2009

IDENTITY AND DIVERSITY OF AGARICOMYCETES (FUNGI: BASIDIOMYCOTA) IN TEMPERATE AGRICULTURAL SOILS

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(Spine title: Diversity of Basidiomycota in temperate agricultural soils)

(Thesis format: Monograph)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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Abstract

The objectives of this study were to identify and to compare the species richness, community composition and genetic diversity of Agaricomycetes in Michigan agricultural soils. Ribosomal DNA sequences were obtained from soils in four treatments: conventional till monocrop, no-till monocrop, historically tilled grassland and never-tilled grassland. The Agaricales dominated the community, with 85 % of all detected taxa. The taxonomic composition of conventional till plots was different from other plots due to the high prevalence of *Pneumatospora* (Cantharellales) and *Lachnella/Calathella* (Agaricales) clades and the absence of *Hygrocybe* (Agaricales) and Polyporales clades. These latter clades dominated less disturbed treatments. Genetic diversity of never-tilled plots was greater than in other treatments. Twenty-seven of the 52 species detected were also detected at this site two years earlier. This rate of re-detection suggests that the fungal community is relatively stable and that results of DNA-based survey techniques are repeatable across years.

Keywords: Basidiomycota, agaricomycotina, soil, agriculture, diversity, taxonomic composition, molecular ecology, phylogenetics, sequencing, DNA, basidiomycetes.

Acknowledgements

Many life changes have happened since I started this project and I am grateful for the understanding and guidance of my supervisor Dr. Greg Thorn. Without his support over these years I would have certainly been overwhelmed with all the other responsibilities in my life and surely not pursued closure on this project.

I would like to thank the members of my committee: Dr. Marc-André Lachance for his support and advice on many issues including many hours spent troubleshooting molecular protocols and the time spent reviewing this thesis, and Dr. Ed Topp for his advice and support.

Within the Thorn lab, I would like to acknowledge the help of Michael Lynch who was always available to answer my many question regarding laboratory protocols and computer programs. I would also like to thank Michael for providing past data for comparison. I am also grateful for the many conversations, technical support and guidance provided by Angela Dang in the Molecular Biodiversity, Ecology and Environmental Sciences (MoBEES) laboratory.

I thank all those who helped coordinate space and equipment at Kellogg Biological Station in Michigan and who offered advice for sampling and data analysis, namely A. Corbin, Dr. G. Philip Robertson and Dr. A. Stuart Grandy. I would also like to thank Kellogg Biological Station LTER for financial support through their student research grant and the LTER Network Office for student conference travel support.

Last but not least I am indebted to Robin Bloom for the hundreds of hours spent discussing this research and its analysis as well as reviewing my written work.

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List of Abbreviations and Symbols

ACE	Abundance-based coverage estimator
ANOVA	Analysis of Variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
B001	Reverse primer with recognition site 1093bp downstream from the 5' end of the nLSU. Specific to Basidiomycota (93)
B2R+	Forward primer, attachment location 1003 upstream from the 3' end of nSSU. Specific to Agaricomycetes (93)
BLAST	Basic Local Alignment Search Tool (4)
Bp	nucleotide base pairs
CT	Conventional Till
D1-D2	Domain 1 and Domain 2, two variable regions of the ribosomal large subunit
DDBJ	DNA Database of Japan
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylene diamine tetraacetic acid, a metal ion chelator
EMBL-Bank	European Molecular Biology Laboratory nucleotide database
GenBank	Nucleotide database of the National Center for Biotechnology Information
HTS	Historically Tilled Successional
Kb	Kilo base pairs = 1000 base pairs
KBS	Kellogg Biological Station
KMO	Kaiser-Meyer-Olkin test
LPI	Lincoln-Peterson Index
LTER	Long Term Ecological Research
MANOVA	Multivariate analysis of variance
MOD-LR3	modified LR3 primer from Vilgalys, R., and M. Hester (141)
NCBI	National Center for Biotechnology Information, Bethesda, MD, USA
nLSU	nuclear ribosomal large subunit
nSSU	nuclear ribosomal small subunit

NT	No Till
NTS	Never Tilled Successional
OTU	Operational Taxonomic Unit
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
Rxn	Reaction
t-RFLP	Terminal Restriction Fragment Length Polymorphism
U	Units

CHAPTER 1: INTRODUCTION

Chapter 1: Introduction

"In spite of all the miracles of technology, we still depend on green plants for our food. The green plants in turn depend on the soil in which they grow. The importance of the dark world under our feet is indisputable." (5)

The relationship between soil ecosystem functioning and aboveground primary productivity is the focus of much of the current interest in soil ecological research. This interest stems from concerns about our ability to maintain crop yields without degrading the soil resource. The large increases in crop yields over the past century have been achieved through external inputs such as inorganic fertilizers, crop genetics, and advanced machinery. These types of inputs are not likely to offer large increases in crop yields in the future (99). As a result more attention is being given to the role of soil biota in providing ecosystem services such as nutrient retention, soil structure and carbon storage, that are necessary for maintaining primary productivity.

Crop yields have continually increased through the 20th century (46), but this trend has required increasing rates of nutrient input over the years (124, 149). As a result Miller (99) notes that "identifying the wellspring from which increased productivity will be forthcoming in the decades ahead is less obvious." The current challenge facing sectors dependent on soil ecosystem services (i.e. agriculture, forestry) is to maintain yields while sustaining the ecological processes of the biological systems on which they depend (91, 99).

In the Ecological Society of America's proposal for the Sustainable Biosphere Initiative (an initiative to determine the role of ecological science in management and maintenance of the Earth's resources), Lubchenco *et al.* (91) identified soil ecosystems as a research priority. In agriculture, crop yields depend heavily on the ability of soil to provide numerous ecosystem services such as pore space for root penetration and nutrient retention. Many of these services are driven by the biotic community in soils (22, 63, 138, 146, 149, 151). However, limited knowledge of the taxonomic composition and interspecific interactions of microbial communities means that we do not yet understand the key players, their individual roles, nor the community dynamics that govern net ecosystem functions. We therefore do not understand how current agricultural practices such as tilling, fertilization and mono-cropping[1] change the community taxonomic composition and related functional capabilities (41, 82, 137). Greater knowledge of the taxonomic identity of the biological community in agricultural soils and how current agricultural practices alter this community composition are increasingly important research questions (91, 99).

Poor understanding of the soil biotic community can at least in part be attributed to the difficulties of studying organisms in the soil (82, 152). *In situ* visual observation of soil organisms and associated processes is not possible due to the opaque nature of the soil matrix. Soil is chemically complex and highly heterogeneous (111, 116), even under long-term disturbance regimes such as tillage that homogenize the soil environment

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[1] Mono-cropping is referred to here as the growing of single crop species on a plot such as wheat, corn or soybean. These monocultures may be planted on a yearly rotation basis.

(121). Analytical methods are further complicated by the small size and high diversity of organisms that live in the soil matrix (39, 50, 116). These small organisms are not easily identifiable as they lack unique features on which reliable morphological identification can be based. This has led to unstable classification schemes and poor taxonomic knowledge of soil organisms (107), and ultimately to a lack of understanding of abundance and distribution of soil microorganisms. In contrast to sampling macro-organisms such as plants, counting of microbial taxa in a given area of soil by microscopic examination of the soil is impractical both because of the number of organisms likely to be encountered and the difficulty in identifying them. Given these factors it is not surprising that knowledge of soil biotic community is limited (41).

Studies of many soil microbial communities have been restricted by the limitations of available techniques (e.g., community composition using culture-based methods and morphological taxonomy) but, following the first successful extraction of DNA from soil (139), the recent adaptation of molecular methods for use in analyzing soil samples has opened new avenues by which to explore these communities (41, 82, 137). Studies employing molecular techniques have identified many taxa in soils that were undetectable by other methods. This new information on soil biotic communities diversity has raised many new questions on how this high diversity relates to maintenance of ecosystem services.

Three main hypotheses regarding the relationship between ecosystem function and species richness have been proposed: i) The "redundancy" hypothesis where species richness does not directly affect ecosystem function as most species overlap in function; ii) The "rivet" and or "linear" hypotheses which postulate that each species contributes to

have attributed important soil functions such as nutrient retention, carbon storage, and particle binding (22, 150) to Agaricomycetes, but they seldom appear in soil biota studies (10, 138).

The Agaricomycetes are key players in the degradation of complex carbon sources. They are among the few organisms that have the capacity to metabolize lignin and thus have obtained a pivotal role in the functioning of terrestrial ecosystems (85). This complex carbon compound makes up a substantial proportion of terrestrial biomass as it is present in all woody plant tissues and provides the rigidity needed by many land plants to stand upright (57). Crawford (26) estimates that lignin is the most abundant aromatic compound on earth and is only second to cellulose in living terrestrial biomass. As easily degradable carbon (cellulose and hemicellulose) is contained within the lignin matrix, lignin degradation is an important rate limiting step in nutrient turnover in soils (57).

The Agaricomycetes are also associated with the stabilization of soils through their filamentous growth form (8) and extracellular exudates (such as glycoproteins) that entrap soil particles in small aggregates, creating pore space (138). Such microstructure allows for water retention and gaseous exchange within soil and facilitates plant root penetration.

Filamentous fungi are capable of moving through soil structures, bridging pore spaces and crossing nutrient-poor areas via their extensive hyphal networks (9). The tough cell walls of many Agaricomycetes make them well suited to crossing both dry and nutrient-poor areas(18).

Agaricomycetes and other macro-fungi are also involved in the transportation of nutrients through soil via their hyphal network (72, 147, 151). The tough cell walls of fungi are also believed to increase nutrient retention in soils based on the supposition that the walls are resistant to decomposition, essentially 'locking up' nutrients within them and causing longer residence times before mineralization (55).

The importance of studying Agaricomycetes

One of the most convincing reasons to conduct studies on diversity of Agaricomycetes diversity in soils is implicit in the contents of the latest edition of the *Compendium of Soil Fungi* (35). Out of many listed species, less than ten belong the Agaricomycetes. In contrast, Lynch and Thorn (93) reported 186 species belonging to this group in Michigan agricultural soils. Given the understanding of the important roles in the soil ecosystem attributed to this group of fungi it is ironic that so few are recorded in the current authoritative text on the subject.

There are several factors that have led to Agaricomycetes being poorly characterized in soil ecosystems. While some factors apply to many soil microorganisms, some are unique to Agaricomycetes. In general these organisms are i) difficult to detect due to inconsistent and ephemeral fruiting and the inability to culture the majority of Agaricomycetes in the lab (60), ii) difficult to enumerate as the concept of 'individual' (or other natural unit of enumeration used in studies) does not apply to their hyphal growth form (131), iii) difficult to PCR-amplify using soil-extracted DNA due to their comparatively low concentrations of DNA in the soil (29) and iv) difficult to identify, except when fruiting, due to a lack of morphological characters (61, 87, 96, 101).

Traditional sampling methods such as soil dispersion plate culturing and fruiting body surveys used in microbial work are unsuited to studying Agaricomycetes. Dispersion plate culturing methods are highly biased towards fungi that produce abundant asexual spores and are capable of quickly colonizing new substrates (principally ascomycetous moulds). Agaricomycetes produce few propagules in soils and colonize slowly making them unsuited for culture methods. Although techniques have been developed for isolating soil Agaricomycetes on media by employing inhibitors and supplying specific substrates (136), such methods can be time consuming due to slow colonization. A further issue with long culture times is that plates may easily become contaminated with other faster-growing species before the Agaricomycetes have developed. This method also may produce cultures of dormant spores, which may be long lasting in the soil environment and non-representative of the active members of the sampled soil community. Thorn *et al.* (136) proposed a soil washing method before plating to reduce spores and increase the recovery of hyphae from the soil matrix and from within decaying plant litter to reduce the culturing of resting spores. Even with Agaricomycete-tailored methods, results obtained from culturing remain biased towards "lab-culturable" species and only a small percentage of this group (approximately 17% of the known diversity of Basidiomycota) are culturable (60, 78); in truth, the proportion – taxonomic or numerical – of soil fungi that can be cultured from soil is largely unknown.

Field surveys of fruiting bodies favour species that that frequently produce conspicuous sexual fruiting bodies. These studies are confounded by yearly variation in environmental condition as well as the reduced probability of encountering the relatively short lived fruiting bodies of many Agaricomycetes during sampling events (58, 133). To

obtain robust community resolution fruiting body surveys require frequent sampling over a multi-year period (e.g. (133)), which is not often feasible.

The main problems with detection in fruiting body surveys and culturing methods are resolved by DNA-based methods. DNA-based methods allows for the detection of an individual regardless of fruiting or culturability in the lab (34), but positive results depend on successful DNA extraction and amplification from soils. A new problem seems to occur in DNA studies with respect to the Agaricomycetes as current studies employing broad-scale rDNA analyses under-represent Agaricomycetes. The low representation of Agaricomycetes in these broad-scale studies is most likely due to this fungal group's low concentrations of DNA compared to other soil organisms (19). Nuclei in Agaricomycetes are often concentrated in the cytoplasm-rich hyphal tips, with much of the area or volume that an individual occupies being filled with empty hyphae. This greatly reduces the ratio of target DNA to soil volume or mass. Methods that filter our non-target taxa are therefore necessary for enumerating these DNA depauperate taxa. Soil washing has been used to decrease the high concentration of bacterial and fast growing fungal species in a few DNA-based studies of Agaricomycetes (29, 93). This method was originally developed for culture-based studies to increase the recovery of Basidiomycota on culture plates (45, 112, 136) and appears to improve the concentration of target DNA in the material used for DNA extraction. Findings of Deacon and Thorn (29) demonstrate the benefits of soil washing in DNA-based studies of basidiomycete diversity. In their study basidiomycete sequences were not amplified using DNA extracted directly from bulk soils (i.e., not washed) but successful amplification was obtained from the same soils that

had been washed. These findings are important when considering the diversity recovered by general DNA-based studies of soil fungi.

Although data at the species-level can be obtained from DNA sequence-based methods, numerous studies on soil fungal community employ intermediate-level resolution methods. Data obtained from these methods are unable to address differences in communities in terms of taxonomic similarity. Examples of intermediate level methods include t-RFLP, ARDRA, and DGGE (see (103, 108, 109, 137) for reviews of these methods). These methods are less time consuming, and inexpensive, but offer limited insights into community structure. Often the data only indicate whether or not two or more communities are different. Without using a higher resolution method (such as cloning and sequencing) differences among community-types cannot be matched to differences or changes in community membership.

The 'gold standard' of molecular techniques for fungal community studies, according to Fitter (41), is DNA sequencing. In environmental samples the PCR product is a mixed template and must be cloned before sequencing. The resulting sequences can be placed in phylogenetic trees or run through sequence similarity searches (e.g., BLAST (4)) for identification. This allows for the recording of taxa, and this level of information can be used to compare results between different studies in space and time. DNA sequences are the baseline data for molecular systematics and ecology, but due to the extensive diversity in soils and the costs and time of DNA sequencing work, exhaustive community sequencing studies are rarely feasible.

As the ability to detect soil basidiomycetes improves, many diversity and community assemblage studies must wrestle with idea of what constitutes an individual

organism. As the growth of hyphal fungi is theoretically unlimited, one individual can extend over a great distance, area or volume in soil. Biomass of such organisms may span several orders of magnitude (eg. (131)). To further add to the difficulty in defining individuals in basidiomycetes is that hyphal cells can act as part of a the larger organism or quite separately on their own, which is very different from what is typically possible for plants or animals. Studying this modular type of organism clearly has its challenges when placed within the framework of the classical, individualistic paradigm of community ecology (117).

Fungal species identification has been a major barrier to the inclusion of fungi in ecological studies as both a great amount of time and expertise is needed to identify fungi by morphological traits (15). Most fungi are impossible to identify morphologically in their vegetative state and fruiting is sporadic, which reduces acquisition rates of identifiable samples. The advent of molecular approaches has improved our ability to identify species, as DNA from vegetative or fruiting stages can be used in molecular identification. The phylogenies resulting from DNA-based methods, however, are very different from traditional morphological phylogenies (64, 87, 101). As a result, placement of a given organism, determined from each of these two approaches, are often not comparable.

DNA-based phylogenies indicate that a high amount of morphological convergent evolution has taken place in the kingdom of fungi (e.g. (68)). This has led to a very volatile period in fungal nomenclature. Our understanding of relatedness among different species, groups and sexual phases (i.e. anamorphic (asexual) and teleomorphic (sexual)) is gradually improving, but comparisons of species diversity between new and older

studies are not straightforward. The volatility in species and family placements of fungi is complicated by the limited amount of sequence data from morphologically identified specimens (104). This makes DNA-based fungal identification difficult and time consuming (34). Hawksworth (59) suggests that fungal taxonomy is approximately 70-100 years behind vascular plant taxonomy.

Given the methodological challenges imposed by the soil environment and past difficulties in detecting Agaricomycetes, it is not surprising that only a handful of studies have reported on their composition and diversity. Citation results from the Biosis literature database using the search term combination of agriculture (agri*), diversity (divers*), basidiomycete (basid*) with the 'AND' boolean operator returned 448 studies. Appendix 1 summarizes the results after filtering out studies focused on one particular group (e.g. mycorrhizal) or single species (often pathogenic fungi) (Appendix 1). Only three studies have applied sequencing to soil-extracted DNA and contain data at the species-level regarding basidiomycete diversity in agricultural or grassland systems (80, 93, 98). All of these studies are recent, with the earliest published in 2004. The aim of the present study was to use molecular tools to study the diversity of Agaricomycetes in agricultural soils exposed to a range of agricultural treatments, and to assess the repeatability of molecular-based methods by comparing the results to data collected at the same site 2 years earlier.

Diversity in the Agaricomycetes (Taxonomy and Nomenclature)

In order to aid the reader in understanding the diversity of taxa reported in this study, the common terminology and names of the major groups are presented. This

information is provided with the caveat that even those in the discipline find it difficult to keep pace with the ongoing changes in nomenclature. For example, several substantial nomenclature changes have been made over the duration of this study alone, as molecular phylogenetic information is greatly altering our understanding of the relationships among fungi (61, 66).

The group of interest in this study is the class Agaricomycetes as defined by Hibbett *et al.* (64, 66); an approximate synonym used earlier is the homobasidiomycetes. These are the fungi colloquially referred to as "mushrooms and toadstools". The homobasidiomycetes, with approximately 13,000 known species, were divided into eight major clades by Hibbett and Thorn (69): the euagarics (Agaricales, with 8,425 known species), Bolete (840), Russuloid (1,000), Polyporoid (1,350), Cantharelloid (170), Thelephoroid (240), Hymenochaetoid (630) and Gomphoid/phalloid (350) clades. While historically each of these clades had distinguishing morphological fruiting body features that defined them, molecular information indicates that these features are often the result of convergent evolution (i.e. two ancestral paths leading to the same form) and thus an overall description of the common fruiting body features of these major clades is complicated and likely uninformative. Certain fruiting body forms such as the puffballs have arisen multiple times, with the result that the former class Gasteromycetes has been dispersed among several of the major clades of Agaricomycetes (66, 68). Nutritional modes vary within and among major clades from saprobic to ectomycorrhizal or pathogenic (69). At least some members of all the major clades of Agaricomycetes occur in soils.

The term "clade", used extensively in current fungal taxonomic literature (14, 66, 69, 96, 102), refers to a monophyletic group of organisms, that is, all of the members descended from a single common ancestor (102). Depending on the ancestor chosen to define the clade, it may be large (for instance, encompassing all Agaricomycetes) or small (encompassing a group of closely related species). A large group effort has been made to reconcile recent phylogenetic results with fungal classification at the levels of orders and above (66). However, many "minor clades" (approximately equivalent to genera) remain without formal taxonomic names (e.g., (102)). This study follows the format outlined by Moncalvo *et al.* (102), who described 117 clades of "agarics" (both Agaricales and other clades of Agaricomycetes). Where a generic name accurately encompasses a clade, its name (capitalized and written in italics) is used as the clade name, but where the clade is comprised of taxa that were not expected to have any natural relationship provisional names are used (102).

Mechanisms by which agricultural practices affect fungal community structure

Modern agricultural practices involve physical and chemical manipulations of the soil environment that are intended to directly improve primary productivity but may interfere with long-term soil sustainability through indirect effects on microbial processes. For example, mechanical tillage of soils may reduce competition between weedy plants and crop species but tillage may significantly retard the establishment of hyphal fungi that would otherwise provide important decomposition services to the system. The practice of mechanical tillage breaks up the soil and associated biological structures such as roots, litter and associated fungi. The extensive hyphal networks of Agaricomycetes are destroyed through fragmentation while species that colonize dead

plant material (saprobes) may become more broadly distributed throughout the soil profile as surface materials are tilled under in the tillage process. Since Agaricomycetes are slow-growing, the frequency of physical soil disturbance may strongly affect their ability to colonize or persist in tilled soils.

Chemical manipulations such as addition of commercial fertilizers to soil (usually nitrogen-phosphorus-potassium mixes) may also impact the composition and abundance of fungal taxa. These impacts may directly reduce or increase fungal richness by increasing mineral nutrients beyond levels that some fungi can tolerate (54) or they may indirectly affect the fungal community by altering the quality and quantity of plant carbon inputs to the soil. For example, changes in the quality of such plant inputs as a result of nitrogen fertilization has been shown to alter the abundance of fungal taxa in boreal forests (3). The effects of fertilization on the Agaricomycete richness, genetic diversity and community composition at KBS-LTER can be examined by comparing each of these community properties between fertilized and non-fertilized treatments.

Most studies on the topic of fungal diversity in soils report on fungi as a group or focus specifically on economically important species (e.g. crop parasites or mycorrhizal taxa). Studies that report on basidiomycetes do so incidentally and have shown both positive and negative effects of fertilization on abundance and species richness (11, 27) The effects of agricultural practices are thus likely system-specific and dependent on the natural history of the taxa being studied. The long-term effects of agriculture practices on communities of Agaricomycetes in temperate agro-ecosystems is not currently known and therefore cannot be inferred from general fungal studies in other areas.

The long-term effects of tillage and fertilization on temperate agro-ecosystems is the focus of research at the Kellogg Biological Station Long-Term Ecological Research site in southwestern Michigan. The KBS-LTER site is a field experiment with replicated tillage, fertilization and control treatments continuously managed since 1989. The site therefore provides an ideal location for addressing the question of how tillage and fertilization affect fungal communities.

