

**Fungal communities of beech (*Fagus sylvatica*) trees:
Heart rot and origins of decay**

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

by

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Dedication

To Andrew, who passed away in October 2015.

A passionate conservationist, who won the category 'Most Beautiful' for his collection of an orange peel fungus (*Aleuria aurantia*) during our first fungal foray together at Moelyci, Gwynedd in 2012.



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Summary

Heart rot and hollowing of tree trunks is an ecologically important phenomenon and is the result of fungal decay of wood. Despite the value of heart rot habitats to thousands of species, globally, little is known about the development of the fungal communities which give rise to them. This thesis explores these aspects in beech (*Fagus sylvatica*) trees.

Destructive and non-destructive methods were used to survey for wood decay fungi in beech trunks. The predominant species identified depended on the survey method employed. In mature trees with no external indicators of heartwood decay, overall incidence of decay was 45%, and a species of *Pholiota* in the *adiposa-aurivella* complex was detected most frequently. In recently fallen and felled beech trunks, however, the ascomycete *Kretzschmaria deusta* appeared most frequently, and was the likely contributor to tree windthrow in most of these cases. Examination of the spatial aspects of fungal communities in trunks permitted observations relating to heartwood patterning and the categorisation of species as principally causing butt or top-rots, and as cavity formers.

Metabarcoding of the universal fungal barcode (ITS), and a second barcode (LSU), revealed the distribution and diversity of endophytes, or latent fungi, in functional sapwood of standing trunks. Together, the metabarcoding approach detected a higher diversity of taxa than did isolation of fungi from wood dust samples. Fungal community composition varied between trees at different sampling sites. Overall, several heart rot fungi were detected throughout functional sapwood of trunks, providing evidence that heart rot can, in principle, develop from latent propagules.

Interactions between heart rot species other beech decay fungi were compared in wood block pairings and on agar media. Species rankings based on scored outcomes revealed different relative combative ability depending on the medium on which the interactions took place. Of the species examined, *Hypholoma fasciculare* and *Fomitopsis pinicola* were the most combative, whilst other heart rot fungi showed no clear trend towards high or low combativeness. Competitive interactions alone were, thus, not sufficient to explain fungal community development in beech trunks.

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Chapter 1

Introduction

1.1. Background

Woody plants comprise much of the 450 Gt of plant matter on land (Bar-On et al., 2018), and this immense carbon and nutrient store is locked up in over three trillion trees (Crowther et al., 2015). Understanding where, when and how carbon moves between the Earth and the atmosphere remains one of the most vital research pursuits in modern-day environmental science, underpinning a global response to the present climate emergency (Ripple et al., 2019).

Fungi are the main agents of wood decay, responsible for turning plant material from recalcitrant structures into smaller, constituent molecules. Beginning in the standing tree, decomposition is completed on the forest floor where wood eventually becomes soil organic matter (Boddy, 2001). As well as functioning to recycle nutrients, fungi alter the availability of resources — both their nature and distribution— and are considered to be ecosystem engineers of forests (Lonsdale et al., 2008).

Various measures also show that species and populations are in decline and going extinct at alarming rates (Ceballos et al., 2017; Eisenhauer et al., 2019). Forest ecosystems remain under anthropogenic pressure, though rates of tree loss have slowed (IPBES, 2019). The ever-increasing demand for forest products results in clearing and degradation of forests. There also exists a lingering perception of dead or decaying wood as an undesirable reservoir for pests and disease (Winter, 1993; van der Wal et al., 2017), which has contributed to extensive forest sanitation practices and ‘dull and tidy’ woodlands (Elton, 1966). In these ecosystems, saproxylic organisms are particularly under threat. These are species which require decaying wood for some of their life cycle (e.g. as a direct food resource or a habitat) (Stokland et al., 2012). Only in recent decades has dead and decaying wood been recognised as component of ecological processes (Franklin et al., 1987), including both small portions such as twigs and branches (known as fine wood debris; FWD), and large portions such as limbs and trunks (coarse woody debris; CWD).

Large, old trees play critical and unique roles which cannot be provided by small trees, across many ecosystem types (Lindenmayer and Laurance, 2016). They

provide habitat for thousands of species of animals, plants and fungi, serving as an 'arboreal megalopolis' (Speight, 1989). Large, old trees contain substantial amounts of decaying wood, hosting saproxylic communities in different parts of a tree, which become further differentiated as decay progresses. Yet, they are a declining resource, subject to high rates of loss and low, or inconsistent rates of recruitment. Despite their importance, large, old trees receive inadequate protection (Lindenmayer et al., 2014).

Given the dual crises of climate change and biodiversity loss, there is a need to understand and better value the processes which play out in large, old trees, and give rise to their exceptional qualities. These processes play out over centuries but begin with stem or trunk decays in standing trees - this is the phenomenon called heart rot.

1.2. Heart rot

There has been interest in wood decaying fungi for as long as people have experienced the inconvenient degradation of various timber products or wood 'in service'. The connection between decay and the appearance of fruiting bodies, hereafter sporocarps, is easily made by the most casual observer. Heart rot, as wood decay in the interior of tree trunks and larger branches is not a disease or pathological condition (Boyce, 1938). Despite this wood not being functional to the tree in terms of water conduction, heart rot is still often regarded as a pathology (Regué et al., 2019) as discolouration or decay in trunks reduces production volumes and profitability in the forestry industry. Thus, since the early 20th century there has been a literature describing the principal fungal decays of timber (Cartwright and Findlay, 1946 and therein) and of heart rots (Wagener and Davidson, 1954 and therein).

1.2.1. Wood structure and heartwood formation

Trees have a complex internal structure comprising different cell types. In woody stems, two kinds of secondary meristem are responsible for cell division which cause lateral growth of the stem. The cork cambium produces cells which make up periderm, which includes bark. Behind the periderm, the vascular cambium produces phloem on the outer side and xylem (wood) on the inner side. In seasonal environments, production of new xylem from spring through to autumn produces

annual growth rings which are easily counted. Xylem comprises tracheary elements and fibres, which are concerned with conducting water, and surrounding these are living parenchyma cells which have a range of functions, including: storage and transportation of nutrients, and plant defence (Morris et al. 2016). Parenchyma cells are arranged axially around vessels and also distally as rays. The bulk of xylem is referred to as sapwood, which contains living cells and reserve materials. By definition it is usually, but not always, functional in water conduction (Evert, 2006).

With increasing tree age, the innermost sapwood is eventually converted to heartwood, and is chemically altered. In contrast to sapwood, heartwood contains reserve substances but, crucially, no living cells (Evert, 2006). The conversion of sapwood to heartwood is a programmed process, although when this begins in the life of a tree varies between species. The proportion of sapwood to heartwood found within a trunk, the chemical differences and visible distinction between the two types of wood, can also vary widely. For example, there can be marked differences in the types and amounts of extractive compounds deposited in the wood (Taylor et al., 2002). For these reasons there are several terms describing or characterising heartwood. One notable distinction is between species that develop a 'true' heartwood and those that form a ripewood. The latter is considered to be a light-coloured heartwood (Bosshard, 1968 in Hillis, 1987), which is without, or with fewer, extractives. The relative susceptibility of ripewood trees to fungal decay is offered as an explanation for the shorter typical lifespan of these species (Lonsdale, 2013).

As heartwood formation is considered a mechanism for nutrient reclamation, heart rot or heartwood decay is a logical extension of this. The development of discrete cavities or large piping hollows within trees is a consequence of heart rot, and is suggested to be an adaptive trait allowing recycling of nutrients (Janzen, 1976; Fig. 1.1a) which may be in short supply as trees become very large. Hollowing has been described as a 'judicious lack of investment' (Ruxton, 2014), whereby increasing volumes of wood, due to incremental sapwood growth, become too metabolically costly to maintain or actively defend from pests or pathogens. Trees with hollows may often maintain their structural integrity far longer than trees without hollows, as evidenced by the high proportion of very old trees in the UK with extensive decay and large hollows.

1.2.2. Constraints on decay development

The theory of compartmentalisation of decay in trees (CODIT) (Shigo & Marx, 1977; Shigo, 1979) has been the prevailing model which explains decay development in trees. It was argued that the concept of heart rot was too simplistic as it failed to explain decay in trees following wounding (Shortle, 1979), or in trees with no heartwood. The CODIT theory changed the focus to the active defence mechanisms of trees; the main assertions of the theory are the existence of four 'walls', of increasing strength, which prevent spread of decay in a tree in the axial, radial and tangential directions.

By demonstrating that growth and activity of wood decay fungi is favoured by aeration, Boddy and Rayner (1983) showed that patterns of decay in living trees need not necessarily be explained by compartmentalisation. They argued that microenvironmental restriction also played a major role. Water conduction in sapwood, and its reciprocal relationship with aeration, is sufficient to explain patterns of decay caused by fungi. It was highlighted that walls 1-3 of CODIT are anatomical hurdles rather than walls, which slow down rather than prevent fungal spread. Even the most effective wall, wall 4, which is produced by sapwood following wounding is eventually breached. Passive environmental restriction and active tree defence need not be mutually exclusive explanations of decay, as explained in detail by Pearce (1996). Now, CODIT remains recognised as approach to explaining and understanding defence processes in trees, and its applicability has been widened to encompass micro- to macro- scale forms of plant defence from leaves through to heartwood (Morris et al. 2019).

1.2.3. Ecology of heart rot fungi

Fungi may enter and establish in living trees through a number of possible routes (Rayner and Boddy, 1988), including: damaged roots, buried branch stubs (Etheridge and Craig, 1976), exposed heartwood through cuts or breakage of limbs (Fig. 1.1b). Fungi seem also to passively accumulate in wood; propagules such as spores or yeasts exist in a dormant phase within sapwood of living trees. These propagules may never become active as mycelium, or only after many years when the microenvironment becomes more favourable, i.e. when water conduction is interrupted and wood becomes aerated. The rapid appearance of strip cankers along beech bark following periods of abiotic stress (drought), provides one example: long cankers caused by *Eutypa spinosa* or *Biscogniauxia nummularia* can

develop in xylem from numerous propagule foci in the trunk (Hendry et al., 1998). Occurrence of other latently present asco- and basidiomycete fungi has also been demonstrated in branch wood under different drying regimes (Chapela and Boddy, 1988a) and by specific primer assays (Parfitt et al., 2010).

Most wood decaying species belong to the Basidiomycota (Naranjo-Ortiz and Gabaldón, 2019) particularly, but not only, within the class Agaricomycetes. This class is capable of substantial lignin decay, with the evolution of peroxidases over 300 million years ago (Floudas et al., 2012). Yet, only a small proportion of wood decaying species are thought to cause heart rot. This is probably due to the specialised microenvironment of heartwood, which is usually unique to tree species. Heart rot fungi are often associated with a particular tree species or genus, with a range of factors likely to be contributing to host specificity. Very close associations of fungi between tree species or genera probably relate to specific wood chemistry. In trees with especially selective heartwoods, very high specificity is shown. For example, it is often reported that *Fistulina hepatica*, closely associated with *Quercus* and *Castanea*, can utilise tannins as its sole carbon source (Cartwright 1937).

High water content and lack of aeration normally limits decay development, although one experimental study of a small number of heart and sap rot fungi found little differences between their responses to low oxygen levels (Highley et al., 1983). Very few other studies examining the growth requirements of heart rot fungi are reported in the literature. There are, however, numerous series of studies describing the wood decay capabilities of a range of species, with a focus on wood properties relevant to arboriculture (Schwarze & Baum, 2000; Baum & Schwarze, 2007; Bari et al. 2015). Such studies also address species ability to colonise sapwood, and regard some heart rot fungi as neither entirely saprotrophic or necrotrophic. Lonsdale (2004) suggests that the age a tree eventually attains may be related to the heart rot fungus present.

Although fungi decay wood components differently, different decay types are implicated in the process heart rot. Broadly, decay fungi are regarded as white- or brown- rots, but this is a simplified paradigm which does not capture the range of fungal decay mechanisms (Eaton, 2000; Riley et al., 2014). White rots followed the evolution of lignin (Ayuso-Fernández et al., 2019), and can degrade lignin preferentially to cellulose components (selective white rots), while others degrade lignin and cellulose simultaneously. Brown rots evolved later, losing some of the genes for the enzymatic arsenal of white rot relatives. Instead, these polymerise cellulose and leave behind lignin residues and wood which is brown and brittle in



Figure 1.1. (A) Large beech pollard featuring large sporocarps of *Ganoderma adspersum*. The large, open hollow contains well-decayed wood, tending towards soil, with aerial roots growing down into this material. (B) Entry and exit routes by fungal sporocarps. (C) Brown rot decay which visually distinguished by dark colour and cubical appearance. (D) The ascomycete *Kretzschmaria deusta*, featuring the anamorphic (non-sexual; white/ grey) and teleomorphic (sexually reproductive; black) stages. (E) White rot fungi of different species forming interaction zone lines between territories.

appearance (Fig. 1.1c). Soft rot types are further identified as a decay type, although the wood superficially appears like a white rot.

1.2.4. Development of heart rot

Heart rot is the result of complex succession involving many organisms. Although animals play a role in the physical breakdown of wood, much of their contribution towards wood decay is via their interactions with fungi; principally the Basidiomycota and some Ascomycota (Fig. 1.1d). Invertebrates (Jacobsen et al., 2015; Ulyshen, 2016) and vertebrates also play a role in propagule dispersal, with woodpeckers shown to be vectors of heart rot fungi (Jusino et al., 2016). There is growing evidence of the role of bacteria in wood decomposition, and of fungus-bacteria interactions in wood, both of which might influence decay processes (Johnston et al., 2016).

After the initial development of decay fungi in wood, communities are considered to be structured by competitive interactions (Boddy, 2000; Hiscox et al., 2018). Fungal competition in wood is competition for space; wood decay fungi occupy distinct territories within which resources are accessible (Fig. 1.1e). On the forest floor, competition for resources results in fierce combat (Boddy, 1993), involving a suite of biochemical activity such as the production of volatile organic compounds (VOCs) (El Arieibi et al., 2016), increased enzyme secretions (Hiscox et al., 2010) and changes in mycelial morphology (Boddy and Hiscox, 2017). After some years of decomposition on the forest floor, little difference is detectable between fungal communities in heartwood and sapwood (Leonhardt et al., 2019). This suggests that, though heartwood might initially contain secondary metabolites which only heart rot fungi can tolerate, these species cannot defend territory against later-colonising species as wood becomes progressively decayed.

1.3. Heart rot habitats and their importance

1.3.1. Hollows and cavities

Trees with heart rot are increasingly recognised as 'keystone structures' (Remm and Löhmus, 2011; Müller et al., 2013) that are valuable for several guilds of wildlife (Fig. 1.2a). The large hollows and cavities or rot-holes of such trees are the result of fungal decay. They appear as the wood becomes progressively decayed, causing what remains to fall away. Often working in concert, animals chew, tunnel into, and

excavate decaying wood. Large hollows provide stable, sheltered dens for bears (Bull et al., 2000; Wei et al., 2019) and roost and nest sites for bats and birds (Kalcounis and Brigham, 1998). The availability of hollows limits population sizes of birds (Newton, 1994), and artificial nest-box provision does not seem to adequately supplement natural hollows formed by fungal decay processes (Le Roux et al., 2016; Ruegger, 2017). Thirty cavity-excavating bird species with have reported associations with fungal decay (Elliott et al. 2019). Birds may be selecting trees with certain decay communities (Jusino et al., 2015), but further evidence suggests they may also facilitate decay development, returning to excavate previously visited trees some years after fungal inoculation (Jusino et al. 2016). In the UK, the three species of woodpecker utilise decaying wood in preference to harder, undecayed wood (Glue & Boswell, 1994) and, as primary excavators, their activity provides nesting habitats for other species.

Saproxyllic invertebrates and fungi are the most diverse groups of organisms supported by heart rot in living trees (Stokland et al., 2012) (Fig. 1.2b). Many invertebrate groups also contain specialist hollow species (Taylor & Ranius 2014). From one large handful of rot-hole contents, invertebrates comprising 13 families were counted (Cuff et al. *unpublished*). Distribution and density of animals is variable, but abundance can be very high, with some studies reporting 500 arthropod individuals per litre of wood from dead branches (Paviour-Smith & Elbourn 1993) and an average 2,500 arthropods per kg of 'wood mould' (Park & Auerbach 1954) - the loose, soily wood and other debris that accumulates in hollows. The most studied component group of saproxyllic communities are beetles, whose larvae comprise most insect biomass in tree hollows (Siitonen and Ranius, 2015). Saproxyllic beetles usually require several years of larval development in decaying wood. A recent IUCN assessment showed 18% of saproxyllic beetles in Europe to be threatened (Cálix et al., 2018).

Invertebrates are suggested to respond to fungal semiochemicals which indicate the presence and types of decaying wood (Davis et al., 2013). Volatile organic compounds (VOCs) from fungi, or suites of compounds, can serve as attractants (Leather et al 2014) and may determine tree-fungus-invertebrate interactions. However, the number of species and VOCs involved in these communities mean it is difficult to unravel the nature of interactions, and the overall importance is unknown (Siebold et al. 2014).



Figure 1.2. (A) Keystone structures: Large, old beech trees. (B) Basal tree hollow with *Armillaria* white rot as home for slugs and worms. (C) *Inonotus cuticularis*, an uncommonly seen fungus but which known to host larval development of the beetle *Dorcatoma ambjoerni*. (D) *Pleurotus cornucopiae*, a species which may only fruit on large diameter beech trunks. (E) *Hericium coralloides*, a tooth fungus, a relative of the protected species *H. erinaceus*.

1.3.2. *Diversity and specificity in heart rot habitats*

Tree hollows host distinct faunal assemblages, likely driven by variation of hollow microclimates (Cuff et al., *unpublished*). For some saproxylic taxa, the type of fungal decay matters, though this has so far been difficult to demonstrate. Distinct assemblages are associated with different decay fungi in given tree hosts (Kaila et al., 1994; Jonsson et al., 2005; Yee et al., 2006). The sporocarps of decay fungi also serve as habitat and food for invertebrates (Alexander 2002; Fig. 1.2c). While sporocarp production is generally seasonal, some fungi produce tough, perennial structures and others are softer and more ephemeral and host different species (Schigel 2007). Such associations between fungal sporocarps and invertebrates may range from generalist to specialist, but the significance of fungi for supporting invertebrate populations is not widely appreciated and detailed research is limited (Schigel 2006; Schigel 2011).

Although all fractions of dead wood are important for fungal diversity, large trunks appear to support heart rot fungi and other specialists that smaller diameter wood does not (Heilmann-Clausen and Christensen, 2004) (Fig. 1.2d). The availability of large volumes of heartwood might provide more opportunities for mycelial development, and space for the persistence of less combative species. Large trees also provide a different microenvironment compared to smaller trees - more stable temperature, moisture and gaseous regimes. The great age of some trees can also provide longer temporal opportunities for the colonisation of decay fungi.

1.4. Fungal communities of beech trees: Heart rot and origins of decay

1.4.1. *Research rationale*

This thesis explores the nature of heart rot fungal communities in beech trees. Beech is a widespread tree in Europe, in its biogeographical history migrated north and northwest from its extent in Italy and the Balkans since the last glacial maximum (Magri, 2008). In the UK, beech is thought to be native to southeast England and southeast Wales (Packham et al., 2012). Nonetheless, it is present across much of the UK, forming a majority component of woodlands of three recognised National Vegetation Classification (NVC) types (Rodwell, 1991) and otherwise is planted in many amenity settings.

Beech is an ecologically important tree species. Several invertebrate species, some plants and fungi found with large, old beech trees have protected status in the UK

under the Wildlife and Countryside Act 1981. Large or old trees can also be termed veteran trees, displaying features such as hollows and cavities that are important for wildlife (Lonsdale, 2013). Areas with large numbers of these trees are also afforded protection. For example, they may be designated as Site of Special Scientific Interest (SSSI) or Special Area of Conservation (SAC) for collections of ancient and veteran trees, or for their saproxylic invertebrate assemblages.

This research examines trees from several protected sites which represent a long continuity of wooded habitat, ancient wood pasture or the practice of pollarding (Appendix 1). Understanding the development of fungal communities in beech trees, may contribute to the conservation management at these sites for individual species and their associates. At Windsor Great Park, for example, the violet click beetle *Limoniscus violaceus* is a particularly rare species which requires basal hollows of beech trees and is found at perhaps only two other sites. Likewise, one of the few protected fungal species *Hericium erinaceus* (Fig. 1.2e) has been recorded from three sites visited.

1.4.2. Thesis outline

The aims of the work are twofold; firstly, to investigate the diversity of species involved in heart rot and subsequent hollowing of beech trunks, and, secondly, to gain insights into routes of colonisation and decay development.

The study reported in Chapter 2 seeks to detect heart rot in asymptomatic trees. This is a preliminary exploration, asking: What are the predominant heart rot fungi in beech trees? At what size or age class can fungi be detected? Trees were sampled with an increment borer at sites across the southern UK. Wood decay species were detected via DNA extraction from wood and isolation of fungi from wood chips from along core lengths.

Chapter 3 describes the fungal communities in trunks of recently fallen or felled trees. Using traditional culture methods and sequencing of isolates, the spatial arrangement and extent of colonisation occupied is determined, providing clues to points of fungal entry and community development.

In Chapter 4, attempts are made to determine which endophytic fungi are present in functional sapwood, and whether heart rot fungi might be latently present. From 10 sites in England and Wales, wood dust samples were collected from both mature trees and small number of young trees. A subset of mature trees were additionally

sampled around the trunk and limbs up into the canopy. A metabarcoding approach using two fungal barcodes was directly compared with cultivation of fungi from the same samples.

In Chapter 5, ecological traits of beech fungi are investigated under laboratory conditions. Hyphal extension rates and wood mass loss capabilities are measured and considered with relation to competitive interactions in wood blocks and agar culture. These findings may aid in the interpretation of heart rot community development through time.

Chapter 6 is a synthesis of work presented in the previous chapters, discussing the wider ecological and practical implications. Key areas for future research are identified and, finally, the main conclusions are summarised.

Chapter 2

A survey of heart rot in standing trees

2.1. Summary

The presence of heart rot in standing trees is difficult to ascertain without the presence of fungal sporocarps, or by destructive sampling. Consequently, it is not known when heart rot begins to develop in trees, and which are the first decay fungi involved in the process. Using an increment borer, this study took samples to survey 55 beech trunks at sites across the southern UK. Fungi were isolated from wood chips excised from wood cores and identified via sequencing of the internal transcribed spacer (ITS) DNA region. The probability of heart rot detection increased with tree size and age, as determined by counting growth rings. A species of the genus *Pholiota* was the most frequent decay fungus, detected in five trees. No decay was detected in trees less than 66 years old. This method is proposed as a means to survey for rare or indicator species.

2.2. Introduction

One of the first signs of heart rot in a standing tree is the appearance of a fungal fruiting body (hereafter, sporocarp) emerging from some portion of the trunk or limbs. Most existing knowledge of fungus-tree associations comes from observations of fungal sporocarps occurring on the tree, and these suggest that many show close associations with particular species or genus (i.e. are host-specific; Boddy *et al.*, 2017).

The likelihood of detection of fungi via sporocarps is known to be problematic, and subject to several biases relating to the timing and frequency of surveys (Halme and Kotiaho, 2011; Straatsma *et al.*, 2001), and the physical size of the taxa observed (Heilmann-Clausen, 2001). Sporocarps are ephemeral structures, and their phenology and persistence is influenced by immediate local conditions. Moreover, seasonal differences across geographical regions make comparisons between surveys difficult (Boddy *et al.*, 2014).

In fallen wood, bark sloughs or decays away and much of the surface-area becomes available for both fungal entry and exit (fruiting) (Dossa *et al.*, 2018). Thus, for coarse and fine woody debris, sporocarp studies have identified decay stage

(Heilmann-Clausen, 2001) and resource size (Abrego and Salcedo, 2011) as two important factors structuring fungal communities.

Fungal colonisation of heartwood is more difficult to infer via sporocarps for several reasons. In a living trunk, heartwood lies at the centre of a trunk comprised of protective bark and a ring of functional, water-conducting sapwood. Heart rot fungi are therefore limited in exit points and can be effectively encased within tree trunks for long periods. Some heart rot fungi also produce large sporocarps, such as woody brackets, and so might fruit only after a critical amount of carbon / nitrogen resource has been acquired, through decay (Moore *et al.*, 2008). Finally, while homokaryons (unmated mycelium) may be long-lived in nature are generally thought to become mated heterokaryons relatively rapidly (Williams *et al.*, 1981). Heartwood colonised by spores could, theoretically however, host homokaryotic mycelia for some time in the absence of other spore inocula, and this could also limit sporocarp production.

Studies which detect mycelium are better for understanding fungal communities. The most informative heart rot surveys at a stand-level have come from forestry plantations, where large numbers of felled trees and stumps are available for study and are more easily sampled. Heart rot fungi identified in this way include: *Chondrosterum purpureum* and *Inonotus radiatus* in *Alnus incarna* (Arhipova *et al.*, 2011a), *Heterobasidion parviporum* in *Picea abies* (Arhipova *et al.*, 2011b), *Phellinus pini* in *Pinus banksiana* (Basham, 1966a), *Inonotus tomentosus* and *Armillaria ostoyae* in *Picea mariana* (Whitney *et al.*, 2002). These studies show some stands to be dominated by one or two basidiomycete species, with a long list of infrequent decay fungi.

Increment borers can be used to assess decay in trunks and are less destructive than felling. Use of cores for studies other than dendrochronology are, to date, limited. Of note are the studies which show that frequency of decay occurrence is substantially underestimated in standing trees (Giordano *et al.*, 2015; Lygis *et al.*, 2005). For example, in *Alnus glutinosa* stands, decay incidence was shown to be 75% with *Inonotus radiatus* present in over 30% of sampled trees (Arhipova *et al.*, 2012).

This chapter describes a non-destructive survey of mature trees. An increment borer was used to extract complete radial cores which could be further used for fungal isolation and annual ring counting. The main aims were to (1) identify the predominant fungi present in the trees, and (2) determine the age at which heart rot

develops. Given the age at which heartwood is said to occur in beech, it is hypothesised that heart rot will only be detected in trees over 80 years old (Hillis, 1987).

2.3. Methods

2.3.1. Sampling and study design

Fifty-five beech (*Fagus sylvatica*) trees were sampled between July and September 2017 from seven sites across the southern UK (Fig. 2.1). Trees deemed visibly healthy (i.e. with no visible decay, wounds or fungal sporocarps), were selected with a minimum distance of 10 m between each individual tree. Trees measured between 50 - 90 cm diameter at breast height (DBH; 1.3 m above ground). This range was chosen to capture the minimum size at which heartwood begins to develop; this was estimated as 50 cm for 80 year old trees (Rozas, 2003). The maximum sampled tree diameter was limited to 90 cm diameter, wherein the pith could be reached with the longest available increment borer (45 cm).

