INVESTIGATING THE ECOLOGY, DIVERSITY AND DISTRIBUTION OF CORD-FORMING FUNGI IN GREAT BRITAIN.

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Submitted for the degree of:

Doctor of Philosophy

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Dedications

For my family, friends and supervisors who have given me their unfailing support and encouragement throughout the research project and creation of this thesis.

Thank you

Plate 1: The cord-forming fungus, *Resinicium bicolor*, foraging as a co-operative network from multiple inoculum blocks. (Pencil drawing by Dr Sarah Simblet and reproduced with permission of Bloomsbury Publishing)

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Abstract

Cord-forming fungi (CFF) are an assemblage of saprotrophic fungi which can use complex foraging organs of longitudinally arranged hyphae to join up disparate substrates in a patchy resource environment. Their importance to woodlands lies, mainly, in their ability to modify nutrient cycling and soil structure. Therefore, in order to enable woodlands to continue to thrive in terms of their health and ecosystem function, it is necessary to understand the factors contributing to the establishment, success and diversity of this group. Whilst work to date on CFF has focussed on their physiology and interactions in laboratory conditions, little work has been carried out on their taxonomy and establishment/presence in the field. The work in this thesis begins to address these crucial unanswered questions in CFF ecology.

By carrying out investigations at a range of scales, from phylogenetic analysis to UK wide Species Distribution Modelling, this thesis reaches a number of surprising results with potentially important implications for woodland management. This is most evident in Chapter 3 where our hypothesis that fungal communities develop over time in plantations of different woodland ages was disproved, illustrating that even 13 years after planting, fungal communities in plantations on ex-agricultural land had not begun to reach those in established ASNW. These unexpected results continue into Chapter 4, where the thesis demonstrates that dominant canopy species has a greater impact on community composition than any other woodland factor. Chapter 5 continues this theme, by showing that removal of invasive species is not always beneficial for the cord-forming fungal communities, especially if it involves removing the woody substrate.

The work described, detailed and analysed in this thesis has initiated further investigations, proposed changes to woodland management practices and laid the foundations for future work relating to CFF and their role and function in British woodlands.

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CORD-FORMING FUNGI IN GREAT BRITAIN: WHAT ARE THEY; WHAT DO THEY DO; AND WHAT ARE THE CRITICAL QUESTIONS?

The role of fungi in woodland systems

Woodlands are maintained and supported by vast assemblages of fungi, which perform vital ecosystem services such as decomposition, nutrient mobilisation and solute transport (Boddy 1993). Some hypothesise that colonisation of land by plants would not have been possible without fungal facilitation (Brundrett 2002; Courty *et al.* 2010; Heckman *et al.* 2001; Simon *et al.* 1993).

The role of fungi in wood decay was first documented by Herman Schacht in 1863 and has received steady interest from the amateur and the professional ever since (Blanchette 1991). Rolstad *et al.* (2004) estimated that more than 1,500 macrofungal species occur in boreal forests with higher numbers expected in tropical regions (Anderson *et al.* 2010). Saprotrophic fungi, indicators of productive and ecologically active woodlands, are often assumed to be passengers on, rather than drivers of such activity. This view is naïve and ignores the huge contribution thatsuch fungi make to woodland nutrient cycling and soil structural modification (Barrett *et al*. 2009).

Cord-forming fungi as a subset of the fungal kingdom

The cord-forming fungi are a functional group of saprotrophic fungi which form wide-ranging networks of cords, ramifying through the soil-litter interface, altering the dynamics within and between species and modifying their external environment (Thompson *et* al. 1983; Blanchette 1991). Whilst many woodland communities have been well studied and

characterised, the fungi – and cord-forming fungi (CFF) in particular - are often omitted from research and management plans.

Cord-forming fungi are arguably the most instrumental assemblage for wood decay and alteration of woodland functioning across the range from tropical (Boddy 1993; Anderson *et al.* 2010) through temperate (Dowson *et al*. 1988a) to boreal (Junninen *et al.* 2006) ecosystems.

Figure 1: *Megacollybia platyphylla cord from ASNW in Warwickshire***.**

Cord-forming fungi are characterised and distinguished by the manner in which their hyphae align parallel to one another into a system of tube-like organs termed cords (Jennings 1987; Boddy 1993). Functional differentiation of the tissues follows; external hyphae become reinforced and encrusted with insoluble oxalates whilst the inner hyphae become better adapted for fluid mass flow by losing cross walls and increasing lumen diameter (Boddy 1993; Wells *et al.* 1998).

As I show in Chapter 2, CFF are polyphyletic: the cord-forming condition appears to have evolved at least twice, involving members of both the ascomycetes (Coates & Rayner 1985a,b,c) and basidiomycetes (Thompson *et al*. 1983). The majority of known CFF fall into the basidiomycete class (Monk *et al.* in review). This huge pool of diversity is potentially critical to the maintenance of functionally active woodlands and a stable climate (Boddy 1993; Boddy & Watkinson 1995; Courty *et al.* 2010; Hackl *et al.* 2005; Kluber *et al.*, 2010) and warrants detailed further study.

Cord-forming fungi as a component of woodland systems

Existing at the soil-litter interface (SLI), CFF colonise woody resources (Dowson *et al.* 1986; Dowson *et al.* 1988b). Current literature states that visible CFF populations take six months to develop on felled wood (Coates *et al*. 1985a,b) although it can take centuries for artificially introduced fungal species to integrate into a pre-existing fungal network (Vacher 2010). Colonisation occurs from a range of sources such as the airborne spore pool; the litter spore and cord reservoir; and animal spore dispersal (Coates *et al.* 1985a,b,c). Some CFF species exist in wood as latent propagules which initiate on a dramatic drop in sap water potential, an indicator of tree death (Boddy 2001).

The growth of CFF occurs as a network and can be compared to the optimal foraging strategy (MacArthur & Pianka 1966; Boddy 1993, Heaton *et al.* 2012). Cords grow at the SLI until they encounter a woody resource. On contact with a resource the system becomes more diffuse and hyphae spread across and into the deadwood, assimilating the nutrients through the use of enzymes including lignin peroxidase (Wells & Boddy 2002; Snajdr *et al.* 2011). Nutrients released from the resource in this way are accessible to other fungal and plant species through cord exudates (Harmon *et al.* 1994). Given that cord networks can extend up to and above 300 m in diameter (Thompson and Rayner 1983) the potential for nutrient redistribution across the forest floor is great. CFF transport accounts for up to 17% total N transported through temperate woodland ecosystems (Tlalka *et al.* 2002) pulsing in a co-ordinated manner, bidirectionally (Tlalka *et al.* 2007), at a rate significantly greater than that which could be achieved by diffusion alone (Tlalka *et al.* 2002). In addition to simple nutrients; sugars, amino acids and oxygen can all be transported through cord networks suspended in water (Jennings 1987; Watkinson 2006).

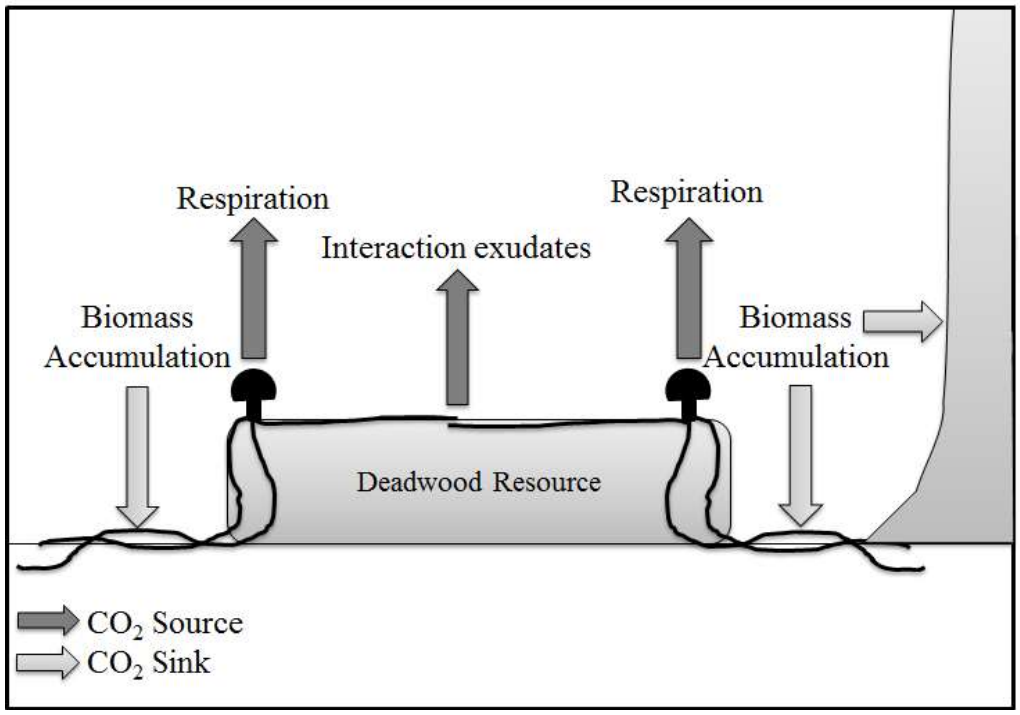

Figure 2: *Carbon dynamics in a woodland ecosystem. Dark arrows indicate carbon loss and light arrows indicate carbon uptake.*

CFF are able to influence the activity and growth of other plants and fungi through exudates and nutrient reallocation (Boddy *et al.* 1985; Dowson *et al.* 1986). At the interaction zone between two cording individuals of the same species, the nutrient-rich cord exudes nutrients. These free nutrients are then taken up by the mycelium through septal pores along the diffusion gradient (Boddy 1999). When this happens in wood, this exudation at the interaction zone can lead to wood discoloration at the contact zone (C-zone) which is seen as spelting. This C-zone is a habitat for other fungal and bacterial species within deadwood (and sometimes living wood), supporting a wide range of saproptrophic woodland species beyond the CFF species alone (Boddy, 1993).

tAs a result of exudate release, CFF canmodify root pathogen activity (Boddy 1993). *Phanerochaete velutina* (PV29; DC: Pers.) Parmasto can retard growth and extension of pathogenic species such as *Heterobasidion annosum* (PV29; DC: Pers) Kumner (Leake *et al.* 2001). Such effects are found in a range of CFF, pathogenic and non-pathogenic, leading to a hierarchy of competitive interactions between them (Boddy & Thompson

1983; Dowson *et al*. 1988; Boddy 1993). When a pathogenic cord system is suppressed by a benign system, the interactions can be associated with reduced virulence of the pathogen (Dowson *et al.* 1988). When species, evenly matched in the competitive hierarchy, come into contact with one another deadlock ensues; neither mycelium is able to extend past the competition (C) zone (Boddy 1993). Therefore, the order of colonisation and the nature of the primary coloniser is a crucial determinant of the trajectory of succession on a woody resource (Hudson 1968; Thompson *et al.* 1982). In many cases, CFF interactions in wood, as opposed to at the SLI, lead to complete replacement of one species by another rather than co-existence (Wells *et al.* 2002).

Perturbations to CFF communities and community dynamics can be highly detrimental to woodlands (Boddy 1993; Dighton 2003). In addition to their role in mobilising nutrients from deadwood and litter resources, CFF constitute a sizeable nutrient pool in their fungal structures, storing 18% P and 20% N of the total soil nutrient pool (Lodge 1993; Markkola *et al.* 1995). CFF also exert a high degree of control over the carbon budget within woodland ecosystems (Watkinson *et al.* 2006). CFF release carbon dioxide as a consequence of decomposition and mycelial interactions (Wells *et al.* 2002). However, they also have the potential to increase the carbon sequestered by the ecosystem as a whole by increasing the growth rate and capacity for growth of all floral and mycofaunal components through fertilisation from cord exudates which in turn increases the decomposition and nutrient mobililsation rate (Edman *et al*., 2004). Therefore, CFF are critical components of nutrient cycling in woodland systems through the equilibrium they maintain between biomass accumulation, respiration and exudation (Fig 2). Increased fungal richness is often associated with increased decay rates as a wider range of specialisms (e.g. cellulytic or delignifying enzyme activities) combine and supplement each other in a decomposition event (Blanchette 1991; Buchanan *et al*. 2001).

Potential implications of research into cord-forming fungi on woodland management practices

An increased understanding of the processes and effects that CFF exert on temperate woodland ecosystems enhances our ability to incorporate CFF into woodland management practices and policies, thus facilitating a more holistic approach to forestry, increasing the likelihood of effective and successful management for woodland productivity and function. Managing woodlands for CFF could be a simple and effective way to mitigate the effects of environmental change on temperate woodlands through their associated effects on nutrient cycling and soil structure (Thompson *et* al. 1983; Blanchette 1991).

Research to date has had tangible effects upon woodland management regimes by accounting for the dynamic nature of fungi (Lonsdale *et al.* 2008; Norden *et al.* 2004a; Norden *et al.* 2004b). Current fungal conservation efforts involve leaving brash piles in woodland areas for fungal colonisation. Such actions are a result of research demonstrating that fungal communities and hyphal networks are constantly changing to accommodate changes in the local resource environment, adapting to a patchy resource environment (Heaton 2010). Other practices such as creating multiple small areas of deadwood patches arise from studies illustrating that the majority of fungal spores fall within a few metres of the fruiting body (Moykkynen *et al.* 1997, Edman *et al.* 2004). Therefore, logs placed in close proximity to colonised logs are more effective at providing colonisation corridors and supporting rare fungi than more remotely scattered deadwood clusters. However, much fungal research is not incorporated into management practice as recommendations are often too abstract, diffuse or absent. Often, the fundamentals are missing, omitting the schema upon which we can base new advances in understanding. For example, currently, woodland practitioners are aware that management practices affect fungi generally and competitive interactions between fungal species by altering temperature or water regimes (Boddy *et al.* 1985). The extension rate of CFF can cease at temperature extremes (less

than 5ºC and more than 30ºC) or water potentials below 1.5MPa. However, the ecological activity of CFF is not factored into management plans as woodland managers are largely unaware of these fungi in their woodlands and the link between visible sporocarp and hidden CFF.

Studies in boreal ecosystems have identified a significant effect of tree species on fungal sporocarp assemblages found on deadwood (Lindhe *et al.* 2004). This differentiation has been known since 1968 when Hudson identified characteristic fungi on different wood species such as the occurrence of *Hypoxylon fragiforme* (Pers.) J. Kickx. f. on beech (*Fagus sylvatica* (L).) and *Daldinia concentrica* (Bolt.:Fr) Erikss, on ash (*Fraxinus excelsior* (L.)). Further work in temperate regions could aid restoration efforts and highlight the fungal species that would be expected in functionally and ecologically active woodlands. Such information would be particularly important in an era of increased planting of woodland on ex-agricultural land, likely to be mycologically depauperate, supporting and enhancing previous work suggesting that fungal species richness does not differ between plantations and ancient semi-natural woodlands (Humphrey *et al.* 2000; Johnson *et al.* in review).

Thesis aims

Major advances have been made regarding our understanding of CFF and the roles they play in woodland ecosystems, but there are still many features that remain unknown. This thesis aims to address some of these gaps in our knowledge with the overall aim of establishing firm theoretical foundations upon which future work into the potential uses and environmental management capacity of CFF can be based.

Context of this thesis

The majority of studies of CFF to date have been laboratory or mesocosm based and have reached a number of important conclusions which were fundamental to the development of the studies in this thesis. Of particular relevance to this volume of work, are the studies carried out by Boddy, Watkinson, Fricker, Tlalka and Bebber, looking at fungal interactions, illustrating that fungal systems translocate Nitrogen, phosphorus, other minerals and sugars across the SLI through the cord system. It is these studies which have provided the impetus for research and the increased understanding of the importance of fungi to woodland ecosystems. Without this work, our knowledge of cord-formers as components of woodland ecosystems would not be as detailed as we would lack the physiological understanding of the purpose and ecosystem benefits of the organ. Subsequent mesocosm work such as those of Boddy, Jones and A'Bear move the field forward to consider the interactions of cord-forming fungi with one another and with invertebrate grazers such as woodlice, springtails and orabatid mites. Again, this work adds depth to the current level of understanding about the fine-scale ecology of CFF in woodland ecosystems. These studies, combined, create a solid foundation for the work carried out in this thesis which seeks to study similar questions of CFF establishment, interactions and ecology, but at a wider field-scale, accounting for all of the woodland community components and the interactions these may have with the components studied in the mesocosms.

A number of field experiments were also pertinent to the thesis. The work of Coates and Rayner (1985a, b, c) underpinned many of the elements of the applied data chapters by examining establishment on woody resources from the airborne pool, below-ground and at ground level. Their findings informed the hypotheses of Chapters 3, 4, and 5. Furthermore, Chapter 6 used this investigation to suggest parameters for inclusion in the model.

The paper by Wells *et al* (1998) showed that CFF interactions in wood, as opposed to the soil-litter interface can lead to the complete replacement of one species by another. This supplements the work of, Lindhe *et al* (2004) which studied the effect of tree species on fungal sporocarp assemblages found on deadwood and found these communities to be different. These studies were fundamental to the assumptions and hypotheses made in Chapter 4, namely that you would expect a range of fungal species to colonise woody resources and lead to the eventual dominance of a single CFF on each woodblock, but that the fungal communities found during these early establishment phases would be expected to be affected by the dominant tree species. Building on these studies, and in combination with the work by Humphrey *et al* (2000) and Johnson *et al* (in review), the concepts for Chapter 3 were identified. These two studies investigated the difference in fungal communities between plantations and ASNW and found there to be no difference. Chapter 3 then sought to build on these by investigating the effect of time on fungal community establishment – how old does a woodland need to be before the plantation fungal community equals that of ASNW.

Therefore, although breaking new ground as to the scale of investigations undertaken in this thesis, there is a large amount of prior work by a range of academics which has provided the inspiration, impetus and knowledge base from which to extend. By increasing the scale from mesocosm to forest- and UK- level effects the aim of this thesis is to lay the foundations for future whole-ecosystem approaches to CFF ecology by posing and answering fundamental unanswered questions and developing putative next steps in the field.

The crucial unanswered questions

This thesis will address a number of fundamental questions regarding the diversity, distribution and ecology of cord-forming fungi in Great Britain which have been investigated using a range of experimental methods and a range of techniques.

These questions are:

1) What is the current known diversity of CFF in Great Britain?
2) Which ecological, environmental and landscape features are characteristic of CFF and determine the presence of CFF in an area?
3) Can CFF distributions be modelled effectively and reliably?

Such questions have been identified as those shortcomings in our understanding of CFF which prevent the investigation of their ecology and their capacity to modify ecosystems and are therefore vitally important within the field of CFF ecology and functioning and to woodland management practices in Great Britain. It is hoped that, through attempting to answer these questions, future work can occur at a deeper level to obtain a full understanding of this important group of ecosystem engineers.

Thesis structure

Questions will be addressed at a range of ecological levels using a variety of techniques: from phylogenetic analysis to Maximum Entropy modelling. The diagram below illustrates the structure of the thesis and the manner in which the individual papers/chapters are interrelated.

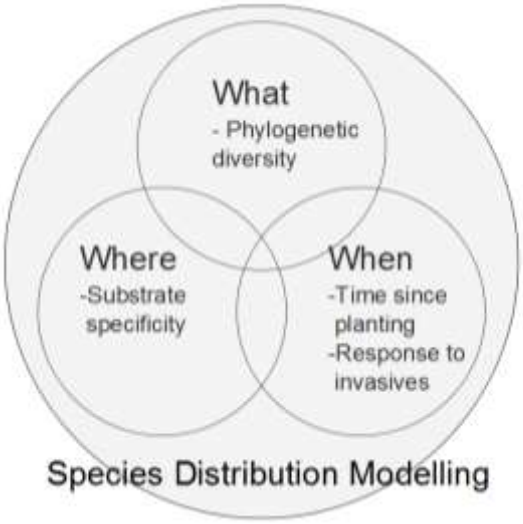

Figure 3: *Diagram illustrating the structure of this thesis illustrating the what, where and when aspects of research and the interrelations and crossovers between them.*

Here I present a thesis by publication in which the data chapters are presented as papers with a link page between the current paper and its predecessor. All chapters in the thesis consist of the majority of my own work; I am first author on each paper.

This first chapter is an introduction to the thesis and also contains a literature review explaining the current state of knowledge of CFF with respect to woodland management.

Chapter Two is a phylogenetic review of CFF. The paper hypothesises putative CFF clades and origins based on data available in NCBI GenBank (Benson *et al.* 2010) and the Silva database (Pruesse *et al.* 2007). The aim is to demonstrate the broad phylogenetic range and evolutionary origins of the CFF condition, drawing attention to the chronic under-sampling of CFF; a theme returned to during the thesis.

Chapter Three moves on to investigate the effect of woodland age on fungal communities and associated ecosystem functions. This extends the scale of the research to study diversity, distribution and ecosystem processes occurring at the stand level. The speed of fungal community colonisation and regeneration on old agricultural land is studied in

collaboration with the Heart of England Forest Ecosystem Project. This is one of three applied strands within the thesis, contrasting with the more theoretical phylogenetics and modelling based chapters.

Chapter Four considers the within-woodland level of diversity and the impact of substrate identity and canopy features therein. This paper presents an investigation into the effect of host and substrate specificity on CFF communities in British temperate woodlands. Presented research takes the form of a manipulation experiment whereby logs of five different species (beech, sycamore, oak, conifer and rhododendron) were transplanted beneath canopies of each of the same five species. The experiment was replicated in two sites in Oxfordshire: Wytham Woods, managed by the University of Oxford; and Bladon Woods, part of the Blenheim Palace Estate. Hypotheses being tested were that tangible patterns of colonisation exist in stands dominated by different species and that such effects could be characterised as originating from the influence of litter species identity (determined by dominant canopy species) or coarse woody debris identity (determined by the species of transplanted log). Such efforts could be important to restoration activities aimed at introducing fungal species to areas by inoculation, identifying species likely to thrive and those likely to suffer from the absence of a suitable substrate.

The second applied chapter, Chapter Five, concerns the restoration and recolonisation of fungi, including CFF, on cleared *Rhododendron* stands. This investigation extends the Heart of England Forest Ecosystem Project to research the effect that the characteristics of newly created bare ground have on fungal succession processes. Lundy, Bristol Channel, provides ideal natural laboratory conditions for this project. In conjunction with high quality *Rhododendron* clearance records, a robust experimental design could be created. This chapter combines an investigation of CFF recolonisation on the removal of a deadwood resource, with the discussion of recolonisation of grassland fungi and the restoration of native fungal assemblages on cleared areas of the Eastern Sidelands.

Chapter Six; the largest geographical scale investigation of the thesis, extends across the United Kingdom. Investigating and evaluating the appropriateness of Maximum Entropy (MaxEnt) modelling approaches for UK fungal distribution modelling and atlas creation, this chapter considers the broad environmental requirements of fungi and the inherent biases in fungal records. This study utilises the Fungal Records Database of Britain and Ireland, curated by the British Mycological Society (Kirk and Cooper, 2009), and processed prior to analysis using a newly devised protocol, using this as a basis for MaxEnt modelling. We critically analyse the protocols in place for SDM modelling of species with a broad environmental niche and determine minimally adequate sample sizes for reliable modelling.

Over the course of the research programme a central thread is followed, that is, understanding the diversity and distribution of cord-forming fungi at a range of scales and in combination with other fungal groups. We can use this information to inform our hypotheses of ecosystem function and the ecology of this group to provide a sound basis for more in-depth work regarding the role and function of CFF in temperate woodland systems.

An additional output of this research is that of rigorous testing of assumed best practice regarding fungal collection, DNA extraction, phylogenetic analysis and species distribution modelling. Through testing and challenging the assumptions inherent in these methods, protocols have been improved and the standard of data and resultant theory will be more robust. Such work facilitates higher quality investigations in the future. It is hoped that this thesis will provide a springboard from which the functional ecology and ecosystem engineering capacity of CFF can launch.

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CHAPTER TWO

TRACING EVOLUTIONARY RELATIONSHIPS IN CORD-FORMING FUNGI: MAPPING CORDS TO THE FUNGAL TREE OF LIFE.

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Declaration:

I confirm that the work contained in this chapter is wholly my own and that the contributions of the other authors to the paper constituted advice regarding experimental design and proof-reading the manuscript prior to submission. This paper has been resubmitted, following revisions, to 'Fungal Ecology'.

Signed:

Kirsty Monk (DPhil candidate) Dr Nick Brown (Supervisor)

TRACING EVOLUTIONARY RELATIONSHIPS IN CORD-FORMING FUNGI: MAPPING CORDS TO THE FUNGAL TREE OF LIFE.

Abstract

Fungal cords, structures formed when hyphae of saprotrophic fungi align and join together along their length to produce rope-like organs, are involved in nutrient transport, carbon dynamics and water cycles in woodland systems. Despite the importance of this trait, little is known about its phylogenetic diversity. Therefore, we undertook a comprehensive literature survey and generated a database of known cord-forming fungi (CFF). Phylogenetic analyses based on the small subunit ribosomal RNA gene (SSU rDNA) show that known CFF are restricted to two clades: one in the ascomycetes (a subset of Xylariales), the other in the basidiomycetes (Agaricomycotina minus Dacrymycetales). Internal transcribed spacer (ITS) rDNA phylogenies of *Armillaria*, *Phanerochaete*, and *Serpula* show the intra-generic phylogenetic distribution of CFF lineages, and whether the cord-forming habit clusters phylogenetically within higher-level taxa. The *Armillaria* phylogeny suggests an association between phylogenetic clustering, cord branching pattern and geographic distance whilst other genera show little or no discernible relationship between the known ability to form cords and phylogenetic clustering. Incomplete data may contribute to the latter observation, as CFF knowledge is strongly biased towards certain groups. The CFF habit is likely employed by many more fungal lineages than previously thought.

Keywords

Cord-forming fungi, rhizomorph, SSU rDNA, ITS rDNA, *Phanerochaete, Agaricomycotina, Armillaria, Xylariales, Boletales*

Introduction

Cord-forming fungi (CFF) are an important yet understudied group of ecosystem engineers whose members are often thought to encompass a wide diversity of the fungal kingdom (Boddy 1993, Fricker 2008). Cords are defined as 'aggregations of longitudinally arranged hyphae' (Boddy 1993) and are specialised for transport of; water, nutrients (sugars, amino acids), and oxygen from the external environment (Watkinson 2006). In CFF fungal hyphae align and fuse parallel to one another before differentiating structurally to create a tubular organ adapted to mass flow (Tlalka *et al.* 2002). The internal vessels are specialised for transport: wide (>20 μm), thin-walled vessel hyphae without cross-walls are interspersed with thick walled 'fibre hyphae' to facilitate the transport of water and water soluble compounds through the network. The outer vessels become reinforced with oxalates, protecting and waterproofing the interior (Jennings 1987; Boddy 1993; Wells 1998). With the exception of the pathogenic agaricomycete *Armillaria*, this study is concerned with the cord-forming habit as an adaptation to facilitate saprotrophy, although many saprotrophic CFF also form ectomycorrhizal associations with plants.

Cord systems are found at the interface between the soil and the litter layer and often on dead wood in both temperate and tropical woodlands and grasslands. Cords grow across the interface until they encounter coarse or fine woody debris. On encounter, the fungus colonises this resource through fanning out the cord system, assimilating the nutrients otherwise trapped in the wood through the use of enzymes such as lignin peroxidase,

making nutrients accessible both to itself and other fungal and plant species through cord exudates containing simple compounds. Cord-forming fungi have been shown to play a role in nutrient cycling in forest ecosystems (Barrett *et al.* 2009) through the equilibrium they maintain between biomass accumulation, respiration and exudation.

CFF are taxonomically diverse, represented by saprotrophs and ectomycorrhizal fungi that are widely distributed in woodland and grassland habitats in temperate, boreal and tropical biomes (Dowson *et al.* 1988; Boddy, 1993; Junninen *et al.* 2010). Known cord-forming lineages all have the ability to form complex fruiting bodies, including mushrooms and toadstools, brackets, clubs, and encrusting fungi. The cords themselves exhibit a wide range of types and morphologies as multi-hyphal structures in different taxa, and can refer to mycelial cords such as those created by the genus *Phanerochaete* (which lack a melanised outer sheath) or melanised rhizomorphs such as those in *Armillaria* which can grow either monopodially (where all side shoots branch off from a single main line) or dichotomously (where the cord branches into two shoots to create a network of cords across a resource space).

Most previous work on CFF has focused on the nutritional and ecological characteristics and dynamics of a few relatively well-known species. Broader knowledge of the diversity and evolutionary relationships of CFF is lacking. In this study we carried out a comprehensive literature survey to identify all fungi known to form cords. We then obtained representative sequences for these taxa from publically available databases and carried out phylogenetic analyses at two levels of resolution (SSU and ITS rDNA) in order to determine the evolutionary origins and diversification of the CFF habit. This knowledge will provide a useful reference point

for future work seeking to understand the functional diversity of CFF activity and its ecological consequences.

The study is based upon the underlying hypothesis that cord-forming can be traced to a small handful of origins and that taxa known to form cords are typically those which form complex fruiting bodies. We further expect that this study will unveil an incomplete knowledge of the CFF condition as cording is likely only to occur in instances where the organ is needed to link up resources in a landscape of disparate woody resource provision.

Materials and Methods

Literature Survey

A comprehensive literature search was carried out on SciVerse Scopus (Elsevier B. V. 2012) and NCBI Pubmed (Geer *et al.* 2010) using the keywords; <cord AND fungi>, <rhizomorphs>, <syrrotia> and <strands>. These words were selected as they represent all terms referring to the cord structure in published literature. This generated a working list of all documented saprotrophic cord-forming fungi identified (Appendix 1). Of the genera known to form cords, the number of species of each represented in NCBI GenBank (Benson *et al.*2013) or the Silva database (Pruesse *et al.* 2007) as ITS rDNA sequence accessions was ascertained and used to inform genus selection for the ITS rDNA trees (Appendices 2 & 3).

Sequence acquisition and alignment

SSU rDNA

The SSU rDNA alignment for the fungal kingdom generated by James *et al*. (2006) was used as a basis for this study. It was modified to ensure representation of all cord-forming

orders, as far as sequences were available, using the sequence dataset described previously. Sequences for incorporation were identified using the Silva database and the associated accession found in NCBI GenBank. Sequences were selected on a basis of their sequence quality as indicated in the Silva database. Selected sequences were aligned using MAFFT version 6.850b with a L-INS-i strategy, default parameters and no additional arguments (Katoh *et al.* 2002) and refined by eye in BioEdit v 7.0.5.3 (Hall 1999). Preliminary trees were generated using RaxML BlackBox v 7.2.7 (Stamatakis 2006) on the Cipres Science Gateway v 3.1 (Miller 2009; Miller *et al.* 2010) using the default parameters and studying the resultant tree for similarities to accepted taxonomic groupings and concordance with James et al. (2006). We refer to this as the 'orders tree' (Fig 1). The same approach was then separately applied to orders containing more than three cord-forming genera and with adequate representation of 18S sequences in the public databases (Boletales, Agaricales and Phallales); = 'genus trees' (Fig 2; Appendices 4 and 5, respectively).

ITS rDNA

A higher resolution tree ('species tree') was generated for a sub-set of known cord-forming genera and orders using the ITS 1 and 2 rDNA regions including 5.8S and the flanking regions of the neighboring SSU rDNA and 28S subunits. The five genera selected (*Phanerochaete* (Fig 3), *Armillaria* (Fig 4), *Xylaria* (Appendix 6), and *Tomentella* (Appendix 7), were chosen on the bases of phylogenetic coverage, lifestyle diversity (saprotroph, ectomycorrhizal, pathogen), and different levels of sampling of cord-forming lineages (Table 1). All unique sequences were sourced from GenBank and Silva; the highest quality accession of adequate length for each species was included in the alignment. Selected sequences were aligned and preliminary phylogenetic trees generated as described above.

Phylogenetic analyses

For the 'orders tree' a Bayesian consensus tree was calculated using MrBayes v 3.1.2 (Ronquist & Huelsenbeck 2003) in parallel mode (Altekar *et al.* 2004). Two separate MC^3 runs with randomly generated starting trees were carried out for 5M generations each with one cold and three heated chains.

