Ecology of the rare oak polypore *Piptoporus quercinus* and the tooth fungi *Hericium cirrhatum, H. coralloides,* and *H. erinaceus* in the UK

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Summary

Although fungi are essential to functioning of forest ecosystems, ecology of wood decay fungi, particularly rare species, is understudied.

Hericium coralloides, on the UK red data list, *H. erinaceus* and *Piptoporus quercinus*, UK BAP species, and *H. cirrhatum* are rare in the UK, existing in isolated populations in areas with a history of continuous tree cover. *Hericium* spp. fruit primarily on beech (*Fagus sylvatica*), and *P. quercinus* exclusively on oak (*Quercus* spp.). Their ecology is unknown, beyond information on fruit body occurence, combative ability against wood decay fungi and extension rates on agar. Their ecology in terms of spore dispersal and germination, and inter- and intraspecific interactions was investigated.

Basidiospore dispersal of *Hericium* spp. was typical of basidiomycetes over the distances investigated (0-100 m from fruit bodies), but basidiospore germination was consistently under 1% in the laboratory. Mating systems of *H. coralloides* and *H. erinaceus* were bifactorial, confirming previous research using North American isolates. *H. cirrhatum'* mating system remains unclear, due to anomalous clamp connections. Mating experiments also showed that *H. coralloides* from different host species can interbreed, and fruit bodies occurring simultaneously on a substrate may originate from a single mycelium. *H. coralloides* was successfully established artificially in living beech, revealed using molecular techniques. Primary mycelia of *H. coralloides* were more combative than secondary, indicating the significance of this lifecycle stage for rare species.

P. quercinus had under 1% spore germination and unifactorial mating. The six populations sampled had only four mating alleles, two being unique to one fruit body. This implies inbreeding, but phenotypic variation (extension rates and colony morphology) prove the population is not clonal.

Results are discussed in relation to ecology of rare fungi in general, possible factors relating to the rarity of *Hericium* spp. and *P. quercinus*, and potential conservation strategies for these species.

Publications

Crockatt, M., A. Ainsworth, D. Parfitt, H. Rogers, and L. Boddy. 2007. Why are the tooth fungi *Hericium cirrhatum, H. coralloides* and *H. erinaceus* rare? Pages 116-118. *World Conference on the Conservation and Sustainable Use of Wild Fungi*. Junta de Andalucia, Cordoba, Spain.

Crockatt, M., G. Pierce, R. Campbell, P. Newell, and L. Boddy. 2008. Homokaryons are more combative than heterokaryons of *Hericium coralloides*. Fungal Ecology. doi:10.1016/j.funeco.2008.01.001

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Abbreviations

ANOVA	analysis of variance
BAP	biodiversity action plan
BMS	British Mycological Society
DGfM	Deutsche Gesellschaft für Mykologie, German Mycological Society
diam	diameter
DNA	deoxyribnucleic acid
dNTP	deoxynucleotide triphosphate
FB	fruit body
FBP	fruit body primordia
FBT	fruit body tissue
fig	figure
FRD	fungal records database
IUCN	International Union for Conservation of Nature
MA	malt agar
Nat Grid Ref	national grid reference
PCR	polymerase chain reaction
RDL	red data list
sec	second
SSI	single spore isolate

Chapter 1 Introduction

1.1 Fungal diversity and conservation

Fungal diversity has been conservatively estimated as at least 1.5 million species (Hawksworth, 1991; 2001), although less than 5% of these have been described (Mueller and Schmit, 2007). Using this estimate, at the rate of current description (ca. 1000-2000 a year), it will be in the region of 1000 years before all species of fungi are described (Hawksworth, 1991). The majority of undescribed species are likely to be microfungi, estimates suggesting that 16-41% of macrofungi have been described (Mueller *et al.*, 2007). Unfortunately as many biodiverse habitats are under threat from destruction, modification and fragmentation, it seems likely that a substantial number of these species, both micro- and macrofungi, will be extinct long before they are described. In the mid-1980s it was estimated that 25% of biodiversity present would be gone within 25 years, i.e. by the early 21st century (Raven, 1988, cited in Hawskworth, 1991), which would include the loss of 376,000 fungal species, more than five times the number presently described (Hawksworth, 1991). As biodiversity hotspots such as tropical rainforests where greatest diversity exists are also often areas for which little data exists regarding the fungal biota (Mueller *et al.*, 2007), it is unknown how many species of fungus have been lost during this time period, and whether this prediction has proved accurate.

Despite being an extremely diverse and relatively understudied group, fungal conservation lags behind protection of other taxa such as mammals, birds and plants, due to a combination of lack of knowledge of so many species, their often relatively uncharismatic appearance compared to e.g. mammals, and the difficulties of assessing fungi using established criteria (Heilmann-Clausen and Vesterholt, 2008). For example, the International Union for Conservation of Nature (IUCN) Red Data List (RDL) uses criteria such as population size for inclusion / categorisation. Even this simple measure is complicated when considering fungi: as it is fruit bodies that are considered, non-fruiting individuals cannot be counted, which may be a substantial proportion of the population (Burnett, 2003). As a further complication, fruit bodies are not predictably produced, the frequency of production differing according to species and environmental conditions (Moore *et al.*, 2008; Rayner and Boddy, 1988).

Although 20% of Europe's fungal species are estimated to be endangered (Dahlberg and Croneborg, 2003), there is no protection for fungi at the European

level, although RDLs for fungi exist in several countries including the UK. The UK's RDL is not yet formalised, the preliminary list (Ing, 1992) having recently been updated to a "preliminary assessment" (Evans *et al.*, 2007). This list contains nearly 400 species, categorised according to current IUCN guidelines (IUCN, 2001), adapted to make them appropriate to the fungal lifestyle. The UK's response to the 1992 Convention on Biological Diversity, the UK Biodiversity Action Plan (BAP; www.ukbap.org.uk), also provides protection for approximately 200 fungal species through creation of Species Action Plans (SAPs), which identify conservation strategies and targets with partner organisations. In addition four species receive legal protection under the 1981 Wildlife and Countryside Act: *Battarraea phalloides, Boletus regius, Hericium erinaceus* and *Buglossoporus pulvinus* (=*Piptoporus quercinus).*

Fungi, a diverse but understudied group, are amongst the most important organisms in the world (Mueller and Schmit, 2007). Fungi are ubiquitous, occurring from polar regions to the tropics, performing a range of functions in these widely varying habitats, as well as being extensively used by humans for food, food additives, medicines and biocontrol agents. As pathogens and parasites they regulate populations of other organisms, are critical to at least 75% of plants as mycorrhizas (Smith and Read, 1996), are a source of food to many groups of animals such as insects and rodents, modify environments and are important decomposers, particularly of wood, and hence crucial to nutrient cycling (Rayner and Boddy, 1988). They are considered essential for the functioning of forest ecosystems (Vasiliauskas *et al.*, 2004; Lonsdale *et al.*, 2008), habitats which support a large proportion of fungi. In Sweden for example, approximately 20% of the 12,000 fungal species are associated with dead wood (Boddy and Heilmann-Clausen, 2008). As a component of biodiversity wood decay fungi have intrinsic value within forest ecosystems. Functionally, they play vital roles, being one of the few groups of organisms capable of utilising the lignin from wood, thus releasing important nutrients into the ecosystem (Rayner and Boddy, 1988). As well as recycling nutrients, by decaying wood they alter availability of resources, both nutritional and spatial, for other organisms, rendering them important "ecosystem engineers" of forests (Lonsdale *et al.*, 2008).

1.2 Forests

Forests, particularly primary forests, are recognised as important reservoirs of biodiversity (Harmon *et al.*, 1986; Siitonen, 2001; Goldberg *et al.*, 2007; Lonsdale *et al.*, 2008). Coarse woody debris (CWD), i.e. dead wood ranging from standing dead trees to fallen branches, was considered primarily as a reservoir for pest species until the mid-1980s (Jonsson and Kruys, 2001). It is now recognised as an important resource, a diverse substratum with hundreds of microhabitats (Siitonen, 2001), that is the main resource for many groups of organisms including rodents, insects living within polypores, birds such as woodpeckers, molluscs (in mangrove forests) and bryophytes (see references in Lonsdale, 2008). For example, a conservative estimate of species dependent on dead wood in Finland alone is 4000-5000 (Siitonen, 2001).

Primary forests, also known as ancient woodland, have higher volumes of coarse woody debris (CWD) than managed forests (Hansen *et al.*, 1991; Siitonen *et al.*, 2000; Shorohova and Shorohov, 2001), which may account for their greater levels of biodiversity: species richness in taxa as diverse as wood decay fungi (Humphrey *et al.*, 2000) and small mammals (Ecke *et al.*, 2001) increase with amount of CWD in a stand. Modern forestry practises result in smaller volumes of CWD within stands, and that present is often of different types (e.g. size, mode of death) compared to natural forests (Siitonen *et al.*, 2000). Primary forest, which remains only in small, fragmented patches in Europe, is therefore an extremely important habitat, the conservation of which is intrinsically linked to the conservation of wood decay fungi. Within the UK such habitat covers less than 2% of the land, and is extremely fragmented (Rackham, 2003).

1.3 Wood as a habitat for fungal growth

Both the physical structure of wood and microclimatic conditions within it affect fungal growth (Rayner and Boddy, 1988), allowing different fungal species to colonise or survive in various host species as decay progresses.

Wood, when dried but not decayed, is composed of approximately 40-50% cellulose, 25-40% hemicellulose, 18-35% lignin and less than 20% lipids, starches, simple sugars, peptides and other easily accessible and assimilable products (Boddy, 1992). These components form vessels and tracheids, which transport water, fibres, which are strengthening elements, and parenchyma, undifferentiated living cells surrounding the more specialised cells. Organisation, relative abundance and size of

these cell types vary between tree species, and are crucial in determining patterns of colonisation (Rayner and Boddy, 1988). Fungal hyphae generally follow the path of least resistance, growing within the pathways created by these elements which results in long, thin, cone-shaped decay columns. This reflects the mainly axial arrangement of transport elements within wood, i.e. vessels and tracheids, with few radial pathways, i.e. parenchyma rays, and extremely few opportunities for tangential spread (Rayner and Boddy, 1988).

On a larger scale, the outer layer of wood is the sapwood, younger wood which functions in water conduction. Older, non-functional wood in the centre of the tree is termed heartwood. In some species, such as oak (*Quercus* spp.) and chestnut (*Castanea* spp.), the heartwood is visually identifiable as a darker central region, due to the presence of extractives. Extractives can act as a carbon source for fungi, and can stimulate or even inhibit their growth. They include waxes, fats, fatty acids, alcohols, steroids and resins, the type, quantity and distribution of which varies between species and even between individuals (Rayner and Boddy, 1988).

Variations in the microclimate within wood, particularly moisture but also aeration and temperature, influence the development of decay (Boddy and Rayner, 1983a; Boddy, 2001). In living trees moisture content is highest where there are living cells and actively conducting tissues, i.e. the sapwood. High moisture content imposes poor aeration, inhibiting aerobic processes; at the other extreme low water availability can inhibit metabolic processes, evidenced by poor growth in the laboratory on wood and agar subjected to low water potentials (e.g. Griffith and Boddy, 1991; Wald *et al.*, 2004a and b). Sapwood, the functional portion of a tree in which water conduction occurs, is therefore a difficult environment for fungal growth; thus in the living tree the central heartwood is a habitat more conducive to fungal growth (Rayner and Boddy, 1988).

Temperature will vary more in smaller diameter substrata such as twigs than in the heartwood of standing trees (Griffith and Boddy, 1991). Although there are species that can withstand extremes of temperature, in general, low temperatures decrease metabolic activity and high temperatures inhibit enzyme function (Cooke and Whipps, 1993).

Wood is a changing, rather than fixed environment. For example, a wound that breaches the sapwood exposes the underlying heartwood, bringing changes in the gaseous regime and moisture, and exposing it to greater variations in temperature. The

environment also changes as decay proceeds (Boddy, 2001), some fungi even having the ability to regulate moisture content of the wood, e.g. *Armillaria* spp. and *Xylaria hypoxylon*, which regulate moisture content *via* their psdeudosclerotial plates (Boddy and Heilmann-Clausen, 2008).

The mode of wood decay, which is accomplished by production of extracellular enzymes, varies between species. The three types of rot cause characteristic appearances in the wood according to which components are broken down (Baldrian, 2008). Brown rot, produced almost exclusively by basidiomycetes, occurs when hemicelluloses and celluloses, but not lignin, have been removed, resulting in wood that is characteristically friable, brown, and cubically cracked. White rot breaks down lignin as well as cellulose and hemicellulose, leaving the wood with a bleached appearance. There are two types of white rot: in selective delignification, caused only by basidiomycetes, the cellulose is broken down after lignin and hemicelluloses; simultaneous white rot, which breaks down all components concurrently, can be caused by some xylariaceous ascomycetes as well as basidiomycetes. Soft rot degrades cellulose and hemicelluloses and slightly alters the lignin. Wood decayed by soft rot has a soft consistency or is brown and crumbly under wet and dry conditions respectively. It generally occurs in conditions of very high or fluctuating moisture, or where treatment by preservatives inhibits growth of brown or white rot fungi, and is caused primarily by ascomycetes.

1.4 Ecology of wood decay fungi

1.4.1 Arrival and exit

The initial stages of colonisation for any wood decay fungus are arrival at a substratum and establishment within it. Arrival can be as mycelium, or as spores, gaining access to woody tissues *via* discontinuities in bark such as wounds or branch stubs (Rayner and Boddy, 1988). They may also develop from latent propagules, i.e. fragments of mycelium or yeasts that are present in functional sapwood but prevented from developing due to high water content and low nutrient availability (Boddy, 2001). As with entry, exit can be effected as mycelium or as spores, the latter following formation of reproductive structures (Rayner and Boddy, 1988).

1.4.2 Life-history strategies

Between the stages of entry and exit, the habitat of wood decay fungi is dynamic: during the progress from uncolonised to fully decomposed wood a range of fungi, and other organisms, will form communities that change and develop as decay proceeds (Rayner and Boddy, 1988). A range of life-history strategies have developed among wood decay fungi that enable them to utilise the various niches available over the course of the decay process (Boddy and Heilmann-Clausen, 2008).

Terminology to describe these strategies was originally developed for plants, but is applicable to fungi (Cooke and Rayner, 1984). This theory is based on the idea that community change is driven by three factors: (i) environmental disturbance; (ii) incidence of competitors, and (iii) environmental stress (Grime, 1977). Individuals better adapted to each of these circumstances are termed ruderal (R-selected), competitive (C-selected) or stress tolerant (S-selected), respectively. It is important to note that an individual may utilise more than one of these strategies at different stages according to circumstances, and therefore that individuals or species cannot be definitively categorised as being R-, S- or C-selected (Rayner and Boddy, 1988).

The first species to colonise a new resource are termed primary colonisers, and typically exhibit R-selected characteristics (Boddy, 2001): efficient dispersal, rapid spore germination, fast growth, and the ability to utilise compounds in previously uncolonised resources (Rayner and Boddy, 1988).

As primary colonisers expand their territories they will eventually come into contact with other fungi. The most common interaction between fungi is competition (Boddy, 2000); thus at this stage C-selected behaviour is required. Fungi arriving at previously colonised resources are termed secondary colonisers, and generally exhibit the C-selected characteristics necessary at this stage (Boddy and Heilmann-Clausen, 2008). These characteristics include good combative ability, and the ability to grow well under relatively unstressed and undisturbed conditions. Thus, in previously colonised resources C-selected characteristics enable an individual to effectively gain territory.

S-selected characteristics are not associated with a particular stage of decomposition, but enable individuals to function or survive under stressful conditions that inhibit growth of most organisms (Boddy and Heilmann-Clausen, 2008). As well as the specific characteristics necessary to survive a particular extreme, for example desiccation or very low pH, S-selected individuals typically have a slow or

intermittent commitment of biomass to reproduction, and may also grow slowly (Boddy and Heilmann-Clausen, 2008).

1.4.3 Inter- and intraspecific interactions

Wood decay fungi do not live in isolation, but will inevitably encounter individuals of the same or different species. Provided individuals are not genetically identical, combative interaction is the most likely outcome of such a meeting (Boddy, 2000). Interactions can result in deadlock, in which neither individual gains territory, or replacement, in which territory is gained. Although species or individuals can be placed in competitive hierarchies (e.g. Holmer and Stenlid, 1997), outcomes and combative ability are not fixed, but vary according to many factors including relative size of mycelium (Holmer and Stenlid, 1993), nuclear status (Fryar *et al.*, 2002) and abiotic conditions such as pH (Wald *et al.*, 2004b).

It is possible for genetically identical individuals to come into contact with each other as ramets, for example following germination of asexually produced spores in the same location. Being genetically identical the colonies will fuse, and function as a single unit. Observations of decay columns too long to have developed within a single season are assumed to have formed in this manner (Boddy and Rayner, 1984), which lead to the theory of development from latent propagules (Boddy, 2001).

1.5 Basidiomycete life cycle

In most basidiomycetes sexual reproduction does not occur *via* specialised reproductive structures, but by fusion and nuclear exchange between sexually compatible mycelia (Burnett, 2003). A number of terms can be applied to the unmated / mated mycelium: primary / secondary, monokaryotic / dikaryotic or homokaryotic / heterokaryotic. Mono- / dikaryotic implies one or two nuclei, i.e. a single nucleus or one from each parent, but as basidiomycetes sometimes have more than two nuclei in a compartment, e.g. in the genera *Coniophora, Stereum*, and *Phaneroctaete* (Ainsworth, 1986), the term is misleading. Homo- / heterokaryotic are more correct as they indicate that all nuclei are similar, or that there is more than one type, i.e. different nuclei from the two parental mycelia. However, the terms primary and secondary mycelia are preferred, as analysis is required to prove that nuclei originate from different parents; unless this has been carried out the terms can be misleading (Ainsworth, 1986).