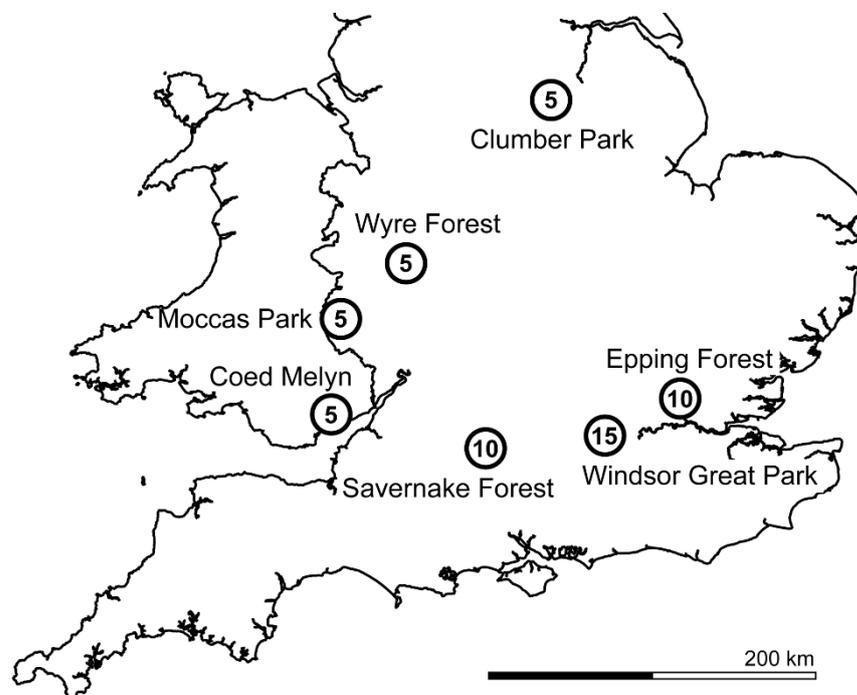


Figure 2.1. Locations of sites within the southern UK. Numbers in symbols indicate number of trees sampled.

2.3.2. Sample Collection

From the south-facing side of each tree, a 5 x 5 cm square of bark was removed, using a hammer and chisel which was wiped with 10% bleach solution and 70% isopropanol immediately prior to use. Care was taken not to touch exposed wood surface when removing the bark square. A 40 or 45 cm length x 5.15 mm diameter increment borer (Haglof) was brushed with 10% bleach, rinsed with 70% isopropanol and then flamed for sterilisation. The borer was left to cool for approximately 1 min before engaging with sapwood, and pushed into the wood, by turning, as far as possible to gain the longest possible core. The borer extractor, sterilised in the same way, was used to extract the core (Fig. 2.2) which was immediately transferred with forceps to an autoclaved plastic straw. Straws were sealed with tape at each end and transported back to the laboratory in an insulated



Figure 2.2. Extraction of wood cores from trunks. (A) Removal of extractor 'spoon' from increment borer, and (B) aseptic handling of wood core prior to insertion into plastic straw. Image courtesy of Dr Jeremy Dagley.

cooler bag. Two adjacent cores per tree were refrigerated at 4°C for less than 24 hours before processing.

2.3.3. Core processing

Cores were handled aseptically in a laminar flow hood and first visually inspected and photographed. The length of each core was noted, together with the extent of wood discolouration and whether decay was suspected. As two cores were extracted per tree, the longer core was used for isolation of fungi and ring counting, while the shorter core was retained in a plastic straw at -80°C for later DNA extraction. Wood chips were excised at ~1 cm intervals along the length of cores (Fig. 2.3). With a sterile scalpel, two cuts, one at a 90° angle and another at 45°, were made to a depth of half the core diameter, yielding wedge-shaped chips. This method allowed both isolation of fungi in relation to the radial position in the tree while retaining observable rings for age estimation. Chips were placed on individual 2% malt extract agar (MA) plates and incubated at 20°C in darkness. These were checked periodically and sub-cultured within 12 weeks to give clean cultures. For trees where no fungal isolations were obtained from the centremost position, the second core, stored at -80°C, was subsequently sampled for DNA extraction. In these cases a chip was excised from either the identified centre of the tree or, if there were visual clues, from the region corresponding with discolouration seen in the first core.

2.3.4. Species identification

Fungal cultures from the centremost position in the tree, or from adjacent discoloured regions, were processed for DNA extraction and sequencing. All other fungal isolations were retained on agar slopes for long term storage at 10°C. DNA

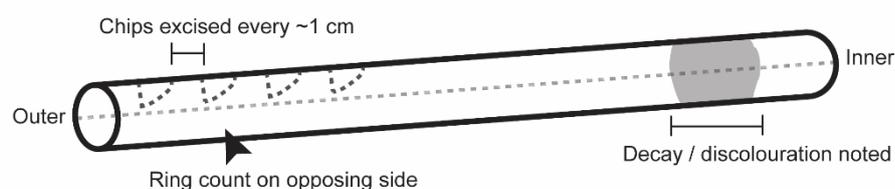


Figure 2.3. Representation of wood chip sampling scheme along wood core length.

was extracted from mycelium grown in pure culture using a DNEasy Plant Mini Kit (Qiagen) following manufacturer's instructions. The full internal transcribed spacer (ITS) region was amplified with forward primer ITS1F (5'CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993) and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.*, 1990) in 25 µl reactions containing: 1.25 µl template DNA, 1 µl of forward and reverse primer (10 µM and 3 µM, respectively), 0.125 µl Taq polymerase with 5 µl buffer (New England Biolabs), and 16.625 µl molecular grade water. The thermocycler program was as follows: 95°C for 3 min, followed by 35 - 37 cycles of: 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, with a final 72°C extension for 10 min. PCR products were visualised on Sybr Safe (Invitrogen) gel, purified with a QIAQuick Purification Kit (Qiagen) and quantified on a Qubit 3.0 fluorometer (Invitrogen). Sequencing was performed in one direction by Eurofins Genomics.

Wood samples were cut into fine fragments with secateurs within a laminar flow hood. DNA was extracted from 0.25 g of this wood with a PowerSoil kit (Qiagen) following manufacturer's instructions, except for a modified homogenisation step in Fast Prep machine (2 x 30 s at 4 ms⁻¹). Long amplicons of the whole fungal ITS region were difficult to amplify from wood, so the shorter ITS2 region was amplified using primer pair gITS7 (5'GTGARTCATCGARTCTTTG-3'; Ihrmark *et al.*, 2012) and ITS4.

Taxa were identified by alignment with sequences in the UNITE database, using the massBLASter program within the PlutoF workbench (Abarenkov *et al.*, 2010). UNITE comprises well-annotated fungal ITS sequences, initially for identification of ectomycorrhizal fungi, but now contains good coverage of basidiomycetes within the northern hemisphere (Rolf Henrik Nilsson *et al.*, 2019). Taxa were assigned to putative ecological roles using the open-source database FUNGuild (Nguyen *et al.*, 2016).

2.3.5. Estimation of tree age

Following extraction of wood chips, cores were gently air-dried for several weeks before being glued into wooden mounts. To produce a flat surface with visible tree rings, cores were successively sanded with rough through to fine sandpaper. Annual rings were counted under a dissecting microscope at x20 magnification. A complete ring count was not possible for some cores, as the centre of the tree was missed or not reached. For these partial cores, tree age was estimated using the

formula:

$$a = c + r/g$$

Where c is the number of counted rings, r is the missing radius length (cm) and g is the growth rate (cm y^{-1}) over the length of the core, as it was measured before air-drying (Ranius *et al.*, 2009).

2.3.6. Statistical analysis

Analysis was performed in R (version 3.4.4) (R Core Team, 2018) within Rstudio (version 1.1.442) using packages dplyr (Wickham *et al.*, 2018). Model and data summary plots were produced using ggplot2 (Wickham, 2016) and cowplot (Wilke, 2019).

A linear regression was fitted to DBH and Age. Initially age estimates from complete and partial ring counts, with interaction terms, were included. This interaction was not significant; whether the age estimates were based on a full or partial ring count did not significantly alter the slope of the line. Thus, the regression line was fitted to all data points.

A generalised linear model ('glm' function in R), using a binomial distribution and 'probit' link was fitted to heart rot presence/ absence data to examine the probability of heart rot using explanatory variables. Initially the variables age, DBH and site, were included. The model was refined using multi-model inference (Burnham and Anderson, 2004) using package 'bbmle' (Bolker and Team, 2017), whereby the best model is identified by ranking models according to information criteria. Due to separation within the predictor variables, penalised logistic regression models were also fitted with the 'logistf' package (Heinze and Ploner, 2018), which uses Firth's bias reduction method of maximum likelihood estimates, to deal with the bias stemming from small sample sizes (Firth, 1993). Likelihood ratio tests were performed to test significance of overall models using 'lmtest' (Zeileis and Hothorn, 2002) or, for penalised models, within 'logistf'.

2.4. Results

2.4.1. Cores summary and tree age

Extracted cores from the 55 trees sampled were a mean 34 cm in length. Core length ranged from 14 to 43 cm, though the length obtained reflected not just the

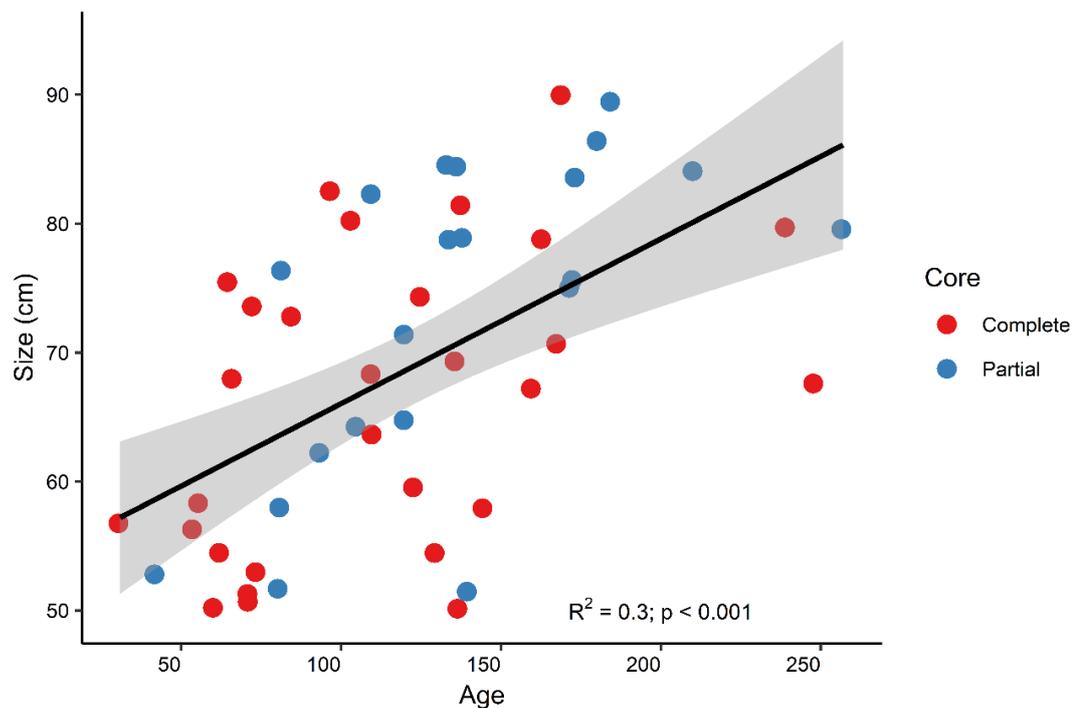


Figure 2.4. Scatterplot showing the relationship between tree age and size, measured as DBH (diameter at 1.3 m above ground). Tree age was determined by ring counts of complete and partial cores. Linear regression line: $\text{Size} = 0.13 \times \text{Age} + 53$.

overall size of the tree, but the success of core extraction. For example, when the increment borer reached areas of more advanced decay an intact core was more difficult to extract. After ring counting, 29 extracted cores were of sufficient length to reach the centre of the tree (i.e., the full radius) or beyond. The mean age of trees across sites was 122 (31 - 257) years, or 112 and 136 years for complete and partially complete cores, respectively. In general, size increased with tree age ($p < 0.001$), but there were large differences in growth rates between trees, since age explained only 30% of the variation in size (Fig. 2.4).

2.4.2. Fungal isolates

Of 55 tree cores, isolation attempts were unsuccessful for six cores and no fungal cultures were obtained (Appendix 2: Fig. S2.2). For the remainder, numerous isolates were found along the length of each core. The number of morphotypes per sampled core ranged from 0 to 11.

Overall, 23 (42%) trees contained a wood decay fungus. Thirty-one cultures were isolated from the centremost region of trees, of which 16 were wood decay fungi (Table 2.1). A further eight fungi were detected through amplification of DNA from

wood, and seven of these were wood decay fungi. Wood decay species were predominantly of the Basidiomycota, with eight genera detected. This could represent nine or more species, due to difficulties in resolving between species in the genera *Armillaria* and *Pholiota* using ITS sequences. Of the sequences examined, taxa in the genus *Pholiota* were problematic to assign to species level. The *Pholiota adiposa-aurivella* complex is not well-circumscribed using ITS sequences and is hereafter referred to as *Pholiota* 'A'. *Pholiota* 'A' was the most frequently detected wood decay fungus and was both isolated into culture and detected as DNA from wood from two sites. Ascomycetes in the order Xylariales, and two notable species recorded frequently on beech wood — *Ascocoryne* sp. and *Neobulgaria* sp. — were also detected. The fungi not thought to be wood decayers all belonged to five classes of Ascomycota and were primarily assigned to pathotroph and saprotroph trophic modes in FUNGuild (Table 2.2).

2.4.3. Trees with heart rot

Based on initial visual inspection of cores, 33 trees were suspected to have heart rot (i.e. had visibly discoloured or decayed wood). Of those suspected, decay fungi were detected in 58% (Fig. 2.5a). No decay fungi were found in trees that were less than 50 years old (Fig. 2.5b), but were found in 48% of trees over 80 years. The highest proportion of trees (92%) with decay fungi was in size class 60 - 70 cm (Fig. 2.5c).

There were differences in the proportion of trees with decay fungi between sites. Moccas Park and Epping Forest had the highest proportion of trees with decay fungi (Fig. 2.5d), though trees at these sites did not have the largest mean size and age (Fig. 2.5a, b). No decay fungi were found in trees of the Wyre Forest. (Fig. 2.5d), where both mean age and size of trees were lowest of all sites (Fig. 2.5a, b).

The best model constructed included age, size, site and interaction terms age + size, and size + site ($\chi^2 = 43.24$, $p < 0.001$). However, this model was not chosen due to data separation, indicated by large standard errors for some terms and fitted probabilities of 1. This was likely due to the relatively few observations for some sites and large differences in mean age (Fig. 2.5a) and size (Fig. 2.5b). With a penalised logistic regression the terms within this model were not significant. Therefore, the simplest model, including only age, was favoured. There was a significant relationship between decay presence and age ($\chi^2 = 4.69$, $p < 0.05$)

Table 2.1. Detection summary of wood decay fungi from the central tissues of beech trees, together with type of decay and sporocarp features (n = 55).

Species	Classification				Decay Mode	Sporocarp			Detection		
	Family	Order	Class	Phylum		Shape	Persistence	Size	No.	%	Method
<i>Neobulgaria sp.</i>	Lachnaceae	Helotiales	Leotiomycetes	Ascomycota		Jelly		S	2	4	Culture
<i>Ascocoryne sp.</i>	Helotiaceae	Helotiales	Leotiomycetes	Ascomycota		Jelly		S	3	6	Culture
<i>Nodulisporium sp.</i>	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota				S	1	2	Culture
<i>Kretzschmaria sp.</i>	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota	Soft	Crust	Long	Small	2	4	Wood
<i>Ganoderma pfeifferi</i>	Ganodermataceae	Polyporales	Agaricomycetes	Basidiomycota	White	Bracket	Perennial	Large	1	2	Culture
<i>Ganoderma adspersum</i>	Ganodermataceae	Polyporales	Agaricomycetes	Basidiomycota	White	Bracket	Perennial	Large	1	2	Culture
<i>Efibula sp.</i>	Phanerochaetaceae	Polyporales	Agaricomycetes	Basidiomycota	White	Crust	Long?	Small	1	2	Culture
<i>Aurantiporus fissilis</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	White	Bracket	Short-lived	Large	1	2	Culture
<i>Laetiporus sulphureus</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	Brown	Bracket	Short-lived	Large	2	4	Culture
<i>Pholiota A</i>	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	White	Mushroom	Short-lived	Small	5	10	Culture / Wood
<i>Armillaria sp.</i>	Physalacriaceae	Agaricales	Agaricomycetes	Basidiomycota	White	Mushroom	Short-lived	Small	2	4	Culture
<i>Coprinellus disseminatus</i>	Psathyrellaceae	Agaricales	Agaricomycetes	Basidiomycota	White	Mushroom	Short-lived	Small	1	2	Wood
<i>Psathyrella cernua</i>	Psathyrellaceae	Agaricales	Agaricomycetes	Basidiomycota		Mushroom	Short-lived	Small	1	2	Wood
									23	45	

Table 2.2. Other fungi (all Ascomycota) detected from the central tissues of beech trees, together with ecological information derived from FUNGuild database (n = 51).

Species	Classification			Trophic Mode	Ecology	Detection		
	Family	Order	Class			Guild	No.	%
<i>Aspergillus niger</i>	Aspergillaceae	Eurotiales	Eurotiomycetes	Pathotroph-Saprotroph	Plant Pathogen-Wood Saprotroph	1	2	Culture
<i>Cadophora malorum</i>	Cadophora	Helotiales	Leotiomycetes	Symbiotroph	Endophyte	1	2	Culture
<i>Cladosporium</i> sp.	Cladosporiaceae	Capnodiales	Dothideomycetes	Pathotroph-Saprotroph-Symbiotroph	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph	1	2	Wood
<i>Clonostachys</i> sp.	Bionectriaceae	Hypocreales	Sordariomycetes	Pathotroph	Plant Pathogen	2	4	Culture
<i>Debaryomyces hansenii</i>	Debaryomycetaceae	Saccharomycetales	Saccharomycetes	Saprotroph	Undefined Saprotroph	1	2	Culture
<i>Gibberella avenacea</i>	Nectriaceae	Hypocreales	Sordariomycetes	Pathotroph	Plant Pathogen	1	2	Culture
<i>Penicillium dierckxii</i>	Aspergillaceae	Eurotiales	Eurotiomycetes	Saprotroph	Undefined Saprotroph	1	2	Culture
<i>Talaromyces purpleogenus</i>	Trichocomaceae	Eurotiales	Eurotiomycetes	Saprotroph	Undefined Saprotroph	1	2	Culture
<i>Tolypocladium cylindrosporum</i>	Ophiocordycipitaceae	Hypocreales	Sordariomycetes	Pathotroph-Symbiotroph	Animal Pathogen-Clavicipitaceous Endophyte-Fungal Parasite	1	2	Culture
	Mycosphaerellaceae	Capnodiales	Dothideomycetes	Pathotroph-Saprotroph	Plant Pathogen-Undefined Saprotroph	1	2	Culture
	Reticulascaceae	Glomerellales	Sordariomycetes			1	2	Culture
			Helotiales	Leotiomycetes			2	4
		Pleosporales	Dothideomycetes			2	4	Culture
						16	31	

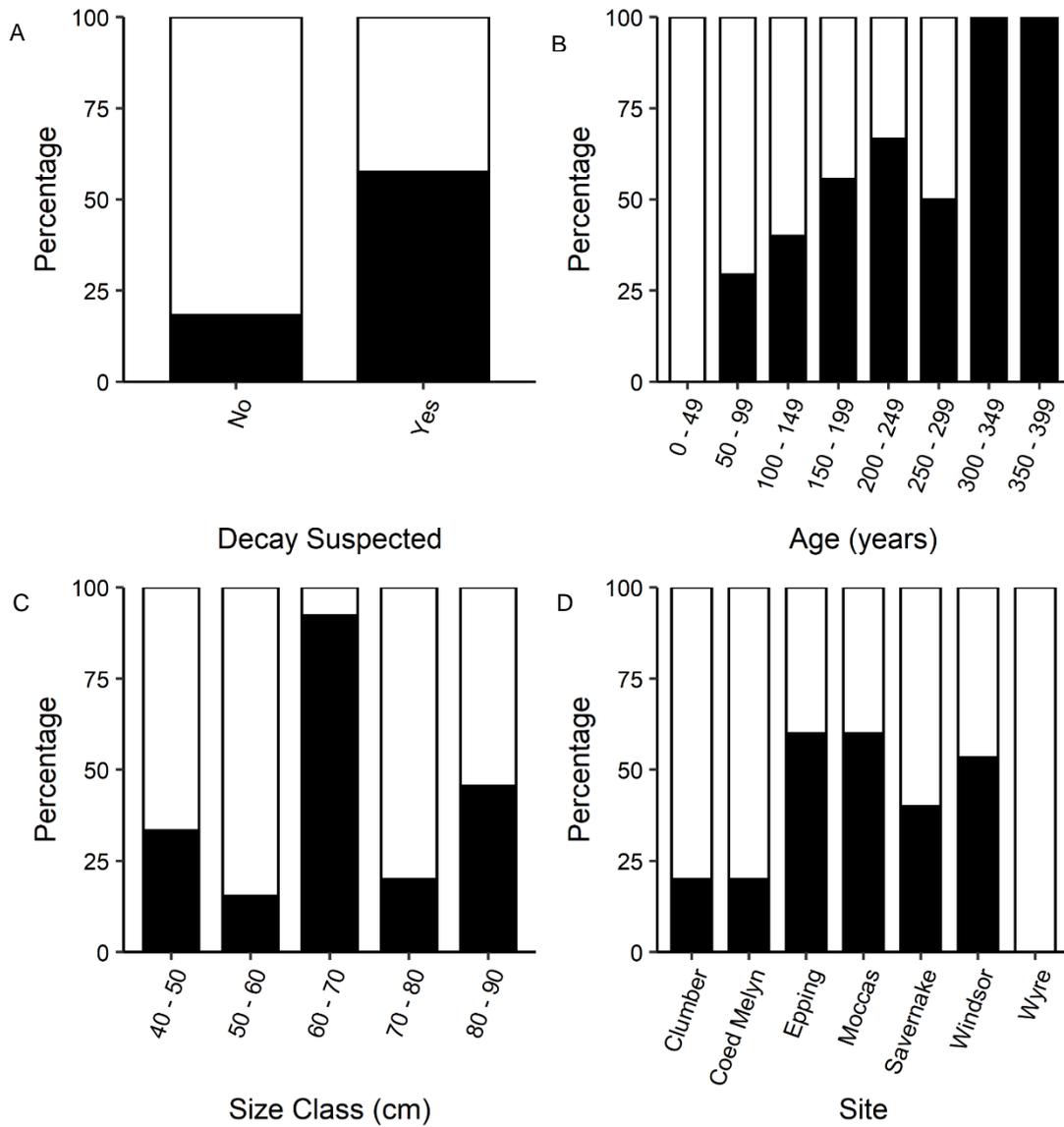


Figure 2.5. Percentage of trees where a decay fungus was detected (black shading), depending on (A) whether decay was suspected based on visual inspection, (B) tree age, (C) tree size and (D) site of sampling.

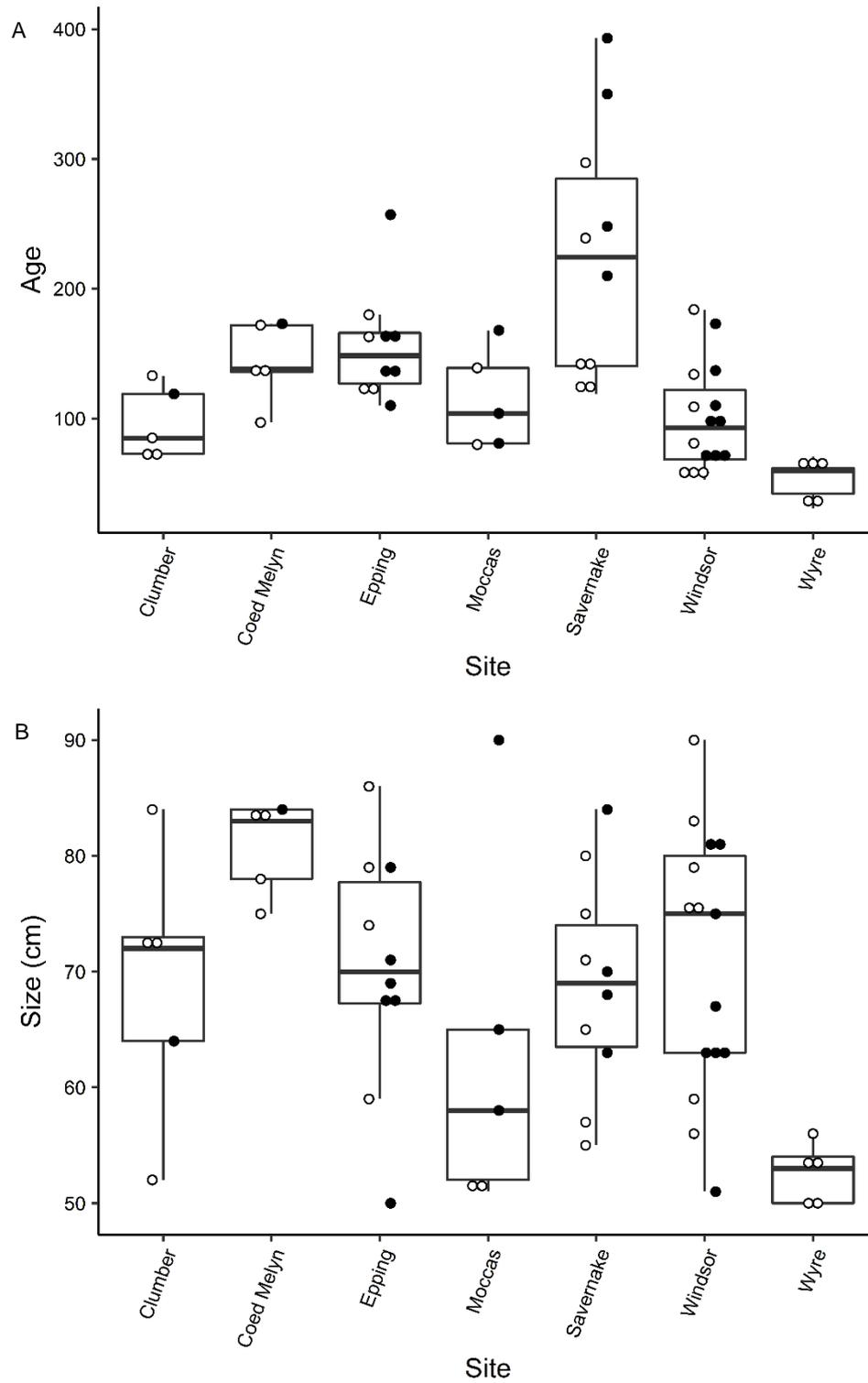


Figure 2.6 Boxplots showing the age (A) and size (B) distributions of trees sampled across sites. Shaded data points are trees where a decay fungus was found.

Table 2.3. Most explanatory binomial model on decay presence in standing trees.

Term	Coefficient	S.E	z value	Pr(> z)
(Intercept)	-0.941	0.390	-2.412	0.016
Age	0.005	0.003	2.078	0.038

Null deviance: 74.767 on 54 degrees of freedom

Residual deviance: 70.080 on 53 degrees of freedom

McFadden's pseudo R²: 0.06

ΔAIC: 10.5; AIC weight: 0.005

(Table 2.3; Appendix 2. Fig. S2.3). According to this model, trees of age 170 years have a 50% chance of containing a decay fungus.