Four treatments were selected to represent agricultural disturbances: conventional till mono-crop (tilled annually), no-till (not tilled since 1989), historically tilled grassland (not tilled since 1989) and never-tilled meadow (native prairie-meadow maintained by annual burning). In addition to tillage history, the CT and NT plots receive annual fertilizer applications.

Analysis of the composition of communities of Agaricomycetes in the four treatments selected for this study will enable a dissection of the potential impacts of agricultural practices related to tillage, fertilization and cover types. For instance, some taxa may be intolerant of the physical disruption caused by tillage, whereas other taxa might become more abundant as a result of such disturbance. It has been noted for example, that tillage can enhance the concentration of fungi in soil that normally infect above-ground plant material (e.g. pathogenic species such as *Sclerotium rolfii* and *Rhizonctonia spp.*) through the burial of infected plant biomass during plowing (28, 100). Tillage distributes propagules of such taxa vertically and horizontally through agricultural soils much more efficiently than natural dispersal (28). In the KBS-LTER context, such disturbance-tolerant taxa would be expected to dominate the CT treatment and to have

reduced prevalence in other treatments. In contrast, taxa that propagate via fragile hyphal networks would be expected to dominate less disturbed treatments.

The effects of cover types on fungal community composition cannot be strictly separated from the effects of fertilization in the experimental design at the KBS-LTER site. This is because both of the monocrop treatments (CT and NT) receive annual fertilizer applications both of the mixed-vegetation treatments do not receive fertilizer applications. In spite of this apparent non-independence of treatments, the relative importance of the treatment factors can be inferred on a taxon-specific basis by considering the requirements and sensitivities of the observed taxa and their prevalence between treatment groupings (i.e. CT + NT versus HTS + NTS). For example, some taxa may be considered generalists from the perspective of nutritional mode (and therefore not affected by variability in cover type) but may be sensitive to fertilization (see for example (7)). In contrast, other taxa may have specialized nutritional modes that require substrates found only in certain cover types (such as wood) without any evidence of sensitivity to fertilization. Use of existing knowledge on species life history is therefore important to interpreting results of this field experiment.

Determining the taxonomic composition of agricultural soils and exploring their affiliations with agricultural treatments is fundamental to advancing our knowledge of how agriculture affects soil biodiversity. In addition to evaluating responses of individual taxa, assessment of species richness and genetic diversity provide useful insights into the structure and complexity of biological communities. This study therefore reports on taxonomic richness, genetic diversity and taxonomic composition in order to provide a multi-faceted exploration of Agaricomyecetes in the study site.

Study objectives

The objectives of this study were:

i) to determine the taxonomic composition of the community of Agaricomycetes in agricultural soils at a study site in central Michigan, USA.

ii) to examine the repeatability of the DNA-based results by comparing taxonomic composition and frequency to those of a previous study at the same site by Lynch and Thorn (93).

iii) to compare the species richness, community composition and depth of genetic diversity of Agaricomycetes among four different agricultural treatments.

These objectives were met by measuring the taxonomic diversity of Agaricomycetes in agricultural soils using molecular techniques that favour their recovery. A similar study was carried out by Lynch and Thorn (93) at the KBS-LTER site in 2002. The data from that study provide a base from which to compare results of the present study. Data for both studies were collected using similar molecular methods. Comparison of these data provides some insight into the repeatability of these methods in field studies. This will be the first study of the diversity of Agaricomycetes in agricultural soils, according to currently available literature, where past molecular data exist with which to make such comparisons.

CHAPTER 2: MATERIALS AND METHODS

Chapter 2: Materials and Methods

Study site description

This project was conducted on experimental agricultural plots at the Kellogg Biological Station Long-Term Ecological Research site (KBS-LTER), located in southwestern Michigan (42° 24' N, 85° 24' W, elevation 288 m). The KBS-LTER study area contains a series of 46 experimental plots, 42 allocated to one of seven management treatments in a randomized complete-block experimental design and 4 plots of the eighth treatment on an adjacent site. This study investigated four of these eight treatments in order to reflect a gradient in management intensity. The four treatments were conventional till (CT), no-till (NT), historically tilled successional (HTS), and never tilled successional (NTS). Conventional till treatment plots were characterized by annual disturbance of the soil profile as a result of mechanical tillage prior to planting monocultures of rotational commercial crops (maize-soybean-wheat, with wheat in the sampling year). In contrast, no-till treatments were not tilled and planting of the same rotation of crops was achieved by seed drilling. Both conventional and no-till treatments included applications of fertilizer and herbicide. More detailed information on agronomic activity on these plots can be found in the Agronomic Field Log table available at http://lter.kbs.msu.edu/datasets/7.

The plant community of the historically tilled successional sites (HTS) is maintained in an early successional state through annual burning, usually performed in March. Based on data collected annually at KBS, the dominant plant species in this

treatment during the year of this study included weedy herbs and grasses such as *Trifolium pratense, Solidago canadensis, Arctium minus, Poa pratensis, Bromus inermis,* and *Phleum pratense.* The never-tilled successional sites (NTS) are maintained in an early successional meadow state by annual mowing in late fall. The dominant vegetation included native herbs such as *Solidago canadensis, Rubus* sp., *Euthamia graminifolia,* and saplings of *Sassafras albidum* with reduced dominance of grasses relative to HTS. More detailed community study information is available in the table KBS019-004 (Non-crop Biomass) located on the KBS website (http://lter.kbs.msu.edu/datasets/22). No fertilizers or herbicides are added to these last two treatments. Four replicates of each of the four treatments were selected for this study, resulting in a total of 16 study plots.

Field sampling

Two soil cores, 2.5 cm diameter and 10 cm deep, were collected at 3 points in each plot using a stainless steel soil corer. Soils were collected on 6 November 2004, after harvest but before the annual burning of HTS and mowing of NTS. Four replicates of four treatments were sampled, totaling 48 soil cores (4 treatments x 4 plots x 3 sampling points = 48). Soil corers were wiped clean with a cloth soaked in 70% ethanol between treatments to minimize cross-plot contamination. Collected soils were packed in sterile polyethylene WhirlPak bags and immediately put into a cooler on ice in the field to induce dormancy of soil organisms. Samples were stored at 4°C in the laboratory until the time of processing.

Soil washing procedure for nucleic acid isolation

Soils were subjected to a washing procedure to reduce the number of bacteria and spores from non-basidiomycetes (e.g. ascomycete anamorphs and Mucorales) that tend to produce spores in high numbers in soil (136). Ten grams of soil (fresh weight) were transferred to an autoclaved jar with lid and 125 mL of 0.1 M sodium pyrophosphate was added. Samples were shaken vigorously for 5 min to homogenize. The resulting mixture was transferred to three stacked sieves of decreasing pore size: #16 (1.18 mm), #60 (0.25 mm), and #270 (0.053 mm). Soil particles were washed through each sieve with distilled water using a hand-held spray bottle. One millilitre of the organic matter remaining on the 53 μm sieve was pipetted into a 1.5 mL centrifuge tube using a broad-bore pipette tip. The tubes were centrifuged for 1 min at 12,000 g to concentrate the organic matter at the bottom and 250 μL of compacted organic matter was used in place of the 250 mg of dry soil suggested in the protocol of the Power Soil DNA extraction Kit ® (Mo Bio laboratories, Inc). Sieves were immersed in 70% ethanol, washed in warm soapy water, and rinsed thoroughly with distilled water between samples to minimize cross-sample contamination.

Molecular protocols

DNA isolation followed the Mo Bio Power Soil DNA protocol with minor adjustments (Mo Bio Laboratories, Solana Beach, CA). Bead beating was carried out in four cycles of 30 sec at setting number 4 in a Fast-Prep 120 (Bio101, QBiogene, Carlsbad, CA) with 5 min on ice between cycles. Elution of the DNA from the filter was completed with two aliquots of 40 μL and the eluent was divided into 7 aliquots of 10 μL each to ensure DNA was not subjected to freeze/thaw cycles.

Amplification was carried out with Platinum *Taq* (Invitrogen) using basidiomycete-specific primers B001 [5'-GCT TTA CCA CAT AAA TCT GA-3'] and B2R+ [5'-TAC CGT TGT AGT CTT AAC AG-3'](93) (Figure 2.1). This yielded an amplification product approximately 2.4 kb in length. The final reaction mix contained 1x Buffer, 2 mM MgCl, 0.25 mM dNTPs, 0.25 mM of each primer, 1.4 Unit/rxn Platinum *Taq*, 1% v/v polyvinyl pyrrolidine, and 3 µL of template DNA in a final volume of 20 µL. Polyvinyl pyrrolidone was added to reduce interference of polyphenols (122). PCR was performed with the following protocol: initial denaturation at 92° C for 120 sec followed by 35 cycles of denaturation at 92° C for 10 sec, annealing at 55° C for 20 sec and extension at 72° C for 120 sec and a final extension at 72° C for 7 min to ensure complete extension of the final product. Each soil sample was amplified separately as a means of reducing amplification biases as well as increasing the likelihood of amplifying rare targets. Amplified products from the three soil samples within a plot were combined and cloned in one reaction with TopoTA cloning (Invitrogen). Twenty clones from each cloning reaction were randomly selected and digested at 37° C for 2 h using 0.5 µL MspI (Promega) restriction enzyme. Fifteen microlitres of the restriction digests were run in a 1.5% agarose gel in Tris-acetate-EDTA buffer at 75 V for 90 min with MassRuler (Fermentas) as the DNA ladder. Gels were photographed, normalized and compared using FingerPrinting II software (BioRad). Gel lanes with unique patterns were sequenced uni-directionally using MOD-LR3 primer [**GGT** CCG TGT TTC AAG AC] based on the LR3 primer described by Vilgalys and Hester [CCG TGT TTC AAG AC**G GGG**] (141) where bolded letters represent the difference between the two primers (Figure 2.1).

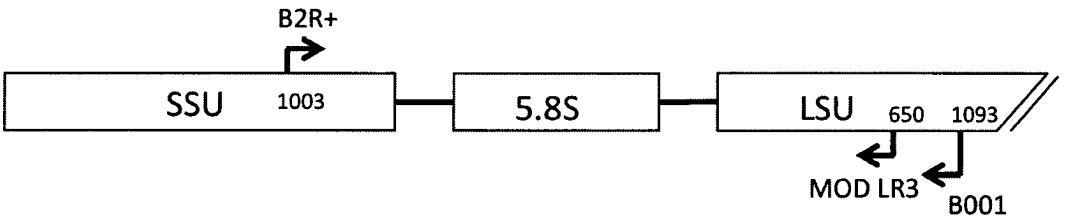

Figure 2.1. Priming sites of basidiomycete specific primers B2R+/B001 and sequencing primer MOD-LR3 in the nuclear ribosomal genes

Soil organic matter content

Oven-dried soils were transferred to pre-weighed crucibles and samples were ashed in a muffle furnace at 450°C for 24 hrs. Final contents were weighed immediately and organic matter content was calculated as the difference in mass between the dry weight and ashed weight of each sample.

(dry weight – ashed weight)/dry weight*100 =% soil organic matter

Site-level data analysis

Sequence analyis

Resulting sequences were aligned with named reference sequences from GenBank using Muscle 3.6 (36). Alignments were refined in Muscle and then verified and edited manually. Sequences were grouped into operational taxonomic units (OTUs) defined at one per cent difference in sequence nucleotides, using DOTUR (127). This was based on a genetic distance matrix created by DNADIST in the Phylip package (38). In taxonomic terms this definition of OTU equates roughly to the species level of identification as sequences sharing >99% similarity in the D1-D2 variable domain of the nLSU have been

found to be conspecific in many fungal groups (113). To place sequences in a clade context the Neighbor-joining method (125) was used to construct a tree using clade-identifying sequences supplied by the MOR site (http://mor.clarku.edu/) (67) and those collected in this study. The Neighbor joining (NJ) method is quicker and makes fewer assumptions than Maximum parsimony (MP) and Maximum likelihood (ML). NJ trees have been found to support most of the same terminal groupings as MP and ML (83, 101) and is therefore an effective method for placing sequences in a clade context. Trees were constructed using PAUP 4.0b10 for Windows (134) and tree stability was assessed using 1000 bootstrap data resampling iterations.

KBS-LTER site richness and diversity analyses

The number of occurrences of the digest pattern(s) associated with a given OTU (as defined above) was used as an index of prevalence of the OTU in each plot. Diversity measures of species richness and rarefaction curves were obtained using the EstimateS program (24). Concordance of species richness estimators was assessed by comparing confidence intervals for values generated by the following estimators: second order Jackknife, abundance-based coverage (ACE), bootstrap, and Chao 1 (95).

Individual sequences were identified using BLAST (http://www.ncbi.nlm.nih.gov/blast/) (94) to match sample sequences from the present study with sequences submitted to GenBank. Top matches were used in pairwise alignments in BioEdit 7.0.5.3 (56), allowing the ends to slide and trimming overhangs prior to calculating similarity. On average 650 bp were used in pairwise similarity tests. Sequences were identified to the clade classification of Hibbett and Thorn (69).

Repeatability of molecular methods analysis

To determine if the data collected provided a representative sample of the Agaricomycetes, the proportions of OTU richness per clade from this study and from Lynch and Thorn (93) were compared to the proportion of known species in each clade calculated from data in Hibbett and Thorn (69). The proportion of species in each clade is an index of clade richness. Patterns of clade richness were compared across studies to assess the extent to which the richness of clades at KBS was proportional to total known richness of clades. This comparison was carried out to determine if there were clades that appeared to be under-represented at KBS and if clade representation was similar between studies at KBS.

Frequency of OTU occurrence among clades was compared to results of a previous study from the same site two years earlier (93). Sampling times differed between Lynch and Thorn and this study but results were deemed useful for comparison as Basidiomycota fungi are known to persist in the soils minimizing effects of sampling time, and because Lynch and Thorn did not detect a difference in community between sampling times (93). The purpose of this comparison was to assess robustness of molecular techniques for determining species composition and diversity. OTUs were individually collated across studies to determine the number of OTUs shared between studies and the number unique to each study. Resulting counts were further partitioned into their corresponding major and minor clades in order to evaluate consistency of clade representation among studies.

Agaricomycete richness was calculated based on data from both studies using the Lincoln-Peterson index (LPI), a mark-recapture index (44). Mark recapture indices are

usually used to estimate population size but can be used to estimate the number of species present in a community (21, 43). The extension of mark-recapture theory from estimation of population size of a single species to the number of species in a local species pool simply requires consideration of the degree to which mark-recapture model assumptions for single species applications are congruent with the multi-species case. A central assumption of mark-recapture estimators of single species population size is that all individuals in the population under study are equally likely to be sampled. In the context of species richness estimation this assumption translates to a belief that all taxa in the local species pool are equally detectable. If this assumption is met, the resulting index should be a reasonable estimate of the total number of species in the community under study. In practice, the distribution of species among samples is rarely uniform meaning that many taxa are relatively rare. In such cases, mark-recapture indices are expected to underestimate actual species richness and are therefore conservative estimators (43). The Lincoln-Peterson index was selected as a computationally simple estimator suited to data resulting from two independent sampling events. This index is calculated by the following formula:

$$n \times N/r$$

Where n = the number of units captured and marked (OTUs identified in 2002) in the first sampling (time 1); N = the number of samples taken at a later point (time 2), r = the number of species obtained at time 2 that were identified at time 1.

The formula used for the standard error was:

$$\mathrm{sqrt}(n^2 \times N(N-r)/r^3)$$

The 95% confidence bounds were estimated as:

$$(\text{LPI}) +/- (1.96 \times \text{standard error})$$

Where 1.96 is the constant from the standard normal curve that corresponds to $p = 0.05$.

Treatment-level data analysis

Soil organic matter analysis

Data on soil organic matter content were not normally distributed within each treatment category. As a result, the Kruskal -Wallis non-parametric test for k-independent samples (SPSS vers. 11.0 (1)) was used to test for differences in per cent soil organic matter content among treatments.

Genetic diversity and species richness analyses

Species richness and rarefaction curves for each treatment were obtained using the EstimateS program (24). Since these estimators were developed for species richness data, sequence data were aggregated into OTUs. Operational Taxonomic Units were defined as sequences sharing 99% or greater similarity. They are therefore abstract taxonomic units that are generally thought to represent distinct species (113). The amount of overlap among 95% confidence limits of richness estimates and rarefaction curves were used to test whether differences among treatments were significant. Groups were considered significantly different if confidence intervals did not overlap (132).

As OTU definition is sensitive to the selected threshold of per cent similarity, genetic diversity across a range of per cent similarities were used in addition to richness of OTUs at 99% similarity to test for differences among treatments. Genetic diversity was calculated using aligned sequence data. From each treatment alignment a distance matrix

was created in Phylip (ver. 3.2 (38)). This matrix was used as input for genetic diversity analysis in DOTUR (127). The measure of evolutionary distance among sequences is inversely related to their similarity. For example a greater relative OTU richness in one treatment at an evolutionary distance of less than 0.01 (99% sequence similarity) indicates greater relative richness at the approximate taxonomic level of species (113). The relative OTU richness is obtained from the formula:

$$\text{Relative OTU Richness} = \frac{\text{\# of OTUs at given evolutionary distance}}{\text{total \# of unique sequences detected in the treatments}}$$

Analysis of covariance (ANCOVA, SPSS vers. 11.0) was used to test for differences in the genetic diversity in each treatment across the observed range of evolutionary distance (expressed as relative OTU richness over increasing dissimilarity). *Post-hoc* tests for differences in proportion of unique OTUs among treatments were conducted using pairwise t-tests with Bonferroni correction of experiment-wise error rate.

Community composition among KBS-LTER agricultural treatments

It is common to obtain a high proportion of singletons at the OTU-level in microbial diversity studies (73, 79), which makes meaningful community comparisons difficult. Therefore, comparisons of community similarity among treatments were carried out by aggregating the occurrence and frequency of each OTU into indices of prevalence for each minor clade (*sensu* Moncalvo *et al* (102)).

Nonparametric MANOVA (ADONIS in the VEGAN package ver.1.11-0 for R (81)) was used to formally test for differences in community composition between treatment cover types (monoculture crop versus successional grassland).

Principal Components Analysis was used to visualize trends in minor clade similarity among treatments by reducing the 20 input variables (minor clades) to a smaller set of derived variables that represent mathematical combinations of minor clades. The sampling adequacy of data for PCA was measured using the KMO test and the Bartlett test of sphericity (1). Test values desired before proceeding with PCA are a KMO value of 0.5 or greater and a significant Bartlett's test (144). The PCA was manually constrained to 2 axes to simplify interpretation based on the assumption that if treatment effects were important, correlations with treatment would be apparent in the first two components. Minor clades occurring in only one plot representing less than 5% of the total clone population were defined as rare and removed prior to applying PCA in order to avoid diluting the correlation structure with variables that add little information (47). Patterns of plot separation were interpreted using 95% confidence ellipses around the treatment centroid: the greater the separation of the ellipses, the greater the difference in community composition.

CHAPTER 3: RESULTS

Chapter 3: Results

Sequence recovery

Successful amplifications were obtained from all 48 soil samples (3 samples from each of 4 replicate plots of 4 treatments). Amplification products from each of the three soil samples within each plot were combined for a total of 16 cloning reactions. Amplicons from one plot were never successfully cloned, therefore sequence data were obtained from 15 of the 16 experimental plots.

A total of 324 clones were digested, and resulted in 98 unique digest patterns, and these yielded 96 unique sequences. Of these 96 sequences 95 were characteristic of Agaricomycetes and one of an ascomycete. The 95 sequences of Agaricomycetes were used in this study. Average sequence length obtained was 910 bp, with 300 bp covering the 5.8S-ITS2 regions and approximately 600 bp of the nLSU D1/D2 divergent domains.

Diversity of sequences and species richness in soils of four agricultural treatments at KBS-LTER

Sixty-four of the 96 unique sequences had 99% or greater matches with sequences in GenBank. Of these 60 had been deposited in a study completed two years prior to the present study at KBS-LTER within the same treatments (93). Thirteen of the 95 sequences had best matches in GenBank from other sources, and four of these were at 99% or greater. Sequence identifications are listed in Appendix II.

At 99% or greater sequence similarity the 95 unique basidiomycete sequences grouped into 52 OTUs. Rarefaction analysis indicated that OTU accumulation declined logarithmically with addition of samples (Figure 3.1). Using the derivative of the logarithmic curve the rate of new OTU accumulation at the maximum observed sample size (n=15) was estimated to be 1 OTU per sample.

The 95% confidence intervals for second order jackknife estimates of total species richness provided significantly higher estimates than those based on bootstrap and ACE. Values derived from these last estimators were tightly grouped at all levels of sampling effort (Figure 3.2). Point estimates of richness based on the Chao 1 (classic) estimator were very similar to those obtained by bootstrap and ACE, but the 95% confidence intervals for values of Chao 1 were much wider and extended beyond error bars of all other estimators. The estimated number of undetected species increased with increasing sampling effort (Figure 3.2). Average values of the three similar estimators suggest a total species richness of 74, indicating that 72% of the species were detected at the maximum level of sampling. No significant differences in species accumulation curves (rarefaction) and richness estimators among treatments were detected (data not shown).

