Lycoperdon pratense* (Lycoperdaceae), *Trametes* spp. (Coriolaceae), and *Orbilia* sp. (Orbiliaceae) for DD sites and *Calvatia cyathiformis* (Lycoperdaceae), *Mycena robusta* (Tricholomataceae), *Exidia* sp. (Exidiaceae), *Uncobasidium* sp. (Meruliaceae), *Sirobasidium* sp. (Sirobasidiaceae) for PR sites, suggesting the ability to serve as well facultative biotrophs with plant roots, as reported for some species recently (Smith et al., 2017). Other indicator species were *Russula vinacea* (Russulaceae) for DD plots and *Trichophaea flavobrunnea* (Pyronemataceae)*, Pestalotiopsis* sp.

(Pestalotiopsidaceae) for PR plots, which putatively were assigned as ectomycorrhiza, bifunction, and uncertainly functions *"incertaine sedis"*, respectively. *Marchandiobasidium* sp. (Corticiaceae) is known as mycoparasite based on the current knowledge. Changes in nutrient availability with other mentioned related factors may synergistically influence the fungal functional guild balance, promoting fungi with wide eco-physiological capabilities. For example, the genus *Pestalotiopsis* has a wide range of enzymatic capabilities, most of which interestingly, are expressed saprotrophically on plastic materials. In addition, the presence of the ectomycorrhizal genus *Russula* in DD plots seems to have a negative influence on the other ectomycorrhizal genera or other guilds in competition for resources, such as N and P (Sun et al., 2015; Bödeker et al., 2016). Linking fungal taxonomic assemblages with functional traits and determining the indicator species with unbalanced competition between various macrofungal assemblages may provide important insights for litter associated fungi and future forest management activities.

4.5.3 Comparisons, correlations and distributions between different macrofungal assemblages and variables across plot sites

The results of the present study clearly demonstrated the fact that the taxonomic and functional richness of macrofungal assemblages were structured by several climatic factors such as temperature or rainfall, and biological features including tree diversity, chemical composition of litter and fungal species interactions, and apparently site characteristics in the Ozark forest ecosystems. Although the temperature was not obviously different at the time of samplings and one month before samplings, it is expected there was a minor influence of this factor individually in this study on different structuring of macrofungal propagule assemblages similar to the results of obtained for litter fungal compositions in deciduous forest across a temperate forest

(Bahnmann et al., 2018). However, differences in monthly precipitation may have an influence. Moisture availability typically is a key factor limiting biological process including leaf litter decomposition (Gessner et al., 2010). In the present study, differences in precipitation between sites before and after leaf litter samplings may explain effects on development of fruiting bodies and difference in community structures based on the inventory method in both sites (Fig.1.7). These results suggest that temperature and precipitation can affect mycelial growth and later fruit body development at the species level, and consequently also fungal community and trait dynamics (Andrew et al., 2016). Tree species could be another essential factor shaping the macrofungal distribution and community structure in decomposing leaf litter as being revealed with litter fungi across a temperate forest dominated by specific tree species (Figs. 3.1 and 3.2; Bahnmann et al., 2018). Similarly, this factor has been recently demonstrated in root -associated fungi (Nelsen, 2017), and ECM fungal communities (Tedersoo et al., 2013). In mixed forest ecosystems, tree species impact decomposition process directly through species-specific litter quality (McClaugherty et al., 1985; Hobbie, 1992) and indirectly through altered microenvironmental conditions and soil biota structure (Joly et al., 2017). Litter species richness and compositions influence fungal litter and soil diversities and community structures because of heterogeneity (Osting et al., 2018). The major component of organic material in forest soil results from the vegetation that is deposited on the soil surface as an organic layer (litter) and is partially dispersed into the soil (McClaugherty et al., 1985; Santa Regina and Tarazona, 2001). The present results revealed potentially that leaf litter nutrient concentrations are an important factor and attributer in structuring of fungal associated assemblages taxonomically and functionally and in interactions between groups (Fig. 4.14). Members of orders in Basidiomycota, known as lignocellulose decomposers, were correlated significantly with

saprotrophic and ectomycorrhizal functions with DD plots where high nutrient concentrations compared to different members of orders mostly related to the Ascomycota known as biopolymer and lignocellulose decomposers with bifunctions and parasites in PR plots. The higher saprotrophic richness in DD plots may increasingly activate organic matter mineralization in combination with improved leaf litter quality explaining the high ectomycorrhizal richness of propagules capable of mineralizing nutrients such as nitrogen and phosphorus with high concentrations. This cascade could lead to progressive increases in the overall fungal diversity specifically (Body et al., 2008; Purahong et al. 2016), and the species competition between the saprotrophs themselves, or with other guilds, such as mycorrhizas for essential energy requirements such as N and P or efficient enzyme production during litter decay (Näsholm et al., 2013; Kyaschenko et al., 2017). In the present study, these genera were highlighted (Fig. 4.11), as important decomposers, facilitating mobilization of nutrients not only from leaf litter but also other complex organic materials with successional establishment of functional guilds based on their capacity to access more complex nutrient pools from litter materials (Purahong et al. 2016; Kyaschenko et al., 2017). On the other hand, dominant mycoparasites such as species of *Tremella* could negatively impact the species from other functions (Sun et al., 2019).

Litter decay communities are especially important since lignocellulolytic fungi with others are key determinants of forest biogeochemical cycling such as carbon sequestration rates and nutrient cycling (Boody et al., 2008). However, isolating the factors driving the community structure and functional heterogeneity are particularly challenging in litter-associated fungi because of the community-assembly dynamics and several abiotic and biotic factors in a microsite strongly influenced by stochastic processes (Purahong et al., 2016). Thus, characterizing the structure and functions of macrofungal communities and the factors driving its

variability provides information for future sustainability managements of the diversity of macrofungi (Senn-Irlet et al., 2007). Therefore, the results obtained in the present study suggest there may be synergic effects with other features influencing on taxonomic structures and functional guilds differentiating communities.

4.6 Conclusions

The metagenomic results provided herein showed that leaf litter of deciduous mixed forests in Ozark harbors distinct macrofungal assemblages and overlapping functions. The leaf associated fungi were diverse taxonomically and functionally in both investigated study sites. Taking into account the unidentified and cryptic species assemblages within the undercurrent taxa, the species richness would be expected to rise from what is revealed from the current results of leaf litter microhabitat. Although the overall richness and dominant functions of saprotrophs did not differ significantly, there were unique species composition in each study site that contributed in clear functional variations for other guilds and the saprotrophic community between both sites. Each site was characterized by a subset of indicator species with high fidelity but relatively low abundance. That suggests the local abiotic-biotic variables in each site could be major drivers for structuring species diversity as well as community composition and functions. The diverse litter species and nutrient contents that reflect the diverse structure of tree species and the dominant tree species in each study site are main factors in those assemblages. In the present study, the smallest overlap between macrofungal communities, based on gathering fruiting body analyses and molecular sampling method, suggests that both approaches should be considered if the goal is to recover the greatest breadth of diversity data.

4.7 References

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4.8 Supplemental Materials

Table 4.1S. Unique molecular identifier tags (MIDs) for samples collected from Pea Ridge (PR) and Devil's Den (DD) sites.

Sample Name	MID Tag Name	MID Sequence
DD1	IonXpress_11	CCTGCCATTCGC
DD2	IonXpress_12	CTTGACACCGC
DD3	IonXpress_13	CTAGGACATTC
DD4	IonXpress_14	CTTCCATAAC
DD5	IonXpress_15	CCAGCCTCAAC
DD6	IonXpress_16	CTTGGTTATTC
DD7	IonXpress_17	TTGGCTGGAC
DD8	IonXpress_18	CCGAACACTTC
DD9	IonXpress_19	TCCTGAATCTC
DD10	IonXpress_20	CTAACCACGGC
PR1	IonXpress_01	TTAAGCGGTC
PR2	IonXpress_02	CTAGGAACCGC
PR3	IonXpress_03	CTTGTCCAATC
PR4	IonXpress_04	TCCTCGAATC
PR5	IonXpress_05	CGGACAGATC
PR6	IonXpress_06	CGGAAGGATGC
PR7	IonXpress_07	TAGGTGGTTC
PR8	IonXpress_08	TCTAACGGAC
PR9	IonXpress_09	CCGCAATCATC
PR10	IonXpress_10	TCTAGAGGTC

Table 4.2S. Sequences of Ion Torrent adaptors and gene primers for sequencing the ITS2 region.

Sequence Name	**Sequence**
Ion Torrent adaptor	
Squencing adaptor P1	5`-CCTCTCTATGGGCAGTCGGTGAT-3`
Squencing adaptor A	5`-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3`
Gene primer	
fITS7-trP1	5`-GTGARTCATCGAATCTTTG-3`
ITS4	5`-TCCTCCGCTTATTGATATGC-3`

Table 4.3S. The summary of raw sequances and read length histograms of the next-generation sequancing for each sample from Devil's Den investigated plots (D1-D10).

Sample ▲	Bases	>=Q20 Bases	Reads	Mean Read Length	Read Length Histogram
D1	31,397,016	28,178,385	140,479	223 bp	
D2	30,570,385	28,008,882	127,283	240 bp	
D3	27,450,528	24,673,466	121,443	226 bp	
D4	34,079,956	30,632,195	155,288	219 bp	
D5	31,055,945	27,769,901	141,881	219 bp	
D6	28,986,548	26,081,249	131,024	221 bp	
D7	24,572,095	22,082,637	93,626	262 bp	
D8	33,434,292	29,858,888	153,310	218 bp	
D9	40,099,853	36,660,257	158,097	254 bp	
D10	30,669,027	27,976,737	131,466	233 bp	

Table 4.4S. The summary of raw sequances and read length histograms of the next-generation sequancing for each sample from Pea Ridge investigated plots (PN1-PN10).

Sample ▲	Bases	>=Q20 Bases	Reads	Mean Read Length	Read Length Histogram
PN1	29,306,926	26,624,624	122,863	239 bp	
PN2	26,681,400	24,375,943	114,210	234 bp	
PN3	29,564,431	27,027,815	121,167	244 bp	
PN4	27,268,852	24,827,622	126,152	216 bp	
PN5	24,858,422	22,542,418	97,298	255 bp	
PN6	30,458,772	28,000,430	120,113	254 bp	
PN7	27,413,160	25,108,795	106,912	256 bp	
PN8	25,232,965	23,080,089	98,207	257 bp	
PN9	27,539,216	25,263,604	109,248	252 bp	
PN10	33,099,452	30,205,095	132,147	250 bp	

Table 4.5S. Macrofungal taxa and their codes for species clustered on a matrix axis after using two-way cluster analysis for DD and PR plots.

Macrofungal Taxa	**Codes**	**Macrofungal Taxa**	**Codes**
Amylocorticiellum	Amylocor	*Hebeloma*	Hebeloma
Antrodia	Antrodia	*Heterobasidion*	Heteroba
Arachnopeziza	Arachnop	*Hohenbuehelia*	Hohenbue
Arrhenia	Arrhenia	*Hygrocybe*	Hygrocybe
Ascobolus	Ascobolu	*Hymenoscyphus*	Hymenosc
Athelia	Athelia	*Hyphoderma*	Hyphoder
Botryobasidium	Botryoba	*Hyphodontia*	Hyphodon
Calocera	Calocera	*Hypochnicium*	Hypochni
Calvatia	Calvatia	*Incrucipulum*	Incrucip
Calyptella	Calyptel	*Inocybe*	Inocybe
Ceraceomyces	Ceraceomy	*Laccaria*	Laccaria
Ceriporiopsis	Ceripori	*Lachnum*	Lachnum
Cistella	Cistella	*Lasiobelonium*	Lasiobel
Claussenomyces	Claussen	*Leptosphaeria*	Leptosph
Claviceps	Clavicep	*Lycoperdon*	Lycoperd
Clitocybe	Clitocybe	*Marasmius*	Marasmiu
Coccomyces	Coccomyc	*Marchandiobasidium*	Marchand
Coprinopsis	Coprinop	*Mycena*	Mycena
Cortinarius	Cortinar	*Mycetinis*	Mycetini
Crepidotus	Crepidot	*Orbilia*	Orbilia
Crinipellis	Crinipel	*Papiliotrema*	Papiliotr
Dacryobolus	Dacryobo	*Parasola*	Parasola
Daedalea	Daedalea	*Peniophorella*	Peniopho
Daldinia	Daldinia	*Pestalotiopsiss*	Pestalot
Ditiola	Ditiola	*Peziza*	Peziza
Dacryobolus	Dacryobo	*Phanerochaete*	Phaneroc
Daedalea	Daedalea	*Phlebia*	Phlebia
Duportella	Duportel	*Phlebiopsis*	Phlebiop
Efibula	Efibula	*Pholiota*	Pholiota
Eriopezia	Eriopez	*Plectania*	Plectania
Exidia	Exidia	*Pluteus*	Pluteus
Exobasidium	Exobasid	*Praetumpfia*	Praetump
Fulvifomes	Fulvifom	*Pseudoclitocybe*	Pseudocl
Galiella	Galiella	*Punctularia*	Punctula
Ganoderma	Ganoderm	*Ramaricium*	Ramarici
Gloeoporus	Gloeopor	*Rhizocybe*	Rhizocyb
Gymnopus	Gymnopus	*Rodwayella*	Rodwayel

Table 2.1. Cont.