The typical basidiomycete lifecycle (Fig 1.1) presents particular challenges for rare wood decay species at certain stages. The first of these is spore dispersal (Chapter 2), which is often achieved via wind or air currents, although water and animals can also act as spore vectors (Rayner and Boddy, 1988). Dispersal by wind or air has the disadvantage that landing is entirely haphazard, the chances of arriving in a habitat suitable for germination and subsequent growth being extremely small (Heilmann-Clausen and Boddy, 2008). For a species with specific requirements for spore germination (Chapter 2 & 6) this stage may therefore act as a bottleneck, with an extremely small proportion of spores produced achieving germination. Following germination the primary mycelium must become established in the environment, potentially another bottleneck, as conditions under which germination has occurred may rapidly alter, or be different to those required for mycelia growth. Once established, primary mycelium faces the same biotic and abiotic challenges within a substratum as secondary mycelium (Chapter 3), but must meet a sexually compatible conspecific (Chapter 4 & 6) to achieve sexual reproduction. For a rare species with fewer individuals, fruit bodies and hence spores, such a meeting is less likely than for a more common species.

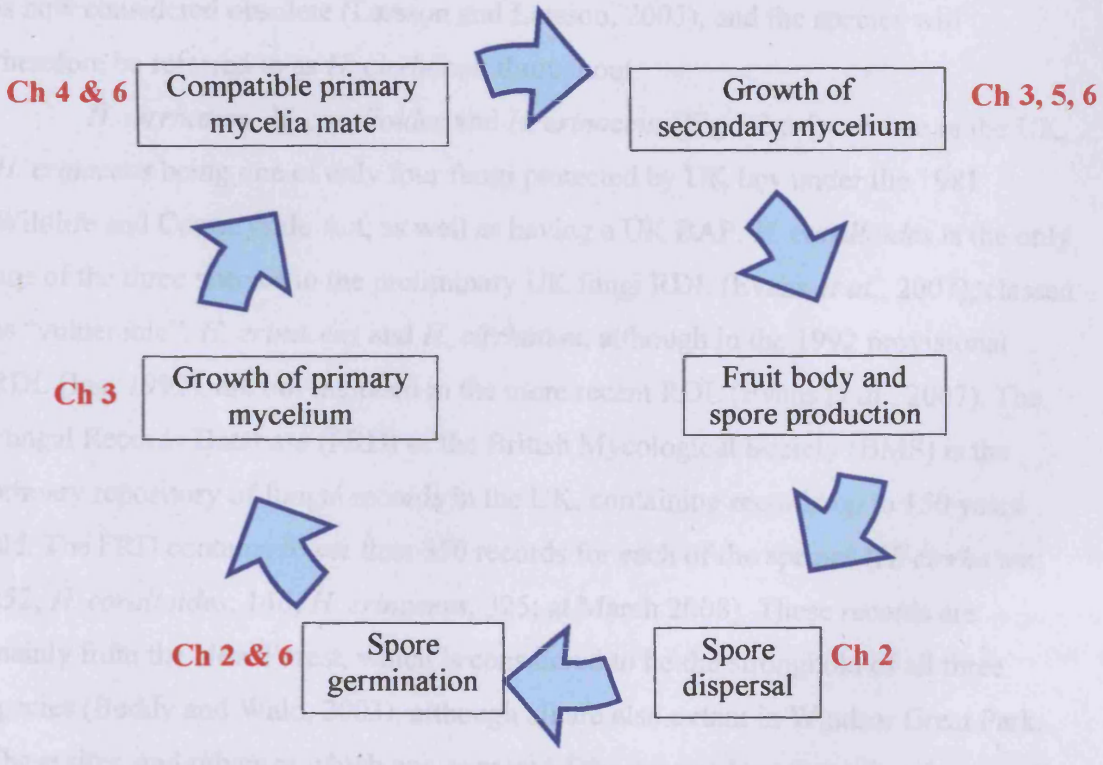


Figure 1.1 Generalised basidiomycete lifecycle
Red text indicates the chapter in which that stage will be investigated.

As the majority of spores land within a few metres of the fruit body (Stenlid, 1994) sibling primary mycelia are likely to meet. Although in most basidiomycetes sexual compatibility is genetically controlled by mating systems that promote outbreeding (Burnett, 2003; Chapter 4 & 6), a rare species with few propagules may be particularly at risk from inbreeding (Chapter 6).

Examples of rare wood decay fungi that may be subject to these particular challenges in the UK include *Hericium cirrhatum, H. coralloides, H. erinaceus* (Boddy and Wald, 2003; Boddy *et al.*, 2004) and *Piptoporus quercinus* (Roberts, 2002), which are the subject of the current study.

1.6 *Hericium* spp. and *Piptoporus quercinus*

1.6.1 Hericium cirrhatum, H. coralloides and H. erinaceus

Hericium spp. are classified as: Basidiomycota, Agaricomycotina, Agaricomycetes, Russulales (Hibbett *et al.*, 2007). The genus *Hericium* includes *H. coralloides, H. erinaceus, H. abietis, H. americanum* and *H. flagellum* (Stalpers, 1996). *H. cirrhatum* has usually been placed in a separate genus as *Creolophus cirrhatus* (Stalpers, 1996), but following observations on spore morphology and phylogenetic analyses this genus is now considered obsolete (Larsson and Larsson, 2003), and the species will therefore be referred to as *H. cirrhatum* throughout.

H. cirrhatum, H. coralloides and *H. erinaceus* (Fig 1.2 a-f) are rare in the UK, *H. erinaceus* being one of only four fungi protected by UK law under the 1981 Wildlife and Countryside Act, as well as having a UK BAP. *H. coralloides* is the only one of the three species in the preliminary UK fungi RDL (Evans *et al.*, 2007), classed as "vulnerable". *H. erinaceus* and *H. cirrhatum*, although in the 1992 provisional RDL (Ing, 1992), are not included in the more recent RDL (Evans *et al.*, 2007). The Fungal Records Database (FRD) of the British Mycological Society (BMS) is the primary repository of fungal records in the UK, containing records up to 150 years old. The FRD contains fewer than 350 records for each of the species (*H. cirrhatum*: 252; *H. coralloides*: 146; *H. erinaceus*, 325; at March 2008). These records are mainly from the New Forest, which is considered to be the stronghold of all three species (Boddy and Wald, 2003), although all are also extant in Windsor Great Park. These sites, and others at which one or more of the species have been found, generally have a long history of continuous tree cover, relatively high levels of dead wood and a

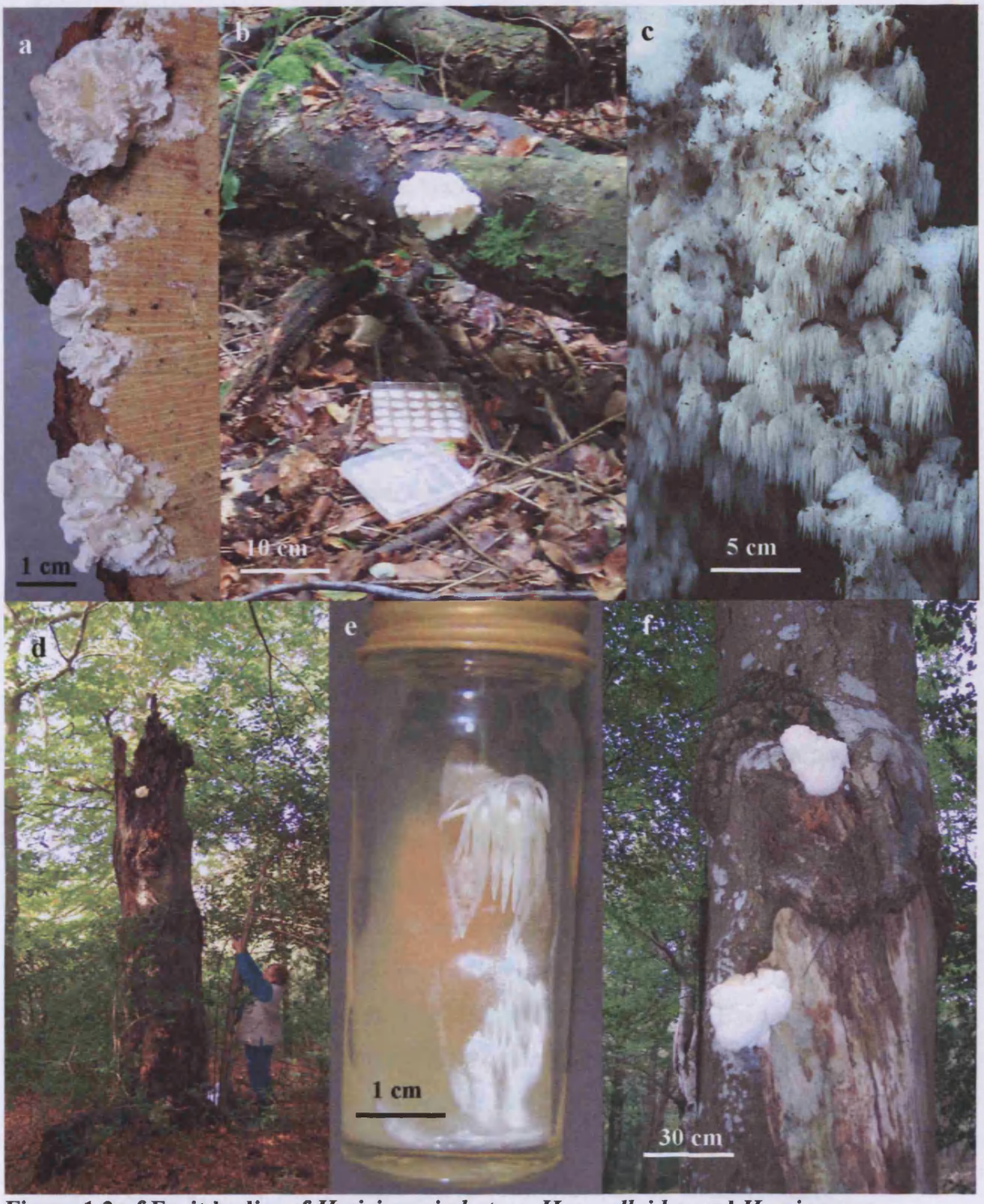


Figure 1.2a-f Fruit bodies of *Hericium cirrhatum, H. coralloides* and *H. erinaceus*
a, immature *H. cirrhatum* fruit bodies on beech slice in the laboratory; b, mature *H. cirrhatum* fruit body on felled beech trunk at Burnham Beeches with spore traps (10 cm^2) below; c, mature *H. coralloides* fruit body in the snow, scale bar 5 cm; d mature *H. coralloides* on beech snag, Busketts Wood, New Forest; e, sporulating *H. erinaceus* fruit body produced on agar slope in the laboratory, spines ca. 1 cm; f, mature *H. erinaceus* fruit bodies on living beech tree in Shaves Wood, New Forest; note spores that have fallen from spines in b. Photo credits: a, © Juliet Hynes; b, d and e © Martha Crockatt; c and d, © Martyn Ainsworth. Scale bars are approximate.

range of tree age classes (Boddy and Wald, 2003), including sites such as Burnham Beeches (nat grid ref: SU98) and Epping Forest (NG reference: TQ49).

Although many of the FRD records, particularly older records, lack information as to host (species / state of host), analysis of them has provided important information (Boddy and Wald, 2003). *Hericium coralloides* and *H. erinaceus* are found fruiting from early September to late November, *H. cirrhatum* having a longer season from mid July to November. Over 80% of records for all three species are on beech (*Fagus sylvatica*) with 14% of *H. coralloides* records on ash (*Fraxinus excelsior*) and occasional findings of all species on other hosts including *Quercus* spp., *Acer* spp. and *Betula* spp. *H. erinaceus* is often found on living beech trees, sometimes fruiting regularly for up to 20 years on a single host (Marren and Dickson, 2000; Boddy and Wald, 2003). In contrast, *H. coralloides* is generally found on fallen logs where it fruits for only a few seasons, and *H. cirrhatum* occurs sporadically on cut stumps, rarely fruiting on the same host twice (Marren and Dickson 2000; Boddy and Wald 2003).

Within Europe, *H. erinaceus* has been recorded from 435 localities in 18 countries, in 15 of which it is a red listed species (Dahlberg and Croneborg, 2003). Further afield, *H. erinaceus* is common in Japan, but *H. cirrhatum* extremely rare, being known from only three localities (Eiji Nagasawa, pers. comm.); *H. erinaceus* is also common in North America (e.g. Guglielmo *et al.*, 2007). Distribution of *H. coralloides* is less well documented, but it is on the red data list of Bulgaria (Gyosheva *et al.*, 2000) and is also rare in Germany, where it received some attention as "Mushroom of the Year 2006" of the German Mycological Society (DGfM).

Beyond fruiting patterns, little is known of these species. Existing ecological research shows that: the three species have average extension rates in agar culture for mesophilic fungi and are average combatants against other wood decay fungi (Wald *et al.*, 2004b); *H. coralloides* can be successfully established in freshly felled beech trunks (Boddy *et al.*, 2004; Chapter 5); *H. coralloides* and *H. erinaceus* have bifactorial mating systems in North America (Hallenberg, 1983; Ginns, 1985; Chapter 4); spore production of *H. erinaceus* is related to temperature and humidity (McCracken, 1970).

H. erinaceus is widely grown commercially for culinary and medicinal purposes, particularly in Japan and China. This has led to several publications on cultivation techniques (e.g. Suzuki and Mizuno, 1997; Stamets, 2000; Figlas *et al.*,

2007) and medicinal properties, including potential anti-cancer (Petrova *et al.*, 2008) and anti-MRSA (Ueda *et al.*, 2007) compounds. *H. coralloides'* fruit bodies are also edible and can be cultivated (Stamets, 2000), although it is not generally grown commercially. As *H. erinaceus* readily produces large fruit bodies in culture (Stamets, 2000; Fig 1.2e), and is common in Japan and North America, its rarity in Britain and the rest of Europe is intriguing.

1.6.2 Piptoporus quercinus

P. quercinus (Fig 1.3a-c) is classified as: Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales (Hibbett *et al.*, 2007). The genus contains only one other species, *P. betulinus*. *P. quercinus* has the common synonyms *Buglossoporus pulvinus* and *B. quercinus*, but *P. quercinus* is the accepted name (Roberts, 2002).

In common with *H. erinaceus*, *P. quercinus* is one of the four fungi protected under the 1981 Wildlife and Countryside Act, and has UK BAP status, but is no longer classed as endangered in the recent RDL (Evans *et al.*, 2007). It is widespread across Europe and Asia, but rare throughout its range, being on the red lists of at least four countries; it is not known from North America (Roberts 2002).

P. quercinus is found fruiting only on exposed oak (*Quercus* spp.) heartwood, generally of veteran (>250 years old) trees (Fig 1.3b-c), in which it causes brown rot (Roberts, 2002). It is not limited to large trees, but is often found on smaller limbs, sometimes long fallen, of as little as 15 cm diam. (Roberts, 2002). It fruits between July and August in sites that have a continuous history of tree cover, typically old oak pasture (Roberts, 2002). Its fruiting situations, for example on snags (standing dead tree) not shaded by other trees (Fig 1.3c) are often highly exposed to extremes of temperature and desiccation, implying it has the ability to colonise or persist within substrates under these adverse conditions (Roberts, 2002). Oak heartwood is itself an inhospitable environment in which few other fungi can grow, having high tannin content (Rayner and Boddy, 1988) and a relatively low pH of approximately 3.75 (Gray, 1958).

The stronghold of the species in the UK is Windsor Great Park, in which there are 95 trees on which *P. quercinus* is known to have fruited (Ainsworth, 2008). The second most common sites for it are The Oaks at Kingston Lacey (NG reference: ST9603), a National Trust property in Dorset, at which there are eight known host trees (Roberts,

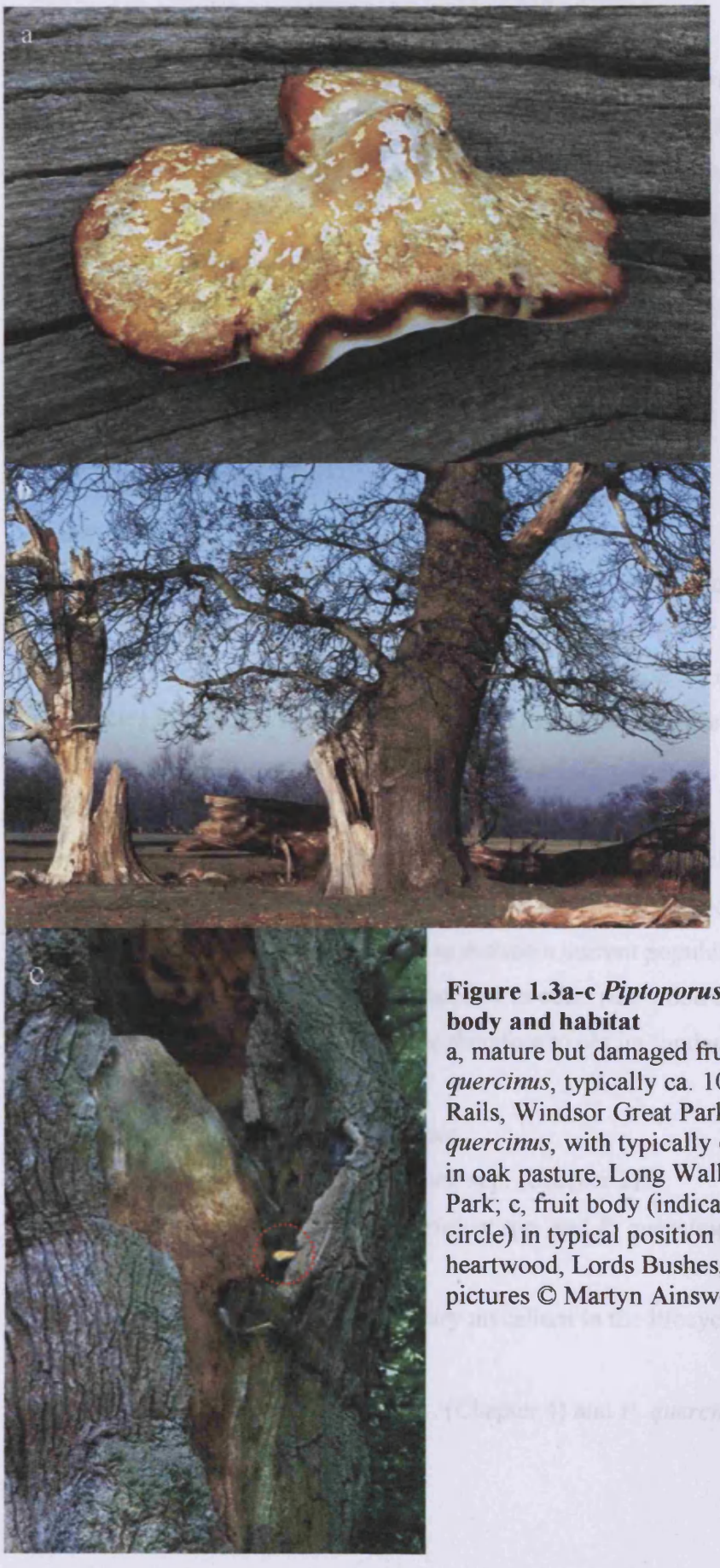


Figure 1.3a-c *Piptoporus quercinus* fruit body and habitat
a, mature but damaged fruit body of *P. quercimus*, typically ca. 10 cm diam., Bears Rails, Windsor Great Park; b, host tree of *P. quercimus*, with typically exposed heartwood in oak pasture, Long Walk, Windsor Great Park; c, fruit body (indicated by dashed red circle) in typical position on exposed heartwood, Lords Bushes, Epping Forest. All pictures © Martyn Ainsworth.