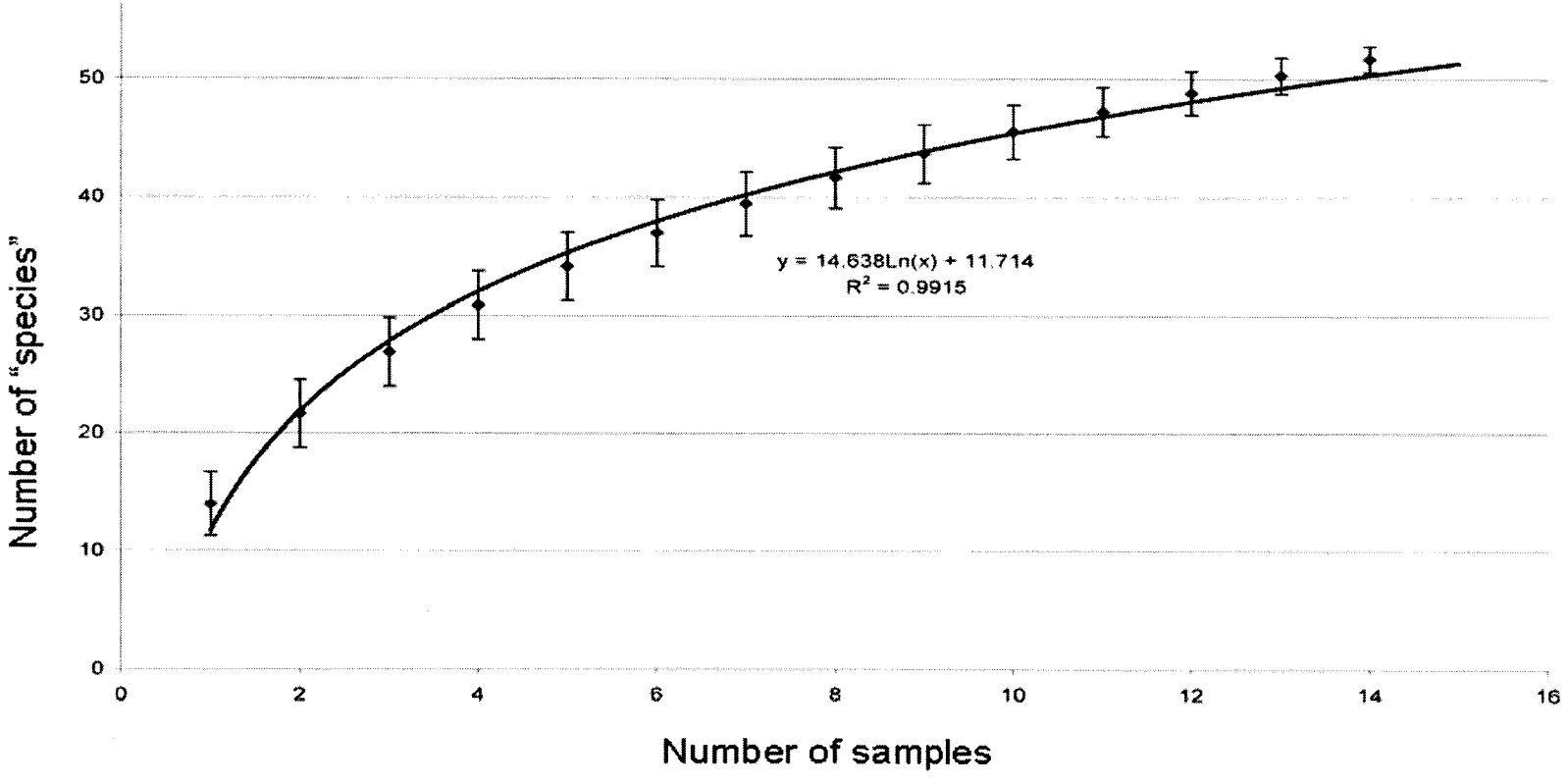

Figure 3.1 Coleman rarefaction curve showing simulated rate of species accumulation with increasing sampling effort based resampling of empirical species occurrence data within pooled samples (n=15). Derived using Mau Tau algorithm in EstimateS (24). Samples collected from KBS-LTER site in November 2004.

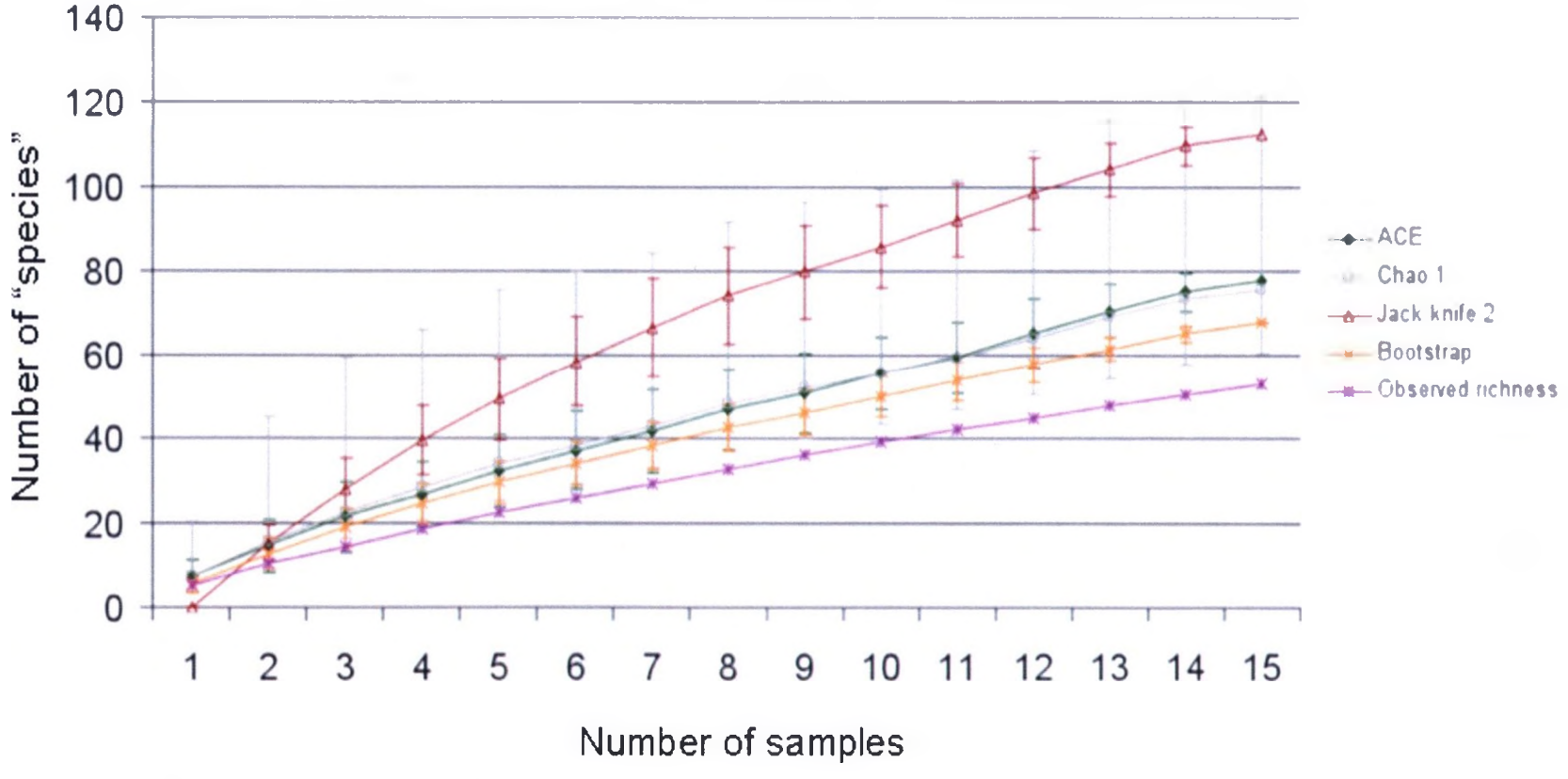

Figure 3.2. Comparison of estimators of total species richness based on number of observed species among pooled samples (n=15) from four agricultural treatments at the KBS-LTER site. Ninety-five per cent confidence limits of the Chao 1 estimator encompassed all other estimators. Results of ACE, Chao 1 and Bootstrap were similar while the Jack knife estimators were significantly higher.

Distribution of sequences in soils from four agricultural treatments at KBS-LTER among major and minor clades

The 95 unique sequences were distributed among five of the eight major clades of Agaricomycetes described by Hibbett and Thorn (69). Representation of major clades within the pool of 324 clones varied from 1% to 69% (Figure 3.3). Within these five major clades 23 minor clades were represented (Figure 3.4).

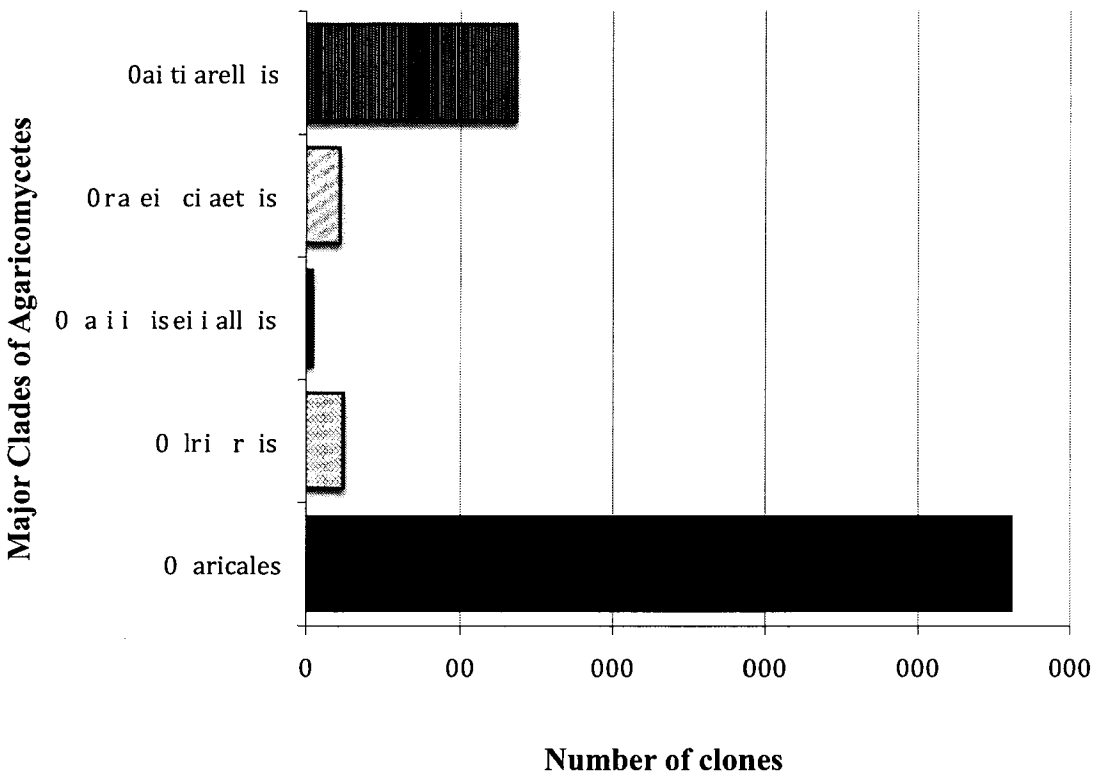

Figure 3.3. Distribution of clones (n=324) among major clades of Agaricomycetes in four agricultural treatments at KBS-LTER site. The Agaricales clade comprised 85% of all sequences sampled.

The Agaricales clade accounted for the greatest proportion of clones of any major clade with 69% of all clones having sequences that fell into this group. Agaricales comprised 17 of the 23 minor clades with the greatest number of clones belonging to the Clavarioids clade. The Polyporoid and Hymenochaetoid clades were each represented by two minor clades and the Cantharelloid and Thelephoroid clades were represented by one minor clade each (Figure 3.4).

To explore the extent of phylogenetic diversity among observed sequences a phylogenetic tree based on the nuclear large subunit (nLSU) rDNA was constructed using the sequences observed in the present study combined with available basidiomycete sequences from GenBank that are considered clade-identifier sequences in MOR (http://mor.clarku.edu/). The following results are based on the tree in Figure 3.5. For visualization purposes the tree is split over two pages. Clades are discussed in decreasing order of OTU richness.

Major clade Agaricales

Agaricales were the most dominant clade in terms of the number of clones attributed to it (as described above) and the number of constituent OTUs. Four-three of the fifty-two OTUs detected belonged to the Agaricales. This clade had seventeen minor clades of which the Clavarioid clade accounted for the largest number of clones (Figure 3.4) and the largest number of OTUs of all minor clades (Figure 3.5).

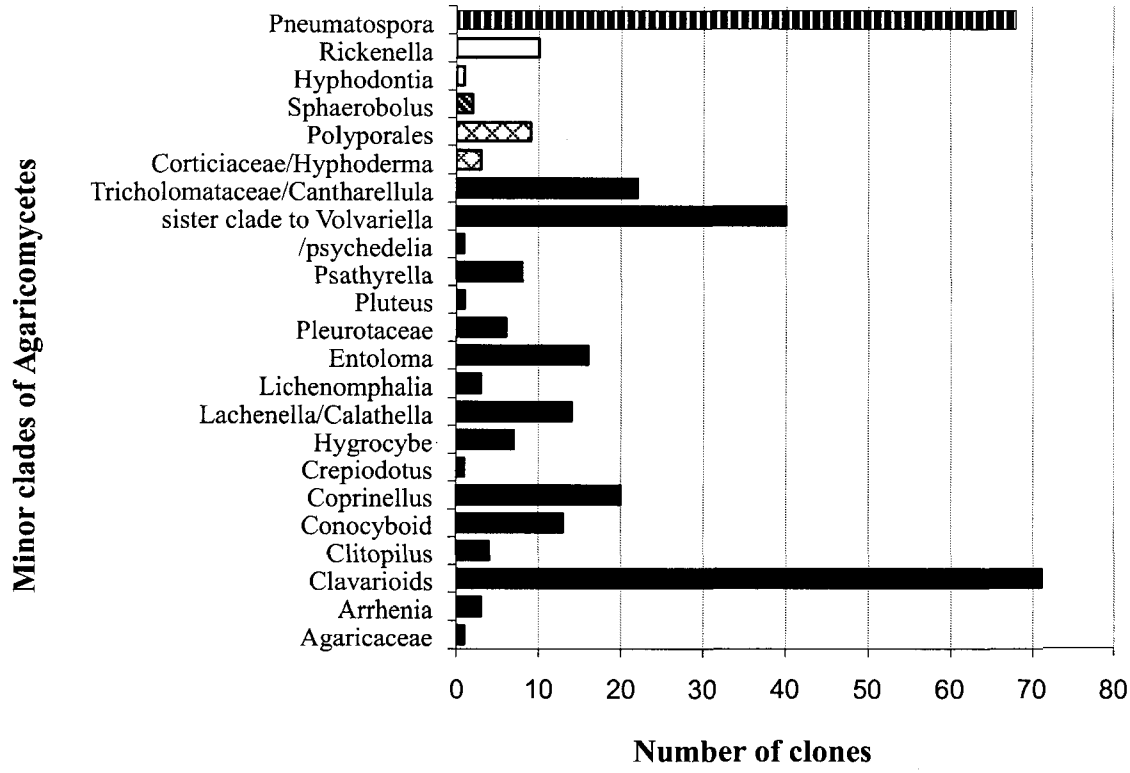

Figure 3.4. Distribution of clones (n=324) among minor clades of Agaricomycetes in four agricultural treatments at the KBS-LTER site. Pneumatospora and Clavarioids were the dominant minor clades comprising approximately 43% of all clones.
Minor clades are grouped into major clades according to the following key: black = Agaricales; large grey hatching = Polyporoid; small black hatching = Gomphoid/Phalloid; white = Hymenochaetoid; vertical black striping = Cantharelloid

The second most OTU-rich minor clade within the Agaricales was labelled the "Sister clade to *Volvariella*" by Lynch and Thorn (93). The clade had strong bootstrap support (95%) but named reference sequences in GenBank were not found (Figure 3.5). This and the Clavarioid clades jointly accounted for 63% of all OTUs detected in this study (23% and 40%, respectively) (Figure 3.5). The Tricholomataceae/*Cantharellula* minor clade ranked third in number of clones but was represented by only one OTU (OTU 2) found in only one plot, where it was the only basidiomycete detected. Two

clades within the family Psathyrellaceae (120) were represented, namely /coprinellus and /psathyrella[2]. Four sequences of the /coprinellus clade were found in four different plots. The similarity between these sequences was high. Therefore, at the defined OTU level of 99% or greater similarity among sequences, all detected /coprinellus sequences grouped into one OTU (OTU 20). Two terminal nodes (OTU 18 &19) were within the /psathyrella clade, and the third (OTU 15) was a sister branch to the /coprinellus and /psathyrella clades. Five sequences representing three OTUs were found in the Nia clade (16).

Four OTUs were placed in the Entolomataceae. OTU 28 is within the monophyletic genus *Clitopilus* contained within the Entolomataceae (102). OTU 25, identified as *Nolanea sericea* (99.4%), was one of the four phylotypes recovered in this study matching an identified GenBank sequence at 99% or greater. The Entolomataceae were represented by 20 clones, approximately 7.5% of all clones analyzed.

The /conocyboid clade of the family Bolbitiaceae (102) is represented by 3 OTUs. None of the OTUs recovered in the /conocyboid clade had GenBank matches greater than 97.6%.

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[2] A forward slash "/" preceding an uncapitalized genus name is used to distinguish a clade label from a generic name, which is capitalized and written in italics in the text (101).

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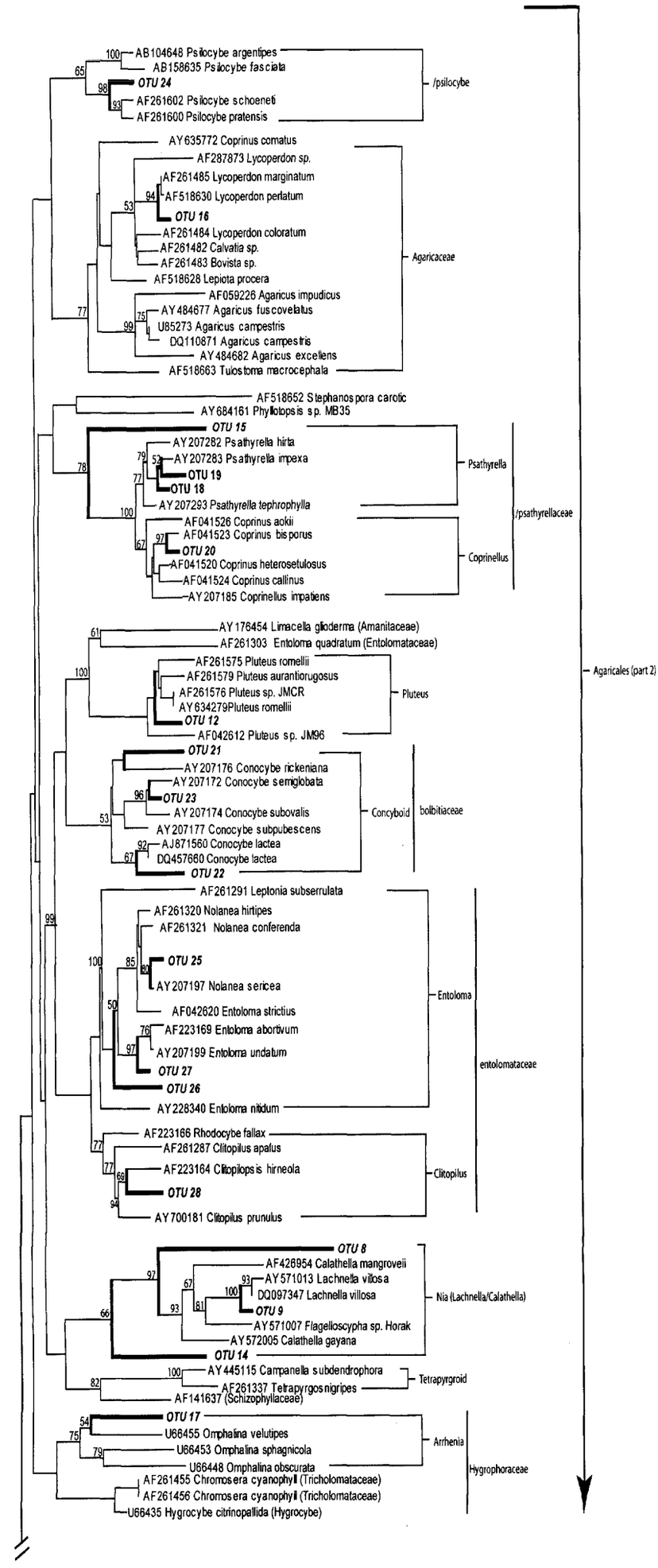
Figure 3.5. Neighbour-Joining tree produced in PAUP 4.0b10 from 650 base pairs of the nuclear LSU rDNA of Agaricomycetes detected in soils of four agricultural at the KBS-LTER site. OTUs represent unique sequences (≥99% similarity) and are shown in relation to selected reference sequences in GenBank. OTUs from this study are prefixed with "OTU"; other taxa are prefixed with GenBank accession numbers. Bolded branches show distance from sampled sequences to identified GenBank sequences. Forward slash (/) is employed to distinguish when a genus label is used to represent a minor clade.