Genus	% in Genbank
Agrocybe	14.00
Agaricus	52.00
Amaurodon	0.60
Antrodia	58.70
Armillaria	68.57
Bjerkandera	100.00
Boletus	5.67
Clitocybe	12.00
Collybia	366.67
Cortinarius	5.00
Coprinus	480.00
Grifola	80.00
Gymnopilus	25.00
Hebeloma	53.33
Heterobasidion	100.00
Hydnum	5.00
Hymenochaete	39.09
Psilocybe	19.33
Hysterangium	18.00
Laccaria	28.00
Leccinum	46.67
Lenzites	83.33
Lopadostoma	0.00
Lycoperdon	46.00
Marasmius	16.20
Megacollybia	800.00
Melanoleuca	92.00
Merulius (now Phlebia)	n/a
Mutinus	8.33
Paxillus	46.67
Phallus	16.67
Phanerochaete	36.92

Genus	% in Genbank
Phlebia	64.00
Pisolithus	160.00
Psathyrella	19.25
Pseudotomentella	50.00
Podosordaria	17.39
Ramaria	25.45
Resinicium	200.00
Rhizopogon	63.33
Rosellinia	13.85
Schizophyllum	66.67
Scleroderma	66.67
Sclerogaster	0.00
Sebacina	31.03
Serpula	250.00
Sistotrema	19.57
Skeletocutis	20.00
Steccherinum	30.30
Stereum	29.63
Stropharia (now Panaeolina)	600.00
Suillus	112.00
Thelephora	20.00
Tomentella	40.00
Trametes	6.00
Trechispora	21.74
Tricholoma	39.00
Tricholomopsis	10.00
Ustulina (now Kretzchmaria)	3.57
Vuilleminia	70.00
Xylaria	29.00

Table 1: *Percentage of species containing each known cord-forming genus represented in GenBank and Silva SSU rDNA databases relative to all species in the genus (as identified from "The Dictionary of the Fungi" 10th Edition (Kirk 2008)). Percentages >100 are a consequence of taxonomic reorganization of species since the sequence was uploaded.*

The evolutionary model applied included a GTR substitution matrix, a four-category auto-correlated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 100 generations. One million generations were discarded as burnin (trees sampled before the likelihood plots reached a plateau) and the consensus tree constructed from the returning sample. Maximum-likelihood (ML) analysis used RAxML-7.0.4 (Stamatakis 2006). One thousand bootstrap replicates were performed using the novel rapid bootstrap algorithm (Stamatakis, unpubl) using the GTR model with CAT approximation (all parameters estimated from the data). The genera and species trees were analysed using RAxML only. RAxML analyses were carried out via the Cipres Science Gateway Portal (Miller 2009). Bootstrap support values were mapped onto the tree with the best likelihood and the Bayes consensus tree using Dendroscope (Huson *et al.* 2007). Accession numbers of sequences used in the phylogenetic analyses are summarized in Appendix 3.

Results

SSU rDNA trees

The 'orders' SSU rDNA tree (Fig 1) includes representative species for all current fungal orders for which sufficiently high quality sequence data were available, and includes all CFF orders for which sequences of sufficient quality and length were publicly available. The topology of our SSU rDNA phylogeny is concordant with that based on six genes in James *et al*. (2006). A total of 60 cord-forming fungal genera are known (Table 1). CFF are restricted to the Dikarya and occur in two clades: A - 17 orders within the basidiomycete subphylum Agaricomycotina, and B - within the ascomycete order Xylariales (Fig 1). Whilst neither clades is supported by strong bootstrap values (>75) or posterior probabilities (>0.75), their concordance with the 6 gene phylogeny of James *et al.* means that we can begin to draw conclusions as to groupings from these. Furthermore,

there is sufficient support in the more basal branches to provide additional evidence for the two clade hypothesis. In the present study, one cord-forming species per order (Appendix 1) was required for the order to be categorised as cord-forming (see Methods). Twelve of the 17 orders in Agaricomycotina had cord-forming members (=5.4% of genera); those that have no known CFF taxa are Dacrymycetales, Atheliales, Auriculariales, Gloeophyllales and Sebacinales. In Xylariales, 2.4% of genera are known to be cord-forming. The incidence of cord-forming in both groups is given in more detail in Table 2. Overall, the percentage of documented cord-forming genera within orders ranges from 2.4% to 22.2%.

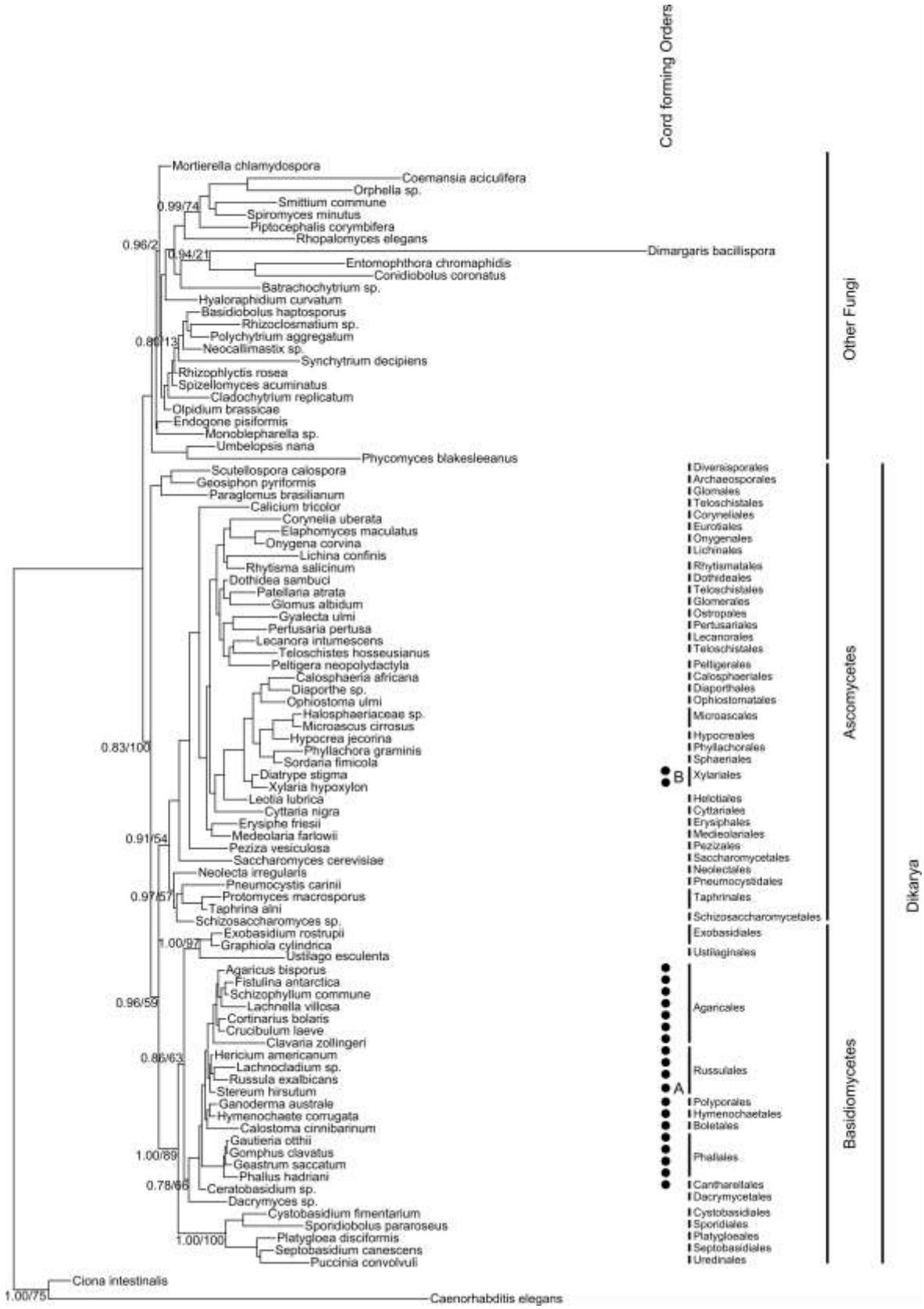

Figure 1 *SSU rDNA phylogeny (1592 positions, 106 sequences) of a broad representation of the fungal kingdom. The phylogeny is rooted on* Animalia*. CFF orders are represented as closed circles. Numbers on branches = (posterior probability/bootstrap value).*

Order	Total # genera	# known CFF genera	% CFF genera	Names of CFF genera
Agaricales	413	20	4.8	*Agaricus, Agrocybe, Armillaria, Clitocybe, Collybia, Coprinus, Cortinarius, Gymnopilus, Hebeloma, Laccaria, Lycoperdon, Marasmius, Megacollybia, Melanoleuca, Panaeolina, Psathyrella, Psilocybe, Schizophyllum, Tricholoma, Tricholomopsis*
Atheliales	22	0	0	-
Auriculariales	32	0	0	-
Boletales	96	8	8.3	*Boletus, Leccinum, Paxillus, Pisolithus, Rhizopogon, Scleroderma, Serpula, Suillus*
Cantharellales	38	2	5.3	*Hydnum, Sistotrema*
Corticiales	29	1	3.4	*Vuilleminia*
Geastrales	7	1	14.3	*Sclerogaster*
Gloeophyllales	8	0	0	-
Gomphales	18	1	5.6	*Ramaria*
Hymenochaetales	48	2	4.2	*Hymenochaete, Resinicium*
Hysterangiales	18	1	5.6	*Hysterangium*
Phallales	26	2	7.7	*Mutinus, Phallus*
Polyporales	216	9	4.2	*Antrodia, Bjerkandera, Grifola, Lenzites, Phanerochaete, Phlebia, Skeletocutis, Steccherinum, Trametes*
Russulales	80	2	2.5	*Heterobasidion, Stereum*
Sebacinales	9	0	0	-
Thelephorales	18	4	22.2	*Amaurodon, Pseudotomentella, Thelephora, Tomentella,*
Trechisporales	15	1	6.7	*Trechispora*
Tremellales	38	1	2.6	*Sebacina (Cristella)*
Xylariales	209	5	2.4	*Kretzschmaria, Lopadostoma, Podosordaria, Rosellinia, Xylaria*

Table 2: *Number of genera containing at least one CFF species (identified in the literature search) and total number of genera (from Kirk, 2008) in each order identified as a CFF containing order. CFF genus identities are included.*

Additional SSU rDNA ('genus') trees were generated to investigate patterns of cord-forming clades at the genus level within three orders for which sufficient sequence data were available and which contained more than three CFF genera (categorised as a genus containing at least one cord-forming species): Boletales (Fig 2), Agaricales (Appendix 4), and Phallales (Appendix 5). Within Boletales, 8.3% of genera are known to contain at least one CFF species. Very uneven taxonomic coverage of the order in GenBank suggests at first sight that this order is dominated by CFF, but in fact the literature survey shows that only 8.3% of its genera are known cord-formers. The Agaricales 'genus' tree (Appendix 4) shows that the CFF is apparently randomly spread across the whole order with no phylogenetic clustering. Only two known CFF genera were available for inclusion in the Phallales tree (Appendix 5), but this is sufficient to show that the CFF habit does not appear to be clustered relative to the shown diversity of the order as a whole.

ITS rDNA ('species') analyses

Phanerochaete

The genus *Phanerochaete* is one of the better known cord-forming genera (Burdsall 1985; De Koker *et al.* 2003), therefore likely to show phylogenetic clustering of the CFF habit if it occurs. Sixty five species are known in total (Kirk 2008) of which 24 were represented in the sequence databases. Of the 24 species included in the phylogenetic analysis (Fig 3), nine were known from the literature to form cords and a further two are reputed to form cords and discussed as cord-forming (open circles; unpubl.). The clade from *P. velutina* down to *P. carnosa* are all cord-formers. However, the trait is scattered across the rest of the tree without any apparent pattern. This phylogeny is congruent with those of De Koker *et al*. (2003). Three other cord-forming species are known, *Phanerochaete salmoneolutea* ,

Rhizochaete brunnea and *Rhizochaete borneensis*. However, ITS sequences were not present in GenBank and therefore are not included in Fig 3.

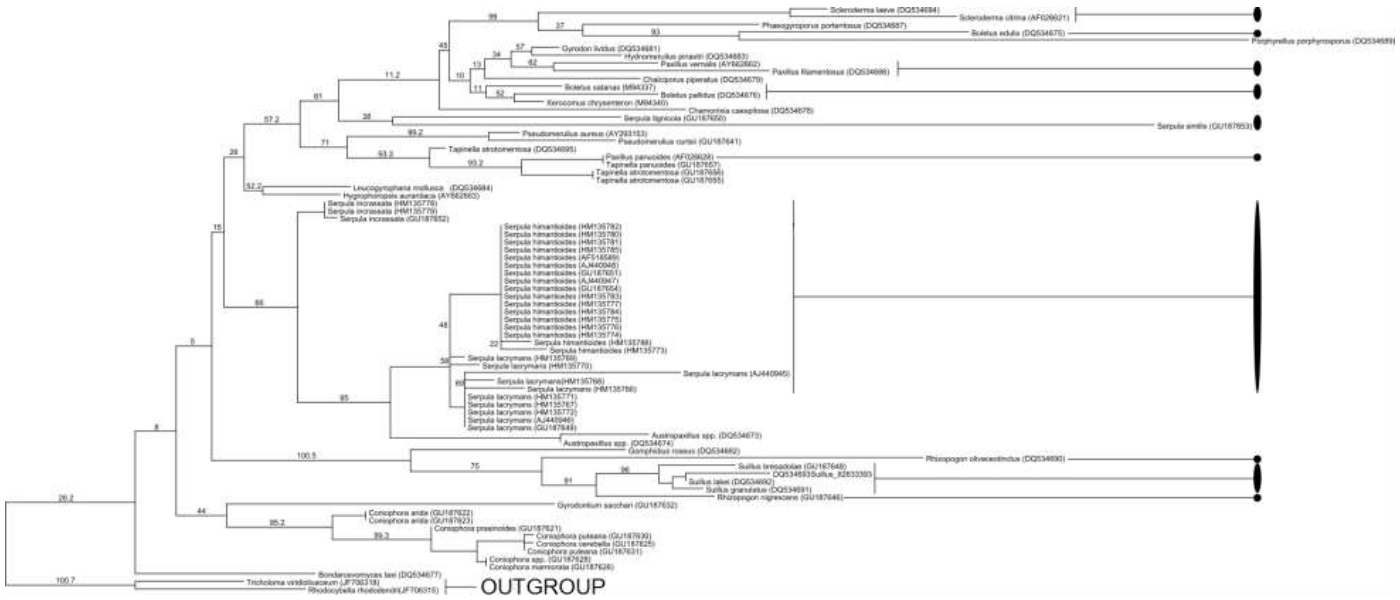

Figure 2 *Boletales SSU rDNA phylogeny (1140 positions, 91 sequences), rooted on* Tricholoma *and* Rhodocybella*. Closed circles (ovals) indicate documented cord formers. Numbers on branches = (posterior probability/bootstrap value).*

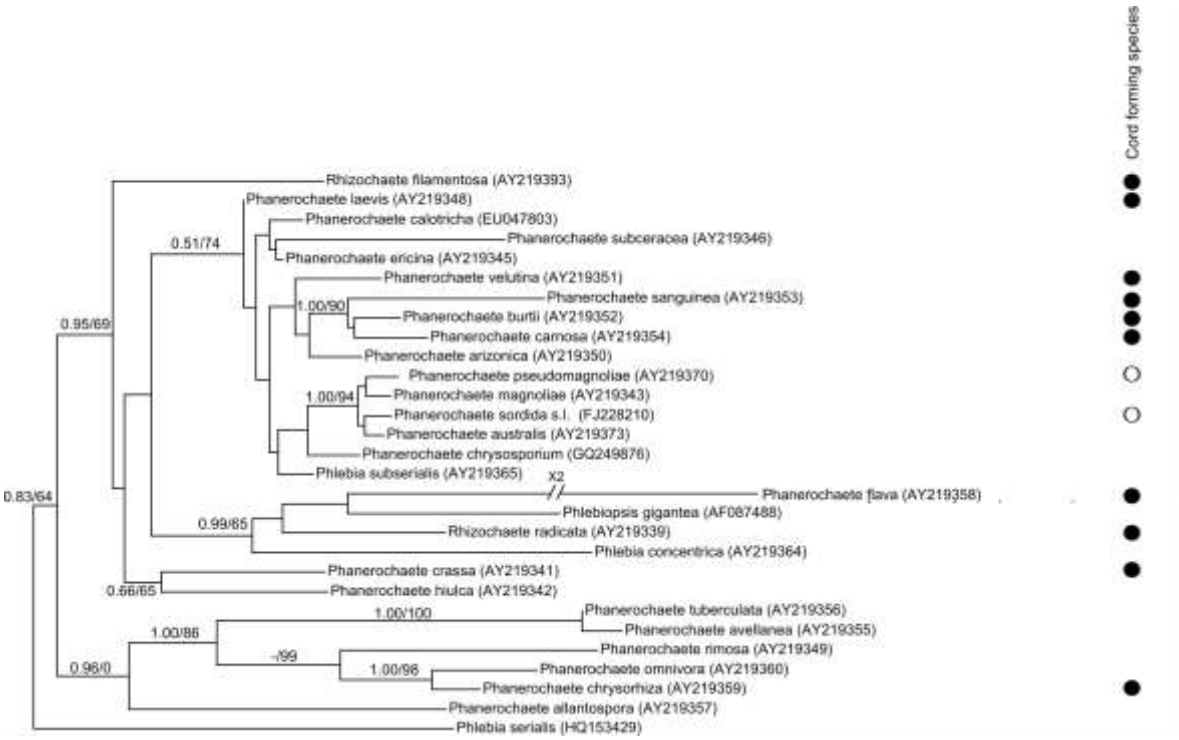

Figure 3 *Phanerochaete ITS1-5.8S-ITS2 rDNA phylogeny (506 positions), rooted on* Phlebia serialis, *with cord-forming species indicated. Closed circles indicate documented cord-formers, open circles indicate species known to form cords but not published as such. Numbers on branches = (posterior probability/bootstrap value).*

Armillaria

This is a rhizomorph-forming genus in which the habit is associated with parasitism - *Armillaria* causes white-rot root disease, and is able to keep feeding saprotrophically on its host once the latter is dead. This is therefore an unusally well-studied genus (Volk 1995). Of 35 *Armillaria* species listed in *The Dictionary of the Fungi* (Kirk 2008), 24 are represented in GenBank, four of which are also present in the Silva database. Eighteen species have been identified as rhizomorph-forming; these are all represented in the phylogenetic analysis (Fig 4). The weakly-supported clade *A. hinnulea* down to *A. ostoyae* are all rhizomorph-forming. Only six of the 24 species in Fig 4 are not known to form rhizomorphs. Furthermore, there are differences in cord morphology between clades. Morrison (2004) showed that CFF branch in two distinct ways, monopodial (all branches

originating from a single arterial cord line) and dichotomous (successive branching of the cord system into a fan network). Our analysis shows that the monopodial growth habit appears restricted to a phylogenetic cluster within the northern hemisphere clade, whereas dichotomous growth (perhaps the ancestral condition in this genus) is found paraphyletically in both northern and southern hemisphere clusters. The evolution of a monopodial growth habit in the Northern Hemisphere may be associated with greater saprotrophic colonisation success and virulence (Morrison 2004).

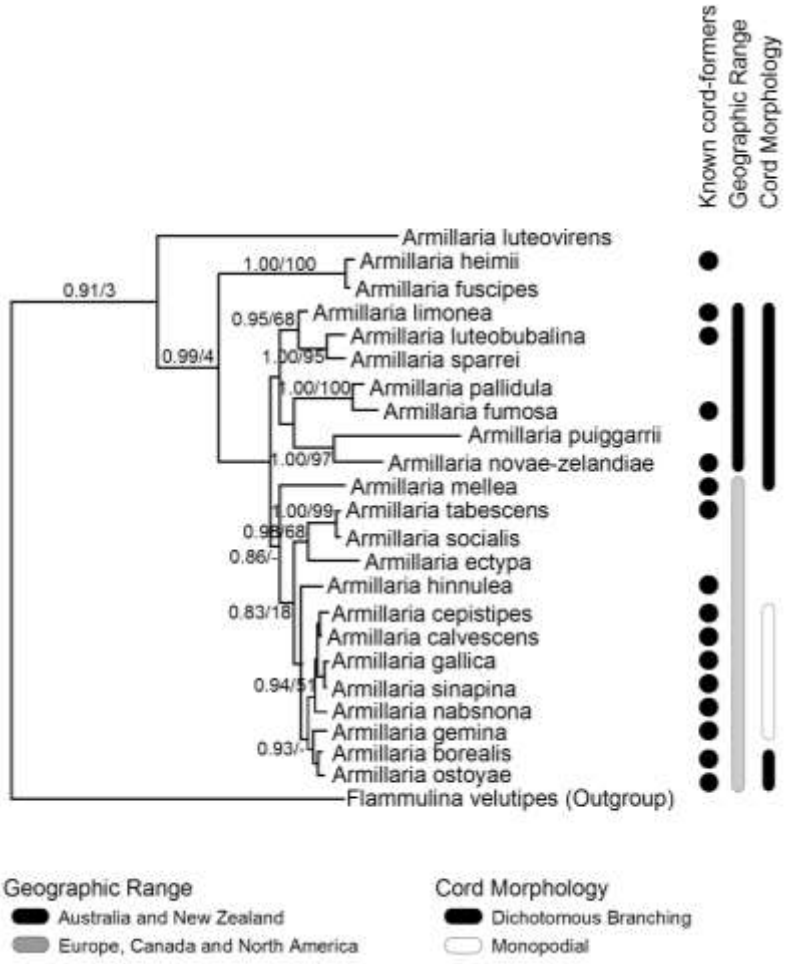

Figure 4 Armillaria *ITS1-5.8S-ITS2 rDNA phylogeny (730 positions), rooted on* Flammulina velutipes, *with CFF indicated as closed circles. Bars show differences in geographic range (central column) and morphology (right column).* Armillaria *species without cord morphology or geographic range indicated do not have this information documented. Accessions of* A. luteovirens, A. heimii *and* A. fuscipes *are from China, Réunion and Africa respectively. Numbers on branches = (posterior probability/bootstrap value).*

Xylaria, Tomentella, and Serpula

Xylaria, an ascomycete CFF genus, is strongly represented in GenBank with 87 ITS1 rDNA sequences, representing 29% of the total number of species currently in the genus (Kirk 2008). However, only two of the 300 species are documented cord-formers. These cord-formers, *X. polymorpha* and *X. hypoxylon*, are as distantly related to each other as to most other *Xylaria* species (Appendix 6). There is no indication or literature to suggest that the histological arrangement or adaptations are different between ascomycete and basidiomycete cord structures. *Tomentella* is another (agaricomycete) genus well represented in GenBank with 32 ITS1 rDNA accessions representing 29% of known species (Kirk 2008). However, as for *Xylaria* only a small proportion of these are known CFF, and show no phylogenetic pattern on our tree (Appendix 7). The CFF species are *T. botryoides, T. ferruginea*, and *T. cinerascens*. To our knowledge this is the first published ITS tree for *Tomentella* and shows that the genus splits into two (albeit weakly supported) clades. Within Serpulaceae only the maximally-supported clade of *Serpula sensu stricto* (the closely related species *S. lacrymans* and *S. himantioides* (Skrede *et al*. 2011)) are known CFF. The deeper-branching species (*S. tignicola*, *S. similis* and *S. incrassata*, which have been re-classified as *Merulius* (Kirk 2008)) and the ectomycorrhizal *Austropaxillus* (which may branch between the last named three species and *Serpula sensu stricto* (Skrede *et al*. 2011)) are not known to form cords.

Discussion

Our analyses of currently available data suggest that across Dikarya the ability to form cords has arisen (or been retained) in two instances: once within ascomycetes with the origin of the Xylariales clade; and once within basidiomycetes, at the base of the

Agaricomycotina clade between Dacrymycetales and the radiation of the rest of the subphylum. The alternative hypothesis is that each CFF lineage or clade within the Xylariales and Agaricomycotina has evolved the ability to form cords independently, in response to ecological selection, is far less parsimonious. The patchy distribution of cords within Xylariales and Agaricomycotina is very likely at least partly due to lack of knowledge of individual taxa, which is too incomplete to know whether non-CFF species form cords or not, or under which conditions they are formed. Many genera, such as *Hygrocybe* and *Clavaria*, have not been grown in isolation in laboratory conditions. Therefore, we have no means of knowing the variety of growth forms they display. In contrast, genera such as *Aspergillus*, have been grown extensively in laboratory conditions and therefore, we can be more confident that they do not readily form cording structures. It is also possible that the ability to form cords has been lost in some lineages in the absence of a selection pressure maintaining it.

Five orders within the basidiomycete subphylum Agaricomycotina are not known to contain any CFF: Dacrymycetales, Atheliales, Auriculariales, Goeophyllales and Sebacinales. These are all saprotrophic fungi that frequently form gelatinous fruiting bodies (Bon *et al.* 1987), although Sebacinales are known to form mycorrhizal associations with a variety of plants (Weiss *et al.* 2011). The cord-forming habit appears to have arisen after the divergence of the jelly fungi Dacrymycetales, perhaps in the common ancestor of all other orders of the subphylum. However, the other non-CFF orders are more derived and branch in several places across the subphylum. It is possible that the development of cord systems was an evolutionary response to nutrient paucity requiring the development of a foraging organ. Smaller, simpler fungi require fewer resources and therefore have less need of foraging apparatus.

Some saprotrophic cord-forming species are also ectomycorrhizal, such as *Suillus* spp. (Boletales). These organisms show similar but distinct structures for cords and ectomycorrhizae (Randall & Grand 1986). Saprotrophic and ectomycorrhizal cord organs are both found in orders and families of fungi comprising complex sporophore-bearing taxa with extensive mycelial networks and significant biomass per individual (Boletales, Gomphales, Thelephorales, Amanitaceae, Cantharellaceae, Cortinariaceae, Russulaceae and Tricholomataceae), suggesting that the propensities for forming these ramifying, exploratory structures are linked. ECM and CFF could be alternative foraging and nutrient assimilation strategies using the similar trait of modified hyphal organs. Skrede *et al*. (2011) suggest that an ability of geophilous fungi to transport water from the ground to a substrate could be a first step towards mycorrhizal symbiosis. The distinction occurs in the resource utilised by these hyphal structures. ECM fungi obtain nutrients from living plant matter through associations with the root systems. By contrast, CFF obtain nutrients saprotrophically from deadwood and litter-fall at the soil litter interface (although *Armillaria* can also obtain nutrients directly from a living host, as can another rhizomorph forming fungus *Rigidoporus lignosus* (a member of the agaricomycete Polyporales). Therefore, CFF structures are required to be hardier and better able to withstand desiccation than hyphae alone, explaining the salt encrusted outer layer of the cord 'tissue'. CFF have only been found in woodland and forest habitats where the desiccation risk is minimised through protection by leaf litter; there are no known instances of cord formers among typically grassland fungi (e.g. *Hygrocybe* spp) although CFF such as *Agrocybe gibberosa* (Agaricales) have been documented in grasslands colonising grass litter (Robinson *et al.* 1993). CFF species have also been observed in tropical forests, where they may form networks trapping litter in the canopy (Hedger 1993). Non-agaricomycete basidiomycetes (Exobasidiales, Ustilaginales, Cystobasidiales, Sporidiales, Platygloeales, Septobasidiales and Uredinales) are mostly yeast, filamentous, parasitic or rust/smut-

forming fungi, none of which are known to form cords. Therefore, CFF appears strongly associated with robust and highly productive sporophore-formers in woodland habitats, and with fungi associated with trees either as saprotrophs, mycorrhizae, or parasites. Even lineages closely related to CFF but not associated with woodlands are not, so far, known to form cords, such as the genus *Bovista*. This underlines the nutritional role of cords in connecting and utilising patchily distributed resources (i.e. fallen dead wood) that are nutritionally rich but relatively difficult to assimilate such as are most frequently found where trees are grouped together.

Within ascomycetes, the orders most closely related to the cord-forming Xylariales are, Sphaeriales, Phyllachorales, Hypocreales, Microascales, and Ophiostomatales, which are saprotrophs and plant parasites. Previous studies by Schoch *et al.* (2009) have placed the saprobic Sordariales and Bolinales as sister groups to the Xylariales. The nature of ascomycete cord-forming is currently considered very similar to that of basidiomycete cord-forming: no morphological or physiological differentiation has so far been made between the ascomycete and basidiomycete cord systems. Within the ascomycetes, ectomycorrhizal and cord-based nutrition are not known to overlap within taxa; ECM fungi are most often Pezizales as opposed to Xylariales, suggesting an alternative relationship between saprotrophic cord-forming and ectomycorrhizal habits in ascomycetes from basidiomycetes (Agerer 2006).

We present phylogenetic trees in a nested taxonomic hierarchy - from one showing fungal orders within higher-level relationships, through genera in trees representing single orders, to species within genera. Our motivation was to build as complete a picture as possible of the phylogenetic distribution of the CFF habit within those groups known to contain them. For example, whether orders known to include CFF are densely populated with them or

whether only a small proportion of taxa are involved, and similarly for the relationship between CFF species within genera. It is perhaps not unexpected that a clear pattern did not emerge from this – in general, the further one investigates down the hierarchy the less is known about each individual lineage. However, we identify the orders Agaricales, Russulales, Phallales, Corticiales, and Boletales as particularly rich in CFF and suggest that the incidence of the trait is much higher in reality than we have been able to gather evidence for here. It is highly likely that the lack of phylogenetic clustering of the cord-forming habit in our sub-order level trees (with the exception of *Armillaria*) is a result of under-sampling/recording cord structures relative to sporocarps. Historically, this is not surprising as cords would hardly ever be the choice of structure to investigate or collect for taxonomic identification, and therefore the study of their diversity has remained neglected well into the period in which molecular taxonomy methods have been increasingly applied to many cryptic groups. We suggest that an unbiased molecular survey of cords would reveal at least many more agaricomycete cord-forming taxa, even if many lineages form cords rarely or only under specific conditions. Nonetheless, our phylogenetic results do suggest that lineages most likely to display the cord-forming condition are macroscopic saprotrophs.

As a commercially important pathogenic cord former, *Armillaria* is an exception in this study and unusual in any case. The majority of *Armillaria* species are causative agents of *Armillaria* root rot, a disease of ancient woodland trees in temperate regions. As a consequence, *Armillaria* has been well studied in terms of both geographic range and cord-morphology (Morrison 2004; Maphosa *et al.* 2006). Relatively intensive work on this group has shown that a high proportion of its members are cord-forming and has identified differences in cord branching patterns, which may relate to functional differences between lineages (Morrison 2004). The ancestral *Armillaria* branching pattern may be

dichotomous; that is, their cord systems split into two at multiple points along the length of the cord, as this pattern is found in both southern and northern hemisphere clades (Fig 4). This theory is supported by a study in 2003 which demonstrated that *A. heimii*, basal to the other characterised species, has a dichotomous or palmately branching rhizomorph growth form. However, within the northern hemisphere radiation a novel monopodial growth form has emerged in which one main cord line dominates and smaller cord filaments branch off from this arterial line. More comprehensive data for *Armillaria* regarding cord-forming capacity and growth form would provide the basis for more informed interpretation of the evolutionary and ecological diversification of the cord-forming habit. Many *Armillaria* species, such as *A. mellea*, form cords rarely and under specific conditions (Sortkjaer & Allerman 1972; Worrall 1986; Baumgartner & Rizzo 2001), therefore members of the genus which are not known to form cords are potentially false negatives. It is possible that all species have the capacity to form cords but rarely have the need for them; the pathogenic nature of many *Armillaria* species reduces the requirement of foraging for new woody resources and provides sufficient nutrients for fruit body production and therefore colonisation of new areas via airborne spores.

Because most work on CFF has been concerned with the ecology and energetics of the cord-forming habit, by definition focusing on fungi already known to form cords (particularly within the genera *Armillaria*, *Phanerochaete* and *Hypholoma* (Boddy 1999; Woodward & Boddy 2008), it could be the case that only a relatively small proportion of CFF have been recognised. Cords themselves generally lack sufficient morphological characters for taxonomic assignment, and therefore do not provide a useful surrogate for the phylogenetic diversity they harbour. We have little idea to what extent even the known diversity of CFF form a functionally redundant assemblage or whether their activities and ecosystem services are more finely subdivided than is currently realised. Further

assumptions about the strong association between the cord-forming habit and fallen dead wood might be restrictive. For example, cords are regularly found on leaf litter unassociated with logs or substantial blocks of dead wood. Cords may be more commonly present as saprotrophs in non-woodland habitats, and/or more associated with living plants, than currently recognised. Indeed, in tropical forests, cord systems exist as networks in the forest canopy, trapping leaf litter before it reaches the forest floor and using this as a nutrient resource (Hedger 1993). Randomised and ecologically comprehensive cord sampling and sequencing campaigns currently in progress will reveal to what extent CFF form part of the hidden/cryptic biodiversity that is thought to dominate ecosystems worldwide (Bickford *et al.* 2007; Pfenninger & Schwenk 2007), and more focused work and experiments will illuminate their ecological and functional diversity.