2002), and Sherwood Forest (NG reference: SK6267), in which nine fruit bodies were recorded in the 2007 season (Howard Williams, pers. comm.).

From the single paper published on the ecology of *P. quercinus,* it is known to grow relatively slowly on agar, and is a poor combatant against other wood decay fungi (Wald *et al.*, 2004a). Optimum conditions for mycelial extension on agar were 25 °C at pH 3.75 (Wald *et al.*, 2004a). It produces large numbers of asexual chlamydospores (Stalpers, 1978), thick walled resting spores that may enable an individual to survive periods of adverse conditions (Rayner and Boddy, 1988). This may explain *P. quercinus*' ability to survive in highly exposed substrates (Roberts, 2002; Wald *et al.*, 2004a).

1.7 Research priorities, aims and objectives

Virtually nothing is known of the ecology of these species or why they are rare. Stages of the lifecycle identified as presenting particular challenges to rare fungi (Fig 1.1) have not been studied: we know nothing of spore dispersal or germination, the extent of the role that primary mycelium plays in the lifecycle, or mating system of these species in the UK. These areas have been identified as research priorities for *Hericium* spp. and *P. quercinus* by Natural England (Boddy *et al.*, 2004), and form the basis for this investigation.

A clearer understanding of the lifecycles and ecology of *Hericium* spp. and *P. quercinus* may give clues as to why they are rare in the UK, should enable effective conservation strategies to be developed to maintain current populations, and may allow lessons to be learned that can be applied to other rare wood decay basidiomycetes. The aim of this thesis is therefore to obtain fundamental information on the ecology of these species.

Specific objectives were to investigate:

i. basidiospore dispersal of *Hericium* spp. (Chapter 2);
ii. basidiospore germination of *Hericium* spp. and *P. quercinus* (Chapters 2 and 6, respectively);
iii. the relative significance of primary mycelium in the lifecycle of *H. coralloides* (Chapter 3);
iv. mating systems of *Hericium spp.* (Chapter 4) and *P. quercinus* (Chapter 6);

v. whether fruit bodies of *Hericium* spp. (Chapter 4) or *P. quercinus* (Chapter 6) occurring simultaneously on the same substrate are produced by a single mycelium;

vi. artificial establishment of *H. coralloides* in living beech (*Fagus sylvatica*) (Chapter 5).

Chapter 2: Spore germination and dispersal in *Hericium cirrhatum*, *H. coralloides* and *H. erinaceus*[1]

2.1 Introduction

For many wood decay fungi spore dispersal is an important, if not the main, method by which new habitats are reached. Sexual spores not only enable fungi to reach new habitats, but also increase genetic diversity. For stable populations as little as one immigrant per generation can prevent alleles becoming fixed through genetic drift (Slatkin, 1987); thus even very low levels of spore dispersal between populations are important in maintaining genetic diversity. Studying spore dispersal and germination is therefore crucial to understanding a species' population dynamics, genetics and distribution patterns (Edman and Gustafsson, 2003).

Poor spore dispersal and / or germination could be a factor contributing to the rarity of a species, by both preventing the species from colonising new resources and by preventing gene flow between existing populations. Moreover, before contributing to the effective population, a spore must germinate, which may require specific conditions (see references in Merrill, 1970). If a species has stringent requirements for spore germination or growth the effects of poor dispersal and germination are exacerbated, as suitable resources may be rare. The rare tooth fungi *Hericium cirrhatum*, *H. coralloides* and *H. erinaceus* could be examples of such species, being found predominantly in old growth woodland, a rare habitat in the UK. As all three species are average to good combatants against other wood decay fungi (Wald *et al.*, 2004b), and fruit readily in culture (Stamets, 2000 [*H. coralloides* and *H. erinaceus*]; Boddy *et al.*, 2004) these factors are unlikely to contribute to the species' rarity, and other factors, such as those outlined above, should be investigated.

The objectives of this study, therefore, were to determine the extent of spore dispersal and germination. The techniques adopted used species specific primary mycelium to trap spores (Adams *et al.*, 1984). This method ensures that only germinable spores are counted, and is well-established in the small field of wood

[1] Published as Crockatt, M., A. Ainsworth, D. Parfitt, H. Rogers, and L. Boddy. 2007. Why are the tooth fungi *Hericium cirrhatum, H. coralloides* and *H. erinaceus* rare? Pages 116-118. *World Conference on the Conservation and Sustainable Use of Wild Fungi.* Junta de Andalucia, Cordoba, Spain.

decay basidiomycete spore trapping (Williams and Todd, 1984; Vilgalys and Sun, 1994; Nordén and Larsson, 2000; Hallenberg and Küffer, 2001; James and Vilgalys, 2001; Edman *et al.*, 2004a; Edman *et al.*, 2004b). This technique was used to determine short distance (0-100 m) spore dispersal, combined with laboratory investigations into spore germination to assess whether these could be factors contributing to the rarity of *Hericium* spp. in the UK.

2.2 Materials and methods

2.2.1 Spore germination

Spores were obtained from 28 fruit bodies at several field sites (Table 2.1), by attaching small pieces of sporulating fruit body to the lid of a Petri dish with petroleum jelly. This was positioned above a glass slide and left to deposit spores overnight. Spores were suspended in sterile distilled water, and spread onto agar medium at a density of ca. 5-40 spores per field of view at x100 magnification. Unless stated otherwise, the medium was 2% malt agar (MA: 15 g Lab M agar no. 1; 20 g Lab M malt extract l^{-1} distilled water; Lab M, Bury, Lancashire, UK) and plates were incubated at 20 °C in darkness. Plates were observed frequently until germination commenced; days taken to germinate and percentage germination were recorded. Percentage germination was counted on several days until a constant level was reached or until colonies grew so large as to obscure the view and prevent accurate counting. A total of 500 spores were counted from at least three of the five replicate plates to obtain the percentage germination.

2.2.2 Germination media and treatments

Preliminary experiments having found that germination was consistently well below 1%, germination was also evaluated under a range of abiotic and biotic conditions (Table 2.2), in an attempt to increase percent germination. Spores from all fruit bodies were spread on 2% MA and incubated at 20 °C, different media and treatments being tested in addition to these control conditions according to the number of spores available. At least five replicate plates were made for spores from each fruit body for each treatment. Media used were as follows: water agar (15 g l^{-1} agar in distilled water); 1% MA (as 2% MA, but only 10 g malt extract); charcoal agar: 2% MA with 0.28 g l^{-1} activated charcoal added prior to autoclaving; beech (*Fagus sylvatica*) agar: 10 g l^{-1} beech sawdust soaked overnight in distilled water, the filtrate of which was

Table 2.1 *Hericium* spp. spore prints collected 2005 – 2007

Species	Fruit body	Date collected	Location	NG reference	Collector
H. ci	AL1[a]	24.07.05	NF	SU3729204148	AL
	MA124	01.10.05	WGP	SU977734	AMA
	MA125	07.10.05	BB	SU94398467	AMA
	MA128	26.10.05	EF	TL42960020	AMA
	SS2[a]	26.07.06	NF	SU3729704146	SS
	AL10	02.08.06	LW	SU224211	AL
	MA130	28.06.07	WGP	SU98277287	AMA
	MA131	28.06.07	WGP	SU97607326	AMA
	MA132	28.06.07	WGP	SU97547331	AMA
	BB1	18.10.07	BB	SU94408471	MEC
H. co	MA126[b]	15.10.05	WGP	SU956740	AMA
	MA127	20.10.05	EF	TQ42329853	AMA
	MA129[b]	03.11.05	WGP	SU956740	AMA
	BW1	16.10.06	NF	SU312111	MEC
	AB1[c]	07.11.06	EF	TL43790042	MEC
	AB2[c]	07.11.06	EF	TL43790042	MEC
H. e	AL3	30.09.05	NF	SU2534106669	AL
	AL4	30.09.05	NF	SU3394305706	AL
	AL5	05.10.05	NF	SU2247515117	AL
	AL6[d]	05.10.05	NF	SU2896811808	AL
	AL7	19.11.05	NF	SU228148	AL
	OckA[e]	16.10.06 30.10.06	NF	SU246115	MEC
	OckB[e]	16.10.06 30.10.06	NF	SU246115	MEC
	OckC[e]	16.10.06 30.10.06	NF	SU246115	MEC
	SW1a	16.10.06	NF	SU28801193	MEC
	SW2[d]	16.10.06	NF	SU28921180	MEC
	Eye 1	30.10.06	NF	SU227154	MEC
	Eye 2	30.10.06 21.11.06	NF	SU22731493	MEC

H. ci, Hericium cirrhatum; H. co, H. coralloides; H. er, H. erinaceus; BB, Burnham Beeches, Slough; EF, Epping Forest; LW, Langley Woods, Wiltshire; NF, New Forest; WGP, Windsor Great Park. AL, Alan Lucas; AMA, A. Martyn Ainsworth; MEC, Martha E. Crockatt; SS, Stuart Skeates; for contact details apply to Prof L Boddy (boddyl@cardiff.ac.uk); where two collection dates are shown two spore prints were taken from the same fruit body on the dates indicated; superscript letters indicate fruit bodies from the same tree. Equivalent Natural England references for *H. erinaceus* are in Appendix I.

used in place of distilled water in normal 2% MA; cellulose agar (modified Eggins and Pugh media (Eggins and Pugh, (1962)), Park, 1973): $(NH_4)_2SO_4$, 0.5 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; Ca Cl_2, 0.1 g; yeast extract, 0.5 g; ball-milled cellulose (72 hr), 10.0 g; agar, 20.0 g l^{-1}; in distilled water (no ball mill being available cellulose was not milled). The effect of mycelium from single spores (obtained by picking single germinating spores and culturing in isolation) and fruit body tissue (obtained by fruit body tissue isolation onto agar medium) of the same species was investigated by taping (Nescofilm®) a plate well colonised with the appropriate mycelium above a plate of spores, allowing any volatiles produced by the mycelium to reach them, or by inoculating directly onto the plate of spores to investigate the effect of any diffusible chemicals produced by the primary or secondary mycelium. Spores from two fruit bodies each of *H. coralloides* and *H. erinaceus* were stored for four, eight, 12 and 24 weeks prior to spreading onto 2% MA and 2% MA plus activated charcoal (0.28 g l^{-1}). Influence of spores from different fruit bodies upon germination was investigated by spreading spores from three fruit bodies on the same plate. Effect of a lowered incubation temperature of 10 °C was also investigated.

2.2.3 Spore dispersal

Traps consisted of 10 x 10 cm vented plastic dishes divided into 25 2 x 2 cm wells (Sterilin, Barloworld Scientific, Staffordshire, UK), filled with 2% MA, each compartment being well-colonised by a primary mycelium (obtained as previously described) of the appropriate species; traps were also made using 5 cm diam dishes (Sterilin). Open traps were placed at various distances around trees bearing fruit bodies for various lengths of time. Petroleum jelly was applied around the top edge of each plate to deter mites. Weather conditions were recorded. Dishes were incubated at 20 °C in the dark for 10-15 days, depending on extent of contamination, and subcultures were made from each compartment onto 2% MA. Resultant cultures were checked for clamps to indicate that mating had occurred, and hence that at least one spore of the species in question had landed and germinated in that compartment. Wells were also briefly checked for lines of antagonism, which could occur if more than one spore had landed and germinated there.

Having found, in a preliminary trial in 2005, clamp connections in all wells at distances of up to 10 m from a *H. coralloides* fruit body, over times from four to eight

hr, distance of traps was increased and exposure times decreased for main experiments. Multi-well traps were placed at 10 and 100 m from the fruit body in the four cardinal compass directions (Fig 2.1) and left open for four or six hr during daylight, ca. 08.30 – 16.30. Two multi-well control traps, one closed and one open, were placed directly beneath the fruit body (=0 m). In some experiments (Table 2.3), 5 cm diam traps (three replicates) were placed at the same points as multi-well traps, and in two cases also up to 800 m from the fruit body (Table 2.3). Ten fruit bodies in the South East of Britain (Table 2.3) were used.

To allow easy comparisons, data were converted to spores arriving per m^2 per hour, with the assumption that a well with clamp connections was the result of a single spore having landed and germinated there.

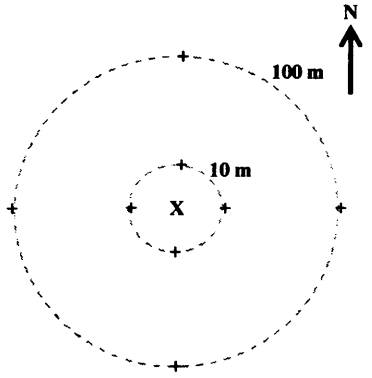


Figure 2.1 Spore trap placement at 0, 10 and 100 m from the fruit body
X = fruit body and 0 m control spore traps; + = spore trap location

2.3 Results

2.3.1 Spore germination

Germination rate was low for all species, some collections failing to germinate after 20 weeks (Table 2.2). In all but eight of the 75 treatments / collections plated from fresh spore collections, germination was < 1%. The highest and most rapid germination was for spores of *H. erinaceus* on a plate which was contaminated by an unidentified yeast; 16.3% germinated within one day, whereas uncontaminated replicates germinated after 13 d with 3.3% success. Inoculating further plates of the same spores with the isolated contaminant, as well as plates of spores from other fruit bodies, failed to result in an increase in speed or success of germination. There was no evidence that composition of the medium, presence of spores from other fruit bodies or volatiles or diffusibles from mycelium from single spores or fruit body tissue affected germination. Age of spores was directly related to time taken to germinate,

H. coralloides and *H. erinaceus* being similarly affected (Fig 2.2). Germination in all cases was again < 1%.

Table 2.2 Time (d) until germination for spores of *Hericium cirrhatum, H. coralloides and H. erinaceus* under different conditions

	water agar	1%MA	2%MA	charcoal[1]	cellulose[2]	beech[3]	+het[4]	+hom[5]	10 °C	Spore mix
H. cirrhatum (10 fruit bodies)										
N	2		10	2			1	1	1	
fastest	30		29	32						
mean			32					92		
slowest	>50*		>146	>50			>146		>85	
H. coralloides (6 fruit bodies)										
N		2	6	2		1	2	3		
fastest		9	8[6]	7[7]			8	8		
mean		9	14[6]	7			8	9		
slowest		9	22	7		>50	>50*	11*		
H. erinaceus (16 fruit bodies)										
N		3	17	4	3	3	3	3		1[†]
fastest		8	1[y], 8	8	15	11	8	8		10
mean		10	11[8]	10	15	15	10	10		10
slowest		11	>50	11	15	19	12	12		10

[1]charcoal agar; [2] modified Eggins and Pugh agar; [3] beech agar; [4] heterokaryon above spores; [5] homokaryon above spores; 10 °C, spores incubated at 10 °C. Full details in text. >, indicates that spores had not germinated by this time, but plates had become contaminated. These times have not been included in the estimate of the mean; [67y8]contain a treatment/ collection with > 1% germination: [6]1.5%, [7]5% and 9.9%, [y]16.3%, [8]3.3%; *heterokaryon or homokaryon inoculated onto the plate; [y]Plates contaminated with a yeast; [†]one combination of spores from three fruit bodies.

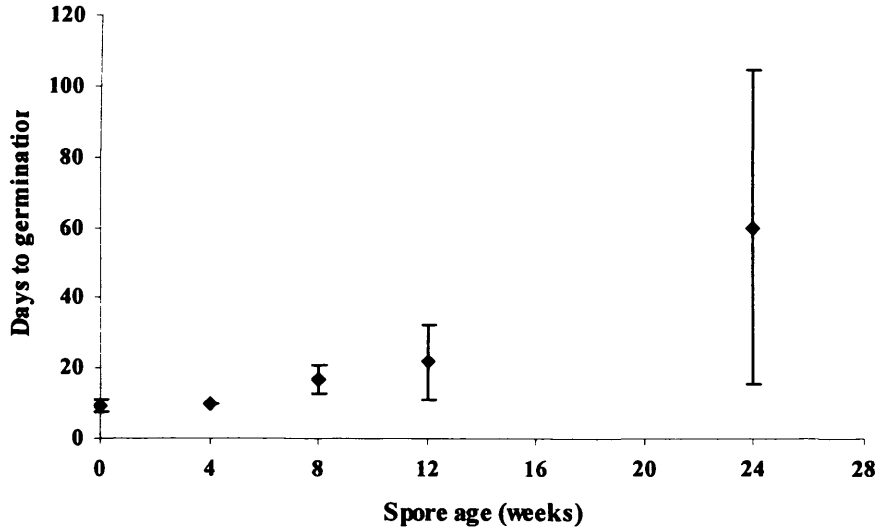


Figure 2.2 Effect of spore age on time until germination of *Hericium coralloides* and *H. erinaceus*
Data are for spores from two fruit bodies of each species. Not all replicates of 24 week old spores germinated so this value is an underestimate. Error bars are SEM.

2.3.2 Spore dispersal

A high proportion of the wells in plates directly beneath fruit bodies had clamp connections (Table 2.3). The proportion of wells with clamps decreased with distance from the fruit body (Fig 2.3), but there was evidence of mating even at 100 m; clamps were not found on traps more than 100 m from the fruit body. Unopened control plates lacked clamp connections. Contamination ranged from 0 to 100%, with an average of 40% (±8.3 SEM).