Macrofungal Taxa	**Codes**
Russula	Russula
Rutstroemia	Rutstroemia
Schizoxylon	Schizoxy
Sclerotinia	Scleroti
Scutellinia	Scutellini
Sebacina	Sebacina
Septobasidiales	Septobas
Serpula	Serpula
Sirobasidium	Sirobasi
Sistotrema	Sistotre
Skeletocutis	Skeletoc
Skvortzovia	Skvortzo
Steccherinum	Steccher
Stilbella	Stilbella
Taphrina	Taphrina
Tomentella	Tomentel
Trametes	Trametes
Trechispora	Trechisp
Tremella	Tremella
Trichaptum	Trichapt
Trichophaea	Trichoph
Uncobasidium	Uncobasi
Wrightoporia	Wrightop
Xylaria	Xylaria

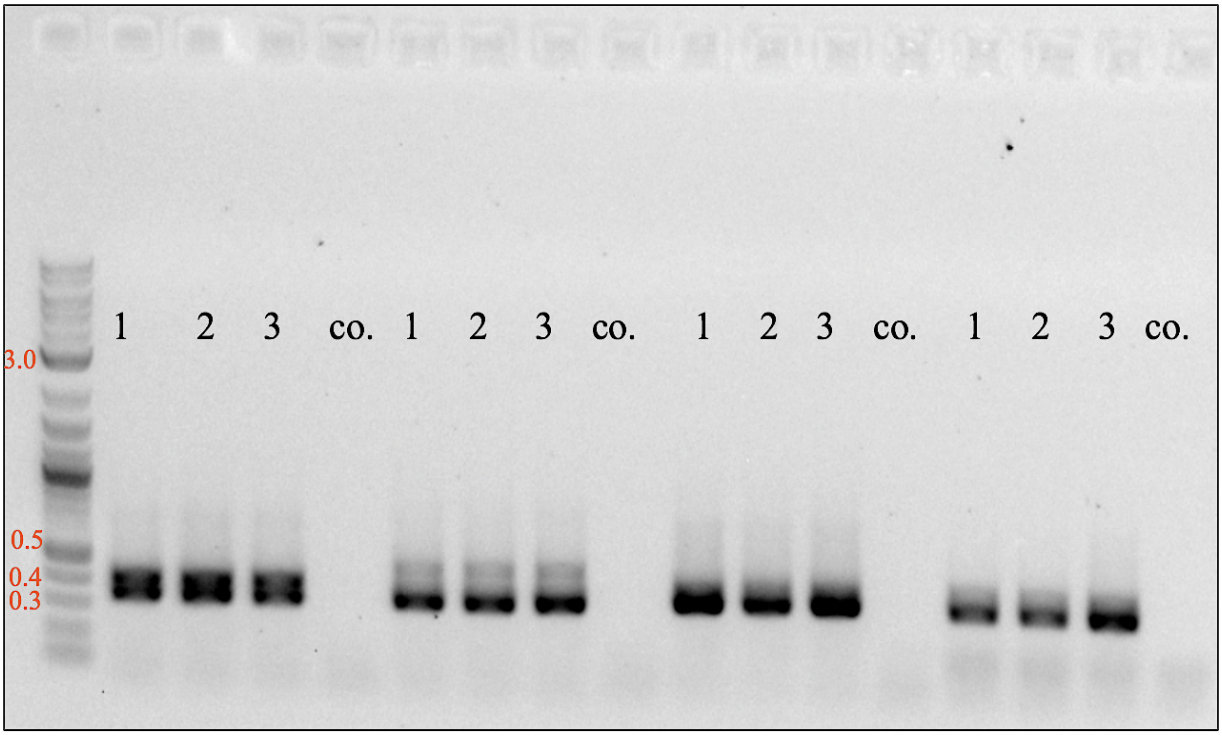

Fig. 4.1S. Representative samples of electrophoreses for triplicate PCR products with negative control (co.) for each sequenced sample. Photograph by the author.

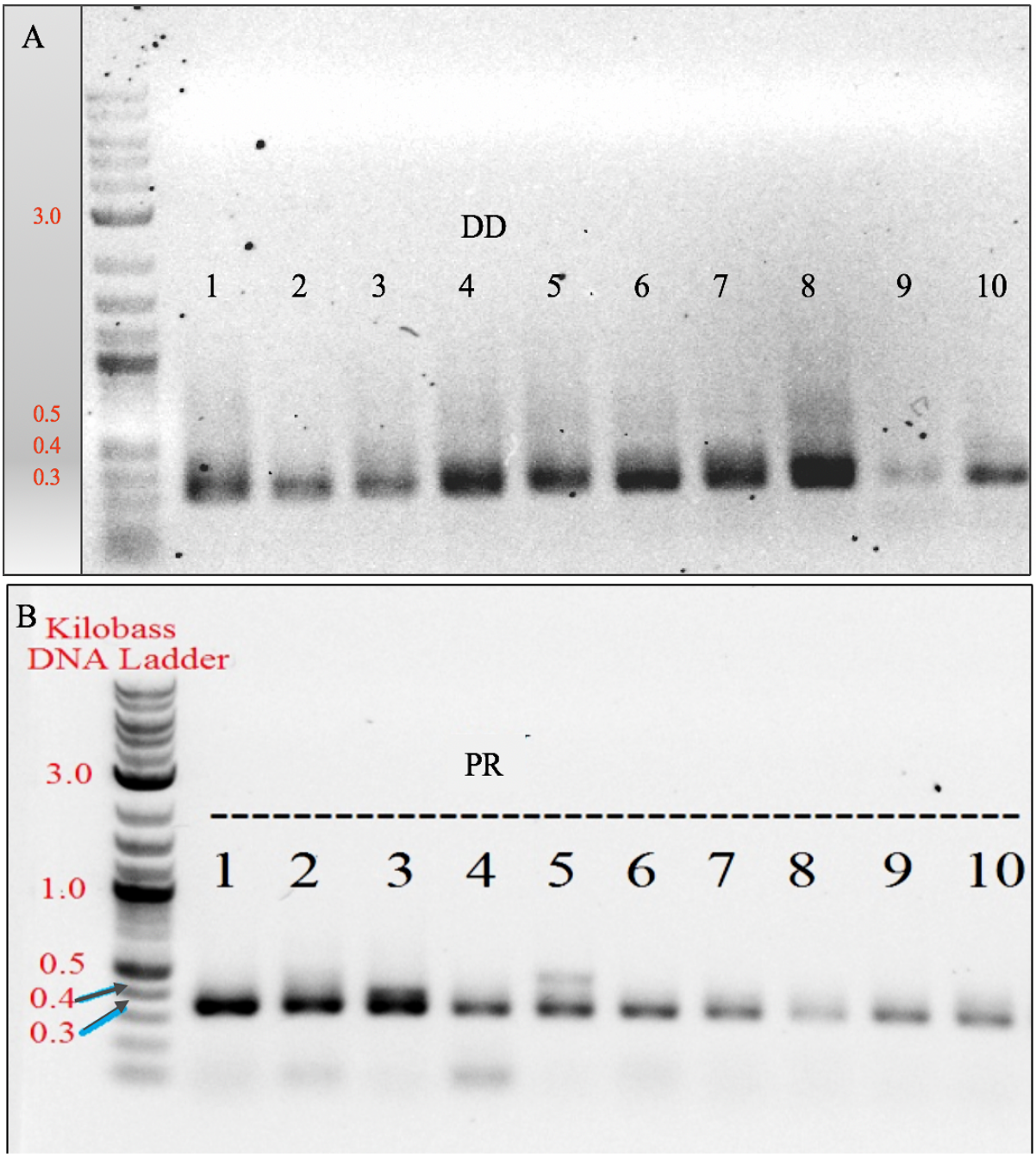

Fig. 4. 2S. Electrophoresis of pooled PCR products for 10 samples of each investigated site; Devil's Den (DD) and Pea Ridge (PR) sites. Photograph by the author.

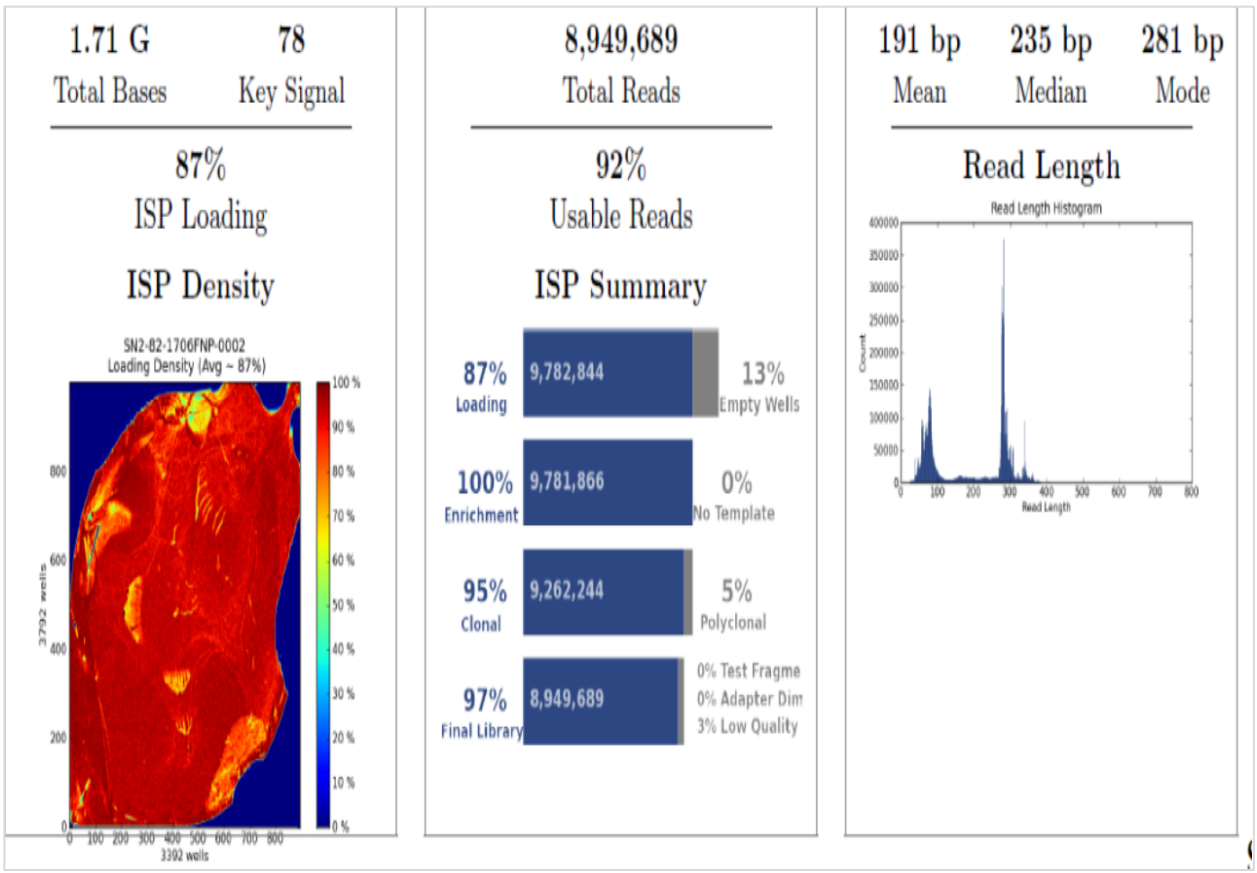

Fig. 4.3S. The raw run summary of Ion Torrent sequencing. The Ion Torrent dataset of the current study contained 2,502,187 reads of total sequence reads with the approximatly median read lengths of 236 bp.