AB104648 Psilocybe argentipes
AB158635 Psilocybe fasciata
OTU 24
AF261602 Psilocybe schoeneti
AF261600 Psilocybe pratensis
/psilocybe
AY635772 Coprinus comatus
AF287873 Lycoperdon sp.
AF261485 Lycoperdon marginatum
AF518630 Lycoperdon perlatum
OTU 16
AF261484 Lycoperdon coloratum
AF261482 Calvatia sp.
AF261483 Bovista sp.
AF518628 Lepiota procera
AF059226 Agaricus impudicus
AY484677 Agaricus fuscovelatus
U85273 Agaricus campestris
DQ110871 Agaricus campestris
AY484682 Agaricus excellens
AF518663 Tulostoma macrocephala
Agaricaceae
AF518652 Stephanospora carotic
AY684161 Phyllotopsis sp. MB35
OTU 15
AY207282 Psathyrella hirta
AY207283 Psathyrella impexa
OTU 19
OTU 18
AY207293 Psathyrella tephrophylla
Psathyrella
AF041526 Coprinus aokii
AF041523 Coprinus bisporus
OTU 20
AF041520 Coprinus heterosetulosus
AF041524 Coprinus callinus
AY207185 Coprinellus impatiens
Coprinellus
/psathyrellaceae
AY176454 Limacella glioderma (Amanitaceae)
AF261303 Entoloma quadratum (Entolomataceae)
AF261575 Pluteus romellii
AF261579 Pluteus aurantiorugosus
AF261576 Pluteus sp. JMCR
AY634279Pluteus romellii
OTU 12
AF042612 Pluteus sp. JM96
Pluteus
OTU 21
AY207176 Conocybe rickeniana
AY207172 Conocybe semiglobata
OTU 23
AY207174 Conocybe subovalis
AY207177 Conocybe subpubescens
AJ871560 Conocybe lactea
DQ457660 Conocybe lactea
OTU 22
Conocyboid
bolbitiaceae
AF261291 Leptonia subserrulata
AF261320 Nolanea hirtipes
AF261321 Nolanea conferenda
OTU 25
AY207197 Nolanea sericea
AF042620 Entoloma strictius
AF223169 Entoloma abortivum
AY207199 Entoloma undatum
OTU 27
OTU 26
AY228340 Entoloma nitidum
Entoloma
AF223166 Rhodocybe fallax
AF261287 Clitopilus apalus
AF223164 Clitopilopsis hirneola
OTU 28
AY700181 Clitopilus prunulus
Clitopilus
entolomataceae
Agaricales (part 2)
OTU 8
AF426954 Calathella mangroveii
AY571013 Lachnella villosa
DQ097347 Lachnella villosa
OTU 9
AY571007 Flagelloscypha sp. Horak
AY572005 Calathella gayana
OTU 14
Nia (Lachnella/Calathella)
AY445115 Campanella subdendrophora
AF261337 Tetrapyrgos nigripes
Tetrapyrgroid
AF141637 (Schizophyllaceae)
OTU 17
U66455 Omphalina velutipes
U66453 Omphalina sphagnicola
U66448 Omphalina obscurata
Arrhenia
AF261455 Chromosera cyanophyll (Tricholomataceae)
AF261456 Chromosera cyanophyll (Tricholomataceae)
U66435 Hygrocybe citrinopallida (Hygrocybe)
Hygrophoraceae
0.1

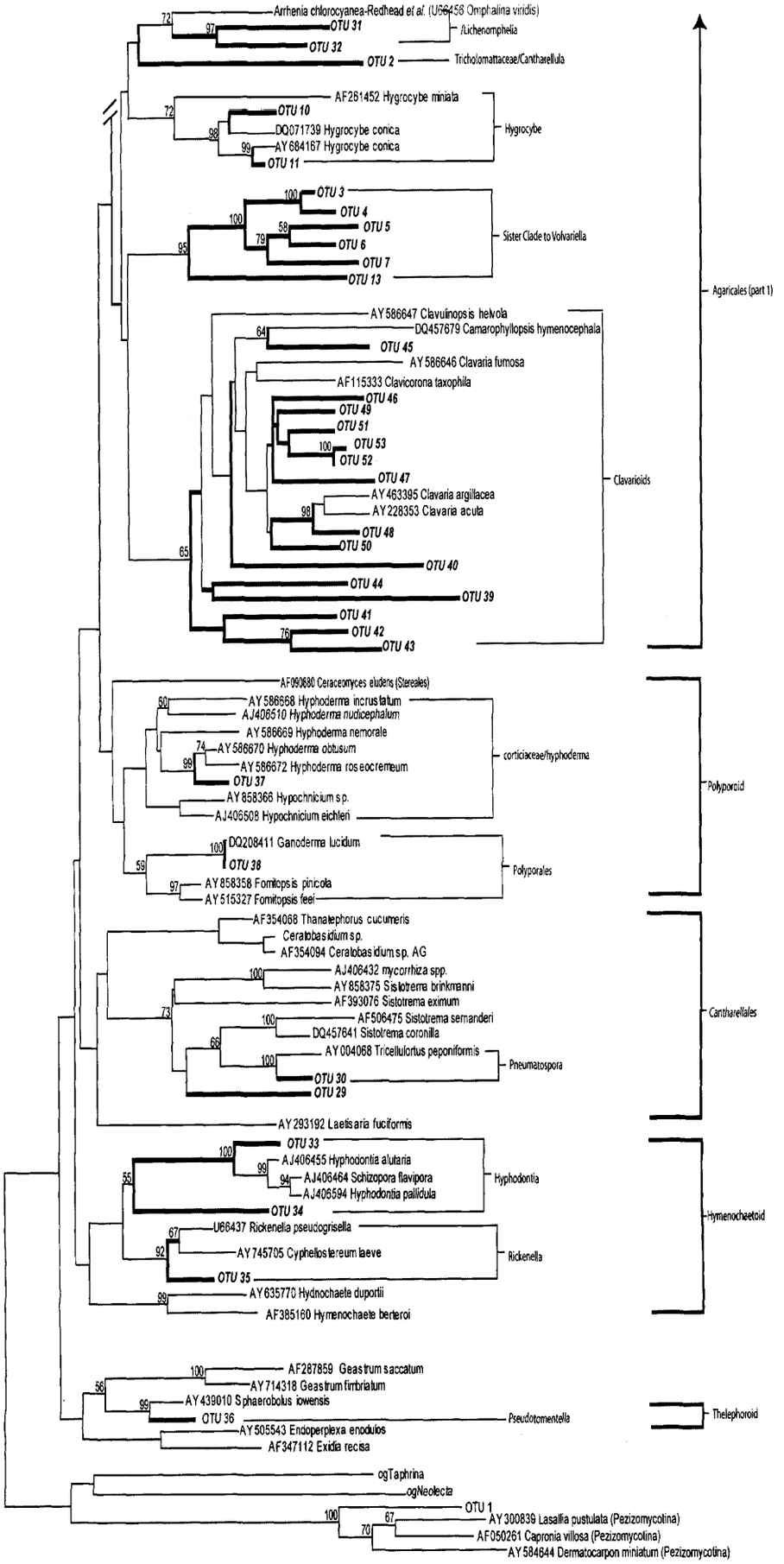


Figure 3.5. continued

The Omphalinoid-hygrophoroid group of Moncalvo *et al.* (102) was represented by five OTUs. Three OTUs are in the *Arrhenia* group with OTU 17 in the /arrhenia clade and OTUs 31 and 32 in the /lichenomphalia clade. Two OTUs were within the /hygrocybe clade.

The remaining three clades of Agaricales were each represented by one OTU. OTU 12 fell into the *Pluteus* clade and matches an OTU of *Pluteus* recovered by Lynch and Thorn in 2002 (similarity 99.7%). OTU 16 was placed within the Agaricaceae clade and was identified as *Lycoperdon perlatum* (sequence similarity 99.3% over 614 bp). OTU 24 is in the /psilocybe clade and the best available database sequence match was *Psilocybe coprophila* (similarity 94.3%).

Major clade Polyporoid

Two OTUs were in the Polyporoid clade. OTU 37 was in the /corticoid clade (102) and shared 98.6% sequence similarity with *Hyphoderma obtusum*. OTU 38 was within the /polyporaceae clade and is identified as *Ganoderma lucidum* (99.2%).

Major clade Cantharellales

The Cantharellales clade was represented by species allied to *Pneumatospora*. Seventeen sequences belonging to the *Pneumatospora* clade were recovered in seven samples. Sixteen of these sequences grouped into one OTU (30), which was highly prevalent among the pool of clones representing 21% of total clones analyzed. This ranks

second to the much more OTU-rich Clavarioid clade of the Agaricales (22% of all clones analyzed).

Major clade Hymenochaetoid

The Hymenochaetoid clade was represented by three OTUs. OTU 33 and 34 are related to species in the *Hyphodontia* group but both branch early and remain at the outer boundaries of the most closely related identified sequences. No available sequences matched OTU 34 at greater than 91% but OTU 33 matched one of the two *Hyphodontia* 'species' from the Lynch and Thorn study (similarity 99.7%) (93). The third OTU (OTU 35) is within *Rickenella* and matches one of the OTUs of *Rickenella* recovered in the Lynch and Thorn study (93) (similarity 99.7%).

Major clade Gomphoid/phalloid

The Gomphoid/phalloid clade, represented by one OTU (OTU 36) that was most closely related to *Sphaerobolus iowensis*. A 100% match was found with one of the two OTUs detected in the Gomphoid/phalloid clade by Lynch and Thorn (93).

Additional taxa

One OTU recovered from these methods was not a basidiomycete and matched sequences of *Lasallia* (93.6%), a lichenized fungus in the *Umbilicariales* of the subphylum *Pezizomycotina* in the Ascomycota.

Proportional clade representation

The proportion of OTUs detected in the five major clades in our study was generally similar to the proportion of known species in each of these clades, with an

over-representation of the Agaricales and under-representation of Polyporales (Table 3.1). Two of the three clades not detected in this study were detected by Lynch and Thorn (93): the Russuloid and Thelephoroid clades, with both occurring at proportions approximately equal to the proportion of known species. The Bolete clade was absent from both studies.

Table 3.1. Major clade representation within species pools observed in two studies at KBS-LTER compared with the proportions of total known taxa of Agaricomycetes in each major clade as reported by Hibbett and Thorn (69).

CLADE	2004	2002	Known proportion
Agaricales	0.85	0.77	0.65
Polyporoid	0.03	0.07	0.10
Russuloid	0	0.015	0.08
Bolete	0	0	0.06
Hymenochaetoid	0.06	0.04	0.05
Gomphoid-phalloid	0.02	0.02	0.03
Thelephoroid	0	0.01	0.02
Cantharelloid	0.04	0.08	0.01

Robustness of taxonomic composition among studies

Comparison of clade diversity in the present study with that estimated by Lynch and Thorn (93) showed that gross patterns of major and minor clade representation were similar (Figure 3.6). Five of seven major clades were represented in both studies. The two major clades not represented in the present study, Russuloid and Thelephoriod, were rarely encountered by Lynch and Thorn (93) (Table 3.1, Figure 3.6).

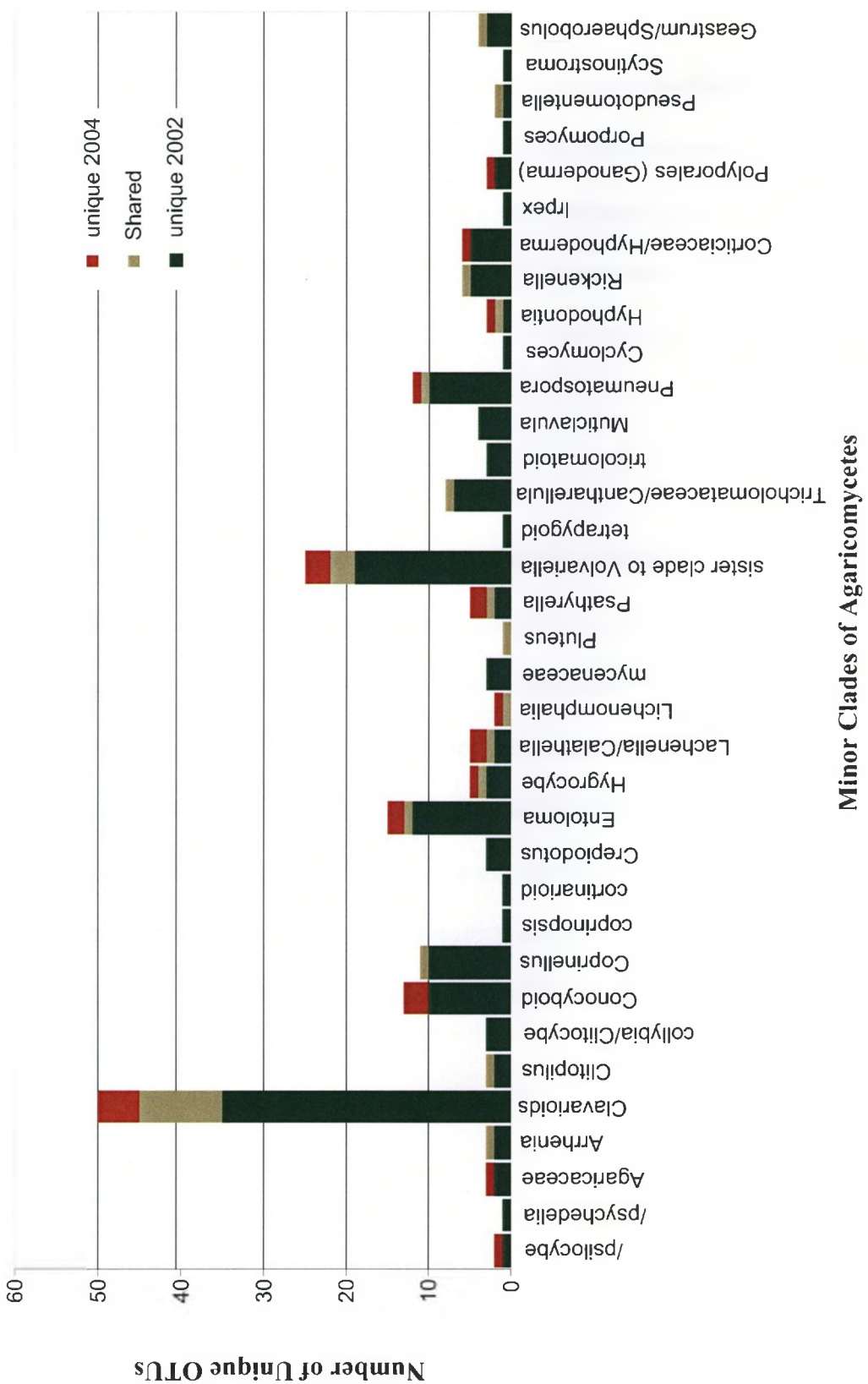
Twenty-two minor clades were detected in both studies and 13 others were detected only in the study of Lynch and Thorn (93). As in the case of the major clades,

the minor clades not detected in the present study were rarely encountered by Lynch and Thorn (93) and varied from proportions of 0.005 to a maximum of 0.02. Of the 21 minor clades represented in both studies, 16 had shared species (Figure 3.6). The two dominant clades (Clavarioids and "Sister clade to *Volvariella*") were the same in both studies. The proportional representation of these clades (*i.e.* the relative number of OTUs in each clade) was similar between the present study and that of Lynch and Thorn (93). In the case of the Clavarioid clade proportions were 0.28 and 0.24 respectively. In the case of the "Sister clade to *Volvariella*" the proportions were equal at 0.11. Representation of the remaining clades detected in both studies showed similar patterns. The average difference in proportions of OTUs representing each of the remaining 14 clades was 0.018 (1.8%).

Species richness estimation based on two studies

Two hundred and thirteen unique OTUs of Agaricomycetes were detected in the two studies. As noted above, 27 OTUs (51%) detected in this study had also been detected by Lynch and Thorn (93). Using the data from both studies, the Lincoln-Peterson index estimates total richness at 359 with upper 95% confidence bound of 484, and lower confidence bound of 292.

Figure 3.6. Proportion of OTUs (defined as sharing ≥ 99% sequence similarity) within each minor clade of Agaricomycetes that were unique to a study in 2002 (93), unique to this study in 2004 and detected in both studies (shared). Rate of OTU re-detection in 2004 was 51%.



Minor Clades of Agaricomycetes

Soil organic matter content

Results of the Kruskal-Wallis test for k-independent samples indicated that only NTS was significantly different from the other three treatments in organic matter content (Chi square = 29.757, df = 3, $p < 0.001$). Based on non-overlapping confidence intervals for treatment means, NTS had significantly higher soil organic matter content values than HTS (3 times the organic matter content of CT and NT and more than twice the organic matter content of HTS (Figure 3.7).

Genetic diversity

Analysis of covariance results indicated that relative OTU richness among treatments varied over increasing evolutionary distance ($F_{(3,115)} = 36.02, p < 0.001$). Examination of the rate of change in relative OTU richness as evolutionary distance increases shows that NTS is characterized by proportionately more genetic diversity up to a threshold of 0.11 evolutionary distance units (Figure 3.8). *Post hoc* examination of treatment means indicated that NTS had a higher overall relative OTU richness than each of the other treatments with significant differences from HTS and CT. Treatments CT and HTS were on average significantly lower than NTS while NT was lower at levels less than 0.11 evolutionary distance units.

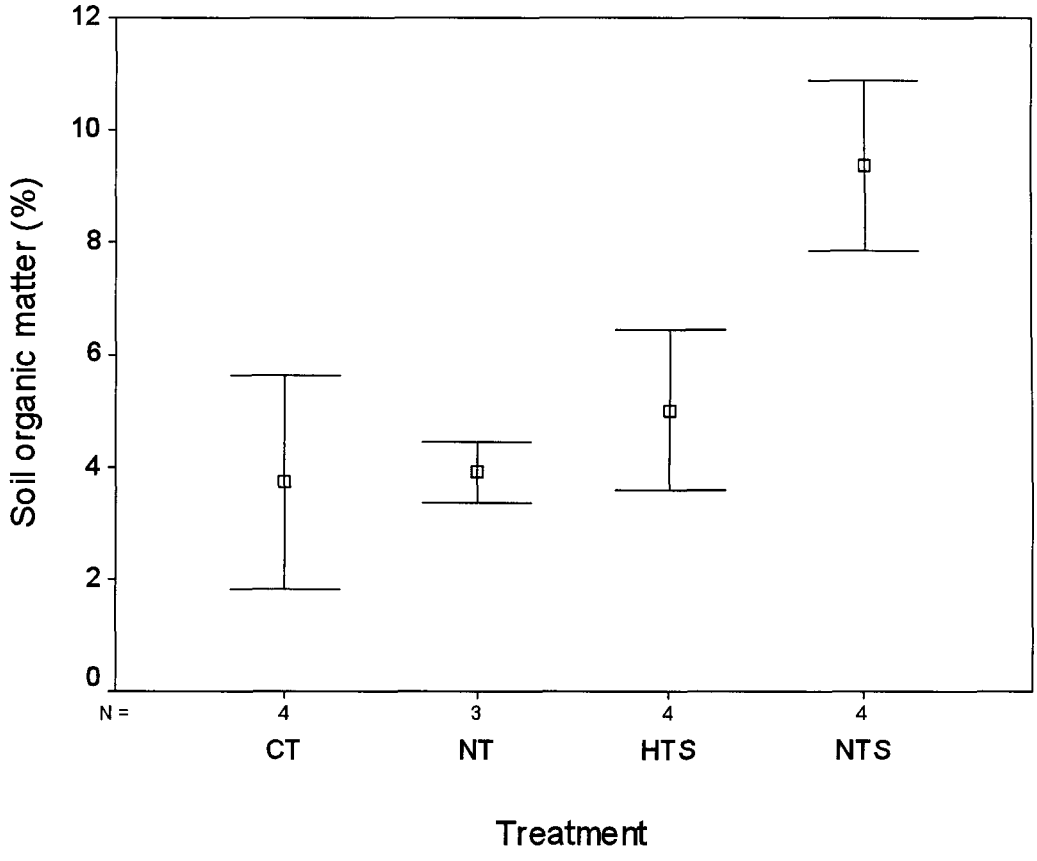


Figure 3.7. Means and confidence intervals for per cent soil organic matter content (SOM) values among four agricultural treatments at KBS-LTER. CT=Conventional Till; NT=No Till; HTS= Historically Tilled Successional; NTS= Never Tilled Successional. Soil organic matter content was significantly higher in Never Tilled plots. Boxes indicate treatment means, and error bars show the 95% confidence intervals.

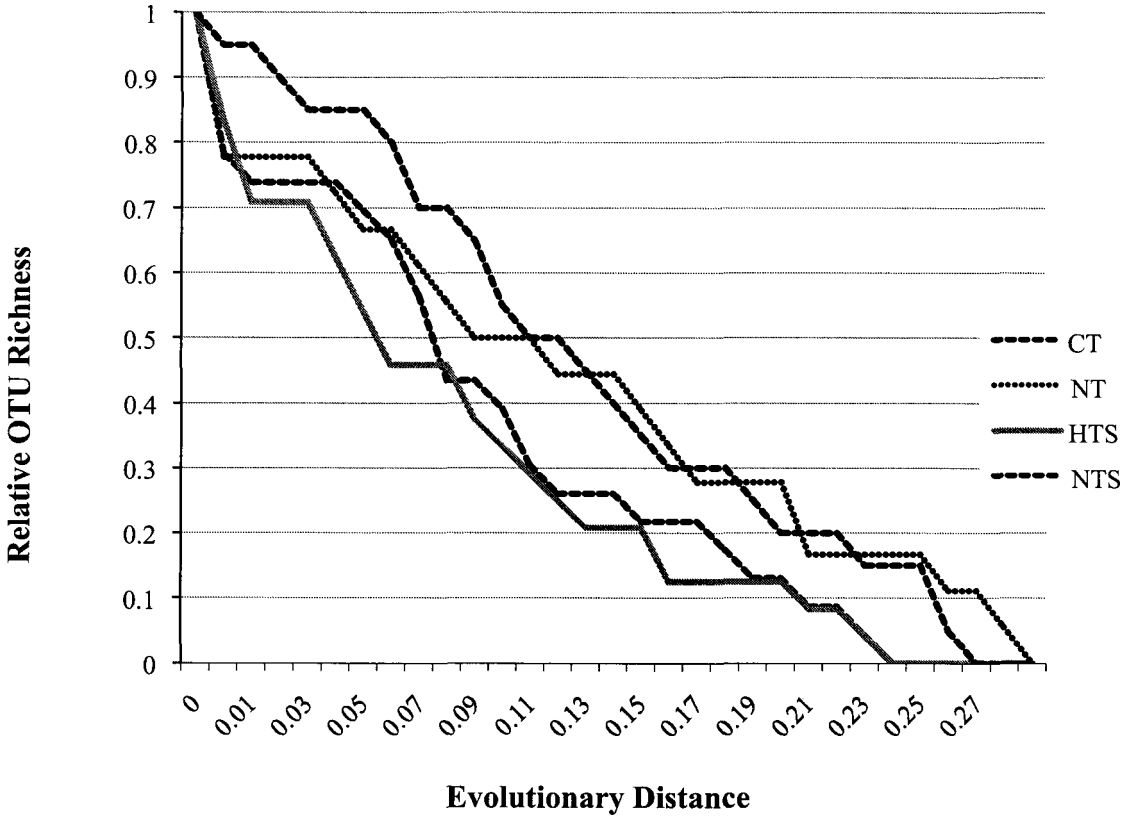


Figure 3.8. Change in relative OTU richness within agricultural treatments at the KBS-LTER site as evolutionary distance increases. The slope of each line indicates the depth of genetic diversity found at a given evolutionary distance. A line that falls quickly indicates most of the genetic diversity was contained in small differences among sequences. Conversely a line that tapers slowly indicates higher genetic diversity among sequences and represents greater diversity at the family level and higher. The evolutionary distance encompassing the total difference in sequences in a given treatments is marked by the x-intercept and represents the total genetic diversity found in a given treatment.

Community composition

Results of non-parametric MANOVA (carried out using Adonis in Vegan package for R) indicated that monoculture treatments (CT and NT) were significantly different from mixed vegetation treatments (HTS and NTS) based on minor clade composition ($F(1,13) = 3.19, p = 0.02$) (Table 3.2). The null hypothesis that agricultural treatments did not affect community composition was therefore rejected. To further interpret patterns in community composition, PCA was used to ordinate plots based on minor clade frequencies.