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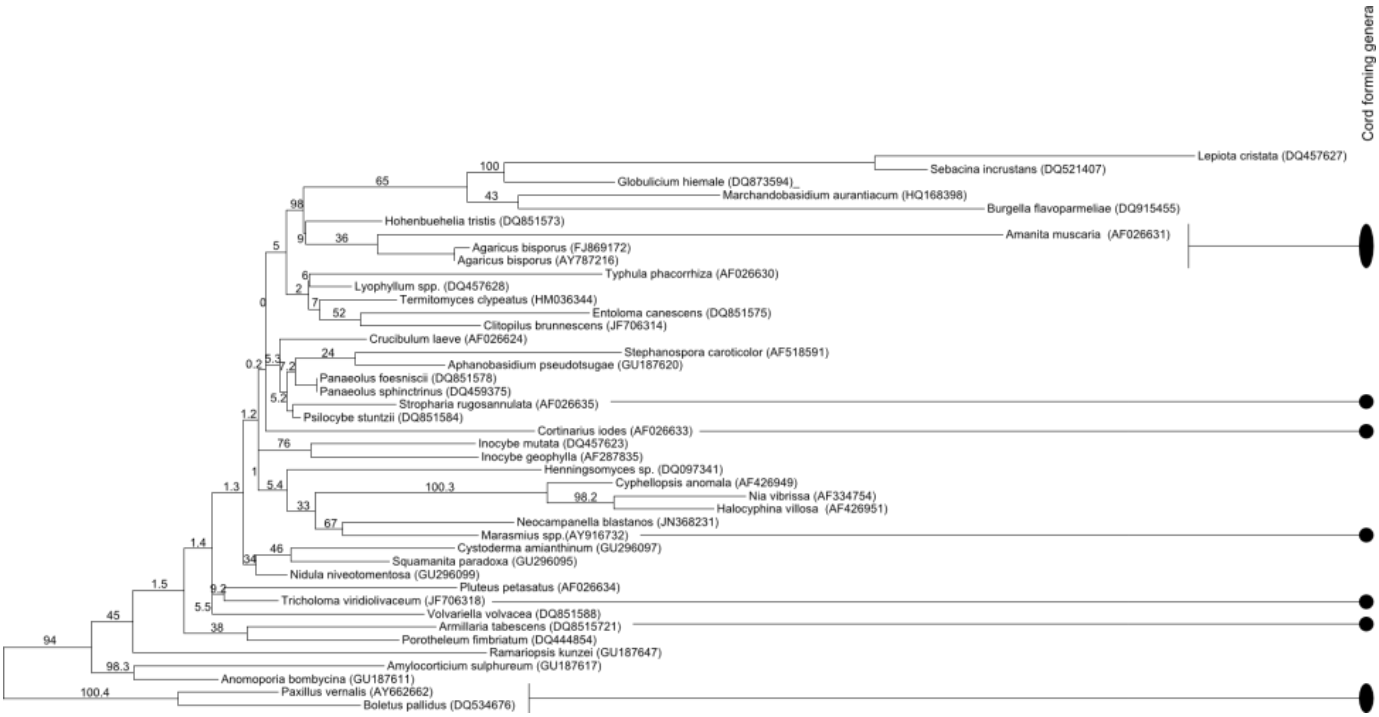
Appendices

Appendices 1 – 3 are available on the CD included (inside front cover of bound thesis).

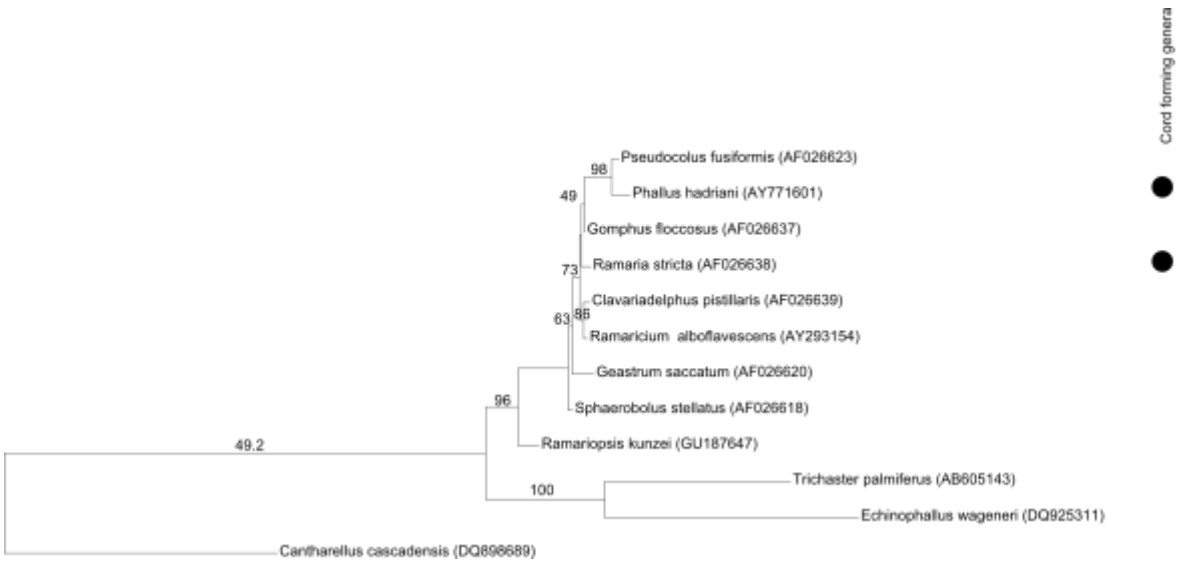
Appendix 1 *Table containing the names and references for all documented CFF. Papers containing CFF were found using the search engines SciVerse Scopus and NCBI PubMed with the Booleian search terms: <cord AND fungus>, <syrrotia>, <strand AND fungus> and <rhizomorph>*

Appendix 2 *Breakdown of cord-forming genera including the total number of species in the genus (according to Kirk, 2008), the number of unique species in both the Silva and NCBI GenBank databases, the total number (and percentage) of known CFF (according to Appendix 1) and the percentage of species for the genus in the nucleotide databases.*

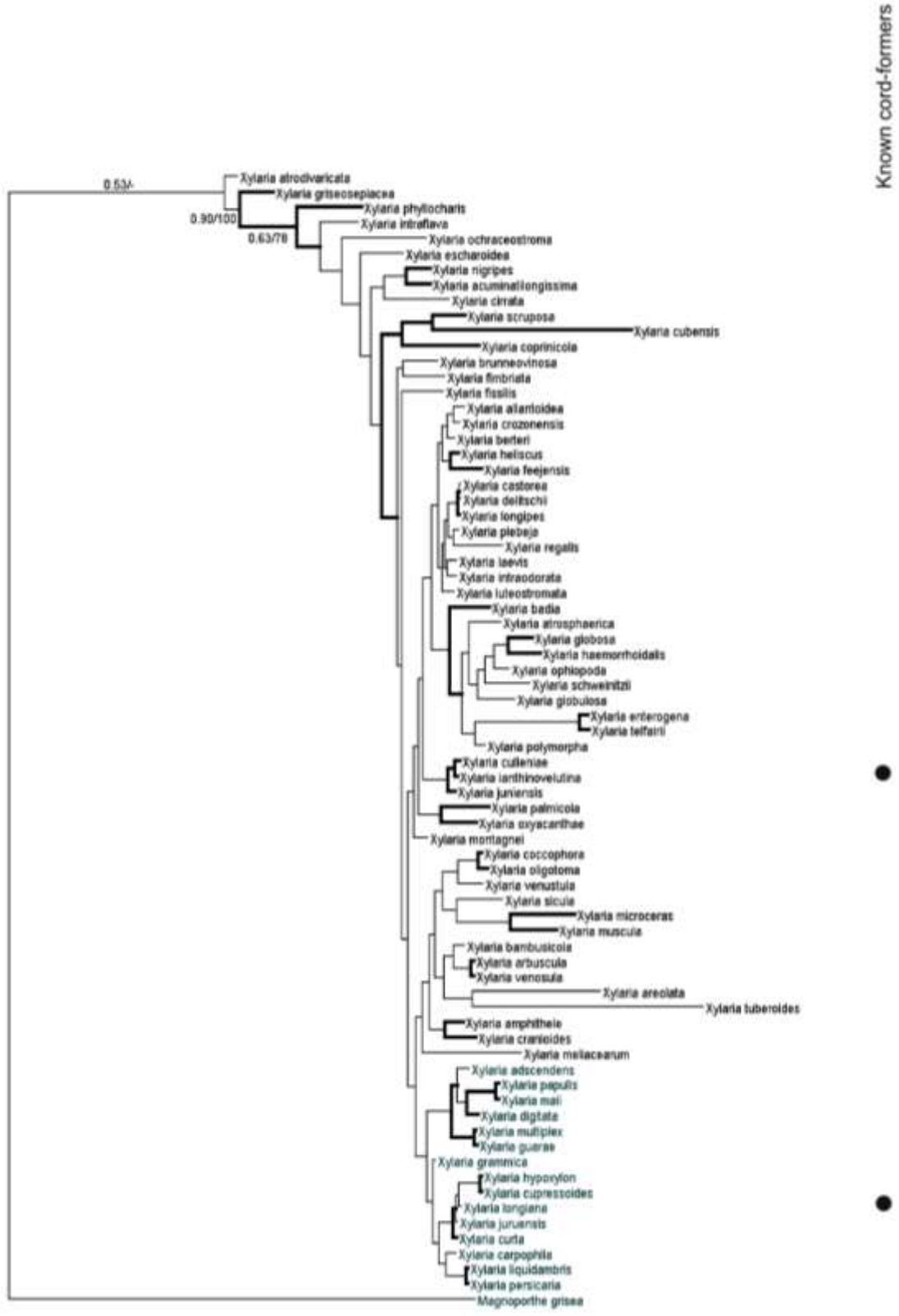
Appendix 3 *Table of accession numbers used to generate the alignments and phylogenies used in the paper. Sequences are all available in NCBI GenBank and ordered by the phylogeny they are incorporated into.*



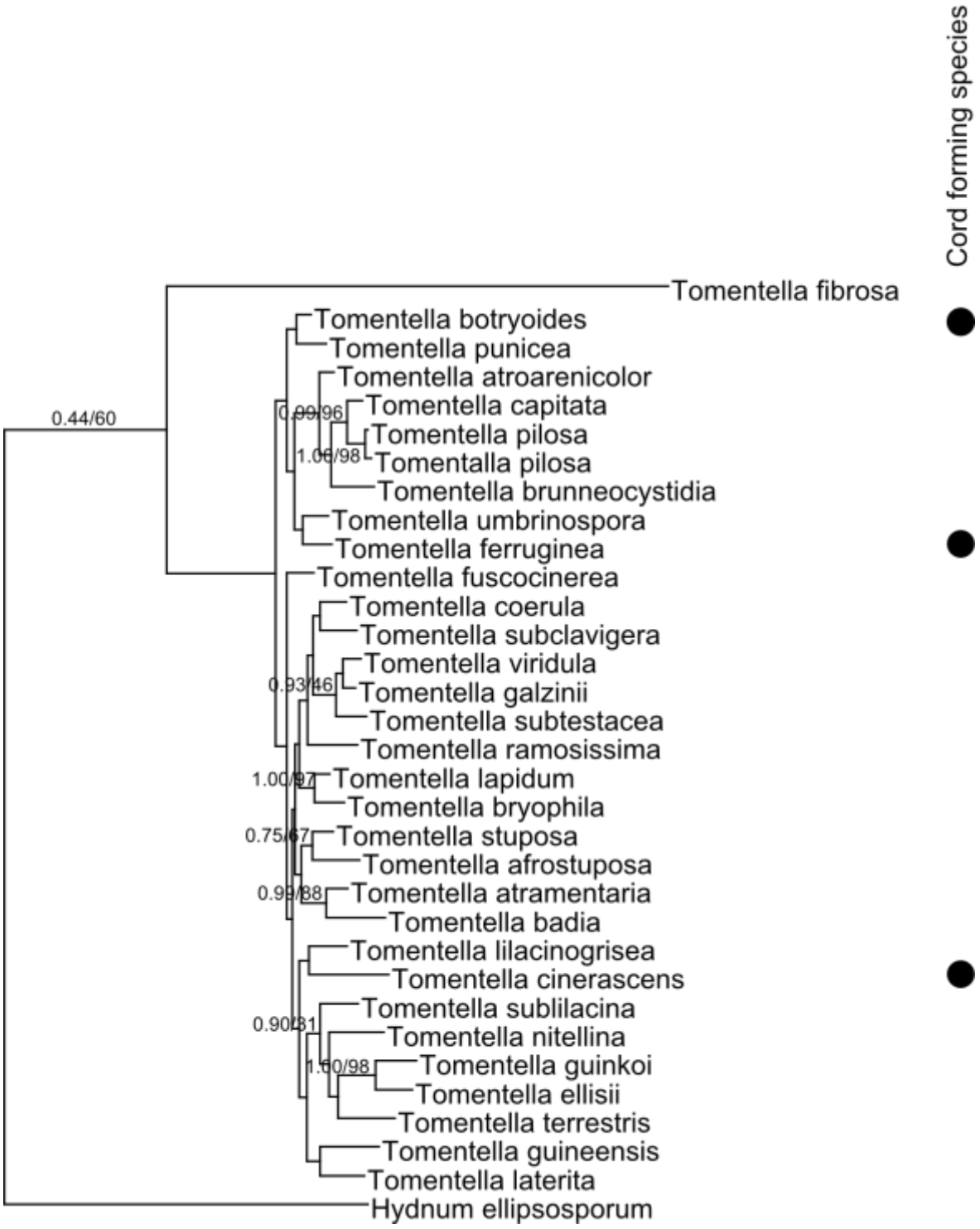
Appendix 4 *Agaricales SSU rDNA phylogeny (1703 positions, 43 sequences), rooted on Boletus and Paxillus. Closed circles/ovals indicate documented cord-formers. Numbers on branches = (posterior probability/bootstrap value).*



Appendix 5 *Phallales SSU rDNA phylogeny (1762 positions, 12 sequences) rooted on Cantharellus. Closed circles indicate documented cord-formers. Numbers on branches = (posterior probability/bootstrap value).*



Appendix 6 *Xylaria ITS1-5.8S-ITS2 rDNA phylogeny (2048 positions) rooted on Magnaporthe grisea. Closed circles/ ovals indicate documented cord-formers. Numbers on branches = (posterior probability/bootstrap value).*



Appendix 7 *Tomentella ITS1-5.8S-ITS2 rDNA phylogeny (531 positions) rooted on Hydnum, with cord-forming species indicated as closed circles. Numbers on branches = (posterior probability/bootstrap value).*

CHAPTER THREE

THE EFFECT OF WOODLAND PLANTATION AGE ON FUNGAL COMMUNITY COMPOSITION.

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Declaration:

I confirm that the work contained in this chapter is wholly my own and that the contributions of the other authors to the paper constituted advice regarding experimental design and proof-reading the manuscript prior to submission. This paper is under review for 'The European Journal of Forest Research'.

Signed:

Kirsty Monk (DPhil candidate) Dr Nick Brown (Supervisor)

THE EFFECT OF WOODLAND PLANTATION AGE ON FUNGAL COMMUNITY COMPOSITION.

Abstract

In an age in which Great Britain is looking to rapidly reforest and afforest, it is vital that planting regimes are focussed around all ecosystem components to generate woodlands conferring the full suite of environmental benefits to their surroundings. In this investigation, we have looked for patterns in fungal community composition in a sequence of woods planted on ex-agricultural land in Warwickshire, UK, and compared them to those in ancient semi-natural woodland nearby. An extensive fungal sampling approach enabled profiles of the fungal communities to be built up, identifying the timescales needed for functional woodland ecosystems to be restored. A key finding was that the richness of woodland fungal species increases with time since tree planting, however woodland fungal community composition was still very different to that characteristic of ancient semi-natural woodland (ASNW) fourteen years after tree planting. We use this data to consider the implications for ecosystem services including decomposition and nutrient cycling and conclude that fungal community composition may be a valuable indicator of forest ecosystem establishment and restoration success. National forest policies aim to enhance ecosystem service provision by creating new native woodland. However, our results suggest that where this is done on ex-agricultural land it may take much more than a decade for fungal communities to resemble those in natural woodland and therefore, additional efforts must be taken to establish fully functional woodlands.

Key Words

ASNW, Cord-forming fungi, Re-colonisation, Restoration

Introduction

The majority of countries in Europe and North America experienced a net increase in forest area in the decade 2000-2010 as a result of afforestation (FAO 2011). From 2005–2010, afforestation added about 5 million hectares of woodland per year, globally. Replanting since 1870 has increased the percentage of woodland cover in Great Britain from 4.7% to 12.8% (Forestry Commission, Forest Service 2012). The Government Forestry and Woodlands Policy Statement (DEFRA, 2013) set an aspiration to plant an average of 5,000 ha per year in England in order to achieve 12% woodland cover by 2060.The Scottish Government aims to achieve 25% woodland cover by 2025 (Iason 2011). A recent national assessment (Read *et al*., 2009) recommended that an additional 14,840 ha of new woodland should be planted per year over the next 40 years as part of plans to ameliorate the effects of climate change.

Tree planting alone, particularly where it is on ex-agricultural or post-industrial land, is unlikely to create diverse, productive woodlands as they typically lack the associated flora, fauna and fungi (Anon 2001, PlantLife 2011). An effective woodland can be defined as one which provides a range of ecosystem services that are important to society. Fungi play a particularly key role in the construction of many woodland ecosystem services. For example, the carbon storage capacity of woodland depends, among other factors, on the balance between net primary productivity and litter decomposition – both processes strongly influenced by fungi (Dighton 2003, Molina *et al.* 1999). Management of the fungal community and the wood-wide web (Helgason *et al.* 1998) in new woodlands may be critical to ensure that the carbon storage function is optimised. This paper examined the fungal communities found in a series of woodlands planted over a period of 14 years and compares them with nearby established woodland. The aim of the project was to see if we might infer patterns of fungal colonisation and succession on newly planted woodlands at

different times since tree planting and to predict how long it might take for fully functioning woodland to develop.

The Heart of England Forest Project (www.heartofenglandforest.com), formerly the Forest of Dennis, is an organisation which has been planting new woodlands for about 20 years on agricultural land adjacent to current woodland sites in Warwickshire, with the aim of creating the largest area of contiguous woodland in the UK. Currently, the new woodland (the Heart of England Forest) extends across about 890 hectares (ha). Planting has been occurring at a rate of roughly 121 ha per year. The species planted are consistent with the National Vegetation Classification (NVC; Rodwell 2006) W8 or W10 (lowland mixed deciduous woodland) and are planted as saplings with additional natural tree establishment occurring from nearby hedgerows. Large rides (cleared pathways through a woodland) are mown into the new woodlands to encourage wildflowers to grow. The fungal diversity of these new, managed woodlands was investigated to enhance understanding of the establishment time and characteristics of fungal communities on land previously under agricultural management. These data will be used to inform woodland management practices to introduce and encourage important fungi into woodlands at an early stage of restoration to speed up the restoration and afforestation process.

Planting on agricultural land comes with inherent problems such as residual effects of fertilisers and pest control (Willoughby & Moffatt 1996). Studies to date have investigated the pitfalls and successes of schemes to increase old-growth elements, such as coarse woody debris and standing deadwood, in temperate forests (Humphrey 2005; Vandekerkhove *et al.* 2011). These have found that fungal communities respond to old growth features of woodlands and that the richness increases associated with them are significant. Humphrey (2005) suggested that old growth characteristics will take more than 100 years to develop in upland spruce plantations, pertinent here as deciduous woodlands tend to be slower growing and their features may, therefore, be expected to take much

longer to develop. This study seeks to understand whether the increase in fungal richness associated with woodland age is detectable in younger planted stands (up to 14 years old) and whether fungal community turnover is occurring at these small timescales.

Of all the fungal components of woodland, saprotrophs are arguably the most important from an ecosystem engineering perspective. Within the saprotrophs, one particular group – cord-forming fungi - are of particular note. In cord-forming fungi, hyphae align to produce a tube-like organ which can move water and dissolved nutrients the woodland floor for over 300 metres in diameter (Boddy 1993, Thompson & Rayner 1983) and take decades to develop.

The present study revolved around a single basic hypothesis, that older plantation woodlands and ASNW would be expected to have a greater proportion of saprotrophic fungal species than newer plantations on agricultural land, as woody resources provide a greater variety of substrates to support such diversity. Furthermore, it would be expected that the old-growth associated conditions of standing and fallen deadwood and increasingly closed canopies would be more conducive to fungal prevalence.

As a result, this study sought to address a number of gaps in our knowledge of fungal regeneration and succession in planted woodlands; firstly to characterise fungal communities in planted stands of different ages and to determine whether and how these communities differed from established woodland ecosystems in the area. An additional aim of the thesis was to determine the factors involved in determining these community differences and examine the extent to which such factors are associated with one another . Therefore, the effectiveness of the current planting regime adopted by the Heart of England Forest for encouraging rapid regeneration of fungal communities in British woodlands planted on old agricultural land could be assessed and methods that may be effective in encouraging more rapid regeneration in such areas can be proposed.

Materials and methods

Site selection

The Heart of England Forest, Warwickshire, is a large area of both existing and newly planted woodland with a well-documented history of planting. It currently covers three main geographic areas separated by towns, roads and agricultural land. We selected three regions within the Forest (centered on Spernall, Dorsington, and Honeybourne). Within each region, we selected between 7 and 15 sites, dependent upon the range of plantation ages available. Site selection aimed to encompass the widest possible range of times since afforestation: between 1 and 13 years. For each year represented at a region, three replicate sites were attempted; no site was sampled more than once (Appendix 1). This information enabled the creation of a long-term monitoring programme to investigate the effects of woodland age upon the fungal communities therein.

The sampling strategy was stratified to incorporate data from as broad an age range as possible over all three sites although full coverage at each site was not possible. A total of 35 sites were surveyed incorporating plantings from 1996 to 2011 as well as three control sites which consisted of existing mixed woodland (Figure 1).

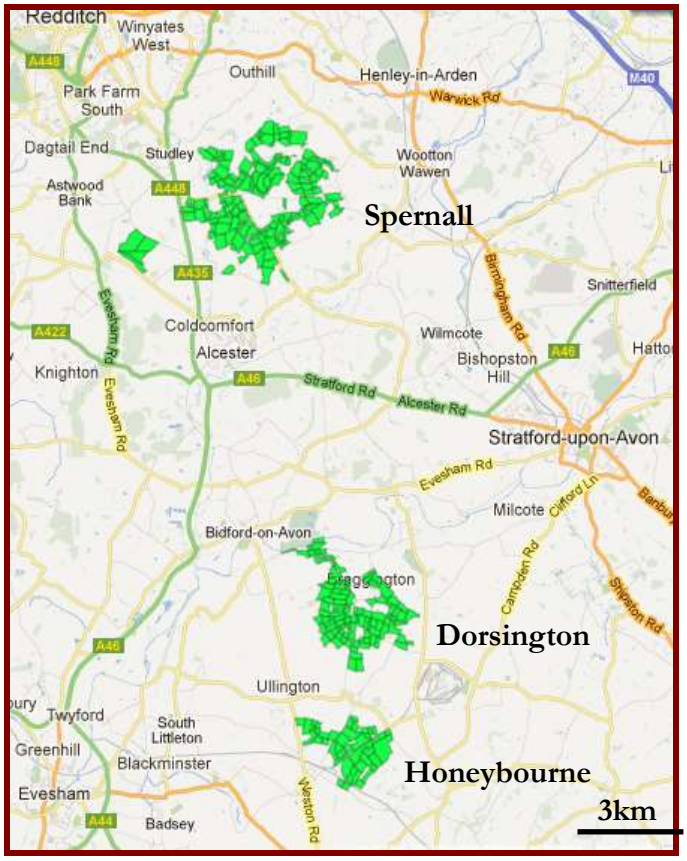

Figure 1 *Map to show experimental site locations. All sites are within 20km of one another and exhibit similar characteristics including ground vegetation and tree species mixes*

Data Collection

A detailed fungal foray was carried out at each site. The sampling and survey technique was based upon a Rapid Botanical Survey protocol (Hawthorne 1995). Within each site four volunteers searched for and sampled unique fungal species for one hour, a total of four man hours per site. Sampling took place between 3rd and 9th September 2012. This approach reduces the chance of uneven sampling in different areas and the risks associated with fungal point surveys, whereby one fungal species may dominate over an area, providing an unrepresentative sample of the site. Fungal richness data were collected in preference to diversity data due to the difficulty of determining a fungal individual (Thompson & Rayner 1982; Dix & Webster 1995) and the resulting potential for confounding results if an abundance measure was incorporated.

Within each site, data were also collected regarding the average diameter at breast height (*dbh*) of trees in the site, average canopy cover, dominant tree species and pH as these are factors likely to be of particular importance to woodland fungal species presence/absence (Högberg *et al.* 2007, Kauserud *et al.* 2010)

Average *dbh* was measured using a diameter tape. Ten trees per site were selected for measurement and were spread across the geographic extent of each site. Canopy cover was measured using a canopy scope (Brown *et al.* 2000). Five canopy scope measurements were taken within each site and a mean calculated to provide a site measure. This was considered a sufficient replication size for these sites as they were not extensive and of relatively homogeneous canopy coverage, the standard errors of the measurements were small. Tree species identifications were carried out in the field, trees and shrubs encountered during the fungal foray were noted.

Five soil samples using a soil core were taken at each site. Soil pH was measured by dissolving 25g soil in 75ml tap water with added calcium chloride to remove impurities. This solution was used as the basis for a pH test using the pH/EC/TDS Waterproof Family meter (Hanna Instruments) which had been calibrated using a range of standard pH solutions.

Fungal Identification

Taxonomic assignations were made on the basis of ribosomal RNA gene sequences (rDNA) to overcome the ambiguities of morphological identification, particularly for cryptic forms such as cords. The growth form of each fungus was noted in the field to enable analysis of the time needed for cord-forming species to establish in newly planted woodlands.

Samples for DNA extraction and analysis were collected in the field in two ways. Sporocarp samples were collected on FTA cards (Whatman: Catalog#WB120411) and

extracted using Whatman FTA protocol BD02 (www.whatman.com). Smaller sporocarps, bracket fungi and mycelia were collected in bags containing silica gel. Dried samples were extracted using the MoBio UltraClean Soil DNA Isolation Kit (MoBio: Catalog#12800-100) using the included maximum yield protocol (www.mobio.com). The ITS region (ITS1, 5.8S and ITS2) of the DNA samples were PCR-amplified using the fungal-specific (biased towards basidiomycetes) primers ITS1F and ITS4B (Bruns *et al.* 1993; Jasalavich, Ostrofsky & Jellison 2000). Amplified samples were cleaned and Sanger-sequenced in the Sequencing Facility of the Natural History Museum, London. Sequence traces were manually edited in FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Taxonomic affiliation was determined on the basis of BLASTn searches of the ITS1-5.8S fragment (using a 90% similarity cut-off, Table S1) in GenBank (NCBI).

Data Analysis

Community similarity was analysed by non-metric multi-dimensional scaling (NMDS) based upon Euclidean distances (PAST V. 2.12, http://folk.uio.no/ohammer/past (Hammer 2001)). Shepard plots were used to compare plotted distances with those in the dissimilarity matrix Fungal species richness data were analysed via a series of generalised linear models with a Poisson distribution assumption and log link function (JMP version 10 SAS Institute Inc., Cary, NC) testing the relationships between the collected variables. Environmental variables were tested for autocorrelation using Wilcoxon tests or Pearson product moment correlation coefficient analysis (JMP version 10 SAS Institute Inc., Cary, NC). Autocorrelated variables were removed from the analysis to provide the most parsimonious model.

Creating the most parsimonious model

Autocorrelation in the variables included in the complete generalised linear model (*Fungal richness = Site + Age + pH + DBH + Canopy + Tree Species Richness*) was tested. The results of these analyses (Table 1) led to the omission of DBH, pH and Canopy from the most parsimonious model. Tree species richness was also omitted as the survey was incomplete. Therefore, the tree species list can inform general conclusions but is not sufficient for statistical analysis.

Variable 1	Variable 2	p-value	Test information	Test
Site	pH	0.0071	Degrees of freedom = 2	Wilcoxon
Site	Canopy	0.1246	Degrees of freedom = 2	Wilcoxon
Site	Diameter	0.1006	Degrees of freedom = 2	Wilcoxon
Canopy	Age	<0.0001	Correlation = - 0.8651	Pearson's product moment correlation
Canopy	Diameter	<0.0001	Correlation = +0.8128	Pearson's product moment correlation
Canopy	pH	0.2611	Correlation = 0.1952	Pearson's product moment correlation
Diameter	pH	0.4890	Correlation = 0.1209	Pearson's product moment correlation
Diameter	Age	<0.0001	Correlation = - 0.8337	Pearson's product moment correlation
Age	pH	0.4517	Correlation = - 0.1314	Pearson's product moment correlation

Table 1: *The results of two-way statistical tests to identify auto-correlation in variables. Shaded rows indicate pairings that are auto-correlated at p=0.05 or below and therefore which need one or both variables to be removed from the most parsimonious model.*

Results

Fungal communities and how they differ between sites of different ages

Our key finding is that established woodlands tend to exhibit similar fungal communities, which differ significantly from those found in newly created woodlands (<= 14 years since planting). Fungal community analysis by NMDS (Figure 2) with the 95% ellipse plotted, showed that, within the Heart of England Forest, planted woodlands were very similar in composition and only established woodland sites were significantly different, a result supported by ANOSIM analysis (p=0.191, 1000 randomisations, sample statistic = 0.242). A Shepard plot demonstrated that the NMDS was a good representation of the dissimilarity matrix. Variables are pooled by year of establishment, which leads to the presence of one point per year on the plot.

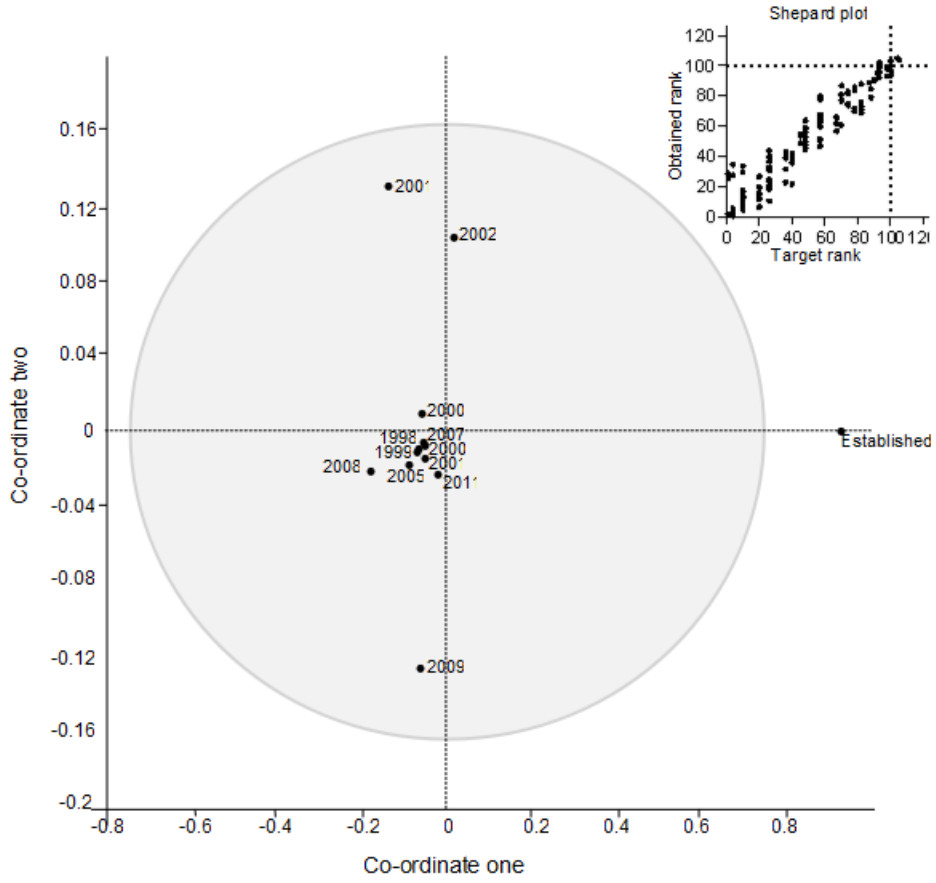

Figure 2 *Non-metric multi-dimensional scaling (NMDS) plot of differences in fungal community composition between sites planted in different years. A 95% probability ellipse (the region in which samples have significant similarity to the group mean fungal composition) has been identified on the plot by the grey circle. Different years are identified by labelled small solid circles. Shepard plot has been included top right and shows good fit to the NMDS approach (Stress=0.1245, R-square axis one = 0.7675, R-square axis two = 0.0815)*

Even 14 years after planting, fungal communities had not begun to approach the community composition of established woodlands. The difference in woodland fungal communities of established and planted areas are most strongly determined by the genera *Laccaria* and *Inocybe*, which seem to characterise newly planted woodlands according to SIMPER analyses (% contribution *Laccaria* = 18.9, % contribution *Inocybe* = 27.4). Re-

running the NMDS plot without established woodlands supported the conclusions that there were no significant differences in fungal communities between the planted sites.