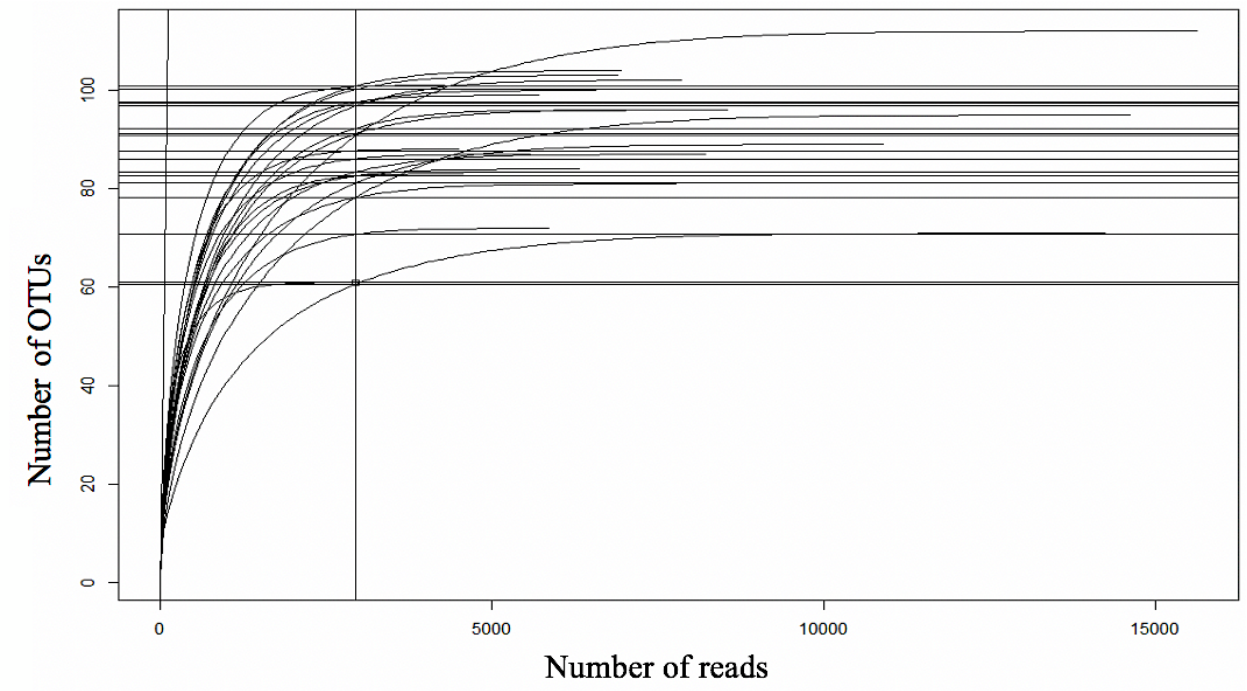

Fig. 4. 4S. Rarefaction curve of sampled leaf litter-associated fungi for DNA sequence reads of ITS2 region across 20 sampled plots of both Pea Ridge (PR) and Devil's Den (DD) sites. All investigated leaf litter samples approached a plateau, suggesting an equally deep OTU recovery and sequenced most of the fungal species in the sampled plots.

Chapter 5. Effect of prescribed burning on the taxonomic and functional diversity of litter-associated macrofungi in the Pea Ridge National Military Park, Arkansas

5.1 Abstract

Litter-decomposing fungi are vital components of forest ecosystems and inhabit multiple microhabitats of this heterogeneous substrate available to them on the forest floor. The expected increase in prescribed burning activity around the world as a consequence of global climate change and long-term fire management in forest ecosystems is likely to impact on litter-decomposing fungi and their contributions to ecosystem services. Herein we present the results of the first effort to document the effect of prescribed burning management practices on the macrofungi associated with the litter layer of a temperate forest in Pea Ridge National Military Park, a historic forest in northwest Arkansas. During the 2016 field season, the fungi associated with the litter layer were collected from twenty selected plots subjected to two different prescribed burning regimes. A total of 91 taxa were identified using ITS Sanger sequencing. Taxonomically, neither total species richness nor total macrofungal productivity were substantially different between compared sites, but prescribed burning shaped the composition of the fungal assemblage on various litter microhabitats. Seven species apparently represent indicator taxa in unburned plots, whereas only one taxon could be characterized as an indicator taxon in burned plots. The creation of new fungal microhabitats after the combustion of much of the leafy litter presumably triggered a compositional shift in the fungal assemblage in addition to a detectable decline in the species richness and numbers of fruiting bodies inhabiting the various microhabitats. Nevertheless, the functional redundancy of saprotrophs in the two sets of plots did not seem to be greatly affected greatly. These results are important for future forest management

decisions to preserve fungal diversity, productivity, microhabitats, and ecological functions with other forest management goals.

5.2 Introduction

Fires are the primary natural disturbance factor in various types of forests and represent an important ecosystem type to consider in evaluating the possible impacts of global climate change. It has been predicted that there will be an increase in the incidence and severity of large global uncontrolled forest fires in the future, and this has already been shown to have a major impact on humans (Pechony and Shindell, 2010; Liu et al., 2010, 2013). Prescribed burning has been carried out for sustainable and restoration purposes, with many uncertainties with respect to ultimate outcomes (Altangerel and Kulla, 2013; Santín and Doerr, 2016). Historically, fire regimes usually shape the composition and structure of oak forests and reduce fuel loads in the eastern United States (Schuler and McClain, 2003; Oliver et al., 2015) and are now applied more frequently across the central and southern Appalachian mountains and plateau regions than in the recent past (Arthur et al., 2017). However, such fires directly or indirectly disturb the forest soil, through their capability to readily and totally consume major constituents of the surficial organic matter (Arthur et al., 2017). Even at low or intermediate fire intensity, fires consume and transform the soil organic horizon, resulting in carbon combustion, nutrient volatilization, microclimate alteration and the mortality of soil organisms (Neary et al., 1999; Hatten and Zabowski, 2010; Greeshma et al., 2016). For example, low-intensity prescribed burning, has been demonstrated to show an impact on inorganic nutrient dynamics and availability such as involving carbon (C) and nitrogen (N) (Dzwonko et al., 2015; Tylor and Midglay, 2018). Thus, fires could be considered as a major soil forming factor in forest ecosystems (Santín and Doerr,

2016) representing a factor with the highest probability with respect to declining and altering soil organismic diversity in the short or long term (Oliver et al., 2015; Sun et al., 2015).

Dead plant organic matter is the most valuable renewable resource in the biosphere and the driving force behind the establishment of the soil profile in forest ecosystems. The litter layer is a major source of organic matter accumulation and soil protection from loss via erosion (Berg and McClaugherty, 2008). This layer is a heterogeneous mixture comprised mainly of leafy and fine woody detritus that carpets the forest floor. It consists mostly of recalcitrant biopolymers related to the physicochemical tree naturality (Osono, 2007; Berg and McClaugherty, 2008). Litterfall is a key pathway for forest ecosystems through the decomposition process and has consequences for biogeochemical cycles based on the high diversities of organismic decomposers and various abiotic factors (Hättenschwiler et al., 2005; Bisht et al., 2014). This detritus provides a microhabitat for a major contributor to soil ecosystem processes, the mycelial networks of litter-decomposing fungi with potential connections both with each other and with the mycelia of other fungi. The litter layer also shelters potential consumers and a variety of other organisms that form a myriad of complex interactions in forest ecosystems (Boddy et al., 2008). As functional components of this system, saprotrophic fungi, along with mycorrhizal fungi, are major contributors to litter degradation, nutrient mobilization and mineralization, due to their ability to effectively colonize and access new resources through a number of different strategies and patterns of foraging mycelia and enzymatic activities (Boddy et al., 2008; Bodeker et al., 2016). However, the composition of the assemblage of litter decomposers and the functional guilds associated with varied litter types under natural field condition remain largely unknown (Baldrian, 2017). Forest fire management may provide strong selective pressure on most forest organisms, fungi in particular (Oliver et al., 2015; Reazin et al., 2016).

Few studies involving substantial observations of fungal responses following fires have been carried out, so little data are available (Greeshma et al., 2016; Dove and Hart, 2017). The distinctive responses of these fungi appear to depend on fire intensity and the season of the year, the time elapsed between burning and sampling, and the forest type involved (Cairney and Bastias, 2007; Dooley and Treseder, 2012). Nevertheless, according to meta-analyses and fire consequence studies, it has been suggested that soil and litter microbes, with their enzymatic activities and ecosystem services, may need at least a decade or more for complete recovery to the pre-burned state after an interruption by fire (Dooley and Treseder, 2012; Köster et al., 2015). With insufficient knowledge about the fungal taxonomic, diversity and functional responses of the assemblage affected by fire (Dove and Hart, 2017), there is no a way to assess the impact of an increase in forest wildfires or prescribed fires due to future warming trends (Liu et al., 2013).

Although litter-decomposing fungi play a central role in decomposition activities, the consequences of recurring burning on the taxonomic and functional abundance and diversity of the assemblage of litter-associated fungi in temperate forests are poorly understood. The present study is the first effort of which we are aware to connect the impact of prescribed burning on the taxonomic composition, structure, and diversity of the assemblage of litter-associated macrofungi in the Pea Ridge National Military Park in northwest Arkansas. This historic park covers an area of approximately 1,740 hectares. The park is subject to prescribed burning to manage upland oak ecosystems and to preserve the forest features considered to be those associated with the natural environment of the 1862 Civil War battle and also those in which earlier prehistoric Native Americans lived (Annis et al., 2011; Arthur et al., 2017).

5.3 Materials and Methods

5.3.1 Study sites and sampled plots

The two forest study sites investigated are located in the northern portion of Pea Ridge National Military Park (Fig. 1.9). This portion of the park is maintained via low to moderate intensity prescribed burns every five years, mostly conducted during the fall (Fig. 1.10). The fuel source is primarily leaf litter, twigs and small pieces of coarse woody debris. The first site (36°27'19" N, 94°01'27" W) was burned six months prior to the beginning of our study (hereafter referred to as the burned site). The second site (36°27'37" N, 94°01'20" W) was burned five years previously (hereafter referred to as the unburned site). The climate of northwest Arkansas is generally hot and humid, with unpredictable rainfall during the summer months. The forest considered in the present study is a temperate deciduous mixed dominated by several species of oak (*Quercus alba* L., *Q. velutina* Lam., and *Q. marilandica* Muenchh.), hickory (*Carya* spp.), winged elm (*Ulmus alata* Michx), and red cedar (*Juniperus virginiana* L.) (Stephenson et al., 2007). The study sites are dominated by oaks and hickories in the overstory (stems ≥10 DBH (cm)), with winged elm, hickories, and oaks in the midstory (≥ 2.5 – 9.9 DBH (cm)). Detailed information on the composition of local vegetations is provided in Fig. 5.1S. The depth (cm) of the litter layer carpeting the forest floor was determined at the four corners and the center of each plot using a ruler prior to collecting fruiting bodies. The mean depths of unburned and burned litter layers were 3.1 and 2.5 cm, respectively and did not differ significantly.

Fruiting bodies of fungi were collected systematically from the two study sites, each of which was divided into ten 10 x 10 m plots in the same manner as described in Lodge et al. (2004). The plots of each site were located along two parallel transects with 35-50 m as interval distances among the plots to obtain fruiting bodies from a wide variety of microsites on the forest floor.

The surveys were limited to macrofungi with a cap diameter ≥ 1 mm and 1 cm for Ascomycota and Basidiomycota, respectively.

5.3.2 Processing and identification of macrofungi

Because fruiting essentially depended on a period of precipitation, sampling for macrofungi was carried out in each site approximately every two weeks from May to September 2016. With few studies on genetically distinct macrofungal individuals generally and litter associated fungi specifically, it was impossible to quantify individual abundance for diversity analysis in an area merely by observing the fruiting bodies. Thus, a number of fruiting bodies for a particular species within a plot was quantified only as reproductive entities with several ecological and physiological functions (López-Quintero et al., 2012; Halbwachs et al., 2016). After recording substrate data, fruiting bodies were photographed in their natural microhabitats and tallied within each plot. Representative specimens were collected and transferred to the laboratory. Litter substrates with which the fungi were associated were segregated into three microhabitat categories. These were leafy litter (LL), very fine woody litter (WL), and mixed litter (ML) as described in detail in Alanbagi et al. (2019).

Specimens were dried for 24 to 48 h at a temperature of 45 ^{o}C on a food dehydrator, placed in small white pasteboard boxes and then deposited in the mycological herbarium of the University of Arkansas (UARK) for future study. The collected fungi were identified morphologically based on various publications (e.g., Breitenbach and Kränzlin, 1984; Elliott and Stephenson, 2017) and this identification was confirmed by amplifying and sequencing the internal transcribed spacer (ITS) region of the fungal ribosomal DNA (rDNA).

5.3.3 DNA extraction, PCR amplification, and Sanger sequencing

DNA extraction, protocol amplification, PCR product visualization, and Sanger sequencing were described in detail elsewhere (Alanbagi et al., 2019). In brief, small fragments of mostly the caps from fruiting bodies were used first to extract DNA and then to process genomic DNA using the Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin), according to the manufacturer's instructions. The extracted DNA was amplified and sequenced by targeting the ITS region using the fungal-specific primer pairs ITS1F–ITS4 or ITS4B (Gardes and Bruns, 1993), along with the universal primers ITS1 and ITS4 (White et al., 1990). Consensus sequences of successful Sanger sequencing were obtained after editing and alignment of the sequencing results using the Geneious program version 9.1.8 (Biomatters Ltd., Newark, New Jersey).

5.3.4 Taxonomic affiliations and functional assignments

The Basic Local Alignment Search Tool (BLAST) available on the website (www.ncbi. nlm.nih.gov/genbank/) of the National Center for Biotechnology Information (NCBI) (GenBank) was used to verify individually the identity of each sequence fragment. Determining taxa of fungi was completed using a query coverage of ≥ 80% and sequence similarity of ≥ 97% based on consideration of the results from the GenBank BLAST search. Fungal taxonomy follows that given on the Index Fungorum database (www.indexfungorum). Based on taxonomic affiliates, the identified taxon was assigned putatively to a functional guild based on the relevant published literature (e.g., Rinaldi et al., 2008; Tedersoo et al., 2014; Morgado et al., 2016).