2.5. Discussion

2.5.1. Heart rot fungi were identifiable from tree core samples

Decay fungi identified were mostly of the Basidiomycota, which is reflective of this taxonomic group being the main agents of wood decomposition (Boddy and Watkinson, 1995). Likewise, the majority of these were white rot fungi, which corresponds with general observations on the decay type seen in standing beech trees. Several species detected are already known to cause heart rot of beech.

Decay of trunks is normally associated with conspicuous brackets in the order Polyporales. Yet, by sampling mycelium, this study detected more agarics relative to polypores. Fungi in the genus *Pholiota* were the most frequently detected. These may be important heart rot fungi that are simply observed on fewer occasions due to their soft, short-lived sporocarps. *Ganoderma adspersum*, by contrast, forms more robust, perennial sporocarps, but was only detected as mycelium in one tree. Previously, *G. adspersum* was described as the most important heart rot fungus of beech (Cartwright and Findlay, 1958).

The detection of *Aurantiporus fissilis* is notable. It is infrequently recorded in the UK and an indicator species for beech forests of conservation value (Ainsworth, 2004). The coring method employed in the present study could be used to survey for indicator species and, indeed, refine notions of indicator species. Additionally, core sampling is useful for testing whether rare or threatened species are truly rare as

mycelium: *Hericiium* spp. and *Creolophus cirrhatus*, thought to be specialists on beech trees (Boddy *et al.*, 2011), were not detected in any trees sampled here.

Other fungi detected had been previously thought to be associated with late-stages of wood decay. For example, *Coprinellus disseminatus* was previously regarded to be a more ruderal species, recorded as sporocarps in tree hollows and assumed to be a rapid coloniser of open tree cavities. One possibility is that decay can progress from early-stage to well-advanced, despite being limited to small volume within a living trunk.

2.5.2. *Age is the best predictor of heart rot*

Heartwood formation is a programmed process which is related to cell age. It is, therefore, unsurprising that tree age was the best predictor of heart rot detection in this study. As trees become older, more tissues are converted to heartwood, allowing more time and opportunities for fungal colonisation to occur.

Age is not a perfect predictor of heart rot occurrence, and this variation between trees may be attributed to two factors: firstly, biotic and abiotic disturbances to trees which expose heartwood or otherwise produce entry courts for fungi, and, secondly, the occurrence of potential fungal colonists in the vicinity of the tree. Large wounds, caused by branch shedding, are an obvious point of entry to heartwood. They accumulate with age, and are linked to tree hollow occurrence (Koch *et al.*, 2008). Additionally, slowing growth rates in older trees reduce the ability of a tree to compartmentalise wounds. The development of decay, and the causal fungi must, however, always depend on the amount and type of inoculum, likely as spore rain, reaching the tree (Edman *et al.*, 2004).

The relationship between tree size and heart rot is perhaps more indirect. Trees with a high growth rate attain a large size, but this growth rate is negatively correlated with wood density and other characteristics. This can explain the presence of heart rot in younger trees which are not old but are relatively large. Edman *et al.* (2006) found fungal growth and decay was higher in fast-grown wood, which was less dense. Interestingly, there were species differences, with the scarce *Fomitopsis rosea* able to decay slow-grown wood more rapidly than other fungi.

2.5.3. *Core extraction success and limitations to the approach.*

This study has demonstrated the utility of an increment borer for estimating the

occurrence of heart rot and the diversity of decay fungi within forest stands. Few studies have attempted to assess heart rot in this way. Trees sampled were those of a size at which early stages of heart rot was expected, though without any external indications, such as large wounds or sporocarps. Of all trees sampled, decay fungi were detected in approximately half of them.

Appropriate technique and sterilisation of increment borers can yield long core samples, from which tree-inhabiting fungi may be isolated and studied. This study shows that heart rot fungi could be detected and related to tree age, and the approach could be further used to compare heart rot communities between tree species and across regions. The approach may also be adapted to search for indicator species of important forest stands, where reliance on sporocarp surveys may fail to identify sites or provide support for site protection. There is precedence in the UK for the use of soil DNA survey to notify a Site of Special Scientific Interest (SSSI) site for grassland fungi (Natural England, 2019).

It was not always possible to reach the centre of the tree, despite using a long-enough increment borer. In some instances, unintended breaking of cores during extraction and the presence of decay, hollows or wood abnormalities prevented the return of full cores i.e. of the complete tree radius. Beyond a certain size of tree, the use of an increment borer will become less effective in identification of heart rot fungi, but where decay hollowing may be safely presumed.

There is a reasonable expectation that decay will be missed when trees are sampled at breast height, as decay columns must be well-developed to reach this height within the trunk. Instead, Stenlid and Wästerlund (1986) recommend taking cores at stump height. While this might be possible for the straight stems of *Picea* spp., this is unfeasible for mature beech trees, and many others, with large buttress roots.

Detection of fungus from core samples might also depend on the shape of heartwood in the stem-radial direction (Wernsdorfer, 2005), so repeated corings from multiple sides might increase rate of detection. Coring is not a preferred option in many circumstances (Tsen *et al.*, 2016), because of concerns of pathogen introduction into trees. If this risk is accepted, however, this work demonstrates that both isolation of fungi and annual ring counts are possible from only one core sample, with minimal injury to the tree.

A further limitation of the approach is that fungal detection does not indicate the extent of colonisation throughout the trunk, nor in branches and root systems. Use

of non-invasive techniques, including acoustic tomography, can be used to estimate the location of hollows and loss of wood density (Deflorio *et al.*, 2008; Goh *et al.*, 2018). These techniques do not yield biological information or fungal cultures for further study, but they could improve the efficacy of this increment borer survey. For example, by informing where trunks should be sampled to avoid hollows or well-decayed pockets. This can maximise likelihood of fungal detection and minimise number of wounds created by sampling.

2.5.4. *Conclusions*

In this survey of standing beech trees, heart rot fungi were shown to be predominantly from the Basidiomycota. Tree age is the most important factor in predicting heart rot, which was detected in trees over 50 years old. Overall incidence of colonisation was 42% of beech trunks but, given the sampling strategy of asymptomatic trees within a limited size range of relatively small trees, this is likely to be an underestimate.

Chapter 3

Fungal community development in beech trunks

3.1. Summary

The spatial arrangement of fungi in trunks is not known and logistically difficult to examine. This study describes three-dimensional fungal community structure in recently felled or fallen trunks by isolating fungi from transverse wood sections. A variety of patterns were seen and key observations included: were that: ingress of water influences distribution of fungi; and, decay fungi are not always present in discoloured wood. *Kretzschmaria deusta* (Ascomycota) was found in the most trunks (47%), which colonised from the roots and was linked to the type of tree failure in these cases. *Ganoderma adspersum* and a *Pholiota* in the *adiposa-aurivella* complex (Basidiomycota) were each also found in 24 % of trunks. Several other wood decay fungi, though found less frequently, occupied long vertical columns (*Pholiota squarrosa*) or a large cross-sectional area (*Trametes versicolor*). As such, fungal succession may proceed following a variety of initial colonists.

3.2. Introduction

Tree trunks may contain a large volume of substantially decayed wood, termed heart rot, before tree death. Heart rot is ecologically essential process (Chapter 1), and a survey using an increment borer has more precisely shown which fungi are active as mycelia in tree trunks (Chapter 2). However, little research has paid attention to whole decay communities and the detail of which species cause hollowing (Stokland et al., 2012), despite the importance of large trees with hollows as stable, long-lived habitats within woodlands (Lindenmayer and Laurance, 2016; Müller et al., 2013).

Decay observed in living trunks and branches follows a spatial organisation explained by its anatomy (Boddy and Rayner, 1984). The primary restriction on wood decay is water (Boddy and Rayner, 1983a). Water-filled conducting xylem, or sapwood, is inimical to hyphal growth, enzymatic action and lignin decomposition – an aerobic process (Rayner and Boddy, 1988). In response to wounding, trees can mount reactions which effectively compartmentalise dysfunctional tissues, forming a barrier between them and new sapwood increments (Shigo and Marx, 1977). This effectively limits the growth of decay fungi, but such barriers are readily penetrated by hyphae in excised wood samples (Schwarze and Baum, 2000). These restrictions work in concert

(Pearce, 1996). Together, active defence, anatomical limitation, and water saturated tissues means that wood decay fungi seldom enter sapwood of living trees, and only heartwood tissues are colonised and undergo decay.

Heart rot fungi are seen to be S-selected species, adapted to the relatively stressful conditions within heartwood (Chapter 1). Although heartwood is generally drier and more aerated than functional sapwood, the presence of a range of compounds—extractives—confers some degree of natural durability (Taylor et al., 2002). Heart rot fungi might therefore be adapted to germinate on heartwood and resist toxicity of extractives (Valette et al., 2017), which vary between tree species.

Fungal entry into the living tree may occur via several routes (Rayner and Boddy, 1988). Heart rot fungi might commonly access heartwood which becomes exposed through damage to the roots or stem, or breakage of large limbs and branches. Heart rot fungi may also colonise as mycelium below-ground, present in decaying wood of neighbouring tree roots or stumps. A further possibility is that fungal propagules gradually accumulate within sapwood throughout the life of a tree and later develop when heartwood forms.

Of all organisms, tree trunks, are large, opaque structures that are logistically difficult to study. To date, living trunks of few tree species have been inventoried for wood-inhabiting fungi (Basham, 1973a, 1966a; Berry and Lombard, 1978; Hopkins et al., 2011; Vasiliauskas and Stenlid, 1998). However, a smaller number of studies have explored the spatial arrangement of fungal communities in tree trunks: accounts of *K. deusta* in an elm, lime and beech tree (Wilkins, 1939, 1943a, 1943b), and detailed descriptions of colonisation by this fungus in four trees. (Guglielmo et al., 2012)

Work pioneered on dead branches attached to trunks showed that decay communities are distinct between branches across tree species, excepting a few common fungi (Boddy and Rayner, 1983b; Boddy et al., 1987; Chapela and Boddy, 1988). Dead branches often show clear demarcation of fungi through the presence of pseudosclerotia, also called 'zone lines', and visual qualities specific to the action of certain fungi. Thus, in branches it is easy to visualise the spatial occurrence of fungi within the wood. Here, similar approaches are used to examine the three-dimensional structure of fungal communities in a single tree species. Beech is a ripewood tree, with no delineation between functional wood and non-functional wood, and sap flow cannot be estimated visually (Lüttschwager and Remus, 2007). Beech can, however, facultatively develop colouration in the central tissues which is referred to as red heart or red heartwood and is regarded as a false heartwood. This can be further

characterised into include more types: wounded heartwood, which is traceable to tree damage, and abnormal and splashing heartwood (Seeling, 1991 cited in Wernsdörfer, 2005). It is possible that the recognition of patterns represents real differences in heartwood formation, but it is not known if these influence fungal community composition or vice versa.

This study builds upon Chapter 2 not only in identifying the main decay fungi within beech trunks, but in mapping the positions of decay fungi in relation to patterns observed in transverse trunk sections. This approach provides clues to routes of colonisation and order of community development.

3.3. Methods

3.3.1. Study Design

The structure of the fungal community was determined for seventeen trunks from thirteen trees, from Windsor Great Park, Epping Forest and in the town of Bridgend, Wales. More trunks than trees were sampled as one tree, a lapsed pollard, comprised four large stems on top of the lower trunk (bole) which were examined separately. Trees had recently fallen or been felled between February 2015 to July 2017 (Table 3.1). They were between 53 cm and 92 cm DBH.

3.3.2. Sample Collection and Processing

Along each tree trunk, a longitudinal reference line was cut with a chainsaw before transverse cross sections were made. These were cut at non-standardised intervals, typically between 1 and 2 m, in order to follow the pattern of decay which could only be viewed as each cut was made. Radial sections were photographed and placed in black plastic bags immediately after cutting.

Trunk sections were transported back to the laboratory where they were stored at room temperature for <24 hours before sampling. Cross sections were photographed and surface sterilised by wiping with 10% domestic bleach. Wood chips (between 4 and 7) were aseptically excised from all of the main visually distinct regions and placed onto 9 cm plates containing 2% malt extract agar. Fungal outgrowth onto agar plates was monitored and subcultured to obtain clean cultures, usually within one week. Where more than one species appeared to be growing from a chip, both were transferred onto separate plates. Often, isolates from the same section or other adjacent sections

Table 3.1. Summary of trees sampled by sectioning.

ID	Location	Lat Long	DBH (cm)	Description	Felled or Fallen	Sample Date & Time Prior
B1	Bridgend Bryntirion campus	51.507 -3.606	92	Amenity tree, with sporocarps at base.	Felled	06/17 <1 month
E1	Epping Forest Staples Hill	51.651 0.052		Very large lapsed pollard. Uprooted.	Fallen	06/15 02/15
E2	Epping Forest	51.653 0.037	70	Suspected instability.	Felled	10/15 <1 month
E3	Epping Forest	51.653 0.037	77	Two inoculated trees appearing as a single stem.	Felled	10/15 <1 day
E4	Epping Forest Furze Ground	51.666 0.073	80	Stem breakage at base.	Fallen	02/16 <1 month
E5	Epping Forest Furze Ground	51.666 0.073	80		Fallen	02/16 <1 month
E6	Epping Forest Jack's Hill	51.675 0.073	53	Uprooted.	Fallen	09/16
E7	Epping Forest Jack's Hill	51.674 0.073	61	Uprooted.	Fallen	09/16
E8	Epping Forest Jack's Hill	51.675 0.074	84	Uprooted.	Fallen	09/16
E9	Epping Forest Jack's Hill	51.674 0.072	60	Uprooted.	Fallen	09/16
W1	Windsor Great Park South Forest	51.437 -0.644	80	Hollow present.	Felled	02/15 <2 months
W2	Windsor Great Park High Standing Hill	51.454 -0.666			Felled	07/15
W3	Windsor Great Park Swinley Forest	51.395 -0.711	81	Large maiden on beech avenue, set among forestry.	Felled	12/15 <1 day

appeared morphologically similar. As an aid to interpretation these were paired against each other to determine if they were same somatically compatible individuals as in Rayner *et al.* (1984).

3.3.3. Identification of species by DNA sequencing

Fungal isolations were retained on agar slopes for long term storage at 10°C. DNA was extracted from mycelium grown in pure culture. DNA extraction, Sanger sequencing and species identification was as described in Chapter 2 (Section 3.4).

As in the previous chapter, taxa in genus *Pholiota* were problematic to assign to species level. Fungi in the *Pholiota adiposa-aurivella* complex are referred to as *Pholiota* 'A' throughout.

3.3.4. Estimation of fungus extents

Simplified section drawings were made using Adobe Illustrator CC 2015. The area colonised by each fungus was drawn by encircling the region from where isolations were confirmed. The cross-sectional area of trunks and area of fungal extents were measured from section drawings in ImageJ 1.52 (Schneider *et al.*, 2012). From these measurements, the percentage colonised area in each cross-section was calculated.

Length of decay column was estimated as the length between highest and lowest point of detection. Where fungi were only encountered in one section, the length of the decay column was estimated as a fixed length of 1 m.

3.4. Results

3.4.1. Fungi isolated

Heart rot was developing or present in twelve trees, or sixteen of the seventeen trunks examined (Fig. 3.1, 3.2 and Appendix 3). In one trunk, no decay fungi were detected via isolation nor DNA amplification from wood (Appendix 3, tree E9). In total, eighteen species of wood decay fungi were detected (Table 3.2). The majority were Basidiomycota, but the most frequent fungus was the ascomycete *Kretzschmaria deusta*. *Ganoderma adspersum* and *Pholiota* 'A' were also frequently detected.

Each trunk contained a mean total of three different species, with a range from zero to six. In many of all sections examined, only one decay fungus was detected. A maximum of three decay species were detected in the same transverse section in two sections of a trunk (Fig. 3.1, sections 2 and 4). Other species regularly recorded, but not

Table 3.2. Decay fungi detected in all trunks, including where each likely colonised the trunk (origin), the vertical extent (length) and maximum colonised cross-sectional area (area).

Fungus	Trunks	Origin		Max. Length (m)	Max.Area (m ²)	Max. Area (% Section)
		Base	Top			
<i>Kretzschmaria deusta</i>	8	8	-	5.2	0.15	43
<i>Ganoderma adspersum</i>	4	4	-	5.6	0.15	26
<i>Pholiota 'A'</i>	4	1	3	1.6	0.01	4
<i>Eutypa spinosa</i>	2	1	1	4.5	0.02	4
<i>Coprinellus disseminatus</i>	2	-	2	1.0	0.01	3
<i>Hypholoma fasciculare</i>	2	2	-	3.2	0.06	13
<i>Stereum hirsutum</i>	2	1	1	1.5	0.01	4
<i>Armillaria gallica</i>	1	1	-	4.8	0.05	8
<i>Bjerkandera adusta</i>	1	-	1	1.0	0.01	3
<i>Cylindrobasidium evolvens</i>	1	-	1	1.0	0.01	9
<i>Mucidula mucida</i>	1	-	1	1.0	0.00	2
<i>Neobulgaria pura</i>	1	-	1	2.0	0.03	1
<i>Oxyporus populinus</i>	1	-	1	1.0	0.00	1
<i>Perenniporia fraxinea</i>	1	-	1	2.1	0.02	4
<i>Pholiota squarrosa</i>	1	1	-	13.2	0.21	32
<i>Pleurotus cornucopiae</i>	1	-	1	2.0	0.05	21
<i>Pleurotus ostreatus</i>	1	-	1	1.0	0.00	1
<i>Trametes versicolor</i>	1	1	-	5.9	0.13	27

Tree W1

Fungus	Length (m)	Area (m ²)
<i>Armillaria gallica</i>	4.8	0.05
<i>Trametes versicolor</i>	5.9	0.13
<i>Hypholoma fasciculare</i>	3.4	0.04
<i>Bjerkandera adusta</i>	1	0.01
<i>Stereum hirsutum</i>	1	0.02
<i>Cylindrobasidium evolvens</i>	1	0.01

Hollowing
 Wet wood

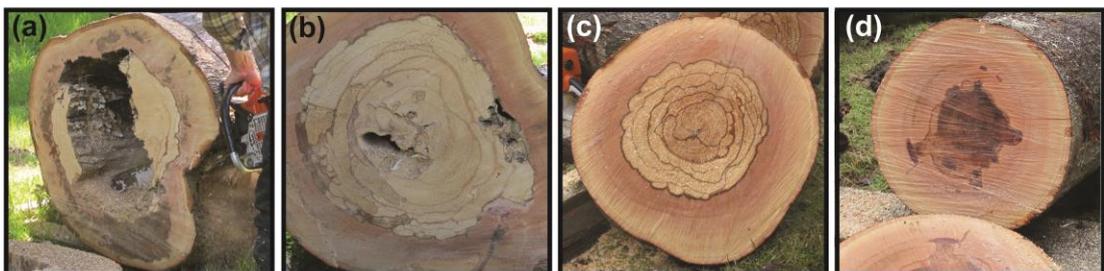
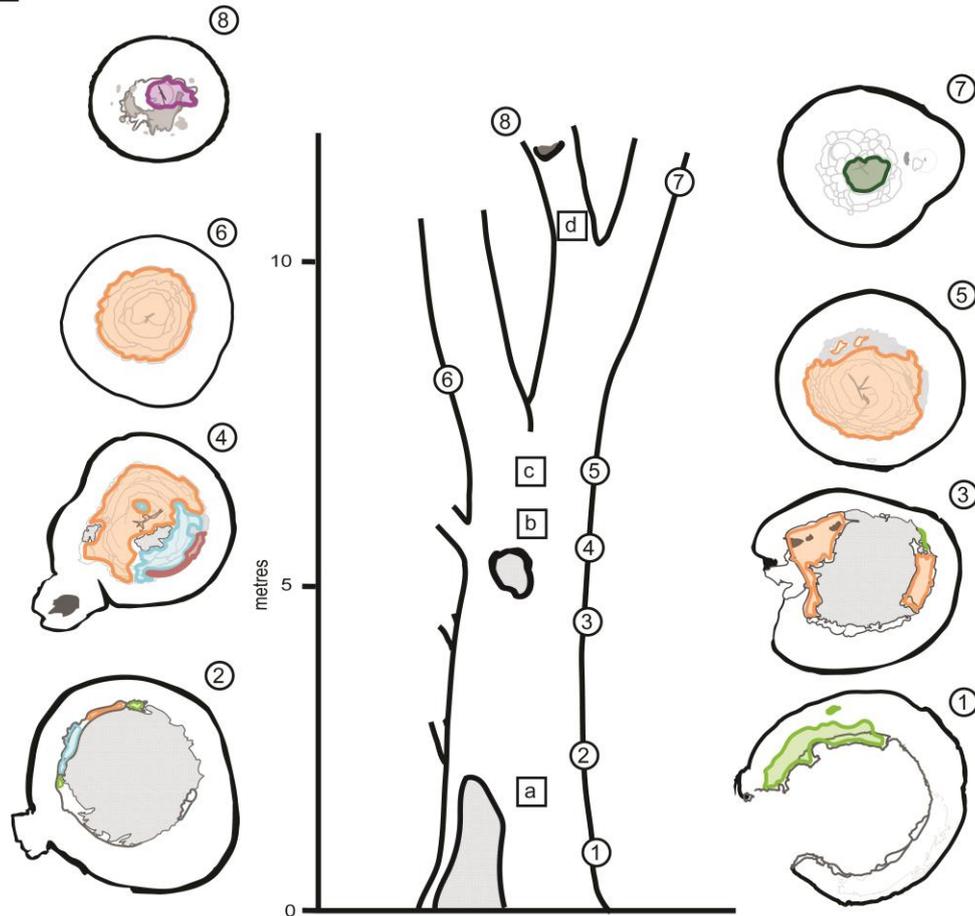


Figure 3.1. Tree with an open, basal hollow, which was felled due to safety concerns. Several decay fungi were found in the heartwood around the hollow periphery (a). Higher, a small cavity developed at the site of an old limb tear-out, joining the central hollow. Hollowing was reduced in extent (b), and by 7 m is no longer present. *Trametes versicolor* dominates for a further 2 m (c). In higher limbs other species develop but are not detected in wet wood pockets below branch wounds (d).

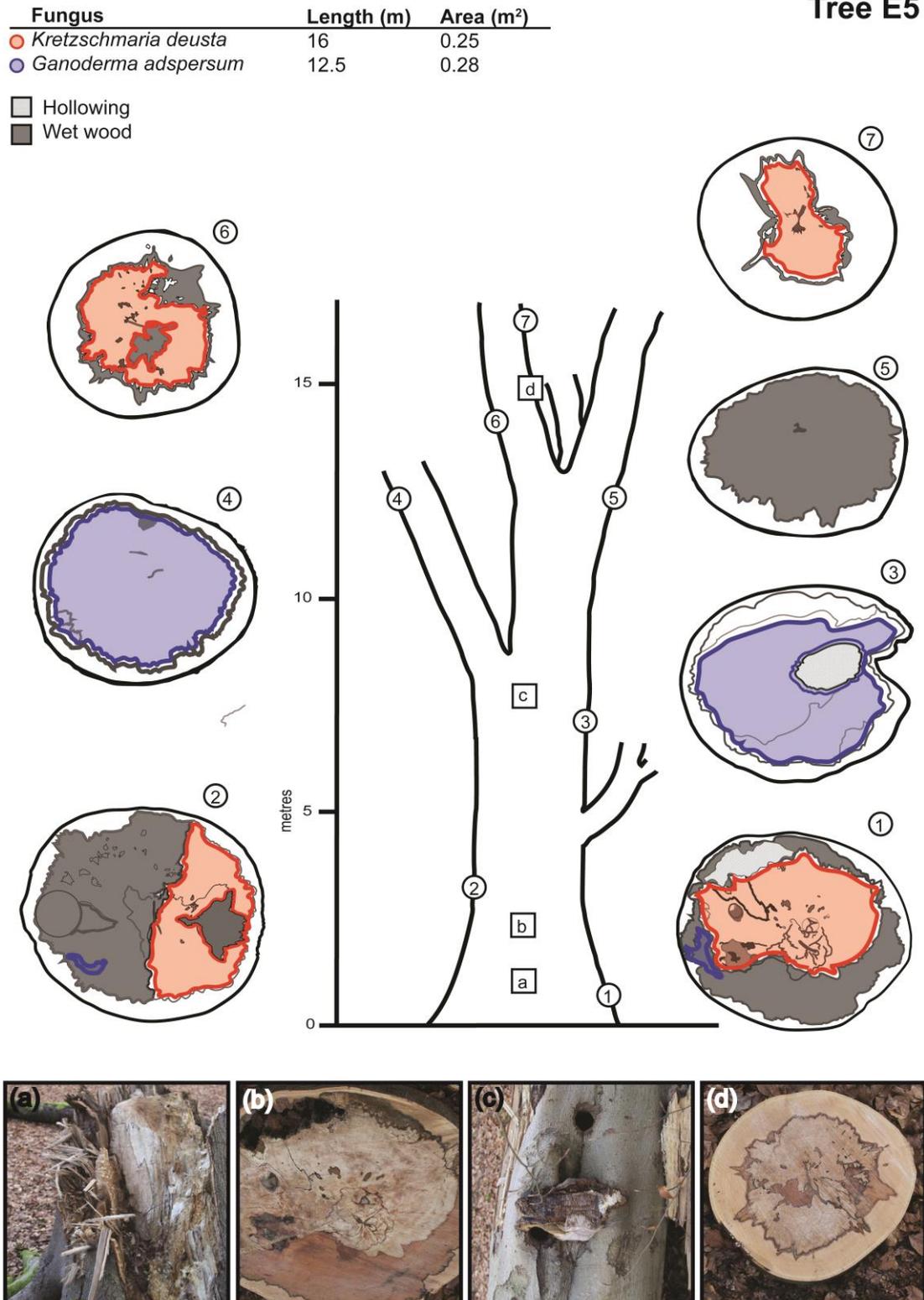


Figure 3.2. Tree which had failed at the base (a). Immediately above the point of failure, the trunk was colonised by both *K. deusta* and *G. adspersum* and featured a small hollow (b). Wet regions of wood were present throughout the trunk and appeared to limit the extent of *K. deusta*. *G. adspersum* became large in extent at 7 m, where the colonised wood was decayed sufficiently for excavation by woodpeckers around the site of sporocarps (c). Only *K. deusta* was found in the leading limbs (d).

considered to be wood decay fungi included: *Trichoderma*, *Penicillium*, *Fusarium*, and *Lopadostoma* spp.

3.4.2. *Extent and origins of decay*

The extent of heart rot varied widely between trees (Fig. 3.1, 3.2, and Appendix 3). Many decay fungi were detected only within one cross-section, with vertical extent estimated at 1 m. At the other extreme, in separate trees *Ganoderma adspersum*, *Pholiota squarrosa* and *Kretzschmaria deusta* occupied decay columns in excess of 10 m. In most cases, the cross-sectional extent of decay was largest towards the base of trees, except in one case where most decay occurred as a top rot caused by *Pleurotus cornucopiae* (Appendix 3. E2). The mean maximum cross-sectional area reached by a single fungus was 17% (range 1-74%). *K. deusta* reached large extents, its maximum area in cross-sections ranging between 25 - 74%. In one case, *G. adspersum* reached 54%, but for the other species cross-sectional area reached no more than 32%.