The effect of woodland age and site on fungal species richness

Fungal species lists were curated to generate a list of woodland fungi, omitting grassland fungi. Woodland fungal richness was used as the response variable in the generalised linear model: *Fungi = Age + Site*. The model was significant (*P*= <0.0001, d.f. = 3, error d.f.=31) with both parameters (age and site) also exhibiting a significant association between themselves and woodland fungal richness (Figure 3).

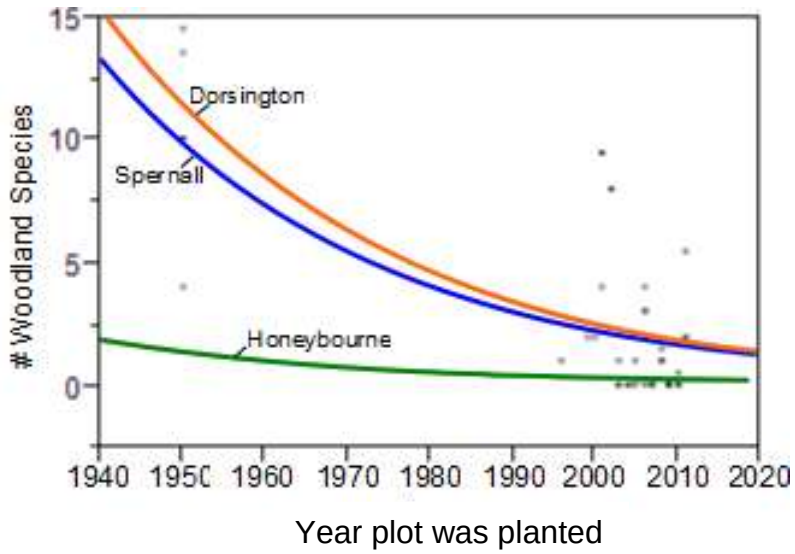

Figure 3 *Regression plot of generalised linear model with a Poisson distribution assumption and log link function. Model: # woodland species = Age + Site. Age is plotted against woodland species. Different sites are identified by different coloured lines on the plot with label attached. Whole model is significant (P=<0.0001, d.f.=3, error d.f.=31) as are the parameters Age (P= <0.0001, d.f. = 1,) and Site (P=0.0431, d.f. = 2)*

Age had a significant positive curvilinear association between the length of time a woodland had been established and the richness of woodland fungi therein (*P*= <0.0001, d.f. = 1). Site was significantly associated with fungal richness (*P*=0.0431, d.f. = 2).

Greatest fungal richness was documented in Spernall (14 species) with lowest richnesses observed in Honeybourne (0 to 3 species). Regression plots suggest that the woodland fungal community richness at the Dorsington and Spernall sites begin to increase rapidly fifty years after planting. In contrast, Honeybourne has not yet begun to display the rapid increase in fungal richness. Honeybourne was significantly different in fungal richness to the other two sites. This result is potentially a result of the increased disturbance associated with sites close to a town and which are in regular use for rough shooting, or due to the age profile around the area which is lower than that of the other two regions (Honeybourne=2005, Spernall=2001, Dorsington=1998).

Cord-forming fungi in newly planted woodlands

Cord-forming fungi were only found in one of the 35 sites. This was an established woodland site in the Spernall area which is not used for commercial activities such as rough shooting and which is not accessible to the public. The site showed no signs of active management and all dead wood appeared to be from natural limb fall. There was a deep litter layer in this woodland and the tree species mix included both coniferous and broadleaved species. Other established woodlands in this area showed evidence of extensive management (ride mowing, pheasant rearing and felling), were disturbed by commercial activities, or were situated next to intensively managed arable land. Other established woodlands included in this study were of similar establishment stages, therefore, the lack of CFF in the other two sites was interesting and future forays at the sites may need an increased sampling intensity to test this finding. These findings suggest that whilst some management practices can be beneficial, many high intensity woodland management practices such as deadwood clearance can drastically reduce fungal communities and fungal establishment in woodlands (Monk & Hemery 2013).

Discussion

Fungal communities in newly planted woodlands

Fungal species richness increases with time since woodland planting on previously non-wooded sites. The number of woodland specialist fungi is greater in established woodlands where the canopy is denser, the litter layer deeper, deadwood volume higher and the soil more alkaline and varies from site to site. Such effects are congruent with previous studies (Pouska, Svoboda & Lepsova 2010; Humphrey 2005) that identified that older woodland sites host higher species richnesses than newer sites, which contain deadwood of an earlier decay stage (Rolstad *et al.* 2004). However, these results are discordant with a 2001 study in which it was found that fungal species richnesses in hazel were lower in older stands (Nordén & Paltto 2001). It may be that the pattern of colonisation differs between mixed and monospecific stands and between tree-based and shrub-based habitat architectures. These results enhance current knowledge by demonstrating that the fungal communities that colonise woodlands newly planted on non-wooded land are not similar to fungal communities in established woodland, even fourteen years after planting.

One of the fungal groups considered most functionally important to British woodland ecosystems (Monk & Hemery 2013), the cord-forming fungi, are not prevalent in woodlands planted on old agricultural land. Cord-forming fungi from seven species were collected in only one of the 35 study sites. These fungi were of the species: *Megacollybia platyphylla, Resinicium bicolor, Mycena galopus, Armillaria, Hebeloma ammophilum, Laccaria laccata* and *Inocybe curvipes.* The majority of these species were from the order Agaricales and were all associated with coarse woody debris (CWD). The site at which the cord-formers were found (SP9) had dense canopy, little disturbance, a large amount of CWD (not quantified), and minimal evidence of woodland management. Cord-forming

fungi were not encountered in other established woodlands. It is likely that disturbances suppress the growth and establishment of fungal communities (Lindblad 1998; Bassler, Muller *et al.* 2012). In particular, the reduction in CWD associated with managed woodlands has been implicated in a regional loss of more than half of the saprotrophic fungal species within woodland (Siitonen, Penttila & Kotiranta 2001). Additionally, fine woody debris, also greater in unmanaged woodlands, is important for supporting fungal diversity (Kuffer & Senn-Irlet 2005), particularly that of ascomycete fungi (Norden *et al.* 2004).

Implications for managers of planted woodlands

Increasing the volume of deadwood in planted woodlands has the potential to encourage colonisation by a greater range and number of fungal species (Rolstad *et al.* 2004; Berglund *et al.* 2011; Blaser *et al.* 2013). However, care would have to be taken to avoid introducing potential invasive fungal species or pathogens to new woodlands (Monk & Hemery 2013). Higher levels of coarse woody debris in woodland will increase the rate of increase in fungal species richness as deadwood of a higher decomposition state hosts a greater richness of fungi of more complex community characteristics (Renvall 1995). Therefore, the authors suggest that fallen and standing deadwood should not be removed from regenerating woodlands to enhance the natural deadwood stocks and accelerate the rate of fungal recolonisation in replanted stands.

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Appendices

Appendix 1 *Geographical locations of study sites by field studied. As surveys were diffuse using a Rapid Fungal Survey approach, exact locations of fungi studied are not included.*

Site	Plot	Latitude	Longitude
Dorsington	1	52°9'31.99"N	1°49'47.53"W
Dorsington	2	52°9'27.18"N	1°49'34.77"W
Dorsington	3	52°9'35.40"N	1°49'21.62"W
Dorsington	4	52°9'25.83"N	1°49'7.55"W
Dorsington	5	52°8'59.57"N	1°49'15.16"W
Dorsington	6	52°8'48.41"N	1°48'56.99"W
Dorsington	7	52°8'53.43"N	1°47'41.03"W
Dorsington	8	52°8'17.53"N	1°47'41.05"W
Dorsington	9	52°8'2.18"N	1°47'54.86"W
Dorsington	10	52°8'16.00"N	1°48'21.42"W
Dorsington	11	52°8'39.11"N	1°49'23.41"W
Dorsington	12	52°9'17.39"N	1°49'7.77"W
Dorsington	13	52°8'36.02"N	1°49'23.07"W
Honeybourne	1	52°6'19.27"N	1°47'45.97"W
Honeybourne	2	52°6'6.88"N	1°47'53.07"W
Honeybourne	3	52°6'26.79"N	1°47'52.84"W
Honeybourne	4	52°6'24.70"N	1°49'21.23"W
Honeybourne	5	52°6'31.50"N	1°49'40.87"W
Honeybourne	6	52°6'21.00"N	1°49'47.01"W
Honeybourne	7	52°6'26.90"N	1°47'34.76"W
Spernall	1	52°15'19.80"N	1°52'52.46"W
Spernall	2	52°15'21.34"N	1°51'54.00"W
Spernall	3	52°15'3.40"N	1°51'26.71"W
Spernall	4	52°14'42.83"N	1°50'49.50"W
Spernall	5	52°14'48.03"N	1°50'34.02"W
Spernall	6	52°14'56.36"N	1°50'36.82"W
Spernall	7	52°15'6.58"N	1°49'44.37"W
Spernall	8	52°15'16.27"N	1°49'30.88"W
Spernall	9	52°15'41.13"N	1°51'32.96"W
Spernall	10	52°15'44.32"N	1°51'17.95"W
Spernall	11	52°14'56.05"N	1°51'37.22"W
Spernall	12	52°15'0.01"N	1°51'51.39"W
Spernall	13	52°15'3.18"N	1°52'8.12"W
Spernall	14	52°15'23.28"N	1°49'16.90"W
Spernall	15	52°15'29.60"N	1°49'7.42"W

CHAPTER FOUR

THE IMPACT OF CANOPY AND WOODY RESOURCE SPECIES UPON THE FUNGAL COMMUNITIES OF LOGS PLACED IN OXFORDSHIRE WOODLANDS.

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Declaration:

I confirm that the work contained in this chapter is wholly my own and that the contributions of the other authors to the paper constituted advice regarding experimental design and proof-reading the manuscript prior to submission. This paper has been submitted to 'The European Journal of Forest Research'.

Signed:

Kirsty Monk (DPhil candidate) Dr Nick Brown (Supervisor)

THE IMPACT OF CANOPY AND WOODY RESOURCE SPECIES UPON THE FUNGAL COMMUNITIES OF LOGS PLACED IN OXFORDSHIRE WOODLANDS.

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ABSTRACT

Saprotrophic fungi, and cord forming fungi in particular, are known to play an important role in woodland decomposition and nutrient cycling. Work to date has focussed on the physiology and inter-fungal behaviours of this group, whilst little is known about their interactions with the wider environment. We investigated the extent to which fungal community composition is determined by the composition of the woodland within which it is found. By using two sites in Oxfordshire, this paper considers the impact of dominant canopy species and the species of coarse woody debris on fungal richness and community composition. Such information will aid the holistic restoration of woodlands by providing

information regarding the specificity and establishment preferences of typical temperate woodland fungal species. The study finds that it is dominant canopy species which influences the fungal richness and presence of cord-forming fungi in an area. No significant effect of either dominant canopy or coarse woody debris species on the community composition was found, suggesting that establishment is dependent upon the local fungal species pool rather than micro-scale habitat features. Such findings suggest that by ensuring that sufficient debris is left on the forest floor, fungi will establish regardless of the species of debris or the canopy type.

Key Words

Cord-forming fungi, Fagus sylvatica, Quercus robur, Rhododendron ponticum, Acer pseudo-platanus, Picea sitchensis, resource specificity, woodland restoration

Introduction

Whilst it is known that saprotrophic fungi provide essential ecosystem services, maintaining soil fertility and carbon cycling (Dighton 2003, Molina *et al.* 1999), we know little about their ecology – particularly their relationships with plants and the environment. This is important information as we know that the composition and structure of UK woodlands is already in a period of rapid change (FAO 2011). Without this information we cannot predict the likely consequences of these changes for soil formation and carbon cycling.

Within the saprotrophic fungi, cord-forming fungi (CFF) are - an important woodland assemblage as a consequence of their ability to transport water and dissolved solutes across the soil litter interface (Boddy 1993). Work to date has focussed upon the physiology, phylogenetic relatedness and inter-fungal ecology of this group (Fricker, 2008). This study extends these investigations to include an examination of the resource specificity of CFF and the relative importance of the litter and coarse woody debris resource pools for determining fungal diversity. Increasing the current level of understanding of resource specificity will enable future work to address the issues of woodland enhancement and restoration. A consequence of introducing host and resource appropriate species should be that the negative downstream effects of introductions are minimised and trees can be planted with their fungal cohorts to speed up establishment and growth in a new setting.

The study considers the colonisation of deadwood and leaf litter by communities of saprotrophic fungi and the specificity with which this is done. In particular, this paper seeks to investigate how the species of dominant canopy tree and/or coarse woody debris influences the diversity and composition of saproptrophic fungal communities. This experiment will take the form of a transplant experiment using five wood types: *Quercus robur* L. (Oak); *Acer pseudoplatanus* L. (Sycamore); *Picea sitchensis* (Bong.) Carr. (Spruce); *Fagus sylvatica* L. (Beech); and *Rhododendron ponticum* L. (Rhododendron). Logs were also placed in grassland sites in Wytham as a control group. The fungal community characteristics of the logs and the plots within which logs were placed was then used to investigate the relative importance of the canopy and coarse woody debris (CWB) features and identities therein. This information is then used to consider the implications of such results for afforestation and reforestation efforts in temperate regions. By

understanding the resource specificity and requirements of ASNW fungal species, management practices will be better able to enhance and encourage the re-colonisation of planted stands by ASNW species.

We hypothesise that fungal communities will establish distinct communities on logs of different species and under canopies of different tree species as a consequence of the different chemical compositions and potential niches that they can provide.

Materials and Methods

Site selection

Bladon Woods, part of the Blenheim Palace estate; and Wytham Woods, owned and managed by the University of Oxford, were used as two independent sites for the study. Sites were selected for the range of woodland types they contained, accessibility and their similarity to one another in terms of species representation, proximity to built-up areas, and human induced disturbance.

Within each site a number of plots were identified in which sets of five logs were placed. Three plots of each canopy type, *F. sylvatica, Q. robur, R. ponticum, P. sitchensis, A. pseudoplatanus*, were identified at each site. Plots were selected to be of similar distance from the main pathways through the woodlands, under similar canopy cover and a suitable distance from setts, burrows or other potential sources of site disturbance. Plot locations were marked within the woodland to prevent disturbance by other researchers in Wytham

or rough shooting parties in Bladon, GPS co-ordinates were recorded for each plot location (Appendix 1).

Log placement

All logs used in the investigation were sourced from Wytham Woods; logs (40cm long, 10cm diameter) were split transversely. In February 2010 five logs, one of each study species, were laid out in a single line buried in the litter layer to 2 inches deep at each plot. The cut faces of the logs were all angled north (Figure 1) to ensure that the effects of wind direction and incident sunlight were minimised.

Figure 1 *Logs in situ at the Wytham Woods site. All logs are facing north and are buried 2 inches into the litter layer.*

The order of logs within the line was determined randomly, counteracting possible edge effects and minimising the sheltering effects of one log upon another. Logs were labelled with aluminium tags in the centre of the uppermost bark surface attached by 5cmx5mm aluminium nails (Figure 2). The logs were visited every six months to ensure they were still intact, to restore the log position in the event of disturbance (often by badgers), and to monitor the progress of fungal colonisation (without recording) so that we could determine an appropriate time to make the first enumeration, minimising the disturbance caused by recording.

Figure 2 *One beech log with an aluminium tag attached by an aluminium wire placed in a beech dominated canopy area.*

Data collection

Logs were revisited in May 2013 and all fungal species which had colonised the introduced logs were collected for later identification. Fungal species were collected in 2 ml Eppendorf tubes and preserved in a freezer at -20ºC. Only macro fungi were collected: macro fungi were taken to be those with visible sporocarps or cord systems on the surface of the log or on the soil in contact with the underside of the log.

All logs were left *in situ* for further collections at two year intervals into the future. Such collections will enable succession on fresh cut logs to be studied to complete decomposition of the woody resource.

A number of plots were lost due to inadvertent destruction by woodland management activity in the woodlands. Namely: two beech plots and three rhododendron plots from Wytham as a result of timber felling; and three oak plots from Bladon due to deadwood clearance.

Plot data was collected by means of a rapid fungal survey. This is based upon the Rapid Botanical Survey approach developed by Hawthorne and Abu-Juan (1995). The approach involves surveying a plot of indeterminate size with the search standardised by time spent searching as opposed to search area. Each plot was surveyed for one hour. The Rapid Fungal Survey approach may also be curtailed should no new collections be taken within 15 minutes of searching.

Fungal identification

Taxonomic assignations were carried out using DNA extraction methods to overcome the issues of cryptic speciation and unreliable identifications. Samples for DNA extraction and analysis were collected in the field in two ways. Sporocarp samples were collected on FTA cards (Whatman: Catalog#WB120411) and extracted using Whatman FTA protocol BD02 (www.whatman.com). Smaller sporocarps, bracket fungi and mycelia were collected in bags containing silica gel and frozen at -20°C. Dried samples were extracted using the MoBio UltraClean Soil DNA Isolation Kit (MoBio: Catalog#12800-100) using the included protocol (www.mobio.com).

The ITS region (ITS1, 5.8S and ITS2) of the DNA samples were PCR-amplified using the eukaryote-biased fungal-specific primers ITS1 and LR21. Amplified samples were then cleaned and Sanger-sequenced at the Sequencing Facility of the Natural History Museum, London. The resulting sequence traces were edited in FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Taxonomic affiliation was determined via BLASTn searches of the ITS1-5.8S fragment (using a 90% similarity cut-off, Table S2) in GenBank (NCBI) and added to the microclimate and plant identification data.

Data analysis

We used GLMs to identify factors affecting fungal species richness.

Fungal Richness = Site + Canopy_Species[Site] + Log_Species[Site] + Canopy_species*Log_species

Cord Richness = Site + Canopy_Species[Site] + Log_Species[Site] + Canopy_Species*Log_Species

Analyses were run using a Poisson distribution function assumption, a Maximum Likelihood expectation and a log link function.

As well as testing the influence of dominant canopy and coarse woody debris species on CFF richness: the effect of such factors on the presence or absence of CFF structures was investigated. A Nominal Logistic Fit test was carried out with 17 iterations; again using the JMP10 software package and the model:

CFF presence = Site + Canopy_Species[Site] + Log_Species[Site] + Canopy_species*Log_species

Differences in fungal community composition beneath different dominant canopy species both within sites and across both sites were examined using Analysis of Similarity (ANOSIM) and Non-metric Multi-Dimensional Scaling (NMDS) analyses. A 95% probability ellipse was applied to the Euclidean distance output of the NMDS analyses to identify species whose community composition was significantly different to that of other groups within the analysis. ANOSIM was executed using the CAP4 community statistics package (Pisces Conservation Ltd. 2007) whilst NMDS was carried out in the PAST 3 software (Hammer *et al.* 2001) using Euclidian distances. Further to these analyses, the CAP4 software package was used to carry out a SIMPER (Similarity percentage) analysis upon the datasets to identify the species which contributed most to any observed difference in the pooled data showing fungal community characteristic under stands of different dominant canopy species.

Subsequently, overall and within-site differences in community composition on and around the logs in a plot were investigated. A series of NMDS analyses based upon Euclidian distances were carried out. These analyses treated the data both as an overall pool across the two sites and then as two separate sites; investigating differences in background fungal community composition at the different plots in the analysis. These analyses used the data collected both from the logs and that collected using a rapid fungal survey approach (Chapter 3) from the surrounding area at the plot location. Similar to the between site

approach, a SIMPER analysis was used to investigate the species contributing to any differences observed.

Results

The effect of dominant canopy and woody substrate species upon fungal richness.

Fungal richness was significantly associated with the dominant canopy species (P=0.0049, χ^2=20.34, df=44). No other factors were shown to be significant in the model (Table 1).

Effect	Degrees of Freedom	χ^2 test statistic	P- value
Whole model	36	42.85	0.201
Canopy_species[Site]	7	2.034	0.005*
Log_species[Site]	8	7.53	0.480
Site	1	1.12	0.290
Canopy_species*Log_species	20	9.92	0.970

Table 1 *– Generalised linear model test statistics, P-values and degrees of freedom for the model: Fungal richness = Site + Canopy_species[Site] + Log_species[Site] + Canopy_species*Log_species. Asterisk in P-value column denotes a significant effect. The model was run using a Poisson approximation, log link and a Maximum Likelihood estimation.*

Figure 3 illustrates that logs placed beneath sycamore, beech and spruce dominated canopies tend to have higher fungal richnesses than those beneath oak and rhododendron canopies or those placed in grassland. A SIMPER analysis demonstrated that the sites showing the greatest difference from one another were Beech and Oak; Oak and Rhododendron; Beech and Grassland; and Grassland and Rhododendron. In all of the comparisons, *Armillaria* or *Radulomyces* were the species responsible for the greatest contribution to that difference (Table 2). In addition, Oak- and Grassland-dominated

communities appear more different to other communities in the sites with higher dissimilarity values. This difference is identified in the SIMPER output as as the result of lower than average *Armillaria* abundance in Oak woodlands, and higher than average *Radulomyces* abundance in grassland communities.

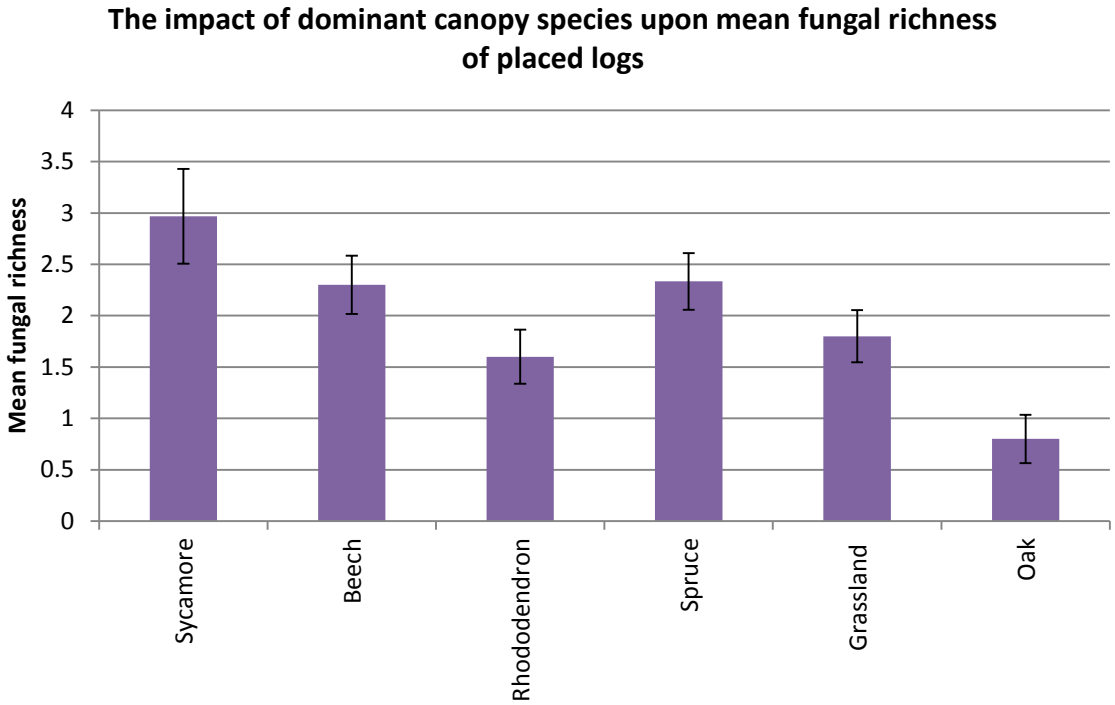

Figure 3 - *The impact of dominant canopy species upon fungal richness of logs placed in two Oxfordshire woodlands. Bars denote mean fungal richness found on logs placed in woodlands for two years and are accompanied with error bars displaying the standard error around the mean of each sample group.*

Species A	Species B	Dissimilarity	Fungal species (average abundance A, average abundance B, % contribution to dissimilarity)
Beech	Grassland	82.14	*Radulomyces* (0,2.66;29.9)
			Armillaria (2.25, 1.33; 17.2)
			Russula (0.75, 0; 6.7)
Beech	Oak	86.1	*Armillaria* (2.25, 1, 19.9)
			Trametes versicolor (0,1.33; 9.51)
			Radulomyces (0,0.67; 7.54)
Beech	Rhododendron	69.36	*Armillaria* (2.25, 1.33; 17.79)
			Xylaria hypoxylon (0.25, 0.67; 9.37)
			Russula (0.75, 0; 9.28)
Beech	Spruce	73.43	*Armillaria* (2.25, 1.83; 12.67)
			Radulomyces (0,1.17; 10.76)
			Phanerochaete sordida (0.5, 0.67; 10.76)
Beech	Sycamore	62.48	*Armillaria* (2.25, 2.67; 16.30)
			Phanerochaete sordida (0.5, 0.5; 8.53)
			Agrocybe praecox (0.5, 0.33; 8.12)
Grassland	Oak	75.74	*Radulomyces* (2.67, 0.67; 28.31)
			Armillaria (1.33, 1; 15.1)
			Trametes versicolor (0.67, 1.33; 19.90)
Grassland	Rhododendron	84.33	*Radulomyces* (2.67, 0; 37.02)
			Armillaria (1.33, 1.33; 13.33)
			Trametes versicolor (0.67, 0; 7.45)
Grassland	Spruce	73.57	*Radulomyces* (2.67, 1.17; 23.15)
			Armillaria (1.33, 1.83; 13.58)
			Phanerochaete sordida (0, 0.67; 6.45)
Grassland	Sycamore	79.83	*Radulomyces* (2.67, 0; 28.45)
			Armillaria (1.33, 2.67; 17.44)
			Trametes versicolor (0.67, 0.17; 6.54)
Oak	Rhododendron	91.4	*Armillaria* (1, 1.33; 15.81)
			Trametes versicolor (1.33, 0; 10.39)
			Radulomyces (0.67, 0; 9.70)
Oak	Spruce	75.06	*Armillaria* (1, 1.83; 14.87)
			Trametes versicolor (1.33, 0.17; 9.39)
			Radulomyces (0.67, 1.17; 8.63)
Oak	Sycamore	80.85	*Armillaria* (1, 2.67; 21.09)
			Trametes versicolor (1.33, 0.17; 9.46)
			Trechispora (0.67, 0.5; 8.82)
Rhododendron	Spruce	76.36	*Radulomyces* (0, 1.17; 12.26)
			Armillaria (1.33, 1.83; 11.76)
			Phanerochaete sordida (0.33, 0.67; 8.14)
Rhododendron	Sycamore	70.91	*Armillaria* (1.33, 2.67; 20.58)
			Xylaria hypoxylon (0.67, 0.33; 9.71)
			Phanerochaete sordida (0.33, 0.5; 8.55)
Spruce	Sycamore	71.21	*Armillaria* (1.83, 2.67; 12.50)

Species A	Species B	Dissimilarity	Fungal species (average abundance A, average abundance B, % contribution to dissimilarity)
			Radulomyces (1.17, 0; 10.43)
			Phanerochaete sordida (0.67, 0.5; 8.35)

Table 2 *SIMPER results comparing plots of different dominant canopy types. Species being compared are in the first two columns with their dissimilarity in column three. Column four contains the three fungal species which contribute most to the dissimilarity observed in order of their percentage contribution to that dissimilarity. Numbers in brackets are the average abundance of the fungal species in Plot A, the average abundance of the fungal species in Plot B and the percentage contribution of that species to the overall dissimilarity value.*

The effect of dominant canopy and woody substrate species upon CFF communities.

There was a significant effect of log species when interacting with dominant canopy species upon the presence or absence of CFF on any log in the study areas (*P*=0.0152, χ^2=36.05, df=20) (Table 3).

Effect	Degrees of Freedom	χ^2 test statistic	*P*- value
Whole model	36	55.07	0.0219*
Canopy_species[Site]	7	21.81	0.003*
Log_species[Site]	8	4.85	0.774
Site	1	$1.17e^{-5}$	0.997
Canopy_species*Log_species	20	36.05	0.015*

Table 3 – *Nominal logistic fit test statistics, P-values and degrees of freedom for the model: Cord presence = Site + Canopy_species[Site] + Log_species[Site] + Canopy_species*Log_species. Asterisks in P-value column denote a significant effect. The model was run with 17 iterations with a generalized R^2 of 0.49.*

However, there was no significant effect of site, log species or dominant canopy species upon the richness of CFF species across the study sites (Table 4).

Effect	Degrees of Freedom	χ^2 test statistic	*P*- value
Whole model	36	20.98	0.978
Canopy_species[Site]	7	6.64	0.468
Log_species[Site]	8	4.93	0.765
Site	1	0.39	0.534
Canopy_species*Log_species	20	10.24	0.964

Table 4 – *Generalised linear model test statistics, P-values and degrees of freedom for the model: Cord richness = Site + Canopy_species[Site] + Log_species[Site] + Canopy_species*Log_species. The model was run using a Poisson approximation, log link and a Maximum Likelihood estimation.*

The impact of dominant canopy and woody substrate species upon fungal community composition within and between sites.

A NMDS analysis of the fungal species found under each canopy type performed across both sites simultaneously demonstrated no significant deviation of any group from the norm in community composition between any of the plots at either site at the 95% significance level (Figure 4).

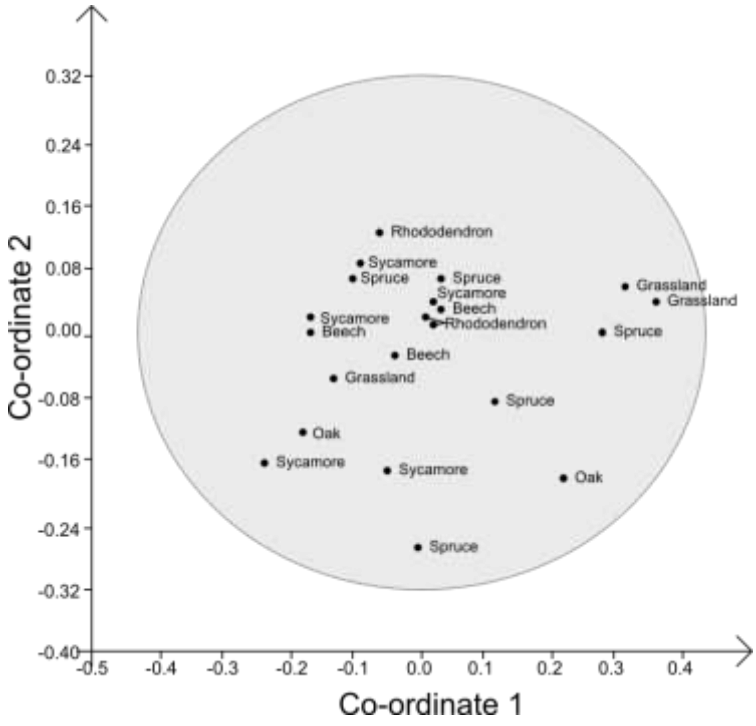

Figure 4 – *NMDS result for pooled information of all fungal species resulting from logs and rapid fungal surveys from Bladon and Wytham. Species identities relate to the dominant canopy species of each plot. The grey circle illustrates the 95% probability density ellipse. All study plots lie within the ellipse illustrating that no plot differs significantly from others in terms of its community composition at the 95% level of significance.*

However, an analysis of similiarity (ANOSIM) and an associated PCA showed Bladon and Wytham to vary significantly in their fungal community compositions (*P*=0.001, 1000 randomisations, >1000000 permutations) as visualised in Figures 5 and 6.