5.3.5 Statistical analyses

Total species richness (number of taxa) was assessed taxonomically and functionally by comparing data on the taxonomic boundaries for burned and unburned sites. For these analyses,

all species of macrofungi in the different litter categories were included in the general category 'litter'. The total productivity of fruiting bodies was quantified for both sites as an indicator of community response to burning disturbance. The results were statically evaluated using analysis of variance (ANOVA). Values of fungal taxa richness and fruiting body productivity obtained for the various litter microhabitats and for the different functional groups were also calculated separately to assess and compare their prevalence between the two sites using factorial analysis of variance (by a split-plot two-way analysis of variance ANOVA). Tukey's test was later performed to compare means, selecting 0.05 as the significance value for all statistical tests. To describe the general taxonomic patterns between both sets of study plots based on the number of species, the distribution of phyla and orders were calculated and visualized. The percentage of macrofungal productivity for each genus was also calculated and compared within and between investigated sites.

The effect of prescribed burning on fungal community assemblages across study sites for various microhabitats was assessed taxonomically and functionally by comparing the dissimilarity in datasets and a visual estimate based on multivariate analyses in PC-Ord version 6.1.2 (McCune and Grace, 2002). Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance was conducted. In brief, the primary species matrix based on binary data was generated. The secondary matrix consisted of categorical values for the two sites with the number of species per taxonomic phylum. The number of species per taxonomic phylum and functional group and for the various litter microhabitats along with the total species richness for both compared plot sets in an additional secondary matrix were analyzed. Variables with $| r | \geq 0.5$ values for either NMDS axis were considered essential for characterizing changes in fungal assemblage structure and overlaid on the ordinations as strength vectors and directions (Roger et al., 2009; Geml et al.,

2014). The multi-response permutation procedure (MRPP), which provides a *P*- value and an *A*-statistic (effect size) was performed based on Bray-Curtis distances for detecting differences in the multivariate data based on treatment type (McCune and Grace, 2002).

Comparisons of patterns of rarity (number of species occurrences) and the distribution of litter-associated species across the twenty investigated plots was carried out and visualized by two-way cluster analysis, using Bray-Curtis distances and flexible beta (ß= -0.25) as the linkage method in PC-Ord as well (McCune and Grace, 2002). Finally, Indicator Species Analysis (Dufrêne and Legendre, 1997) was tested to detect any preferences of individual species for a particular treatment type and a significance of observed indicator values through Monte Carlo test with permutations (4999) using PC-Ord ($P < 0.05$).

5.4 Results

5.4.1 Taxonomical identifications and community structures

A total of 91 taxa of litter-associated fungi were morphologically identified and genetically confirmed, of which seven species (8%) were documented in both sets of study sites after collecting or recording a total of 1,646 fruiting bodies. These represented 42 and 56 of taxa with 941 and 705 records of fruiting bodies in the unburned and burned sites, respectively. Taxonomically, the identified taxa mostly identified to species belonging to 48 genera, 12 of which (26%) were recorded in both types of study sites, but with distinct differences in fruiting body productivity (Fig. 5.1). The genera were classified into 31 families, 10 of which were recorded from both unburned and burned study sites. These taxa were assigned to 11 orders, with five shared between the two study sites, with substantial differences in their proportional contributions to overall fungal community assemblages (Fig. 5.2A and B).

The taxonomic patterns described above were apparently driven by the members of the Ascomycota and Basidiomycota (Fig. 5.2C and D) due to fire management. Members of the Basidiomycota were more abounded in both study sites (94% and 70 % of unburned and burned plots, respectively), where the total belonging to the order Agaricales (88 % and 75% for unburned and burned plots, respectively) made up the majority of the identified fungi. However, members of the Ascomycota were more prolific for burned plots (30%) than for unburned plots (6%, Fig. 5.2A and B). Several orders in the phylum Ascomycota were recognized as litter-inhabiting fungi, including examples in the Pezizales (11%), Helotiales (4%) and Orbiliales (1%) with higher percentages for burned litter plots (Fig. 5.2A). In addition, there was an appreciable number of specimens that yielded sequences that could not be matched beyond genus with anything in GenBank. It seems likely that the majority of these are either relatively rare taxa yet to be sequenced or taxa new to science.

The proportion of species recorded on burned plots that were unique to this study site was 89%. Species in genera such as *Clitocybe*, along with *Mycetinis opacus* (Berk. & M.A. Curtis) A.W. Wilson & Desjardin and *Lactarius camphoratus* (Bull.) Fr., which were present in unburned plots, were replaced in burned plots by different taxa such as *C. subditopoda* Peck, *M. scorodonius* (Fr.) A.W. Wilson & Desjardin, *L. pterosporus* Romagn, and *L. subserifluus* Longyear. Increases in the fruiting body productivity of some species such as *Crucibulum leave* (Huds.) Kambly and *Sarcoscypha occidentalis* (Schwein.) Sacc or a reduction in the macrofungal productivity of leaf-inhabiting taxa such as species of *Crinipellis*, *Gymnopus*, *Marasmius* and *Schizophyllum* were also apparent (Fig. 5.1).

5.4.2 Species richness and productivity patterns for litter microhabitats and ecological functions

The total species richness and productivity responses did not differ substantially for the two fungal assemblages (Fig. 5.3). However, there were significantly different for microhabitat and functional measures (Figs 5.4 and 5.5). At microhabitat scales, the recently prescribed burning consumed mostly dead leaves and partially eliminated some fine debris and exposed small pieces of wood previously covered by newly fallen leaves. The depths of the litter layers did not significantly differ between both unburned and burned sites. The fungal responses among plot sites differed depending on the microhabitat prevalence of particular taxa. The macrofungal richness and productivity of leafy litter (LL) species were significantly greater in the unburned plots compared to burned plots. The richness of fine woody litter (WL) species did not vary appreciably compared with the species associated with other microhabitats, while the macrofungal production of the former was significantly greater in burned plots (Fig. 5.4). Although species diversity completely shifted, the dominant functions were not altered. Saprotrophic redundancy was prevalent in the two investigated study areas, regardless of unassigned functional guilds for a number of taxa. The saprotrophic richness and the total number of produced fruiting bodies were statistically greater than for other detected functional groups in the two types of study sites. The population assemblages of saprotrophs decreased in unburned as opposed to burned plots sites. There was low productivity of most fungal ectomycorrhizas in unburned sites, but their richness and productivity did not differ appreciably between the two types of study sites (Fig. 5.5). Most of the saprotrophs recorded belong to the Basidomycota, within the Agaricales most prevalent in both study sites and the Orbiliales in the

Ascomycota in the burned plots (Fig. 5.2). The majority of these were collected predominantly from leafy and woody litter in unburned and burned plots, respectively (Fig.5.4).

5.4.3 Comparing macrofungal community assemblies across investigated sites

The NMDS-ordinations of binary data revealed a strong structuring of the fungal litter assemblage across sites with a three-dimensional solution. The final stress of 12.7894 and final instability < 0.00022 resulted from NMDS. The fungal assemblages of the burned site cluster were more scattered than those of the unburned site cluster without overlap (Fig. 5.6 and Table 5.2S). This was statistically confirmed by MRPP conclusions (A = 0.1275, P = 0.0000033). At the taxonomic family level, the Omphalotaceae (r = -0.929), Marasmiaceae (r = -0.556), Tricholomataceae (r = -0.579), Hydnangiaceae (r = -0.515), and Sebacinaceae (r = -0.462), which mostly colonized leafy litter, with negatively correlated with axis 1. Conversely, the Bolbitiaceae (r = 0.613), Agaricaceae (r = 0.566), Pluteaceae (r = 0.537), and Thelephoraceae (r = 0.525) and other families which largely occupied woody debris showed a positive correlation with NMDS axis 1 (Fig. 5.6A and Fig. 5.1S). The microhabitats and functional differences in species assemblages in the plots being compared were also remarkedly displayed on NMDS axes in which the saprotrophs were dominant for unburned plots and ectomycorrhizas clustered separately for burned plot assemblages on heterogenous microhabitats (Fig. 5.6B and Table 5.1S).

The two-way cluster analysis confirmed the NMDS-ordinations and statistical results in which the macrofungal assemblages strongly clustered as a response to prescribed burning (Fig. 5.7 and Table. 5.2S). Species that grouped closely on the x-axis dendrogram distributed in similar plots and plots that assembled tightly on the y-axis dendrogram had similar species assemblages.

Burned plots displayed more patchy distribution of species assemblages, suggesting that a number of species were unique to specific plots.

Indicator species analysis showed that *Gymnopus androsaceus* (L.) J.L. Mata & R.H. Petersen, *G. disjunctus* R.H. Petersen & K.W. Hughes, *G. subnudus* (Ellis ex Peck) Halling, *G. spongiosus* (Berk. & M.A. Curtis) Halling, *Infundibulicybe gibba* (Pers.) Harmaja, and *Mycena albiceps* (Peck) Gilliam, with a perfect indication value for *Mycetinis opacus*, were significantly reliable indicators for the unburned plots. These dominant species are members of the families Omphalotaceae, Mycenaceae, and Tricholomataceae. Conversely, only *Crucibulum leave*, a member of the Agaricaceae, was a reliable indication for the burned sites ($P \leq 0.05$, Table 5.1 and Fig. 5.7).

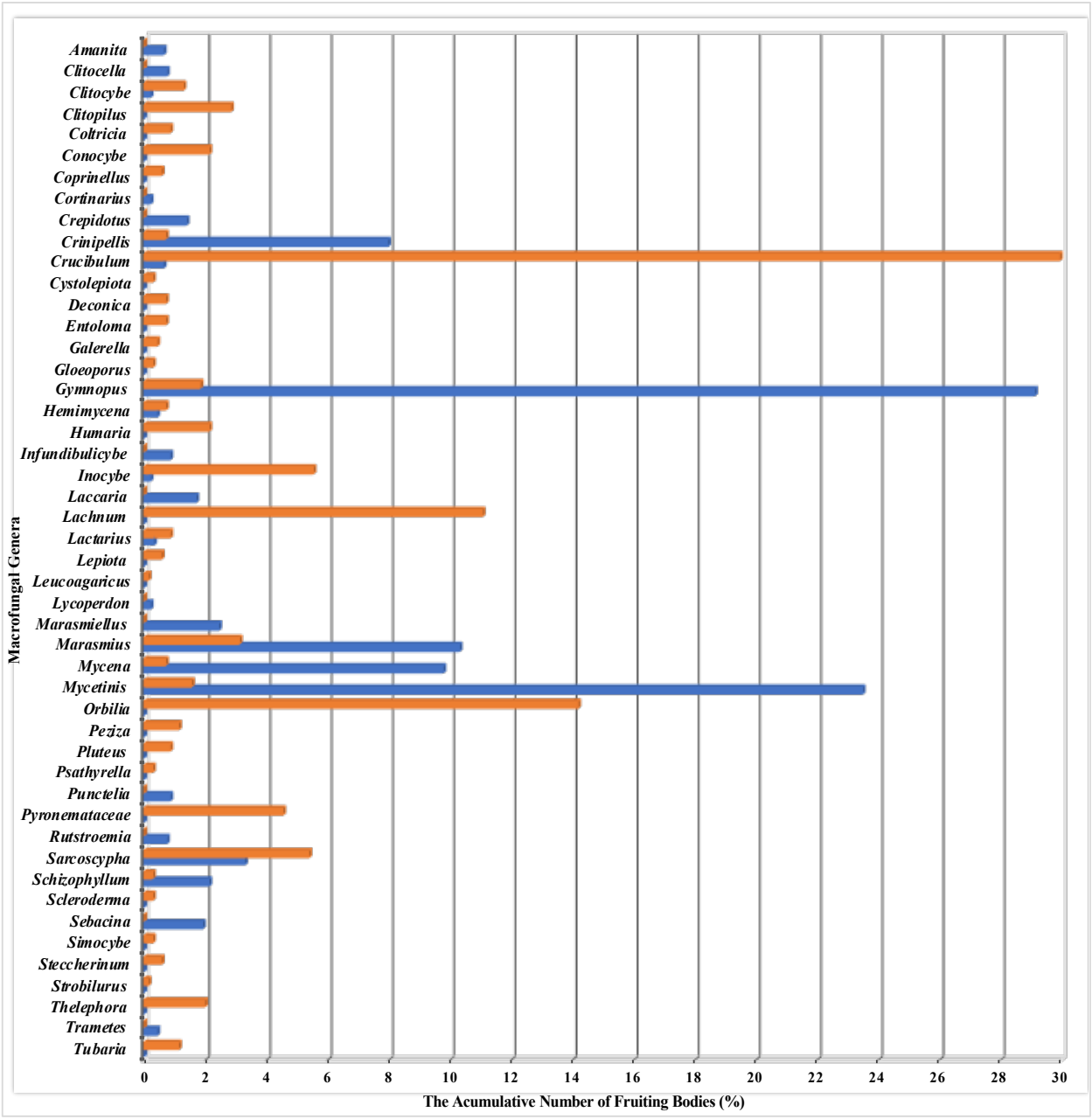

Fig. 5.1. The estimated percentages of the macrofungal genus productivity for burned (orange bars) and unburned (blue bars) sites based on a number of fruiting bodies produced by a particular species.