In general, fungi detected were closest to the base of the tree and could thus be referred to as causing a butt or basal rots. *Coprinellus disseminatus* was associated with a broken limb in both cases of its detection (Appendix 3. B1, E8). Some decay columns did not have an obvious point of origin. Notably, *Pholiota* 'A' was detected in the mid-trunk region in three cases, and did not appear to relate to any other decay column or wound. Only one tree examined was previously managed as a pollard (Appendix 3. E1), and colonisation into the pole regrowth (now large stems) continued from the main trunk below. In this case *K. deusta* was present in the four stems, but co-occurred with different species in each of them.

In trees with evidence of hollows or developing cavities the associated fungi were: *Armillaria gallica*, *Trametes versicolor* and *Hypholoma fasciculare* (Fig 3.1), *G. adspersum* (Fig. 3.2), *P. cornucopiae* (Appendix 3. E2), *C. disseminatus* (Appendix 3. E8), and *P. squarrosa* (Appendix 3. W3).

3.4.3. *Observations of trunk patterns*

False or facultative heartwood patterns were observed which matched descriptions from other texts (Fig. 3.4). Red heart was frequently seen, forming irregular, cloudy patterns of different sizes (Fig. 3.1, 3.4. a & b; Appendix 3. W3, E9). It was not associated with a particular fungus species. Wounded heartwood occurred immediately below a broken limb and featured small cavities (Fig. 3.4. c); the associated fungus was *C. disseminatus*.

Patterning termed abnormal red heart was observed (Fig. 3.4. e) and seemed to comprise areas of water-saturated wood, overlaying the red heart pattern (Appendix 3. E4, E5). No trees sampled featured the splashing heartwood pattern (Fig. 3.4. f).

Zone lines produced by fungi were not regularly present in trunks and so decay columns were not readily estimated by visual means. As wood becomes progressively decayed it is possible to distinguish colonised from sound wood, and between species, by differences in colour and texture (Fig. 3.4. g, h). However, in one example heart rot was in such an early stage that it was not suspected prior to evidence of isolations from wood (Appendix 3. E6).

Wood chips taken from sapwood often yielded no fungal cultures, but successful isolations of fungi and bacteria did occur sporadically. When heartwood patterning was present, heart rot fungi appeared to be confined to these areas. This became evident after wood incubated for 2-3 days, when mycelial outgrowth originated from only within of the presumed heartwood-sapwood boundary (Fig. 3.4. i).

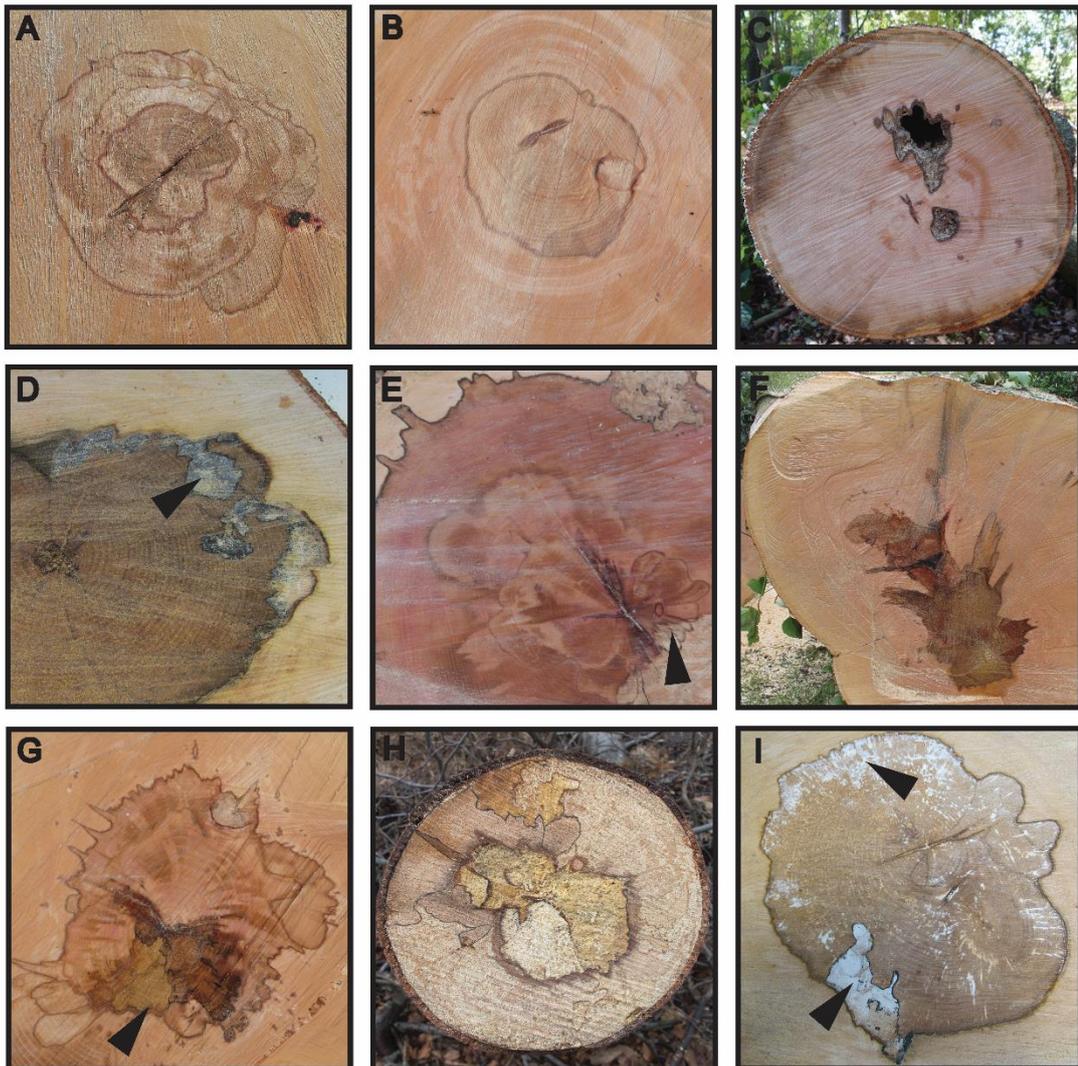


Figure 3.3. Patterns in heartwood of beech trunks. (A) Red heart and (B) early stage red heart of smaller extent with no detectable fungi. (C) Wounded and (D) splashing heartwood. (E) Outgrowth of mycelium restricted by wet wood. (F) Red heart pattern (arrowed) beneath wet wood. (G) Heartwood with some decay patterning, allowing some estimation of fungal colony extent. (H) Decayed beech branch with clear demarcation of fungi by zone lines and differing wood colouration. (I) Outgrowth of mycelium by two species (arrowed), following incubation at room temperature.

3.5. Discussion

This series of case studies shows decay in beech trunks to be spatially heterogeneous and varying widely in extent. Considered together, heart rot of beech trees originates with a few common species, but a larger suite of potential colonists can give rise to varying combinations of decay fungi from tree-to-tree.

3.5.1. Few species are found frequently as heart rot fungi.

Most of the decay fungi detected were well-known beech associates (Cartwright and Findlay, 1946). There is an obvious congruency with these results and existing knowledge from sporocarp observations. Conversely, the absence of a wider range of species reinforces the impression that heart rot fungi show host-specific preferences. Some species, such as *Trametes versicolor*, *Mucidula mucida* and *Hypoxylon fragiforme*, were also found to be in common with attached beech branches (Chapela et al., 1988). This suggests that microclimatic differences, even between branches and trunks of the same host tree, affects mycelial distribution within wood, rather than simply producing conditions which determine sporocarp appearance.

The predominance of *Kretzschmaria deusta* is unsurprising given the sampling strategy which focused on wind-thrown trees. *K. deusta* can shorten life of trees in promoting uprooting or root breakage. The likelihood of this depends on growing circumstances of trees, for example, in urban settings where root health is poor and more prone to death. On sites with shallow soils and water-stress, as at Epping Forest, this might explain the high frequency of *K. deusta* occurrence. However, there were also many examples of very old trees with evidence of this fungus.

Observations on *K. deusta* mirror those of (Guglielmo et al., 2012) which examined four trees including *Acer* and *Platanus* as well as *Fagus*. As in that study, *K. deusta* appeared to colonise from the base of the tree. In some cases colonisation was likely by spore infection, as evidenced by multiple genets of the fungus and numerous zone lines closest to the base. These zone lines become fewer in transverse sections higher up the trunk - a likely result of intraspecific competition. In trees where few genets are found, colonisation via mycelium is possible. However, neither the present study nor that of Guglielmo et al. (2012) adequately demonstrates this, since the presence of a single genet at the base of a tree would also be explained as the outcome of intraspecific competition which had already played out in the past.

The fungus *Ascocoryne* was often co-isolated with *K. deusta* and other fungi - a phenomenon also reported by Basham (1966b, 1973b). The presence of *Ascocoryne* in

living stems of different tree species, through to decaying logs, is of interest. Given its potential as a long-term component of fungal communities in wood, further enquiries on the ecology of *Ascocoryne* are recommended.

The species *Hypholoma fasciculare* and *T. versicolor*, are typically regarded as secondary colonisers, with combative capabilities to displace and hold territory against competitors. As strong combatants these fungi may have more of an impact on the subsequent community development (Hiscox et al., 2015). *H. fasciculare* has also been reported from around hollows in *Tilia* (Terho et al., 2007), but there it was also hypothesised that other species were first involved in decay, with *H. fasciculare* colonising later.

3.5.2. Heart rot species colonise differently and can remain spatially distinct

By contrast to branches, fungal community dynamics in trunks may play out on larger time and spatial scales. Probable sites of entry by fungi were observed via several points along trunks but, in most cases, decay was detected close to the base of the tree, where both the connection to the root system and oldest tissues occur.

Colonisation could be both via root system or by past injury to base of the tree, for example, animal or mechanical damage. It was not possible to sample roots in this study, and it is also difficult to locate past injuries, especially where decay is well-advanced. The exact mode of entry of species could be better determined by further examination of heart rot in earliest stages.

If colonisation occurs in at different locations within the living trees, there is potential for fungi to persist for a long time without fungal interactions occurring. Further, when more than one species found in cross section some seemed not to share any interaction boundaries and remained spatially separate. Spatial distance between fungi can be explained via inhibitory effects from a distance. Volatile organic compounds or other semiochemicals could service to reduce instances of direct contact between species by inhibiting hyphal growth in the direction of a competitor (Heilmann-Clausen and Boddy, 2005).

3.5.3. Patterns in trunks should be interpreted with additional evidence

Decay columns may be smaller than can be estimated on visual inspection. In this sense, red heart patterning poses difficulty in estimating extent of heart rot. Guglielmo *et al.* (2012) also report absence of the colonising fungus, *K. deusta*, in higher regions up the trunk, despite visible discolouration which would suggest its presence.

Conversely, the presence of pseudosclerotial plates (PSPs) can be good indicators of fungal presence, and can function as barriers to maintain favourable growth conditions (Rayner and Boddy, 1988), and otherwise as interaction zones between species competing for space. Further investigation is usually required to determine species identity and number. This work demonstrates PSPs as zone lines between individuals of the same species, such as *K. deusta* (also seen in Guglielmo *et al.* (2012)). Additionally, PSPs can be present as 'ghost' lines which do not appear to demarcate any current boundaries but are evidence of previous interactions or territory held.

Sapwood samples often yielded no fungal cultures, which is expected since sapwood, when functional, is inimical to fungal growth. It is important to note, however, that sapwood width is difficult to determine in ripewood trees where there is no clear distinction between sapwood and heartwood. It is estimated that most sap flow in beech trees occurs in the outer 40% of the trunk radius (Lüttschwager and Remus, 2007), but this can vary considerably. This variation must be borne in mind when interpreting whether decay fungi, when reaching large extent in trunk cross-section, has impact on the sap flow capacity of trees.

Finally, the importance of wet wood as a determinant of fungal extent in living trees must be acknowledged. Wet regions within heartwood often contain bacteria (Johnston *et al.*, 2016) and no wood decay fungi. The low oxygen availability in wet wood, as in sapwood, is likely the reason for the absence of fungi. It has been previously noted that wet heartwoods limit the spread of decay (Weber and Mattheck, 2003), though wet wood may have different origins across tree species. In the beech trees presented here, the ingress of water was traceable to stem forks or broken branches.

3.5.4. *Ecological implications*

Many of the uprooted trees sampled were those with *K. deusta* at the base of the trunk or observed in the roots, thereby providing a link between type of tree failure and predominant fungus. This is also observed in mainland Europe, where *Fomes fomentarius* causes mid-trunk breakages (Heilmann-Clausen, 2005). Hoppe *et al.* (2016) suggest that co-occurrence of species which each cause high levels of mass loss in wood is an example of functional redundancy in wood decay communities. However, if the spatial niches of these fungi are considered, the identity of species has important, lasting ecological impact, particularly in determining types of tree failure (uprooting, trunk snap, limb tear-out), and therefore the nature of woody debris inputs to the forest floor.

3.5.5. *Conclusions*

This culture-based study has yielded many useful observations from which to generate further hypotheses regarding fungal community development in trees. This approach can complement other methodologies, including metabarcoding, which are used for approximating whole communities including difficult-to-culture species, but lack spatial resolution that is key to understanding the ecology of wood decay fungi in standing trees.

Chapter 4

Fungi in functional sapwood: Endophytes and origins of decay

4.1. Summary

Sapwood comprises most above-ground forest biomass, but its fungal microbiome is largely unknown. Here, a sterile method is used to sample functional sapwood of young and mature beech trees, including at different sides of tree trunks and heights up into the crown. Wood decay fungi were detected in approximately half of all samples. The number of all fungal OTUs did not correlate with tree size or location. However, the number of OTUs appeared to increase with height above ground. ITS and LSU metabarcoding detected more distinct taxa than isolation of fungi into culture. Community composition differed by survey approach; LSU detected more taxa at the class-level than did ITS, but the ITS barcode detected a larger relative proportion of taxa in the class Agaricomycetes. Multiple survey approaches give confidence in results of microbiome studies.

4.2. Introduction

Sapwood is the water-conducting wood of living trees and is the vital connection from roots to leaves. Sapwood (xylem) is primarily composed of long, non-living tube elements through which water is conducted. It is also dynamic – containing parenchyma cells capable of nutrient storage and mobilisation, and of defence responses (Morris et al., 2016; Pearce, 1996). Despite being the predominant feature of the world's three trillion trees (Crowther et al., 2015) and constituting most of the above-ground forest biomass, sapwood is one of the least explored forest habitats (Baldrian, 2017). Little is known about the contribution of the sapwood microbiome to tree functions. In particular, the diversity and ecological roles of wood-inhabiting fungal endophytes, often termed latent fungi, remains largely unstudied.

The plant-endophyte symbiosis is a continuum from mutualism to parasitism (Schulz and Boyle, 2005; Sieber, 2007). Trees are sessile and long-lived organisms which can adapt to environmental stressors slowly relative to an endophytic microbiota,

and it has been suggested that they may favour mutualistic associations (Petit and Hampe, 2006).

Some sapwood endophytes are typically organisms that begin the process of wood decomposition (Boddy and Griffith, 1989). Wood decay fungi might pre-empt niche space in sapwood, in advance of suitable conditions developing there. They may also function across the transition from living to decaying wood (Song et al., 2016). As decay progresses, early colonists are replaced by more combative secondary colonisers, but their initial occupancy could determine the succession of the fungal community (Cline et al., 2018; Lindner et al., 2011). Endophytes might directly inhibit other fungi (Heilmann-Clausen and Boddy, 2005), or they may do so indirectly by modifying their resource (Hiscox et al., 2015; Schilling et al., 2015). Regardless of the mechanisms, the identity and distribution of endophytes can impact the pattern and extent of decay in the living tree. Decades later, these endophytes may influence the rate and succession of wood decay when a tree dies and falls to the ground (Van Der Wal et al., 2015).

Several culture-based studies, in which small chips have been excised from functional sapwood and placed on artificial media, have documented fungi in a range of tree species. In general, these have revealed communities low in diversity, comprising mostly of Ascomycota and many other sterile isolates non-identifiable based on morphological characters. Basidiomycetes, including wood decay species, comprised a small fraction, though Martin et al. (2015) found much higher diversity in the tropical host, *Hevea*. Such culture-based approaches are, however, heavily biased toward taxa that germinate and grow rapidly. Studies where freshly cut wood was incubated in the absence of spore colonisation have yielded a more complete picture. Different early decay communities developed from cut branches and twigs under varying moisture, gaseous and temperature regimes, but in the absence of spore colonisation (Chapela and Boddy, 1988a; Hendry et al., 2002). The same method applied to young stems revealed the heart rot fungus *Fomes fomentarius* in *Betula* spp. and *Fagus sylvatica* (Baum et al., 2003; Danby, 2000). This suggests that subsets of species can develop from a larger, endophytic pool of decay fungi, depending on the prevailing environment of wood in its surroundings. Further, PCR specific primers detected eleven wood decay basidio- and ascomycetes in a range of angiosperm trees (Parfitt et al., 2010).

High throughput sequencing (HTS) studies are likely to reveal greater diversity in wood than is detected in culture-based or sporocarp survey approaches. Indeed, this has already been seen to be the case in HTS studies of decomposing wood on the

forest floor (Kubartová et al., 2012; Ovaskainen et al., 2013). Different approaches also show contrasting community composition in foliar endophytes (Johnston et al., 2017; Siddique et al., 2017): Accumulating studies of fungi in leaves show these communities to be influenced by tissue age, altitude (Siddique and Unterseher, 2016), site, and height above ground (Harrison et al., 2016).

This chapter aimed to characterise fungi in functional, water-conducting xylem of the widespread tree, European beech (*Fagus sylvatica*). It was hypothesised that wood saprotrophs would be a large component of endophyte communities, but that whole communities would vary by site, tree size and height up the trunk. This study tests our hypotheses using a combination of DNA metabarcoding and isolation of fungi into culture.

4.3. Methods

4.3.1. Study design

Between July and September 2017, 66 beech trees were sampled from ten sites across the southern UK (Fig. 4.1). These comprised 51 of the trees cored in Chapter 2, whereas trees at Coed Esgynfa, Buckfast Abbey and Wytham Woods were new to this study. Trees selected were visibly healthy, with no obvious decaying regions, wounds, or fungal sporocarps. At three sites, young trees were selected, measuring 10-30 cm diameter at breast height (DBH: 1.3 m), whilst at the seven other sites the trees were mature, between 50 and 90 cm DBH. Each tree was sampled from one point on the trunk; the south aspect at 1.3 m above the ground. A subset of five mature trees were studied more intensively, with samples taken at ascending points up the trunk, and along larger diameter branches at the lowest and highest positions in the crown (Fig. 4.8a).

4.3.2. Field sampling

To ensure that fungi detected in trees were from within functional sapwood rather than from aerial and bark contamination, sterility was essential during sampling. Simply adapting the practice of catching sawdust falling from drill bits, commonly used for decaying wood studies, is not sufficient to exclude contaminants, based on preliminary investigations and sequencing runs. At each tree, a square of bark (5 x 5 cm) was aseptically removed to expose sapwood using a hammer and chisel, which was first wiped with 10% bleach solution followed by 70% isopropanol. Using a cordless drill and a 4 mm diameter drill bit, wood dust was collected into a sterile

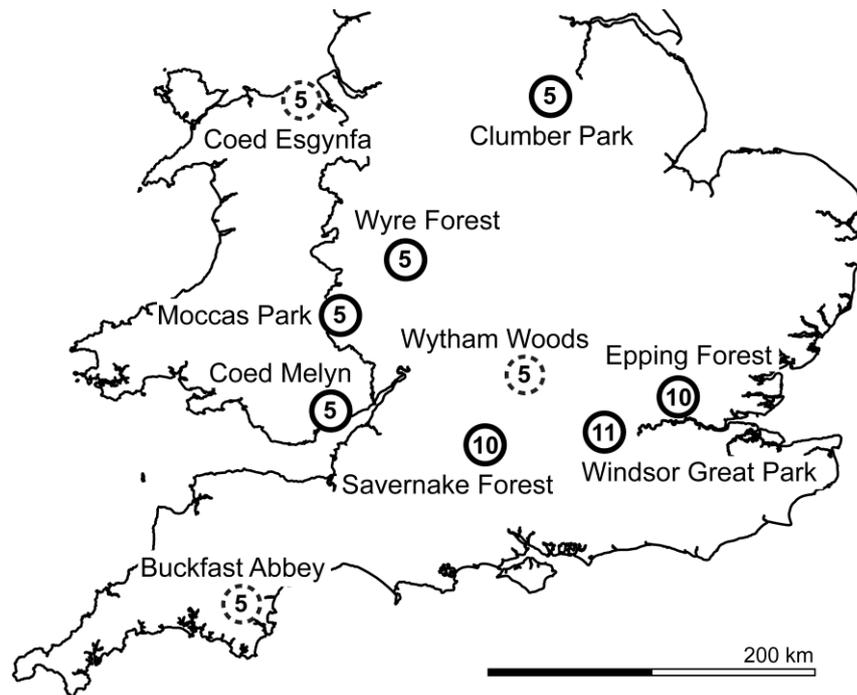


Figure 4.1. Locations of sampling sites within the southern UK. Numbers in symbols indicate number of trees. Dashed lines around circles indicate sites where young trees where only young trees (10-30 cm DBH) were sampled.

microfunnel from depth of 4 cm into the trunk. Sterile drill bits were prior assembled with sterile microfunnels and were transported to the field in individual sterile falcon tubes (Fig. 4.2). The contents of the tube were then carefully emptied into a sterile 1.5 ml microtube under cover of a sterile box. Sampling was repeated at each corner of the exposed sapwood to provide four microtubes per square. These were placed into a cool bag containing freezer blocks and transported to the laboratory. Within 12 h, 1 ml of filter-sterilized cell lysis solution (CLS; Lindner and Banik, 2009) was added to the first tube and frozen at -80°C . The remaining three were processed immediately for isolation of fungi. Field control samples were taken by following this sampling procedure approximately 2 cm from the surface of a standing tree trunk, chosen at random.

4.3.3. Isolation of fungi into culture

Microtubes containing wood dust were weighed and one third of contents from each tube was pooled. Sterile distilled water was added to wood dust to produce 1%



Figure 4.2. Drill bit preparation and field sampling. (A) Assembly of drill bits in sterile falcon tubes for transportation (top), and arrangement within the falcon tube of drill bit in relation to the collecting microtube (bottom). (B) Tree trunk after bark square removal, showing location of four drilled holes in each corner, whilst in the centre two larger core holes from increment borer sampling in Chapter 2. (C) Collecting of wood dust sample into microtube held against exposed sapwood. (D) Emptying of wood dust into clean microtube under cover of sterile box.

(weight/ volume) suspension. Suspensions were then plated by spreading 1 ml onto: (i) 2% malt extract agar (MA), (ii) 2% MA with kanamycin. Poured plates were made using 2% MA with (iii) 1 ml of suspension and, lastly, (iv) dry wood dust. 9 cm Petri dishes were sealed and incubated in darkness for 12 weeks. Petri dishes were checked periodically and developing mycelia subcultured where necessary.

Isolates were grouped by morphotype, examined microscopically to confirm similarity, and then processed for DNA sequencing. The full ITS region was sequenced in one direction using primer pair ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al., 1990). PCR conditions were conducted as follows: 95°C for 3 min, 35-37 cycles of: 95 °C for 30 sec, 56°C for 30 sec, 72°C for 1 min, followed with a final 10 min elongation at 72°C. PCR products were checked on 1.5% agarose gels stained with Sybr Safe (Invitrogen) and purified with a QIAquick Purification Kit (Qiagen). DNA was quantified on a Qubit 2.0 fluorometer (Invitrogen) and sequenced by Eurofins Genomics UK.

4.3.4. *Processing of wood dust for metabarcoding*

Samples in CLS at -80°C were first defrosted at room temperature and then heated at 65°C for 3 h. 100 µl of supernatant was used for DNA extraction, using the protocol detailed in Lindner and Banik (2009) with modifications in Brazeo and Lindner (2013).

Two barcode regions were, separately, amplified per sample to capture greater fungal diversity; part of the internal transcribed spacer gene (ITS2) – the universal fungal barcode (Schoch et al., 2012) – and the flanking region LSU (large subunit). We generated barcoded HTS amplicons using one PCR step. Mock fungal community controls for each barcode – ITS2 (SynMock) and LSU (LSU Mock) – were used for bioinformatics parameterization, including clustering efficiency, estimation of index bleed and recovery of a known community (Palmer et al., 2018). Field sampling and DNA extraction controls were also included at this stage.

For ITS2, the forward primer was composed of the Ion A adapter sequence, followed by the Ion key signal sequence, a unique Ion Xpress Barcode sequence (10-12 bp), a single base-pair linker (A), and the fITS7 primer (5'-GTGARTCATCGAATCTTTG-3'; Ihrmark et al., 2012). The reverse primer was composed of a trP1 adapter followed by ITS4 (White et al., 1990). Together, this primer pair amplifies a region on around 264 bp, but this is quite variable between

fungal groups. Amplification was performed in 15 µl reactions with 1 µl of (1x or 1:10 dilution) of template and 0.1 µl GoTaq Polymerase. PCR conditions were: initial denaturation at 94°C for 3 min, 10 cycles of 94°C for 30 s, 60°C for 30 s (-0.5°C per cycle), and extension at 72°C for 1 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 minute, with a final extension at 72°C for 7 min.

For LSU, the forward primer was LR0R (5'-ACCCGCTGAACTTAAGC-3'; Vilgalys and Hester, 1990) and reverse primer was JH-LSU-369rc (5'-CTTCCCTTTCAACAATTTTAC-3'; You et al., 2015). Both were adapted for HTS as above. PCR conditions were: initial denaturation at 94°C for 3 minutes, 40 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 90 s, with a final extension at 72°C for 7 min.

Amplicons were confirmed for DNA on 1.5% agarose gels stained with ethidium bromide. Barcoded amplicons were then cleaned of residual primers using Zymo Select-A-Size DNA Clean & Concentrator kits (Zymo Research). Cleaned amplicons were then quantified on a Qubit 2.0 fluorometer using the high-sensitivity DNA kit. Following quantification, the cleaned amplicons were equilibrated to (2000 pM), then pooled in equimolar concentrations to form a combined library. Two combined libraries, one for ITS and one for LSU, were sequenced over two runs on an Ion Torrent PGM with 318 v2 chips.

4.3.5. *Bioinformatics*

High throughput sequencing data were processed using AMPtk v1.1.3 (Palmer et al., 2018). Reads were first demultiplexed, then reads with forward and reverse primers were retained and primer sequences removed. Reads shorter than 125 bp were discarded and those remaining were truncated. Clustering into OTUs was performed with UPARSE (Edgar, 2013) at 97% similarity for ITS, but for LSU this was performed with DADA2 (Callahan et al., 2016) which performs better for this barcode (Skelton et al., 2019). Index bleed percentage in and out of the mock community samples were calculated, then read counts within the range of index bleed were removed from the OTU table. All non-fungal OTUs were removed at this stage prior to further analysis.

4.3.6. *Identification and determination of ecological roles*

Taxonomy was assigned to OTUs using the built-in ITS and LSU databases by means of a hybrid approach using global alignment, UTAH and SINTAX. Distinct

taxon assignments were identified as wood saprotrophs using the open-source database FUNGuild (Nguyen et al., 2016), within the AMPtk pipeline. Taxon lists were also manually examined, and taxa known to be involved in wood decay were noted. Taxon assignments for wood saprotrophs were further investigated by searching genus or family-level assignments against the Fungal Records Database of Britain and Ireland (FRDBI) and Genbank, where *Fagus* was the associated organism.