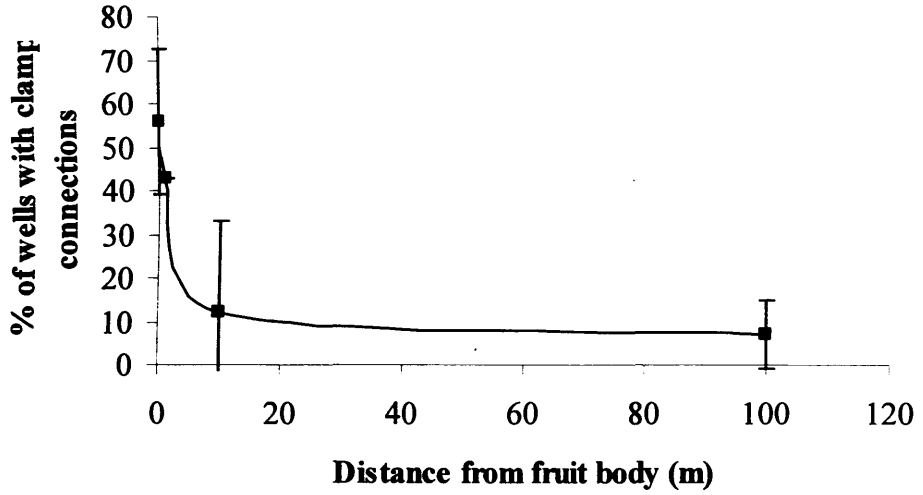


Figure 2.3 Average percentage of wells with clamp connections at 0-100 m from fruit body
Averages are for all fruit bodies and times at that distance; error bars are SEM; there are no error bars at 1 m as there was only one replicate.

In one instance (*H. erinaceus* VW1) clamps were found in 5 cm diam dishes, but not in multi-well dishes at the same point (10 m, 8 hr). No clamps were found in the 5 cm traps placed over 100 m away. Clamps were also absent in 5 cm diam dishes used for *H. cirrhatum* BB1, and from the only two uncontaminated dishes from *H. coralloides* AB1.

2.4 Discussion

Spores from most fruit bodies germinated, although percent germination of all three *Hericium* spp. was consistently low. This is a common situation for wood decay basidiomycetes, whose spores generally germinate in the laboratory (Merrill, 1970; Fries, 1987; Petersen, 1997), although often at low percentages (see references in

Table 2.3 Spore dispersal at 0 – 100 m from *Hericium* spp. fruit bodies expressed as percentage of wells with clamp connections and number of spores landing per m^2 per hour

Date	Location	FB Code	Substratum	Weather (time of day)	0 m 6 hr		10 m 4 hr		10 m 6 hr		100 m 6 hr	
					% clamped	spores m^2 hr^{-1}	% clamped	spores m^2 hr^{-1}	% clamped	spores m^2 hr^{-1}	% clamped	spores m^2 hr^{-1}
H. cirrhatum												
18.10.06	Burnham Beeches	BB1[a]	Fallen beech trunk	LR (PM)	0[b]	0[b]	33[b]	208[b]	0[b]	0[b]	5	19
H. coralloides												
01.11.05	WGP	MA129[c]	Standing ash	D	nd	nd	nd	nd	nd	nd	nd	nd
30.10.06	Buskett's Wood, NF	BW1	Standing dead beech	D	100	≥417	74	463	57	236	21	88
21.11.06	Buskett's Wood, NF	BW1	Standing dead beech	R	All contaminated							
07.11.06	Ambresbury Banks, EF	AB1	Standing dead beech	D	33[b]	139[b]	2	13	5[b]	19[b]	0	0
H. erinaceus												
03.10.06	Virginia Water, WGP	VW1[a]	Fallen beech trunk	LR (PM) W	50	156	nd	nd	nd	0	0[d]	0[d]
16.10.06	Ocknell, NF	OckA	Fallen beech trunk	D	8	33	nd	nd	0	0	2	6
31.10.06	Eyeworth, NF	Eye1	Fallen beech trunk	D	100	≥417	nd	nd	8	33	nd	nd
31.10.06	Eyeworth, NF	Eye2	Fallen beech trunk	D	100	≥417	43	179[e]	13	52	nd	nd
21.11.06	Eyeworth, NF	Eye2	Fallen beech trunk	R	All contaminated							

% clamped excludes contaminated wells; ≥, if 100% of clean wells have clamps then the number of spores fallen per m^2 per hour is the minimum; nd, not determined; [a] 5 cm diam dishes placed > 100 m (400 and 800 m (BB1); 200, 300 and 400 m (VW1)); [b] 5 cm diam dishes also placed; [c] results in text; [d] 100 m for 8 hr; [e] 1m for 6 hr; NF, New Forest; EF, Epping Forest; WGP, Windsor Great Park; LR, light rain; D, dry; R, rain; W, wind; AM, midnight to noon; PM, noon to midnight. NG references see Table 2.1, except *H. erinaceus* VW1, which is SU958693.

Merrill, 1970). However, some species have germination in excess of 90% (Schmidt and French, 1983). Both percentage of spores germinating and time taken to germinate are subject to many factors, including age of fruit body at spore release (Schmidt and French, 1983), age of spores (e.g. Aime and Miller, 2002) and temperature (e.g. Sautour *et al.*, 2001). Such studies have frequently shown that germination can be increased, i.e. spore viability is much higher than germination under sub-optimal conditions. For example, *Polyporus dryophilus* (=*Inonotus dryophilus*) had low percentages of erratic germination (Bailey, 1941, cited in Merrill, 1970), which increased to 92% when CO_2 was increased or volatiles produced by that species' mycelium were present (Morton, 1964, cited in Merrill, 1970). That germination rate can be high in the *Hericium* spp. was evidenced by the five-fold increase in percent germination of *H. erinaceus* on the plate contaminated with a yeast. Further, the spore traps provided evidence that germination frequently occurs in the field if established primary mycelia are present. Spores that do not germinate may be either nonviable or merely dormant. Future research should investigate spore viability, for example using vital stains such as propidium iodide and fluorescein diacetate. Successful germination of spores up to 24 weeks indicates that these species could remain dormant while environmental conditions are unsatisfactory. Dormancy is probably exogenous as with other basidiospores (Rayner and Boddy, 1988).

The majority of basidiospores from wood decay basidiomycetes fall within 1 m of the fruit body, with few travelling further than 100 m (Kallio, 1970; Stenlid, 1994; Nordén and Larsson, 2000). *Hericium* spp. appear to be no different. There is evidence that spores of wood decay fungi, albeit a small number, regularly travel greater than 1 km (Kallio, 1970; Stenlid, 1994; Nordén, 1997; see references in Nordén and Larsson, 2000), and occasionally up to 1000 km (Hallenberg and Küffer, 2001). If *Hericium* spp. behave similarly this implies that spore dispersal is not a contributing factor to their rarity. It is important to note, however, that dispersal may not be followed by germination, let alone growth within a resource. The technique used here, of trapping spores with a primary mycelium, has the advantage of counting only germinable spores, but it must be remembered that such a situation may be more favourable for germination than the vast majority of possible landing places. Thus it is dispersal of germinable spores and colonisation potential that is measured; this must not be confused with actual colonisation.

It is not easy to meaningfully compare the number of spores settling per m^2 per hour with other studies, there being such wide variation amongst species in the total

number of spores produced. In addition, when 100% of wells had clamp connections the number of spores landing is almost certainly an underestimate. Sporulation is also likely to vary with age and condition of fruit body and environmental conditions such as temperature and humidity (Rayner and Boddy, 1988). This is certainly true for *H. erinaceus* in the USA, where it was found that spore production varied inversely with relative humidity, and had an optimum temperature of 24-27 °C (McCracken, 1970), illustrating the importance of taking environmental variables into account. Further studies of spore dispersal combined with investigation of spore production of *Hericium* spp. in the UK, using existing data on *H. erinaceus* as a starting point, could provide more precise information regarding dispersal potential of these species.

The technique of using species specific primary mycelium as a bait for spores proved extremely useful for experiments using relatively short exposure times. The problem of contamination is difficult to avoid due to the rich agar medium – other studies have found similar problems (Vilgalys and Sun, 1994). To investigate longer distance spore dispersal,4 wood traps colonised with primary mycelium would be more appropriate, as these are inconspicuous and can be left unattended in woodland locations for several weeks (Edman *et al.*, 2004a).

Although it has been shown that *Hericium* spp. are similar to other wood decay basidiomycetes in terms of short distance spore dispersal and percentage germination, the effects of long distance spore dispersal may be of greater importance to these rare fungi whose populations are widespread. Short distance spore dispersal and establishment will maintain populations on a local scale, but to counteract inbreeding it is important for populations to retain gene flow between them, i.e. for spores to reach and establish in other populations. Isolated populations of rare fungi sometimes have lower spore germination, possibly due to inbreeding (Högberg, 1998 cited in Högberg and Stenlid, 1999; Edman *et al.*, 2004a). It is therefore vital to investigate long-distance spore dispersal of *Hericium* spp. which, according to fruit body distribution, exist in the UK in relatively small, distant populations that are not connected by suitable habitat corridors (Boddy and Wald, 2003). Combining this with investigations into spore viability using vital stains (see above) would bring us closer to understanding the possible effects of spore germination and dispersal on *Hericium* spp. in the UK.

Chapter 3: Combative ability and extension rates of *Hericium coralloides* primary and secondary mycelia[1]

3.1 Introduction

The lifecycle of a typical basidiomycete comprises spore germination producing a primary mycelium, followed at some point by fusion and exchange of nuclei with a mating-type compatible primary mycelium to yield a secondary mycelium. Subsequently haploid sexual spores are produced following karyogamy and meiosis, and/or asexual haploid spores, oidia, are derived usually incorporating single nuclei. Primary mycelia are generally considered short-lived (e.g. Kauserud *et al.*, 2006), and the secondary mycelium assumed to be the dominant phase, although there is little evidence for this. Indeed, some primary mycelia, for example those of the wood-rotting species *Trametes versicolor* and *Heterobasidion annosum*, can persist in the field for several years, and possibly much longer (Coates & Rayner, 1985; Garbelotto *et al.*, 1997; Redfern *et al.*, 2001). There is also evidence of an inverse relationship between number of colonies of a species in the field and the number that are primary mycelia (Stenlid, 1994). Thus, for a rare species mating may be delayed due to low numbers of potential mates.

The vast majority of studies on fungal ecophysiology and the development and functioning of communities in dead organic resources have employed isolates obtained from colonized resources or from fruit body tissues, i.e. secondary cultures, and largely ignored the primary phase. However, there is evidence that primary and secondary mycelia exhibit differences in performance (Table 3.1), for example in terms of extension rate (e.g. Simchen, 1966; Hansen, 1979; Fryar *et al.*, 2002), wood decay rate (e.g. Platt *et al.*, 1965; Amburgey, 1970; Elliott *et al.*, 1979), and interspecific combative ability (Fryar *et al.*, 2002). So far there does not appear to be a trend for primary or secondary mycelia to out-perform the other in any particular activity.

[1] Crockatt, M., G. Pierce, R. Campbell, P. Newell, and L. Boddy. 2008. Homokaryons are more combative than heterokaryons of *Hericium coralloides*. Fungal Ecology. doi:10.1016/j.funeco.2008.01.001

Studies investigating differences between primary and secondary mycelia

Author/Date	Characteristics investigated	Isolates used	Primary/secondary mycelia related?	Results[a]
Fryar *et al.*, 2002	Competitive ability and extension rate of four wood decay basidiomycetes	One PM and one SM (progeny of PM) of each species	N	SM>PM; SM<PM[b]
Redfern *et al.*, 2001	Area of stump colonisation by *Heterobasidion annosum*	Natural colonisation by airborne spores; other spores used unspecified	Y	SM>PM
Sakamoto *et al.*, 2001	Protein expression in *Flammulina velutipes*	Two PM and derived SM	N	Non-quantitative differences
Garbelotto *et al.*, 1997	Colonisation of roots by *Heterobasidion annosum*	Four PM and four SM	N	SM= PM
Nguyen *et al.*, 1992	Phosphate solubilising activity of *Laccaria bicolour*	PM and 20 derived SM	N	SM=PM
Kope and Fortin, 1991	Variation in antifungal activity of *Pisolithus arhizus*	32 PM, 41 derived SM	N	SM>PM
Meysselle *et al.*, 1991	Relative acid phosphatase activity of *Hebeloma cylindrosporum*	11 SM; 20 sibling PM and 50 derived SM	N	PM=SM
Elliott *et al.*, 1979	Wood decay rate of *Serpula lacrymans*	Three SM from FBT, 40 PM derived from the three FBs, 138 SM synthesised from PM	Partially	PM>SM
Hansen, 1979	Wood decay and extension rate of *Phellinus weirii*	Six SM and six PM (from four fruit bodies)	Y	SM>PM
Bezemer, 1973	Extension rate of *Gloeophyllum trabeum*	13 SM and their constituent PM	N	SM>PM
Amburgey, 1970	Extension and decay rates of *Gloeophyllum trabeum*	Eight SM from FBT; 24 PM from the FBs; 15 SM synthesised from the PM; chemically induced PM from the synthesised SM	Partially	SM>PM / SM<PM[b]
Simchen, 1966	Extension rates of *Schizophyllum commune*	Six SM from FBT; 24 PM from the six FB; 72 derived SM	Y	SM>PM / SM=PM / SM<PM[b]
Platt *et al.*, 1965	Wood decay rate by *Heterobasidion annosum*	16 SM and 10 PM from one fruit body	Y	SM>PM

PM, primary mycelia; SM, secondary mycelia; sig diff = significant differences; FB(T) = fruit body (tissue); [a]SM>PM, SM outperformed PM; PM=SM no significant differences between PM and SM; SM<PM, PM outperformed SM; [b]different results for different species/isolates.

For saprotrophic species that rarely produce basidiocarps, such as *Hericium coralloides*, there will be relatively fewer airborne propagules compared to frequently fruiting species. In these cases the primary phase may be of greater significance than for a common species, as this phase may be relatively long, and it will be the primary mycelium that establishes the individual in dead organic resources. Thus, primary mycelia of rare species must be particularly 'fit' to survive and mate, or poor survival of primary mycelia may contribute to the rarity of the species.

In the following study *H. coralloides* was used as a model species to examine relative 'fitness' of primary and secondary mycelia of rare wood decay basidiomycetes. Secondary mycelia of *H. coralloides* obtained from fruit body tissue isolation are average to good combatants, not losing any territory to most members of the early stage decay community against which they were tested (Wald *et al.*, 2004b). Rarity is not, therefore, likely to result from poor combative ability of established secondary mycelia. Experiments were therefore designed to test the hypotheses that: (1) primary mycelia grow more slowly than secondary mycelia (in agar culture) and (2) primary mycelia are less combative than secondary mycelia.

3.2 Materials and methods

3.2.1 Cultures

Primary and secondary mycelia of *Hericium coralloides* and other wood decay Ascomycota and Basidiomycota (Table 3.2) were maintained on 2% or 0.5% (w/v) malt agar (MA; 15 g l^{-1} Lab M agar no. 2 (LabM, Bury, Lancashire, UK) with either 20 or 5 g l^{-1} Munton & Fison Spray Malt Light (Munton Plc, Stowmarket, Suffolk, UK), respectively).

Primary mycelia were obtained by isolation of single germinating spores. Small portions of mature hymenium were stuck to the lid of a Petri dish with petroleum jelly and positioned over a glass slide onto which spores were allowed to deposit overnight. Spores were suspended in sterile distilled water (SDW) and diluted so that when spread on 9 cm non-vented Petri dishes (Greiner Bio-One, Austria) of high clarity 2% MA (20 g Lab M agar no. 1; 15 g Lab M malt extract; l^{-1} distilled water) there was approximately one spore per field of view at x100 magnification. Plates were sealed with Nescofilm®, incubated at 20 °C in darkness and checked regularly until germination commenced. The location of single, well spaced

Table 3.2. Details of *Hericium coralloides* isolates, and isolates against which they were paired in agar culture

Ecological role	Species	Strain	Source	Isolated by	Date isolated/ created
Tooth fungus[a]	*Hericium coralloides*	**Secondary mycelia**			
		MA1	*Fagus sylvatica*, Windsor Great Park	*A .M. Ainsworth*	Feb 2002
		MA102	*F. sylvatica*, Windsor Great Park	*J. Hynes*	Oct 2003
		331	Sweden	FCUG[c]	
		424	Yugoslavia	FCUG[c]	
		1229	Sweden	FCUG[c]	
		Primary mycelia			
		MA126[b]			
		1, 6, 7, 10, 13	*Fraxinus excelsior*, Windsor Great Park	*M. Crockatt*	Nov 2005
		MA127			
		2, 10, 14, 25, 27	*F. sylvatica*, Epping forest	*M. Crockatt*	Nov 2005
		MA129[b]			
		9, 18, 20, 25, 29	*Fr. excelsior*, Windsor Great Park	*M. Crockatt*	Dec 2005
		Artificial secondary Mycelia			
		MA126 1 x MA127 27			Aug 2006
		MA126 1 x MA129 9			Aug 2006
		MA127 2 x MA127 9			Aug 2006
		MA126 10 x MA127 2			Aug 2006
		MA127 27 x MA129 25			Aug 2006
		MA126 1 x MA126 10			Aug 2006
		MA127 2 x MA127 25			Aug 2006
		MA129 9 x MA129 29			Aug 2006
		MA129 20 x MA129 29			Aug 2006

Table 3.2, continued

Ecological role	Species	Strain	Source	Isolated by	Date isolated/ created
Primary colonizers, latently present in standing trunks and attached branches	*Coniophora puteana*	Cput1	*F. sylvatica*	*S. J. Hendry*	Sep 2005
	Daldinia concentrica[e]				
	Eutypa spinosa[e]	Es1	*F. sylvatica*	*S. J. Hendry*	
	Stereum gausapatum	Sg1	*Quercus robur,*	*L. Boddy*	
	Vuilleminia comedens	Vc1	*Quercus robur*	*L. Boddy*	
Heart rotters	*Fomes fomentarius*	JHC001-201	*F. sylvatica,* Denmark	*J. Heilmann-Clausen*	
	Ganoderma applanatum	JHC-GA	*F. sylvatica,* Denmark	*J. Heilmann-Clausen*	
	Laetiporus sulphureus	MA123	Windsor Great Park	*M. Crockatt*	Oct 2006
Early secondary colonizers on standing and fallen wood	*Trametes versicolor*	D2	*Quercus robur*	*L. Boddy*	
Later secondary colonizing cord-formers	*Hypholoma fasciculare*	GTWV2	Wenvoe, S. Wales	*G. Tordoff*	2003
Later stage fungi whose ecological strategies have been little studied	*Ceriporiopsis gilvescens*	CgJHC1	*F. sylvatica,* Denmark	*J. Heilmann-Clausen*	
Ecological strategy unknown	*Panus conchatus*	Pc1	Windsor Great Park	*M. Crockatt*	Oct 2006
	Pholiota alnicola[f]	Pa1	Windsor Great Park	*M. Crockatt*	Oct 2006

Unless stated otherwise all cultures were obtained from within the UK and are maintained at Cardiff University. [a] Ecological role not yet entirely clear; found both in central heart regions and outer sapwood particularly on beech (Boddy and Wald, 2003); [b] Specimens obtained from same tree; [c] Supplied by Fungal Cultures Collection, University of Gothenburg (FCUG)/Nils Hallenberg; [e] *Ascomycota*, all others are *Basidiomycota;* [f] Although primarily on alder (*Alnus* spp.), *P. alnicola* is found occasionally on beech

germinating spores was marked with a dummy objective (a thin metal tube mounted on an objective lens that can be lowered into the agar to cut a plug of agar that directly corresponds to the field of view through the x10 objective lens). The plug of agar containing the germinating spore was then transferred to a fresh agar plate under a low power microscope (x10 magnification) with a needle of sharpened tungsten wire (tungsten wire pulled through molten sodium nitrite in a steel container). It was important that germinating spores were not touching any other mycelium, or had any spores in the same field of view, to ensure that mating could not occur. However, as germination was generally well below 1%, with often only a single germinating spore found on an entire plate, in order to obtain sufficient primary mycelia for experiments it was often necessary to transfer germinating spores that had (ungerminated) spores in the same field of view. To avoid possible mating that could occur if one of these spores germinated, as soon as sufficient mycelium was visible this was subcultured onto a fresh agar plate, where it was checked for presence of clamp connections. Cultures with clamp connections were discarded.