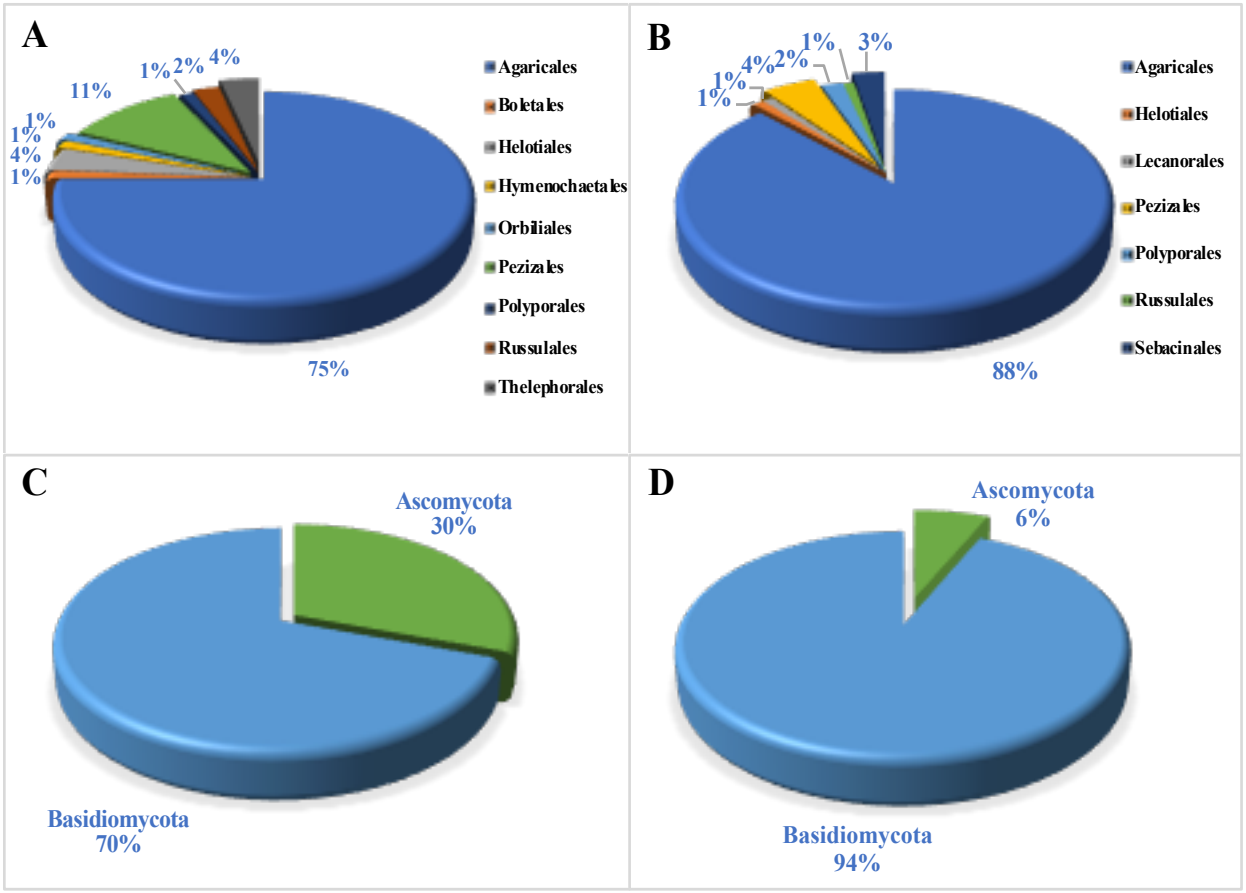

Fig. 5.2. The taxonomic comparisons of the fungal community assemblages in the burned and unburned sites based on the number of species per taxon. The percentages of orders in the (A) burned and (B) unburned sites and phyla in the (C) burned and (D) unburned sites.

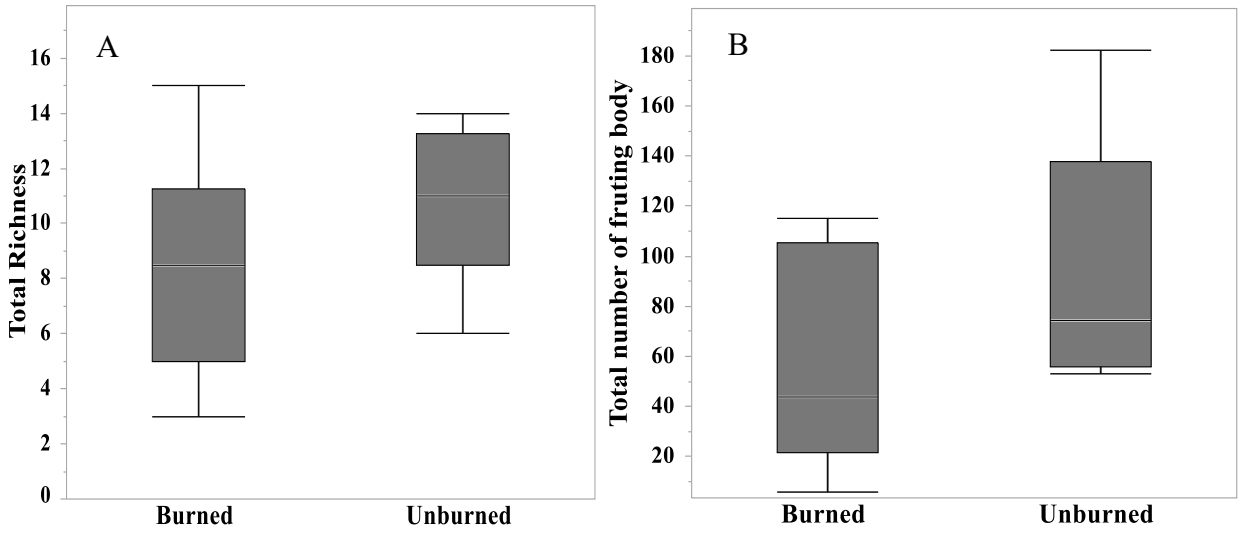

Fig. 5.3. The total species richness (A) and number of fruiting bodies (B) of litter-associated macrofungi for both investigated sites with no significant differences apparent between sites based on the pairwise Tukey's test at $P < 0.05$.

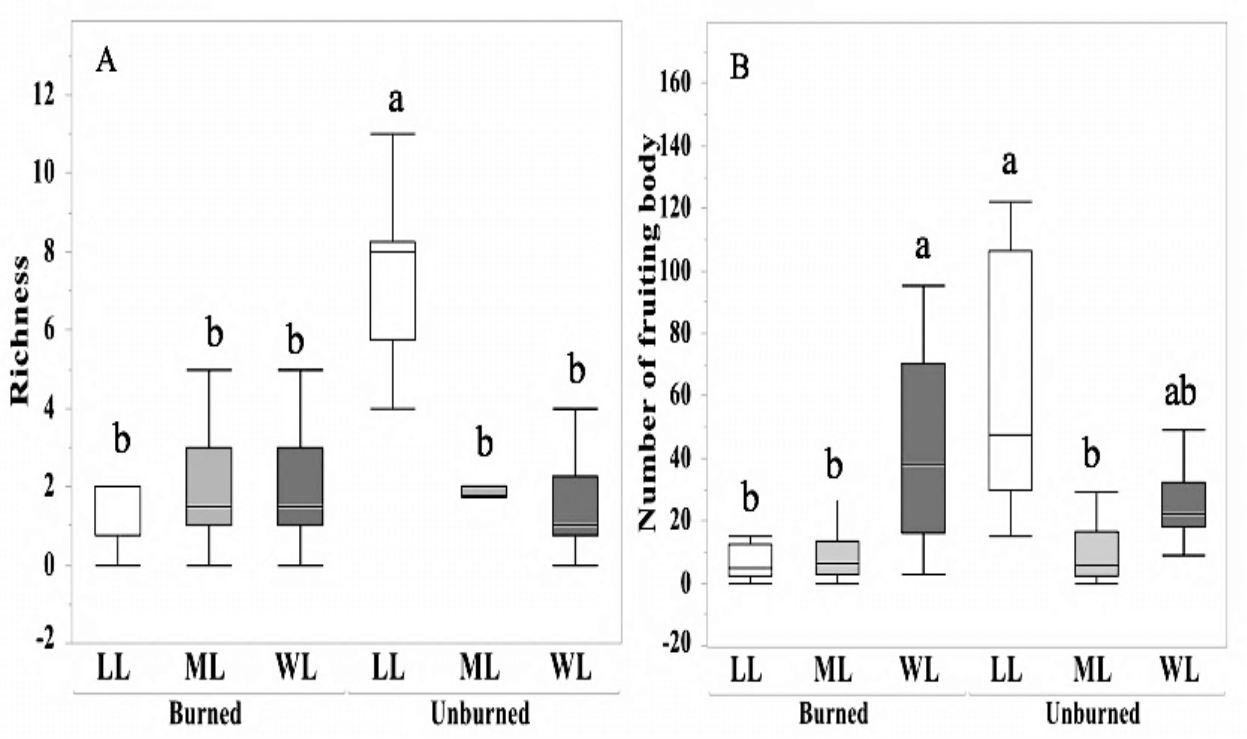

Fig. 5.4. The species richness (A) and number of fruiting bodies (B) of litter-associated macrofungal assemblages on the various microhabitats, leafy litter (LL), woody litter (WL) and mixed litter (ML), in burned and unburned sites. Bars without shared letters indicate significant differences as determined by the pairwise Tukey's test at $P < 0.05$.

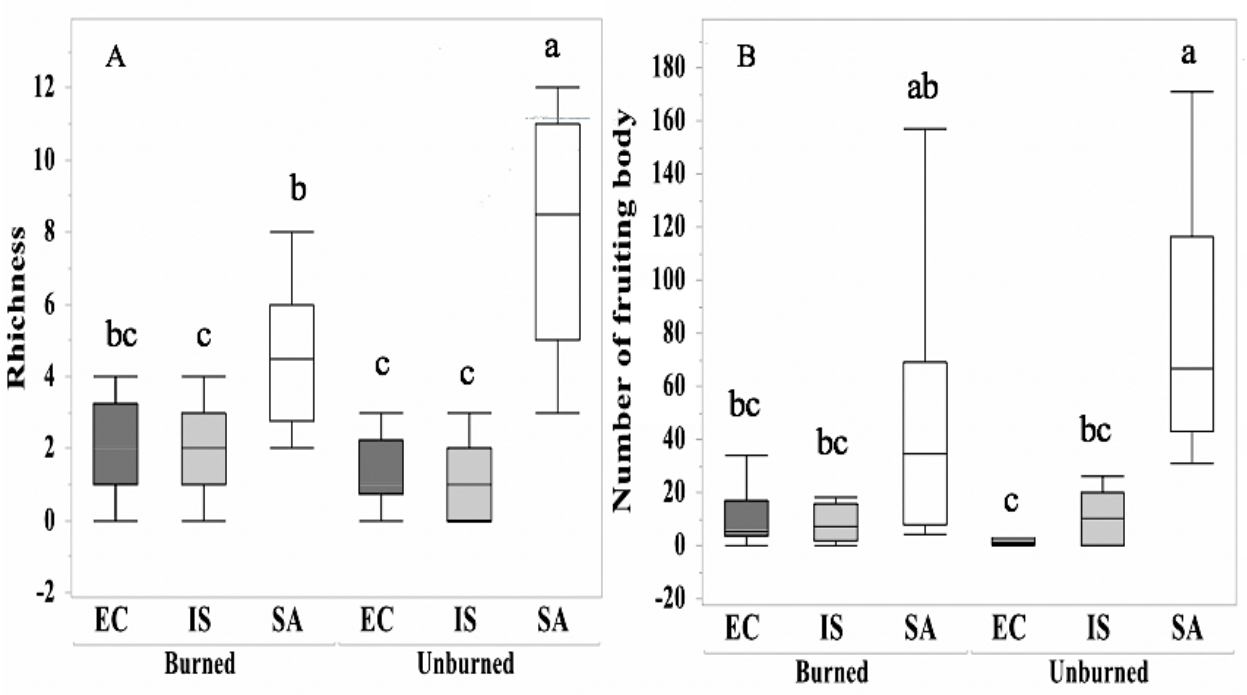

Fig. 5.5. Comparisons of the putative ecological functions of litter-associated macrofungi, ectomycorrhizas (EC), saprotrophs (SA) and *incertae sedis* (IS) guilds, in relation to species richness (A), and productivity of fruiting bodies (B). Bars without shared letters indicate significant differences as determined by the pairwise Tukey's test at $P < 0.05$. Lichenized fungi were excluded from the functional data due to low richness.