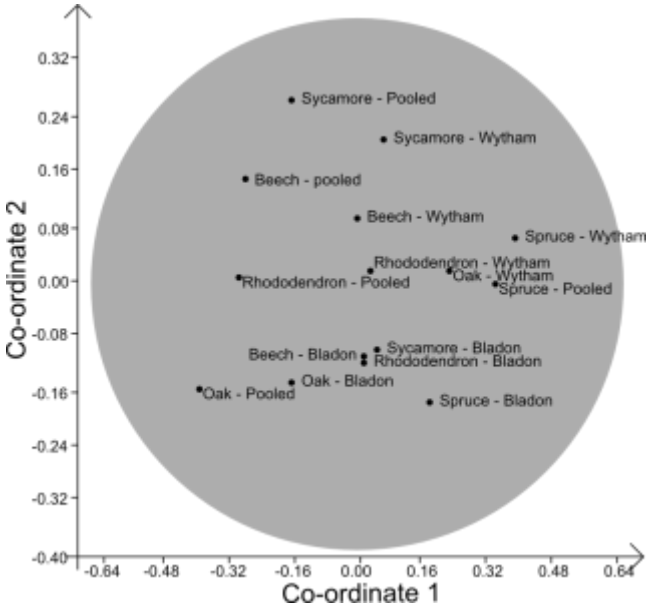

Figure 5 *– NMDS result for pooled information from logs alone from Bladon and Wytham. Species identities relate to the species of woody resource from which the fungi were taken. The grey circle illustrates the 95% probability density ellipse. All study plots lie within the ellipse illustrating that no plot differs significantly from others in community composition.*

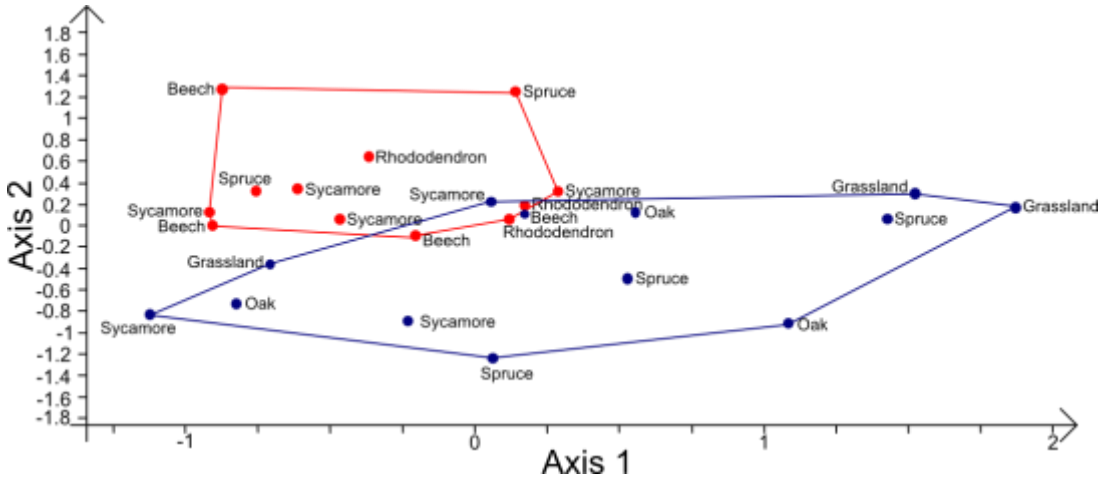

Figure 6 *– PCA plot showing differences in community composition of plots from Wytham (blue) and Bladon (red).* R^2*=91.24*

SIMPER analysis showed that the species contributing most to these differences were *Armillaria, Radulomyces, Trametes, Phanerochaete* and *Trechispora* with *Armillaria* and *Phanerochaete* being more abundant in Bladon and *Radulomyces, Trametes* and *Trechispora* more abundant in Wytham (Table 5).

As a result of these differences, NMDS analyses and an ANOSIM were carried out on both sites independently to test for effects which may have been masked by the differences at the site level. These analyses showed no significant difference in fungal community composition between any plots within the sites at the 95% significance level, supporting the previous findings from across the two sites (Figures 6 and 7).

Species	Average abundance (Bladon)	Average abundance (Wytham)	Average dissimilarity	% contribution
Armillaria spp.	2.33	1.46	12.0	14.9
Radulomyces spp.	0.08	1.23	10.4	12.9
Trechispora spp.	0.08	0.69	5.15	6.40
Phanerochete sordida	0.67	0.15	4.98	6.19
Trametes versicolor	0	0.62	4.00	4.97
Capronia fungicola	0	0.54	3.89	4.82
Phanerochaete carnosa	0	0.54	3.59	4.46
Xylaria hypoxylon	0.33	0.23	3.37	4.18
Phallus impudicus	0.42	0	3.25	4.03
Agrocybe praecox	0.33	0	2.45	3.04
Stereum hirsutum	0.25	0.08	2.45	3.04
Peniophora aurantiaca	0.17	0.23	2.44	3.03
Amylostereum chailletii	0	0.38	2.33	2.89
Mutinus caninus	0	0.31	1.98	2.46
Datronia mollis	0	0.23	1.87	2.32
Hyphoderma setigerum	0.25	0	1.83	2.28
Russula spp.	0.25	0	1.66	2.06
Polyporus gayanus	0.17	0.08	1.56	1.94
Ceratobasidium spp.	0.017	0	1.42	1.76
Scleroderma areolatum	0.17	0	1.42	1.76
Trimmatostroma betulinum	0	0.23	1.41	1.75

***Table 5** – SIMPER results comparing the fungal communities of Bladon and Wytham, species are listed in the table in order of their percentage contribution to the dissimilarity between the composition of the fungal communities at Bladon and Wytham.*

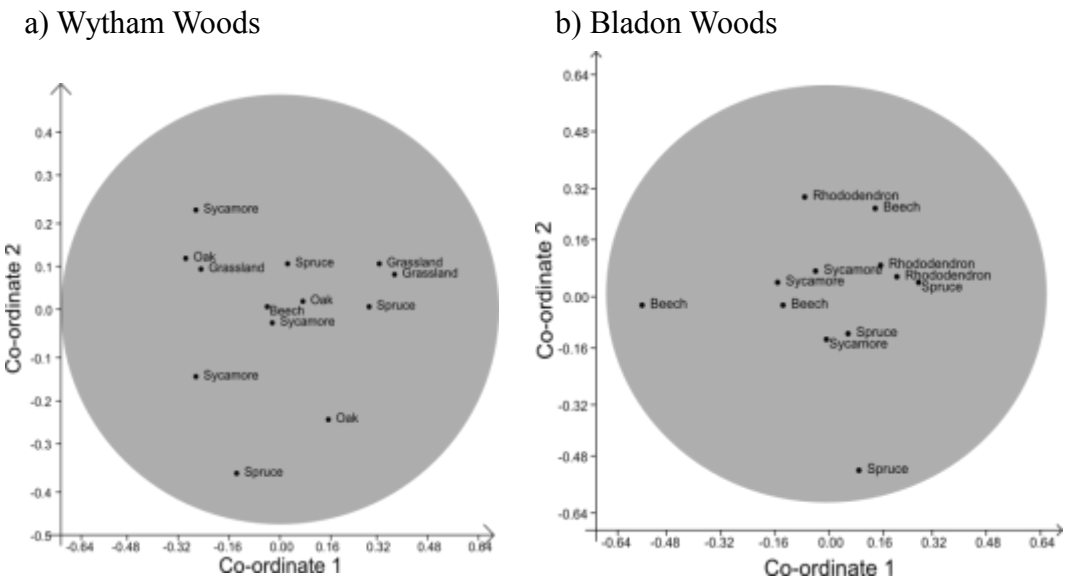

Figure 7 *– NMDS results illustrating the effect of dominant canopy species separated by plot (Wytham or Bladon). Species identities relate to the species of dominant canopy species at the site. The grey circle illustrates the 95% probability density ellipse. All study plots lie within the ellipse illustrating that no plot differs significantly from others in terms of its community composition at the 95% level of significance.*

Discussion

Fungal dynamics in newly planted woodlands are governed by a range of factors both expected and unexpected. The number of fungal species colonising a log placed in a wooded stand is associated with the tree species dominating the canopy. This may be a result of different humidity levels in woodlands caused by different densities of canopy cover. It may also be a consequence of the different strategies which need to be employed to digest different leaf litter types.

The SIMPER analysis carried out on the plots to compare the fungal community make-up under different dominant canopy species suggests that the communities tend to be determined by either the presence of *Armillaria* in the more native dominated stands, or *Radulomyces* in the grassland and rhododendron sites. This is likely to be a consequence of the surrounding fungal species pool which has adapted for the different challenges associated with the litter layer in these environments. Interestingly, when the native vs. non-native effect of dominant canopy species on fungal community composition is considered, the results obtained are in stark contrast to previous work on insect diversity by Southwood in 1961. Southwood found that insect diversity was lower on the exotic coniferous species and higher in oak dominated woodlands. However, in this study, it was found that the diversity of fungi was higher in the coniferous woodlands compared to the oak-dominated woodlands. Rhododendron stands have very close canopies and highly acidic litter layers. This means that they are often considered inhospitable to fungi. However, this is refuted in work considering the regeneration of grassland fungi after rhododendron clearance on Lundy Island, Bristol Channel (Monk *et al.* 2011) which found that many ASNW fungal species including cord-forming fungi were associated with the Rhododendron patches due to the coarse woody debris it provided. This provides further evidence to suggest that many ASNW fungal species are generalists and do not exhibit strong preferences for particular species of woody resource if the growth conditions are right. In contrast, grassland, a much less humid environment with a poorly developed litter layer, was dominated by *Radulomyces* which appeared to be being cultivated on the underside of the logs by ants.

Conclusion

Fungal communities are, above all else, determined by the fungal species pool surrounding the area in which they establish. Whilst the dominant canopy species is an important component of the woodland with regards the fungal species richness on coarse woody debris, it does not have the capacity to override the effect of the background species pool, only to bring with it and encourage additional species which may be better suited to decomposing the fine woody debris and leaf litter associated with that tree species.

This knowledge supports existing knowledge regarding fungal establishment rates in newly planted woodlands (Chapter 3) to inform woodland management practices, suggesting that it is the presence of deadwood as opposed to the species identity or provenance of that wood which is important for the establishment of area specific fungal species. Therefore, in afforestation and reforestation efforts, the background fungal communities should be considered and, if necessary, enhanced to encourage the establishment of fungi typical of ancient semi-natural temperate woodland. This becomes increasingly important when the functional diversity of the fungal communities is considered. The lower diversity of communities under stands such those dominated by Rhododendron and the reduced functional diversity therein could be considered an indicator of lower resilience to future environmental change. It is vital to consider the diverse components of woodlands, their ecosystem roles and the functional diversity they represent if we are to maintain the health and ecosystem function of British woodlands now and in an uncertain future.

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Appendices

Appendix 1 GPS locations of sites in Wytham and Bladon. The Rhododendron plots for Wytham have been removed as the site was never recovered.

Site	Dominant Canopy Species	Site Code	Latitude:Longitude
Wytham	Oak	L1	N51.777:W1.337
Wytham	Oak	L2	N51.777:W1.340
Wytham	Oak	L3	N51.778:W1.341
Wytham	Beech	L4	N51.768:W1.337
Wytham	Beech	L5	N51.766:W1.337
Wytham	Beech	L6	N51.767:W1.336
Wytham	Sycamore	L7	N51.774:W1.325
Wytham	Sycamore	L8	N51.776:W1.327
Wytham	Sycamore	L9	N51.774:W1.328
Wytham	Spruce	L10	N51.763:W1.320
Wytham	Spruce	L11	N51.764:W1.319
Wytham	Spruce	L12	N51.764:W1.318
Wytham	Grassland	L16	N51.764:W1.324
Wytham	Grassland	L17	N51.767:W1.328
Wytham	Grassland	L18	N51.767:W1.327
Bladon	Oak	001	N 51.822:W 1.343
Bladon	Oak	002	N 51.822:W 1.343
Bladon	Oak	003	N 51.822:W 1.343
Bladon	Sycamore	004	N 51.821:W 1.341
Bladon	Sycamore	005	N 51.821:W 1.341
Bladon	Sycamore	006	N 51.821:W 1.339
Bladon	Beech	007	N 51.821:W 1.341
Bladon	Beech	008	N 51.821:W 1.341
Bladon	Beech	009	N 51.821:W 1.341
Bladon	Spruce	010	N 51.821:W 1.337
Bladon	Spruce	011	N 51.822:W 1.337
Bladon	Spruce	012	N 51.822:W 1.336
Bladon	Rhododendron	013	N 51.822:W 1.344
Bladon	Rhododendron	014	N 51.822:W 1.344
Bladon	Rhododendron	015	N 51.822:W 1.344
Bladon	Rhododendron	016	N51.823:W 1.341
Bladon	Rhododendron	017	N51.824:W 1.341

CHAPTER FIVE

POST-CLEARANCE EFFECTS OF RHODODENDRON ON THE FUNGAL COMMUNITIES OF THE EASTERN SIDELANDS OF LUNDY, BRISTOL CHANNEL.

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Declaration:

I confirm that the work contained in this chapter is wholly my own and that the contributions of the other authors to the paper constituted advice regarding experimental design and proof-reading the manuscript prior to submission. This paper has been published in 'The Journal of the Lundy Field Society' (2014).

Signed:

Kirsty Monk (DPhil candidate) Dr Nick Brown (Supervisor)

POST-CLEARANCE EFFECTS OF RHODODENDRON ON THE FUNGAL COMMUNITIES OF THE EASTERN SIDE LANDS OF LUNDY, BRISTOL CHANNEL.

by

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ABSTRACT

Rhododendron ponticum is a constant problem on Lundy. We present the results of an experiment investigating the lasting legacy of *Rhododendron* following clearance and the time needed for natural regeneration of the plants and fungi in cleared areas. These results are discussed with particular reference to their implications for management and clearance on Lundy and in other areas in the future.

Key Words: *Rhododendron, fungi, acidification, regeneration, restoration, soils, time-series*

INTRODUCTION

The issue

Rhododendron ponticum L. is a rapidly spreading invasive shrub, introduced to the UK for its aesthetic beauty. The shrub has a tendency to spread outside of an introduction site and into adjacent ancient semi-natural woodland and grassland communities. As a result of many features of its biology, such as acidic leaf litter, dense canopy cover and allelopathic foliage; it has the capacity to alter the environmental conditions and the associated vegetation (Cronk *et al*., 1995). In particularly sensitive areas in Britain, efforts are being made to manage the spread of the shrub and/or eradicate it (Dehnen-Schmutz *et al*., 2004; Edwards, 2006). Lundy is one such area and efforts to eradicate the shrub are proving successful. Here we investigate the aftermath of *Rhododendron* colonisation on fungal communities and attempt to identify the timescale involved in recovery of the native flora and mycota.

Rhododendron as an invasive species

The high colonisation potential of *R. ponticum* is a result of the great number of seeds that it produces. Every flower head produces many thousands of seeds, which are efficiently dispersed by the wind. These swamp the seed bank for kilometres around the plant and cause it to out-compete the less abundant seeds and seedlings of native species (Stout, 2007).

In addition, the leaf litter of *R. ponticum* is highly acidic, similar to those of another ornamental shrubs also belonging to the genus, the azalea (*Rhododendron* subgen *Tsutsuji* L.). As *R. ponticum* cover increases and its leaves begin to dominate the litter layer, the soil becomes altered in a way that makes it unsuitable for other species. *R. ponticum* is evergreen, so it sheds and regrows its leaves all year-round exerting a constant stress on the

ground vegetation beneath the stand (Cronk *et al*., 1995). This stress comes in two forms. Firstly, the presence of a thick evergreen shrub layer leads to reduced light levels, suppressing ground vegetation through light limitation. Secondly, the year-round supply of new leaves shed from the shrub leads to continuous addition of acidic leaf litter to the soil.

Rhododendron and Lundy

Rhododendron ponticum was first introduced to Lundy in the Millcombe valley in the early Nineteenth Century (Marren, 1971) from where it began to spread beyond the areas where it was originally planted (Charter, 1877). In 1926, a wildfire cleared several large areas on the eastern side of the island providing a bare post-fire habitat, well suited for colonisation by *R. ponticum*. Once this fire had passed, the native vegetation was unable to regenerate as rapidly as *R. ponticum* which is adapted to fire-dominated landscapes such as those of its natural range in the Northern Mediterranean (Tabbush & Williamson, 1987; Compton, 1998).

The ideal conditions for *R. ponticum* colonisation, unfortunately, closely resemble those necessary for the healthy continuation of the Lundy Cabbage (*Coincya wrightii* O. E. Schulz), an endemic plant with its own associated fauna including the Lundy cabbage weevil (*Ceutorhynchus contractus pallipes* Crotch), the cabbage stem beetle (*Psyllioides chrysocephala* L.) and the Lundy cabbage flea beetle (*Psylliodes luridipennis* Kutschera, 1864).

The destructive potential of *R. ponticum* on the flora of Lundy was recognised in the 1940s when the then owner, Martin Coles Harman approached the Lundy Field Society to request the implementation of *Rhododendron* control measures (Harman, 1950). Early attempts to control and reduce the spread of the shrub were unsuccessful due to regrowth from cut stumps, a result of the failure to apply herbicide to the cut surface.

In 1998, more concerted control and clearance efforts began with support from English Nature and a volunteer workforce. Eradication methods involved co-ordinated action of both staff and volunteers clearing the thickets and treating cut stumps with herbicide to kill the rootstocks (Compton, 1998). Twenty years on, these efforts have had a marked effect on the landscape, with the almost complete eradication of *Rhododendron* from the Eastern Sidelands.

Predicted impacts of *Rhododendron* colonisation

The acidic effect of *Rhododendron* informed the hypotheses of this study regarding fungal community responses following clearance of *R. ponticum* (Figure 1).

We hypothesise that acidification of the soil will have a similar effect to the application of fungicide or herbicide, reducing the number of species capable of surviving in such an extreme environment, selecting for species able to cope with such hostile conditions.

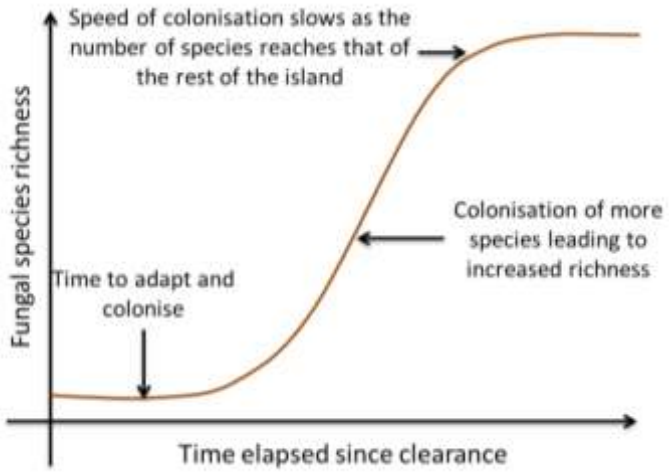

Figure 1: The predicted effects of time since clearance on fungal communities of cleared *Rhododendron* stands.

Once the impact of acidic leaf fall is reduced or removed altogether (by removing the plant) the community would be expected to follow the pattern in Figure 1. Initially, there would be residual effects of the acidity on the soil. This means that existing saprotrophic

fungi associated with the *Rhododendron* resource would take time to return soil pH to a level at which native fungi can colonise. When fungi begin to colonise, they have a stabilising effect upon the soil and bring the acidity closer to that of the non-colonised areas allowing more species of both fungi and plants to recolonise and grow in the area as fungal diversity is closely linked to plant diversity. This is shown in Figure 1 by the curve of increasing steepness over time. However, this rate of increasing species richness (the number of species in a defined area) over time cannot be sustained. Eventually one of two things may happen. Firstly, there may be insufficient resources in the soil, such as nutrients, water or space, to support any additional fungi so the number of new species will tail off. Alternatively, the fungal community may reach the point whereby it has representative species from all of the fungal species present on the island due to the absence of inoculum of particular fungi in the vicinity. Such an effect is likely to be exacerbated in a small island habitat and may cause the plateau to be less pronounced as new spore migrations occur across the Bristol Channel from North Devon and South Wales.

Relevance of the study

Lundy is rich in fungi and is of particular importance for its high diversity of waxcaps (*Hygrocybe* spp.) (Hedger, 2010). These fungi are highly sensitive to the fertilisation and ploughing of soils; and thus are found only on unimproved grassland soils (Griffith *et al.*, 2002). As a result of agricultural intensification, waxcaps have become a conservation concern in the United Kingdom. Of all *Hygrocybe* species, 89% appear on IUCN red lists within Europe (Arnolds, 1993).

Understanding the soil processes and regeneration patterns on cleared land will enable the Eastern Sidelands to be managed for such rare species to encourage their persistence and reduce the risk of their loss through persisting effects of acidification on the island.

METHODS AND MATERIALS

Site Selection

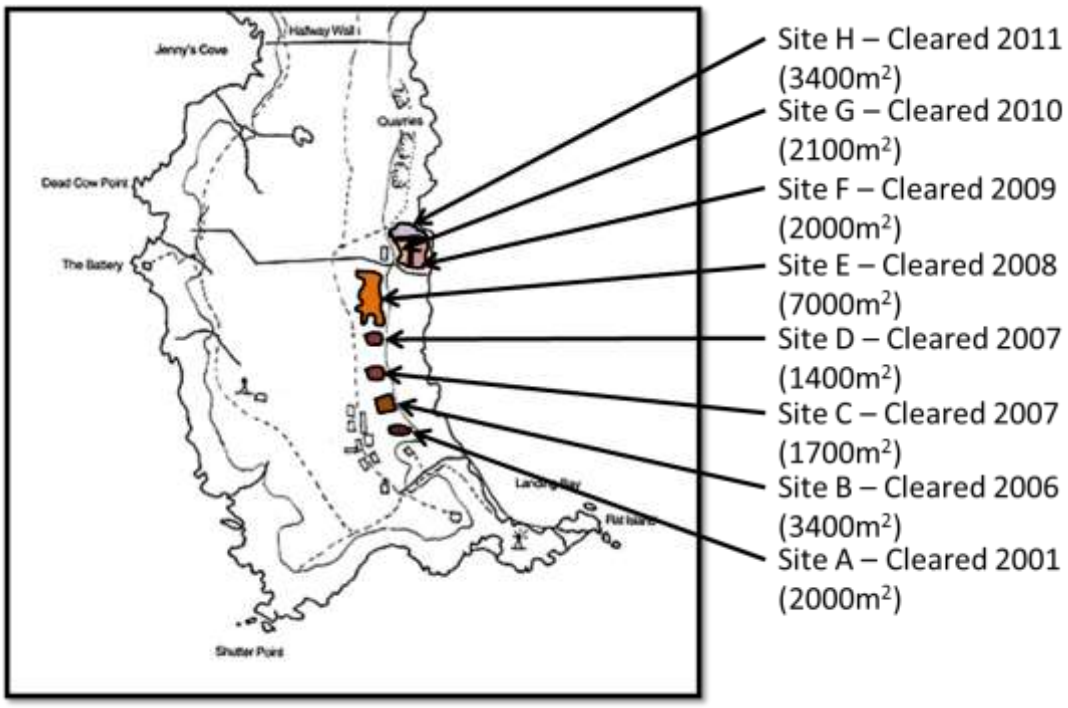

Figure 2: Location of fieldwork sites on the Eastern Sidelands of Lundy, Bristol Channel. Labels indicate the site identification; the year in which the site was cleared and the approximate area. Site locations were identified in collaboration with the Lundy Ranger, Steve Pratt.

A map of the history of *R. ponticum* clearance on Lundy over the past 12 years was obtained from Mr Steve Pratt (Lundy Ranger). One of the unique aspects of Lundy as a study site is the detailed records and maps available describing and recording eradication efforts undertaken since the 1990s, instigated by English Nature (now Natural England) in 1998 (Compton, 1998). This map was used to create a sampling strategy incorporating the year of clearance as a stratified variable and controlling for other environmental variables such as aspect, distance from village and slope (Figure 2).

Lundy provides ideal conditions for a controlled experiment. All the *R. ponticum* stands were located on the Eastern Side lands and were sited on similar soils, and exposed to similar wind conditions, salt spray and temperature variation.

Field Measurements

Five quadrats were located within each of the eight sites (labelled A-H; see Figure 2). $1m^2$ quadrats were placed at positions selected by means of a random walk using the most south-easterly point of the site as the origin. A random walk uses a list of random numbers to determine the steps taken along and up a grid from a pre-determined origin to cover the area in an unbiased manner. Within each quadrat, the overall number of plant and fungal species, and their identities, were recorded. Plant identifications were carried out in the field whilst samples of each discrete fungal species were collected for DNA-barcode based identification at a later date.

Microclimate information was collected for each quadrat. Recorded variables were: aspect; slope; deadwood diameters; and soil pH. Soil pH was measured by dissolving 25g soil in 75ml tap water with added calcium chloride to remove impurities. This solution was then used as the basis for a pH test using the pH/EC/TDS Waterproof Family meter (Hanna Instruments) which had been calibrated using a range of standard pH solutions. Soil cores were taken to a depth of 10cm where possible or to the rocky substrate is this was shallower. The soil cores were homogenised prior to removal of the 25g sample.

Fungal identification

Fungal identifications were carried out using DNA extraction methods to overcome the issues of cryptic speciation and unreliable identifications (Dentinger *et al.*, 2010).

Samples for DNA extraction and analysis were collected in the field in two ways. Sporocarp samples were collected on FTA cards (Whatman PLC: Catalog#WB120411) and extracted using Whatman FTA protocol BD02 (www.whatman.com). Smaller sporocarps, bracket fungi and mycelia were collected in bags containing silica gel. Dried samples were extracted using the MoBio UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, California: Catalog#12800-100) using the included protocol (www.mobio.com).

The ITS region (ITS1, 5.8S and ITS2) of the DNA samples were amplified using PCR and the specific primers ITS1 and LR21. Amplified samples were then sequenced using the facility at the Natural History Museum, London and edited in FinchTV version 1.4.0 (Geospiza Inc.). Species identities were obtained via reverse BLAST searches on GenBank (Benson *et al.*, 2007; Benson *et al.*, 2010) and added to the microclimate and plant identification data.

Data analysis

The parameter, fungal richness, was chosen for analysis as it circumvents many of the issues inherent in the use of biodiversity indices for fungal analysis. Fungal richness refers to the number of unique fungal species within a given unit area. In these analyses fungal richness is either by site (across all five quadrats), as in the Wilcoxon test; or an average value of all of the sites cleared in the same year, as in the community bar graph.

We tested for a relationship between fungal richness at each site and environmental variables using a generalized linear model (JMP version 10 SAS Institute Inc., Cary, NC). Soil pH was transformed from a logarithmic to a linear variable and therefore represents the concentration of hydrogen ions in the soil, a more direct measure of acidity. A higher concentration of hydrogen ions ([H+]) represents higher acidity and thus a lower pH, the therefore [H+] will be used throughout the results and discussion. A Wilcoxon test (JMP

version 10 SAS Institute Inc., Cary, NC) was used to test for a relationship between soil acidity and time since clearance.

RESULTS

Regeneration of fungal species on cleared *R. ponticum* stands is variable. Some sites display high levels of fungal richness which seems independent of age. Fungal species mixes were different in more recently cleared sites, a consequence of the resources available at a site. Grassland specific fungi were not observed in any sites illustrating that the process of restoration and regeneration is incomplete. Plant communities had also yet to stabilise although this was not explicitly analysed in this investigation.

Factors associated with fungal richness

Visually the eight sample sites were very different both in terms of the vegetation present and the macrofungal occurrences (Plate 1). Sites which had been cleared longer had greater vegetation cover whilst patterns in the fungal morphotypes present were not clear cut. There was no significant effect of the site area and the fungal richness within the quadrats selected therein.

Plate 1: Photographs illustrating variations in plant communities in quadrats at different times since clearance. In each photograph a metre square quadrat is apparent for scale. Year of clearance is displayed in the top left of each photograph.

A generalized linear model with a Poisson distribution assumption was run using all microclimatic variables, separated by site, and their effect upon fungal richness (the number of different fungal species in a quadrat). Table 1 shows the results of this model. P-values of less than 0.05 indicate a significant association between the factor and fungal richness in the study sites on Lundy. The overall model was not significant (p=0.0512, $d.f.$ =5, 39) indicating that more variables and a greater intensity of sampling may be required in future field seasons, however, some individual variable effects were significant.

Factor	Degrees Of Freedom	Chi-Square	P-Value
Year of Clearance	1	0.0025038	0.9601
H+ Concentration	1	5.9683075	0.0146
Average Deadwood Diameter	1	2.3666393	0.1240
Slope	1	1.0614588	0.3029
Aspect	1	0.518348	0.4715
Model (Difference)	5	11.0081	0.0512

Table 1: Results of a generalized linear model investigating the associations between multiple factors and fungal richness on Lundy, Bristol Channel. P-values of less than 0.05 indicate a significant association.

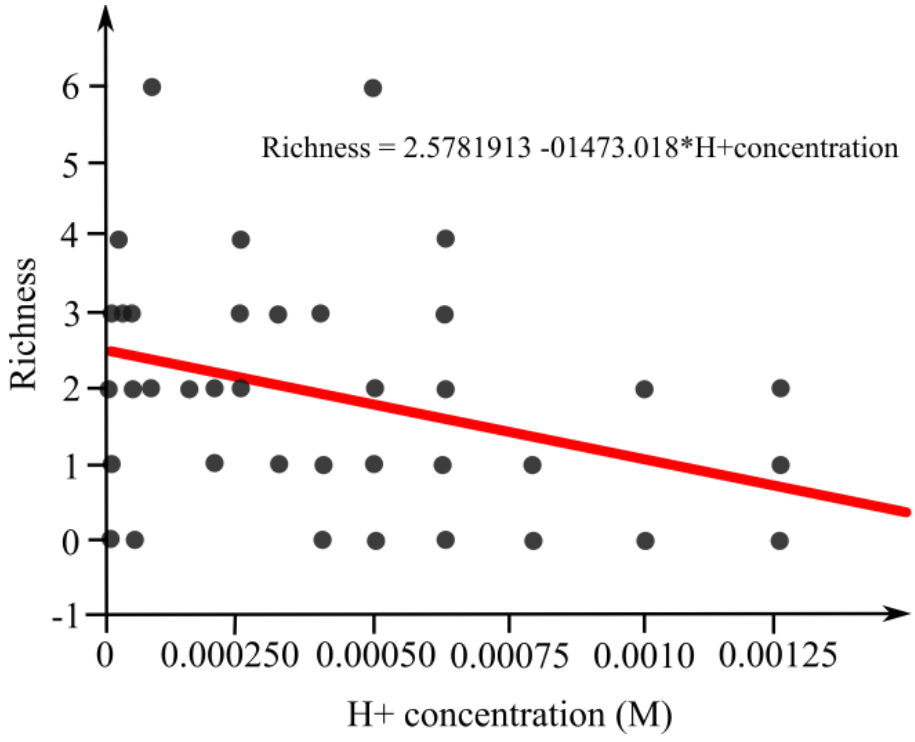

Figure 3: Scatterplot showing the association between acidity (concentration of hydrogen ions) and fungal richness. A line of best fit has been added (red line) and the correlation is significant (p= 0.0020, Spearman's rho = -0.4478).

Table 1 shows a significant association between fungal richness and acidity (p=0.0146, *d.f.* =1) although there is no support for an association between year of clearance and fungal richness (p = 0.9601, *d.f.* = 1). A scatterplot (Figure 3) visualising this effect shows that fungal richness decreases with increasing acidity. A Spearman's rank correlation testing this association was also significant (p= 0.0020, Spearman's rho= -0.4478).

No significant interaction was found between [H+] concentration and the year of clearance so an interaction term and was therefore not included in the final model. The analysis (Table 1), using the generalized linear model, demonstrated no significant associations between any of the other factors included in the model although further work with a greater sample size would be useful in determining whether deadwood diameter or volume was an important variable in determining the richness of fungi in a site as the *p*-value is around 0.1 (p=0.1240, *d.f.* =1).

Links between soil pH and time since clearance

pH did not follow the trend we had anticipated. Acidity increased in the time following *R. ponticum* removal; sites which had been bare for longer displayed much lower pH soils than more newly cleared areas.

As discussed, soil acidity is significantly associated with fungal richness. It is assumed that time since clearance would have an impact upon soil pH as the breakdown of acidic leaf litter by colonising organisms leads to a reduction in acidity (an increase in pH). Across the sites, a pH range of 2.9 – 6.2 units was observed.