Table 3.2. Results of non-parametric multi-variate analysis of variance of minor clade composition of Agariomycetes in four different agricultural treatments at KBS-LTER

	df	*Sums of Squares*	*Mean Square*	*F ratio*	R^2	*p*
Source of Variation	1	0.9241	0.9241	2.9583	0.20	0.02
Cover Type Residuals	13	3.7485	0.3124			
Total	14	4.6726				

The Bartlett Test of Sphericity was highly significant ($X^2(91) = 153.5, p < 0.001$) resulting in the rejection of the null hypothesis that the variables in the correlation matrix are uncorrelated. However the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.22, lower than the recommended value of 0.5 or greater. PCA was applied noting that the KMO test indicated that the correlation structure represented by the resulting PCA axes would be low (Figure 3.9). Principle Component Analysis generated two vectors of plot scores. Total variance explained by the two vectors (Factor 1 &2) was 41%. Graph results of PCA showed a high degree of spread across Factor 1, which was caused mostly

by separation of CT plots from plots of other treatments. Plot scores on axis 1 were correlated with soil organic matter content (SOM) (r_s= -0.54, n=15, p= 0.037).

Taxonomic representation in treatments studied

Sequences represented taxa from five of the eight major clades of homobasidiomycetes (69) referred to here as Agaricomycetes (*sensu* (64)).The majority of the sequences were within the Agaricales clade (71%) (Figure 3.10). Agaricales and Cantharelloid clades were represented in all treatments. The Polyporoid clade was represented in all treatments except no till. The Gomphoid/Phalloid clade was only represented in NTS and the Hymenochaetoid clade was represented in the two monoculture treatments of CT and NT.

The five major clades detected covered 23 minor clades. Ten of the 23 minor clades were detected in only one of the four treatments. Four minor clades occurred in two treatments: the Polyporoid and *Hygrocybe* clades in HTS and NTS and the *Lachnella/Calathella* and *Omphalina* clades in CT and NT. Three clades occurred in three treatments: *Coprinellus* and *Psathyrella* clades in CT, NT and HTS and the Conocyboid clade in CT, NTS and HTS. The remaining four occurred in all treatments: the Clavarioid, *Entoloma*, "Sister clade to *Volvariella*" and *Pneumatospora* clades (Figure 3.11-3.14).

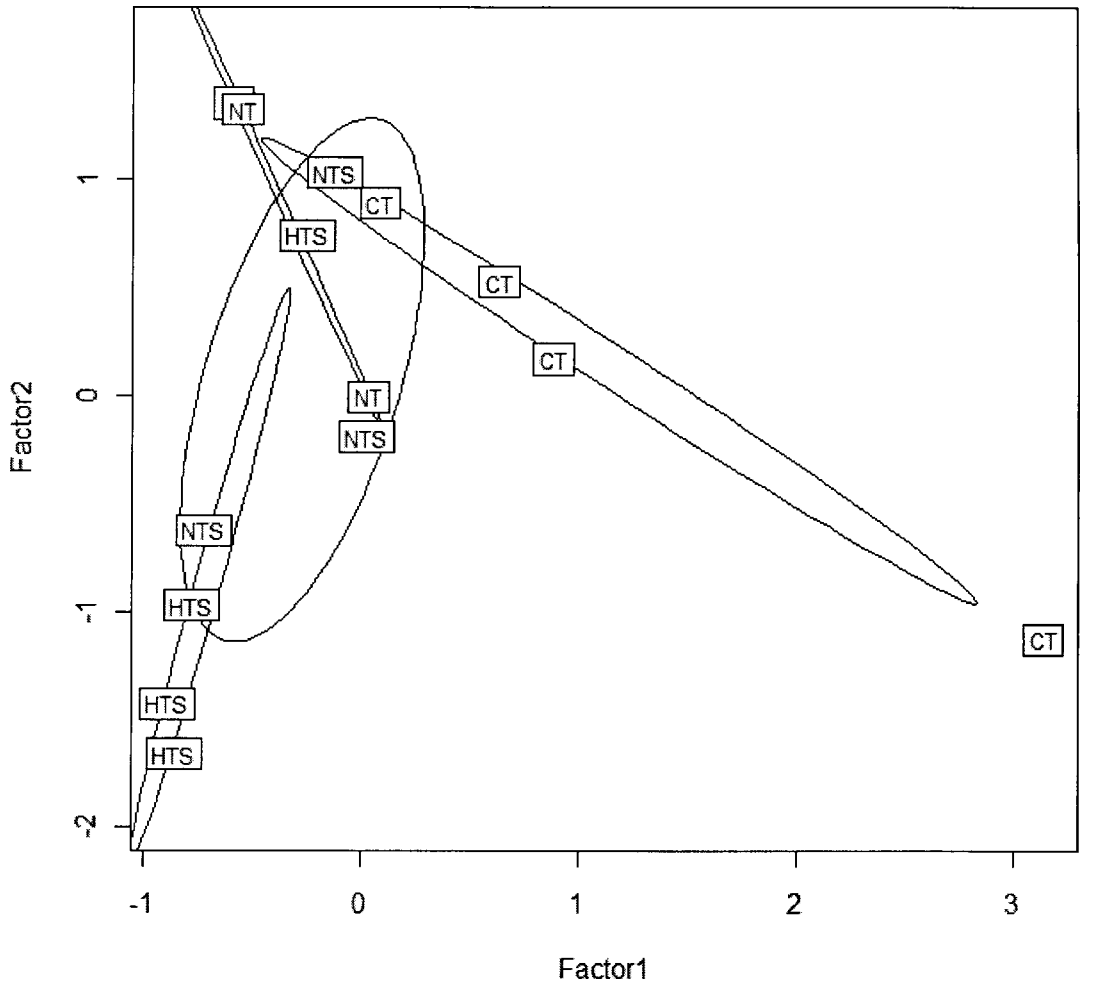


Figure 3.9. Principal component analysis ordination showing separation of agricultural treatment plots at the KBS-LTER based on minor clade similarity with 95% confidence ellipses for treatment centroids.

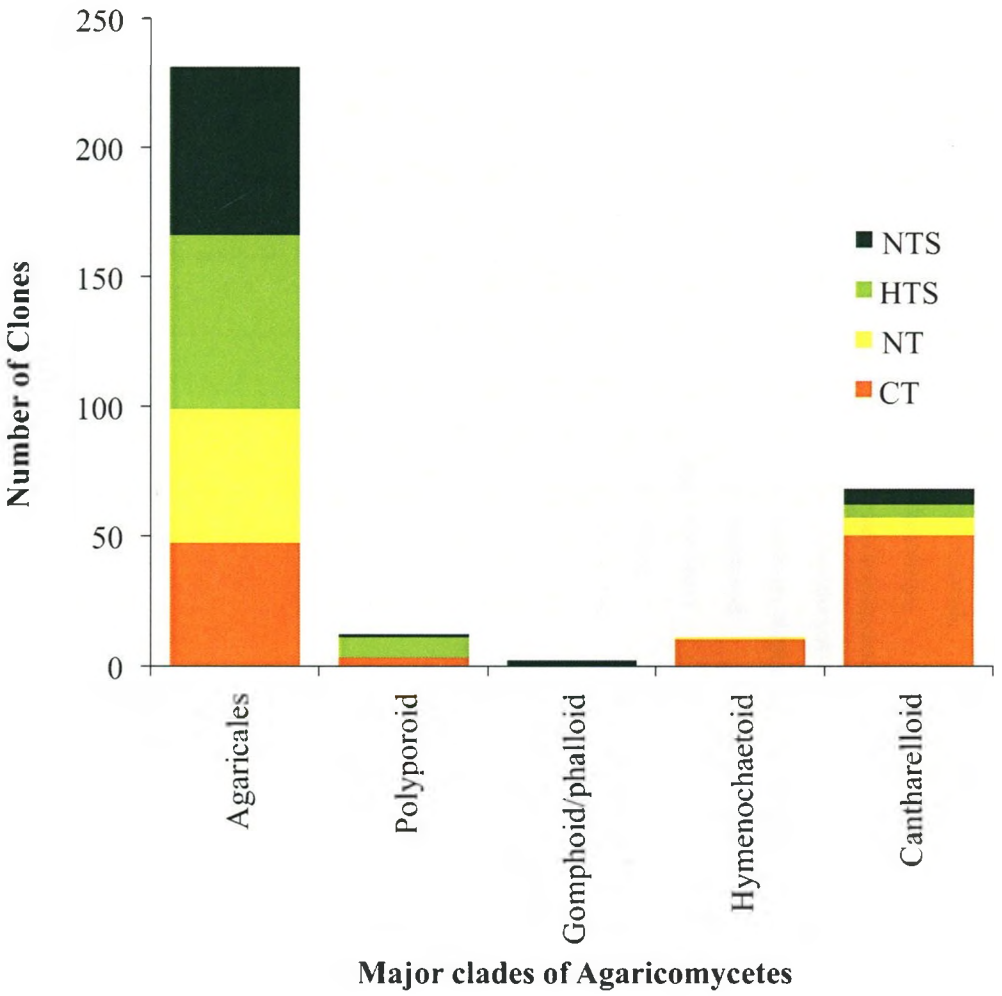


Figure 3.10. Representation of the major clades of Agaricomycetes in four agricultural treatments at the KBS-LTER site. The Agaricales clade showed relatively equal prevalence among treatments. In contrast other clades such as Gomphoid/phalloid and Hymenochaetoid clades were rare and detected in only one or two treatments.

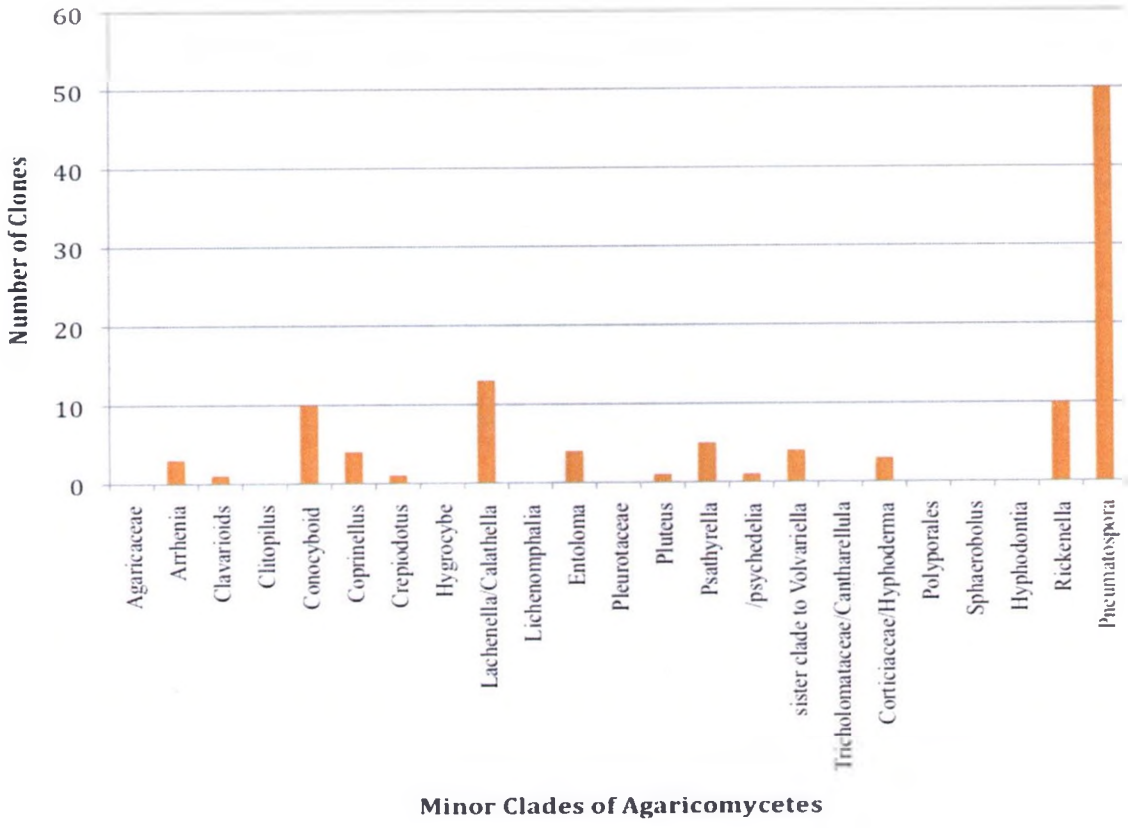


Figure 3.11. Occurrence of minor clades of Agaricomycetes in the Conventional Till treatment at KBS-LTER site. Number of clones is an index of prevalence of each clade within the treatment

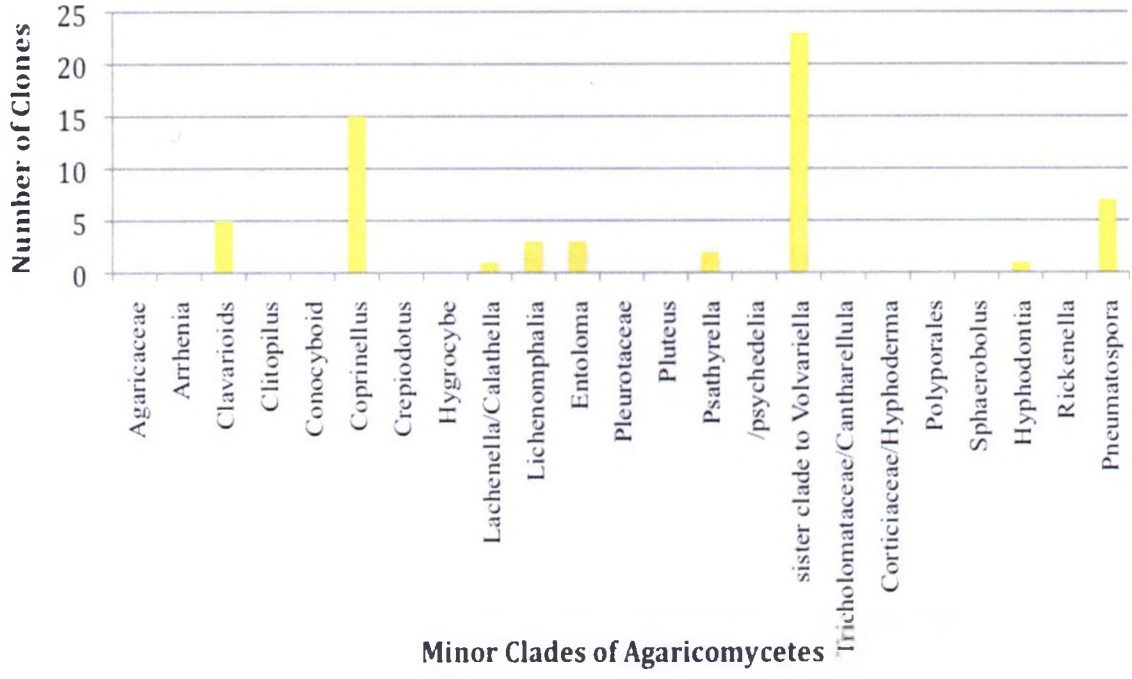


Figure 3.12. Occurrence of minor clades of Agaricomycetes in the No Till treatment at KBS-LTER site. Number of clones is an index of prevalence of each clade within the treatment

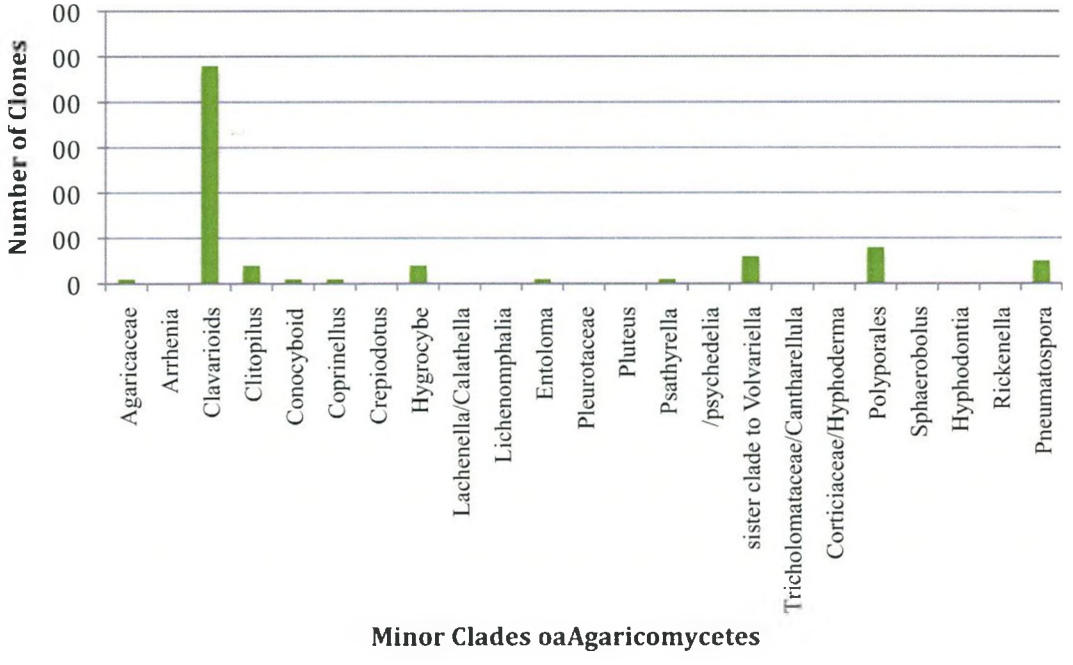


Figure 3.13. Occurrence of minor clades of Agaricomycetes in the Historically Tilled Successional treatment at KBS-LTER site. Number of clones is an index of prevalence of each clade within the treatment

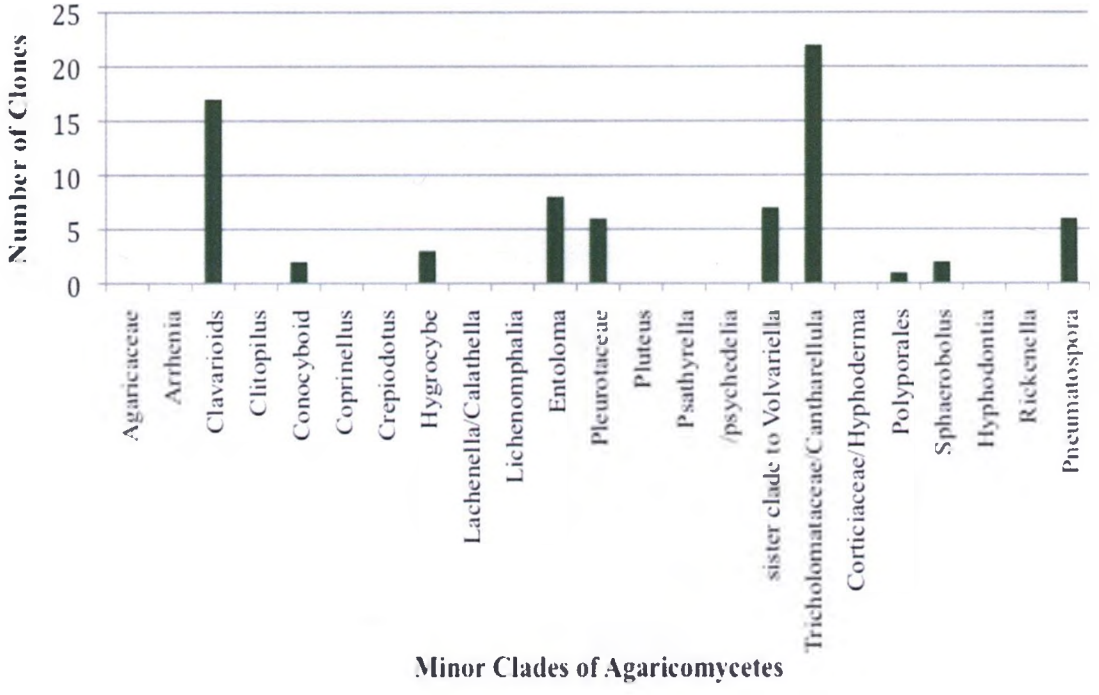


Figure 3.14. Occurrence of minor clades of Agaricomycetes in the Never Tilled Successional treatment at KBS-LTER site. Number of clones is an index of prevalence of each clade within the treatment

Representation in Conventional Till plots

Examination of patterns of minor clades among treatments indicated that six of the 21 minor clades were most prevalent in CT: *Pneumatospora, Lachnella/Calathella* (*Nia* sensu (16)), Conocyboid (Bolbitiaceae), *Rickenella, Psathyrella* and occurred in at least

one other treatment (Figure 3.11-3.14). The most common clades in CT were *Pneumatospora* and *Lachnella/Calathella* (*Nia*).

The highly prevalent *Pneumatospora* clade of Cantharellales accounted for 44% of all CT clones and was represented by two OTUs. Sixteen sequences grouped into one OTU while the second OTU in this minor clade was represented by one sequence. The closest identified GenBank match for the more prevalent OTU was *Minimedusa obcoronata* (33).

The *Lachnella/Calathella* clade was represented by a total of three OTUs, two of which were detected in CT. These OTUs accounted for 12% of all CT clones analyzed. In the present study all the OTUs detected in this clade were sampled from monoculture treatments (CT and NT).

The Conocyboid clade was detected in CT, NTS and HTS with 77% of the clade occurrences in CT. This clade represented nine per cent of all CT clones analyzed.

One OTU was in *Rickenella*, part of the Hymenochaetoid Clade (69). This OTU was represented by one sequence and accounted for nine per cent of all CT clones.

Five of the eight occurrences of the *Psathyrella* clade were detected in CT treatments representing five per cent of all CT clones analyzed. In the present study, the *Psathyrella* clade was detected in CT, NT and HTS (in order of decreasing prevalence) but was not detected in NTS.

The Corticiaceae/*Hyphoderma* clade, part of the Polyporoid clade, was represented by two sequences grouping into one OTU. Clones belonging to this clade in CT composed three per cent of all CT clones analyzed.

Representation in No Till plots

Two of the minor clades detected had the majority of their occurrences in the NT sites: the "Sister clade to *Volvariella*" and *Coprinellus* clade. The most prevalent was the "Sister clade to *Volvariella*". Twenty-three of the 40 clones represented in 4 of the 6 OTUs in the "Sister clade to *Volvariella*" were sampled from NT sites. This clade was detected in all treatments. Two sequences from NT represented by 15 clones were grouped into one OTU within the *Coprinellus* clade. A total of 20 clones were identified as this OTU, with detections also in CT and HTS.

Representation in CT and NT plots (cropped with inputs)

One minor clade was equally represented in CT and NT plots, *Omphalina*. There were three OTUs detected in the *Omphalina* clade with two occurring in separate plots of NT representing five per cent of all NT clones analyzed. The third OTU was detected in CT.

Representation in Historically Tilled Successional plots

Two minor clades had the highest prevalence in historically tilled sites (HTS): Clavarioid and *Clitopilus*. The Clavarioid clade was the most prevalent in this treatment representing 61% of all HTS clones analyzed and 68% of all clones detected in the clade. This minor clade occurred in all treatment with 14 OTU in HTS, six OTUs detected in NTS, two in NT and one in CT.