Spore prints originated from three fruit bodies: MA126 and MA129, which were produced on the same ash tree in Windsor Great Park, Berkshire, UK (Nat. Grid ref. SU956740), and MA127 and on a beech tree in Epping Forest, U.K. (Nat. Grid ref. TQ42329853).

Secondary mycelia were obtained by excising small pieces of tissue (avoiding the hymenium) from within fruit bodies collected from the field, which were surface sterilised by flaming before being placed onto 2% MA. In addition, ten artificial secondary mycelia were created by pairing selected primary mycelia on agar plates to produce stable secondary mycelium (Table 3.2).

3.2.2 Extension rates

Extension rates were determined on 0.5% MA for all 15 primary mycelia and the five natural secondary mycelia (Table 3.2) at 5, 10, 15, 20, 25, 30 and 35 °C, and for artificial secondary mycelia at 10 and 20 °C, with four replicates of each isolate at each temperature. Plugs (6 mm diam) from the growing margin of the colony, were inoculated centrally on 0.5% MA in 9 cm non-vented dishes and colony diameter in two dimensions perpendicular to each other was measured regularly during the log phase of extension. Measurements were made to 0.1 mm using Dialmax vernier

calipers (Swiss Precision Instruments Inc, Garden Grove, CA, USA). Extension rates were determined by linear regression and compared with one-way ANOVA, or Kruskal-Wallis if data were not normally distributed, using Minitab.

3.2.3 Interspecific interactions

Pairings between the 20 natural *H. coralloides* isolates and the 13 other wood decay species were made on 0.5% MA in 9 cm non-vented dishes. Fungi were inoculated (6 mm diam plugs) 3 cm apart at different times according to their extension rates so that they met in the centre of the dish. Plates were incubated in darkness at 20 °C (four replicates per combination). Once colonies had met, interactions were observed weekly, and final outcomes recorded after 12 to 14 weeks as either deadlock (where neither isolate captured territory from the other), replacement (where one fungus had grown over and through the other so that it was no longer recoverable by isolation) or partial replacement (where one fungus was recoverable from some but not all of the territory originally held). Outcomes were confirmed by subculturing from the base of the agar.

As an aid to comparison of overall combative ability, outcome of each replicate of each pairing was given a score: replacement of the antagonist by *H. coralloides* was assigned +2; partial replacement of the antagonist, +1; deadlock, 0; partial replacement of *H. coralloides*, -1; complete replacement of *H. coralloides*, -2. Cumulative values were determined for each isolate.

3.3 Results

3.3.1 Extension Rates

Optimum temperature for radial extension rate was 25 °C for 17 of the 20 natural isolates, exceptions being MA126 6 and 13, and 10, which had optima of 20 °C, and 30 °C respectively. All isolates grew at 5 °C. Maximum temperature for growth was between 30 °C and 35 °C for the majority of isolates, although two secondary and five primary mycelia did grow at 35 °C. No primary mycelia from MA127 grew at 35 °C, and MA126 6 did not grow at 30 °C. The duration of lag phase following inoculation decreased as incubation temperature approached the optimum.

Some isolates were consistently fast or slow growers (e.g. secondary mycelia 331 and 1229, respectively), and at 15 °C primary mycelia extended at significantly

greater rates than secondary mycelia (Fig 3.1). There were also differences between primary mycelia isolated from different fruit bodies (Fig 3.2). However, there were no consistent differences across all temperatures.

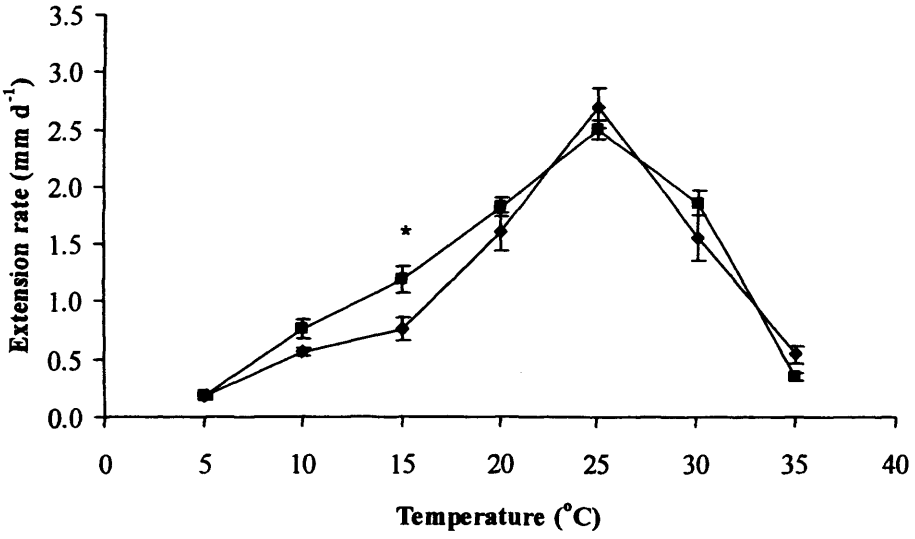


Figure 3.1 Extension rates of *Hericium coralloides* primary and secondary mycelia

▪ , primary mycelia; ◆ , secondary mycelia; error bars are SEM. Significant differences between extension rates, * $P \leq 0.05$

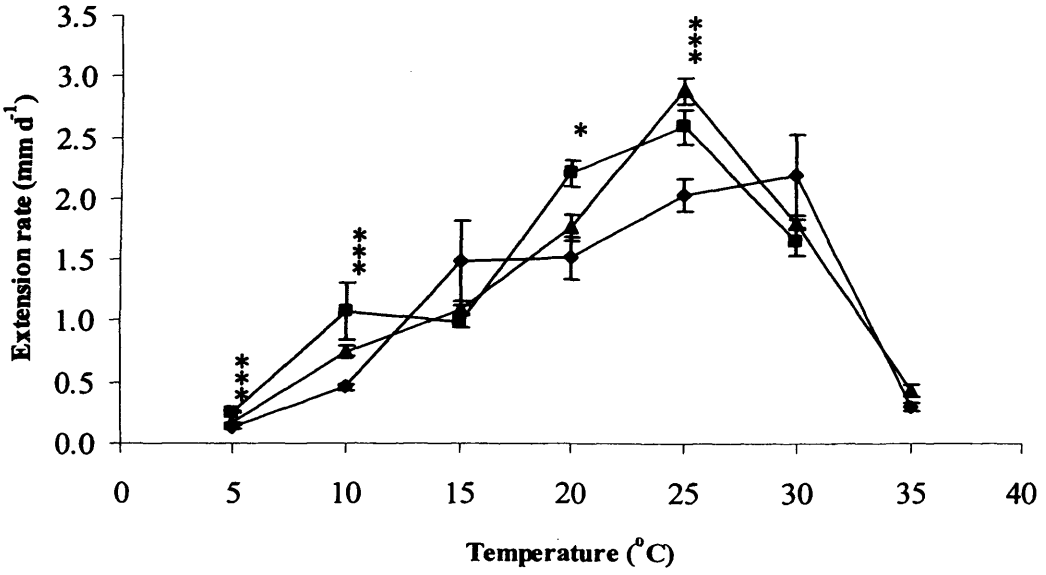


Figure 3.2 Extension rates of *Hericium coralloides* primary mycelia grouped by fruit body

▪ , MA127; ◆ , MA126; ▲ , MA129; error bars are SEM. No MA127 primary mycelia grew at 30 °C. Significant differences between extension rates: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. 5 °C: MA126 *vs* MA127 ***; MA126 *vs* MA129 ***. 10 °C: MA126 *vs* MA127 ***; MA127 *vs* MA129 ***. 20 °C: MA126 *vs* MA127 **; MA127 *vs* MA129 *. 25 °C: MA126 *vs* MA127 **; MA127 *vs* MA129 **.

At neither 10 nor 20 °C were there any consistent patterns between extension rates of artificial secondary mycelia and their parental primary mycelia (Fig 3.3). Results ranged from no significant difference between parent and progeny (e.g. MA129 9 x MA129 29 at 20 °C), to progeny having significantly greater extension rates than either parent (e.g. MA126 1 x MA127 27 at 10 °C).

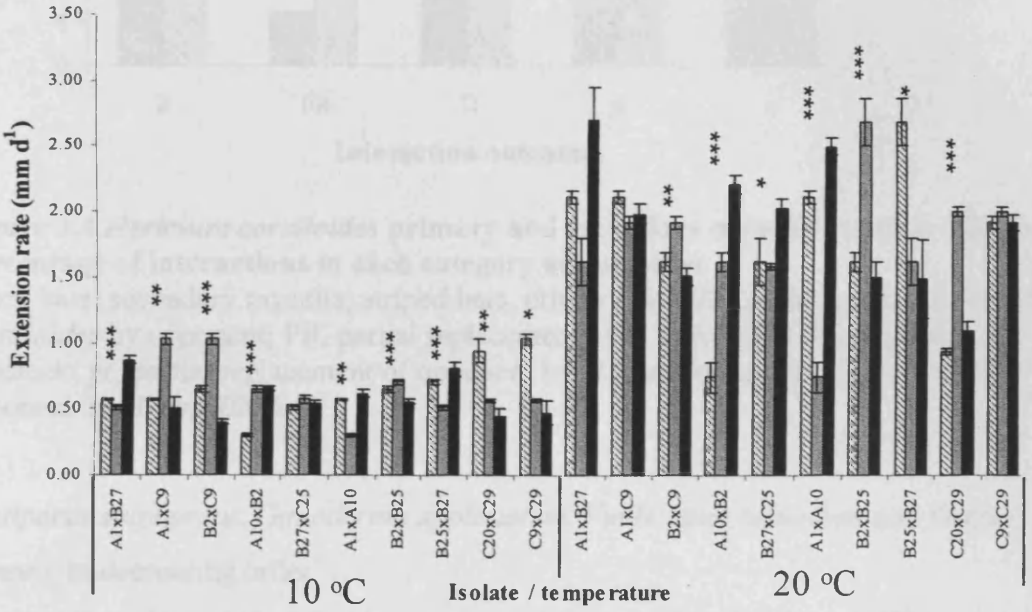


Figure 3.3 Extension rates of artificial secondary mycelia and parental primary mycelia

Striped bars, 1st parental primary mycelia; grey bars, 2nd parental primary mycelia; black bars, artificially created secondary mycelia; e.g. in far left set of bars A1 is striped bar (1st parental mycelium), B27 is grey bar (2nd parental mycelium) and the progeny secondary mycelium is the black bar. A, MA126; B, MA127; C, MA129, e.g. A1xB27 is MA126 1 crossed with MA127 27; bars are SEM; significant differences between extension rates: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.3.2 Outcome of interactions

Hericium coralloides performed well, either gaining territory or reaching deadlock in over half of all interactions, and with 12 of the 20 isolates having positive cumulative scores (mean score for all isolates was 2.6). Deadlock and replacement generally followed gross mycelial contact, although non-contact inhibition occurred with MA126 6 against seven of the 13 opponent species. Overall *H. coralloides* was most successful (large positive cumulative score; Table 3.3) against *Fomes fomentarius*,

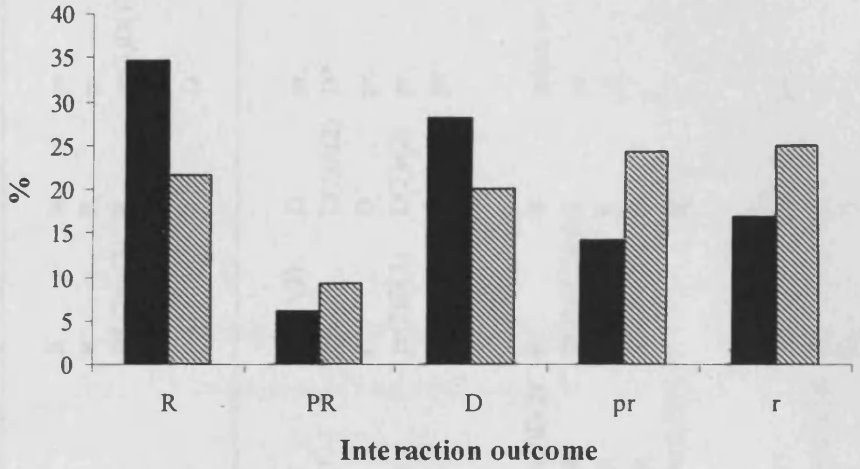


Figure 3.4 *Hericium coralloides* primary and secondary mycelia *vs.* other species: percentage of interactions in each category of outcomes
Black bars, secondary mycelia; striped bars, primary mycelia; R, replacement of *H. coralloides* by opponent; PR, partial replacement of *H. coralloides* by opponent; D, deadlock; pr, partial replacement of opponent by *H. coralloides;* r, replacement of opponent by *H. coralloides.*

Laetiporus sulphureus, *Ganoderma applanatum*, *Vuilleminia comedens* and *Eutypa spinosa*, in decreasing order.

All pairings against *Daldinia concentrica* resulted in deadlock, while *Hypholoma fasciculare* and *Coniophora puteana* always replaced *H. coralloides*.

The weakest combatant, as indicated by the cumulative scores, was MA126 6. Overall, however, secondary mycelia performed less well than primary mycelia: all of the former had negative cumulative scores, compared to only three of the 15 latter (Table 3.3); secondary mycelia gained territory in 31% of interactions, and primary mycelia in 49% (Fig 3.4). Primary mycelia were notably better than secondary mycelia in interactions against *G. applanatum*.

There was sometimes extreme variation in outcomes of interactions between different isolates. For example, four isolates replaced *Ceriporiopsis gilvescens*, while two were completely replaced by the latter. Sometimes there were different outcomes between replicates. This occurred to an equal extent in primary and secondary mycelia. Outcomes varied between both extremes, e.g. MA127 10 against *Stereum gausapatum*, though more commonly there was only a slight shift in outcome, e.g. deadlock to partial replacement.

Table 3.3 Outcomes of interactions between *Hericium coralloides* secondary/primary mycelia and 13 antagonist species

	Isolate	*Ceriporiopsis gilvescens*	*Coniophora puteana*	*Daldinia concentrica*	*Eutypa spinosa*	*Fomes fomentarius*	*Ganoderma applanatum*	*Hypholoma fasciculare*	*Laetiporus sulphureus*	*Panus conchatus*	*Pholiota alnicola*	*Stereum gausapatum*	*Trametes versicolor*	*Vuilleminia comedens*	Cumulative scores
Secondary mycelia	MA1	PR	R	D	Pr	r	R	R	R	r	r	R	R	pr	-4
	MA102	r	R	D	PR(2)D(2)	r	R(3)D(1)	R	r(3)R(1)	pr	r	R	R	pr	-4
	331	D	R	D	D	D	D	R	R	pr	R	PR(2)pr(2)	R	pr(3)D(1)	-17
	424	R	R	D	D	pr(3)R(1)	r(2)D(1)R(1)	R	r(3)pr(1)	D	D	R	R	pr	-26
	1229	PR	R	D	PR	D	D	R	Pr	D	D	r	R	D	-20
Primary mycelia	MA126														
	1	D	R	D	Pr	r	r	R	D	pr	D	r(2)D(2)	D	pr	16
	6	R	R	D*	D*	pr	D*	R	D*	D*	R*	PR	D(2)R(2)	D*	-36
	7	pr	R	D	R	r	r	R	R	pr	r	r	D	pr	44
	10	pr	R	D	Pr	D	r	R	D	D	pr	pr(2)D(2)	D(2)r(2)	pr	14
	13	r	R	D	Pr	PR	r	R	D	PR	R	r	D	pr	0
	MA127														
	2	PR	R	D	pr(3)D	r	r	R	R	pr(2)D(2)	pr(2)D(2)	R	R	pr(3)D(1)	-2
	10	pr	R	D	R	r	r	R	R	pr	PR	R(2)r(1)D(1)	R	pr	14
	14	R(2)PR(2)	R	D	r(3)pr(1)	r	r	R	R	pr(3)D(1)	PR	R	R	pr	-4
	25	r	R	D	D(3)pr(1)	r	r	R	r(3)pr(1)	pr	PR	r	R	r	24
	27	PR	R	D	R	pr	r	R	Pr	D	PR(2)D(2)	r	R	r	10
	MA129														
	9	PR	R	D	R	r	D(3)pr(1)	R	R	D	PR	PR	D	pr	1
	18	PR	R	D	Pr	r	r	R	Pr	D	PR(2)D(2)	PR	D	pr	2
	20	pr	R	D	Pr	r	r	R	Pr	pr	pr	PR	D	pr	20
	25	r	R	D	Pr	r	r	R	r(2)D(2)	pr(2)D(2)	D(3)pr(1)	R	D	pr	15
	29	pr	R	D	D	r	r	R	r(2)D(2)	D	PR	PR	D	r	8
Cumulative Scores		6	-160	0	62	109	93	-160	98	39	-13	-24	-80	82	

Abbreviations: D, deadlock; R, replacement of *H. coralloides* by opponent; PR, partial replacement of *H. coralloides* by opponent; r, replacement of opponent by *H. coralloides*; pr, partial replacement of opponent by *H. coralloides*. * with non-contact inhibition..Figures in brackets indicate how many of the four replicates represented each outcome, for those interactions that gave different outcomes amongst replicates. Scores for each isolate are cumulative for all replicates against ll antagonists. Scores for each replicate were -2 when *H. coralloides* was replaced, -1 when it was partially replaced, 0 for outcomes of deadlock, 1 when *H. coralloides* partially replaced its opponent and 2 when it completely replaced its opponent.