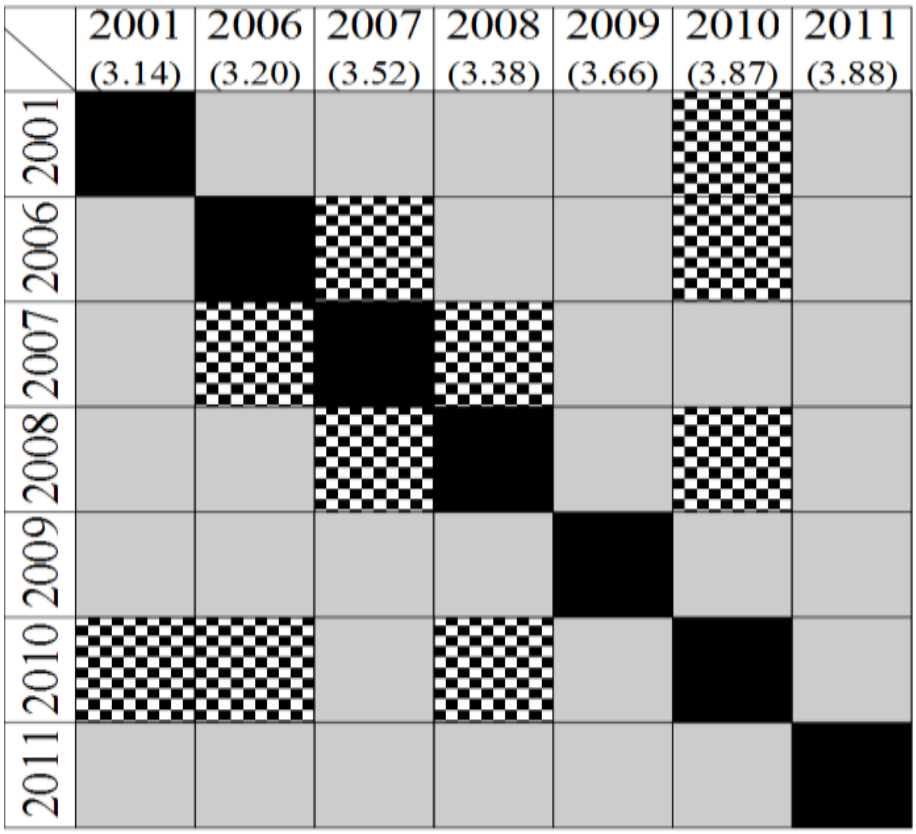

Figure 4: Matrix illustrating the pairwise Wilcoxon test results by year cleared. Black squares indicate a non-relevant test, pale grey squares indicate a non-significant result ($p>0.05$) and chequerboard hatched squares indicate a significant difference ($p<0.05$) between the acidities of soils in the two groups observed (row year and column year). Mean pH is indicated in brackets beneath the year of clearance

Again, the inverse logarithm of pH was used to ensure that the relationship between acidity (concentration of hydrogen ions ([H+])) and the year of clearance was tested. Since [H+] was not normally distributed, and could not be transformed to fit a normal distribution, a Wilcoxon test was used to analyse the data. The model was highly significant (p=0.0305, *d.f.* =6). A Wilcoxon non-parametric comparison for each pair was carried out and the results are summarised in Figure 4. The pairwise comparisons do not wholly support the hypothesis however this is a consequence of the large range of values observed in the 2011 plots (Figure 5). This stark difference is potentially a consequence of the increased exposure of the 2011 site, which was on a cliff edge, to salt spray. When the 2011 plots

were removed from the analysis, the trend of decreasing pH with age is consistent across years and the overall significance is increased (p=0.0086, $d.f.$ =5). The significant differences observed between the values towards the centre of the range of years are indicative of soil processes working to alter the acidity.

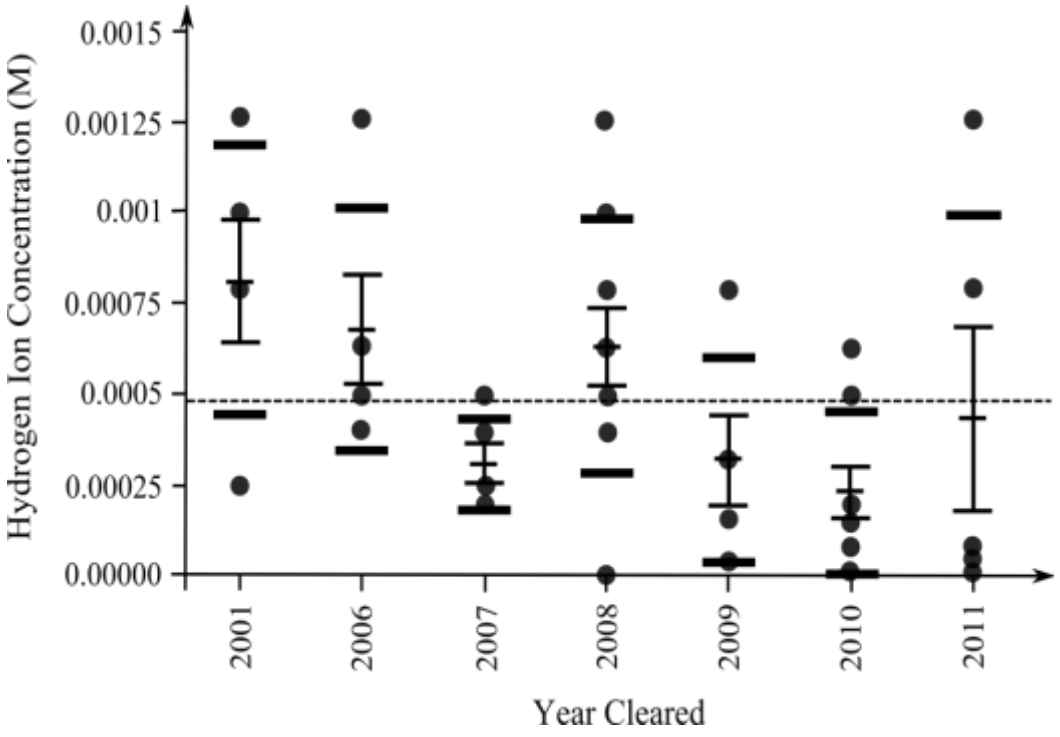

Figure 5: Plot of year cleared against soil acidity (hydrogen ion concentration). Filled circles indicate raw data, thin lines show the mean value and error bar for each year. Thick lines show two standard deviations around the mean. The dashed line represents the grand mean of the dataset. Hydrogen ion concentration has been presented to reflect the results discussed in the text.

A graphical representation of the trend shows that it functions in the opposite direction to that predicted (Figure 5). A Spearman's rank correlation test on the data was significant (p= 0.0027, Spearman's rho = -0.4368). Control sites which had never been colonised by *Rhododendron* were not included in the analysis as a year of clearance could not be reliably entered, skewing the results, but demonstrated a natural pH of 5 ([H+] of

0.00001M). Therefore, even 11 years after clearance, soil pH had not begun to return to levels equivalent to those which had never experienced colonisation by *R. ponticum*.

Successional patterns in species occurrence

The study aimed to produce accurate fungal identifications from DNA samples, a relatively novel approach to ecological investigations of fungi. DNA identifications were cross referenced with morphological identifications carried out in the field to validate the method. Such activities enabled the investigation of successional patterns in species occurrence.

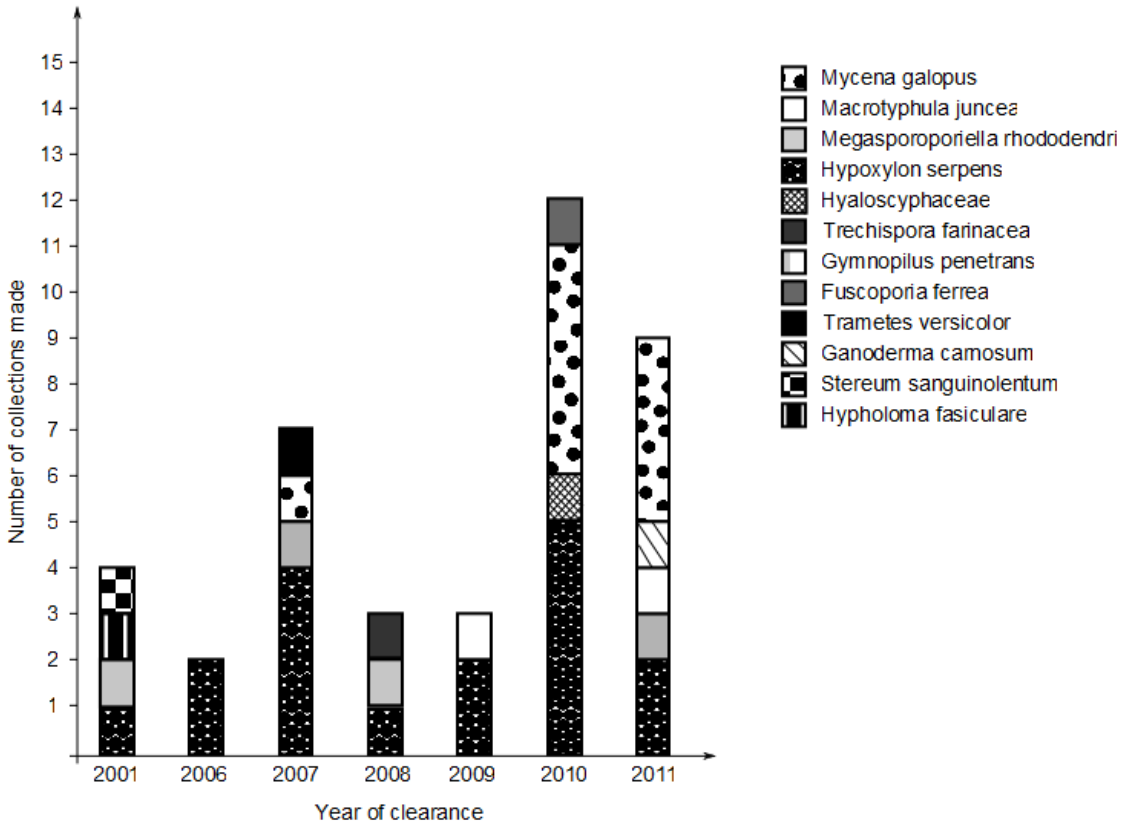

Figure 6: Bar chart illustrating the fungal species present in sites at different times elapsed since clearance. All fungal species named are associated with woodland sites and are found on leaf litter and woody debris. Species were identified from DNA specimens, of the ITS1-5.8S-ITS2 region, collected from fruit bodies and other fungal macrostructures in the field and amplified in the lab. Bacterial species were omitted from the results.

This study seems to have captured the very beginning of the succession from saprotrophic, wood-decaying species to the grassland species typical of the island. The fungal richness declines sharply two years after clearance (Figure 6). Longer term studies and return surveying efforts in the future will capture the succession in progress providing quantitative information regarding regeneration on acidified land.

The addition of a survey of soil fungal species and those without easily observable and collectable fungal structures will enhance this aspect of the study still further adding greater breadth and depth to the survey. The decline two years after clearance could be a consequence of the increased prevalence of grassland species which tend to have more less-visible mycelia and are ephemeral fruiters.

DISCUSSION

The effect of time since *R. ponticum* clearance is more complicated than previously thought. Rather than following classical models of regeneration on bare ground, the fungal community suffers from lasting effects following the removal of *R. ponticum.* Such effects contradict our predictions and show that the fungal richness on cleared stands tends to decrease with time since clearance. However, it would appear that this effect is a consequence of soil pH changes as opposed to time since clearance alone. Such an effect underlines the importance of long term monitoring in situations such as this. It is important to understand the continuing effects of *R. ponticum* colonisation on the soil system and its associated mycota.

The results of these studies are partially explained by the characteristics of the fungi identified in the plots (Figure 6). Where sporocarps and cords are present, they are of saprotrophic, wood decaying fungi such as *Trametes versicolor* (L.) Lloyd and *Rhododendron* specialists such as *Megasporoporiella rhododendri* (Y.C. Dai & Y.L. Wei)

B.K. Cui & Hai J. Li (Plate 2). Such fungi would not be expected to continue in a plot where the wood resource has been removed. Therefore, a reduction in saprotrophic species could be expected over time with a resultant shift from woodland to grassland species. The length of time covered by this experiment was insufficient to follow this transition and captures only the reduction in saprotrophic species over time. The grassland species are yet to recolonise in cleared areas. Future studies, revisiting the sites, should identify the time needed for this shift in habitat to occur informing future management plans for areas similarly affected by *R. ponticum* invasion.

Plate 2: Dead rhododendron branch with white filamentous cords of *Megasporoporiella rhododendri* protruding from dead wood (photograph courtesy of Mr C I Griggs).

The theory that acidic leaf litter leads to increased soil acidity on and around *R. ponticum* stands is contradicted by these data. Acidity follows a trend opposite to that expected, namely, acidity increases with time since clearance. This could be a residual effect of the remaining leaf litter, root stocks and brash, all of which may release acidic compounds as

they break down. These results support previous work by Mitchell *et al* which investigated heathland restoration after *Rhododendron* invasion. The study found that unless areas were litter stripped, the nutrient levels and pHs were unlikely to return to pre-invasion levels (Mitchell *et al*., 1999). Mitchell *et al* also suggest that deep root stocks and soil toxins may reduce the capacity for recovery at cleared *Rhododendron* sites. Further work, investigating the mechanism behind this phenomenon, would be crucial to furthering our understanding of how plant and fungal communities would be expected to recolonise bare ground after *R. ponticum* clearance.

The plant communities in the area, although not presented explicitly in this paper, were also different from site to site and did not achieve the characteristics of the un-colonised land at any point within the observed 11 year clearance window. The most mature cleared areas were dominated by sheep's sorrel *Rumex acetosella* L. and grasses, having passed through bare ground and fern-dominated communities prior to this state (Plate 1).

Vegetation, such as the Lundy cabbage and several native grasses, such as *Holcus lanatus* L., are sensitive to the high pH of the land, even ten years after clearance. Therefore, it may be prudent to pilot a controlled liming experiment to test whether the soils can be restored to their pre-colonisation state, causing succession to become equivalent to that of the un-colonised side lands. However, this method may prove detrimental to one of the groups of organisms the project is seeking to protect, the waxcaps. Waxcaps are very sensitive to anthropogenic changes in their habitats and have been shown to decline in areas where land has been limed (Griffith *et al*., 2002). Therefore, although liming initially appears to be an ideal way of rescuing the landscape, its long-term effects could be far reaching.

A similar rescue and protection effect could be achieved by encouraging scrub growth by gorse (*Ulex* spp. L.) and bramble (*Rubus fruticosus* L. agg.) on cleared areas. Spiny shrubs

protect young plants and fungi from grazing by Sika deer (*Cervus nippon* Temminck, 1838) and feral goats present on the island. It may be that bare ground highlights the presence of succulent young plants and fungi reducing their chance of establishment prior to grazing. By reducing grazing pressure, species may become released from suppression and succession can proceed more rapidly.

A final restoration option would be simply to wait. Soils on the island have an average pH of 5. Many of the plants are acidophilic and may be able to tolerate soils of a wider range of pH than they are currently observed to be growing in. Only by waiting to observe the complete series of colonisations and losses can the baseline situation be understood. Without such a baseline other potential methods to increase the rate of restoration will not have anything against which to be judged, therefore reducing their potential use.

Therefore, we can be sure that there are changes in both plant and fungal communities on old *R. ponticum* stands in the years after clearance. As plants and fungi begin to recolonise they have a stabilising effect on these landscapes. This work is a critical first step in addressing the issue of the persistence of negative effects of *R. ponticum* leaf litter and associated activity on the soil, mycota and flora in an area. Future work, addressing the mechanisms of such longevity and extending this study into the future will enable this question to be answered more completely.

There are, of course, implications resulting from the sampling method used in this study. As a consequence of our research emphasis on cord forming fungi we sampled visible fungal structures and macro-fungi. Therefore, we were unable to detect non-fruiting fungal species with diffuse mycelia or many mycorrhizae. Therefore, our sampling methods may have had a bias towards the collections of woodland fungal species which are more likely to display these macro features. We are, however, confident that our methods were appropriate for this study and provide a vital first step towards developing an

understanding of the processes occurring post-rhododendron clearance. Future work, such as the inclusion of soil fungal screens of soil cores, will fill in the gaps in this study and enhance our knowledge further creating a longitudinal study of the area with high scientific potential.

A better understanding of this process will enable protocols and policies to be developed and implemented to mitigate any detrimental effects and encourage the return of native flora in these areas without potentially damaging the rest of the system. The investigation reported here could be extended to other areas in which programmes of clearance are occurring to test how representative these processes and patterns are. Unfortunately, many other areas do not tend to have complete records, such as those for Lundy, which reduces the potential for a study to be as reliable and detailed. Lundy is therefore a critical site for *Rhododendron* research in Great Britain with particular relevance for acidic grasslands such as those in other granite areas such as Dartmoor and the Scottish Highlands.

CONCLUSIONS

This study hints at some practical management practises for mitigating the effects of *R. ponticum* colonisation post clearance. In an area such as Lundy, the scars on the landscape caused by clearance can detract from its aesthetic beauty. Therefore, restoring the native vegetation and fungal communities cover is a priority.

Fungal and plant communities are slow to recolonise an area post-clearance and do not return to pre-colonisation communities within the twelve year timescale studied.

The low significance of many of the effects on the fungal communities can be explained by the measurement used, fungal richness, which removes species identity and functional trait identity from the analysis. Plotting the change in fungal species over time illustrates the

community turnover occurring in the plots (Figure 6). Plant communities remain suppressed post-clearance and begin to demonstrate shrub encroachment eleven years after clearance.

Lundy provides an opportunity for a long-term monitoring experiment were this experiment to be repeated into the future and include a litter stripping component. The island and the location of *R. ponticum* with reference to aspect, wind and sea spray conditions makes it an excellent natural laboratory, controlling for many variables which would otherwise be hard to account for. It would be comforting to think that the uncontrolled expansion of an ornamental shrub out onto the eastern side lands could be of benefit to our understanding of post-invasion restoration enabling something positive to result from this ecological misadventure.

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CHAPTER SIX

WHAT, WHERE AND WHEN: ADDRESSING THE ISSUES SURROUNDING MAXIMUM ENTROPY MODELLING OF UK FUNGAL DISTRIBUTIONS.

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Declaration:

I confirm that the work contained in this chapter is wholly my own and that the contributions of the other authors to the paper constituted advice regarding experimental design and proof-reading the manuscript prior to submission. This work is in preparation for submission to 'Fungal Ecology'.

Signed:

Kirsty Monk (DPhil candidate) Dr Nick Brown (Supervisor)

WHAT, WHERE AND WHEN: ADDRESSING THE ISSUES SURROUNDING MAXIMUM ENTROPY MODELLING OF UK FUNGAL DISTRIBUTIONS.

Abstract

Fungi fulfil many roles, from economically important pathogens to saprotrophs providing crucial ecosystem services. Understanding the distributions of these organisms will enable ecosystem service provision and disease susceptibility to be mapped. This paper seeks to understand the challenges associated with predicting fungal distributions from patchy foray data and investigates the extent to which they can be overcome to produce reliable species distribution models with high support using publicly available survey data from the Fungal Records Database of Britain and Ireland (FRDBI). Such foray data contain inherent problems such as geographic and sampling effort biases. Using AUC and Jacknife values, we assess the relative merits of different methods to mitigate these biases before investigating the minimum adequate sample size for consistent fungal distribution models. We conclude that distributions can be predicted relatively accurately and reliably with the use of appropriate parameters, cross validation and an adequate sample size.

Key Words:

Atlas, Bias background, *Daldinia concentrica*, FRDBI, MaxEnt, sample size, Species Distribution Model

Introduction:

Species distribution modelling (SDM) is a powerful tool for predicting distributions of well sampled species. It works particularly well for rare species with specialised niches which have been collected infrequently (Hernandez *et al.*, 2006; Stockwell & Peterson, 2002). The geographic occurrence of fungal species is much less well known than that of vascular plants, probably because fungal sporocarps (mushrooms) are ephemeral, rarely collected and many are difficult to identify. SDM, also known as ecological niche or environmental envelope modelling, is a method of predicting the current distribution of species based on correlations between known occurrence data and combinations of and interactions between mapped environmental variables that are likely to affect the species' growth and survival (Pearson *et al.*, 2007). SDM approaches assume that the sample of species occurrences used in a correlative model incorporates the full range of environments that the whole species population occupies (Higgins *et al*., 2012). It is therefore important to have a large sample of records for those species with broad environmental tolerances. However, Pearson *et al.* (2007) have demonstrated that samples of fewer than ten occurrences can give an adequate model of the distribution of a species. This analysis included 11 species of *Uroplatus* leaf-tailed geckos (Gekkonidae, Reptilia) endemic to Madagascar, each associated with a specific primary vegetation type and elevation range. The sample size recommendation from Pearson et al. (2007) is appropriate for rare species for which there are typically few observations, as most rare species have narrow environmental ranges (Brown, 1984).

A very specific problem for fungi is that there are few observations of some widespread (and probably rather abundant) cryptic species. These are likely to have broad environmental ranges and hence be less well modelled from a very small number of

observations. Furthermore, fungal sporocarps may be produced only under specific environmental circumstances which may add to the environmental bias of fungal foray samples. In this paper, we examine whether it is possible to compile robust environmental envelope models of important UK fungi. Fungi have been much less well collected and recorded in the UK than vascular plants and animals. Those collections that do exist are typically spatially (and therefore probably environmentally) biased. For example, very few records are found in urban areas and their suburbs. This may be an artefact of reduced sampling effort in these locations. It is pertinent to discover whether the environmental envelope-type approach can be used to fill in the blanks in patchy data, enabling the production of reliable and accurate distribution maps.

We have used data from the Fungal Records Database of Britain and Ireland (FRDBI), curated by the British Mycological Society (BMS) (Kirk & Cooper, 2009) and available from the National Biodiversity Network (NBN) gateway (data.nbn.org.uk). The FRDBI contains data from fungal forays the 20th and 21st Centuries and lists location data, species identity, habitat type and host species, where appropriate. Dynamic mapping of species records is available through the NBN Gateway. As with many large species compiled by many collectors over long periods of time, records are of variable quality (Bird *et al.* 2013). We have used stringent quality criteria to eliminate those records that we judged to be inaccurate.

Fungal foray record data are accompanied by inherent issues, namely that the records are based almost exclusively on fruiting bodies. Since sporocarps may be produced only infrequently and persist for a short period of time, the absence of any records of a species at a site does not constitute evidence that it was not present. As a result, it is necessary to use a presence-only model such as MaxEnt (Elith *et al.*, 2011) to account for this.

Furthermore, the uneven sampling associated with foray records adds further complexity as it breaks the fundamental assumption of SDM, that the sample of species occurrences includes the full range of environments and conditions that the population occupies. Modifications can be made to the approach to correct for such effects (De Giovanni *et al.*, 2012). This paper seeks to identify the extent to which breaking this assumption impacts the validity of the resulting prediction for an ephemeral and patchily distributed organism.

This study uses six study species valued for their ecological and/or environmental importance each falling into one of three functional groups. 1) Latently-present fungi (those which are present as dormant spores in sapwood and initiate on tree death (Johannesson, 2000; Boddy, 2001): *Auricularia auricula-judae* and *Daldinia concentrica* have high host species specificity. *A. auricula-judae* favours elder (*Sambucus nigra*) whilst *D. concentrica* is most commonly associated with ash (*Fraxinus excelsior*). 2) Saprotrophs: *Phallus impudicus* (L.) Pers. and *Phanerochaete velutina* (DC.:Pers.) Parmasto, are both cord-forming fungi with roles in nutrient transport across the soil-litter interface (Tlalka *et al.*, 2002; Watkinson, 2006) assumed to enhance and promote woodland health and productivity. 3) Pathogens: *Armillaria mellea* and *Heterobasidion annosum* (Butt rot) are of high economic importance. *H. annosum*, predominantly a disease of coniferous woodlands, has an estimated economic cost to European forestry of €800,000,000 a year (Asiegbu *et al.*, 2005). Saprotrophic fungi are prized for their perceived role in ecosystem service provision and nutrient cycling. All of these are basidiomycetes, within the sub-phylum Agaricomycotina, and are easily identified species with low likelihood of misidentification, with the exception of *Armillaria mellea* (Honey Fungus) which has been split into ten separate species (Watling *et al.*, 1982; Watling, 1991).

There is growing awareness of the critical ecosystem functions provided by fungi and their important role in plant and animal health. Many fungi are cryptic and difficult to identify with the majority of the life cycle occurring underground and therefore not recorded by foray data (Whalley & Watling, 1982; Gange *et al.,* 2011). It would be very useful to know which fungi are putatively present in any part of the country and to use this information to create regional checklists. This will assist in identification, conservation and disease control. Furthermore, rapid environmental change will undoubtedly result in the distributions of some fungi changing over the coming decades. Reliable environmental envelope models will assist in making predictions about which species are likely to colonise new areas of the country and the associated impacts of such movements.

Due to the nature of the dataset and the uncertainties inherent in fungal presence/absence records, it is necessary to use presence-only models (Whalley & Watling, 1982; Gange *et al.*, 2011). Prior work by Elith *et al.* (2011) compared 16 modelling methods for 226 species worldwide, testing presence only with independently collected presence/absence data. This study concluded that the best methods for presence-only single species modelling were generalised dissimilarity modelling for single species (GDM-SS), boosted regression trees (BRT) and Maximum Entropy (MaxEnt) although they are not without fault which has been accounted for during the analysis (Yackulic *et al.,* 2013). MaxEnt v. 3.1 (http://www.cs.princeton.edu/~schapire/maxent; Phillips *et al.,* 2004; Phillips *et al.,* 2006) was selected for this study as it is best able to deal with patchiness and collection bias through inclusion of backgrounds which can provide higher AUC (area under curve) and COR (coefficient of restitution) values (Smith *et al.* 2013; Phillips *et al.,* 2009), indicators of a better fit to the model and an increased potential for the model to be generalised.

The inherent spatial biases associated with foray data require the use of a bias cancelling background (Kramer-Schadt *et al.* 2013). In MaxEnt there are three ways of achieving this. A Target Group Background (TGB) uses only grid squares with at least one record and then projects the data to squares for which no information is available, therefore displaying the same bias as the data being analysed. The TGB approach risks ignoring important sources of variation or artificial constriction of the species' expected niche but reduces the risk of artificially inflating a square's importance by the number of records held within it. An alternative approach is the Bias Background (BB) which weights squares by the number of records held therein, not discarding any squares and retaining the full extent of potential niche space but risking exaggerating the importance of some variables by recording effort induced biases. Finally, the Average Bias Background (ABB) creates grid squares weighted by the average number of records collected from each location within it, reducing but not eliminating the risk of over-inflation in popular collecting regions. By considering the modelling approach, the use of a bias-cancelling background and the quality and quantity of data put into the model, this work should provide an evaluation of SDM as a means of generating current and future predictive distribution models for UK fungi.

The hypothesis of this study is that it is possible to create a reliable and accurate species distribution models for fungi from existing fungal datasets, however the sample size needed for this will be much greater than that needed to create accurate and reliable species distribution models for rare species of restricted niches and ranges such as geckos in Madagascar.

Methods:

Dataset development

Datasets were created for each study species from the main FRDBI database. Datasets were cleaned and geo-referencing checks and improvements were carried out in accordance with stringent protocols to ensure a $10km^2$ resolution. Cleaning the data involved cross-referencing the vice-county, 6-figure grid reference and latitude and longitude data with the location description to ensure their accuracy and consistency. In cases of ambiguity in one or more descriptor, the other descriptors were used to correct the inconsistency. Where only a location name was available, the centroid of the location was identified and used as the data-point site. Data were then mapped in ArcGIS 10 (ESRI 2011) to check for further anomalies and correct them.

The datasets contained information for all foray records of a particular species and included data such as latitude/longitude, species identity, year of recording, host species and ecosystem identity. Not all records contained all data, but data provided was used to cross-check resulting models unless deemed unreliable. Datasets were assessed for volume of samples and only used if the number of records in the FRDBI exceeded 250 and that, post-processing the number of records was still greater than 200. This enabled cross-validation of models using an adequate sample (>20 records). All records collected between 1961 and 2011 were included in the analysis with the exception of *A. mellea* whose records prior to 1991 were discarded due to uncertain taxonomy caused by recent taxonomic revision of species delimitations (Watling *et al.*, 1982, Watling, 1991). Cleaned datasets were used in downstream modelling, model calibration and model testing.

Model creation

The TGB, BB and ABB were generated from all geo-referenced records for the study species in the FRDBI to reflect sampling and geographic biases in the data. These three backgrounds and a background free-model (random background) were all run and compared for *H. annosum* and *D. concentrica*. The results were compared within species to identify the optimal background and between species to test how easily the optimal background can be generalised across the fungi.

MaxEnt uses a range of combinations of variables and assumptions relating to their relationships, termed features. The default model includes all possible combinations and assumptions therein; however, this is not always the most appropriate scenario. Too many features can result in an over-fitted model, fitting the training dataset very well but with a reduced test AUC, indicating reduced potential for the model to be generalised. *Heterobasidion annosum* and *Daldinia concentrica* were used as pilot species to test the features of MaxEnt for fungal datasets. Omission graphs, test AUC and jack-knifed output variables were used to assess the best model to enable within and between species comparisons.

Reliable species distribution modelling requires both biotic and abiotic variables to be included but also that models should not be over-fitted (Merow *et al.* 2013). Knowledge of the ecology and biology of fungi were used to inform variable selection. For example, trees under stress through herbivory, disease or drought are particularly vulnerable to attack by saprotrophs and pathogens (James, 1980; Boddy & Rayner, 1983; Stambaugh, 1989). Furthermore, reductions in phloem water potential associated with plant stress stimulate latently present propagules to develop (Johannesson, 2000; Boddy, 2001). Therefore,

precipitation, temperature and soil type were included in the model as proxy determinants of drought stress.

Ten environmental variables were selected to train the model. These were sourced as BIOCLIM variables (Busby, 1991) from the WORLDCLIM dataset (Hijmans *et al.*, 2005) supplemented by eight abiotic variables from the European Soil Portal (http://eusoils.jrc.europa.eu) (Appendix 1). In initial model runs, host species data from the BSBI hectad map scheme (www.bsbi.org.uk/maps-scheme.html) was investigated as a parameter; however the ubiquitous distribution of named hosts for many fungi across Great Britain meant this variable did not contribute greatly to model accuracy and was eliminated from later models. For *D. concentrica* models, host preferences were demonstrated to have strong geographic differentiation, therefore in the model testing phase, only *F. excelsior* associated records were included. The whole *D. concentrica* dataset was then analysed as a case study to investigate the ability of MaxEnt models of fungal distributions to answer ecological questions. In this case, the question was whether the models supported the idea that *D. concentrica* should be divided into two species on the basis of its geographic distribution, an idea first posed by Whalley and Watling in 1982.

Model testing

Models and their output distribution maps were tested for overall performance, accuracy and individual variable effects. Overall performance was assessed using omission graphs, test AUC and jack-knifed output variables. AUC or the area under the receiver operating curve is the most commonly used test for model accuracy and suitability of a presence only model. The AUC represents a ratio of the actual probability of presence in a square compared to the predicted probability. As the AUC is affected by the total area covered by the study species, it is difficult to identify a 'good' AUC value. Species occupying a large proportion of the study area tend to have smaller AUC values (Lobo *et al.*, 2008, Wollan *et*

al., 2008). Therefore, it is difficult to compare AUC values between species; however, comparison within a species for different models will be informative.

Accuracy of the models was assessed via cross-validation of the dataset using 10 sub-groups of the test data. Ten subgroups ensured that each test run contained 40 records even in the least recorded species, *Phanerochaete velutina*. Jack-knife plots and omission graphs were used to assess the individual variable effects. The percentage contribution of each variable was calculated by randomly permuting the values for each variable among all the training points and measuring the subsequent decrease in AUC. Data from the ten model runs for each species were used to investigate the generality of fungal environmental preferences. Analyses of variance (JMP10 (SAS Institute Inc.)) and a principal components analysis (CAP 3.0 (Pisces Conservation Ltd.)) were used to identify differences between guilds in terms of environmental variable importance.

Determining an appropriate sample size

The minimal sample size required for a generalist species with a broad environmental range was assessed through model simulations. MaxEnt models, using the parameters outlined previously and an appropriate number of cross-validation runs (to provide model datasets of greater than twenty accessions), were created using random subsets of the data for three fungal species: *Auricularia auricula-judae*; *Phallus impudicus*; and *Heterobasidion annosum.* These species were selected to span the three functional guilds included in the study.

Within each species, eight models were run to cover a range of sample sizes from ten to 2000 accessions with ten replicates of each model run using a different randomly selected subset of the data. These models were plotted in ArcMap10 (ESRI 2011) and the

continuous probability distribution data was used to generate categorised likelihood values from 1-9 across a raster image of Great Britain with cell size 10km^2. Sample raster datasets were subtracted from the complete model (the model created from the complete dataset available for that species, assumed to be the most accurate model for the data) to provide a raster image of difference from the complete model. The associated attribute table was used to create a dataset containing the samples of difference greater than |5| and the directional differences. These data were then used to investigate prediction errors and the magnitude of area predicted wrongly at each level of sampling for each species using methods similar to those of Hanberry *et al.* (2011). A regression with natural logarithm transformations on both axes (JMP version 10 SAS Institute Inc., Cary, NC) was fitted to the data to identify the level of sampling at which the quality of the model fit relative to the complete model stabilised.