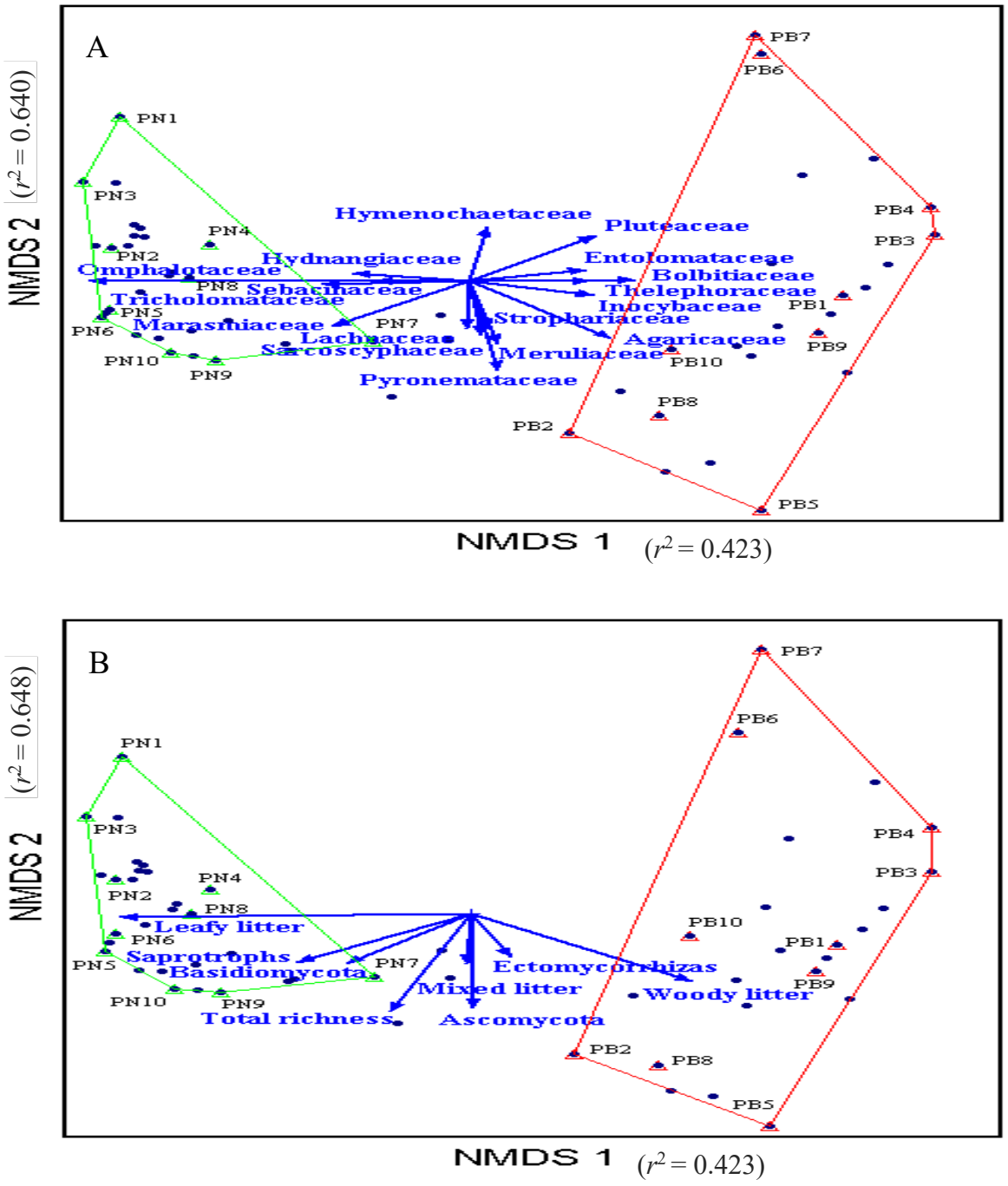

Fig. 5.6. Non-metric multidimensional scaling (NMDS) ordinations for (A) family compositions and (B) fungal structure variables of environmental variables overlaid as vectors on investigated plots, based on the richness data. Vector direction and length designate strengths of correlations within the ordinations.

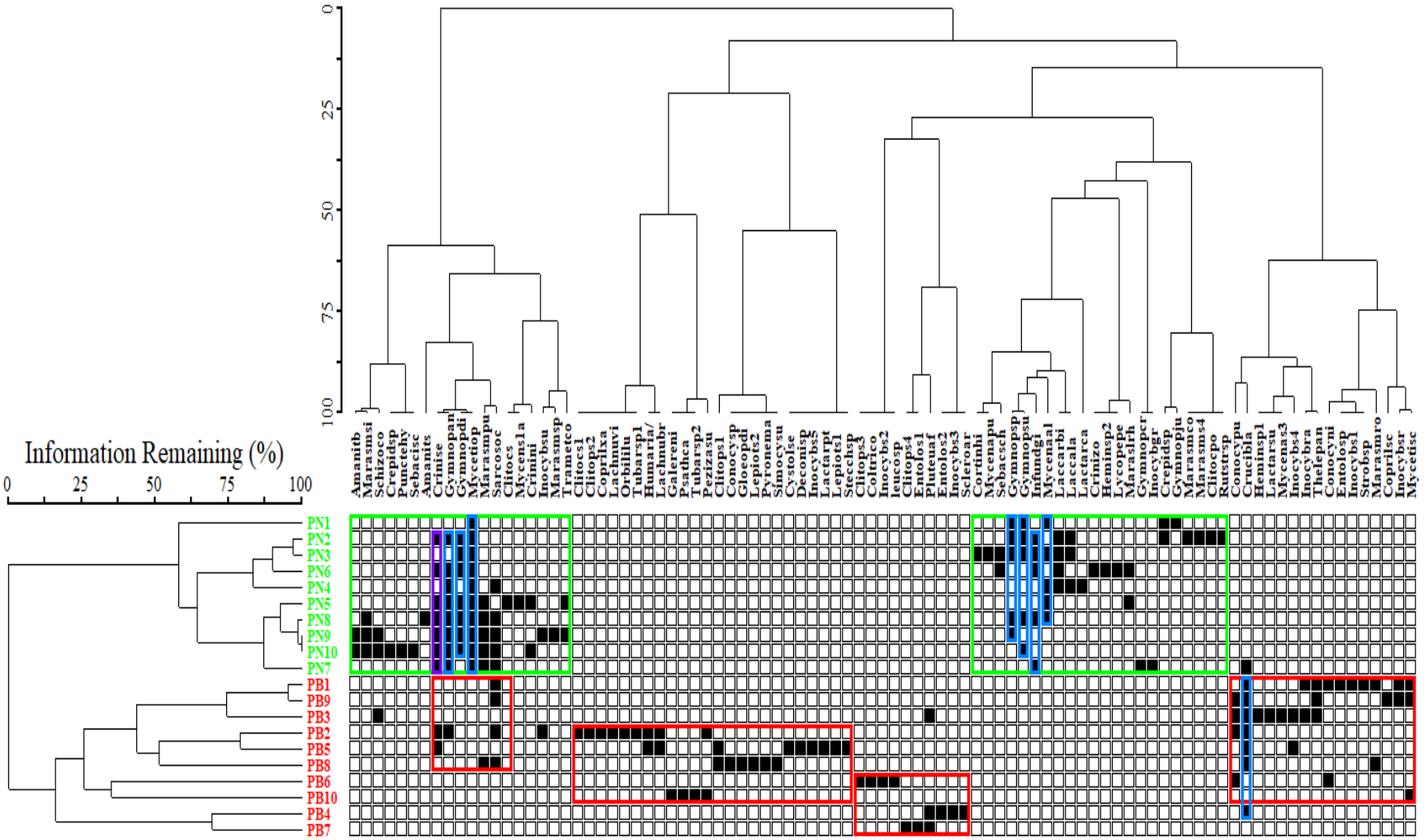

Fig. 5.7. The species distribution of 91 macrofungi, clustered on the horizontal matrix axis, across 20 investigated plots for unburned (green color) and burned (red color) sites and grouped on the vertical axis using two-way cluster analysis. The species coded the first letters of genus and species (see Table 4S for species names). The blue color represents indicator species for both study sites, with *P*-values ≤ 0.05, while purple indicates the species with a *P*-value = 0.066.

Table 5.1. The indicator values (IV%), standard deviation (S.D) and probabilities of litter indicator species with *P*-values ≤ 0.05 for both investigated sites, (Unburned (PN) and Burned (PB) sites.

Indicator Species	**Taxonomic Family**	**Microhabitat**	**Site**	**IV**	**S. D**	***P*-value**
Crucibulum laeve	Agaricaceae	Woody litter	PB	61	9.55	0.0220
Crinipellis setipes	Marasmiaceae	Leafy litter	PN	54	8.24	0.0656*
Gymnopus androsaceus	Omphalotaceae	Leafy litter	PN	71	8.32	0.0062
Gymnopus disjunctus	Omphalotaceae	Leafy litter	PN	70	8.31	0.0034
Gymnopus spongiosus	Omphalotaceae	Leafy litter	PN	50	7.86	0.0316
Gymnopus subnuda	Omphalotaceae	Mixed litter	PN	50	7.83	0.0312
Infundibulicybe gibba	Tricholomataceae	Leafy litter	PN	50	7.93	0.0318
Mycena albiceps	Mycenaceae	Leafy litter	PN	50	8.04	0.0348
Mycetinis opacus	Omphalotaceae	Mixed litter	PN	100	9.26	0.0002

**Crinipellis setipes* was included due to be the nearest *P*-value to the indicator value.

5.5 Discussion

5.5.1 Assemblages of litter-associated fungi: overall taxonomic diversity

The data presented herein provide a body of fundamental data on the assemblage of fungi associated with the forest floor litter, a largely understudied group (Baldrian, 2017). With the dominance of the Basidiomycota in both sets of plots, along with the Ascomycota in recently burned plots, litter-associated fungi clearly are characterized by a high level of taxonomic diversity and fruiting body productivity (Figs. 5.1 and 5.2). Presumably, diverse assemblages of tree species for the two sites in the current study could be attributed to providing various litter microhabitats for fungi to exploit, proliferate, develop an assemblage of taxa to function as imperative members of the forest ecosystems (Berg and McClaugherty, 2008; Baldrian, 2017; Elliott and Stephenson, 2018). Taking into account the cryptic species assemblages within these species, the species richness would be expected to rise from what the present results (Hawksworth and Rossman, 1997). Later, recycling biomass of litter associated fungi would add to the complexity of food web structures and exemplifies a large pool of the forest organic matter with potentially rapid turnover (Clemmensen et al., 2013; López-Mondéjar et al., 2018).

In the present study, prescribed burning apparently did not reduce the total species richness (Fig. 5.3), probably due to the contribution of post-fire assemblages of ascomycetes, displaying a greater fire severity tolerance than basidiomycetes (Claridge et al., 2009; Holden et al., 2013), and the survival or arrival of new propagules with a low level of fruiting body productivity (Robinson et al., 2008), as observed in the present study (Fig. 5.1). Reductions in the total fruiting body production for the burned study plots were recorded and likely are related to a compositional shift (Robinson et al., 2008) and reproductive traits (e.g. fruiting body size and number) of distinct macrofungal assemblages (Bassler et al., 2015). After prescribed burning, the

higher fruiting body production mostly depended on a single species, *Orbilia* as in the ascomycetes and *Crucibulum* for the basidiomycetes. Two species of ascomycetes *Lachnum* inhibited only woody litter. Conversely, the higher fruiting body productivity in the unburned plots resulted in the productivity of an assemblage of species of the dominant genera, likely leading to complementarity in the litter microhabitat use and a high level of litter decomposition. Additionally, macrofungal assemblages of burned plots were comprised mostly of species characterized by a large fruiting body size with a solitary occurrence or a few clustered fruiting bodies. The example included *Leucoagaricus* sp, *Pluteus leoninus* sensu Rea, Cooke, and *Psathyrella candolleana* (Fr.) Maire in the present study. Another reason could be weak competitors of the primary colonizers of unoccupied substrates such as woody litter, which are ultimately replaced by aggressive competitors such as basidiomycetous with hyphal cords (Boddy et al., 2008; Peay et al., 2013).

As already noted, few studies have described the responses of the macrofungi associated with litter to prescribed burning in temperate forests. Thus, the results obtained in the present study were compared to other studies carried out in other biomes, different fire types, fungal substrates, geographic regions, and techniques. Our results concur with reports of the responses of epigeous macrofungal richness in Australian eucalypt forests (Robinson et al., 2008), with few studies directed towards the exact microhabitats of litter fungi (Alanbagi et al., 2019). Similarly, dramatic responses noted for species richness and assembly shifts of soil fungi were detected previously after frequent prescribed fires (Brown et al., 2013) and different fire intensities in the pine forests of the United State (Reazin et al., 2016). It seems likely that the fungal assemblage was directly impacted by extremely high temperatures and that this type of disturbance could fully destroy fungal propagules, similar to what has been reported on soil and ectomycorrhizal

fungi, beginning with temperature as low as 50–60 ºC (Neary et al., 1999; Kipfer et al., 2010). In contrast to our results, increases in species richness and diversity of soil fungi were also recorded two years after wildfires across a boreal forest (Sun et al., 2015), and soil fungal diversity was not affected by controlled burns in pine forests in the United States (Oliver et al., 2015). However, distinct fungal assemblages with the prevalent functional redundancy of a particular assemblage would determine ecosystem process rates (Nielsen et al., 2011).