The *Clitopilus* clade was only detected in HTS plots. They represented five per cent of all HTS clones analyzed and grouped into one OTU.

Representation in Never Tilled Successional plots

One clade was detected only in NTS plots, the Gomphoid/phalloid clade and another clade had the highest prevalence in NTS: the Entolomataceae minor clade of the major Agaricales Clade. The detected OTU in the Gomphoid/Phalloid clade grouped with *Sphaerobolus iowensis*. This OTU was also recorded by Lynch and Thorn (93) in the 2002 sampling in the same plot. Three OTUs represented by 8 sequences detected in NTS were grouped in the Entolomataceae clade. This clade was present in all treatments but highest prevalence was found in NTS

Representation of mixed vegetation with no inputs (HTS and NTS)

Out of the 23 (21, 23?) minor clades, two were detected only in the mixed vegetation plots of HTS and NTS: the *Hygrocybe* and Polyporales clades. Two OTUs representing a total of seven sequences were detected in *Hygrocybe* clade. One OTU was found in each treatment type. The OTU detected in HTS matched a GenBank sequences for *Hygrocybe conica* (AY684167.1) at 99% similarity. The OTU detected in the NTS was also detected in 2002 in the same treatment by Lynch and Thorn (93).

Two sequences, one from each HTS and NTS, were detected in the Polyporales clade. These sequences grouped into one OTU that represented 14 clones. The OTU detected matched *Ganoderma lucidum* (DQ208411) at 99% similarity.

CHAPTER 4: DISCUSSION

Chapter 4: Discussion

Sampling adequacy

This study did not sample beyond the linear growth phase of species accumulation as the rate of new OTU accumulation at the maximum observed sample size (n=15) was estimated to be one per sample (Figure 3.1). As a result, sampling adequacy was insufficient to draw conclusions about total species richness in the site based on resampling methods. This conclusion is shared by Lynch and Thorn (93) whose sampling effort was greater than this study. The Chao 1 estimator of species richness indicated that both studies together were able to capture approximately 66 per cent of the expected diversity, resulting in a large discrepancy of estimated total richness between studies. Given this rate of under-sampling it is likely that total species richness as estimated by Chao 1 (25) is an underestimation of actual richness (73, 79, 95). Methods such as those used in the Chao 1 estimator derive estimates of species richness by mathematically resampling the source data. In addition to this resampling approach, data from two independent samples were used to estimate richness based on mark-recapture methods. A mark-recapture approach includes the total species pool observed in both studies providing more data and information (recapture events) from which estimate calculations are made. The Lincoln-Peterson mark-recapture index predicted higher diversity than each individual study. Using two sampling periods (i.e. data from the two studies) is likely a more robust calculation of total richness. Most remarkable is that an agricultural/ grassland site could contain upwards of 359 Species of Agaricomycetes, as

estimated by the Lincoln-Peterson index. This estimate is likely conservative since the assumption that species are equally "available" (i.e. equally prevalent) in the soil is not met. For example, taxa occurring in low numbers at the site would be less likely to be sampled than more abundant taxa. The variability in the prevalence of taxa within clades (Figures 3.3 and 3.4) indicates unequal capture probability of each species at the study site.

Molecular technique variation and repeatability of results

Comparison between the results of this study and those of the previous study by Lynch and Thorn (93) provides insight regarding the sensitivity of molecular methods in assessing fungal diversity in soils. Similar patterns of species richness within major and minor clades were detected in both studies (Figure 3.7). This indicates that combining soil samples to plot level and reducing the number of clones screened for restriction fragment length polymorphisms in the present study as compared to that of the Lynch and Thorn study (93) produced comparable community composition patterns. Site composition was similar both in the proportion of observed OTUs and the phylogenetic diversity represented within each clade. Differences between study methods are apparent at the OTU level; Lynch and Thorn (93) recovered 3.5 times greater richness, excluding the Heterobasidiomycetes and the Ceratobasidiales. As these groups have several mismatches to the B001 primer (93) it is likely that they were not amplified by the more stringent PCR protocol employed in the current study; hence they were omitted in the calculation of richness differences between studies.

Although overall patterns of clade diversity were similar, variations between the two studies' molecular methods and in the intensity at which the plots were sampled are likely important factors leading to the large difference in total richness. The key differences in methods between studies were i) the amount of soil from which DNA was successfully amplified (980 g compared to 450 g in present study) ii) the ratio of amplification to cloning reactions (1:1 ratio compared to 3:1 ratio in the present study) and iii) the number of clones analyzed (1,146 compared to 324 in the present study).

To test if the larger number of soil samples was the cause of the difference in richness, which would be expected, the number of OTUs detected as a function of soil mass was calculated. In the present study calculations indicate that these methods were able to detect approximately 1.2 OTUs per 10 g sample. Application of this calculation to homobasidiomycete (syn. Agaricomycetes) data reported by Lynch and Thorn (93) resulted in 1.9 OTUs per 10 g sample. Thus if sample size was the same as for the current study to that of Lynch and Thorn, 117 OTUs would be expected in this dataset (based on 1.2 OTUs per sample). The remaining difference between this corrected species richness (117) with the richness reported by Lynch and Thorn (186) indicates that differences in richness were not simply a function of number of samples, but were also influenced by variation in the molecular methods.

In order to minimize the cost of conducting molecular work, the original methods employed by Lynch and Thorn (93) were altered. One significant change was to pool PCR products at the sample level before cloning as a way of reducing the number of cloning reactions from one reaction per soil sample to one reaction per plot (*i.e.* 3 soil samples). At the time it was not known if this modification would have an effect on the

diversity of sequences cloned. In theory, combined amplification products may reduce the representation of rarely occurring sequences as the dominant sequences in each of the three reactions would have a higher probability of being cloned. Comparison of the number of OTUs to number of clones in each study indicated that both methods resulted in identical richness among clones (16 OTUs/100 clones). The similarity in this ratio indicated that differences in OTU richness between these studies are due to the different number of clones analyzed per unit soil (12 clones/10 g soil as compared to 7 clones/10 g of soil). Thus the lower number of clones analyzed in this study along with the smaller amount of soils sampled account for the 3.5 times difference in richness that exists between studies

Diversity in agricultural soils at the KBS-LTER site

The high degree of similarity between the frequency distribution of observed OTUs among clades and the distribution of all known species among clades was unexpected (Table 3.1). Given the small amount of soil collected and the rather specific habitat conditions of the site proportions of OTUs in each clade would have been expected to deviate somewhat from the distribution of all known taxa as a result of sampling bias, colonization limitation, and habitat requirements. The findings of this study suggest that the majority of clades contain a broad spectrum of species, some of which can tolerate agricultural conditions. However, there are clear deviations between observed proportions and known proportions particularly in three clades: Agaricales, Boletales and Russuloid. The latter two clades of basidiomycetes were not detected in this study and are predominantly ectomycorrhizal. Species in these clades likely lack suitable hosts in agricultural systems. The Agaricales clade was more highly represented and while this

clade contains the greatest richness among known sequences, it also appears to be favoured in the KBS experimental site. The broad diversity detected in this study suggests that the PCR-produced bias was minimal, which is expected when using highly diverse environmental templates (143). Approximately half of the 52 OTUs of Agaricomycetes reported here were also detected by Lynch and Thorn (93). Considering that these studies were conducted in different years, that soil microbial communities tend to be highly heterogeneous at fine spatial scales, and that total volume of soil samples was small, this degree of concordance is higher than expected.

Agaricales clade

The Agaricales clade, containing 69% of observed OTUs, was dominant in KBS agricultural soils. The OTUs belonged primarily to two minor clades within the Agaricales: the Clavarioids and The "Sister clade to *Volvariella*", which indicates that these clades are probably dominant components of the basidiomycete community at the KBS study site. Clavarioids are primarily considered saprobic (32) although the nutritional mode for many taxa in this group is not yet known (96). They are a common group of fungi in grasslands (97) and are one of the taxa that has been proposed as indicators of grassland health (97). The Clavarioid taxa detected in this study are dispersed throughout the clade as defined by identified sequences from GenBank, similar to the finding of Lynch and Thorn (93). The structure seen in the phylogenetic tree (Figure 2.6) suggests that more fine-scale diversity exists than is currently recognized for this clade. Of the 15 OTUs detected in the clavarioid clade, 10 matched sequences from Lynch and Thorn (93) at greater than 99%. The "Sister clade to *Volvariella*" is named for the group's close relation to the genus *Volvariella*. Currently thought to be a small genus

of saprobes, *Volvariella* is perhaps best known for its commercially important species, the straw mushroom (*V. volvacea*). Little appears in the literature regarding the ecology of this genus apart from work concerning its cultivation (23), but the eight *Volvariella* species known in the Netherlands are lignicolous or terrestrial, on litter and rich soils in forests and grasslands (17). The absence of any identified sequences in this clade suggests they have been overlooked by traditional morphological taxonomy, due perhaps to the fact that they form minute or hypogeous fruiting bodies, or fruit rarely. This clade was also detected by Lynch and Thorn (93), which indicates that it is persistent in these soils.

The single OTU detected in the Tricholomataceae/Cantharellula clade was represented abundantly in one sample. This clade contains numerous nutritional modes and includes at least 10 ectomycorrhizal lineages, but also many that are saprobic (96). The best match to GenBank reference sequences was to a sequence obtained by Lynch and Thorn (93) from the same plot. Repeated occurrence of the OTU at a given sampling point suggests the presence of an established hyphal network in this particular location.

The majority of species in the Psathyrellaceae (120), formerly treated in Coprinaceae, are saprotrophic (130). They are thought to act as primary or secondary decomposers in terrestrial ecosystems (110). This study detected four OTUs, with one sharing identity (>99%) to a sequence deposited by Lynch and Thorn (93). Two OTUs in this minor clade lacked close matches to GenBank sequences. This suggests that much of the diversity of soil fungi is not yet characterized in the deposited sequences in GenBank, which is not at all surprising when comparing the number of described species to those represented by sequences in Genbank.

The omphalinoid-hygrophoroid group of Moncalvo *et al.* (102) encompasses *Omphalina* and the Hygrophoraceae. OTU 17 is part of the *Arrhenia* group (102) that contains the core of the non-lichenized *Omphalina.* Almost all known species in the /*arrhenia* clade and many species of *Omphalina,* including those closely related to OTUs 31 and 32, are bryophilous, i.e., parasitic on or closely associated with mosses or liverworts (92).

Species of Hygrophoraceae are common in grasslands and some are particularly sensitive to fertilizers. Although the group also contains ectomycorrhizal taxa, the genus detected in this study, *Hygrocybe*, is considered saprotrophic (97, 102). Arnolds (7) raised concerns about the loss of *Hygrocybe* diversity in European grasslands resulting from long term fertilization of these areas and it is now used as an indicator of grassland health (123). In this study one OTU was identified to a species matching the deposited sequence in GenBank for *Hygrocybe conica* (99%). According to Evans (37) *Hygrocybe conica* is considered one of the species tolerant to fertilization.

The *Nia* clade (16), referred to as the *Lachnella/Calathella* clade by Hibbett and Thorn (69), contains most of the cyphelloid members of Agaricales (16), which have minute, cup-shaped fruiting bodies without gills. In this study five sequences representing three OTUs were detected. Two of the OTUs in this clade appear separated from the main group of identified sequences, perhaps suggesting a greater extent of diversity for this clade than is reflected in current databases. The diversity of the *Nia* clade has likely been underrepresented in field surveys owing to its very small fruiting bodies.

The Entolomataceae are considered one of the most species-rich families of Agaricales (12). Given the diversity that exists in this clade, it is interesting that one OTU detected in the current study was recovered by Lynch and Thorn (93) and the same genus (*Clitopilus*) was observed in culture-based studies conducted by Thorn *et al.* (136). *Entoloma* is one of the taxa used to assess grassland health (97).

The three OTUs detected in the family Bolbitiaceae (102) are all within the conocyboid group. *Conocybe* species are saprotrophic and are found worldwide in association with fertile soils such as lawns.

Polyporoid clade

The Polyporoid clade definition applied in this analysis includes two distinct groups: the Polyporales and the Corticiaceae/hyphoderma. Both of these groups consist of lignicolous saprotrophs (white-rot fungi) (69, 89). They are decomposers of woody material and, in the absence of woody vegetation in these disturbed sites are likely associated with lignin-rich coarse textured herbaceous plants such as *Solidago* and *Arctium* that dominated the non-cropped sites.

Cantharellales clade

The most prevalent taxon (21% of all clones) detected in this study was in the Cantharellales. It was sampled repeatedly among the plots suggesting a possible ubiquitous distribution in the KBS site. It matched an OTU detected by Lynch and Thorn (93). The phylogenetic analysis of this study placed this OTU in *Pneumatospora* with closest identified sequence match to *Pneumatospora obcoronata*. Diederich and Lawrey (33) proposed that *Pneumatospora* be regarded as a synonym of *Minimedusa* in

accordance with a recent phylogenetic analysis of this group. *Pneumatospora obcoronata* has been recorded on leaf litter in Japan, Barbados, and Jamaica (33) and is considered a saprotroph.

Hymenochaetoid clade

The Hymenochaetoid clade is highly diverse morphologically. Despite the wide variety of fruiting body structures encompassed by this clade, the constituent taxa all seem to share a saprobic lignicolous life strategy. In the phylogenetic tree presented in Figure 3.5 the Hymenochaetoids are split into two groups: *Hyphodontia* and *Rickenella*. *Hyphodontia* is the largest genus of corticoid basidiomycetes (90), is often found on dead plant material, and is regarded as primarly lignicolous (128). *Rickenella* and other gilled taxa in this clade are bryophilous (119). Whether the association with bryophytes is mutualistic or parasitic is not yet known (90). This study detected one OTU in *Rickenella* and two in *Hyphodontia*.

Gomphoid/phalloid clade

The OTU detected in this clade was most closely related to *Sphaerobolus iowensis*. An identical sequence to this OTU, along with another in the same clade, was detected at the same sampling site (NTS-3) by Lynch and Thorn (93). Species of *Sphaerobolus* are accomplished white-rotters of straw and woody debris (48).

Boletes, Russuloid and Thelephoroid

The Boletes, Russuloid and Thelephoroid clades all consist primarily of ectomycorrhizal species (69, 77, 88). While the Russuloid and Thelephoroid clades were

recovered infrequently by Lynch and Thorn (93) they were not detected in the current study. Their absence in this study is probably related to a lack of suitable host species.

Diversity among agricultural treatments

Soil organic matter

Soil organic matter (SOM) is an important substrate for basidiomycetes as they are heterotrophs and rely on organic matter as an energy resource. SOM is also positively correlated with water holding capacity and aggregate formation which has been attributed to fungal activity in the soil (31, 138). Thus the relatively large range in SOM across treatments in this study (3.8% - 9%, Figure 3.7) may be expected to affect community richness but differences in richness among treatments were not detected in this study. These results are likely related to the detection of insufficient numbers of taxa to see a pattern between variation in SOM and species richness. Richness estimators indicated that the accumulation of taxa within treatments was in the linear phase which makes estimation of total richness difficult (95). This is expressed by the large confidence intervals around richness estimation numbers in each treatment. In contrast, a relationship between community composition within plots and SOM was observed.

The separation of treatments along the x-axis of PCA is correlated with SOM levels and suggests that even though richness did not varying among SOM levels, organic matter content may be an important factor affecting taxonomic composition within plots. Although SOM content may contribute to the separation of plots along PCA axis 1 it does not fully explain their placements. If SOM was the principal structuring factor and affected community composition through a linear relationship, plots CT, NT and HTS

would be expected to group quite closely together as SOM was not significantly different among these treatments. NTS plots would be expected to separate out from the other plots as they contained significantly higher levels of SOM. As Factor 1 shows CT separated from the remaining three treatments, other factors unique to CT, such as tillage, appear to be more influential drivers of community composition.

Although not significant at the sampling intensity of this study, the higher levels of SOM found in HTS compared to CT and NT indicates partial replenishment of these properties since the abandonment of cropping, fertilizing and tillage. However, the large difference between SOM levels in HTS and NTS plots remaining after 16 years since cropping on HTS suggests recovery of SOM levels to pre-tillage conditions for these soils is a relatively long process and has not yet occurred.

Richness and genetic diversity

Estimates of richness (e.g. ACE, Chao 1) did not differ among treatments. Analysis of genetic diversity as opposed to species diversity indicated that NTS contained significantly greater genetic diversity up to a threshold of 0.11 evolutionary distance units (Figure 3.8). This threshold represents a greater diversity in NTS at a taxonomic level above species (e.g. genus or family), as species-level genetic diversity for the particular DNA region analyzed is represented by 0.01 evolutionary distance (99% sequence similarity). Thus OTU richness in NTS was higher at fine scale levels of diversity in comparison to the other treatments. What this suggests is a smattering of broad diversity in CT, NT, HTS, but a greater depth of diversity in NTS. The absolute breadth of diversity (x-intercept in Figure 3.8) is approximately equal among treatments.

The variety of substrates present in NTS such as woody debris, perennial roots, and mixed vegetation offer a broad selection of substrate types not available in the mono-culture plots. The added dimension in NTS of an undisturbed soil profile may create a greater diversity of microenvironments within a given area promoting more fine-scale diversity. The potential for greater diversity in niches and substrates in this treatment seems to relate to diverse genetic representation.

Community composition

Ordination results showed that patterns of minor clade occurrence among sampling units is highly variable. Eight of the 21 minor clades were so rare that they were not informative for quantifying the overall patterns of taxonomic co-occurrence explored using PCA. Even after omitting these rare taxa from the PCA, the representation of clades among sampling units appears highly idiosyncratic at the level of sampling that was carried out in this study. Nonetheless, multivariate results provided some useful insights into potential factors affecting taxonomic composition.

Mono-culture and successional grassland cover types were significantly different in taxonomic composition. The results from PCA suggest that differences between CT and the other treatments may be driving the significant difference between mono-culture and successional grasslands. The closer grouping of NT with HTS and NTS compared to CT suggests that soil disturbance may be an important influence on the community of Agaricomycetes. These findings differ from those reported by Buckley and Schmidt (20) who found that microbial composition was not discernibly different between cultivated and historically cultivated soils at the KBS-LTER site. This study focused on prokaryote microbes and did not specifically report on fungi.

The overlap of HTS plots and NTS plots indicates that the community of Agaricomycetes detected in HTS plots (last tilled 16 years ago) may be recovering to pre-disturbance communities. The lack of complete overlap in ordination ellipses of HTS and NTS suggests returning to a base line community state requires more than a decade, if it occurs at all. The significant difference in SOM levels between HTS and NTS suggests that these two treatments remain quite distinct in terms of physical soil characteristics.

The affinity of minor clades to particular treatments and to treatment groupings based on cover type is likely due to treatment-induced habitat properties that act as selective filters on the pool of available clades. To gain further insight into the differences detected in communities among agricultural treatments, clades showing strong associations with different treatments are discussed with respect to available information on life history requirements.

Conventional Till plots

Two clades showed strong affinity for CT plots relative to other treatments, and are therefore likely drivers of the separation of CT plots from other plots in PCA. The *Pneumatospora* clade of Cantharellales was the most prevalent minor clade in CT and was three times more prevalent in CT than any other treatment (Figure 3.11). This clade accounted for 44% of all clones obtained from CT plots. All sequences of this minor clade grouped closely to a species, *Minimedusa obcoronata* (33), recently identified from litter and leaves collected in Malaysia, Jamaica and Barbados. The *Minimedusa* genus has been noted to form a vegetative propagule. Spread of these propagules via tillage is a probable explanation for its the broad distribution in CT. Patterns of prevalence and

diversity of *Pneumatospora* were very similar between study years (i.e. 2002 (93) and 2004) with a large number of clones representing many OTUs with limited diversity (all grouping near *M. obcoronata*). Both studies detected this minor clade in all treatments with highest prevalence in CT (57% of all *Pnuematospora* clones occurred in CT in 2004 and 50% in 2002). Together these studies suggest that *Pneumatospora* is limited in diversity in agricultural treatments, but some species appear to do very well under conventional tillage practices. Its nutritional mode is believed to be saprobic, therefore its high prevalence in CT plots may be due to a preference for substrates associated with CT (crop residue) or stimulation by tillage. The close matching to a recently described species suggests that the diversity of species within *Pneumatospora* may be greater than currently described. It is interesting to note that the particular OTUs of *Pneumatospora* detected at KBS have not yet been described within available sequence data even though they are very prevalent in the soils at this site.

The *Lachnella/Calathella* clade was also a prevalent minor clade in CT, and all the OTUs detected in this clade were sampled from the monoculture treatments CT and NT. Similar results were recorded in 2002 where all but one OTU was detected in the mono-culture treatments (4-CT; 2-NT; 1- HTS). The cyphelloid fungi grouping together in *Lachnella/Calathella* are readily culturable saprotrophs occurring on wood and diverse plant litter (2).

Minor clades prevalent in CT must tolerate frequent soil disturbance and additions of nitrogen and phosphorus. The main difference between CT and NT is a reduction in the intensity of physical soil disturbance, thus minor clades showing strongest affinity for CT would be expected to exhibit strategies better suited to disturbed soils. In absence of life

history information about these taxa I cannot specifically explain the apparent preference for CT plots of OTUs in these clades. Perhaps they are relatively quick to colonize and establish after disturbance and could be considered ruderal, early colonizers. Alternatively, these species may tolerate disturbance and in this manner continue to persist and dominate this type of treatment. For example taxa that initially colonize above-ground plant material may increase in abundance and distribution in tilled plots since tillage integrates and homogenizes plant biomass (and associated symbionts) into the soil profile.

No Till plots

The most prevalent clade in NT was the "Sister clade to *Volvariella*". Although this clade was detected in all treatments, both in 2002 (93) and in the present study over 50% of occurrences were in NT. In this study, it accounted for 38% of all NT clones analyzed. It is therefore likely an important clade responsible for the separation of NT plots from others in PCA. This clade was well represented in diversity in both sampling years. Four of the six OTUs detected in the current study were among the 22 detected in 2002 by Lynch and Thorn (93), suggesting that even more diversity exists in this clade. No identified sequences from GenBank grouped with the detected sequences in this group, making it difficult to comment on the ecology of this group. The ubiquitous distribution and represented diversity of this unknown clade in the agricultural soils of this site warrants further attention to its ecological role.