3.4 Discussion

The relationship between temperature and extension rate of *H. coralloides* isolates was typical of that of mesothermic fungi, which generally have cardinal temperatures of approximately 5, 25 and 40 °C (Rayner & Boddy, 1988); the maximum rates of around 2.5 – 3.0 mm d^{-1} are low to average for wood decay fungi (Boddy, 1983; Rayner & Boddy, 1988; Magan, 2008). The hypothesis (1) that primary grow more slowly than secondary mycelia is rejected, primary mycelia averaging a more rapid extension rate than secondary, though the difference was only significant at 15 °C. The possibility that extension rate results could be simply due to genetic variation between isolates, rather than karyotic status, is rejected, as is shown by comparisons between parent primary mycelia and artificially created secondary mycelia. The lack of significant differences between primary and secondary mycelia (with the exception of significant differences at 15 °C) contrasts with previous studies, all be they few, in which secondary mycelia of *Gloeophyllum trabeum* and *Phellinus weirii* grew more rapidly than primary mycelia (Bezemer, 1973; Hansen, 1979). A primary mycelium of a *Peniophora* species grew faster than a secondary, but only one isolate of each was tested (Fryar *et al.*, 2002), preventing generalisation for this species. Examining other characteristics, wood decay rate by *Serpula lacrymans* was less in 57% (of 138), intermediate in 33% and more in 10% of secondary mycelia than their component primary mycelia (Elliott *et al.*, 1979). Secondary mycelia of *Gloeophyllum trabeum* generally decayed wood more rapidly than primary mycelia from which they were synthesized (Amburgey, 1970). However, the relationships between karyotic states in this species were not straightforward, as about half of the primary mycelia generated by dedikaryotisation of two wild types decayed wood more rapidly than their progenitor. In *Heterobasidion annosum* primary and secondary mycelia could both be divided into two groups based on decay rate, that of the fast group being similar in both, and likewise that of the slow group (Platt *et al.*, 1965). In the present study, variation in optimum and maximum temperatures for growth and actual extension rates was broad, but was as large within groups (primary and secondary mycelia from different sources) as between. There do not appear to be any comparable published data for other basidiomycetes.

The second hypothesis that secondary mycelia are more combative than primary was also rejected, since all primary mycelia, except MA126 6, had higher cumulative scores than secondary mycelia. In the only other study to have examined

relative competitive ability of primary and secondary mycelia (based on area of agar covered after 58 d; Fryar *et al.*, 2002), outcomes for three species were variable, but only one primary and one secondary mycelium of each species was used.

Both primary and secondary mycelia mostly replaced, or at least deadlocked with, the heart rot species (*Ganoderma applanatum*, *Fomes fomentarius* and *Laetiporus sulphureus*) and early colonizers (*Daldinia concentrica, Eutypa spinosa* and *Vuilleminia comedens*), though not *Stereum gausapatum* or *Coniophora puteana.* Thus, the combative ability of primary mycelia probably does not contribute to the apparent rarity of the species. As in extensive previous studies with secondary mycelia (Wald *et al.*, 2004a,b), there were sometimes differences in outcome between replicates, presumably reflecting closely matched combatants and a delicate balance in the interplay between microenvironment and combative ability.

The scoring system was valuable for summarising combative ability against a range of opponents. It is always easy to spot very good and very poor combatants in a matrix of outcomes, but less easy to ascertain relative combative ability when outcomes range from replacement of antagonist, through deadlock, to replacement by the antagonist. A scoring system has been used previously with grain storage fungi (Wicklow *et al.*, 1980; Magan & Lacey, 1984; Marin *et al.*, 1998a,b), which involved assigning codes for different outcomes, e.g. mutual antagonism on contact (2) and mutual antagonism at a distance (3), and then adding them. That system, however, suffers from the drawback that it is completely qualitative, yet the codes are treated quantitatively. This is inappropriate as, for example, there is no reason why mutual antagonism at a distance should have a higher value than mutual antagonism following contact. Even though it is valid to use our scoring system quantitatively, numerical scoring on its own masks variation in outcomes and similar scores can be obtained for vastly different combinations of outcome, e.g. five replacements of other fungi plus five replacements by other fungi would yield the same score as ten deadlocks, but the ecological consequences would be very different.

Clearly, in the present study primary mycelia performed at least as well as secondary mycelia, though it should be noted that the primary mycelia used were obtained from single spore isolates and, therefore, had not had an independent existence in the field. Also, there is an inherent bias towards spores that germinate under artificial laboratory conditions, which may be particularly significant in a species such as *H. coralloides* that has a germination rate of less than 1 % in the lab

(Chapter 2). Ideally, primary mycelia isolated from wood should be used, though these are extremely difficult to obtain for rare fungi. Alternatively, dedikaryotization could have yielded constituent primary mycelia. Another possible source of bias is having used primary mycelia derived from spores from two fruit bodies on the same tree (MA126 and MA129), which could have been produced by the same mycelium in which case primary mycelia would all have been siblings.

It should be noted that abiotic conditions, including microclimate and venue of interaction (e.g. agar, wood or soil) affect interaction outcome (e.g. Boddy, 2000). The present study concerned variation between primary and secondary mycelia, which can validly be compared on agar medium, though outcome of interactions may differ slightly in more natural conditions. The suggestion that primary mycelia of these rare fungi may have to survive for longer than those of more common species, has not been refuted by these experiments. As primary mycelia of *H. coralloides* are better combatants than secondary, and have at least equal extension rates over a range of temperatures, once established they are likely to be able to survive for long enough to meet a mate and become stable secondary mycelia.

Chapter 4: Mating systems and somatic compatibility of *Hericium cirrhatum, H. coralloides* and *H. erinaceus*

4.1 Introduction

Sexual reproduction is responsible for creating and maintaining genetic diversity. In basidiomycetes sexual compatibility is governed by genetic controls called mating systems. These mating systems have been referred to as "arguably the most complex mating system of all known organisms" (James *et al.*, 2004); luckily for researchers although the systems themselves may be complex, elucidating them is often relatively straightforward.

Although approximately 10% of homobasidiomycetes are self fertile (homothallic; Esser, 1967, based on analysis of mating systems of 335 species, cited in Hibbett and Donaghue, 2001), the majority have diaphoromictic mating systems, which favour outbreeding. In these systems sexual compatibility is governed by one or two unlinked multiallelic loci, referred to as unifactorial or bifactorial mating systems, respectively. Genetic exchange can occur if primary mycelia have different alleles at the locus or loci. In a typical basidiomycete lifecycle the haploid basidiospores produced by a single fruit body will therefore be of either two (unifactorial) or four (bifactorial) mating types. Hence between sibling primary mycelia there will be 50 or 25% sexual compatibility for unifactorial or bifactorial systems, respectively. However as there are many alleles within a population there will be close to 100% compatibility between non-sibling primary mycelia. Thus mating systems promote outbreeding; without such a system there would be 100% compatibility between all primary mycelia regardless of relatedness. The majority of homobasidiomycetes are bifactorial, which promotes a greater degree of outbreeding than a unifactorial system (distribution is 65 and 25%, respectively; Esser, 1967, based on analysis of mating systems of 335 species, cited in Hibbett and Donaghue, 2001).

The significance of in- or outbreeding is likely to be greater for species with few fruit bodies (such as *Hericium* spp. in the UK) than species whose fruit bodies are common, as it is less likely that primary mycelia from the former will encounter other conspecific primary mycelia. As the majority of spores fall within a few metres of the fruit body (Kallio, 1970; Stenlid, 1994; Nordén and Larsson, 1999) it is more likely

that primary mycelia of rare fungi will encounter siblings than non-siblings. A mating system, which favours outbreeding, may be advantageous in such a situation where inbreeding would otherwise be extremely common, thus preventing development of genetic variability which would be brought about by genetic exchange between populations. However, in such populations mating between closely related individuals may be the only opportunity for sexual reproduction, thus inbreeding can also have advantages.

As rare species existing in isolated populations in the UK (Boddy and Wald, 2003) *H. coralloides, H. erinaceus* and *H. cirrhatum* provide examples of basidiomycetes in which inbreeding could be a significant issue. It is therefore crucial to understand their mating systems, i.e. how genetic exchange is regulated.

As a first step to understanding genetic diversity within populations of these species, somatic compatibility of cultures isolated from fruit bodies of each species that appear simultaneously on a substrate was investigated. This was to determine if they originated from a single mycelium or whether genetically different individuals of the same species ever occupy the same substrate, thus testing the hypothesis that a single individual of *Hericium* spp. can produce multiple fruit bodies on the same tree.

Mating systems were also investigated for all three species. North American isolates of *H. coralloides* and *H. erinaceus* are bifactorial (Ginns, 1985), and there are indications that *H. coralloides* within Europe is also bifactorial (Hallenberg, 1983), although this is based on a very small sample size. It has also been suggested that *H. coralloides* may be homothallic, due to observations of primary mycelia with clamp connections, which are generally only seen on mated mycelia. However, unmated mycelia of some genera produce clamp connections (Ainsworth, 1986), and it would be unwise to accept the claim of homothallism without further investigations into nuclear behaviour. The hypothesis that they are bifactorial in the UK, as they are in the North America, was thus tested. The mating system of the closely related *H. cirrhatum* has not been investigated, so was a priority for this study.

4.2 Materials and methods

4.2.1 Cultures

Primary and secondary mycelia (Table 4.1) were obtained as described in Chapter 3.

Table 4.1 *Hericium cirrhatum, H. coralloides* and *H. erinaceus* isolates used in mating system and somatic compatibility experiments

Species	Fruit body	Date collected	Substrate	Collector	Location	National grid ref	Experiments used in*	
							Somatic compatibility	Mating system
H. cirrhatum	AL1	24.07.05	*Fagus sylvatica*	AL	NF	SU37290414		Y (22)
	MA125	07.10.05	*F. sylvatica*	AMA	BB	SU94398467		Y (22)
	MA128	26.10.05	*F. sylvatica*	AMA	EF	TL42960020		Y (12)
H. coralloides	AB1[a]	07.11.06	*F. sylvatica*	MEC	EF	TL43790042	Y	
	AB2[a]	07.11.06	*F. sylvatica*	MEC	EF	TL43790042	Y	
	MA115	24.10.04	*Fraxinus excelsior*	AMA	WGP	SU950751	Y	
	MA116	24.10.04	*F. sylvatica*	AMA	WGP	SU926746	Y	
	MA126[b]	15.10.05	*Fr. excelsior*	AMA	WGP	SU956740		Y (11)
	MA127	20.10.05	*F. sylvatica*	AMA	EF	TQ42329853		Y (10)
	MA129[b]	03.11.05	*Fr. excelsior*	AMA	WGP	SU956740		Y (14)
H. erinaceus	AL7	19.11.05	*F. sylvatica*	AL	NF	SU228148		Y (6)
	Eye 2	21.11.06	*F. sylvatica*	MEC	NF	SU22731493		Y (2)
	SW1a	16.10.06	*F. sylvatica*	MEC	NF	SU28801193		Y (16)
	SW2	16.10.06	*F. sylvatica*	MEC	NF	SU28921180		Y (2)
	Ock A[c]	16.10.06	*F. sylvatica*	MEC	NF	SU246115	Y	Y (2)
	Ock B[c]	16.10.06	*F. sylvatica*	MEC	NF	SU246115	Y	Y (20)
	Ock C[c]	16.10.06	*F. sylvatica*	MEC	NF	SU246115	Y	
	480	Unknown	*F. sylvatica*	PT	EC	SU9727	Y	
	P[†]						Y	

* Y = used in that experiment; numbers in brackets in mating system column are number of primary mycelia obtained from that fruit body. [†]commercial strain obtained from Ann Miller, Aberdeen. Sets of superscript letters indicate fruit bodies occurring simultaneously on the same substrate. AB1 was ca. 80 cm above AB2; MA126 was ca. 1 m above MA129; OckC was 50 cm above OckB, which was 2 m above OckA. Collector abbreviations: AL, Alan Lucas; AMA, A Martyn Ainsworth; MEC, Martha E. Crockatt; PT, P. Thomas; for contact information apply to Prof L Boddy (boddyl@cf.ac.uk). Location abbreviations: NF, New Forest; BB, Burnham Beeches; EF, Epping Forest; WGP, Windsor Great Park, EC, Ebernoe Common. Equivalent Natural England codes for *H. erinaceus* are in Appendix I.

4.2.2 Somatic compatibility

Tissue isolates of *H. coralloides* and *H. erinaceus* isolated from two or more fruit bodies occurring simultaneously on the same substrate (Table 4.1) were paired against each other to investigate somatic compatibility. They were also paired against other isolates of the same species from different locations (Table 4.1). Plugs (6 mm diam) of agar and mycelium from the edge of the colony were placed 3 cm apart on a 9 cm Petri dish of 2% MA, sealed with Nescofilm® and incubated at 20 °C in darkness. Controls were self pairings. Four replicates were made of each pairing including controls (self pairings). Plates were sealed with Nescofilm®, incubated at 20 °C in the dark and checked weekly for signs of somatic incompatibility such as inhibition or stimulation of growth, pigment production or changes in colony morphology such as barrage production. Observations continued until a point was reached where there had been no change in an interaction for four weeks.

4.2.3 Mating system

Non-sibling pairings of primary mycelia were performed to determine if a species was outcrossing. Up to five primary mycelia were randomly selected from each fruit body, and all cultures paired in all combinations. Pairings were made on 2% MA as described above except that plugs were placed 1.5 cm apart to allow colonies greater growth before meeting. Not less than one week after colonies had met, cultures were checked microscopically (x100-400 magnification) for presence of clamp connections, which would indicate that nuclear exchange had occurred and that the primary mycelia were sexually compatible, as clamp connections were found on isolates from fruit body isolates, but never on primary mycelia. Subcultures were made from compatible pairings, from the interaction zone and edges of each colony. When grown out these were again checked for clamps to ascertain whether a stable secondary mycelium had developed.

If greater than ten primary mycelia had been obtained from a fruit body, then sibling pairings were performed to ascertain whether the species had a unifactorial or bifactorial mating system. Two such experiments were performed for *H. cirrhatum* (MA125 and AL1) and *H. erinaceus* (OckB and SW1a) and three for *H. coralloides* (MA126, MA127 and MA129). Up to 22 primary mycelia from a single fruit body (Table 4.1) were paired in all combinations as described above, including pairing with self for controls. Compatible interactions were subcultured and re-checked for clamps

as described above. Results were recorded in a matrix which could be re-arranged so that compatible interactions were grouped together, allowing primary mycelia to be assigned to groups according to which mating allele(s) they possessed.

4.3 Results

4.3.1 Somatic compatibility

H. coralloides fruit body isolate pairings highlighted a similarity in mycelial morphology between AB1 and AB2, compared to MA115 and MA116 (Fig 4.1a-j). Both AB1 and AB2 invariably produced fruit body primordia at the colony margins, whereas MA115 produced clumps of floccose aerial mycelium around the inoculum plug and colony margin, and MA116 had very little aerial mycelium. Pairings of AB1 *vs.* AB2 and control pairings of both were all indistinguishable from each other. Non-contact inhibition was not seen in any pairings, and by six weeks (ca. three weeks after colonies had met) no differences in colony morphology were apparent between any control and experimental pairings, all isolates having met in the centre of the plate and apparently reaching deadlock without overt signs of antagonism.

In fruit body tissue pairings of *H. erinaceus*, isolate P overgrew all other isolates by 40 d, while other pairings were just meeting. By 58 d all colonies had met, but there were no signs of somatic incompatibility between any isolates; 81 d after pairing there were still no signs of somatic incompatibility. Colony morphology of isolates varied greatly even within replicates of the same pairing, including controls. This made it impossible to draw conclusions as to similarity or dissimilarity of OckA, B and C based on colony morphology (Fig 4.2a-e).

4.3.2 Mating system

4.3.2.1 H. cirrhatum

H. cirrhatum clamp connections varied greatly in shape (Fig 4.3a-e) and frequency both within and between pairings, but were occasionally present at most septa and easily interpreted as being clamps. Inconsistency in frequency and appearance of clamp connections made it extremely difficult to decide if a pairing was sexually compatible. Subculturing compatible interactions to confirm secondary mycelium was stable showed that clamps were always present in the central zone, but not always around the margins of both colonies.