According to previous studies, the impact of prescribed burning as an important driver shaping macrofungal assemblages and levels of diversity in forest ecosystems cannot be substantiated because too few studies have been carried out. Most of the available data on soil fungi from European and Australian ecosystems were directed toward wildfire responses, so they may not be completely applicable to the forests of the eastern central United States.

5.5.2 Distributions of litter macrofungal communities on their microhabitats

The distribution of litter-associated macrofungi in relation to the available microhabitats varied in both study sites, and most of the macrofungi, are known to be lignin degraders (Osono, 2007; Boddy et al., 2008) because of efficiently secreted lignin enzymes (e.g., Berg et al., 2013; Voříšková et al., 2014). The species richness and the numbers of fruiting bodies of the fungal taxa that decompose leafy litter were significantly greater for unburned plots compared to burned plots (Figs. 5.1 and 5.3) and were clearly clustered on the NMDS axis 2 (Fig. 5.6B). These basidiomycete taxa are mostly members of the families Omphalotaceae, Marasmiaceae, and Mycenaceae known to be ligninolytic species, which are efficiently colonizers and decomposers, and are most of the individual components of the relatively homogeneous continuous leaf litter layer (Osono, 2007; Boddy et al., 2008).

Most of the indicator species for unburned plots were inhabitants of leaf litter, including *Gymnopus androsaceus*, *G. disjunctus*, *G. subnuda*, *G. spongiosus*, *Infundibulicybe gibba*, and *Mycena albiceps* (Fig. 5.7 and Table 5.1). These taxa displayed similar responses, but different species were noted in other observations following fires (Robinson and Tunsell, 2007; Greeshma et al., 2016). These data suggest that fire reduced or eradicated the inoculum potential availability among leaf litter fungi due to the high surface temperatures associated with litter combustion (Neary et al., 1999; Kipfer et al., 2010) and shifted the vertical distribution of those fungi that survived or recolonized litter at greater soil depths instead of the producing fruiting bodies (Clemmensen et al., 2015). Declining assemblages of surviving leaf litter fungi in the burned plots also could be a result of antagonistic or the greater competitive interactions of the abundant fine woody-inhabiting or inter-guild fungi (e.g., Osono, 2007; Bodeker et al., 2016) or even other groups of organisms (e.g., bacteria) (Tláskal et al., 2016).

Although significant quantities of very fine woody debris are often found exclusively on the forest floor, few studies have focused on the macrofungal community assemblages on this microhabitat (Küffer and Senn–Irlet, 2005). The present results indicated that a diverse array of species with a considerable number of fruiting bodies inhabited only woody litters or mixed (both woody and leafy litter) litter were found in both sites, due to tree species being present (Küffer and Senn-Irlet, 2005; Baldrian, 2017; Cline and Zak, 2015).

In contrast to leafy litter species in unburned plots, woody litter fungi and ectomycorrhizas were represented by the highest fruiting body productivity in burned plots (Figs. 5.5 and 5.6). The creation of new niches for very fine woody litter (WL) involving properties such as small size, closer contact with wetter soil and changed nutrient resources following fire, would likely favor those ascomycetes with fungi associated with wood (Pietikäinen et al., 2000; van der Wal et al.,

2007), especially those species with the greatest fire tolerance (Peay et al., 2013; Holden et al., 2016). Although the survival of fungal propagules remains unclear following fires, the inoculum of WL fungi presumably would be supported by mycelia that survive the fire and resistant propagules from the unburned patches or areas, deep soil profile, or the pre-fire soil spore bank (Figs1.5C and D; Cairney and Bastias, 2007; Claridge et al., 2009). This would trigger a greater abundance and richness of woody litter-inhabiting fungi and preferred ectomycorrhizas in the burned plots and their interactions with other colonizers (e.g., Dahlberg, 2002; Pietikäinen et al., 2000). Thus, the mycelia of woody litter species and ectomycorrhizas later foraged and recolonized such nutrient-rich resources as newly fallen leaves and contained the mycelia of initial decomposers in order to develop growth and competition strategies (Fig.1.5C; Robinson et al., 2008; Hardoim et al., 2015, Baldrian, 2017; Dove and Hart, 2017), enhancing niche overlap and increasing species richness of mixed microhabitat taxa in the burned plots (Figs. 5.4 and 5.6B). Only *Crucibulum leave*, frequently colonizing woody litter and known as a ligninolytic fungus (Casieri et al., 2010) was an indicator species for burned plots (Fig. 5.7 and Table 5.1), suggesting that it is survived vegetatively.

The changes in litter microhabitat composition and redistribution patterns of fungi on preferable microhabitats in the burned plots may need several years to recolonize the same microhabitats and fully recover their biological activities, and these responses also influence the functional redundancy of the litter macrofungal assemblages, including those affecting the litter decomposition process (Holden et al., 2013; Köster et al., 2015).

5.5.3 Comparison of functional diversities of litter-associated macrofungi

In spite of a complete shift in the macrofungal assemblages in burned microhabitats (Figs. 5.2 and 5.4), the dominant functional group, the saprotrophs, were not altered, but such a functional

change in fungal assemblages was also documented in both investigated sites (Fig. 5.5). That suggests distinct taxonomical and functional assemblages on various microhabitats may share similar genetic profiles for active members of fungal litter decomposition (Sun et al., 2015; Bahnmann et al., 2018). The species richness and fruiting body production of functional groups reported herein theoretically preserved saprotrophic stability for the primary decomposers in the various litter microhabitats (Voříšková et al., 2014; Alanbagi et al., 2019) as well as serving as facultative biotrophs with plant roots, as reported recently (Smith et al., 2017). The ability of saprotrophic mycelia for productions of fruiting bodies displayed a drastically greater response to fire than ectomycorrhizal fungi, suggesting differing tolerances to fire between the two guilds and the probable survival of soil or substrate propagules of certain functional species (Cairney and Bastias, 2007; Robinson et al., 2008; Sun et al., 2015). The reduced richness and shifting the vertical distribution of the assemblage of saprotrophs on burned litter may allow the proliferation of opportunistic saprotrophs and a switch in functional guilds, as ectomycorrhizas possess less efficient decomposer abilities (Bodeker et al., 2016; Clemmensen et al., 2015). Some ectomycorrhizal species or some fungi in the "*incertaine sedis*" group may switch from symbiosis to saprotrophism, for example, to support their energy needs for N and other nutrients (Dahlberg, 2002; Sun et al., 2015; Bodeker et al., 2016) based on dominant or available microhabitats in both types of study plots and thus develop fruiting bodies across accumulative heterogenous litter (Küffer and Senn-Irlet, 2005; Verbruggen et al., 2017).

Sharing microhabitats and altered functions for previous reasons by ectomycorrhizal fungi were documented in both study sites, with insignificant differences (Figs. 5.4 and 5.5), but spatially characterized the assemblage of litter-associated ectomycorrhizas on burned plots (Fig. 5.6). Some of these genera were also previously recorded on oak litter (Voříšková et al., 2014;

Verbruggen et al., 2017). In the upper horizons of burned plots, the ectomycorrhizal response is likely due to the lowered richness and mycelial growth required to sustain fruiting body formation by saprotrophic competitors (Fig. 5.5), and the sudden availability of supposedly uncolonized substrates, mostly leafy litter (Fig. 5.4). Photosynthate allocation during the summer would probably meet the ectomycorrhizal carbon needs for litter decomposition and fruiting body formation (Voříšková et al., 2014; Verbruggen et al., 2017). Those observations not only provide evidence of overlapping niches and functions (Sun et al., 2015; Bodeker et al., 2016) but also suggest shifts in the fungal competitive balance in that complex transitions toward increasing nutrient resources and potentially unfavorable microclimate alterations after fire disturbance (e.g., Cairney and Bastias, 2007; Clemmensen et al., 2015). In that case, reduction in mycorrhizal root colonization would be expected (Treseder, 2004; Taylor and Midgley, 2018), but no correlations between fruiting body formation and ectomycorrhizal root tips of leguminous trees were observed in undisturbed forests by Smith et al. (2017). Overall, the fruiting body responses of mycorrhizal fungi to fire disturbances are still uncertain (Taylor and Midgley, 2018), and their influence on litter decomposition under natural field conditions is much more problematic (Fernandez and Kennedy, 2016).

5.6 Conclusions

A high level of fungal taxonomic diversity exists for various litter microhabitats in both types of investigated plots (recent burned and unburned plots) in the present study. Prescribed burning altered the structure of fungal assemblages. Although total species richness did not differ between the two sites, significant variation was apparent in macrofungal species richness on the various litter microhabitats, probably in response to fewer propagules and lower competitive

abilities of growing mycelia, microclimate conditions, and a type of microhabitat in which fungi produced fruiting bodies. The fruiting body productivity of Ascomycota increased significantly following fire compared. Although the functional redundancy, as saprotrophs, did not change, a functional shift in fungal assemblages was documented between investigated plots, likely a response to meet energy needs during fruiting body productions. The fungal litter assemblage changed from mostly basidiomycete leafy litter species to a mixture of ascomycete and basidiomycete woody litter species, with a dominance of saprotrophs as a result of burning. Ectomycorrhizal fungi associated mostly with leafy and mixed litter-microhabitats and contributed to the increase in richness and fruiting body production of the burned plot fungal assemblage.

5.7 References

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5.8 Supplemental Materials

Table 5.1S. The results of prescribed burning effects based on richness of fungal taxonomic group across study plots (n=20) as revealed by correlation coefficients between fungal families recorded (A) and community variables (B) with ordination axes using two separated nonmetric multidimensional scalings (NMDS). Negative and positive values indicate strongly corelated with ordination axes at $|r| > 0.5$ and are plotted in the NMDS figures. Weak correlations with NMDS axes are not included in the table.

Variables	**NMDS1 Correlation**	**NMDS2 Correlation**
A- Fungal families		
Agaricaseae	0.566	-0.493
Bolbitiaceae	0.613	0.057
Entolomataceae	0.515	0.211
Inocybaceae	0.532	-0.248
Pluteaceae	0.537	0.435
Thelephoraceae	0.525	-0.023
Meruliaceae	0.254	-0.516
Pyronemataceae	0.257	-0.615
Stropharaceae	0.211	-0.448
Hymenochaetaceae	0.208	0.478
Hydnangiaceae	-0.515	0.181
Marasmiaceae	-0.556	-0.434
Omphalotaceae	-0.929	-0.006
Sarcoscyphaceae	-0.056	-0.448
Sebacinaceae	-0.462	-0.001
Tricholomataceae	-0.579	-0.127
B- Fungal community variables		
Ascomycota	0.081	-0.628
Ectomycorrhizas	0.299	-0.424
Woody litter	0.699	-0.533
Basidiomycota	-0.525	-0.455
Leafy litter	-0.879	-0.126
Mixed litter	-0.103	-0.455
Saprotrophs	-0.621	-0.45
Total richness	-0.417	-0.641

Table 5.2S. Macrofungal taxa with their codes for the two-way cluster analysis.

Macrofungal Taxa	**Code**
Amanita bisporigera	Amanitb
Amanita sp.	Amanits
Clitocybe sp.1	Clitocs1
Clitocybe subditopoda	Clitocs
Clitopilus sp. 1	Clitops1
Clitopilus sp. 2	Clitops2
Clitopilus sp. 3	Clitops3
Clitopilus sp. 4	Clitops4
Coltricia confluens	Coltrico
Conocybe pubescens	Conocypu
Conocybe sp.	Conocysp
Conocybe nigrescens	Conocyni
Coprinellus xanthothrix	Coprilxa
Coprinellus sclerocystidiosus	Coprilsc
Cortinarius hinnuleoarmillatus	Cortinhi
Crepidotus sp.	Crepidsp
Crepidotus sp. 2	Crepidsp2
Crinipellis nigricaulis	Crinini
Crinipellis setipes	Crinise
Crinipellis zonata	Crinizo
Crucibulum laeve	Crucibla
Cystolepiota seminuda	Cystolse
Deconica sp.	Deconisp
Entoloma sp. 1	Entolos1
Entoloma sp. 2	Entolos2
Entoloma sp. 3	Entolosp3
Galerella nigeriensis	Galereni
Humaria sp.	Humarisp
Gloeoporus dichrous	Gloeopdi
Gymnopus androsaceus	Gymnopan
Gymnopus cremeostipitatus	Gymnopcr
Gymnopus disjunctus	Gymnopdi
Gymnopus junquilleus	Gymnopju
Gymnopus spongiosus	Gymnopsp
Gymnopus subnuda	Gymnopsu
Hemimycena sp.	Hemimysp1
Hemimycena sp. 2	Hemimysp2
Infundibulicybe gibba	Infundgi
Inocybe sp. 4	Inocybs4
Inocybe grammata	Inocybgr
Inocybe radiata	Inocybra
Inocybe sp. 1	Inocybs1
Inocybe sp. 2	Inocybs2
Inocybe sp. 3	Inocybs3
Inocybe sp. 5	Inocybs5

Table 5.2S Cont.