4.3.7. Statistical analysis

Fungal richness is reported as taxonomic richness – the number of unique OTUs detected from each sample. Diversity indices based on sequence abundance are not reported. Sequence abundances, though might be used cautiously, are known to be unreliable indicators of biological abundance; they vary unpredictably due to primer biases, PCR randomness, amplicon/ barcode size variation and read-copy number (Bálint et al., 2016; Jusino et al., 2017; Lofgren et al., 2019; Palmer et al., 2018).

Fungal community composition of samples were visualised using non-metric multidimensional scaling (NMDS), on three axes, using the metaMDS function in vegan package (Oksanen et al., 2018). Community dissimilarities were calculated on a modified Raup-Crick distance matrix (Chase et al., 2011). Community dissimilarity PERMANOVA was performed to test for no significant community differences by location and tree size. As this analysis is sensitive to nonnormality (Anderson, 2006), a further test for homogeneity of dispersion among groups was performed with the betadisper function.

Pearson product-moment correlation tests were performed for OTU number and wood diameter for the five whole-tree samples (i.e. including branches and trunks), and for OTU and height above ground. A further correlation test was performed for OTU number and for all trunks sampled at breast height.

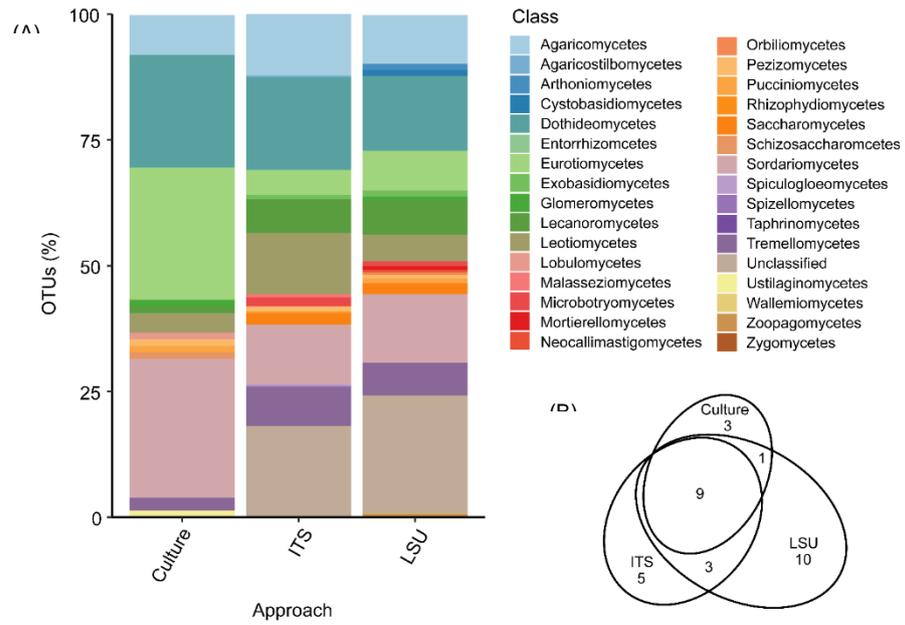


Figure 4.3. Comparison of distinct taxa (OTUs), defined at class-level, found by the different survey approaches. (A) Relative proportion of classes within each dataset, (B) Number of classes unique or in common to each dataset.

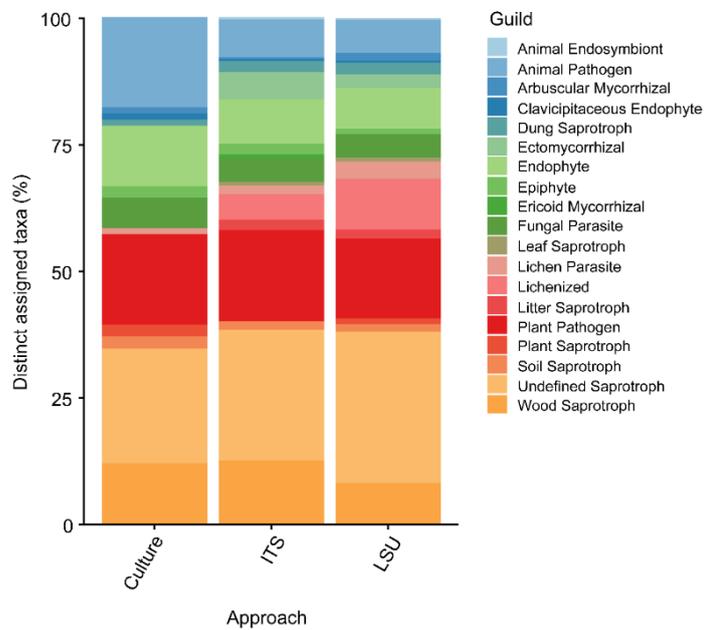


Figure 4.4. Functional guild assignments as a percentage of distinct taxa by ITS or LSU sequencing and culture-based approaches.

4.4. Results

4.4.1. Data summary and taxonomic classifications

In all samples, a total 328 and 412 fungal OTUs were found using ITS and LSU primer sets, respectively. Accumulation curves for each suggest that sampling did not exhaust the fungal diversity detectable within beech sapwood (Appendix 4. S4.1). Sanger sequencing of fungal cultures generated 76 OTUs. The ITS and LSU sequencing datasets had more OTUs in common than either did to the culture dataset. The LSU barcode detected 23 fungal classes, whilst ITS detected 17 (Fig. 4.3a). Culturing detected 13 classes and, of these three fungal classes – the Lobulomycetes, Schizosaccharomycetes and Ustilaginomycetes – were missed by metabarcoding (Fig. 4.3b). Conversely, metabarcoding detected three classes which were not found by the culture-based approach - the Exobasidiomycetes, Microbotryomycetes, and Saccharomycetes. Five and 12 OTUs at genus and family-level, respectively, were common to all approaches. Interestingly, no species-level OTUs were found by all three. The ITS and culture datasets, however, both had the species *Clonostachys rosea* in common, whilst the LSU and culture datasets had *Aspergillus fumigatus*, *A. niger*, *Aureobasidium pullulans* and *Cladosporium langeronii* in common.

4.4.2. Endophytes and their ecological roles

For ITS and LSU sequencing, 145 and 166 distinct taxa, respectively, were assigned to 18 functional guilds. From cultures, 43 taxa were assigned to 13 guilds. In total, 19 functional guilds were detected, and the relative proportions of fungal guilds across the three approaches were broadly similar (Fig. 4.4). 'Undefined Saprotroph' and 'Plant Pathogen' were the most common guild across all approaches. 'Animal Pathogen' and 'Endophyte' guilds occurred as a greater proportion of assigned taxa in the culture dataset than in the HTS sequencing datasets.

4.4.3. Detection of wood saprotrophs

Wood decay fungi were found in almost half of all samples but were a small proportion of all OTUs. Taxa identified as causing a white rot decay type were most frequently identified overall, and with both ITS and LSU sequencing (Table 4.1). The culture-based approach identified more soft rot taxa than white or brown rot taxa. ITS sequencing detected the most wood decay fungi (38) *via* the FUNGuild database (3), and a further 8 were identified with manual inspection. LSU detected

Table 4.1. Taxa identified as wood saprotrophs, together with decay mode, as given in FUNGuild database. Taxa denoted by ■ were not identified as wood saprotrophs but were added in by authors. Traits denoted by - were not given. FRDBI (Fungal Records Database of Britain and Ireland) and GenBank columns indicate if taxon was present in a search of either database with *Fagus* listed as the associated organism. NB, Family-level taxa not searched for in FRDBI.

Taxon	Barcode	Trait	FRDBI	Genbank
<i>Acremonium</i>	ITS, Culture	Soft Rot	✓	✓
<i>Alternaria</i>	ITS, Culture	Soft Rot	✓	✓
■ <i>Amyloporia</i>	ITS	White Rot	✓	x
<i>Anteaglonium</i>	LSU	-	x	x
<i>Antrodia</i>	ITS	Brown Rot	✓	x
<i>Ascocoryne</i>	Culture	-		
<i>Athelia</i>	ITS	-	✓	✓
■ <i>Biscogniauxia</i>	ITS	-	✓	✓
<i>Cephalothecaceae</i>	ITS	-		x
<i>Ceriporiopsis</i>	ITS	White Rot	✓	x
<i>Chaetomium</i>	LSU	Soft Rot	✓	✓
<i>Chaetosphaeria</i>	ITS	-	✓	x
<i>Chaetosphaeriaceae</i>	LSU	-		✓
<i>Cladosporiaceae</i>	ITS	-		✓
(<i>Verrucocladosporium</i>)	ITS	-	x	x
<i>Cladosporium</i>	ITS, LSU, Culture	-	✓	✓
■ <i>Collybia</i>	LSU	-	✓	x
<i>Coniophora</i>	ITS	Brown Rot	✓	✓
<i>Coniothyrium</i>	ITS	Soft Rot	✓	x
■ <i>Cylindrobasidium</i>	LSU	-	✓	✓
<i>Discula</i>	LSU	Soft Rot	✓	✓
■ <i>Eutypa</i>	ITS	-	✓	✓
<i>Flavodon</i>	LSU	-	x	x
<i>Fomitopsis</i>	ITS	Brown Rot	✓	x
<i>Fusarium</i>	LSU	-	✓	✓
<i>Ganodermataceae</i>	ITS, Culture	White Rot		✓
(<i>Ganoderma</i>)	ITS	White Rot	✓	✓
<i>Helotiaceae</i>	ITS, LSU	-		✓
(<i>Chloroscypha</i>)	LSU	-	x	x
<i>Heterobasidion</i>	LSU	White Rot	✓	✓
<i>Humicola</i>	ITS	Soft Rot	✓	✓
<i>Hyaloscyphaceae</i>	ITS	-		✓
■ <i>Hyphodontia</i>	LSU	White Rot	✓	✓
<i>Hypholoma</i>	Culture	White Rot		
<i>Inonotus</i>	ITS, LSU	White Rot	✓	✓
■ <i>Kretzschmaria</i>	ITS	-	✓	✓
■ <i>Lagarobasidium</i>	LSU	White Rot	x	x
<i>Laetiporus</i>	Culture	Brown Rot		
<i>Lentinus</i>	ITS	White Rot	✓	x
<i>Meripilus</i>	ITS	White Rot	✓	✓
<i>Mycena</i>	ITS, LSU	-	✓	✓
<i>Orbilia</i>	LSU	-	✓	✓
<i>Penicillium</i>	LSU	Soft Rot	✓	✓
<i>Peniophora</i>	Culture	White Rot	✓	✓
■ <i>Peniophorella</i>	ITS, LSU	White Rot	✓	x
<i>Periconia</i>	ITS	Soft Rot	✓	x
<i>Phallaceae</i>	ITS, LSU	-		x
(<i>Phallus</i>)	ITS, LSU	-	✓	x
<i>Phialophora</i>	ITS, LSU	-	✓	✓
<i>Phomopsis</i>	LSU	Soft Rot	✓	✓
<i>Pseudeurotiaceae</i>	ITS	-		✓
■ <i>Pseudoinonotus</i>	LSU	White Rot	✓	x
<i>Pyrenochaeta</i>	LSU	Soft Rot	✓	✓
■ <i>Radulomyces</i>	LSU	White Rot	✓	✓
<i>Rhinocladiella</i>	ITS	Soft Rot	✓	✓
<i>Scytalidium</i>	LSU	Soft Rot	✓	x
<i>Serpula</i>	ITS	Brown Rot	✓	x
<i>Sistotrema</i>	ITS	White Rot	✓	✓

▪ Sistotremastrum	ITS	White Rot	✓	x
Stereaceae	LSU	-		x
<i>Torula</i>	LSU	Soft Rot	✓	x
<i>Trametes</i>	ITS	White Rot	✓	✓
<i>Trichaptum</i>	ITS	White Rot	✓	x
<i>Trichoderma</i>	LSU	-	✓	✓
▪ <i>Vuilleminia</i>	LSU	White Rot	✓	x
<i>Xylaria</i>	ITS, LSU	Soft Rot	✓	✓
<i>Xylodon</i>	ITS	White Rot	x	x

30 (21/ 9), whilst culturing detected 13 (10/ 3). The wood decay genera *Ceriporiopsis*, *Inonotus*, *Meripilus*, *Mycena*, *Phallus* and *Xylaria* were found by both ITS and LSU sequencing. *Ganoderma* was found by both ITS sequencing and culturing.

4.4.4. Site and tree size effects on community composition and richness

Fungal communities in trees at the same site did appear to be more similar than to trees at different sites (Fig. 4.5). There was a significant effect of site on community composition for both barcodes (PERMANOVA. ITS: $F = 1.49$, $R^2 = 0.25$, $p = 0.05$. LSU: $F = 1.64$, $R^2 = 0.22$, $p < 0.05$). This effect was not due to very different dispersions between site groupings. There was no significant effect of diameter class on community composition (Fig. 4.6) (ITS: $F = 1.77$, $R^2 = 0.07$, $p = 0.11$. LSU: $F = 1.55$, $R^2 = 0.05$, $p = 0.2$).

The mean OTU richness per sample taken at breast height was 5.0 ± 0.8 (SE units) and 9.3 ± 0.8 , for ITS and LSU respectively. There was almost no correlation between tree size and OTU richness for ITS ($r(64) = 0.02$, $p > 0.05$ and LSU ($r(64) = -0.12$, $p > 0.05$) (Appendix 4. S4.2).

4.4.5. Fungi within trees

For the five trees sampled at multiple points there was no effect of height nor location (i.e. trunk or branch) on fungal community composition (Fig. 4.7). For these trees the mean number of OTUs detected per sample was 7.7 ± 1.3 for ITS and 11.7 ± 1.1 for LSU. OTU richness appeared to increase with height for both barcodes (Fig. 4.8b; Appendix 4. Fig. S4.3), but these correlations were significant only for ITS (ITS: $r(46) = 0.31$, $p < 0.05$. LSU: $r(47) = 0.10$, $p > 0.05$).

Considering only basidiomycetes, wood decay fungi were detected throughout the trunks and branches sampled (Fig. 4.8b). These were mostly in the orders

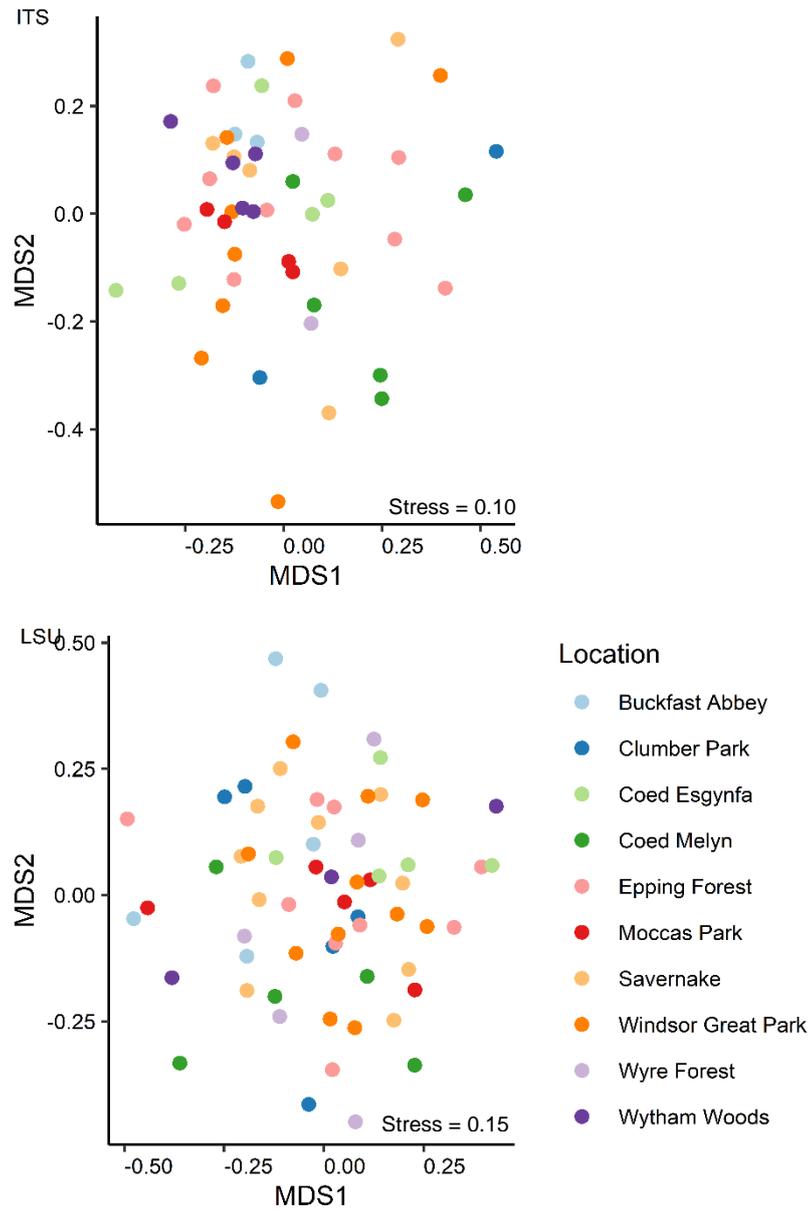


Figure 4.5. NMDS plots of fungal communities of trees sampled once at breast height (~1.3 m). Plots according to ITS and LSU barcodes are coloured by site location.

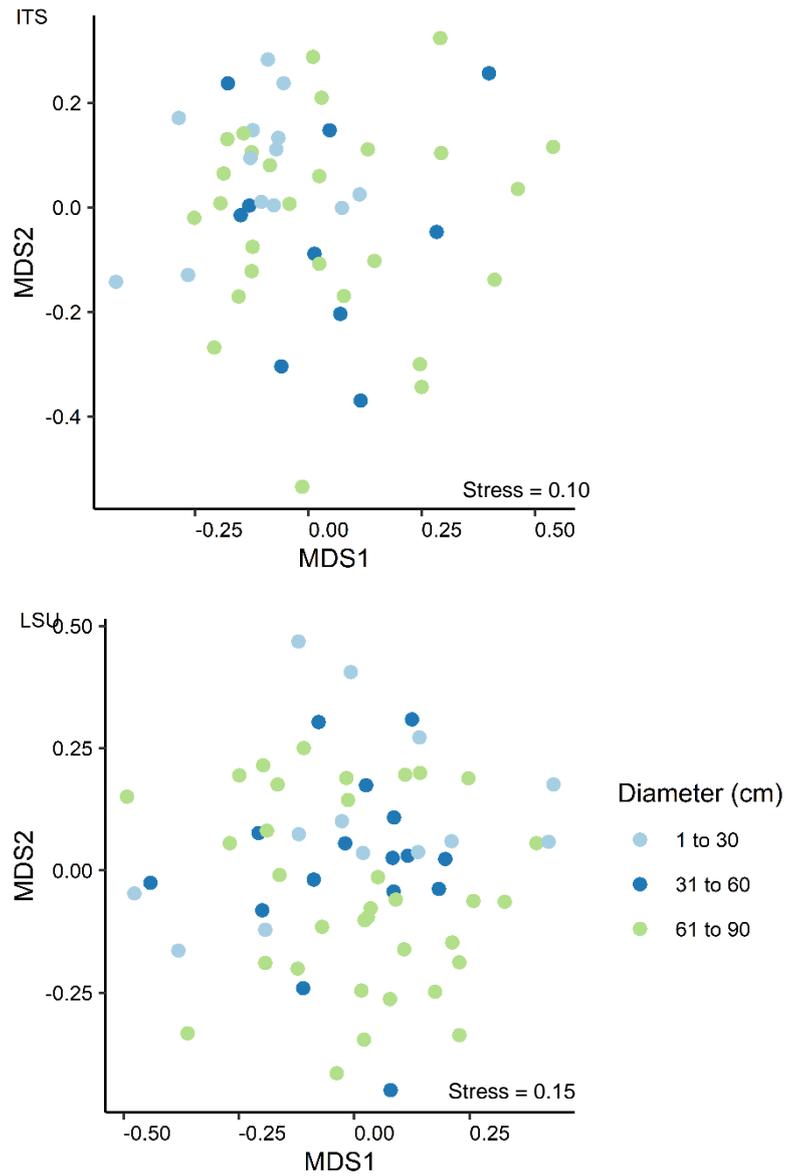


Figure 4.6. NMDS plots of fungal communities of trees sampled once at breast height (~1.3 m). Plots according to ITS and LSU barcodes are coloured by diameter.

Agaricales, Hymenochaetales and Polyporales. They included known beech associates as well as generalists and those more often recorded with other tree hosts.

4.5. Discussion

4.5.1. Wood saprotrophs are present, but not frequently detected, in sapwood

Wood decay taxa were not detected in all wood dust samples collected for this study. Of the wood decay taxa found, however, these were both Asco- and Basidiomycota, and represented white, brown and soft rot decay modes. For trees sampled at multiple points on the trunk and large branches, wood decay species were detectable throughout. These results show that living trees are already colonised by taxa capable of causing significant decay of all wood components, potentially many years before tree death. The majority of wood decay taxa were found in a search of the FRDBI with *Fagus* as an associated organism. This lends support to the idea that fungal taxonomic assignments are not spurious and realistically reflect communities in beech trees, at least at the genus-level. Fewer matches were made in GenBank, but DNA sequence databases span fewer years than do fungal sporocarp records.

Heart rot fungi are thought to have strong associations with particular tree hosts. For example, the species *Ganoderma adspersum*, *Kretzschmaria deusta* and *Meripilus giganteus* are among the most important heart rot fungi of beech in the UK (Cartwright and Findlay, 1946). *Eutypa spinosa* and *Biscogniauxia nummularia* are other known beech associates, causing strip cankers many metres high up trunks following drought conditions (Hendry et al., 1998). These species were all detected in functional sapwood and, though they may frequently colonise trees via other routes, are now confirmed as latently present.

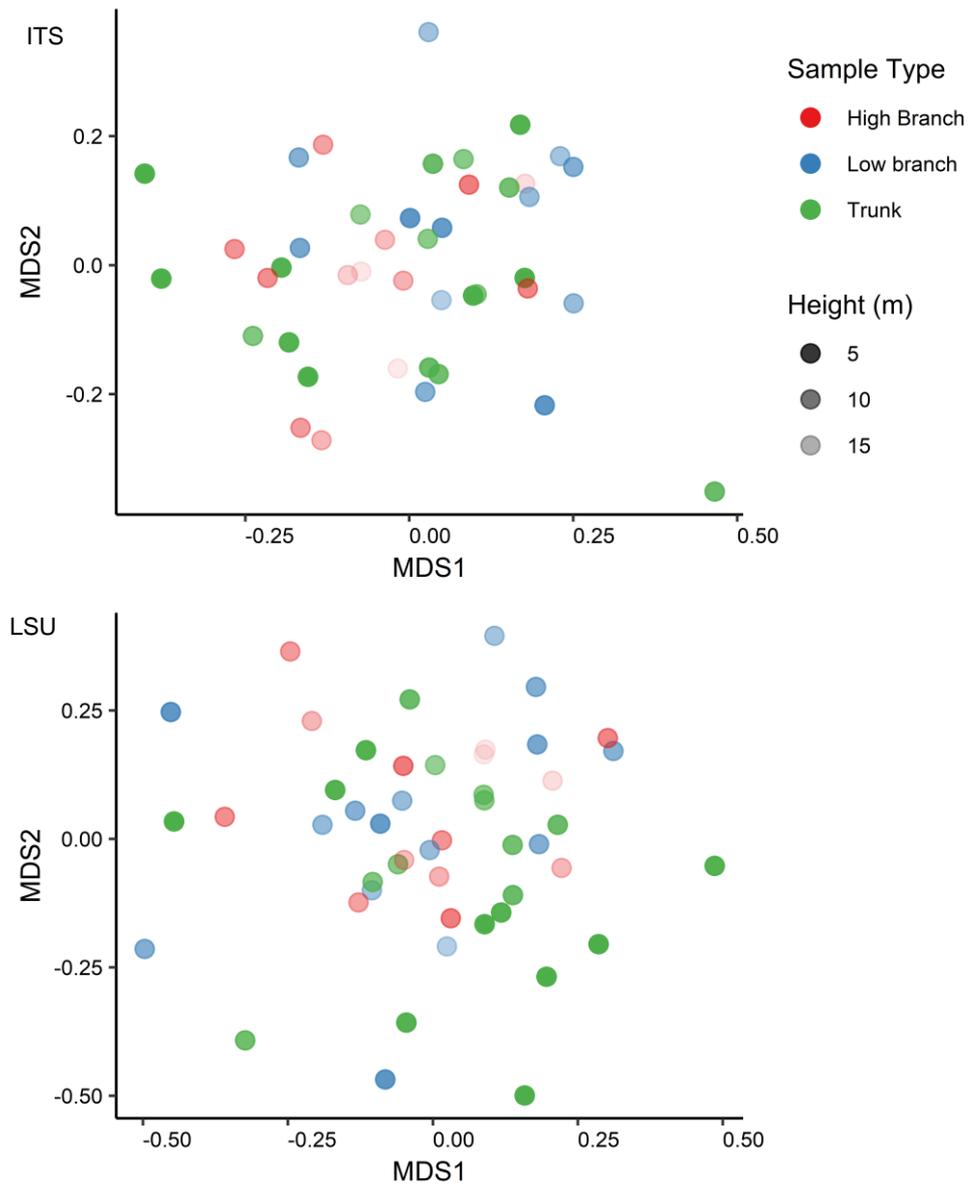


Figure 4.7. NMDS plot of fungal communities of five trees sampled at different heights and positions, according to ITS and LSU barcodes.