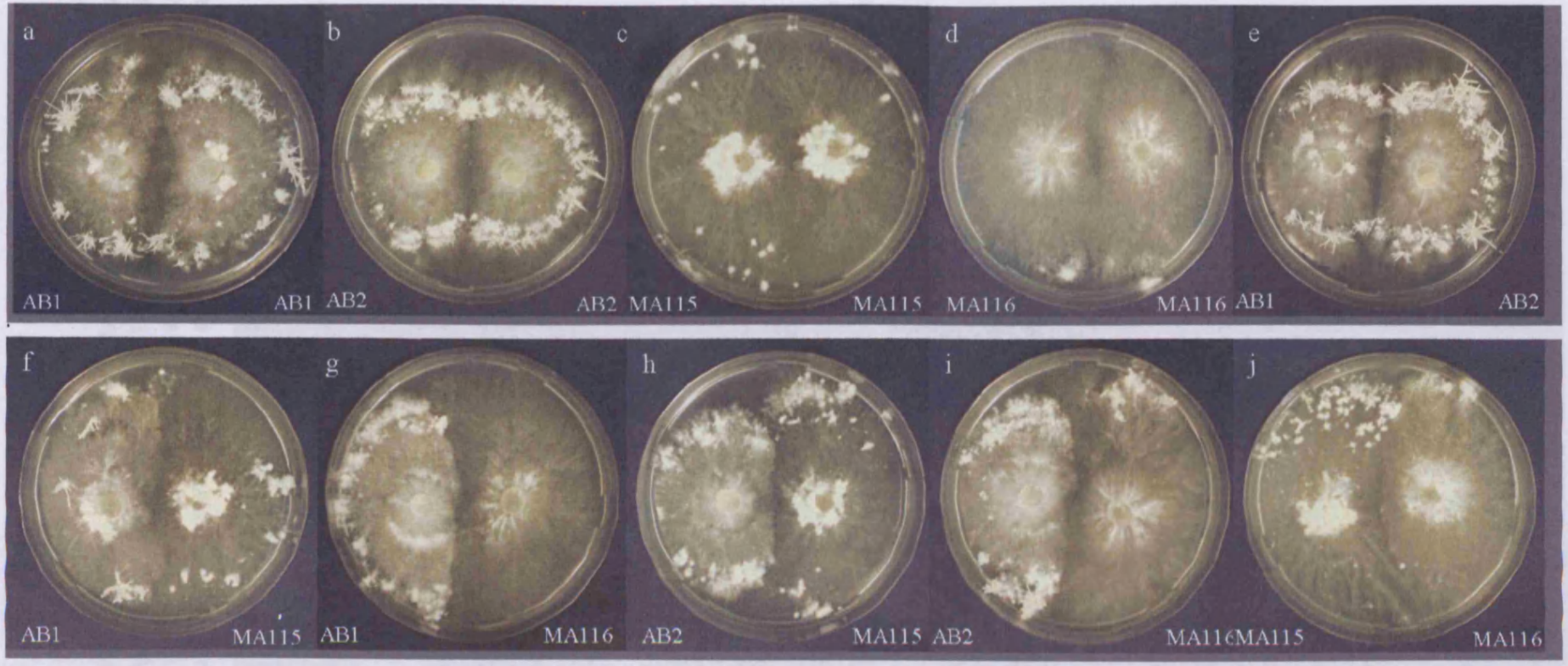


Figure 4.1 a-j *Hericium coralloides* fruit body isolate pairings at 41 d
a-d are control pairings of the four isolates used. e-j are experimental pairings; note similarity of e (AB1 *vs* AB2) to a and b (AB1 and AB2 controls, respectively), and variation in colony morphology on experimental compared to control pairings.

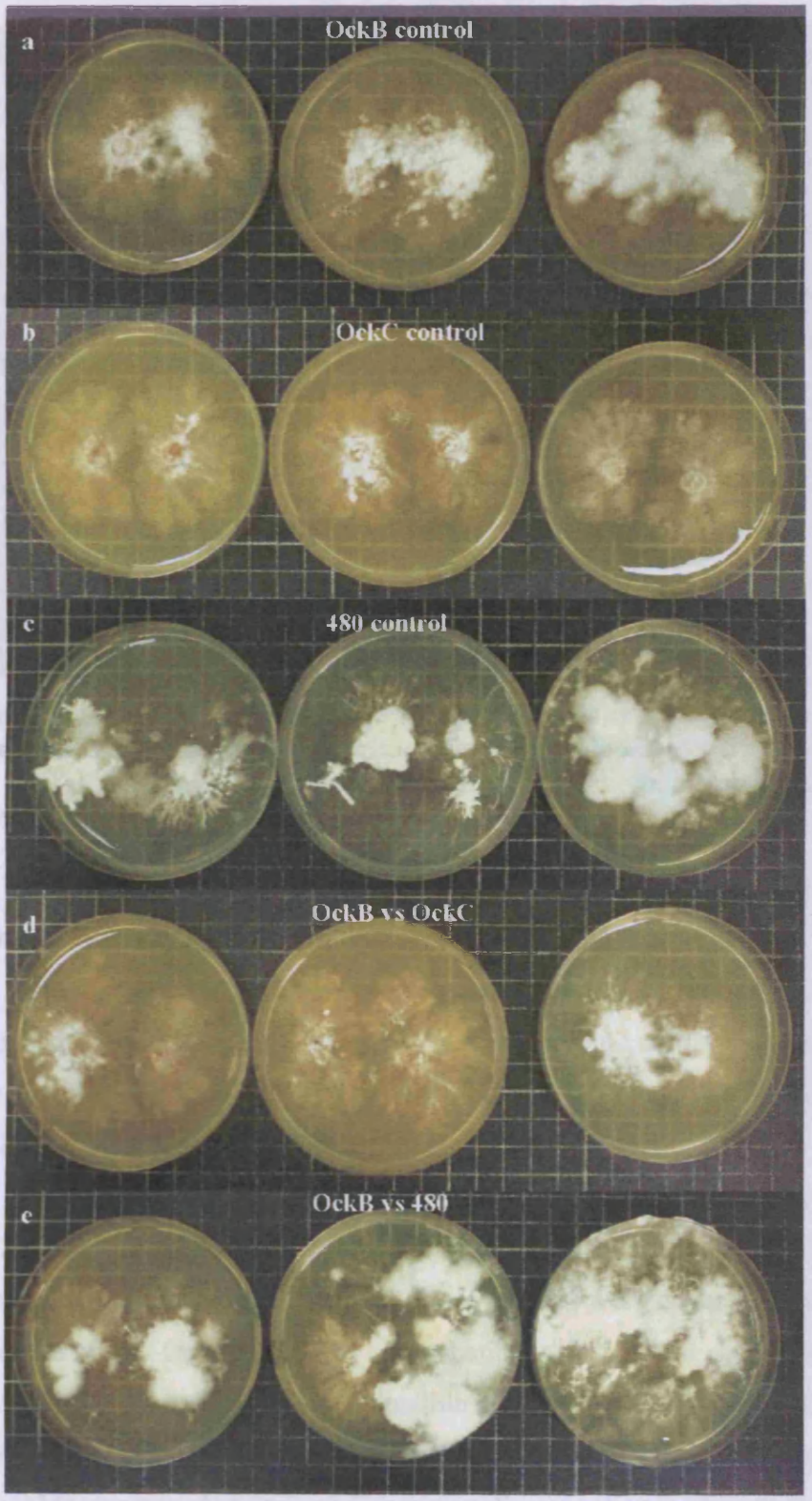


Figure 4.2a-e *Hericium erinaceus* fruit body isolate pairings at 84 d
Each picture shows three replicates of the same pairings. Figs a to c, control self pairings of named isolate; Figs d and e, first isolate is on the left of the plate, second isolate on the right. Note variation in colony morphology between replicate plates in control (a-c) and experimental (d-e) pairings.

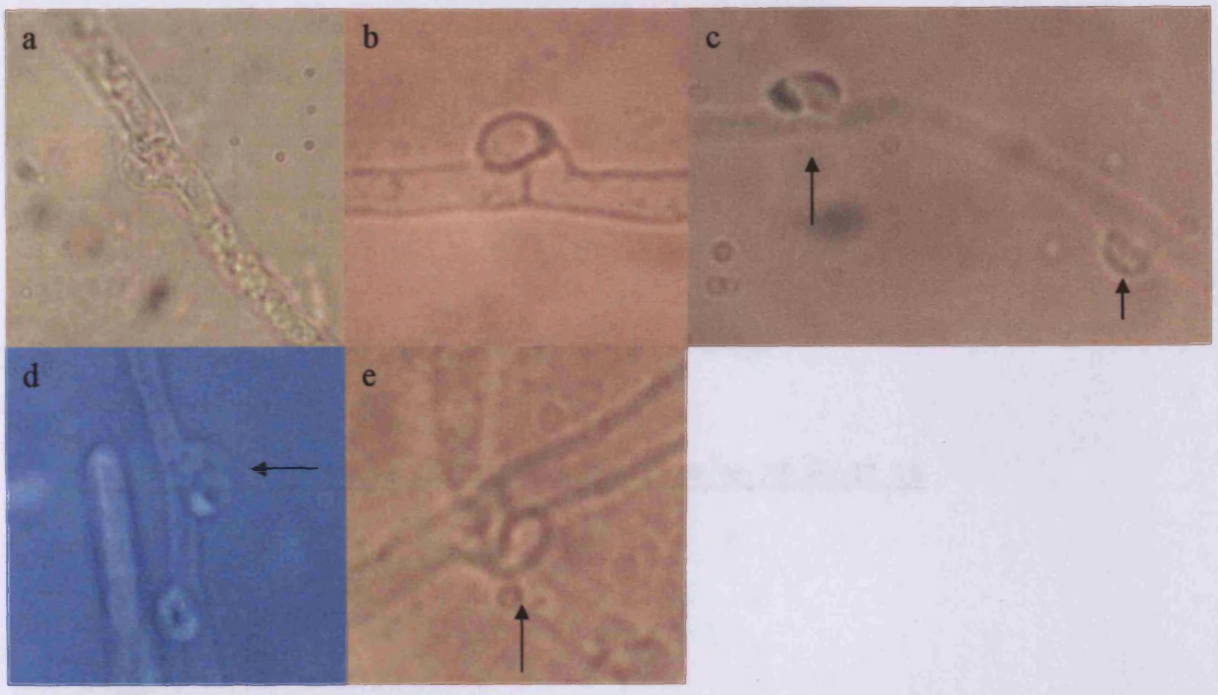


Figure 4.3 a-e Definite and possible clamp connections from interaction zone of pairings of *Hericium cirrhatum* primary mycelia (x400 magnification)
a is a typical, unambiguous clamp connection; b is a definite clamp connection; c-e are possible clamp connections.

H. cirrhatum non-sibling pairings approached 100% compatibility, there being just one incompatible pairing within the matrix (Table 4.2a).

Sibling pairings could not be easily interpreted. *H. cirrhatum* AL1 mating matrix had 25% compatibility, but primary mycelia could not be grouped by mating allele (Table 4.2b). Fruit bodies were found on 60% of compatible interactions, but only 2% of incompatible pairings.

Following high levels of contamination in the first attempt, *H. cirrhatum* MA125 matrix (Table 4.2c) was repeated, thus yielding data on the reproducibility of pairing outcomes. In the first attempt 24% of pairings were compatible, compared to 51% in the repeat. Only half (55%) of compatible pairings were again compatible when repeated. 22 pairings were compatible in the repeat that had been incompatible in the initial attempt. Neither the initial nor repeated matrix, or a combination of the two, could be arranged to group primary mycelia according to their mating alleles. An arrangement counting only pairings that produced regularly shaped, frequent clamps as being compatible did not produce more satisfactory results than when all clamp producing pairings were included.

Table 4.2a-c *Hericium cirrhatum* mating matrices

FB		AL1		MA125		MA128		AL10	
SSI no.		12	16	6	8	17	23	11	13
AL10	13	+	+	+	+	+	+	+	\
	11	+		+	+	+	+	\	
MA128	23	+	+	+	+		\		
	17	+	+	+	+	\			
MA125	8	+	+	+	\				
	6	+	+	\					
AL1	16	+	\						
	12	\							

4.2a *H. cirrhatum* non sibling pairings

SSI no.	2	5	8	9	10	12	15	16	17	18	19	20	22	24	25	26	27	28
28																		\
27			+				+							+			\	
26		+			+											\		
25		+			+						+		+		\			
24				+	+					+	+		+	\				
22									+	+			\					
20												\						
19			+				+				\							
18			+				+			\								
17		+	+		+				+	\								
16			+					\										
15				+			\											
12						\												
10					\													
9			+	\														
8	+		\															
5		\																
2	\																	

4.2b *H. cirrhatum* AL1 sibling pairings

no.	6	8	11	14	17	18	19	21	22	25	32	45	47	48
48		+	+	++	+	+		+	+		+	+		\
47		+				+					++	++	\	
45		+		++	++		+	+		+		\		
32	+		+	+		+	+	+		+	\			
25		+			+		+	+		\				
22	+		+		++		++	+	\					
21	+	+	+	+	+	++	+	\						
19	++	+		+		+	\							
18		+				\								
17	++	+	+	+	\									
14	+	++		\										
11	+	++	\											
8	+	\												
6	\													

4.2c *H. cirrhatum* MA125 sibling pairings

FB, fruit body; SSI no., single spore isolate number; +, stable secondary mycelium found; blanks, secondary mycelium not formed; \, control self pairing. Table 4.2c only: +, stable secondary mycelium present in first experiment only; +, stable secondary mycelium in repeat experiment only; ++, stable clamp connections present in both experiments

Table 4.3a-c *Hericium coralloides* mating matrices

FB		MA126					MA127					MA129				
	SSI no.	1	7	9	10	15	2	4	14	25	28	9	18	20	25	29
MA129	29	+		+	+		+	+	+	+	+					\
	25						+	+	+	+	+				\	
	20	+	+			+	+	+	+	+	+			\		
	18						+	+	+	+	+		\			
	9	+	+			+	+	+	+	+	+	\				
MA127	28	+	+	+	+	+					\					
	25	+	+	+	+	+				\						
	14	+	+	+	+	+			\							
	4	+	+	+	+	+		\								
	2	+	+	+	+	+	\									
MA126	15					\										
	10	+			\											
	9	+		\												
	7		\													
	1	\														

4.3a *H. coralloides* non sibling pairings

SSI no.	4	10	14	16	25	2	22	27	28	38
38	+	+	+		+					\
28	+	+	+	+					\	
27				+	+			\		
22				+	+		\			
2				+	+	\				
25					\					
16				\						
14			\							
10		\								
4	\									

4.3b *H. coralloides* MA127 sibling pairings

SSI no.	6'	9'	10'	13'	3"	9"	20"	4'	7'	8'	14'	12"	18"	25"	28"	11"	15'	12'	1'	1"	24"	29"
29'		+	+	+	+	+	+	+														\
24'		+	+	+	+	+	+	+													\	
1"		+	+	+	+		+													\		
1'	+	+	+	+	+	+	+													\		
12'	+	+	+	+	+	+	+	+											\			
15'	+							+							+	+	\					
11"									+		+	+	+			\						
28"									+	+	+	+	+	+	\							
25"														\								
18"													\									
12"												\										
14'											\											
8'								+		\												
7'									\													
4'								\														
20"							\															
9"						\																
3"					\																	
13'				\																		
10'			\																			
9'		\																				
6'	\																					

4.3c *H. coralloides* MA126 and MA129 combined pairings

Legend: as Table 4.2a-c. Boxes indicate primary mycelia with the same mating alleles. Table 4.3c only: primary mycelia from fruit bodies MA126 and MA129, which occurred on the same substrate, were pooled and a matrix with all primary mycelia performed; x', primary mycelium from MA126; x", primary mycelium from MA129; e.g. 6' is primary mycelium 6, from MA126; 3'' is primary mycelium 3 from MA129.

4.3.2.2 H. coralloides

Non-sibling pairings of *H. coralloides* MA127 against both MA126 and MA129 were 100% compatible, but compatibility was only 16% between MA126 and MA129 primary mycelia (Table 4.3a); compatibility within each fruit body was close to 25%. Fruit bodies were observed on almost all compatible pairings (99%). No definite fruit bodies were seen on incompatible pairings, although there were two plates with possible fruit body primordia.

H. coralloides MA127 sibling pairings had 31% compatibility, and fell into four clear groups (Table 4.3b). Fruit body formation was not noted on these pairings. Following the inter fruit body pairing results, it was decided to combine the MA126 and MA129 matrices, as it seemed likely that the fruit bodies originated from the same mycelium and all primary mycelia would be siblings. Overall, pairings had 23% success. Although there were anomalies, possible groups according to mating alleles could be seen (Table 4.3c). *H. coralloides* MA129 sibling pairings had fruit bodies on 79% of compatible pairings and possible fruit bodies on four incompatible pairings. Fruit body primordia were noted on only one sibling pairing for MA126 (7 vs. 14), which was incompatible.

4.3.2.3 H. erinaceus

H. erinaceus non-sibling pairings had 87.5% compatibility. Of the five incompatible pairings three were between OckA and OckB (Table 4.4a), which were from the same tree.

Sibling pairings of *H. erinaceus* provided easily interpreted matrices in which primary mycelia could easily be arranged according to mating allele (Tables 4.4 b and c). As with other matrices, a few compatible pairings fell outside the compatibility groups (one in Ock B and two in SW1a). Conversely, some incompatible pairings fell within these groups. A few pairings in each matrix (five in OckB and two in SW1a) formed transient secondary mycelia, i.e. the subcultured compatible pairing did not have clamp connections.

4.4 Discussion

There is strong evidence that the sampled *H. coralloides* fruit bodies occurring on the same tree were produced by a single mycelium. This evidence comes from the somatic compatibility experiments, in which pairings of isolates from fruit bodies

Table 4.4a-c *Hericium erinaceus* mating matrices

FB	SSI no.	Eye 2		Ock A		Ock B		SW1a		SW2	
		6	12	3	4	27	32	18	22	2	5
SW2	5	+	+	+	+	+	+		+		\
	2	+	+	+	+	+	+	+		\	
SW1a	22	+	+	+	+	+	+	+	\		
	18	+	+	+	+	+	+	\			
Ock B	32	+	+		+	+	\				
	27	+	+			\					
Ock A	4	+	+		\						
	3	+	+	\							
Eye 2	12		\								
	6	\									

4.4a *H. erinaceus* non sibling pairings

SSI no.	4	15	16	29	32	34	38	18	9	28	19	25	27	6	13	20	31	33	36	39
39		+	+	+	+	+														\
36	+		+	+	+														\	
33	+	+	+	+	+	+	+											\		
31	+	+	+	+	+	+	+										\			
20	+	+	+	+	+	+	+									\				
13	+	+	+	+	+	+	+								\					
6	-	+	+	+	+	+	+							\						
27								+		+	+		\							
25								+				\								
19				+				+	+		\									
28										\										
9									\											
18								\												
38							\													
34						\														
32					\															
29				\																
16			\																	
15		\																		
4	\																			

4.4b *H. erinaceus* OckB sibling pairings

SSI no.	2	10	22	25	26	4	12	3	18	24	1	5	7	8	28	27
28	+	+	+	+	+											\
27	+	+	+	+	+										\	
8	+	+	+	+	+									\		
7		+				+	+						\			
5						+						\				
1			+			+	+				\					
24						+	+			\						
18							+		\							
3						+		\								
12							\									
4						\										
26					\											
25				\												
22			\													
10		\														
2	\															

4.4c *H. erinaceus* SW1a sibling pairings

Legend: as Table 4.2a-c; boxes indicate primary mycelia with the same mating alleles.

located approximately 80 cm apart on the same tree were indistinguishable from control pairings. There is additional evidence from the pooled matrix of MA126 and MA129 primary mycelia pairings, which shows that the two fruit bodies shared the same four mating alleles, implying that they were either very closely related or produced by a single mycelium.