The *Coprinellus* clade was the second most prevalent minor clade in NT. While in this study the *Coprinellus* clade was most prevalent in NT, with low representation in CT

and HTS, Lynch and Thorn (93) detected the majority of the *Coprinellus* clade in HTS with representation in both CT and NT and low detection rates in NTS. Overall this suggests that although it was highly prevalent in NT in the present study, this minor clade seems more generally associated with NT and HTS. These treatments share conditions such as nutrient enhanced soils with no tillage. Ecologically, *Coprinellus* (recently segregated from *Coprinus*) is saprobic. It often occurs in lawns and on a variety of organic debris, suggesting it has some tolerance to nutrient addition and occasional soil disturbance and likely can use numerous substrates as a carbon source (74).

Both of the minor clades discussed above were also found to occur in CT at low levels ("Sister clade to *Volvariella*" = 4% of CT clones; *Coprinellus* = 4% of CT clones). This suggests factors such as fertilizer additions and mono-culture above ground vegetation shared by these treatments may be important to these groups; however, the apparent prevalence in no till plots suggests that they may be sensitive to tillage.

Cropped with inputs plots (CT & NT)

The *Omphalina* clade (118) (*Arrhenia sensu* Moncalvo et al. (102)) was equally represented in CT and NT. This minor clade contains both lichenized and non-lichenized species. The three OTUs detected are part of the non-lichenized *Omphalina*, which may be associated with bryophytes or algae (118), or parasitic on wheat and other grass crops (75); the latter might better explain its occurrence in CT and NT.

Historically Tilled Successional plots

The Clavarioid clade was well represented among treatments and in diversity of OTUs with the largest number (68%) occurring in HTS, where they represented 66% of

all HTS clones analyzed. This was the only clade that showed preference for HTS. This clade includes saprobic, mutualistic (mycorrhizal and algal-associated) and parasitic forms (32). The patterns of detection among treatments noted in the present study were similar to data from Lynch and Thorn (93) except that Lynch and Thorn detected the highest prevalence in NTS, whereas in the current study only 25% of clavarioid sequences were detected in NTS. Irrespective of these differences, both study results indicate that Clavarioids are more prevalent in less disturbed soils with mixed perennial vegetation. McHughs (97) identifies the family Clavariaceae along with several other fungi as important for conservation in grasslands. Most of the fungi listed as being of conservation value appear to be negatively affected by fertilization as is supported in the findings from this study and that of Lynch and Thorn(93) .

Never Tilled Successional plots

Never tilled successional plots had one minor clade that was not detected in any other treatments. This minor clade was represented by one OTU that matched a sequence detected by Lynch and Thorn (93) and grouped as Tricholomataceae/*Cantharellula*. *Cantharellula* is an associate of mosses, but its closest relatives are saprobic on wood (119). Although prevalent, this OTU only occurred in one plot suggesting that the original extraction was highly saturated with one type of DNA. Twenty-two clones of this OTU were detected in a single plot (NTS-1), which may be a result of amplification and cloning bias and not representative of the plot-level clade affinities. However, Lynch and Thorn (93) also detected this minor clade in all NTS plots with 50% of the OTUs from NTS-1. This minor clade shows affinity for NTS possibly as a result of greater

prevalence of woody plant hosts (*Rubus sp.*, *Rosa sp.* and *Sassafras sp.*) in this treatment and appears well established within one of the treatment plots (NTS-1).

The major Gomphoid/phalloid clade was only detected in NTS-3 and was represented by one OTU that grouped with *Sphaerobolus iowensis*. This species, one of the cannonball fungi, often occurs on woody substrates, where it causes a white-rot (49). Lynch and Thorn (unpublished) detected a matching OTU in the same plot during their study suggesting a well-established hyphal system that was perhaps sampled repeatedly.

Species occurring in NTS and in no other treatment likely require specific perennial hosts and may therefore be late successional taxa. They may be sensitive to fertilization and are likely negatively affected by tillage given their association with perennial plant communities.

Mixed vegetation with no inputs (NTS and HTS)

Two minor clades, *Hygrocybe* and Polyporales, were present only in NTS and HTS plots. The *Hygrocybe* clade contains what are referred to as the waxcap fungi of grasslands (97), many species of which are important indicators of habitat quality since they are sensitive to fertilizer applications (53), although other species are tolerant [49]. The one OTU of *Hygrocybe* detected in both HTS and NTS in the current study matched a GenBank sequences for *Hygrocybe conica,* which is considered tolerant to fertilization (37). The presence of this species in these no-input plots therefore is unlikely related to fertilization regimes and does not signify a recovering of HTS plots to pre-tilled conditions as might be indicated from the detection of more fertilizer sensitive *Hygrocybe*

species. Occurrence of *Hygrocybe conica* is likely more related to preference for undisturbed soils.

The Polyporales clade was detected only in HTS and NTS in the current study and principally in HTS and NTS in 2002 with only one OTU outside these treatments. The Polyporales, one of the two minor clades of the Polyporoid clade, (102) consists of wood-decaying species, in particular lignin degrading taxa. As HTS and NTS have perennial mixed-vegetation there are likely to be substrate sources above and below ground for this wood-decaying group. The detected sequences were matched to *Ganoderma lucidum,* which is thought to persist in soils and attack stressed or injured hardwood trees at the base, and this species (or a close relative) also causes a disease of coconut trees and oil palms (42). Lynch and Thorn (93) also detected this species. The host species in these plots is not known.

While there is evidence of preference of the *Hygrocybe* and Polyporales clades for HTS and NTS plots, the possible cause of these associations are not clear. It is likely that both taxa share preferences for untilled soils with perennial heterogeneous vegetation cover.

Summary of findings

The results of this study indicate that molecular methods were able to detect gross patterns of community richness from a relatively small volume of soil. This conclusion is supported by data collected at the same site two years prior by Lynch and Thorn (93). The OTUs detected were diverse in evolutionary history, fruiting body morphology, and included saprobic, mycorrhizal, and possibly parasitic life strategies. The overall

similarity between this molecular study and that of Lynch and Thorn conducted in 2002 (93) suggests that the community is relatively stable. Phylogenetic analysis indicated that more genetic diversity exists than is yet documented in GenBank.

In the four treatments examined in this study, tillage appears to cause a detectable shift in basidiomycete community composition. As basidiomycetes typically form extensive mycelia composed of filamentous hyphae, it is reasonable that the physical disturbance of tillage may have important structuring effects on the community composition (e.g. (129)). Tillage has been noted to shorten the length of hyphae which appears to reduce the fungi's ability to colonize thus limiting the extent of growth (13, 145).

Similar overall patterns of minor clade distribution among treatments were found in this study and Lynch and Thorn's study (93) conducted in 2002. Numerous unknown species were detected in multiple plots within each study year. Findings such as this are not unusual for molecular-based studies (83, 126) and indicate the ability of molecular approaches to detect undescribed or unsequenced but relatively abundant taxa. Of particular interest at the KBS-LTER site is the prevalence of the "sister clade to *Volvariella*". While the sequences in this clade show deep branching diversity and appear to be well distributed among treatments no known sequences grouped within this clade. The "sister clade to *Volvariella*" was the third most prevalent clade detected, occurring in all treatments. Another large group of unidentified sequences of interest from this study occurred within the Clavarioids, the most common clade detected in the present study.

The number of fungal species represented in sequence databases is relatively small compared to what is currently described (86) thus the unknown taxa in this study

could be a result of poor representation of known species in sequence databases. However the extent of unidentified diversity that were observed supports the argument that much of the fungal diversity is yet to be discovered (60). These unidentified taxa likely fruit infrequently and are probably not culturable under traditional laboratory conditions and thus have escaped detection via traditional sampling methods. Further identification, description and life history information for these taxa are needed as a precursor to understanding how their diversity contributes to soil functioning. Even commonly identified taxa are little understood in the way of life history strategies (34, 41). Gathering life history information will likely prove challenging especially for these unseen and unculturable species, but techniques such as stable-isotope probing (SIP) may aid in deciphering the roles and activity levels these unknown taxa may have in the soil ecosystem (114, 115).

CHAPTER 5: GENERAL DISCUSSION

Chapter 5: General discussion

The results presented provide insights into the fungal biodiversity of the KBS-LTER study area and into associations of specific taxa among experimental agricultural treatments. In addition to these site-specific findings, evidence of large gaps in our current knowledge of fungal diversity (as represented in GenBank) was noted. In this chapter, I briefly consider the implications of these results in relation to agro-ecosystem management and fungal biogeography in general.

The methods employed facilitated estimation of total taxonomic composition through the use of analytical resampling. The observed species pool of Agaricomycetes included fifty-two taxa whereas the expected total species pool corrected for heterogeneity in detectability of taxa is estimated to contain seventy-four: the difference between the observed and predicted richness is caused by sampling effort. The greater sampling effort of Lynch and Thorn (93) detected 201 Agaricomycetes (corrected to 186, per. comm. Lynch 2008). Using the observed species pool of Agaricomycetes from both studies, estimated richness was 359.

Despite being able to estimate alpha diversity for the KBS-LTER study site, the interpretation of the local richness estimator is somewhat limited. There is no means by which to assess basic ecological descriptors such as species saturation of the study site relative to a potential species pool since a comprehensive regional or continental list of potential taxa is not available. As a result, there is no objective benchmark for describing the diversity of Agaricomycetes in the study site and comparing it to another. In fact this assessment of site-specific alpha diversity could be regarded as trivial if the identities and

distributions of target taxa were better known (as is the case for vertebrates and plants for example). In the context of mycological research, however, this study (in concert with previous studies at the site (93)) makes several useful contributions.

Molecular-based techniques for surveying fungal biodiversity are a relatively new development. As such, there is significant uncertainty regarding how variations of methods at various stages of analysis can be manipulated to characterize true biodiversity in the study system with minimal analytical costs. At present, there are few empirical data on which to base methodological standards for sampling intensity. At the time of writing, this study, in combination with results of Lynch and Thorn (93) represents the first case in which permanent sample plots have been studied repeatedly using similar molecular sample processing and analysis protocols across years. Whereas this may seem a trivial achievement, the results that 50% of sequences in this study were already detected in the larger study conducted by Lynch and Thorn (70) is much better than expected under a purely idiosyncratic or random model of community composition. This relatively high consistency in taxonomic representation between this study and that of Lynch and Thorn in 2002 (93) provides evidence of a relatively stable basidomycete community in the KBS-LTER site soils, and that molecular methods are capable of repeatably characterizing communities across years. Although few clear associations of OTUs and clades among treatments were found in either sampling periods, there were some surprising instances of taxonomic fidelity to specific plots. For example, two sequences, one matching *Sphaerobolus iowensis* and another related to *Pseudotomentella* sp., were detected only once, each in the same plot in both studies. The probability of both studies detecting these taxa in the same plots is very low under the null model of

random distribution. A more likely explanation would be that there are important local (i.e. sub-plot) characteristics that determine the presence and persistence of at least some of the taxa occupying these sites. This leads to the hypothesis that fine-scale processes operating within plots may explain much of the apparent idiosyncratic occurrence of taxa observed within treatments.

In this study as with other studies involving amplification and sequencing of DNA extracted from soil (83, 126), numerous sequences were not identifiable. These sequences most likely represent as-yet uncultured and uncharacterized taxa or a lack of representation of known fungi in GenBank. As the recovery of novel species in molecular-based studies is not uncommon it appears that much of the true fungal diversity remains to be explored (62, 76, 83, 105). Describing such organisms remains a challenge.

As discussed above, lack of basic information on fungal species distribution at regional levels means that the observed species richness and composition at the KBS-LTER site cannot be assessed relative to expected or available taxonomic regional lists (41). However, the total known pool of taxa as reported in Chapter 3 provides a framework within which the fungal diversity of the experimental treatment plots at KBS can be measured and interpreted (Chapter 3). Although it is recognized that the species detected in this study may be a biased sample of the total species pool (i.e. the DNA of detected taxa may be easier to extract and amplify), the assumption is made that detectability is consistent among treatments. Sampling bias, if present, is therefore expected to cause a systematic under-representation of total diversity but would not affect interpretation of the prevalence of the taxa that were included in the analysis of treatment effects.

Clear patterns of OTU fidelity to specific treatments were not distinguishable as many OTUs occurred only once (singletons) and were therefore not informative for statistical analysis. Even grouping OTUs into a higher-level classification of clades showed highly variable representation among treatments, although at this level of classification statistical significant differences in species composition were detected.

The observed lack of pattern in OTU level fidelity to a given treatment may be driven by highly random occurrence of species within the soil or the result of small sample size. That being said, the sample volumes used for this study are typical of those used in analysis of soil samples by other investigators (40, 83, 98, 105) and were dictated by pragmatic constraints on the cost and feasibility of soil sample processing, DNA extraction and sequence analysis. Few taxa showed clear patterns of consistent presence or absence in particular treatments, but those that did are highlighted here as examples of taxa potentially affected by agricultural intensification.

The *Lachnella/Calathella* and *Pneumatospora* minor clades appear to be facilitated by high intensity disturbance as they had the greatest representation in CT plots. Little is known about the biology of these minor clades other than the vegetative propagule of *Pneumatospora,* morphological characteristics of fruiting bodies and general substrate associations, thus exploring the reasons for the fidelity of these clades to CT is not possible.

The clades that appear to be most affected by high intensity disturbance (i.e. tillage) include the Clavarioid clade and the "sister clade to *Volvariella*". While these clades occur in all treatments, they have only minimal representation in CT plots, perhaps suggesting sensitivity to physical disruption of their mycelium (6). Statistical differences

in taxonomic composition of CT and NT plots combined versus HTS and NTS plots combined, suggests that factors such as mono-culture vegetation and fertilization, also affect clade occurrence. In particular, the Polyporales and *Hygrocybe* clades were only detected in HTS and NTS treatments. While these and other patterns of occurrence were detected among treatments, it is not known whether the presence or absence of a given group will affect the productivity of the local system, as species life-history strategies are not well understood for many fungal taxa.

Contributions of molecular work to understanding fungal diversity

This study is one of few that report on the use of molecular survey methods in a way that facilitates direct comparison among studies by obtaining sequence data. Sequencing provides information at the base-pair level of a single piece of DNA. All sequencing techniques result in base-pair data and thus can be compared among difference studies. Comparison may be made by directly comparing sequences or by using the taxonomic identities obtained from the sequence data. Several repository databases exist for facilitating the dissemination of sequence information (e.g. Genbank, DDBJ, EMBL). In contrast, results obtained from intermediate-level techniques such as Denaturing Gradient Gel Electrophoresis or Restriction Length Fragment Polymorphisms provide community 'fingerprints' or profiles (76). As this information (often a banding pattern) can vary depending on protocol it is not useful for comparison across studies.

Of further importance to the taxonomic identification of sequences is the placement of sequences in phylogenetic trees. Many of the sequences recovered in studies of soil DNA are yet to be formally described in sequence databases (83).

Phylogenetic placement of such unknown taxa that are "missing" from GenBank allows the sequences to be described relative to known sequences. Employing sequence similarity searches such as BLAST (4, 94) to identify sequences can be problematic as the databases often do not contain sequence matches for positive identification and some entries are incorrectly identified (104). Constructing phylogenies using well-characterized sequences to define taxonomic groups not only allows for the unidentified sequences to be placed in a phylogenetic context but also avoids the previously mentioned problems associated with alignment searches (67). This approach therefore allows comparison of phylogenetic diversity among sites and studies.

If phylogenies are constructed, comparisons among studies are possible even when species identity is lacking and different regions of the DNA are chosen for amplification and sequencing. Table 1 (Appendix1) shows that only 1 out of 4 molecular-based studies of soil basidiomycete diversity placed sequences in a phylogenetic tree. As the majority of sequences recovered from soil do not match sufficiently to known and sequenced taxa, phylogenetic trees would allow for an improved understanding of diversity in soils by providing a taxonomic context for unidentified sequences.

Limitations of fungal diversity studies

Studies investigating patterns of taxonomic composition often contrast local species lists (observed species pools) with potential species lists (regional species pools). This paradigm is central to the study of community assembly and the elucidation of 'rules' by which locally occurring taxa settle and sort into ecological communities (148). This

approach is analogous to the single-species research paradigms of habitat selection and species occurrence prediction that are prevalent in the biodiversity management literature (106). Although these paradigms are powerful for interpreting the abundance and distribution of taxa, the information requirements preclude adoption of these approaches for assessing and predicting fungal species distributions (41). The basic limitation of fungal data is the lack of comparability across studies and scales as well as the difficulty of capturing true site diversity with traditional methods. In other disciplines that are based on direct observation of their subjects (as in plant ecology and vertebrate ecology for example), determination of the presence of an organism in a given sample is a relatively simple matter of physical examination of a specimen to arrive at an accurate identification. In contrast, obtaining accurate identification of fungi using molecular methods is achieved without the benefit of direct observation. Inferring the presence of a given fungal taxon using molecular methods relies on a series of indirect steps. The degree to which these indirect steps obscure or distort the identification results is poorly known and will only become more apparent as studies continue to use molecular methods (114).

As a result, although conceptual frameworks for analyzing species distributions have been in place for vertebrates and plants for several decades, no such conventions have yet developed for the analysis of fungal species occurrence. Our understanding of species distributions is therefore limited to collection locations. The limitations of interpretation and use of such information as a basis for interpreting biodiversity and identifying conservation needs are well known (106). These limitations reveal an emerging imperative for mycologists with an applied interest in biodiversity.

It is now well recognized that fungi are a cornerstone component of stable and productive ecosystems (34, 135, 142). In spite of this recognition, cohesion of analytical approaches and synthesis of knowledge are lacking. Development and funding of research agenda that aim to provide basic knowledge of life history characteristics and biogeography for major fungal taxa could perhaps be a catalyst for formalizing fungal ecology as an applied discipline. Progress in developing such fundamental knowledge may yield a better understanding of how the rapid rate of intensification of agriculture is impacting the functioning of the fungal community in soils.

Recommendations for future research

Repeating molecular diversity studies at regular intervals at the KBS-LTER site to allow assessment of repeatability of results and to explore inter-annual and intra-plot variation as a source of noise relative to treatment effects would be a useful direction for future research. It may also be necessary to identify and sample finer scale sample units within plots to focus in on the factors that more proximally affect taxonomic composition and persistence in this system. For example, rather than randomly selecting, extracting, and homogenizing soil cores as sample units, partitioning sample cores into substrate types and extracting DNA from these sub-samples may provide insights into the importance of fine-scale microhabitat conditions relative to plot-level conditions as determinants of species composition and richness.

Longer-term data would provide a more robust assessment of stability of results among years and would help to clarify the poorly-characterized regional soil fungi species pool (84). Developing regional species lists using culture-independent methods (i.e.

molecular tools) will be essential to creating a framework from which to understand species presence and absence in soils under varying management regimes (41). Furthermore combining molecular methods with other methods such as stable isotope probing (114, 115) to identify active community DNA will offer a clearer understanding of community dynamics.

The ability to compare species composition and richness among studies is central to advancing the current rudimentary knowledge of fungal biogeography. Fundamental questions remain such as whether or not fungi even have a biogeography (41). Unfortunately, arbitrary differences in the level of molecular analysis completed and differences in reporting of results prevent the comparison of taxonomic composition and species richness among fungal studies. In order for knowledge of fungal biodiversity to accumulate among studies, standards for reporting and identifying environmental sequence-based results are needed (104). Such standards exist for other ecological disciplines and are voluntarily adopted among communities of practice to ensure data comparability. Examples include metadata standards for documenting biological collections (Ecological Metadata Language (EML)) and reporting observational data (Darwin Core (DwC)). Analogous conventions for reporting on molecular mycology would go a long way toward facilitating comparison and synthesis of biodiversity information across studies and over time.

Culture-independent methods are needed for gaining insight into the life history and ecology of species. Until more information is available regarding the basic biology and ecology of fungi, links between species composition and aspects of ecosystem

function cannot be explored (76, 98). Such linkages need to be understood in order to expand the field of mycology from a descriptive science to a more management-oriented discipline.

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150. **Went, F. W., and N. Stark.** 1968. The biological and mechanical role of soil fungi. Proceedings of the National Academy of Sciences of the United States of America **60:**497-504.

151. **Wright, C. A., C. A. Reddy, and E. A. Paul.** 1997. Basidiomycete diversity: Impacts of perturbation. Bulletin of the Ecological Society of America **78:**333.

152. **Young, I. M., and J. W. Crawford.** 2004. Interactions and self-organization in the soil-microbe complex. Science **304:**1634-1637.

Appendix 1. Summary of Biosis literature results

Summary of Biosis literature results for a combination of three separate searches of i) agriculture (agri*), ii) diversity (divers*), iii) basidiomycete (basid*) with the 'AND' boolean operator (total number 448) after filtering out studies focused on one particular group or single species.

Abbreviations: asco = ascomycetes; basid = basidiomycetes; zygo = zygomycetes; oomyc =oomycetes (Heterokonta); seq = sequences.