Case-study: *Daldinia concentrica*

Initial maps of *D. concentrica* distributions split by host showed a north/south dichotomy in dominant host (Fig 1).

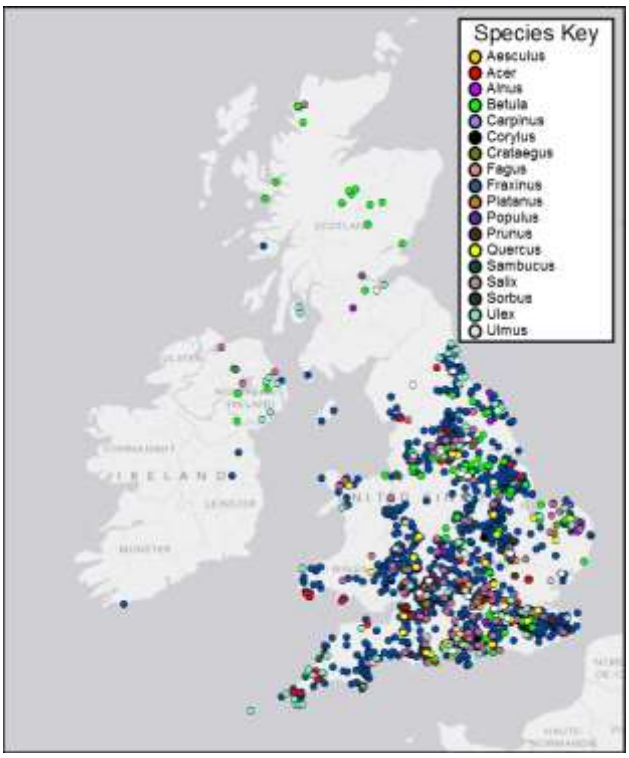

Fig 1: *Mapped locations of Daldinia concentrica (Bolton) Cesati & De Notaris records by host species identification. Colour determined by species key. Records projected as OSGB36 using ESRI ArcMap10 software.*

In southern regions, *D. concentrica* predominates on ash (*Fraxinus excelsior*) whereas further north the principal host becomes birch (*Betula* spp.). Such distributions complement theory developed in 1982 by Whalley and Watling which proposed to split the species in two to create a true *D. concentrica* species, dominant in the south, and a new division *Daldinia vernicosa*, specialised on birch, which dominates in the northern regions. To investigate this phenomenon further, we separated the *D. concentrica* species by host preference, either *Betula* or *F. excelsior*. Those species with no, or alternative, host associations were discarded from the analysis. The species records for *D. concentrica* were analysed as two separate datasets using the optimal MaxEnt conditions identified for the species.

Results

Model settings

A combination of hinge (a stepped response which allows for a break in the angle of the otherwise linear curve) and quadratic response settings result in the highest test AUC values and therefore are taken forwards to the further analyses (Table 1). The trends apparent in *D. concentrica* were constant across *H. annosum* despite their different guild affiliations, suggesting that the observed trends can be generalised across our six study species.

Features	**Test AUC**
Linear	0.670
Hinge	0.669
Hinge, Quadratic	0.672
Hinge, Threshold	0.661
Hinge, Quadratic, Threshold	0.662
All	0.658

Table 1: *AUC values for models run using different feature combinations for the Daldinia concentrica (Bolton) Cesati & De Notaris dataset.*

The best background, as identified from the test AUC, is the random background (Table 2).

Background	**Test AUC**
Random	0.687
Target group	0.670
Bias	0.617
Average	0.559

Table 2: *Test AUC scores for models run using four different backgrounds on the Daldinia concentrica (Bolton) Cesati & De Notaris dataset.*

This result is misleading. The random background would be expected to demonstrate the highest AUC as the test data contains the same uncorrected biases as the training set,

artificially inflating the AUC value. Once the random background is discounted, the next highest AUC score is attributed to the target group background. However, its similarity to the random background suggests that it does little to remove the biases present before application of the background (borne out in examination of the prediction maps), as may be expected from the function of the background considered with the fact that there are very few squares at the 10km grid level containing no records. On examining the jack-knife plots for the average and bias backgrounds, the average bias background provides a much better fit of training and test omission to the predicted omission (Fig 2). We therefore conclude that although the average bias background performs worst in terms of AUC, it is in fact the most accurate means of predicting presence of fungal samples across space.

It could be said that both bias backgrounds are flawed in that they do not account for variation across the grid square. It is likely that these microhabitat-led fluctuations will be at a smaller scale than the $10km^2$ grid. Therefore, all grids will provide an average relative to the other squares.

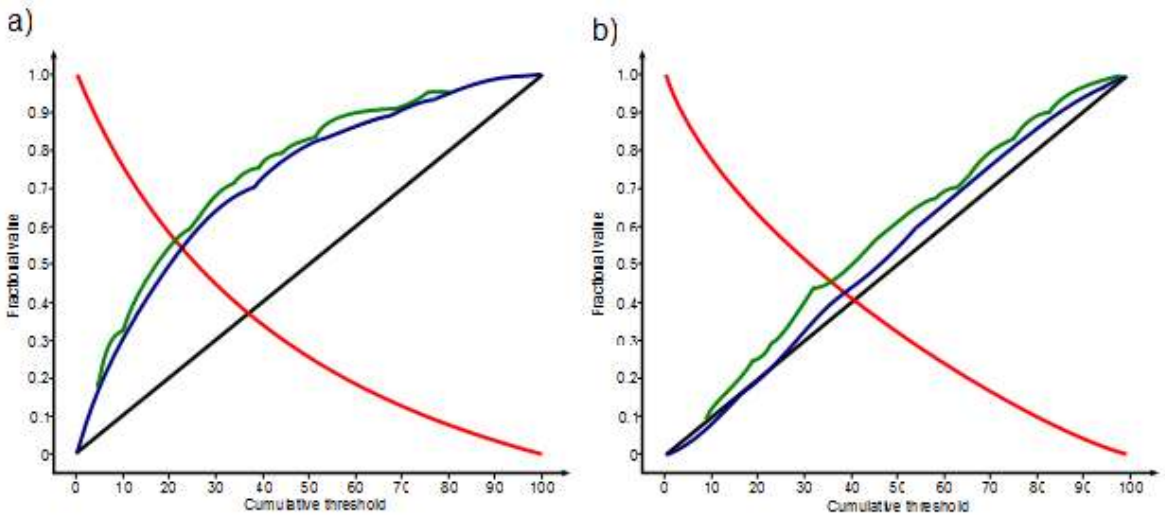

Fig 2: *Omission predicted area graphs for* H. annosum *models run with the bias (a) and average bias (b) backgrounds. Close fit of the training (blue) and test (green) omissions to the predicted omission indicates a better, more accurate model, as in graph b. Fraction of background predicted is indicated by the red line.*

Individual variable effects

Cross-correlation between variables was detected by the greatly reduced AUC associated with the addition of cli38 to the model and notably similar individual variable response curves for the two variables (Fig 3) and led to the exclusion of cli38 (precipitation of the warmest quarter) from the analysis due to its correlation with cli37 (precipitation in the driest quarter). No other variables exhibited significant cross-correlation with one another.

The percentage contribution of each environmental variable to the model for each species suggests that climatic variables tend to be more important to fungal distribution modelling than other factors (Table 3). Across all models cli30, maximum temperature of the warmest month, is consistently one of the four most important factors. With the exception

of cli30, there are neither consistently important variables across all species nor trends across guilds.

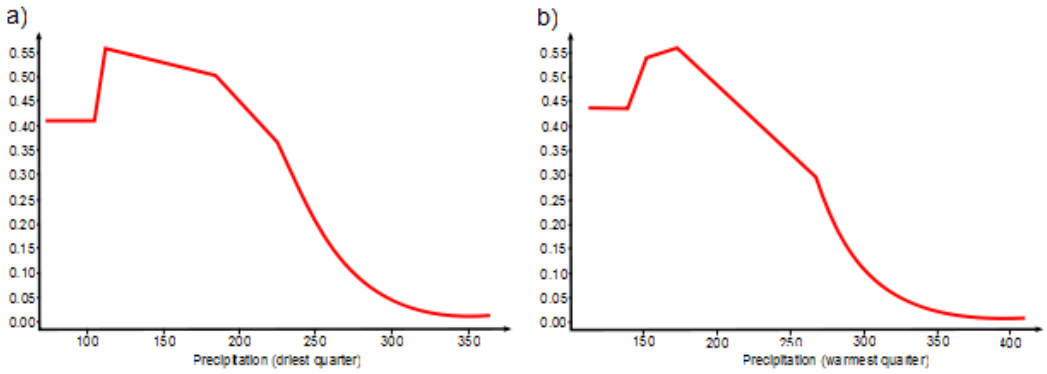

Fig 3: *Marginal response curves for two cross-correlated variables, precipitation of the driest quarter (a) and precipitation of the warmest quarter (b), for the* Daldinia concentrica *(Bolton) Cesati & De Notaris model. These curves are generated from* Daldinia concentrica *models created using only the variable of interest. Similarity in curve shape supports a conclusion of cross-correlation between the variables and omission of one of these variables from subsequent models.*

	Armillaria mellea	Heterobasidion annosum	Daldinia concentrica	Auricularia auricula-judae	Phallus impudicus	Phanerochaete velutina
Max temperature (warmest month)	**34.8**	**33.2**	**9.4**	**45.9**	**35.4**	**12.9**
Min. temperature (coldest month)	1.2	4.1	**14.3**	**20.5**	**12.4**	2.2
Mean temperature (wettest quarter)	1.3	0.2	5.2	0.2	0	3.4
Mean temperature (driest quarter)	6	**4.5**	6.7	**4.5**	7	3
Mean temperature (warmest quarter)	**16.8**	0	**23.3**	0	0	**4.3**
Mean temperature (coldest quarter)	0.2	0	0.5	0.2	0	0.1
Precipitation (wettest quarter)	**16.7**	4.3	**17.3**	**14.2**	**21.6**	3
Precipitation (driest quarter)	**12.3**	**37.6**	**7.5**	3.5	**11.3**	**31.7**
Precipitation (coldest quarter)	0	1.1	6	**4.4**	0.8	**6.4**
Min. elevation above sea level	2.9	0	1.3	1.7	2.2	3.8
Max. elevation above sea level	0.2	**7.2**	3.8	0.2	0	**18.6**
Dominant land use	1	0.3	0	0.1	0	**4.1**
Secondary land use	**3.9**	1.5	4	3.8	0	3.6
Regrouped landuse class	1.6	0.5	0.2	0	1.9	0
Topsoil organic carbon content	0.6	0	0.2	0.2	0	0
Dominant surface textural class	0.5	**5.6**	0.3	0.6	**7.3**	3

Table 3: *Percentage contribution of permuted environmental variables to the overall model of each fungal species. Values are calculated by randomly permuting the values for each variable among all training points and measuring the subsequent decrease in AUC. The five most important variables to a species are indicated in bold text.*

A principal components analysis (PCA) shows that good groupings exist within a species, indicating that the cross-validation groups have good consensus as to the model predictions and responses (Fig 4). However, there is no trend to group species together by guild in response to the environmental variables when the data are projected in two axes. When the groupings visualised in Fig 4 were tested for their statistical significance using an analysis of similarity (ANOSIM) in the CAP3 software packages no grouping by guild was indicated, only grouping by species (P=0.001). Such results support the view that models of fungal species distributions cannot be generalised across guilds or kingdoms and must be tailored to the specific needs of the species of interest due to their diverse environmental requirements.

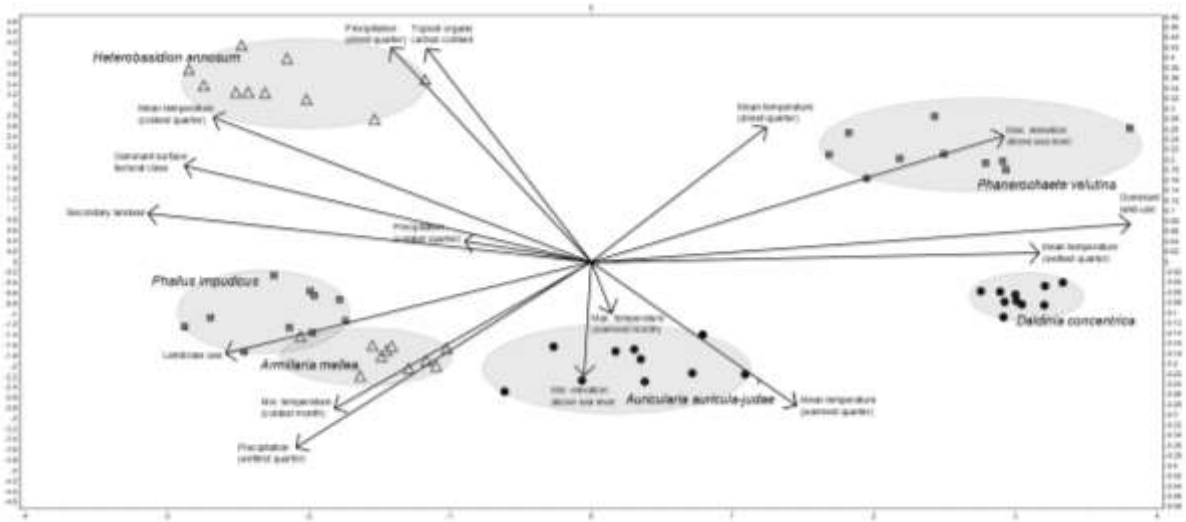

Fig 4: *Principal components analysis (PCA) plot for all model runs for each species. Cross-validation of runs divided each species' dataset into ten equally sized subsets with models run for each (identified with the grey filled ovals). Guild identity is illustrated by shape and shade of marker. Variable effects are depicted as black arrows with variable labels at the tip. Significance of different groupings by species (ANOSIM) P=0.001* R^2*=92.29.*

Establishing a minimally adequate sample size

The minimally adequate sample size required for a reliable and representative model is not consistent across the three species. *Armillaria mellea* fits a logarithmic curve (*P*=<0.0001, d.f. = 1, error d.f. =58, R-square=0.538) (Fig 5). This fit suggests that the minimally adequate sample size for this species is roughly 600 accessions. Looking in more depth at the manner of the prediction errors (incorrect absence prediction or incorrect presence prediction), it becomes apparent that the errors being reduced are those predicting absence in cells where the complete model predicts presence. Fitting the number of cells in which an incorrect absence has been predicted results in an almost identical curve to that produced by all prediction errors, the fit of a logarithmic curve and the associated regression analysis confirm that this pattern is also significant (*P*=0.0001, d.f. = 1, error d.f. = 58, R-square=0.540).

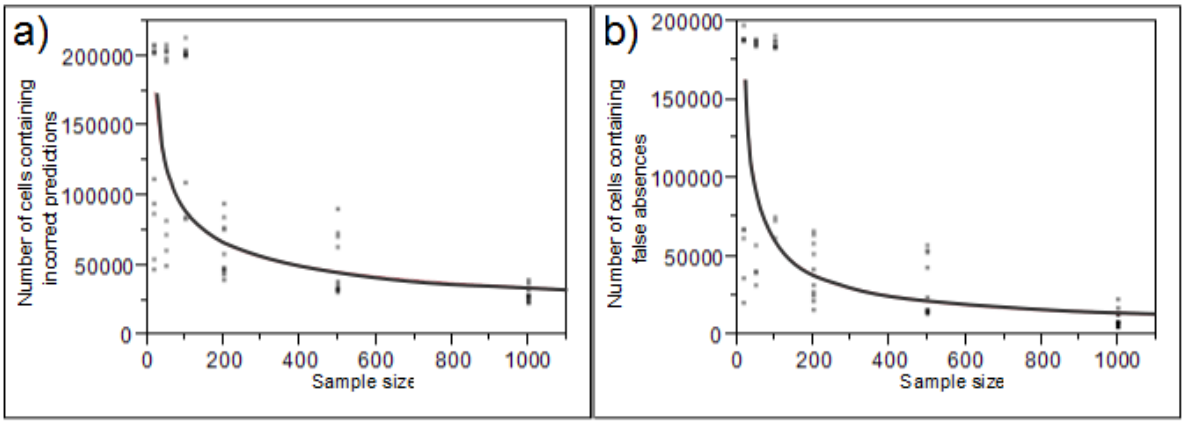

Fig 5: *Logarithmic fit curves for* Armillaria mellea *(Vahl) P. Kumm (Honey Fungus) model plotting sample size against the number of cells in which incorrect predictions had been made. a) the number of total prediction errors (false presences and false absences) made by models of different sample sizes. b) the number of incorrect absences predicted only. Both models are significant (P=<0.0001, d.f.=1, error d.f.=58).*

In contrast, regression analyses for *Auricularia auricula-judae* and *Phallus impudicus* show no significant association between sample size and the number of significantly different cells. *Auricularia auricula-judae* did not show the expected relationship or levelling off of the prediction errors (*P*=0.8437, d.f.=1, error d.f.=68, R-square=0.001). Individual fits of the nature of the prediction error did not prove significant either. Therefore we conclude that much larger sample sizes or additional model parameters would be required in order to accurately predict the distribution of this species.

The models for *Phallus impudicus* show a weak logarithmic association between the sample size and the number of cells containing prediction errors (P=0.0880, d.f,=1, error d.f.= 66, R-square=0.043) suggesting that a near-constant error rate is achieved with sample sizes of roughly 300 accessions however the spread of values around this fit is large (Fig 6) suggesting the models are not consistent and many replicates would be necessary to provide reliable model predictions. Investigating the effect of sample size upon each type of error shows that there is a significant decrease in the number of incorrect absences predicted with larger sample sizes (P=0.0004, d.f.=1, error d.f.= 66, R-square=0.173) which is masked by large but non-significant variation in the number of incorrect presences predicted when the two forms of error are combined. The 'incorrect absence model' suggests that, for *Phallus impudicus*, a sample size of roughly 1000 accessions is required for a consistent model, however the variation around this fit is still great (Fig 6). Therefore, replicate models would be advisable to increase model reliability.

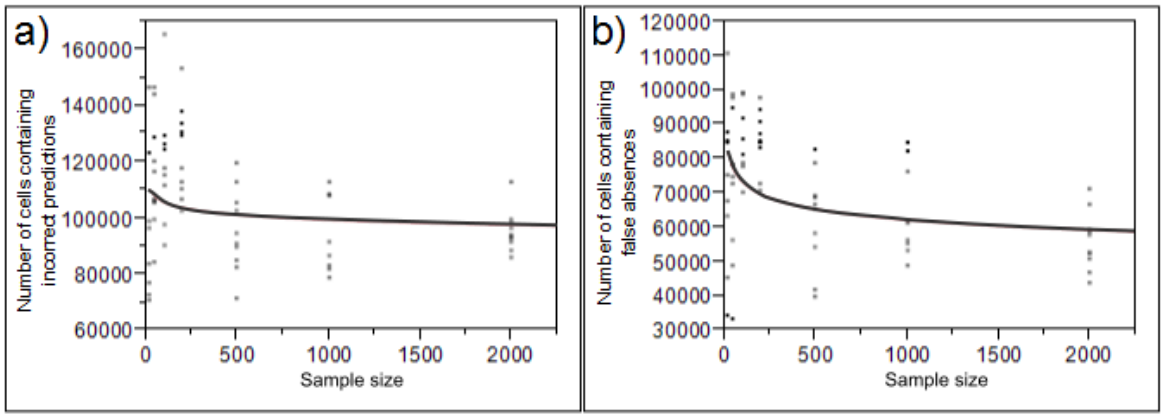

Fig 6: *Logarithmic fits curves for* Phallus impudicus *(L.) Pers. model plotting sample size against the number of cells in which incorrect predictions had been made. a) the number of total prediction errors (false presences and false absences) made by models of different sample sizes. b) the number of incorrect absences predicted only. Fit a) is not significant (P=0.0880, d.f.=1, error d.f.=66) whilst fit b) is (P=0.0004, d.f.=1, error d.f.=66).*

***Daldinia concentrica* case-study**

When *D. concentrica* is modelled as two separate varieties the AUC value for the *F. excelsior* only model increases from 0.0676 to 0.706 and the distribution map becomes more, but not entirely, restricted to southern Britain (Fig 7). The AUC change for the *Betula* only model is small, 0.676 to 0.675, despite the reduction in distribution which would normally lead to an increase in AUC. This effect is likely an artefact of the small number of records, 104 down from 5408 in the unsegregated model.

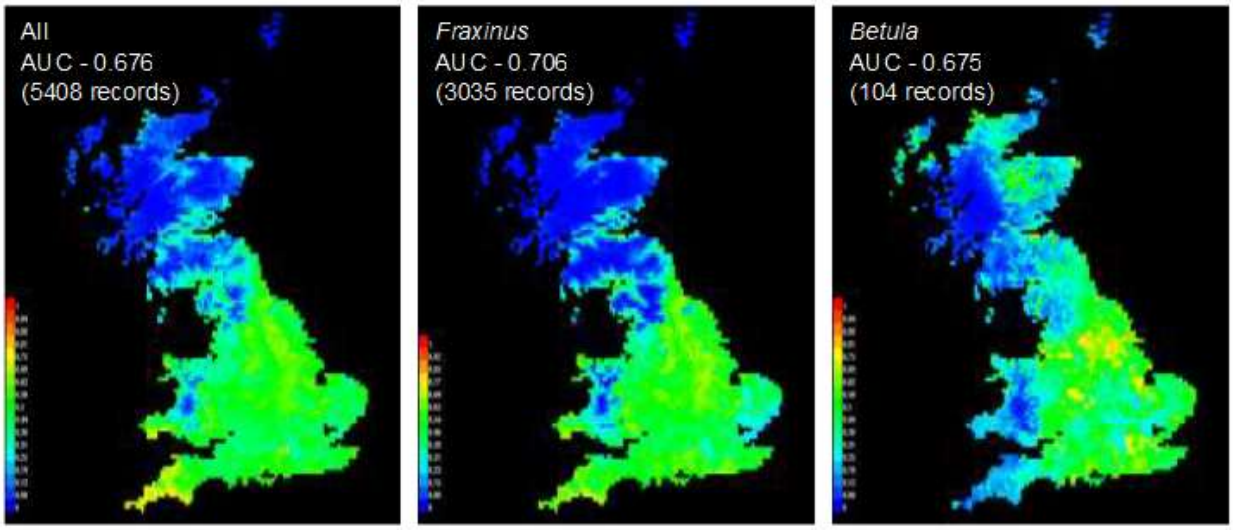

Fig 7: *MaxEnt distribution heat maps for* D. concentrica *for all records (far left) those associated with* F. excelsior *(centre) and those associated with* Betula *only (far right). Predicted distributions are identified by more intense green/yellow/red shading in accordance with the heat key in the bottom left of each tile. Range restrictions are seen in the host specific models. AUC values associated with each model and the number of records are shown in the upper left of each tile.*

Discussion

Despite the issues inherent in fungal foray datasets, it is possible to create reasonably accurate species distribution models with good fits of the training and test data to the predictions, as shown by the good AUC values in the case-study (*D. concentrica*) models. That is not to say that MaxEnt models are the optimal situation or that they are without fault. Models, whist adequately supported by AUC values, are not up to the standard of well-defined species with narrow environmental niches. Furthermore, the number of records necessary to obtain these values is between one and two orders of magnitude

greater than the 10 records suggested by the Pearson *Uroplatus* dataset (Pearson *et al.*, 2007).

Variability in minimally adequate sample size is a consequence of both species specificity and geographic range as opposed to the results of previous studies which omitted the impact of ecological characteristics on model accuracy (Chen & Lei, 2012). As demonstrated by our data, the majority of sampling errors are false negatives relative to the complete model. This suggests that the more restricted the range of a species, the fewer records are needed to define its range. Conversely, more promiscuous and widespread species require much larger datasets to provide a comprehensive range of all habitats and environmental parameters defining the species' range. As demonstrated by the *D. concentrica* case study, more detailed investigations into geographically linked host preferences may improve model predictions and reliability and could be particularly useful to increasing the power of the *A. auricula-judae* model as host is perceived to be an important determinant of the species range and current work suggests a shift or broadening of host preference in response to environmental change (Gange *et al.*, 2011).

When dealing with differences in range size it is necessary to use support values and accuracy measures in combination with one another. The AUC value is dependent upon the background used and can be artificially enhanced by omitting a bias background, predicting biases well as opposed to actual distributions moderately well. In this analysis, care was taken not to use AUC values in isolation; rather in combination with other measures of accuracy and predictive power such as jack-knife plots and omission graphs. Therefore, we can be fairly certain that the results we have presented here are real and true explorations of the limitations of MaxEnt for fungal distribution modelling.

The ideal scenario would be one in which all foray data biases could be overcome in the field, where the asymptote of the species discovery curve is reached for every grid square in Britain. However, without a national effort to record all fungi in Britain over a period of ten years or more, this is unlikely to occur even with the excellent fungal groups that exist in the UK.

This analysis used only data from the FRDBI curated by the BMS. There is however, another surveying and recording body, the Association of British Fungal Groups (ABFG), which curates and visualises its accessions in CATE2. For distribution mapping work to progress as rapidly as possible it will become necessary to combine these databases incorporating SDM for their visualisation, enabling the creation of an Atlas of British Fungi.

Fungal distribution models require the inclusion of multiple additional factors to improve their predictive power and accuracy. In an island situation, such as that of Great Britain, knowledge of the country's mycofauna alone is not sufficient. Fungal distribution data from neighbouring countries such as France, Scandinavia and the Netherlands would be required in order to fill in the blanks left when fungal distributions shift.

Additionally, to model spread and shifting distribution of species over time accurately (a potential offshoot of this work) it would be necessary to include information regarding the modes and extents of spread of each species. This is likely to be complex, accounting for vegetative growth, airborne spore dispersal and vector mediated spore dispersal. Incorporating dispersal potential into prediction models will enable mitigation and introduction efforts with respect to future changes in fungal and vegetative distributions to be better informed and more accurately targeted.

Fungal foray data has been the collection method of choice for more than a century. However, in the advent of rapid, cheap and easy DNA based analyses it is likely that a molecular approach to fungal diversity monitoring will increasingly complement morphological surveys. Technologies such as Whatman FTA cards (PMID: 16792516) will facilitate more accurate identification of cryptic species and vegetative growth structures. This knowledge, in combination with soil fungal DNA surveys will add more depth and certainty to our knowledge of fungal distributions. However, over reliance on these molecular methods is discouraged. DNA contamination can occur from yeasts and on poorly cleaned macro-fungi. More severe is the question of what constitutes fungal presence? DNA surveys will pick up dormant spores which may occur in the soil environment from air and vector based dispersal but which do not experience the correct conditions for initiation and development. In such cases, their inclusion in models will artificially broaden the environmental envelope of the species, reducing its accuracy and predictive power.

Conclusions

Fungal distribution modelling presents a number of unique issues stemming from fungal life history traits and the way in which fungal record databases have been compiled from fungal forays. It is possible to create adequate species distribution modelling using MaxEnt for these species although the model predictions could be improved through the inclusion of additional variables or the use of a community modelling approach. Accurate modelling of fungal distributions will enable the production of predictive distribution maps, regional checklists, focussed investigations into fungal ecosystem service provision, and a baseline

against which to measure future distributional shifts. MaxEnt provides a means of creating such models relatively rapidly and easily.

By virtue of decades of heavy amateur involvement and recording networks backed up by the work on natural history and field mycology societies, British fungi are possibly more thoroughly recorded than anywhere else in the world. The potential of this resource could be further realised by combining data from the FRDBI and CATE2 databases of the BMS and ABFG respectively, and applying distribution models such as those discussed in this paper to identify gaps in the data that can be filled by further directed effort and ground-truthing, for example via citizen science initiatives.

Acknowledgements

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Appendices

Appendix S1: Environmental variables included in the model and subsequent analyses.

BIOCLIM/ ESBD code	**Description**	**Type**	**Study Code**
BIO5	Maximum temperature of warmest month	Continuous	Cli30
BIO6	Minimum temperature of coldest month	Continuous	Cli31
BIO8	Mean temperature of wettest quarter	Continuous	Cli32
BIO9	Mean temperature of driest quarter	Continuous	Cli33
BIO10	Mean temperature of warmest quarter	Continuous	Cli34
BIO11	Mean temperature of coldest quarter	Continuous	Cli35
BIO16	Precipitation of wettest quarter	Continuous	Cli36
BIO17	Precipitation of driest quarter	Continuous	Cli37
BIO18	Precipitation of warmest quarter	Continuous	Cli38
BIO19	Precipitation of coldest quarter	Continuous	Cli39
ZMIN	Minimum elevation above sea level	Continuous	Env30
ZMAX	Maximum elevation above sea level	Continuous	Env31
USE-DOM	Dominant land use	Categorical	Env32
USE-SEC	Secondary land use	Categorical	Env33
USE	Regrouped land class use	Categorical	Env36
OC_TOP	Topsoil organic carbon content	Categorical	Env37
TEXT	Dominant surface textural class	Categorical	Env38

DISCUSSION: HOW CAN NEW TECHNIQUES AND EXISTING TECHNOLOGIES ANSWER FUNDAMENTAL QUESTIONS REGARDING THE DIVERSITY, DISTRIBUTION AND ECOLOGY OF CORD-FORMING FUNGI IN GREAT BRITAIN?

KIRSTY K MONK

The work reported in this thesis has revolved around the fundamental importance of the role of cord-forming fungi (CFF) within British woodlands. Ever since Herman Schacht first documented the role of fungi in wood decay in 1863 (Blanchette, 1991), interest has been increasing, leading to a greater knowledge of the diversity of fungi in, and out of, boreal forests. The CFF and associated saprotrophic fungi studied over the course of the previous five papers are all predominantly temperate woodland and/or grassland species. The work presented illustrates that saprotrophic fungi are integral components of woodland ecosystem dynamics whose diversity cannot be satisfactorily extricated from the other factors dominating the establishment of Ancient Semi-Natural Woodlands (ASNW) in Great Britain. Therefore, it seems that the initial stance taken in this thesis hold true. That is, that the current assumptions made about fungi in woodland systems are naïve and do not take into account the true ecosystem importance of fungi. Therefore, the overall aim of this thesis was to provide the foundations upon which future work could be based to increase the academic understanding of the role, ecology, diversity and distribution of fungi in temperate woodlands.

The ecology, diversity and distribution of cord-forming fungi: a synthesis.

An underlying theme throughout the work has been the application of the laboratory-based concepts of CFF ecology and physiology to larger-scale, field-based investigations. Chapter 2 starts this process by asking how CFF map to the fungal phylogeny and the implications this has for their shared ecology. Although the study has demonstrated that CFF may have originated twice (once in Ascomycetes and one in Basidiomycetes), a more important result is that CFF are only found in saprotrophic lineages capable of producing complex fruiting bodies. With this new knowledge, the Chapters 3, 4 and 5 could be better targeted towards finding CFF in the field by looking for these complex fruiting bodies. These three subsequent chapters aim to apply theories developed in laboratories or at meso-scales, to macro-scale field experiments. Building on the work of Lynne Boddy, Alan Rayner, Wendy Thompson and Sarah Watkinson, these chapters investigate the interactions and establishment of fungal communities, and CFF communities in particular, in a range of settings and scenarios from the impact of woodland age (Chapter 2) through the effect of log or litter substrate (Chapter 3) to the effect of invasive species removal on CFF (Chapter 4). A number of outcomes have resulted from this and these have generally not been those expected at the outset of the projects, changing the way we think about succession in CFF on ex-agricultural land, newly cleared land and in new areas of ASNW.

All of this information was used to inform Chapter 5, which sought to increase the scale of the investigation still further and identify the factors which determine CFF establishment, presence and diversity at a country-wide scale. This area has been fruitful and has led to the development of new projects to address the inherent biases in fungal datasets which should also provide the means of adding information to the phylogenetic and applied elements of the thesis as well.

Answering the crucial unanswered questions in CFF ecology

At its outset, this research programme intended to provide the answers to some of the major questions facing fungal ecologists attempting to carry out in depth analyses of the physiology and ecology of fungi and woodlands in Great Britain. The research programme addressed questions from the molecular (phylogenetic diversity) to landscape (species distribution modelling) scales. The elements of this thesis do not, and could not be expected to, provide definitive answers to these questions. However, they do open the dialogue and hint towards themes and patterns in the ecology, diversity and distribution of CFF and associated saprotrophic fungi; challenging long-standing assumptions.

What is the current known diversity of CFF in Great Britain?