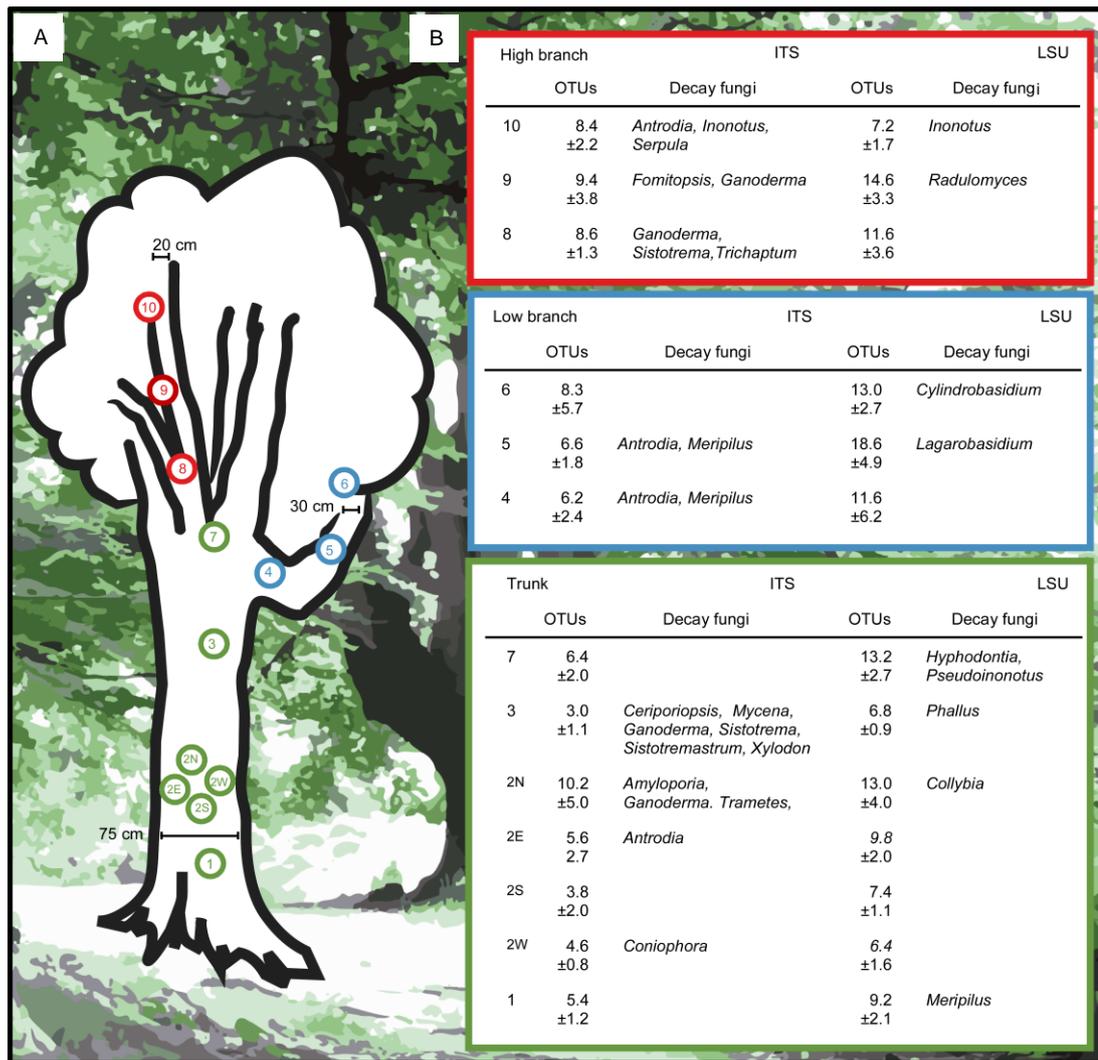


Figure 4.8. Summary of sampling scheme for five mature trees sampled at multiple points. (A) Relative locations on the tree, including at cardinal points, up the trunk and along the lowest branch and highest branch larger 10 cm diameter. (B) Average number of OTUs with standard error at each sampling point, and taxa identified as putative wood decay fungi (basidiomycetes only).

Strong associations between wood decay fungi and 11 tree species were demonstrated even after three years of decomposition (Purahong et al., 2018a). Across living tree species, host associations might be stronger, since the chemistry and structure of the wood, together with interacting plant cells represent a more selective environment than decaying wood. This hypothesis remains to be fully tested for wood sampled from living trees. Work from Lee et al. (2019), however, has commenced this line of enquiry, suggesting that fungal community distance increases with host phylogenetic distance for 22 recently harvested woody species.

4.5.2. *Endophytes and their ecological roles*

Approximately one-third of all taxa were assigned as wood or undefined saprotrophs, confirming the initial hypothesis that these would be a large component of endophyte communities. Of the further assignments, those classified as 'endophyte' by FUNGuild, provided little insight to their role within functional sapwood. That is, except that they have been detected in plants previously. Investigations into ecological roles of cultured endophytes are encouraged to improve open-source databases like FUNGuild and to better understand these communities. As an example, *Aureobasidium pullulans* was detected by all survey approaches, and is a ubiquitous black yeast found with a variety of plant tissue types (Cooke, 1959; Pugh and Buckley, 1971 and therein). Despite the wide distribution of this fungus, its ecology remains unclear. A more holistic understanding of microbial communities and their interactions within plants would be facilitated by combining basic ecological studies and experiments *in planta* with (improving) methods to detect active fungi (R. Henrik Nilsson et al., 2019).

4.5.3. *Influence of site and tree size on fungal communities*

Sites represent fungal species pools from which trees can be colonised. Differences in these species pools between sites can cause spatial variation in present community structure and influence later successional communities via priority effects (Hiscox et al., 2016b). The presented results support this, but further work comparing, for example, air spora between sites would lend strength to notion.

Similarly, large trees, having been subject to longer colonisation time might and harbour different communities than smaller trees. This was not supported by the results, but this could be due to the sampling strategy employed here. By collecting wood dust from a fixed depth into sapwood, the age of wood sampled would be

expected to be similar for both large and small trees. Thus, more exhaustive sampling is recommended to understand where and how fungal communities might differ between trees of different ages and sizes.

4.5.4. Fungal richness and taxonomic classification varies by survey approach

Metabarcoding generated more OTUs than did the culture-based approach and, at all taxonomic levels, ITS and LSU sequencing had more OTUs in common. Taking all samples together, LSU detected more OTUs than ITS. Each approach, even at the class level, detected classes that the other approaches missed. All survey approaches suggest that detection of fungi was stochastic at single sampling points. Number of OTUs varied widely. Multiple sampling points, taken together, yielded more species.

Primers and primer sets are known to have biases, with ecologists cautioned to interpret patterns carefully (Schadt & Rosling (2015). Here, it was shown that the ITS universal fungal barcode is sub-optimal for use with samples containing low fungal biomass (Nilsson et al 2018). This issue was counteracted by separately using a second primer set (LSU) which amplified less plant host DNA, more total OTUs and a larger range of fungal. As such, LSU might be promoted as a better barcode for sapwood fungal communities but, of significance to the evaluation of these survey approaches, is that of the beech heart rot fungi noted in section 4.5.1, all were found by metabarcoding of ITS.

Cultures yielded a lower number of OTUs, lower range of fungal classes and detected proportionately different functional guilds. Overall, the approach supports the interpretation of metabarcoding results, with less uncertainty than with metabarcoding results alone. Culturing methodologies vary, and can impact the number and identity of fungi isolated from a wood substrate (Unterseher and Schnittler, 2009). Wood dust, a highly comminuted sample provides a markedly different microenvironment compared with a larger wood samples like wood chips. Any fungal hyphae, if present in the wood, are inevitably broken during the sampling process, perhaps reducing the number of isolations made. It was, however, deemed an acceptable trade-off so that the same sample type could be used for metabarcoding. Multiple culture methods are ideally employed, but culturing in general is labour-intensive and of declining overall use in fungal studies.

4.5.5. Conclusions

The identity and number of decay fungi detected suggests that entry into functional sapwood is a regular event, though propagules seem sparsely distributed in wood. The presence of wood saprotrophs as endophytes could affect decay outcomes (i.e. white vs brown rot) (Cline et al., 2018) at later successional stages. These priority effects can cascade down to forest floor and beyond (Fukasawa, 2012).

Endophytes are increasingly relevant for study. In sapwood, which is physiologically vital for tree health, fungal endophytes are likely ecologically important. For example, endophytes have been shown to interact directly with *Phytophthora* pathogens in leaves (Arnold et al., 2003) and other pathogens in roots (Terhonen et al., 2016). In sapwood, where maintenance of physiological integrity is so important, mutualisms may be more common compared with leaves and fine roots and which undergo frequent turnover.

This study presents a baseline fungal microbiome ('mycobiome') of sapwood of healthy trees. Together, ITS and LSU barcodes recovered a wide diversity of fungi, but HTS generated more total OTUs than Sanger sequencing of fungal cultures, including species which are, as yet, unculturable. Despite adopting many of the recommended practices for HTS sequencing (Zinger et al., 2019) to ensure robustness of results, the additional fungal cultures corroborate the true presence of the shared taxa in sapwood, demonstrating the value of triangulating results.

Chapter 5

Interactions between heart rot fungi

5.1. Summary

Pairing species in simplified systems is an established method for determining competitive relations between wood decay fungi. The outcome of interactions, however, is known to be context-dependent, with numerous other factors influencing the result of fungal competition. Pairwise competitive combinations of eighteen species, isolated from beech wood and sporocarps, were assembled in wood blocks and on agar culture. Secondary colonising species were shown to have good competitive ability, whilst primary colonising species were the poorest competitors. However, fungi grouped ecologically as heart rots rot ranked among the highest and lowest when scored on overall competitive ability and showed a variation of growth and decay potential. These data indicate that the basic ecology of heart rot fungi should be further explored, and may be used to interpret heart rot community surveys.

5.2. Introduction

Competition is a major determinant of fungal communities in wood; it is a spatially defined resource where occupation of territory determines access to nutrient resources contained within (Boddy, 2000; Hiscox et al., 2018). Fungi differ in their competitive capabilities, but also in ability to cope with abiotic stress and disturbance events. As such, fungi can be classified under a modified C-S-R model (*sensu* Grime (1979)) model of plant ecology (Rayner and Cooke, 1984). The three primary fungal life-history strategies are: S-selected (stress tolerant), R-selected (ruderal), and C-selected (competitive). C-selected species are an informal, but well-studied grouping (Boddy, 1993)

In the living tree, opportunities for fungal colonisation are presented by two main routes: 1) into dead or dying attached branches and, 2) into tree heartwood. Elsewhere, functional sapwood, which is filled with water and low in oxygen, poses a major restriction on growth and activity of decay fungi. In dead branches, decay fungi have been shown to be ruderal species, adapted to arrive first to the substratum by spore rain or by latent colonisation, to grow and reproduce rapidly. In tree heartwood, it is proposed that the gaseous regime is more favourable to fungal

growth than in sapwood but remains relatively restrictive. However, the main restriction on fungal growth is the presence of extractives – myriad compounds that are inhibitory or toxic to fungi (Hillis, 1987). Given this, fungi found in heartwood tissues are regarded as stress-tolerators which are adapted to cope with extractives (Valette et al., 2017), but otherwise might grow and reproduce slowly, and possess limited combative capabilities.

Under this model, alleviation of stress in tree heartwood would lead to succession from stress-tolerant fungi to competitive species. This alleviation might progress by detoxification and degradation of extractives by heart rot fungi, and improvements in gaseous regime as heartwood becomes exposed through wounds or formation of hollows. Thus, as trees age and the duration of colonisation by heart rot fungi increases, so too does the likelihood of competitive replacement by C-selected species.

The outcome of species interactions is context-dependent, with several factors contributing to the structure and development of communities. Several studies now document numerous interacting factors which imply that predicting fungal community assembly is almost impossible (Boddy, 2000). These factors are biotic, including the presence of invertebrates (A'Bear et al., 2014) and bacteria (Johnston et al., unpublished), and abiotic, including temperature (Hiscox et al., 2016a), water potential, gaseous regime (Boddy et al., 1985) and current wood decay state (Fukasawa et al., 2011). More recent studies have found orientation across wood vessels (O'Leary et al., 2018) and relative volume of territory occupied (Song et al., 2015) add complexity to fungal-fungal interactions. Despite forming the basis of our understanding of competition in wood decay fungal communities, the species investigated have mostly included primary colonising fungi, or late-stage cord-forming species, with the exception of Wald et al. (2004) in the investigation of rare *Hericium* and *Creolophus* species.

Exploring fungal interactions is a common method of establishing competitive relationships. In this chapter, pairwise interactions between heart rot fungi, most of which were detected in beech trunks in Chapters 2 and 3, together with some other beech-associated fungi.

5.3. Methods

5.3.1. Study overview

Eighteen species of wood decay fungi were used in agar and wood block competition experiments, and for measurement of mycelial extension rates and wood mass loss capability (Table 5.1). These species were selected prior to completion of studies in Chapters 2 and 3, but twelve of the eighteen species were detected in beech trunks surveyed. Eleven species were considered heart rot fungi of beech trees, based on existing literature. The remainder were also relevant beech associates, though in other ecological roles. All isolates were obtained from wood or sporocarps shortly before the study, except *Fomes fomentarius*, which was taken from the culture collection at Cardiff University.

5.3.2. Mycelium extension rates

6 mm plugs were inoculated centrally onto 2% (w/v) malt extract agar in 9 cm Petri dishes. These plugs were cut from the growing margin of each fungus growing on the same media. Plates were incubated in the dark at 20°C with three replicates per species. The radial extension of colonies was measured across two perpendicular diameters using a vernier calliper, every 1 - 7 days depending on speed of growth. A minimum of five measurements were taken for each plate. The time taken to obtain five measurements ranged from six to 40 days.

5.3.3. Wood density loss

Beech wood blocks (2 x 2 x 2 cm) were defrosted from storage at -20°C then soaked briefly in sterile distilled water before placing in autoclave bags. To sterilise, these were autoclaved 3 times at 24 hour intervals. Blocks were then aseptically placed on mature fungal cultures on 0.5% malt extract agar and left to colonise for 112 days (16 weeks). Relative density was determined for 3 replicates by measurement of oven dried weight / wet volume (g/ cm³). Three further replicates were then removed from agar culture and incubated in sterile plastic pots containing 30 ml sterile perlite at 20°C in darkness. After 84 days (3 months) density was determined for these replicates. Wood block density after initial precolonisation was compared with density after a further 84 days to calculate decay rate, expressed as mg cm³ day⁻¹.

5.3.4. Interactions in wood

Beech wood blocks were colonised for 112 days (16 weeks) as in section 5.3.3. To set up species interactions, colonised blocks were scraped free of adhering mycelium and placed in pairs secured by an elastic band so that wood vessels

aligned. Paired blocks were placed in sterile plastic pots for the duration of the interaction. Pots contained 30 ml of perlite, moistened with 5 ml distilled water, and the wall of the pot featured a ~1 mm hole covered with microporous tape to allow aeration. Elastic bands were removed after 10 days, and perlite was remoistened with 5 ml distilled water after 28 and 56 days to maintain water availability.

After 84 weeks, each pair of interacting blocks were split apart. Each block was chiselled in half, perpendicular to the contact surface. Chips were excised from the split face at 4, 8, 12 and 16 mm from the surface in contact with the adjacent block. Chips were placed on 2 % malt extract agar and incubated at 20°C in the dark for 1-2 weeks until mycelial growth could be identified morphologically.

The outcome of interactions was estimated as territory of the two blocks occupied by a species with relation to its competitor. Outcomes were assigned a score as deadlock (0), where neither species gained territory from the other, and otherwise as partial (-1 or +1) or complete replacement (-2 or +2). Competitive rankings were determined as the sum of interaction outcomes for each species.

5.3.5. Interactions on agar

Interactions between the same species were examined for pairings grown on 2% malt extract agar in 9 cm Petri dishes. 6 mm plugs were cut from the growing margin of each fungus growing in pure culture, and were inoculated 2.5 cm apart. Due to the range of extension rates displayed by each species, plugs were inoculated in a staggered manner, ensuring each species had attained 10 mm in radius when meeting its opponent. Plates were incubated in the dark at 20°C with three replicates per species. After 70 to 84 days, pairings were visually recorded as deadlock, partial replacement and complete replacement, and scored as in section 5.3.4. Ambiguous outcomes were confirmed by re-isolation of the interacting species from plugs taken from the reverse side of the agar.

Table 5.1. Summary of species used in investigations. Species listed in alphabetical order together with name abbreviations, taxonomic placement, culture isolation source and ecology, as determined from literature listed below.

		Classification				Isolate		Ecology	
Species	Family	Order	Class	Phylum	Source	Location	Decay Mode	General Role ^{1,2}	
A.g	<i>Armillaria gallica</i>	Physalacriaceae	Agaricales	Agaricomycetes	Basidiomycota	Wood	Windsor Great Park	White ⁺	Late secondary coloniser (cord-forming)
B.n	<i>Biscogniauxia nummularia</i>	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota	Wood	Coed y Bedw	White	Primary coloniser
B.a	<i>Bjerkandera adusta</i>	Meruliaceae	Polyporales	Agaricomycetes	Basidiomycota	Sporocarp	Windsor Great Park	White	Early secondary coloniser
C.p	<i>Chondrostereum purpureum</i>	Meruliaceae	Polyporales	Agaricomycetes	Basidiomycota	Wood	Windsor Great Park	White	Primary coloniser
F.f	<i>Fomes fomentarius</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	Wood	(JHC isolate)	White	Heart rot
F.p	<i>Fomitopsis pinicola</i>	Fomitopsidaceae	Polyporales	Agaricomycetes	Basidiomycota	Wood	Epping Forest	Brown	Heart rot
G.a	<i>Ganoderma adspersum</i>	Ganodermataceae	Polyporales	Agaricomycetes	Basidiomycota	Sporocarp	Windsor Great Park	White ⁺	Heart rot
G.p	<i>Ganoderma pfeifferi</i>	Ganodermataceae	Polyporales	Agaricomycetes	Basidiomycota	Sporocarp	Epping Forest	White	Heart rot
H.e	<i>Hericium erinaceus</i>	Hericiaceae	Russulales	Agaricomycetes	Basidiomycota	Wood	Naphill Common	White	Heart rot
H.f	<i>Hypholoma fasciculare</i>	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	Wood	Windsor Great Park	White	Late secondary coloniser (cord-forming)
K.d	<i>Kretzschmaria deusta</i>	Xylariaceae	Xylariales	Sordariomycetes	Ascomycota	Wood	Epping Forest	Soft	Heart rot
L.s	<i>Laetiporus sulphureus</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	Wood	Epping Forest	Brown	Heart rot
M.g	<i>Meripilus giganteus</i>	Meripilaceae	Polyporales	Agaricomycetes	Basidiomycota	Sporocarp	Bryn Garw Park	White ⁺	Heart rot (butt)
M.m	<i>Mucidula mucida</i>	Physalacriaceae	Agaricales	Agaricomycetes	Basidiomycota	Sporocarp	Windsor Great Park	White [*]	Primary coloniser
P.f	<i>Perenniporia fraxinea</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	Sporocarp	Epping Forest	White	Heart rot
P.a	<i>Pholiota adiposa</i>	Strophariaceae	Agaricales	Agaricomycetes	Basidiomycota	Sporocarp	Epping Forest	White	Heart rot
P.o	<i>Pleurotus ostreatus</i>	Pleurotaceae	Agaricales	Agaricomycetes	Basidiomycota	Sporocarp	Windsor Great Park	White	Heart rot
T.v	<i>Trametes versicolor</i>	Polyporaceae	Polyporales	Agaricomycetes	Basidiomycota	Wood	Windsor Great Park	White	Early secondary coloniser

* Soft rot reported by Daniel et al. (1992); ⁺ Soft rot capability also reported in Schwarze, (2007). Roles suggested in ¹ Cartwright and Findlay (1958), ² Boddy and Heilmann-Clausen (2008).

5.3.6. Statistical analysis

Analyses were performed in R (version 3.4.4) (R Core Team, 2018) within Rstudio (version 1.1.442) using packages dplyr (Wickham et al., 2018).

Species extension rates on agar were determined by linear regression of hyphal length over time for each replicate. Bar plots showing extension rates and decay rates were produced using ggplot2 (Wickham, 2016).

A chi-squared test was performed to determine whether the proportion of interaction outcomes observed was dependent on the medium of the interaction (wood or agar). The sum of interaction scores per species were ranked from highest to lowest, including ties, for each medium. Correlation of extension and decay rates with interaction scores was then assessed using Spearman's rank correlation.

Network plots were produced with the igraph package (Csardi and Nepusz, 2006) to visualise the possible direction of community succession. Plots arranged, and scaled, species on a grid according to their competitive ranking in wood.

5.4. Results

5.4.1. Outcome of interactions

Interacting in wood, *Hypholoma fasciculare* was ranked as the most competitive species, which either replaced (16) or partially replaced (1) all other species (Fig. 5.1, Table 5.2). The next best competitors were *Fomitopsis pinicola*, *Hericium erinaceus*, *Pholiota sp.* and *Bjerkandera adusta*. These species replaced, were replaced by, and also deadlocked against competitors. By contrast, the ascomycete *Biscogniauxia nummularia* was replaced (13) and partially replaced (4) by all species.

On agar media, *F. pinicola* was the best competitor, reversing the position with *H. fasciculare*. *Armillaria gallica* was ranked as the least competitive, which was replaced by 11 competitors. On agar, neither *A. gallica* nor *H. erinaceus* were able to replace, or partially replace, a competitor.

The distribution of interaction outcomes was significantly different on agar compared to wood blocks $\chi^2 (4) = 22.6$, $p < 0.001$. Of note, however, is that the number of deadlock outcomes was only marginally higher (56) on agar than in wood (52) (Table 5.2). The rank assignments were broadly comparable between interactions in wood and on agar. However, some species ranks were markedly different:

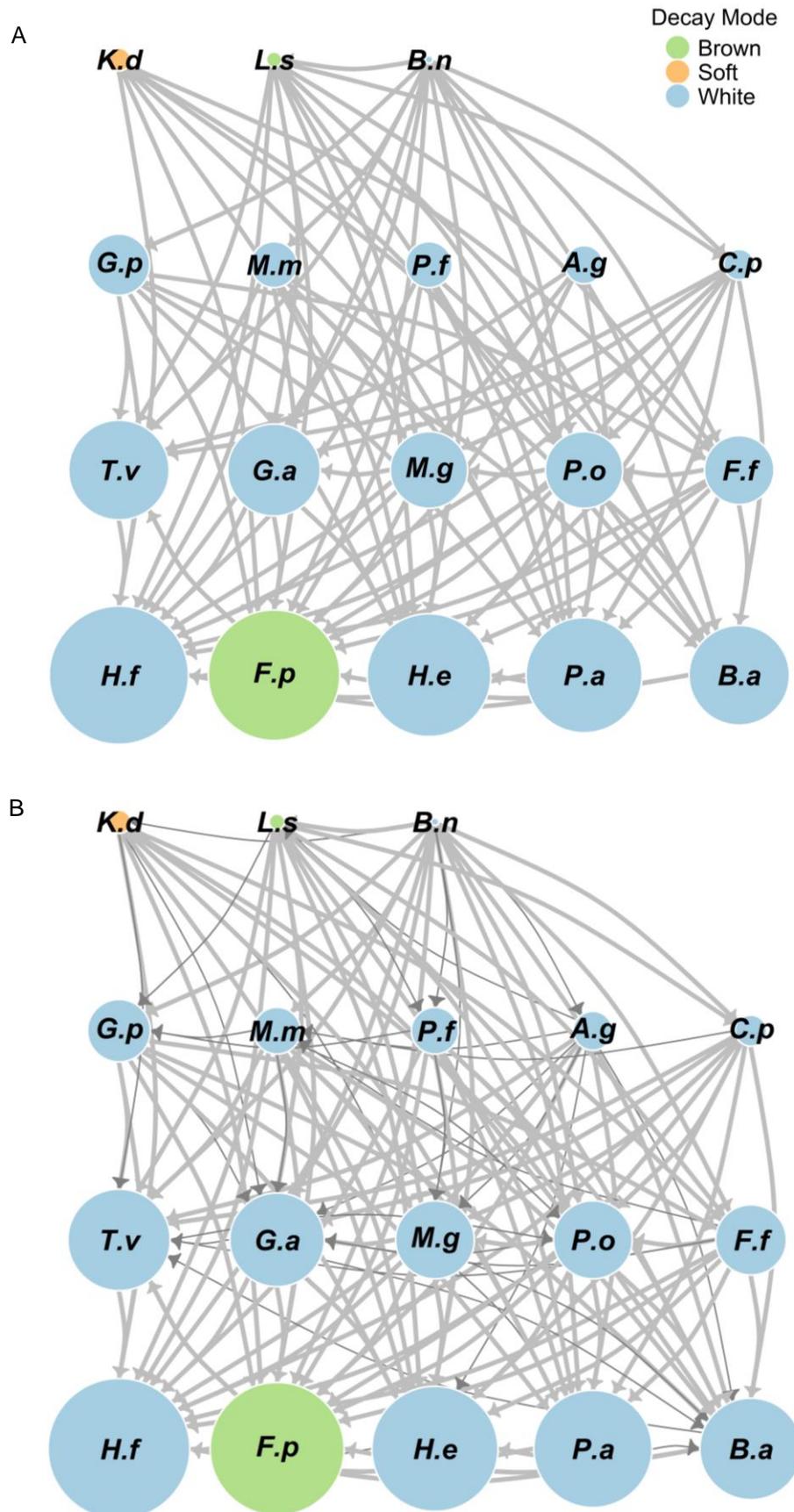


Figure 5.1. Network showing direction of community development in wood blocks, indicating (A) complete replacements only and (B) both partial (dark line) and complete (light line) replacements. Arrows represent the direction of travel of the community, i.e. point to the winner of the interaction. Size of nodes are scaled according to competitive ranking. Abbreviations of species names are indicated in Table 5.1.

Table 5.2. Interspecific outcomes summary by each species on wood and agar media. Outcome abbreviations: PR: partial replacement of competitor, R: replacement of competitor, D: deadlock with competitor, pr: partial replacement of fungus by its competitor, r: replacement of fungus by its competitor. Values in brackets indicate number of interactions with each overall outcome.

Species	Wood		Culture	
	Outcomes	Rank	Outcomes	Rank
<i>Armillaria gallica</i>	PR (1), D (5), pr (6), r (4)	14	D (1), pr (5), r (11)	18
<i>Biscogniauxia nummularia</i>	pr (4), r (13)	18	PR (1), R (1), D (3), pr (7), r (5)	16
<i>Bjerkandera adusta</i>	PR (4), R (14), pr (1), r (3)	5	PR (4), R (7), D (1), pr (4), r (1)	4
<i>Chondrostereum purpureum</i>	R (2), D (4), pr (1), r (9)	15	PR (9), D (2), pr (4), r (1)	9
<i>Fomes fomentarius</i>	PR (1), R (3), D (4), pr (3), r (6)	10	PR (1), R (5), D (1), pr (3), r (7)	10
<i>Fomitopsis pinicola</i>	R (13), D (2), r (2)	2	R (15), pr (2)	1
<i>Ganoderma adspersum</i>	PR (6), R (4), D (2), pr (2), r (3)	7	PR (3), R (1), D (5), pr (3), r (7)	11
<i>Ganoderma pfeifferi</i>	PR (3), R (1), D (3), pr (2), r (6)	11	PR (1), R (8), D (3), pr (2)	5
<i>Hericium erinaceus</i>	PR (1), R (11), D (3), pr (2)	3	D (9), pr (5), r (3)	14
<i>Hypholoma fasciculare</i>	PR (1), R (16)	1	PR (7), R (9), r (1)	2
<i>Kretzschmaria deusta</i>	PR (1), D (5), pr (3), r (7)	16	PR (8), R (1), D (4), pr (2), r (2)	8
<i>Laetiporus sulphureus</i>	PR (1), R (1), D (2), pr (2), r (11)	17	PR (1), D (8), pr (5), r (3)	13
<i>Meripilus giganteus</i>	PR (4), R (4), D (3), pr (1), r (5)	8	PR (2), R (1), D (5), pr (3), r (6)	14
<i>Mucidula mucida</i>	PR (3), R (1), D (3), pr (3), r (7)	12	PR (2), D (5), pr (3), r (7)	17
<i>Pholiota adiposa</i>	R (11), D (3), pr (1), r (2)	4	PR (4), R (5), D (1), pr (4), r (2)	12
<i>Perenniporia fraxinea</i>	PR (2), D (7), pr (2), r (6)	12	PR (4), D (5), pr (3), r (5)	7
<i>Pleurotus ostreatus</i>	PR (2), R (6), D (2), pr (1), r (6)	8	PR (2), R (6), D (3), pr (4), r (1)	6
<i>Trametes versicolor</i>	PR (4), R (7), D (4), pr (1), r (1)	5	PR (7), R (8), pr (2)	3

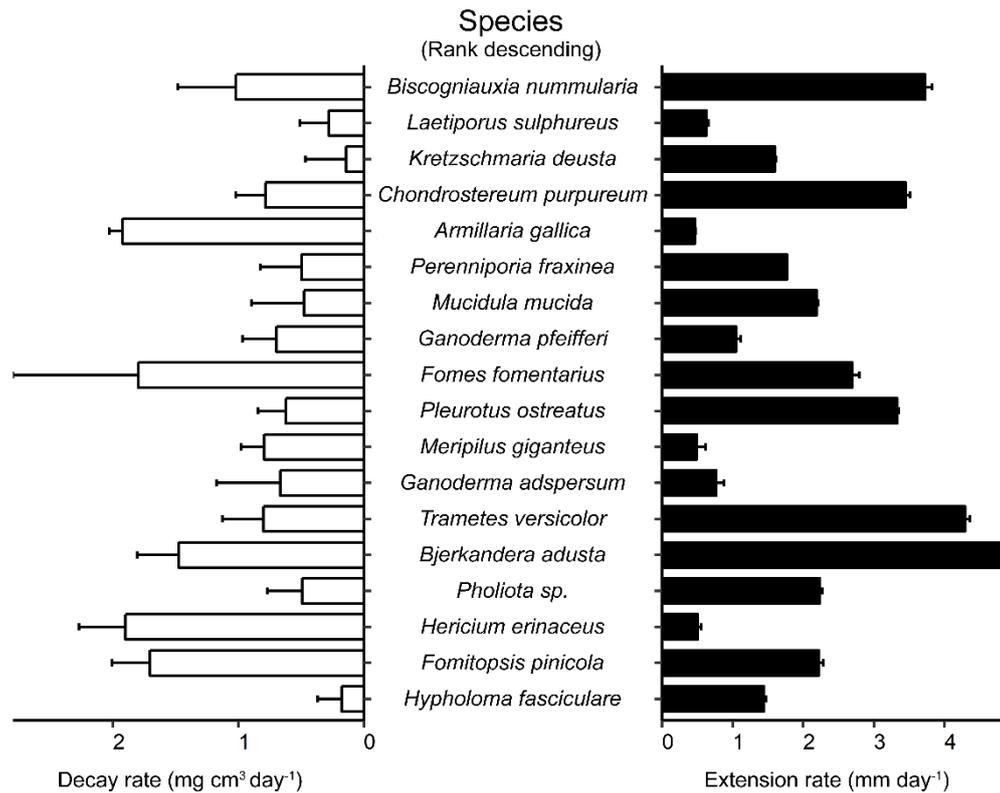


Figure 5.2. Wood decay rate and mycelial extension rates on 2% agar media. Species presented in rank order from least competitive (top) to most competitive (bottom), as calculated from interactions on wood blocks. Bars indicate means \pm standard error.