Macrofungal Taxa	**Code**
Inocybe subfulva	Inocybsu
Inocybe subradiata	Inocybsr
Laccaria bicolor	Laccarbi
Laccaria laccata	Laccala
Lachnum brevipilosum	Lachnubr
Lachnum virgineum	Lachnuvi
Lactarius camphoratus	Lactarca
Lactarius pterosporus	Lactarpt
Lactarius subserifluus	Lactarsu
Lepiota sp. 1	Lepiots1
Lepiota sp. 2	Lepiots2
leucoagaricus sp.	leucosp
Lycoperdon perlatum	Lycopepe
Marasmiellus rhizomorphigenus	Maraslrh
Marasmius conchiformis	Marasmco
Marasmius fulvoferrugineu	Marasmsi
Marasmius sp. 2	Marasms2
Marasmius sp. 4	Marasms4
Marasmius pulcherripes	Marasmpu
Marasmius rotula	Marasmro
Mycena albiceps	Mycenaal
Mycena pura	Mycenapu
Mycena sp. 3	Mycenas3
Mycena sp.1a	Mycens1a
Mycetinis opacus	Mycetiop
Mycetinius scorodonius	Mycetisc
Orbilia luteorubella	Orbililu
Peziza succosa	Pezizasu
Pluteus leoninus	Pluteuaf
Psathyrella candolleana	Psathca
Pyronemataceae sp.	Pyronesp.
Punctelia hypoleucites	Punctehy
Clitocybe popinalis	Clitocpo
Rutstroemia sp.	Rutstrsp
Sarcoscypha occidentalis	Sarcosoc
Schizophyllum commune	Schizoco
Scleroderma areolatum	Scleroar
Sebacina schweinitzii	Sebacsch
Sebacina cadida	Sebacisc
Simocybe sumptuosa	Simocysu
Steccherinum sp.	Stecchsp
Strobilurus sp.	Strobsp
Thelephora anthocephala	Thelepan
Tubaria sp.1	Tubarsp1
Tubaria sp.2	Tubarsp2
Trametes conchifer	Trametco

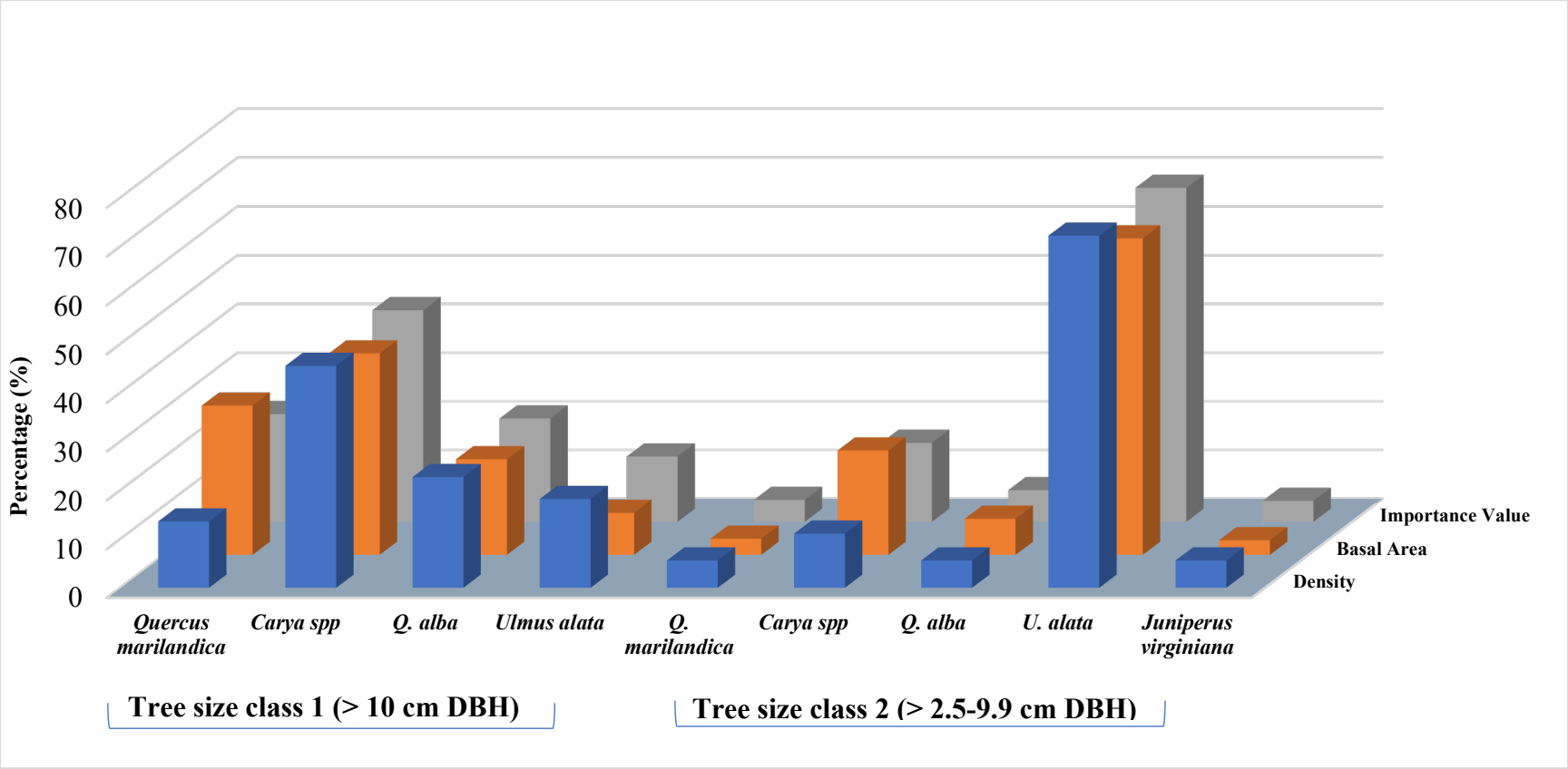

Fig. 5.1S. The forest composition of the sampled sites after dividing trees into large tress with stems ≥ 10 cm diameter at breast height (DBH) and small trees with stems ≥ 2.5-9.9 cm DBH.

Chapter 6. General Conclusions

The dissertation projects generated datasets on the taxonomic and functional diversity of litter-associated fungi in the forest floor litter of two temperate deciduous forests in northwest Arkansas, including macrofungal distribution on various litter microhabitats. It characterized fungal communities and the compared effects of such factors as vegetation, various type of microhabitats, and the environmental conditions related to the investigated sites on both macrofungal species richness and productivity as well as on community assemblages. The findings outlined herein provide a deeper insight into life the in the forest floor litter microhabitat of temperate forests, using data from a field-based fruiting body inventory, incubation chambers, Sanger sequencing and DNA-metabarcoding techniques.

The inventory and incubation chamber methods for obtaining fruiting bodies yielded a total of 126 taxa and showed that the fungi associated with the forest floor litter habitat are taxonomically diverse and make up dynamic assemblages. A high level of fungal taxonomic diversity was determined for heterogenous litter. The nutrient-rich microhabitats of the latter probably support specific populations that play essential roles in decomposition. More than 60% of the taxa recorded species were unique to one of the investigated sites, although the total species richness and number of fruiting body did not differ significantly between the two study sites. Each specific type of litter microhabitat was characterized by a unique fungal community structure. However, species that are known to be associated with leafy litter colonized most of these microhabitats. The litter layers of study sites were apparently inhabited by diverse hidden foraging mycelia that obviously succeed in sexually reproducing and form fruiting bodies on various litter microhabitats under suitable environmental conditions.

Litter-associated fungi have achieved a high degree of ecological flexibility to grow and reproduce in a wide range of litter microhabitats, and these reflect their diverse lifestyle patterns as documented in the present study. The contribution of fungi to the overall ecology of forest ecosystems should not be ignored, since this group occupies a series of functionally diverse guilds with various litter microhabitat preferences. Although the nature of the growth and the development of litter-associated fungi along with their eco-physiological features differ considerably, the results obtained in this research suggest that they contribute greatly to the structural stability, nutrient availability, productivity and other critical aspects of ecosystem functioning. Their influences are similar to those of the assemblages of fungi associated with other substrates such as coarse woody debris. The present study documented these hidden assemblages of fungi for the first time in northwest Arkansas, using Sanger sequencing technology to identify the various taxa present. Several of these undoubtedly represent new records for both northwest Arkansas and the Ozark region of the central United States.

In fact, Sanger sequence analyses of fruiting bodies detected a considerable number of specimens (approximately 41%) that could not be matched beyond the genus level with anything in GenBank. It seems likely that the majority of these are either relatively rare taxa yet to be sequenced or possibly taxa new to science. Taking into the account the cryptic species that exist within a particular species complex, noticeably greater levels of species richness, taxonomic and functional diversity, and microhabitat distribution may exist for litter-associated fungi than currently realized. This is suggested from the results obtained in the current study, based on the sampling of fruiting bodies and the partial sequences of the internal transcribed spacer 1 and 2 along with the complete sequence of the 5.8S ribosomal RNA gene. The assemblage of litter-associated fungi needs to be investigated further in order to resolve species complex using

different molecular approaches. Several studies have been estimated the number of fungal species on the earth to be 1.5- 12 million, but these investigations described above are likely to reveal an even greater diversity when combining data from traditional and molecular methods along with specific methods required to detect cryptic species.

The metagenomic results using next-generation sequencing provide a deeper insight into fungal identification and diversity in different microhabitats that are primarily limited by methodology related to the traditional inventory and Sanger sequencing approaches, sometimes complemented by culture-dependent procedures. In the research described herein, rDNA-metabarcoding of the fungal ITS2 region generated a more meaningful body of data through direct samplings of leaf litter as the main component of the litter forest floor habitat in these deciduous forests. Through the use of manually derived taxonomic identifications, the metataxonomic analyses detected 415 taxa. However, it should be noted that leaf litter materials preserve and store macrofungal propagules from diverse sources and macrofungal habitats. These propagules certainly include those produced by non-litter inhabiting fungi. Nevertheless, barcoding approaches scale up the discovery of hidden fungal diversity. The number of the identified OTUs (hypothesis species) was substantially greater in the rDNA metabarcoding sequencing data than the fruiting body inventory method for the same sampling sites. The overlap proportions of sequences gained with rDNA-metabarcoding methods compared with those obtained with the classical method was only18.56% for genera and 2.84% for species. Several reasons could explain this distinct difference between direct and indirect sequencing of leaf litter associated fungi. The inventory method detects only species that most commonly colonize, decompose, and reproduce in/on the forest floor litter habitat, with a distinct preference for particular litter microhabitats and often restricted to a specific type or just one part of the

overall litter habitat. In contrast, the rDNA barcoding techniques identified the majority of the hyphal foraging species in/on the leaf litter microhabitat. Numerous saprotrophs of litter and wood have been revealed to use mycelial strands, mycelial cords, and rhizomorphs to enhance their exploratory abilities. Those with diverse dispersal patterns with huge numbers of macrofungal spores are more likely to reach the forest floor litter habitat. This presumably explains one of the reasons for the greater number of taxa in the DNA-based leaf litter sampling than in inventory methods. In addition, fruiting bodies do not necessarily reflect actual fungal proliferation. Detecting species that rarely produce fruiting bodies in forest floor litter generally requires different methods from the classic inventory method, and the persistence their propagules or genetic materials on leaf litter is more likely to be identified meta-taxonomically using metabarcoding techniques. In the present study, the smallest overlap between macrofungal communities, based on gathering fruiting bodies and molecular sampling, suggests that both approaches should be considered if the goal is to recover the greatest breadth of taxa and thus to gain a more complete understanding of fungal diversity data. More molecular methodologies are also needed for describing the species hidden in each species complex.

The research described herein also provided a profound body of data relating to the impact of repeated prescribed burning on macrofungal community composition, the richness of the taxonomic assemblages and functional groups and their distribution on various litter microhabitats. Compositionally, prescribed burning shifted the structure of fungal assemblages. Although total species richness did not differ between the two study sites investigated, significant variation was apparent in macrofungal species richness on the various litter microhabitats. The fungal litter assemblage shifted from mostly basidiomycete leafy litter species to a mixture of ascomycete and basidiomycete woody litter species as a result of burning. That is

probably a response to fewer propagules and lower competitive abilities to grow mycelia, microclimatic conditions, and the type of microhabitat in which particular species of fungi actually produces fruiting bodies. Although the functional redundancy, as saprotrophs, did not change, a functional shift in fungal assemblages was documented between the investigated plots, likely a response to meet energy needs during the production of fruiting bodies.

Overall, considerable background knowledge—including the macrofungal community structure, litter microhabitats and functional assemblages in the heterogeneous litter layer—is essential for assessing macrofungal diversity. This knowledge also is essential for future forest management decisions with the objective of preserving fungal richness, diversity, productivity, microhabitats, and ecological function along with the more obvious forest management goals of maintaining and restoring both natural forests and planted forests.