A comprehensive literature review undertaken at the start of the phylogenetic analysis identified all of the known CFF discussed in the academic literature (Chapter Two). From this standpoint, it is possible to understand and explore how CFF can be defined as a subset of the fungal kingdom. The phylogenies generated by this research programme identified two clades of CFF in the fungal phylogeny: one clade branching at the base of the Xylariales, and another near the base of the Agaricomycotina. These groups were well-supported and suggested that the presence of complex fruiting bodies and a saprotrophic lifestyle is a key feature associated with CFF capacity in fungi. However, in as much as this study crystallised some of the ideas surrounding the phylogenetic diversity of CFF, it also highlighted some more known-unknowns in the completeness of our understanding and identification of CFF potential in species. By increasing the use of molecular identification techniques in combination with field observations, an increasingly exhaustive list of fungi with the capacity to form cords can be curated.

Throughout the course of work undertaken during this research programme, the list of known CFF has been extended as a result of the two pronged collection approach (molecular identification in combination with field observations). During the three field-based components of the research programme, four novel genera of CFF were identified using molecular identification techniques (Table 1). These CFF all lay within the known CFF orders and therefore do not alter the conclusions of the phylogeny chapter.

Ch.	Taxonomic Affiliation	Family	Order	Novel?
4	*Armillaria spp.*	Physalacriaceae	Agaricales	No
4	*Megacollybia platyphylla*	Tricholomataceae	Agaricales	No
4	*Mutinus caninus*	Phallaceae	Phallales	No
4	*Phallus impudicus*	Phallaceae	Phallales	No
4	*Phanerochaete carnosa*	Phanerochaetaceae	Polyporales	No
4	*Phanerochaete sordida*	Phanerochaetaceae	Polyporales	No
4	*Phanerochaete velutina*	Phanerochaetaceae	Polyporales	No
4	*Radulomyces spp.*	Pterulaceae	Agaricales	Yes
4	*Trechispora spp.*	Hydnodontaceae	Trechisporales	Yes
3	*Megacollybia platyphylla*	Tricholomataceae	Agaricales	No
3	*Resinicium bicolor*	Meripilaceae	Polyporales	No
3	*Armillaria spp.*	Physalacriaceae	Agaricales	No
3	*Hebeloma ammophilum*	Hymenogastraceae	Agaricales	No
3	*Laccaria laccata*	Hydnangiaceae	Agaricales	No
3	*Inocybe curvipes*	Inocybaceae	Agaricales	Yes
5	*Megasporoporiella rhododendri*	Polyporaceae	Polyporales	Yes

Table 1 *CFF identified through molecular classification of field-collected samples. Column 1 contains the chapter of the thesis to which the identification relates. Columns 2 – 4 comprise the taxonomic assignations of the CFF and Column 5 explains whether the genus of CFF identified is new (i.e. not contained in the initial literature review of known CFF).*

Increasing the level of understanding and the curation of an increasingly extensive list of species capable of forming cords will help to challenge the ideas surrounding the putative

origins of cord-forming as a condition. As more species are identified as cord-forming (or not) the evolutionary history of CFF can be identified addressing the debate as to whether cord-forming has evolved multiple times or perhaps once or twice with multiple subsequent losses.

Therefore, with increased molecular identification of field-harvested CFF, these phylogenies can be supplemented to test the new theories relating to the evolutionary history of CFF. These techniques are already being incorporated into new field studies and therefore, in time, our understanding of the evolution of the cord-forming condition will be increased. Theories can be tested and disproved until the most parsimonious explanation is acquired and supported by more extensive data, thereby providing a clearer picture.

Which ecological, environmental and landscape features are characteristic of CFF and determine the presence of CFF?

Ecological specificity of CFF (Lundy, Felix Dennis and Logs)

This research programme has identified a number of ways in which CFF demonstrate specific ecological features and indicates the potential woodland function of CFF.

During the study on Lundy Island, Bristol Channel, CFF were identified in a location they had not been recorded previously and where traditional wisdom would not have expected them to occur; as *Rhododendron* was cleared and successional trajectories were followed by the plant and fungal communities on barren land (Chapter 5). Whilst the purpose of this study was to explore the re-colonisation of these areas and the fungal and plant communities that re-established, and their features and identities; a beneficial side effect of the project was to increase our understanding of CFF in remote areas. The study identified a CFF (*Megasporoporiella rhododendri*) growing within the woody stumps of cleared

Rhododendron where deadwood was arranged into windrows. This supports the theory that CFF lie dormant as latently-present propagules within the phloem sap, and growth initiates when the water potential of the sap drops as a result of stress (Boddy, 2001). As there was no other incidence of the CFF species on the island despite it having been the subject of numerous highly detailed fungal surveys, we can suppose that the propagules did indeed arrive within the *Rhododendron* wood stock when it was planted as an ornamental shrub prior to its escape and uncontrolled colonisation of the Eastern Sidelands.

In addition to this work, other components of the research programme have contributed to our understanding of which fungal species would be expected in functionally and ecologically active woodlands. One particularly striking result from the investigation into woodland age was the finding that CFF were only present in established ASNW. Therefore, it could be assumed that establishment of CFF takes in excess of 13 years within a woodland and is dependent upon the depth of litter and presence of coarse woody debris.

Resource and woodland age specificity

Research undertaken as parts of the fieldwork components of this volume has suggested a number of factors that appear to be important to the development of CFF within woodland. Whilst the Heart of England Forest Ecosystem Project (HOEFEP) suggests that CFF are only found in ASNW (Chapter 3), the Lundy project and the Wytham resource identity project (Chapter 4) suggest this is refutable. In both of the latter research projects, CFF were found in areas discordant with the features of ASNW. On Lundy Island, this took the form of *Rhododendron* stumps that had colonised acidic nitrogen-rich grasslands; whilst in Wytham Woods, the CFF *Radulomyces* was found beneath and upon logs placed in grassland sites. However, neither of these are sufficient evidence to dispute the findings of the HOEFEP as the *Rhododendron* had developed gradually into an established stand with

a closed canopy and ready supply of coarse woody debris, mimicking the characteristics of ASNW. Similarly, the grassland sites surveyed as part of the Wytham Woods project were within 50m of the woodland edge and received some oak litter fall as a consequence. Therefore it cannot be categorically stated that these fungi would have initiated were the logs placed in a woodland site well-removed from the ASNW stands in Wytham Woods. In order to untangle this further, more sites should be added to the long term monitoring study on grassland at Wytham Woods, spaced increasingly distant from the woodland. This is a tangible possibility due to the position and land ownership of Wytham Woods and would require the Upper Seeds site and adjacent fields to be recruited into the study. The outcomes from the research project would suggest that this would be a worthwhile addition to the project; distinguishing the woodland edge effects on fungi from the fungal communities that could be expected to develop at a grassland site and whether CFF would be likely to colonise grasslands.

Whilst extending the Wytham Woods logs project would increase its research value, the project has not been valueless to date. Analysis of the data collected at the first harvest point have identified that both coarse woody debris and litter layer species identity are important contributors to the fungal richness that would be expected in a woodland. Furthermore, it is the leaf litter that is identified consistently as a significant factor to fungal species richness and CFF presence/absence. It could be argued that this may be a result of the higher variety of compounds present in leaves as opposed to wood and therefore the increased variety of fungal niches available in the leaf litter pool. However, this was not a focus of the study and therefore cannot be stated as fact. It does however suggest another potentially interesting avenue for further study in the future; considering the physiology and chemical composition of leaf litter and the richness of fungi found upon and within them.

Both the HOEFEP and the Wytham logs project also suggest an important role of pre-existing networks in CFF development. When investigating the effect of plantation age on fungal communities, CFF were only found in one plot; the ASNW plot in which coarse woody debris was left *in situ* and standing deadwood was allowed to remain. This result was unexpected as it was anticipated that CFF would develop in the plantations on agricultural land within the thirteen year time-frame studied. This suggests that existing fungal networks are critical in determining the communities that are likely to develop in newly-planted woodlands, a theory supported by work undertaken as part of the Wytham logs project where distinctive communities in the two woodlands studied were borne out in the site level analyses. Further work is already being undertaken in this field in collaboration with other research teams at Cardiff University (pers. comm.). This work looks to identify the impact of existing fungal communities on fungal development on logs inoculated with novel species. The results of this work are not yet complete, but suggest an important role of the existing networks on placed woodblocks.

Landscape type specificity

All of the field-based elements of the research project combine to paint the bigger picture of CFF as a group of organisms that are at the periphery of our scientific knowledge and which are permanently throwing up new surprises; challenging the assumptions on which fungal investigations have been based previously.

The landscape specificity of fungi is one such area. Whilst some of the investigations (such as the Lundy project) have identified fungi where they would not be expected; others have not found fungi where they would have expected them to be located. One such example is the HOEFEP, which found that newly-planted woodland on ex-agricultural land appears to be mycologically depauperate compared to ASNW. The continuation of this project over subsequent years will enable a more complete picture of the mycological communities to

be created. Although the rapid fungal survey approach created for this thesis is a more reliable way of collecting fungal community information for a site than the quadrat approach, it still does not provide a complete picture of the fungal communities present. Increasing the survey size by adding more collections will increase the likelihood of all species present being detected and thus improve the accuracy and reliability of resulting analyses.

The HOEFEP and the Wytham logs project both suggest that fungal communities differ between grassland, plantation and ASNW. Supporting the work of Humphrey *et al.* (2003, 2005), when comparing plantations on ex-agricultural land and ASNW, the HOEFEP found that species richness and community compositions were significantly different in plantations of all ages when analysed against those of ASNW. Similarly, the Wytham logs project identified that the fungal communities present in grassland sites (even on the woodland edge) were significantly different to those within woodlands with a higher abundance of *Radulomyces* and a lower abundance of saprotrophic pathogens such as *Armillaria*.

Can CFF distributions be modelled effectively and reliably?

The modelling component of the analysis made tangible progress towards informing the creation of species distribution models to map current and to predict future fungal distributions (Monk, in prep (d)). By considering the pitfalls of fungal foray data and the processes by which such data is collected, this work has helped to suggest ways in which these issues can be overcome to generate distribution maps and to direct future foray efforts for target fungi.

As a result of these analyses and models, including a worked case-study, it will become increasingly possible to map and model the current and future distributions of fungi in response to specific questions. Such abilities may enable analyses of the spread, across

Great Britain, of pathogenic saprotrophs such as *Armillaria*, enabling mitigation and/or preventative action to be taken to reduce the economic or ecological impact of such range expansions. Similarly, it would be possible also to consider possible range reductions in many species with specific environmental envelopes enabling rescue or conservation efforts to be implemented. Although these are still only within the realms of possibility, it is these opportunities that have led to the take up and funding of FungiWatch (a spin-off project created in response to these findings) by The Natural History Museum, London.

Having explored the set-backs and limitations of existing collection practices and protocols, FungiWatch will enable the reworking and reorganisation of fungal distribution data collection in the field, making the most of both the skills of interested amateurs and recent advances in smartphone technology, to improve the resolution and reliability of geo-referenced collections and field observations. However, despite highlighting the need for increased collection efforts for some CFF species, it is also true that these analyses have shown that the necessary sample size for an optimal model is between 300 and 1000 records. For many species in the Fungal Records Database of Britain and Ireland (FRDBI) this level of coverage has been reached already and therefore, by using the existing data (subject to cleaning and checking) it may be that novel records can be used to provide an accurate, reliable and comprehensive ground-truthing capacity.

<u>What are the outcomes of the rigorous testing of assumed best practice regarding fungal collection, DNA extraction, phylogenetic analysis and species distribution modelling?</u>

A key theme running throughout the research project is the testing of assumed best practice and the use of novel methods to collect and analyse data. One of the major techniques developed and implemented was the Rapid Fungal Survey (RFS), which was used in the Wytham logs, Lundy and HOEFEP elements. This approach was inspired and based upon

the rapid botanical survey (RBS) approach of Hawthorne and Abu-Juam (1995), which suggested a time-constrained as opposed to a space-constrained survey approach to increase the likelihood of discovering less common species when surveying an area. This approach was invaluable during the course of the HOEFEP when fungal species were highly-dispersed across space and in which a highly-changeable number of volunteers would have made a space-constrained survey approach difficult. Therefore, controlling survey effort by man hours proved highly effective in the field and led to the discovery of a wider range of fungi from an increasingly diffuse area. A key recommendation arising from this research programme, when overall fungal community composition is the objective of a survey, is that the RFS be used with the caveat that samples must be taken of each fungus identified to enable molecular identification to occur, increasing the reliability of identifications and providing a greater volume of data for use in further phylogenetic analyses.

When collecting fungal samples for molecular identification, the research programme further tested a number of different preservation and extraction techniques. Ultimately, the use of FTA cards for field sampling was found to be a highly-reliable method for collecting fungal sporocarp data. However, it was not a consistently successful method for the collection of cord samples due to their much lower water content. During the testing phase of the thesis work, it was identified that cord samples were best preserved by collection in Eppendorfs before being cleaned and stored at -20°C as soon as practicable. During the HOEFEP and the Lundy project it was not possible to freeze the samples on the day of collection and was necessary to use an alternative but slightly less robust preservation method: drying samples with silica gel. This method resulted in a slightly higher loss and contamination rates. However, in the absence of access to freezers, this research project still considers it to be the optimal collection strategy in longer length field collection situations.

The final advances made during the research project, are those relating to the modelling of fungal distributions and the curation of fungal distribution data. As discussed previously, suggested improvements and approaches to species distribution modelling for fungi have been made. In addition, the project considered the need for improvements to the fungal distribution datasets. At the outset, fungal distribution data was collected in one of two depositories; the Fungal Records Database of Britain and Ireland (FRDBI), or CATE 2. The FRDBI was used in this project as a result of it being available through a link with the British Mycological Society (BMS). When the quality of this data was interrogated, large inconsistencies were found in the standard of the geo-referencing of records in the database. Many records of fungi collected had latitude/longitude and 6 figure grid references that were unrelated to one another, and cleaning was required to increase the usability of the data for modelling. This cleaning approach is likely to be used to create baseline datasets for a number of other 'stories' for use in the FungiWatch crowd-sourcing programme, initiated as a consequence of this work. Therefore, improvements to the quality of geo-referenced data will lead to increases in the robustness and reliability of pre-existing records in the database with tangible uses and results for fungal projects in the future.

What further work should be done in order to enhance our knowledge of CFF ecology, diversity and distribution in the future? What are the new crucial unanswered questions?

A number of projects have already been implemented in response to the research programme, some of which have been alluded to already. These tend to revolve around further questions, developed in response to the findings. These questions can be defined broadly as:

1. What are the origins of the CFF condition and how many times have CFF arisen?

2. What impact do existing fungal networks have on fungal introductions in a woodland area?
3. What is the impact of resource compound variety on the species richness of saprotrophic fungi colonising it?
4. Do CFF develop on grasslands which are not found at woodland edges?
5. How can models be supplemented with reliable geo-referenced data and accurately and comprehensively ground-truthed?

These questions and current responses to them are discussed in detail below.

1) What are the origins of the CFF condition and how many times has CFF arisen?

By incorporating molecular identifications into the majority of fungal fieldwork, it will be possible to test the hypotheses generated in the research programme relating to the possible origins of the cord-forming condition.

When samples of CFF are analysed and their DNA sequenced and placed in GenBank, the size of dataset available for phylogenetic analysis will be much greater. Therefore, it would be expected that this would lead to much greater resolution and coverage across the fungal kingdom. Having investigated appropriate preservation and extraction protocols, it is hoped that the number of field investigations incorporating molecular identification techniques will rise. By increasing the awareness of the need for increased fungal DNA records, it is hoped that subsequent phylogenetic analyses will be much better able to prove or disprove the ideas mooted in this thesis.

2) What impact do existing fungal networks have on fungal introductions in a woodland area?

Work currently underway in combination with Cardiff University (pers. comm.) is considering the impact of existing CFF networks on the success and succession

characteristics of introduced fungi on inoculated wood blocks. In order to achieve this, a 20x20m plot has been mapped and samples of all CFF systems taken and identified using molecular methods. Sites have been revisited and analyses will be carried out by the team from Cardiff University to identify the extent to which samples from the inoculated wood blocks were able to colonise the area and extend to new woody resources.

Such work will continue to establish the foundations for fungal conservation and management efforts in temperate woodlands by identifying the extent to which efforts to introduce woodland specific fungi would be successful.

3) **What is the impact of resource compound variety on the species richness of saprotrophic fungi colonising it?**

As leaf litter appears to be a significant factor influencing the species richness of CFF communities establishing on and around coarse woody debris, it would be a prudent next step to consider the causes of this effect. A hypothesis may be that the effects seen are a result of the variety of compounds found in leaf litter and the associated niche spaces available to CFF. This hypothesis is partly a consequence of the lack of significance of CWD species identity on fungal species richness, possible a result of the lower variability of CWD compounds.

In order to address these questions, further collections from both the Wytham logs and the HOEFEP sites could be accompanied by leaf collections. These leaves could then be analysed using gas chromatography mass spectroscopy and then used in the reanalysis as an additional effect in the model.

4) **Do CFF develop on grasslands which are not found at woodland edges?**

The Wytham Woods logs experiment uncovered CFF in a grassland habitat. This was unexpected and therefore a source of additional questions. Namely, were CFF found

because the grassland site was situated on the woodland edge and therefore affected by litter fall and other edge effects? One proposal would be to extend the existing Wytham logs experiment to encompass more grassland sites at increasing distances from the woodland edge. This would be able to investigate woodland edge effects in combination with the factors already studied.

By understanding the diversity of habitats in which CFF can occur, it will be possible to maintain appropriate assumptions in future investigations and consider the full suite of benefits and roles that CFF may have in temperate ecosystems.

5) How can models be supplemented with reliable geo-references data and accurately and comprehensively ground-truthed?

One of the most exciting by-products of the research project has been a result of the modelling element. During the course of this project, it became clear that the coverage, resolution and quality of fungal records taken within Great Britain are not currently of a high enough standard to generate consistently high quality current and future predictions. Therefore, it is necessary to consider ways in which the quality and quantity of records can be increased from this point onwards so that modelling all fungal species becomes a real possibility. These requirements and ideas led to the concept of a crowd-sourced, smartphone app-based recording programme. This programme, currently named FungiWatch, is in the early pilot stages (funded by The Natural History Museum, London) and aims to use the reliability of GPS technology and tap into the public interest in fungal science to fill gaps in our knowledge of fungal distributions across the United Kingdom. Such a directed, technology-based approach should also yield the additional benefit of providing a cohort of recorders capable of ground-truthing models rapidly, increasing the speed with which many mapping, modelling and prediction studies can occur.

Providing the basis for woodland ecological theory using novel collection protocols to investigate the role of cord-forming fungi as a component of woodland systems

This research programme has generated findings which test and challenge a number of assumptions. Such assumptions have been held true, as in the case of the assumption that CFF are predominantly a group of organisms associated with ASNW; an idea supported by the HOEFEP and Wytham logs projects. Similarly, the idea that CFF is an organism that requires closed canopy, native species mixes to establish has been called into question by the Lundy project, which identified the CFF *Megasporoporiella rhododendri* on standing deadwood of cleared *Rhododendron* stands.

Other assumptions have been challenged by the findings of the project. One such result is that deadwood identity is critical for CFF. In fact, the results of our analyses suggest that providing fallen and standing dead wood is present CFF can colonise. This result has been found both as a part of the Lundy and Wytham logs projects. Also, the idea of CFF as having multiple origins has been brought into question by the phylogenetic elements of this work. Our analyses shed some doubt on this hypothesis. This is by no means a conclusive analysis of the evolutionary history of the CFF. However, it is sufficient to initiate further dialogue on the subject.

The research undertaken in this project provides a talking point and an initial challenge for widely-held assumptions in the field of fungal ecology. It constitutes a crucial first step in increasing the rigour and reliability of fungal field studies, having tested and used a range of techniques to identify the most effective means of collecting data in the field and analysing off-site. Additionally, the subsequent projects would contribute greatly to the public and academic understanding of CFF and their ecology in Great Britain.

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APPENDIX 1: CORD-FORMING FUNGI IN BRITISH WOODLANDS: WHAT ARE THEY AND WHAT DO THEY DO?

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Declaration:

This work was completed in collaboration with Dr Gabriel Hemery with the majority of work being carried out by myself. The paper has been published in 'The Quarterly Journal of Forestry' where it has won the RFS James Cup (awarded to the best article in 'The Quarterly Journal of Forestry' each year). The introduction is largely based on information contained in this paper.

Signed:

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CORD-FORMING FUNGI IN BRITISH WOODLANDS

WHAT THEY ARE AND WHAT THEY DO

Kirsty Monk and **Gabriel Hemery** investigate the role and importance of this lesser known group of ecosystem engineers in British woodlands, examine the extent of our fungal knowledge and discuss their implications for forestry in the future.

Forests and woodlands are dynamic ecosystems dependent upon a vast array of assemblages, guilds and communities (see Glossary below) to maintain their health and productivity. Many communities, such as trees and ground level vegetation, have been well characterised in the literature, but this level of knowledge does not extend to all components of a woodland.

Saprotrophic fungi, often considered to be indicators of healthy and productive woodlands, are assumed to be passengers rather than drivers of such success. This review refutes this idea, proposing that wood decay fungi are critical ecosystem engineers; the fundamental components maintaining and enhancing energy flows and nutrient dynamics across the woodland network.

The role of fungi for wood decomposition was discussed first by Herman Schacht in 1863 (Blanchette, 1991). Despite extensive research examining fungal dynamics, importance and ecology; many woodland management practices, large scale experiments and policy decisions do not account for fungi and their associated mechanisms.

Fungi should be considered in new plantings and, in particular, for restoration activities where their ability to maintain and modify soil structure, facilitate colonisation by tree species and moderate nutrient cycling can be the difference between success and failure.

Cord-forming fungi

Cord-forming fungi are arguably the most instrumental saprotrophic assemblage, both in

Glossary

Assemblage – a collection of species as a subset of a community which are not all from the same species and which do not all perform the same function within the community.

Community – a collection of species within a geographically defined region as a subset of an ecosystem whose functions and niches complement one another and which are not all of the same taxonomic group.

Guild – a collection of species as a subset of a community which are not all from the same species but which perform a similar function within the community.

Mycorrhization – The attachment of mycorrhizal fungi to plant roots creating a mutually beneficial scenario whereby the fungus enhances the ability of the plant to harvest soil nutrients and the plant supplements the fungal carbohydrate stock as the phloem sap is tapped by fungal hyphae.

Propagule – a dormant fungal life stage, an uninitiated spore.

Sporocarp – The visible portion of a fungus that extends above ground producing spores, the means of dispersal of a fungus.

Figure 1. *Megacollybia platyphylla* cord from an ASNW in Warwickshire.

terms of wood decay and woodland responses to disturbance. The white fragile threads or hyphae of fungi will be a familiar sight to anyone who has brushed aside rotting leaf litter on the forest floor. Searching more thoroughly at the interface between leaf litter and the top soil layer will often reveal instances where the hyphae have come together to form thicker tube-like organs called 'cords' as in Figure 1.

Cord-forming ability is not found in all fungi and seems to be restricted to two groups: firstly the ascomycetes are those fungi that form sac-like structures (asci) to hold their spores, e.g. *Xylaria polymorpha* (Dead man's fingers) (Figure 2), and secondly; the basidiomycetes are those fungi whose spores are held on the end of a club-like cell (basidium) e.g. *Hydnum repandum* (Hedgehog fungus) (Figure 3). Within the cord organ the outer hyphae, waterproofed by reinforcement with salts, protect the inner hyphae, which become adapted to mass flow by loss of cross walls.

Perturbations to cord-forming fungal communities may be detrimental to woodland functioning, which is the subject for a project established recently in the New Forest by the Forestry Commission. In addition to their role in mobilising nutrients from deadwood and litter resources, cord-forming fungi themselves constitute a vital nutrient pool as they incorporate a proportion of mobilised nutrients into their networks and sporocarps, storing 18% phosphate (P) and 20% nitrogen (N) of that contained in the soil nutrient pool (Wells and Boddy, 2002).

Cord-forming fungal interactions, both with other fungi and with other taxa such as vascular plants, have cascading effects across trophic levels, extending from biotic systems to abiotic factors such as nutrient regimes, environmental change and carbon sequestration potential. Consideration of cord-forming fungi when managing woodlands could be a simple yet effective way to mitigate the effects of environmental change on temperate woodlands.

Decomposition

As saprotrophs, cord-forming fungi are most often described as decomposers. Higher fungal species richness is associated with increased decay rates as different decomposition specialities, such as delignifying or cellulytic enzyme activities, supplement one another. Many fungi are present in wood as latent propagules, developing once sap water potential drops, indicating imminent tree death.

Mycorrhization, the attachment of fungal hyphae to plant roots in a symbiotic association, further increases the rates of decomposition of fallen and attached deadwood. The enhanced nutritive value of the association is caused by the extra N and P that are taken up by the fungus and passed on to the plant. The more nutrient-rich timber means that the deadwood is more rapidly degraded as it is a preferred substrate for saprotrophic organisms.

Nutrient Cycling

Cord-forming fungi have the capacity to act as source, sink and reservoir for nutrients, extending across the soil-litter interface connecting resource islands such as logs and stumps on the forest floor. Cord-forming fungi have been tracked over 300m across a forest

floor from a decomposing log, linking up other deadwood and foraging for new resources (Thompson and Rayner, 1983). Nutrient transfer occurs via a mass flow process similar to the phloem of vascular plants. Sugars, amino acids and oxygen are all transported suspended in water.

Interactions with other fungi releases nutrients that the competitor or other soil microbes can take up and sequester, or which may end up enriching the soil nutrient pool.

Cord-forming fungi transport accounts for up to 17% of the total N transported in the woodland ecosystem (Tlalka et al, 2002). Cord-forming fungal networks shuttle N around the woodland floor pulsing at a rate significantly greater than that which can be achieved by diffusion alone. Cord-forming colonies co-ordinate nutrient pulses to enable rapid source-to-sink cycling. Sinks tend to be carbon-rich growing tips or expansion fronts where N supports rapid production of biomass.

Using cord-forming fungi for restoration

It may take centuries for introduced fungal species to integrate fully into pre-existing fungal networks. However, interaction dynamics and their downstream effects are present from the first incidence of colonisation.

Cord-forming fungi interact with one another hierarchically and are able to influence the activity and growth of other individuals through exudates and nutrient reallocation. Some cord-forming fungi grow more vigorously than others and are able to displace less aggressive species on woody resources. When two evenly-matched fungal competitors come into contact with one another in a woody resource, they exude volatile organic compounds creating a competition zone (C-Zone) beyond which neither mycelia extends (Boddy, 1993). It is this C-zone that creates the spalting effect in beech (*Fagus sylvatica*) and other tree species.

Fungal colonisation can occur from multiple sources, including: the airborne spore pool, the soil reservoir and animal dispersal. It is also possible to introduce specific fungi through inoculated wood blocks.

Fungal species richness does not differ significantly between plantations on ancient woodland sites (PAWS) and ancient semi-natural woodland (ASNW). However, the species composition in such regions does differ along with the functional diversity of PAWS and

Figure 2. *Xylaria polymorpha* on oak stump in Warwickshire.

Figure 3. *Hydnum repandum* on deciduous litter in Warwickshire.

ASNW fungal flora. On-going research is addressing this issue and suggests currently that there is enhanced ecosystem engineering activity in ASNW sites (Monk, unpublished).

Economics and Disease resistance

Cord-forming fungi may affect root pathogen activity as a consequence of their exudates, making them potentially important biocontrol agents. For example, *Hypholoma fasciculare* (HFDD2; Huds: Fr) Kumner and *Phanerochaete velutina* (PV29; DC: Pers.) Parmasto are effective suppressants of *Armillaria* root rot (Figure 2). Such actions mean that cord-forming fungi are economically important organisms. In South Carolina alone *Armillaria* root rot is estimated to directly cost the peach industry $4,000,000 per year.

Informing woodland practices and policy in the light of environmental change.

Cord-forming fungi are a pivotal component of carbon cycling and global carbon budgets (Figure 3). Carbon dioxide is released as a consequence of decomposition, although manipulation and promotion of plant and fungal growth has the potential to increase the overall biomass of the woodland system. Carbon dioxide production increases during mycelial interactions of cord-forming fungi as the energy cost of interactions exceeds the capacity for carbon sequestration (Schlesinger, 1977).

Figure 4. *Armillaria* rhizomorphs on fallen oak in the New Forest, Hampshire.

It could be argued that the first stages of succession are the most species-rich and therefore worth conserving in order to maintain maximal functional diversity across a woodland. However, it is also important to conserve habitats across the succession continuum to prevent the loss of potentially crucial ecosystem services from woodlands.

Current fungal conservation efforts involve leaving brash piles in woodland areas for fungal colonisation. As the majority of fungal spores fall within a few metres of the fruiting body, logs placed in close proximity to colonised logs are more effective at providing colonisation corridors and supporting rare fungi than less spatially dense deadwood clusters.

Research has identified many other features of cord-forming fungi that can be used to encourage their establishment in woodlands. Dowson et al. (1988) noted that significantly higher cord extension rates were observed in the spring. Therefore, in order to more rapidly integrate cord systems into a woodland network, introduction efforts would be better advised in the spring months. Introductions can be further enhanced by providing the correct substrates for growth. Lindhe's (2004) study found that cord-forming fungal richness is correlated positively with log diameter and the availability of logs as a substrate rather than stumps. Both of these means of increasing cord-forming fungal diversity are simply achieved and could be incorporated readily into management regimes in British woodlands.

Current work by the lead author seeks to understand the processes occurring in newly-planted woodlands on ex-agricultural land. It is hoped that, when complete, this work will provide the evidence base for informing planting regimes that could lead to more robust new woodlands.

In addition to the ways in which management can actively promote fungal colonisation, it may also inhibit or otherwise alter natural regeneration. Management regimes may affect fungi and their competitive interactions indirectly by altering temperature or water regimes. As the extension rate of cord-forming fungi is highly dependent upon these factors, extremes <5º and >30ºC and water potentials <1.5MPa lead to cessation of growth. It is imperative that we consider fungal requirements and dynamics in woodland management planning, for example, by creating wind breaks and planting saplings closer together to increase humidity and temperature in newly planted woodlands.

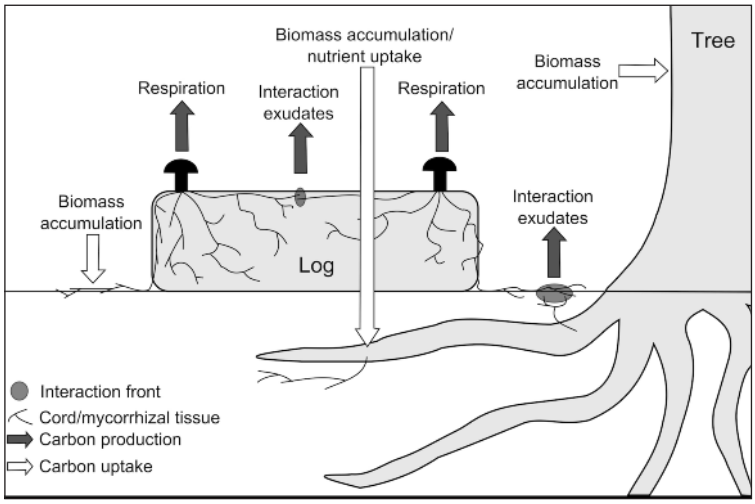

Figure 5. Carbon dynamics in a woodland ecosystem. Dark arrows indicate carbon loss and light arrows indicate carbon uptake.

Fungal communities are dynamic and their hyphal networks are changing constantly to accommodate fluctuations in local environmental conditions and the patchy nature of deadwood resources. By remaining open minded about woodland management strategies and the manner in which these can be modified to support fungal communities, we can create dynamic solutions to promote fungal diversity and retain the full range of ecosystem engineers in the woodland system.

Conclusions

On-going research is uncovering the numerous ways in which cord-forming fungi enhance and encourage woodland growth, health and productivity. Incorporating our knowledge of fungal systems and their ecosystem roles into woodland management planning will help maintain healthy and productive woodlands now and in a future of uncertain environmental change.

The roles of fungi in woodlands are broad, ranging from nutrient cycling, through decomposition to disease control. Simple steps to manage woodlands for fungi need not be costly or time consuming and may make significant differences to the economics and health of woodlands in Britain.

Small changes such as increasing the amount of deadwood on the woodland floor and its spatial distribution can encourage cord-forming fungi colonisation and perpetuation. Encouraging colonisation of specific fungal guilds into an area may endow woodland with the ecosystem functions it lacks, an activity specifically useful to new plantings.

The time has come to consider all components of woodland ecosystems when managing for timber or woodland products. Future improvements to timber yields and woodland health will lie in improving nutrient cycling and woodland resilience, especially in the light of projected environmental change and the uncertainty it presents to woodland owners and managers.

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