H. erinaceus and *Pholiota sp.* were good competitors in wood but performed much more poorly on agar media.

5.4.2. Mycelium extension rates and wood decay ability

When measured on agar, the mean hyphal extension rates across all species was 2.1 mm day⁻¹, and these rates were significantly different ($F(17, 36) = 432.7, p < 0.001$). Extension rate varied between from 0.46 mm day⁻¹ (*Armillaria gallica*) to 4.88 mm day⁻¹ (*Bjerkandera adusta*).

The decay rate of wood blocks following was significantly different between species ($F(17, 36) = 2.371, p < 0.05$), varying from 1.8 (*Fomes fomentarius*) to 0.3 mg cm³ day⁻¹ (*Laetiporus sulphureus*) (Fig. 5.2).

5.4.3. Relationship between competitive ability, extension rate and decay rate

Hyphal extension rates and wood decay rates both poorly correlated with species competitive ranking on wood blocks, and neither correlations were significant ($r_s = -0.03$, $p > 0.05$; $r_s = -0.17$, $p > 0.05$).

Wood decay rates also poorly correlated with competitive ranking on agar media ($r_s = 0.10$, $p > 0.05$). Importantly, however, hyphal extension rates negatively correlated with competitive ranking on agar media ($r_s = -4.50$, $p < 0.001$) (Appendix 5. Fig. S5.1).

5.5. Discussion

It is generally regarded that competitive abilities are broadly correlated with position of species in succession (Boddy, 2000). Here, the highest ranked competitor, *Hypholoma fasciculare*, replaced all other species encountered in wood blocks. This is an unsurprising result as it is a known later stage cord-forming coloniser (Boddy, 1993). Likewise, the primary colonising species (*Biscogniauxia nummularia*, *Chondrostereum purpureum*, *Mucidula mucida*) were amongst the lowest ranked competitors. However, fungi considered to be heart rot species ranked both highly and poorly as combatants. Clearly, the importance of combative ranking requires further exploration in heart rot communities. Combat is unlikely to be the only aspect of competition in establishment of heart rot. The ability to cope with initially poor aeration (Highley et al., 1983; Highley and Kirk, 1979), and to gain access to heartwood tissues (Merrill, 1970) are perhaps more important.

Heart rot fungi can occupy large volumes of wood in standing trees (Chapter 3). The amount of territory occupied by heart rot fungi, relative to secondary colonisers, may explain the persistence of heart rot species within trees, as size of inoculum has previously been shown to effect the outcomes of fungal interactions (Holmer and Stenlid, 1997). O'Leary et al. (2018) found that even relatively weak competitors could co-exist with strong competitors, if the former had a larger adjacent territory. Further, the orientation in wood within which two species meet, i.e. within or across xylem vessels, add to the possible range of interaction outcomes for otherwise weak competitors.

By visualising interactions in a network, it appeared that fungal communities might develop somewhat predictably from species that are predominantly replaced to those which predominantly replace competitors, i.e. in a transitive hierarchy. However, though 60% of all outcomes were complete replacement, the remainder of

outcomes (40%) were either deadlock or partial replacement. This large proportion of deadlocked and partial replacement outcomes shows that species interactions need not result in competitive exclusion and loss of species, but that species may effectively co-exist for undetermined time-periods. Bruns (2018) also noted that non-transitive competition can result in more diverse communities, in reference to the large proportion of species deadlocks also observed in the work of Maynard et al. (2017).

This assessment of competitive interactions on wood and agar media shows that consideration of realism is important for simple experimental systems. Outcomes between species pairs in wood blocks did not correspond well to interactions on agar, although in both systems the two most competitive species were *H. fasciculare* and *Fomitopsis pinicola*. A notable discrepancy is *H. erinaceus*, a slow-growing species which either deadlocks or is replaced by competitors on agar media. In wood blocks, by contrast, *H. erinaceus* ranked as a strong competitor, concurring with Wald et al. (2004). Further hypotheses pertaining to the rarity of these tooth fungi remain to be explored (Boddy et al., 2011).

Interaction outcomes between fungal species on agar were an aid to interpretation of those in wood blocks. Species interactions in wood are difficult to visualise, but on agar the qualities of mycelium can be better seen and interpreted (Appendix S5.2). Studies on agar media, therefore, remain an important first pass in the study of fungal ecology (Crowther et al., 2018). It is important to note, however, that hyphal extension rates did not correlate with competitive rankings calculated from interactions in wood blocks but negatively correlated with rankings on agar. Given this, results of such studies on agar should be treated with caution.

In this chapter, species chosen were those that are likely to interact in nature. It is unlikely that fungal combat is the most important factor structuring heart rot fungal communities, but the late stage fungi were better combatants than many of the early colonisers. Nonetheless, these results should be borne in mind when interpreting assemblages observed in nature.

Chapter 6

Synthesis

Fungal decay and the resultant hollowing of trunks, termed heart rot, is ecologically essential. The collection of studies presented in this thesis provide the most detailed examination of heart rot communities in beech trees to date. Here, existing knowledge regarding heart rot is validated, and aspects of heart rot detection, extent and origins are explored. Further, this work provides insight into the development of heart rot fungal communities via interactions with competing species.

6.1. Heart rot fungi

Chapters 2 and 3 confirmed that, although sporocarp surveys give some indication of the fungi present in standing trees, they are poor indicators of mycelial distribution in wood. These chapters confirm the importance of certain species as heart rot agents of beech trunks, but also report a wider range of colonists that have not so far been discussed in this context in the scientific literature (Table 6.1).

Ganoderma adspersum and the ascomycete *Kretzschmaria deusta* occurred as heart rots of beech trunks and were also detected in functional sapwood. Together, with the ascomycete *Ascocoryne* sp., they were the only heart rot fungi to be detected in all three survey Chapters (2, 3 and 4). *G. adspersum* and *K. deusta* are well-known species and are considered to be important heart rot fungi (Lonsdale, 2013; Weber and Mattheck, 2003). They occurred, however, far less frequently within standing the trees examined here (Chapter 2), than their obvious, long-lasting sporocarps might suggest. In wood block interactions, these two species did not emerge as strong competitors, being placed in the middle of a competitive ranking of 18 beech wood fungi (Chapter 5).

Of 30 beech saprotroph indicator species proposed in Ainsworth (2004), *Eutypa spinosa*, *Aurantiporus fissilis* and *Ganoderma pfeifferi* were found by coring and dissection of trunks, and these fungi had already been previously recorded as sporocarps at the study sites. The survey methods employed here generally failed to detect rare heart rot fungi, such as the tooth fungi *Hericium* and *Creolophus* spp. (Boddy and Wald, 2002), or *Podoscypha multizonata* (Dahlberg and Croneborg, n.d.; Overall and Mottram, 2010). This suggests that these species do not merely fruit rarely but are actually rare in standing trunks. Although this wasn't tested

Table 6.1. Summary of heart rot fungi detected in beech trunks (✓) throughout this thesis, including use for interactions experiments (■) in Chapter 5. Literature reporting species: (1) Cartwright and Findlay (1958), (2) Lonsdale (2013), (3) Ainsworth (2004).

Species	Chapters				Literature
	2	3	4	5	
<i>Armillaria gallica</i>		✓		■	
<i>Armillaria sp.</i>	✓	✓			
<i>Ascocoryne sp.</i>	✓	✓	✓		
<i>Aurantiporus fissilis</i>	✓				3
<i>Biscogniauxia nummularia</i>			✓	■	
<i>Bjerkandera adusta</i>		✓		■	2
<i>Chondrostereum purpureum</i>				■	1
<i>Coprinellus disseminatus</i>	✓	✓			
<i>Cylindrobasidium evolvens</i>		✓			
<i>Efibula sp.</i>	✓				
<i>Eutypa spinosa</i>		✓	✓		3
<i>Fomes fomentarius</i>				■	1, 2
<i>Fomitopsis pinicola</i>			✓	■	
<i>Ganoderma adspersum</i>	✓	✓	✓	■	1, 2
<i>Ganoderma pfeifferi</i>	✓		✓	■	2, 3
<i>Hypholoma fasciculare</i>		✓	✓	■	
<i>Kretzschmaria deusta</i>	✓	✓	✓	■	1, 2
<i>Laetiporus sulphureus</i>	✓		✓	■	1
<i>Meripilus giganteus</i>			✓	■	1, 2
<i>Mucidula mucida</i>		✓		■	1
<i>Neobulgaria sp.</i>	✓	✓			
<i>Nodulisporium sp.</i>	✓				
<i>Oxyporus populinus</i>		✓			
<i>Perenniporia fraxinea</i>		✓		■	2
<i>Pholiota 'A'</i>	✓	✓		■	1
<i>Pholiota squarrosa</i>		✓			
<i>Pleurotus cornucopiae</i>		✓			
<i>Pleurotus ostreatus</i>		✓		■	1, 2
<i>Psathyrella cernua</i>	✓				
<i>Stereum hirsutum</i>		✓			
<i>Trametes versicolor</i>		✓	✓	■	1, 2

directly, these species may genuinely be locally distributed and dispersal-limited (Stenlid and Gustafsson, 2001).

Several potentially important heart rot fungi of beech were either not detected, or detected infrequently, during the course of these studies. Species such as *Polyporus squamosus*, *Fomitopsis pinicola* and *Fomes fomentarius* were not detected. There are a several reasons why this could be the case, but the most obvious is a restricted sample size; as an example, there are over 15,000 mature beech pollards in Epping Forest (Dagley and Froud, 2012). A sample of 10 tree cores from this location could, thereby, not be expected to capture the range of fungi inhabiting trees. It is also possible that some species sometimes regarded as heart rot fungi occupy spatial niches that were missed. *Mucidula mucida*, though sometimes observed on beech trunks, mostly occupies smaller diameter branches (Rawlings, 2018). *Meripilus giganteus*, a conspicuous species fruiting around the base of beech trees, is perhaps restricted to the very lowest portions of trunks, below the standard sampling height of 1.3 m above ground-level.

6.2. Ecological Implications

A conclusion that emerges from these surveys is that wood decomposition may proceed following dozens of potential first colonists of beech trunks. Priority effects have been demonstrated in wood decay communities previously (Dickie et al., 2012; Fukami et al., 2010; Hiscox et al., 2016b, 2015). It was, however, not until recently that the role of latently present fungi have been investigated as the species exerting priority effects (Cline et al., 2018; Song et al., 2016).

To not recognise decay in standing trees is to overlook a centuries-long period of wood colonisation. In a study of decaying beech logs, Hoppe et al. (2016) detected species that likely originated in the standing, living tree. Many taxa from their study were species detected in standing trees (Chapter 2) and in recently fallen trees (Chapter 3). These species, it appears, are among the first colonisers of trees, but can also hold territory some years following tree death. This prospect has large implications for later decay stages, especially if these first colonists are those with very high combative ability (*Hypholoma fasciculare*, *Fomitopsis pinicola*; Chapter 5), or are negatively associated with other desirable taxa (Ottosson et al., 2014).

Some heart rot species increase tendencies for trees to fail in different places (Chapter 3), with consequences for subsequent colonisation of organisms, habitat development, and rate of decomposition. Hollow beech trees are keystone

structures, but some fungi can create more or less stable structures than others. According to Müller *et al.* (2013) this should be considered when selecting trees to be set aside for habitat. For example, under certain circumstances an increment borer survey could be used to identify trees to be felled or retained.

If some heart rot fungi are truly rare, one practical consequence may be the need for assisted colonisation of threatened species (Abrego *et al.*, 2016). In forest restoration schemes, action to increase the volume and type of dead wood may be insufficient to deliver on biodiversity objectives when dispersal, germination and establishment of rare species does not follow. A further opportunity of assisted colonisation is not just conservation of fungi in their own right, but in using fungi to create or provide habitats. This could include different types of decay (e.g. brown rot in beech trunks), cavity formers such as *Pholiot*a species, or those with sporocarps that host or feed saproxylic beetles (Schigel *et al.*, 2006, 2004; Schigel, 2007).

6.3. Methodological aspects

Metabarcoding had generated a new understanding of the vast diversity of fungi, and other microbiota, in wood (Hoppe *et al.*, 2016; Kubartová *et al.*, 2012; Purahong *et al.*, 2018a, 2018b). However, ever longer species lists are limited in use and lack the spatial resolution required for good ecological interpretation. Indeed, we are learning less about more as high throughput sequencing lavishes data on fungal ecology as a field. In Chapter 4, much of the results go uninterpreted because so little is known of the basic ecology of all but a few fungal groups. Most sequences, once assigned to a taxon and functional guild, receive little more than a passing acknowledgement. Indeed, Peay (2014) powerfully called for more biological detail about fewer organisms and places, with the reincorporation of intensive, natural history approaches into fungal ecology – Chapters 2, 3 and 5 have attempted to do this. In particular, the descriptive approach adopted in Chapter 3 has significantly contributed to the understanding of heart rot. In constructing spatial maps of trunks, patterns in trunks have been related to routes of fungal entry, ingress of water and tree damage and anatomy.

6.4. Major questions and future directions

In a Europe-wide sporocarp survey, Abrego *et al.* (2016a) highlighted *Ganoderma adspersum* as a predominant, and distinctive, feature of beech woodlands in the UK, proposing geographical isolation, climate and land-use differences to explain

differences between fungal communities in the UK and continental Europe. The work presented here, however, suggests this fungus to be less frequent than sporocarp records suggest. It is important to learn how well sporocarp inventories reflect the presence and activity of fungi across Europe, and the approaches used here could be extended geographically to address this.

With the confirmed presence of heart rot species in functional sapwood, the future lines of enquiry should begin with establishing this outcome for other tree species; does fungal diversity and community composition in functional sapwood differ with tree host species? The answer to this question is an important first step in understanding more about the entry of fungi into sapwood, as it is not currently known whether fungal propagules may enter via selective or non-selective means. Further, with ongoing improvements in metabarcoding methodologies and technology there are opportunities to use metabarcoding to survey functional sapwood more extensively than ever. Large-scale sampling of sapwood represents a novel means to explore geographical aspects of fungal diversity and distribution. It may also be applied to identify woodland sites that warrant further survey work, and address distributional uncertainties which currently hinder conservation (Molina et al., 2011) and red-listing efforts (Dahlberg and Mueller, 2011).

6.5. Conclusions

The ecology and interactions of fungi in trees are of vital importance to the habitat provision of thousands of organisms, including other saproxylic invertebrates, larger animals and other fungi. Although there are many remaining questions relating to the development of heart rot in trees, the findings presented in this thesis presents foundational information on the identity and distribution of heart rot fungi in trees, which are understudied and under threat.

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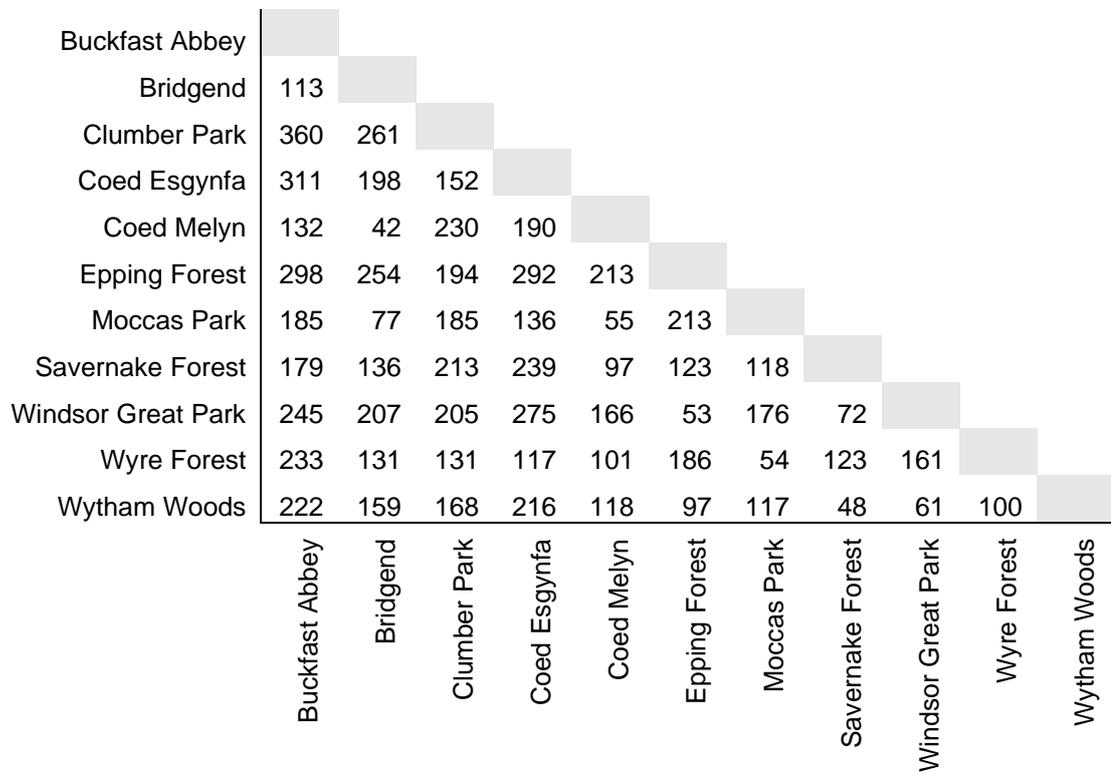


Figure S1.1. Distances (km) between sites visited throughout the thesis.

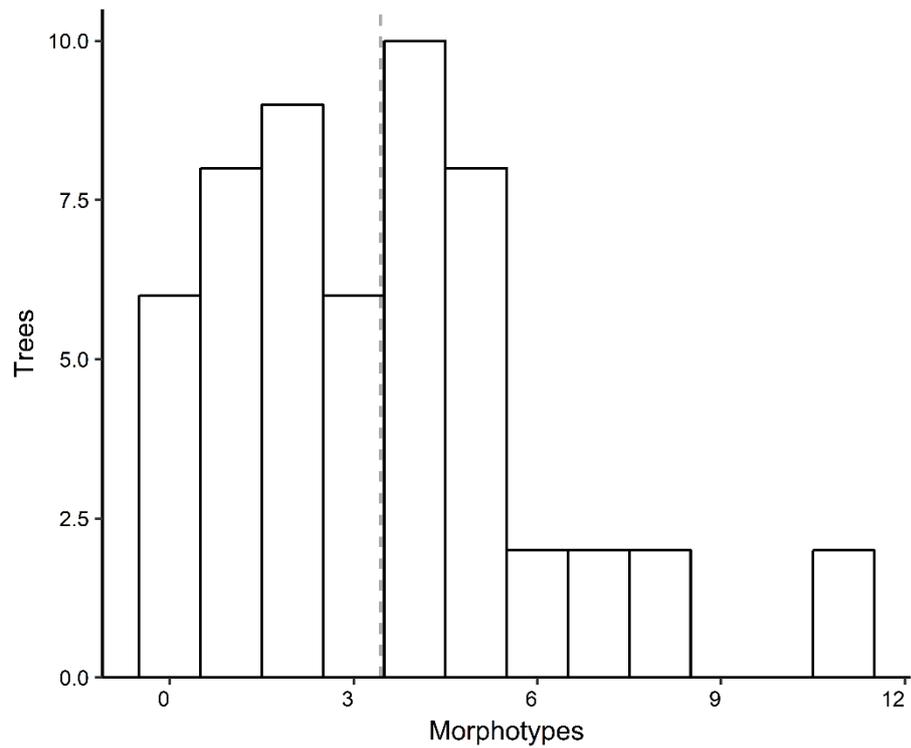


Figure S2.1. Number of fungal morphotypes isolated from cores of each sampled tree. As the length of the core varied between trees, the number of morphotypes is calculated per cm of core length multiplied by the mean overall core length (34 cm).

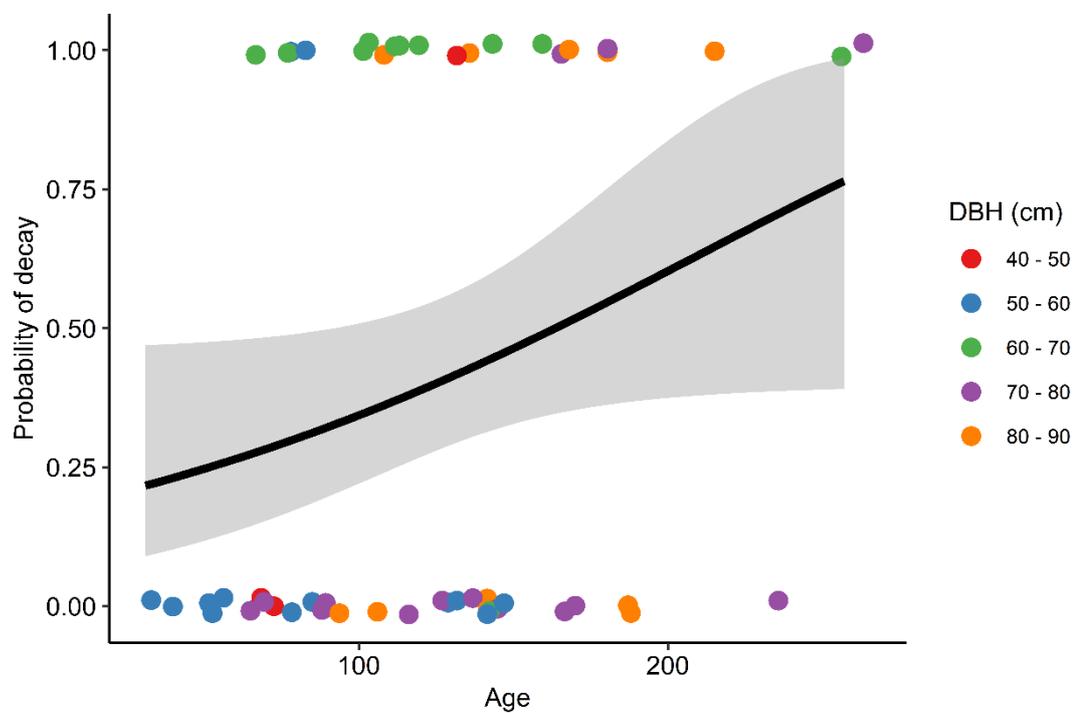
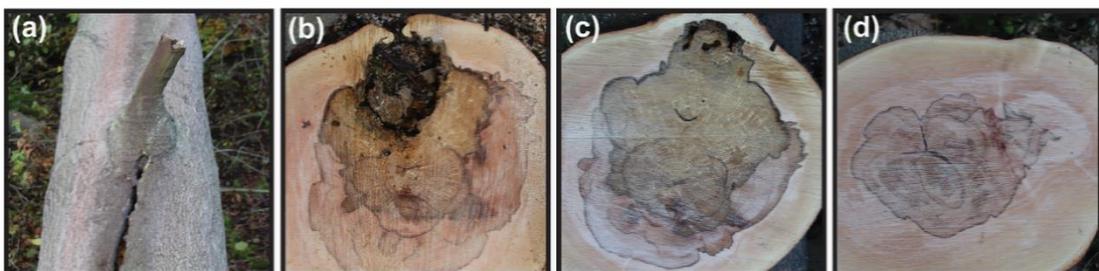
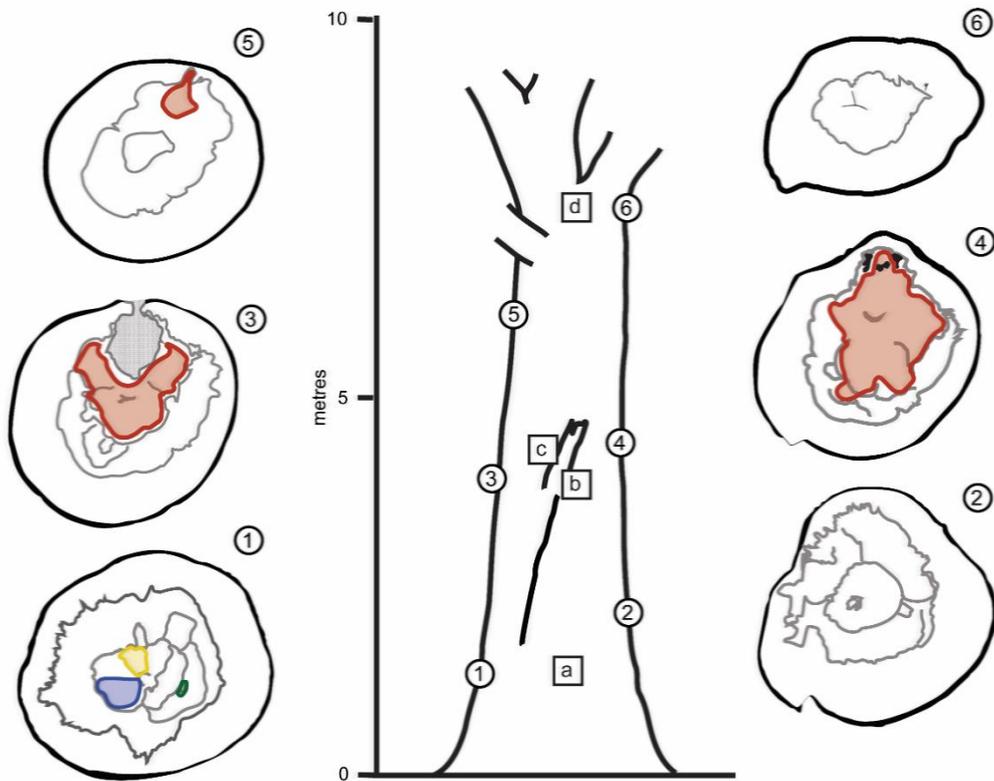


Figure S2.2. Probability of the presence of a decay fungus with increasing tree age. Probability line was fitted using binomial GLM, using tree age as the sole explanatory variable. Actual data points showing decay presence (1) and absence (0) are coloured according to the diameter of the tree.