Research type	Methods	Reference	Year	Ecosystem context	Taxonomic representation	Comments
Traditional	Fruiting body survey	(10)	1993	Agriculture - USA	12 basids; 1 asco. Most common *Coprinus plicatilis, Cyathus olla, Marasmius graminum, Arachnion album, Lycoperdon curtisii, Panaeolina foenisecii, Sphaerobolus stellatus*	
Traditional	Culturing	(136)	1996	Agriculture - USA	67 basidiomycete isolates from washing and selective media recognized as 51 morphotypes, of which 43 were only represented by one isolate	Identified *Clitopilus* sp., *Coprinus* sp., *Irpex* sp. *Ceratobasidium* sp. Found *Clitopilus* isolated at 5 different sites. Main purpose was to develop method. Sampling not structured. [Ectomycorrhizal fungi, not expected due to habitat, would not grow on the selective medium]
Traditional	Culturing	(80)	2004	Grassland (grazed) UK	*Penicillium; Trichoderma* (2 most abundant); *Absidia; Fusarium*. No isolates identifiable as basidiomycetes based on morphology	58 OTUs identified based on morphology

Research type	Methods	Reference	Year	Ecosystem context	Taxonomic representation	Comments
Traditional	Culturing	(30)	2006	Grassland (Sourhope) UK	48 isolates, including 10 basidiomycetes: *Nolanea* sp, *Psilocybe* sp., *Mycena* spp., *Clavulinopsis* sp, *Galerina* spp., *Bovista* sp., *Panaeolus* sp.	
Traditional +	Cultured soil fungi + Molecular profiling (RFLP of ITS)	(140)	2000	Agriculture - France	67 fungal colonies picked; mainly *Penicillium, Trichoderma, Fusarium*= 29 RFLPs. 28 asco; 1 basid (*Cryptococcus* sp)	
Molecular - profiling	Molecular profiling (RFLP of ITS) Soil extracted DNA	(140)	2000	Agriculture - France	30 distinct RFLPs; one the same as culturable fungi & 12 not fungi. 8 asco; 1 basid; 1 zygo; 2 oomy; 6 plasmodiophorids	Problems with primer set not specific. Cloned smears from soil amplification (350-1000bp) amplifying many non fungal groups (almost 1/2 of RFLPs)
Molecular - profiling	Molecular profiling- Amplified Ribosomal DNA restriction analysis (ARDRA)	(80)	2004	Grassland (grazed) - UK	20 ARDRA groups - improved; 24 ARDRA groups unimproved	
Molecular - profiling	Molecular profiling- Terminal RFLP (TRFLP)	(98)	2007	Grassland, agricultural, forest	16 to 115 TRFLP/site [?]	Ceratobasidiales common in arable soils, similar to [57]. Both sampled 5-15cm

Research type	Methods	Reference	Year	Ecosystem context	Taxonomic representation	Comments
Molecular - sequencing	Molecular sequencing BLAST	(80)	2004	Grassland (grazed)- UK	Recovered Zygo, Asco & basid from DNA extraction. Recovered only Asco from culturing. Basid 54%/Asco 21% of clones. Asco 100% of cultures	28 clones sequenced
Molecular - sequencing	Molecular sequencing	(105)	2005	Temperate forest	Species-level identifications not presented	975 seq = 454 OTUs between 30-60% basid phylotypes depending on habitat sampled
Molecular - sequencing	Molecular sequencing & phylogenies	(93)	2006	Agriculture - USA	Hetero and homobasidiomycetes, including 7 of the 8 major clades of homobasidiomycetes	241 basidiomycete phylotypes
Molecular - sequencing	Molecular sequencing (screened by ARDRA)	(98)	2007	Grassland, agricultural, forest	Thelephorales; Pisolithus; Cantharellales; Ceratobasidiales; 25% not identifiable below homobasidiomycete	Found abundance of Thelephoraceae (*Tomentella* spp) unusual as only 10 sp of this genus [?] are known in NSW; 67 basid OTUs via ARDRA

Appendix 2. Pairwise similarity of best sequence match from Genbank to sequences recovered in this study. Matches with Genbank sequences not from Lynch and Thorn[3] are highlighted in light grey. Same treatment matches between Lynch and Thorn study and this study are shaded. Double bar indicates the point at which matches are less than 99% similar.

	Genbank Match			***KBS Treatment-Replicate***	
Clone ID	***GB accession no.***	***Description***	***Percent similarity***	***2002***	***2004***
T8R3019	*gi|85002472|gb|DQ341782.1|*	Uncultured basidiomycete clone 074a_05	100.00%	*T8R3*	*T8R3*
T8R2009	*gi|85002646|gb|DQ341956.1|*	Uncultured basidiomycete clone 143a_03	100.00%	*T8R1*	*T8R2*
T8R2016	*gi|85002646|gb|DQ341956.1|*	Uncultured basidiomycete clone 143a_03	100.00%	*T8R1*	*T8R2*
T7R3019	*gi|85002664|gb|DQ341974.1|*	Uncultured basidiomycete clone 151a_02	100.00%	*T8R4*	*T7R3*
T7R1008	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	100.00%	*T7R1*	*T7R1*
T2R5017	*gi|85002606|gb|DQ341916.1|*	Uncultured basidiomycete clone 129a_03	100.00%	*T2R2*	*T2R5*
T2R5025	*gi|85002606|gb|DQ341916.1|*	Uncultured basidiomycete clone 129a_03	100.00%	*T2R2*	*T2R5*
T8R2018	*gi|85002646|gb|DQ341956.1|*	Uncultured basidiomycete clone 143a_03	99.85%	*T8R1*	*T8R2*
T7R3004	*gi|85002664|gb|DQ341974.1|*	Uncultured basidiomycete clone 151a_02	99.85%	*T8R4*	*T7R3*
T7R3012	*gi|85002664|gb|DQ341974.1|*	Uncultured basidiomycete clone 151a_02	99.85%	*T8R4*	*T7R3*
T7R1007	*gi|85002606|gb|DQ341916.1|*	Uncultured basidiomycete clone 129a_03	99.84%	*T2R2*	*T7R1*
T2R3010	*gi|85002591|gb|DQ341901.1|*	Uncultured basidiomycete clone 126a_05	99.84%	*T2R2*	*T2R3*

[3] **Lynch, M. D. J., and R. G. Thorn.** 2006. Diversity of basidiomycetes in Michigan agricultural soils. Applied and Environmental Microbiology **72:**7050-7056.

	Genbank Match			KBS Treatment-Replicate	
Clone ID	*GB accession no.*	*Description*	*Percent similarity*	*2002*	*2004*
T1R6025	*gi|85002392|gb|DQ341702.1|*	Uncultured basidiomycete clone 054a_06	99.84%	*T7R6*	*T1R6*
T7R6004	*gi|85002606|gb|DQ341916.1|*	Uncultured basidiomycete clone 129a_03	99.84%	*T2R2*	*T7R6*
T2R3009	*gi|85002612|gb|DQ341922.1|*	Uncultured basidiomycete clone 132a_01	99.84%	*T1R1*	*T2R3*
T7R3017	*gi|85002544|gb|DQ341854.1|*	Uncultured basidiomycete clone 109a_03	99.83%	*T7R3*	*T7R3*
T2R3001	*gi|85002622|gb|DQ341932.1|*	Uncultured basidiomycete clone 138a_05	99.69%	*T7R1*	*T2R3*
T2R1007	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.69%	*T2?*	*T2R1*
T2R5004	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.69%	*T2?*	*T2R5*
T2R5024	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.69%	*T2?*	*T2R5*
T8R4003	*gi|85002591|gb|DQ341901.1|*	Uncultured basidiomycete clone 126a_05	99.69%	*T2R2*	*T8R4*
T2R5013	*gi|85002606|gb|DQ341916.1|*	Uncultured basidiomycete clone 129a_03	99.69%	*T2R2*	*T2R5*
T2R1002	*gi|85002583|gb|DQ341893.1|*	Uncultured basidiomycete clone 123a_01	99.69%	*T2R3*	*T2R1*
T1R1020	*gi|85002665|gb|DQ341975.1|*	Uncultured basidiomycete clone 151a_09	99.69%	*T8R4*	*T1R1*
T1R3013	*gi|85002500|gb|DQ341810.1|*	Uncultured basidiomycete clone 097a_01	99.69%	*T2R1*	*T1R3*
T7R5014	*gi|85002382|gb|DQ341692.1|*	Uncultured basidiomycete clone 053a_01	99.68%	*T7R6*	*T7R5*
T2R3019	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.68%	*T7R1*	*T2R3*
T7R1006	*gi|75863837|gb|DQ112556.1|*	Vascellum cf. intermedium MJ5858	99.67%		*T7R1*
T2R1019	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.54%	*T2?*	*T2R1*
T2R5021	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.54%	*T2?*	*T2R5*
T1R3017	*gi|85002583|gb|DQ341893.1|*	Uncultured basidiomycete clone 123a_01	99.54%	*T2R3*	*T1R3*
T2R3021	*gi|85002591|gb|DQ341901.1|*	Uncultured basidiomycete clone 126a_05	99.54%	*T2R2*	*T2R3*
T8R3002	*gi|85002691|gb|DQ342001.1|*	Uncultured basidiomycete clone 158a_13	99.53%	*T8R4*	*T8R3*
T2R5006	*gi|85002624|gb|DQ341934.1|*	Uncultured basidiomycete clone 138a_10	99.53%	*T7R1*	*T2R5*
T1R3005	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.52%	*T7R1*	*T1R3*

	Genbank Match			KBS Treatment-Replicate	
Clone ID	*GB accession no.*	*Description*	*Percent similarity*	*2002*	*2004*
T8R4002	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.52%	*T7R1*	*T8R4*
T8R3005	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.52%	*T7R1*	*T8R3*
T8R3008	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.52%	*T7R1*	*T8R3*
T7R6008	*gi|85002441|gb|DQ341751.1|*	Uncultured basidiomycete clone 067	99.52%	*T8R2*	*T7R6*
T7R6016	*gi|85002441|gb|DQ341751.1|*	Uncultured basidiomycete clone 067	99.51%	*T8R2*	*T7R6*
T7R6019	*gi|85002441|gb|DQ341751.1|*	Uncultured basidiomycete clone 067	99.51%	*T8R2*	*T7R6*
T7R5012	*gi|85002382|gb|DQ341692.1|*	Uncultured basidiomycete clone 053a_01	99.51%	*T7R6*	*T7R5*
T1R6020	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.51%	*T7R1*	*T1R6*
T1R5007	*gi|85002403|gb|DQ341713.1|*	Uncultured basidiomycete clone 058a_02	99.51%	*T2R6*	*T1R5*
T7R5010	*gi|85002382|gb|DQ341692.1|*	Uncultured basidiomycete clone 053a_01	99.51%	*T7R6*	*T7R5*
T7R3015	*gi|85002612|gb|DQ341922.1|*	Uncultured basidiomycete clone 132a_01	99.51%	*T1R1*	*T7R3*
T2R5023	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	99.38%	*T2?*	*T2R5*
T8R4001	*gi|929595|emb|X78777.1|GBRS2 5S*	G.boninense	99.38%		*T8R4*
T8R2008	*gi|7188667|gb|AF223171.1|*	Nolanea sericea strain CBS	99.38%		*T8R2*
T7R5019	*gi|85002583|gb|DQ341893.1|*	Uncultured basidiomycete clone 123a_01	99.38%	*T2R3*	*T7R5*
T2R1010	*gi|85002583|gb|DQ341893.1|*	Uncultured basidiomycete clone 123a_01	99.38%	*T2R3*	*T2R1*
T7R5001	*gi|85002545|gb|DQ341855.1|*	Uncultured basidiomycete clone 109	99.36%	*T7R3*	*T7R5*
T1R5001	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.36%	*T7R1*	*T1R5*
T1R5003	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.36%	*T7R1*	*T1R5*
T1R1022	*gi|85002628|gb|DQ341938.1|*	Uncultured basidiomycete clone 138a_15	99.36%	*T7R1*	*T1R1*
T7R6021	*gi|85002441|gb|DQ341751.1|*	Uncultured basidiomycete clone 067	99.35%	*T8R2*	*T7R6*
T7R1003	*gi|929595|emb|X78777.1|GBRS2*	G.boninense	99.23%		*T7R1*

	Genbank Match			KBS Treatment-Replicate	
Clone ID	*GB accession no.*	*Description*	*Percent similarity*	*2002*	*2004*
	5S				
T8R3011	*gi|85002480|gb|DQ341790.1|*	Uncultured basidiomycete clone 076a_06	99.23%	*T8R4*	*T8R3*
T7R5015	*gi|85002623|gb|DQ341933.1|*	Uncultured basidiomycete clone 138a_09	99.22%	*T7R1*	*T7R5*
T2R3020	*gi|85002623|gb|DQ341933.1|*	Uncultured basidiomycete clone 138a_09	99.22%	*T7R1*	*T2R3*
T1R3015	*gi|85002623|gb|DQ341933.1|*	Uncultured basidiomycete clone 138a_09	99.22%	*T7R1*	*T1R3*
T2R3022	*gi|85002699|gb|DQ342009.1|*	Uncultured basidiomycete clone 160a_05	99.20%	*T8R4*	*T2R3*
T1R5024	*gi|85002403|gb|DQ341713.1|*	Uncultured basidiomycete clone 058a_02	99.20%	*T2R6*	*T1R5*
T7R1004	*gi|85002612|gb|DQ341922.1|*	Uncultured basidiomycete clone 132a_01	99.02%	*T1R1*	*T7R1*
T1R1017	*gi|85002403|gb|DQ341713.1|*	Uncultured basidiomycete clone 058a_02	98.91%	*T2R6*	*T1R1*
T8R3014	*gi|85002459|gb|DQ341769.1|*	Uncultured basidiomycete clone 070a_14	98.85%	*T8R2*	*T8R3*
T8R1017	*gi|85002416|gb|DQ341726.1|*	Uncultured basidiomycete clone 061a_10	98.79%	*T8R1*	*T8R1*
T1R1008	*DQ341612*	Uncultured basidiomycete clone 16a_01	98.76%	*T2R4*	*T1R1*
T8R2019	*gi|85002328|gb|DQ341638.1|*	Uncultured basidiomycete clone 031a_01	98.75%	*T2?*	*T8R2*
T7R1003	*gi|51449890|gb|AY684167.1|*	Hygrocybe conica isolate AFTOL-ID	98.71%		
T1R3009	*gi|46402593|gb|AY586670.1|*	Hyphoderma obtusum 28S ribosomal RNA	98.61%		*T1R3*
T8R4018	*gi|46402593|gb|AY586670.1|*	Hyphoderma obtusum 28S ribosomal RNA	98.60%		*T8R4*
T7R6002	*gi|85002332|gb|DQ341642.1|*	Uncultured basidiomycete clone 031a_07	98.60%	*T2?*	*T7R6*
T7R1001	*gi|85002639|gb|DQ341949.1|*	Uncultured basidiomycete clone 141a_14	98.55%	*T8R1*	*T7R1*
T7R1001	*gi|85002639|gb|DQ341949.1|*	Uncultured basidiomycete clone 141a_14	98.55%	*T8R1*	*T7R1*
T2R5003	*gi|85002561|gb|DQ341871.1|*	Uncultured basidiomycete clone 113a_05	98.21%	*T1R4*	*T2R5*
T8R3017	*gi|85002461|gb|DQ341771.1|*	Uncultured basidiomycete clone 071a_02	97.65%	*T8R3*	*T8R3*
T1R5020	*gi|85002558|gb|DQ341868.1|*	Uncultured basidiomycete clone 113a_02	97.64%	*T1R4*	*T1R5*
T7R5017	*gi|85002544|gb|DQ341854.1|*	Uncultured basidiomycete clone 109a_03	97.61%	*T7R3*	*T7R5*

	Genbank Match			KBS Treatment-Replicate	
Clone ID	*GB accession no.*	*Description*	*Percent similarity*	*2002*	*2004*
T8R3015	*gi|7188667|gb|AF223171.1|*	Nolanea sericea strain CBS	97.53%		*T8R3*
T1R3003	*gi|85002356|gb|DQ341666.1|*	Uncultured basidiomycete clone 041a_012	97.42%	*T1R6*	*T1R3*
T1R3003	*gi|85002356|gb|DQ341666.1|*	Uncultured basidiomycete clone 041a_012	97.42%	*T1R6*	*T1R3*
T1R3012	*gi|85002591|gb|DQ341901.1|*	Uncultured basidiomycete clone 126a_05	96.29%	*T2R2*	*T1R3*
T8R3018	*gi|85002481|gb|DQ341791.1|*	Uncultured basidiomycete clone 076a_12	96.15%	*T8R4*	*T8R3*
T8R3007	*gi|85002545|gb|DQ341855.1|*	Uncultured basidiomycete clone 109a_06	94.71%	*T7R3*	*T8R3*
T7R6001	*gi|30420950|gb|AY228352.1|*	Psathyrella cf. gracilis UBC F1429	94.47%		*T7R6*
T1R5022	*gi|50058148|dbj|AB158634.1|*	Psilocybe coprophila	94.28%		*T1R5*
T8R2003	*gi|85002384|gb|DQ341694.1|*	Uncultured basidiomycete clone 053a_04	94.00%	*T7R6*	*T8R2*
T8R3009	*gi|37960798|gb|AY300839.1|*	Lentinellia pustulata 28S ribosomal RNA	93.65%		*T8R3*
T1R1009	*gi|45861590|gb|AY571012.1|*	Lachnella alboviolascens strain	93.57%		*T1R1*
T1R1007	*gi|85002328|gb|DQ341638.1|*	Uncultured basidiomycete clone 031a_01	93.43%	*T2?*	*T1R1*
T1R1012	*DQ341917*	Uncultured basidiomycete clone 129a_04	92.90%	*T2R2*	*T1R1*
T2R1005	*gi|85002624|gb|DQ341934.1|*	Uncultured basidiomycete clone 138a_10	92.49%	*T7R1*	*T2R1*
T1R1014	*DQ341872*	Uncultured basidiomycete clone 113a_06	91.36%	*T1R4*	*T1R1*
T2R3017	*gi|45861594|gb|AY571016.1|*	Merismodes fasciculata strain PB34	91.23%		*T2R3*
T7R3020	*gi|85002664|gb|DQ341974.1|*	Uncultured basidiomycete clone 151a_02	90.88%	*T8R4*	*T7R3*
T7R3008	*gi|85002548|gb|DQ341858.1|*	Uncultured basidiomycete clone 109a_15	90.78%	*T7R3*	*T7R3*
T2R3002	*gi|85002384|gb|DQ341694.1|*	Uncultured basidiomycete clone 053a_04	88.66%	*T7R6*	*T2R3*

Appendix 3. Placement of sequenced clones into operational taxonomic units (OTUs) defined as sequence similarity ≥99%.

(SCV= "sister clade to *Volvariella*")

OTU	*Sequenced Clone*	*Similar Pattern*	*Minor Clade*
1	***T8R3009***	1	Ascomycete
2	***T8R1017***	22	Tricholomattaceae/Cantharellula
3	***T7R6002***	1	SCV
4	***T2R1007***	1	SCV
4	***T2R5004***	2	SCV
4	***T2R1019***	4	SCV
4	***T2R5021***	2	SCV
4	***T2R5023***	4	SCV
5	***T2R5017***	3	SCV
5	***T2R5025***	2	SCV
5	***T7R1007***	3	SCV
5	***T7R6004***	2	SCV
5	***T2R5013***	1	SCV
6	***T2R3010***	2	SCV
6	***T8R4003***	6	SCV
6	***T2R3021***	1	SCV
7	***T1R3012***	3	SCV
8	***T2R3017***	1	Lachnella/Calathella
9	***T1R5007***	7	Lachnella/Calathella
9	***T1R5024***	1	Lachnella/Calathella
9	***T1R1017***	5	Lachnella/Calathella
10	***T8R3011***	3	hygrocybe
11	***T7R1003***	4	hygrocybe
12	***T1R3013***	1	Pluteus
13	***T2R5003***	1	SCV
14	***T1R1009***	1	Lachnella/Calathella
15	***T1R1012***	5	psathyrella
16	***T7R1006***	1	Agaricus
17	***T1R1008***	3	Arrhenia
18	***T2R5024***	1	SCV
19	***T7R6001***	1	Psathyrella
20	***T2R1002***	5	Coprinellus
20	***T1R3017***	4	Coprinellus
20	***T2R1010***	10	Coprinellus
20	***T7R5019***	1	Coprinellus
21	***T1R3003***	2	Conocyboid
21	***T1R3003***	1	Conocyboid
22	***T1R5020***	8	Conocyboid
23	***T8R3017***	2	Concyboid
24	***T1R5022***	1	Psilocybe
25	***T8R2008***	4	Entoloma
26	***T1R3015***	4	Entoloma
26	***T2R3020***	6	Entoloma
26	***T7R5015***	1	Entoloma
26	***T8R2019***	4	Entoloma
27	***T8R3015***	1	Entoloma

OTU	Sequenced Clone	Similar Pattern	Minor Clade
28	*T7R3017*	2	Clitopilus
28	*T7R5017*	2	Clitopilus
29	*T1R1007*	1	Tricellulortus/Pnuematospora
30	*T7R1008*	1	Tricellulortus/Pneumatospora
30	*T2R3009*	2	Tricellulortus/Pnuematospora
30	*T2R3019*	2	Tricellulortus/Pnuematospora
30	*T1R3005*	1	Tricellulortus/Pnuematospora
30	*T1R6009*	9	Tricellulortus/Pnuematospora
30	*T1R6015*	6	Tricellulortus/Pnuematospora
30	*T8R3005*	2	Tricellulortus/Pneumatospora
30	*T8R3008*	1	Tricellulortus/Pneumatospora
30	*T8R4002*	3	Tricellulortus/Pneumatospora
30	*T1R6020*	5	Tricellulortus/Pnuematospora
30	*T7R3015*	3	Tricellulortus/Pnuematospora
30	*T1R1022*	1	Tricellulortus/Pnuematospora
30	*T1R5001*	14	Tricellulortus/Pnuematospora
30	*T1R5003*	9	Tricellulortus/Pnuematospora
30	*T7R1004*	1	Tricellulortus/Pneumatospora
30	*T1R5012*	1	Tricellulortus/Pnuematospora
30	*T1R5014*	3	Tricellulortus/Pnuematospora
31	*T2R1005*	2	Lichenomphalia
32	*T2R5006*	1	Lichenomphalia
33	*T2R3001*	1	Hyphodontia
34	*T1R1014*	1	SCV
35	*T1R1020*	10	Rickenella
36	*T8R3019*	2	Pseudotomentella
37	*T1R3009*	3	Corticiaceae/hyphoderma
37	*T8R4018*	1	Corticiaceae/hyphoderma
38	*T8R4001*	6	Polyporales
38	*T7R1005*	8	Polyporales
39	*T2R3002*	2	Clavarioids
40	*T8R2009*	3	Clavarioids
40	*T8R2016*	4	Clavarioids
40	*T8R2018*	1	Clavarioids
41	*T7R3020*	2	Clavarioids
42	*T7R3008*	1	Clavarioids
43	*T7R3019*	2	Clavarioids
43	*T7R3004*	3	Clavarioids
43	*T7R3012*	1	Clavarioids
43	*T7R3012*	9	Clavarioids
44	*T8R3014*	1	Clavarioids
45	*T1R6025*	1	Clavarioids
46	*T8R2003*	2	Clavarioids
47	*T8R3007*	1	Clavarioids
48	*T8R3002*	3	Clavarioids
48	*T2R3022*	3	Clavarioids
49	*T7R5014*	8	Clavarioids
49	*T7R5012*	3	Clavarioids
49	*T7R5010*	0	Clavarioids
50	*T7R1001*	1	Clavarioids
50	*T7R1001*	2	Clavarioids

OTU	*Sequenced Clone*	*Similar Pattern*	*Minor Clade*
51	***T8R3018***	1	Clavarioids
52	***T7R6008***	7	Clavarioids
52	***T7R6016***	3	Clavarioids
52	***T7R6019***	2	Clavarioids
52	***T7R6021***	3	Clavarioids
53	***T7R5001***	2	Clavarioids