Tree E2

Fungus	Length (m)	Area (m ²)
● <i>Ganoderma adpersum</i>	2	0.01
● <i>Pholiota 'A'</i>	2	0.01
● <i>Stereum hirsutum</i>	2	0.01
● <i>Pleurotus cornucopiae</i>	2	0.05

Hollowing

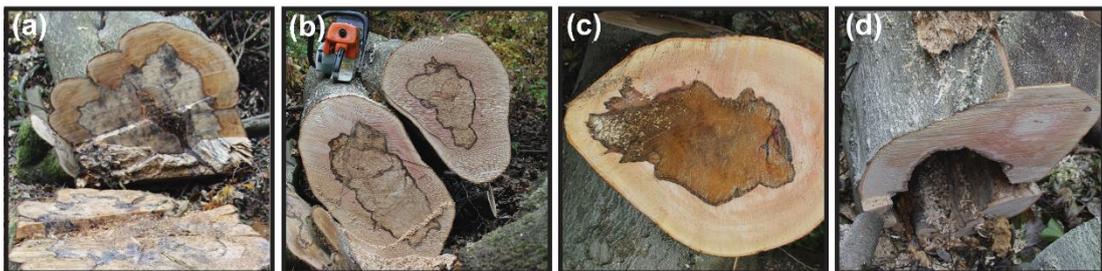
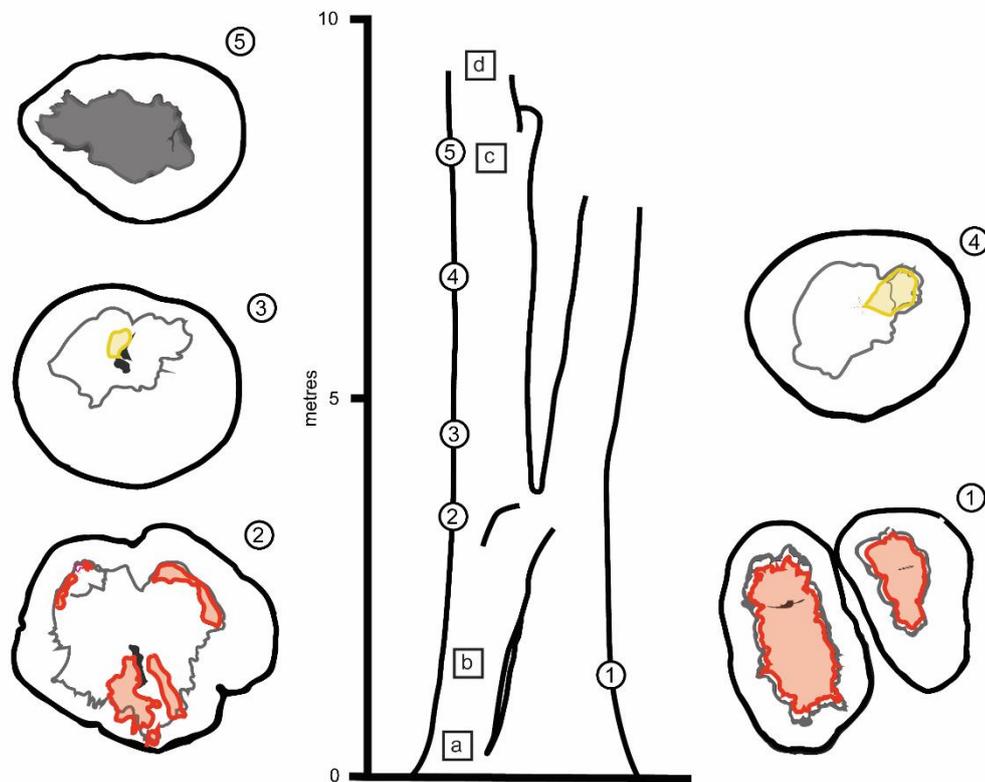


A tree felled due to suspected instability. A split in the trunk below a branch stub (a) indicated considerable decay. A cavity (b) was present immediately below the branch stub, with white rot decay caused by *Pleurotus cornucopiae* clearly visible in cross-section (c). By 7 m up the trunk, no decay fungi could be detected despite red-heart patterning (d).

Tree E3

Fungus	Length (m)	Area (m ²)
○ <i>Kretzschmaria deusta</i>	3.5	0.11
○ <i>Pholiota 'A'</i>	2.5	0.01

■ Wet wood

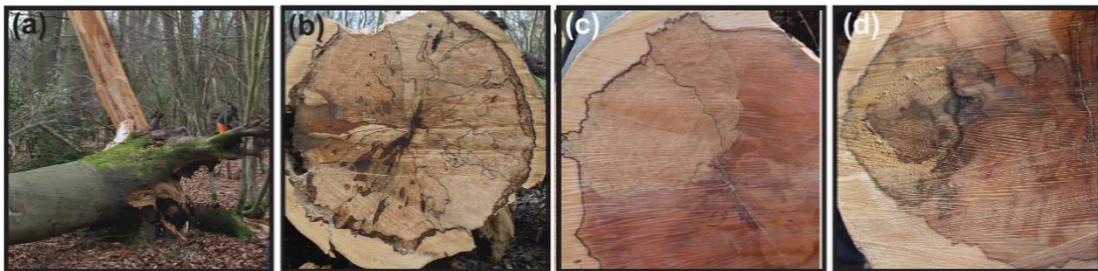
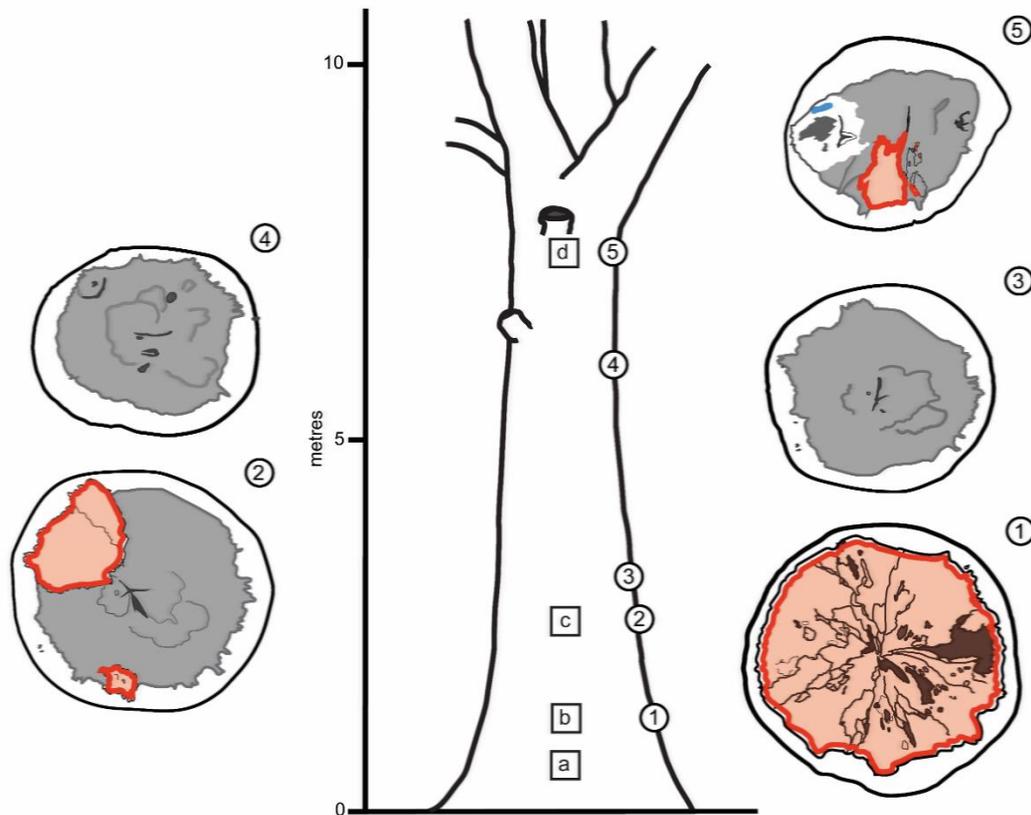


A tree felled with cut base (a) visibly colonised by *Kretzschmaria deusta*. Above the base, the trunk appeared as separate trees in cross-section (b), indicating the twin-stemmed growth of the tree earlier in its life. Wood (c) below a cavity (d) had become saturated due to ingress of water from above. No decay fungi were detected in this section (5), but the cavity may have been caused by *Pholiota 'A'* which was found in the wood below (sections 3 and 4).

Tree E4

Fungus	Length (m)	Area (m ²)
○ <i>Kretzschmaria deusta</i>	8	0.35
● <i>Pleurotus ostreatus</i>	1	0.01

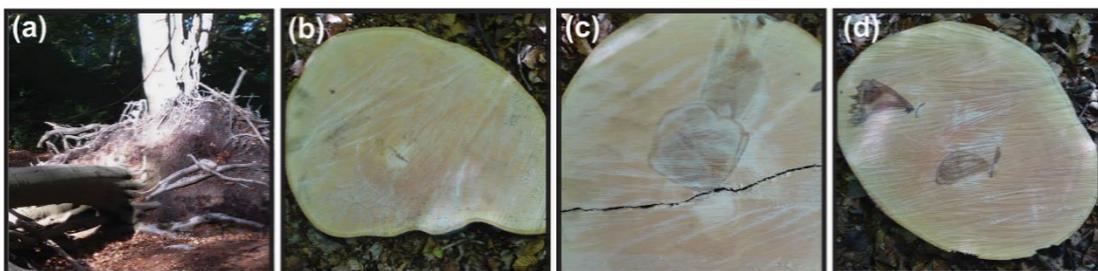
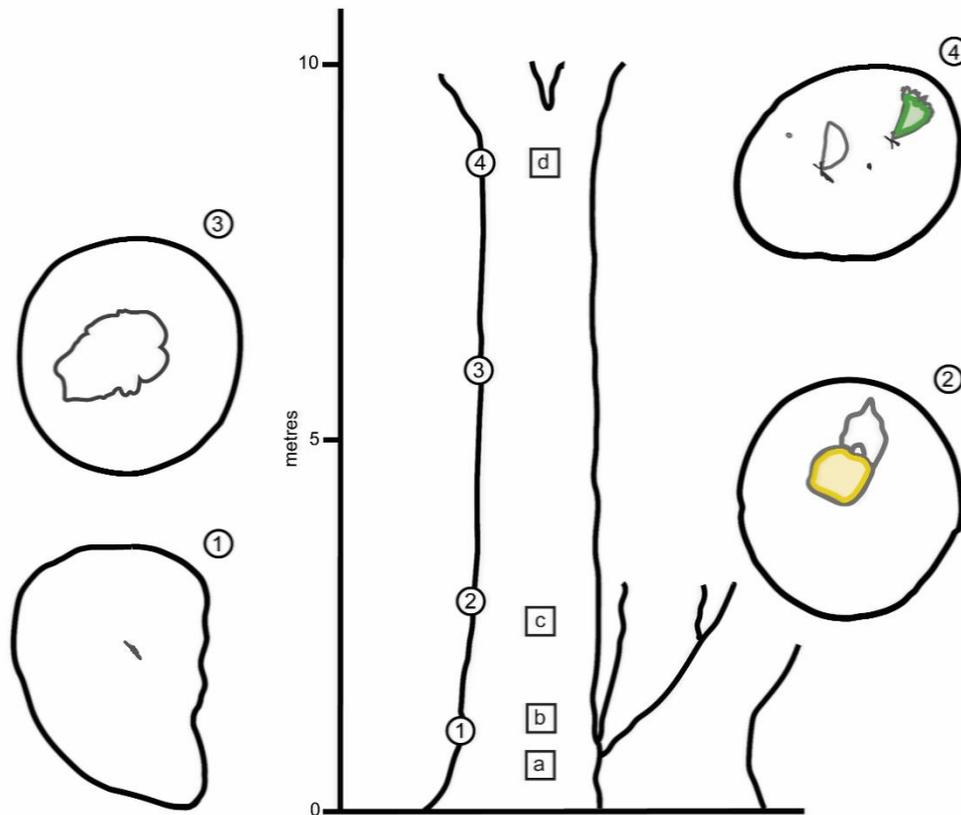
■ Wet wood



A tree trunk which failed at the base during a storm. (a) Large roots, decayed by *Kretzschmaria deusta*, fractured on one side, leaving part of the split trunk standing. (b) The base of the tree was colonised by multiple genets of *K. deusta*, as evidenced by numerous pseudosclerotial plates. (c) A large area of wet wood, was adjacent to a drier region where *K. deusta* was present. (d) Wet wood was also present adjacent to a region colonised by *Pleurotus ostreatus*, which appeared to colonise from a branch stub.

Tree E6

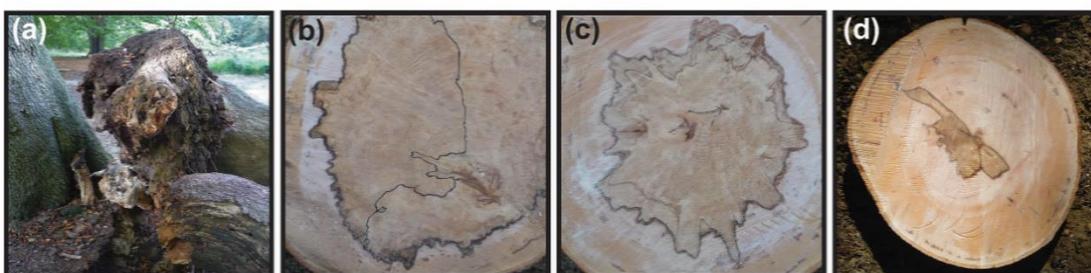
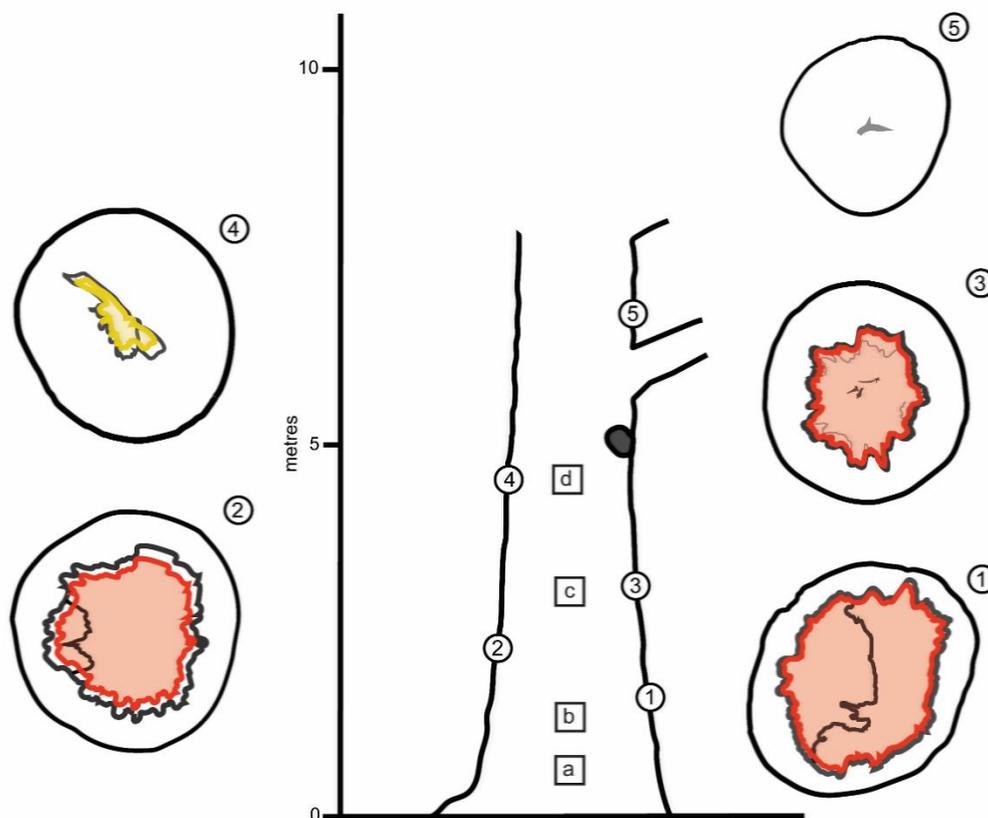
Fungus	Length (m)	Area (m ²)
● <i>Pholiota</i> 'A'	1	0.01
● <i>Eutypa spinosa</i>	1	0.01



A tree, part of a cluster of closely-grown trees, uprooted during high winds. (a) Shallow-rooting of the cluster likely contributed to wind-throw. (b) No indication of heartwood colouration or fungal decay, was observed at the base. (c) A small area of heartwood, colonised by *Pholiota* 'A', was visible at approximately 3 metres, and appeared unrelated to any sign of entry points on the trunk. (d) A further pocket of decay, caused by *Eutypa spinosa*, was detected on one side of the tree fork.

Tree E7

Fungus	Length (m)	Area (m ²)
● <i>Kretzschmaria deusta</i>	3	0.12
● <i>Pholiota</i> 'A'	1	0.01



A tree which had collapsed due to advanced decay of roots by *Kretzschmaria deusta*. (b) Wood was colonised by two individuals, clearly demarcated by a two closely-aligned pseudosclerotial plates. (c) The territory of one individual decreased further up the trunk.

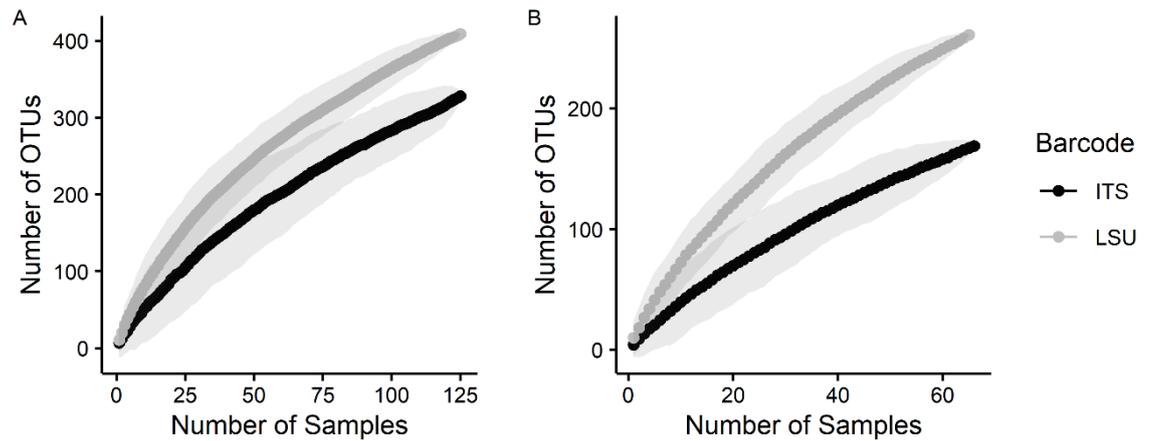


Figure S4.1. OTU accumulation curves for wood dust samples sequenced using two fungal barcodes. (A) Curves generated for all samples in study (126). (B) Curves generated for 66 samples (1 sample per tree) collected from trunks at 1.3 m above ground. Curves were generated by adding samples in a random order, while line ribbons indicate 95% confidence intervals.

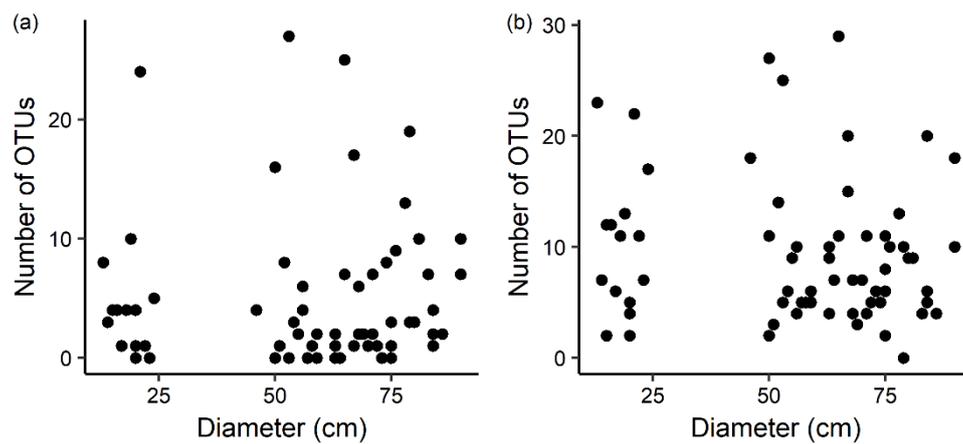


Figure S4.2. Scatter plots of OTU richness in relation to tree diameter at 1.3 m above ground for (a) ITS and (b) LSU.

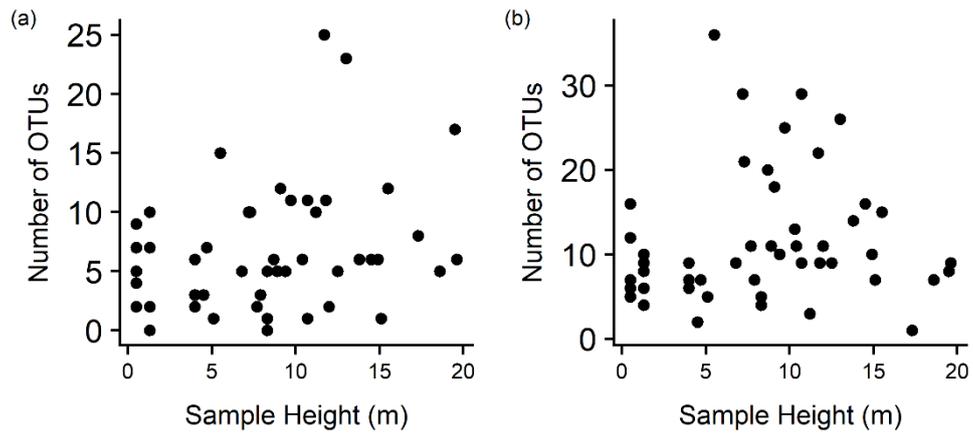


Figure S4.3. Scatter plots of OTU richness and sampling height for five trees sampled at multiple locations for (a) ITS and (b) LSU.

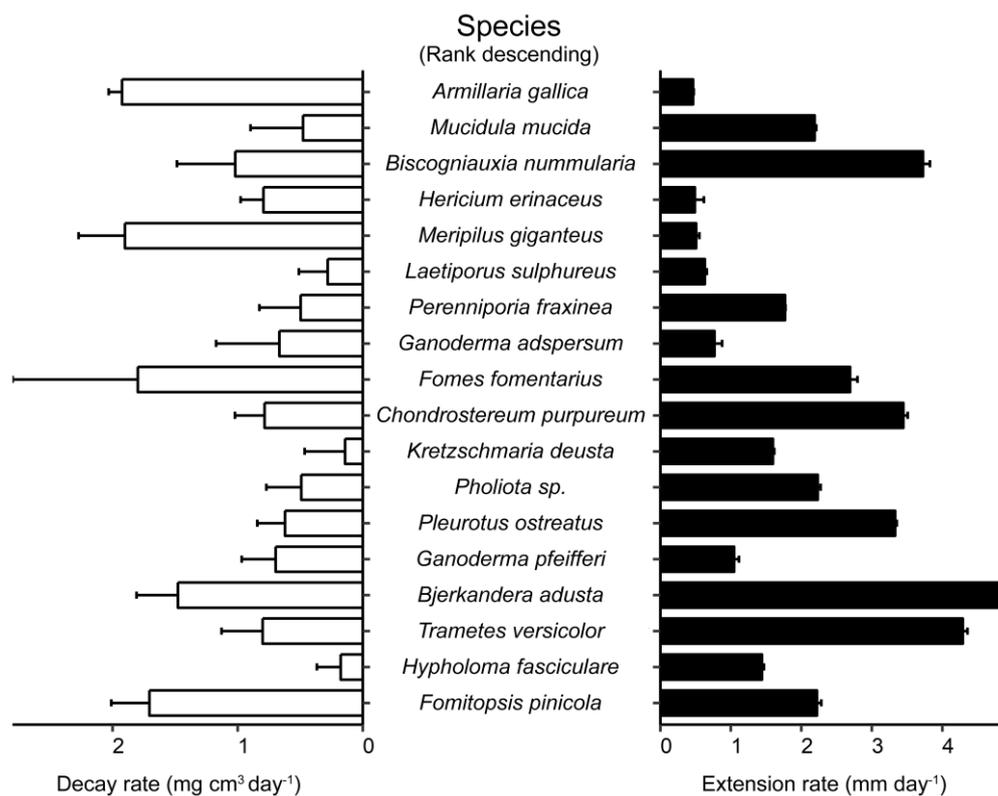


Figure S5.1. Wood decay rate and mycelial extension rates on 2% agar media. Species presented in rank order from least competitive (top) to most competitive (bottom), as calculated from interactions on agar media. Bars indicate means \pm standard error.

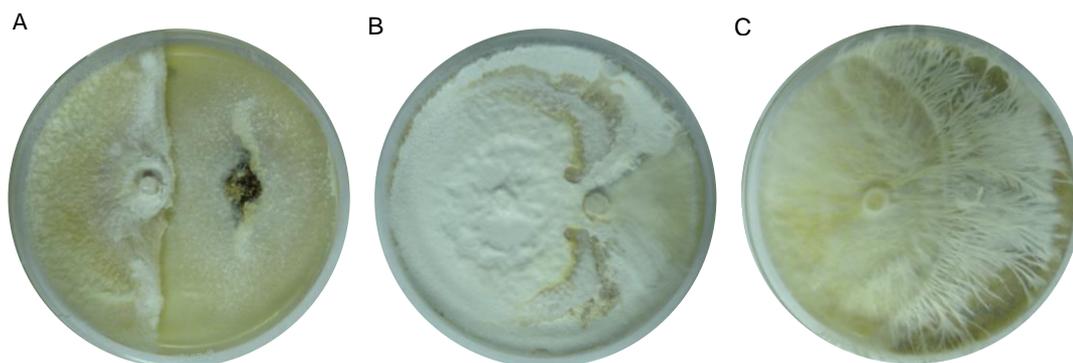


Figure S5.2. Interaction outcomes on agar media. (A) Deadlock between *Ganoderma adspersum* and *Mucidula mucida*, (B) partial replacement of *Fomes fomentarius* by *Ganoderma pfeifferi* and, (C) complete replacement of *Biscogniauxia nummularia* by *Hypholoma fasciculare*.