H. erinaceus may also produce multiple fruit bodies from a single mycelium, but somatic compatibility experiments were inconclusive. Furthermore, although it appears fruit bodies on the same tree may possess the same mating alleles, any conclusions must remain tentative because of the extremely small number of primary mycelia available from such fruit bodies.

In the UK *H. coralloides* and *H. erinaceus* have bifactorial mating systems, which confirms findings in North America and continental Europe (Hallenberg, 1983; Ginns, 1985). The mating system of *H. cirrhatum* remains unknown, although it appears to be outbreeding as sexual compatibility between non-sibling primary mycelia approached 100%, but was much less than 100% between siblings.

The compatibility of pairings between primary mycelia from *H. coralloides* MA126/129 and MA127, which fruited on ash and beech respectively, shows there are no barriers to gene flow between individuals fruiting on different trees. This is important, as such barriers have been found in other wood decay basidiomycetes such as *Laetiporus sulphureus*, for which there is a sexual compatibility barrier between individuals fruiting on broadleaved and coniferous hosts (Rogers *et al.*, 1999). Although *H. coralloides* is usually found fruiting on beech in the UK (Boddy and Wald, 2003), it is useful for conservation of this species to know that individuals on ash, the second most common host, are not sexually separated from the main population.

The low percentage of compatibility between MA126 and MA129 primary mycelia shows that the parental fruit bodies had identical mating alleles. This indicates that they were either very closely related, or had both been produced by a single mycelium. Unfortunately fruit body tissue isolates were not made, so somatic compatibility of the two fruit bodies could not be tested. Knowing whether the fruit bodies were produced by one or two mycelia would be of great interest, as at present it is not known how much wood a typical individual occupies, or whether different individuals ever occupy the same substrate. Somatic compatibility experiments imply that AB1 and AB2, which were approximately 80 cm apart, were produced by the

same mycelium. This shows it is possible for a single mycelium of *H. coralloides* to produce more than one fruit body on a tree, but this does not preclude the possibility of different genotypes coexisting within a substrate. Decay columns of basidiomycetes can extend over several metres (Boddy, 2001), therefore fruit bodies could occur 80 cm apart on the same mycelium. It is also therefore feasible that *H. erinaceus* OckA, OckB and OckC, which occurred over a total distance of 2.5 m on a single trunk, were produced by a single mycelium.

Contrary to previous reports (Hallenberg, 1983), no indications of homothallism were seen in *H. coralloides*. The claim of homothallism was based purely on possession of clamp connections by single spore isolates, assumed to be self-mated; however, as unmated (primary) mycelia of several genera have clamps, e.g. *Stereum*, *Phanerochaete* and *Coniophora* (Ainsworth, 1986) the claim is illfounded. Unfortunately, due to low percentage germination it was necessary to include germinating spores which may have had contact with other spores. Therefore it was possible that clamps on cultures from germinating spores were due to mating having occurred.

H. erinaceus illustrated a textbook bifactorial mating system, with close to 100% sexual compatibility in non-sibling pairings, and approximately 25% compatibility in sibling pairings. Primary mycelia in both sibling matrices were readily grouped according to the four combinations of mating alleles, although as with *H. coralloides,* there were some compatible pairings that occurred outside the compatibility groups. As with *H. coralloides,* there are hints that fruit bodies on the same tree may be produced by the same mycelium, as shown by the incompatibility between primary mycelia from fruit bodies OckA and OckB. This is backed up by the lack of signs of somatic incompatibility between OckA, OckB and OckC.

H. cirrhatum matrix patterns were unclear, although the relatively low proportion of compatible sibling *versus* non-sibling pairings implies the existence of an outbreeding bias. The lack of clarity in matrices may be due to inconsistent appearance of clamp connections, which made scoring difficult, rather than *H. cirrhatum* possessing an unusual mating system. When presence or absence of clamp connections cannot be used to distinguish between primary and secondary mycelium, as in the present case, there are alternative possibilities. For example, in some species clear morphological differences exist between primary and secondary mycelia, which can be used to investigate mating systems (e.g. Rogers *et al.*, 1999; Boddy and

Rayner, 1982). Molecular techniques can also distinguish between primary (homokaryotic) and secondary (heterokaryotic) mycelia by investigating heterozygosity at known polymorphic loci (e.g. de Fine Licht *et al.*, 2005). A more detailed study of primary and secondary mycelia of these species, perhaps using a nuclear stain such as DAPI (4,6-diamidino-2-phenyl-indole dihydrochloride), would be the next logical step towards understanding the mating system of *H. cirrhatum*.

Confirmation of the bifactorial mating systems of UK *H. coralloides* and *H. erinaceus* and the discovery that *H. coralloides* on ash and beech can interbreed are significant advances in understanding potential gene flow within these species. It is interesting that a single mycelium of *H. coralloides*, and possibly also *H. erinaceus*, can produce more than one fruit body, as this indicates that the mycelium can occupy a large volume of wood. Obviously these findings apply only to the trees studied, and do not in any way exclude the possibility that two genotypes could occupy the same substrate. The mating system of *H. cirrhatum* remains unknown, and alternative strategies outlined above may be required to investigate it further.

Chapter 5: Establishment of *Hericium coralloides* in living beech *(Fagus sylvatica)* trees

5.1 Introduction

Fungal communities can develop and decomposition occur within branches still attached to living trees and in standing trunks (Boddy, 2001). There are several ways in which fungi gain access including *via* exposed heartwood resulting from branch breakage; through wounds that allow the bark to be breached; active pathogenesis; and latent propagules (Boddy, 2001). Pre-1980s it was thought that wood decay fungi were unable to colonise branches and trunks unless they had been pre-conditioned by growth of bacteria and non-decay fungi. This has since been proved incorrect, and that wood decay fungi can establish naturally and by artificial inoculation in previously undamaged wood (e.g. Boddy and Rayner, 1984; Hendry, 1993; Boddy, 2001).

H. coralloides is usually found fruiting on large, fallen beech (*Fagus sylvatica)* logs in old forests (Boddy and Wald, 2003), and can be artificially established within freshly felled beech logs, but not in logs that have been felled for some time, presumably due to primary colonising fungi that have already gained a foothold (Boddy *et al.*, 2004). Its ease of establishment in appropriate conditions is confirmed by the fact that it is readily cultivated for commercial purposes (Stamets, 2000). *Hericium* spp. have been found, using PCR amplification, as latent propagules in beech, ash (*Fraxinus excelsior)*, hazel (*Corylus avellana)*, maple (*Acer campestris)* and oak (*Quercus robur)* (D. Parfitt, unpublished). It is possible that the propagules were *H. coralloides*, but the PCR primers used for this work detect *H. erinaceus* and *H. alpestre* as well as *H. coralloides* (Parfitt *et al.*, 2005).

Although *H. coralloides* may have been found as latent propagules, and can readily establish in freshly felled logs, whether it can establish in living trees remains unknown. To investigate this, beech trees were artificially inoculated with *H. coralloides* (Boddy *et al.*, 2004), and subsequently harvested to see whether it had successfully established, and to analyse colonisation patterns. A combination of traditional isolation onto agar and PCR analysis, using the above mentioned primers, were used. This tested the hypothesis that *H. coralloides* could establish in living trees.

5.2 Materials and methods

5.2.1 Inoculation of Hericium coralloides into beech trees

In November 2002 oak dowels (8.5 x 25 mm; Anne Miller, Aberdeen) colonised with *H. coralloides* (obtained from fruit body tissue isolation) were inoculated (by P.M. Wald) into 15 standing living beech trees and 3 ash trees, (ca. 10 cm d.b.h.) at Coed Gorllwyn, Bangor (Nat. Grid Ref. SH589422). Inoculations were made at 0.5, 1.0, 1.5 and 2.0 m above ground, with two inocula at each height 180° from each other, and 90° from those above and below. Staggering inocula in this way minimises the possibility of decay columns interfering with each other (Hendry, 1993). An electric hand drill, the bit sterilised with methylated spirits prior to each use, was used to create holes (9 mm diam and 30 mm deep) into which the colonised dowels were hammered. Electrical tape was wrapped around the trunk to cover inoculation points. Six of the 15 trees were controls, inoculated as above but with uncolonised, sterile dowels.

5.2.2 Sampling

Four beech trees were harvested: one experimental tree on 2nd March 2007 (T4) and a further two experimental and one control tree on 12th August 2007 (T8/T12 and T15, respectively), the rest being left for future sampling. After felling, the lower 2.5 m of each tree was taken for sampling. Trunks were cut into four sections, each one containing a set of inoculation points, then sliced into quarters lengthwise, attempting to cut through at least one inoculation point. Each quarter of each section was assigned a letter arbitrarily, with A-D closest to the ground and Q-T the highest section. After photographing each section, samples were taken from putative decay columns arising from inocula. The area for sampling was swabbed with 5% household bleach, the top 3-4 mm of wood removed with a sterile chisel and a small (ca. 3-5 mm^3) sample taken with a sterile chisel from the newly exposed surface. Samples were stored at -80 °C until required, and trunk sections were stored at -20 °C. Duplicate samples were taken from T4 at each sampling point, one of which was stored at -80 °C for molecular analysis as above, the other being placed on 2% malt agar (MA: 20 g Lab M agar no. 1; 15 g Lab M malt extract l^{-1} distilled water; Lab M, Bury, Lancashire, UK) and incubated for up to three weeks to check for presence of *H. coralloides*. Any mycelium growing from the chip was checked for clamp connections, indicative of a basidiomycete. Sampling was focussed around dowels

(three or four per experimental tree and two for control) that could be clearly seen in cross section – not all were visible in this way due to the difficulty of accurately manipulating large logs through a band saw. Based on PCR results more samples were taken closer or further from the inoculum to determine the extent of the mycelium within wood, with a total of up to six samples taken from around each inoculum. The exception to this was T4, for which all samples were taken at once, and from which a greater number of samples were taken (Table 5.1).

5.2.3 DNA extraction from wood

DNA extraction followed the protocol developed by Cenis (1992). Samples were wrapped in foil and immersed in liquid nitrogen for 5 min then transferred to a sterile, cooled mortar in which they were ground to a find powder using liquid nitrogen with the addition of 600 µl extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, adjusted to pH 8.5 using HCl; Raeder and Broda, 1985). The powder was transferred to a sterile 1.5 ml microcentrifuge tube and the mortar rinsed with 600 µl 0.4% w/v dried skimmed milk which was then transferred to the same microcentrifuge tube. Samples were vortexed for 15 sec, shaken for 30 min and centrifuged (17000 g for 5 min) in an Eppendorf Minispin microcentrifuge (Eppendorf, Cambridge, UK). Supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and the centrifuging was repeated. After being transferred to a fresh 1.5 ml microcentrifuge tube half the volume of supernatant of 3M sodium acetate (pH 8.5) was added and the samples were mixed by inversion and placed at -20 °C for 10 min. Following another 5 min centrifuge (17000 g) the supernatant was transferred to a 14 ml Falcon tube and the DNA purified using spin columns (QIAquick PCR purification kit, Qiagen, Crawley, UK) according to the manufacturer's instructions, with the following exception. Extracted DNA was stored at -20 °C for short term and -80 °C for longer term storage. Due to the large volume of supernatant, it was necessary to run the sample through the spin columns in successive batches of 750 µl each.

5.2.4 DNA extraction from mycelium

DNA was extracted from mycelial cultures of *H. coralloides* and *H. cirrhatum* grown on agar, to be used as positive and negative controls for PCR amplification of DNA extracted from wood (see below).

Table 5.1 Presence of *Hericium coralloides* according to PCR products of extracted DNA and wood chip isolation onto agar

Tree	Sample	Section[a]	Distance from inoculum (cm)[b]	PUV2/ PUV4[c]	HER2F/HER2R[c]			*Hericium* present?	
				1:10	neat	1:10	1:100	Molecular	Agar
T4	1	N	19	+	++	--	--	Y	X
	2	N	8	+	--	--	--	N	N
	3	N	0.5	+	++	+-	--	Y	N
	4	N	4	+	--	--	--	N	X
	5[d]	O	12						X
	6	O	5	+	--	--	+-+	Y	N
	7	O	1	+	++	--	--	Y	C
	8	O	7	+	--	--	--	N	N
	9[d]	I	3						C
	10[d]	I	6						N
	11[d]	I	15						N
	12[d]	I	25						X
	13[d]	J	27						N
	14	J	11	+	--	--	--	N	N
	15	J	3	+	--	--	--	N	X
	16	H	1	+	--	--	--	N	X
	17[d]	H	0.5						N
	18	H	8	+	(+)-	--	--	(Y)	N
	19	H	25	+	(+)-	--	--	(Y)	N
	20	G	17	+	(+)-	--	(+)-	(Y)	N
	21	E	27	+	--	--	--	N	X
	22	A	1	+	--	--	--	N	N
	23	B	1	+	--	--	--	N	N
	24	B	4	+	--	--	--	N	N
	25	C	6	+	--	--	--	N	X
	26	D	12	+	--	--	--	N	N
T8	1	C	1.0	+	--	--	--	N	
	2	C	8.5	+	--	--	--	N	
	3	C	5.0	+	--	--	--	N	
	4	L	1.5	+	--	--	--	N	
	5	L	2.5	+	--	--	--	N	
	6	L	6.0	+	--	--	--	N	
	7	M	7.0	+	--	--	--	N	
	8	M	5.0	+	--	--	--	N	
	9	C	8.5	+	--	--	--	N	
	10	C	1.5	+	--	--	--	N	
	11	L	1.5	+	--	--	--	N	
	12	L	9.5	+	--	--	--	N	
	13	M	22.0	+	--	--	--	N	
	14	M	1.5	+	--	--	--	N	
	15[e]	M	15.5						
T12	1	K	6.5	+	--	--	--	N	
	2	K	14.5	+	--	--	--	N	
	3	K	2.0	+	--	--	--	N	
	4	M	0.5	+	--	--	--	N	
	5	Q	60.0	+	--	--	--	N	
	6	K	1.5	+	--	--	--	N	
	7	K	1.5	+	--	--	--	N	

Table 5.1 cont'd

Tree	Sample	Section[a]	Distance from inoculum (cm)[b]	PUV2/ PUV4[c] 1:10	HER2F/HER2R[c] neat	1:10	1:100	*Hericium* present? Molecular	Agar
	8	M	0.5	+	--	--	--	N	
	9	M	3.5	+	--	--	--	N	
	10	M	5.0	+	--	--	--	N	
	11	M	10.5	+	--	--	--	N	
T15	1	B	1.0	+	--	--	--	N	
	2	H	0.5	+	--	--	--	N	
	3	H	6.0	+	--	--	--	N	
	4	H	3.0	+	--	--	--	N	
	5	H	3.5	+	--	--	--	N	
	6	H	7.0	+	--	--	--	N	

+ band present; - band absent; (+) weak band present; N, *Hericium* not present; Y, *Hericium* present; X, sample on agar contaminated; C, clamp connections present, but became contaminated so could not be further investigated; [a]lowest section is A-D, highest Q-T; [b] estimated from photographs for T4; [c] primers, details intext; [d] only sample for growth on agar taken; [e] sample discarded due to chemical contamination.

Mycelium was scraped from approximately 20 cm^2 agar and transferred to a sterile 1.5 ml microcentrifuge tube. Extraction buffer (as above) and 0.4% (w/v) dried skimmed milk (300 µl each) were added. Samples were vortexed for 15 sec and centrifuged (17000 g for 5 min). After being transferred to a fresh 1.5 ml microcentrifuge tube, half the volume of supernatant of 3M sodium acetate (pH 8.5) was added and the samples were mixed by inversion and placed at -20 °C for 10 min. Samples were centrifuged, the supernatant transferred to a fresh 1.5 ml microcentrifuge tube and an equal volume of isopropanol added. After standing at room temperature for 5 min the samples were again centrifuged after which the supernatant was discarded and the pellet remaining in the microcentrifuge tube was washed in 500 µl 70% v/v ethanol. A final microcentrifugation (17000 g for 5 min) was followed by removal of the ethanol and the pellet being air dried before being re-suspended in 50 µl sterile distilled water. Storage of extracted DNA was as above.

5.2.5 PCR amplification

For each sample duplicate PCRs were performed with template DNA undiluted and at one in ten and one in 100 concentrations, i.e. a total of six PCRs for each sample. In addition, all samples were tested with the general plant primers PUV2 (TTCCATGCTAATGTATTCAGAG) 5'-3' and PUV4

(ATGGTGGTGACGGGTGAC) 5'-3', which would detect beech ribosomal RNA, to confirm that DNA extraction had been successful.

Each PCR reaction contained 2.5 µl 10x buffer (Qiagen), 0.5 µl dNTPs (10 mM), 1 µl of each primer (100 mM), 0.125 µl (0.625 U) HotStar *Taq* (as supplied by Qiagen) and1 µl template DNA, made up to 25 µl with sterile distilled water. The Techne Flexigene thermal cycler (Barloworld Scientific, Staffordshire, UK) was used according to the manufacturer's instructions with the following programme: 96 °C for 15 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min. Primers used were HER2F (ATCTCATCCATCTTACACC) 5'-3' and HER2R (CTCATAACAAGAGGATTGA) 5'-3' (Parfitt *et al.*, 2005), which distinguish *Hericium coralloides, H. erinaceus* and *H. alpestre* from the closely related *H. cirrhatum* and other wood decay asco- and basidiomycetes. Programme for use with primers PUV2/PUV4 was as for HER2F/HER2R. Included in each HER2F/HER2R PCR run were six controls, as follows. A positive control with *H. coralloides* DNA, to prove the PCR and primers were working; a reaction with *H. cirrhatum* DNA and HER2F/HER2R primers to show that the primers were acting specifically, i.e. a negative control; a second reaction with *H. cirrhatum* DNA, but with the species specific combination of primers HER2F and HER3R (CATATGACAGAGGATCGA) 5'-3' (Parfitt *et al.*, 2005) to prove that this DNA was of PCR quality; a reaction with HER2F/HER3R primers and *H. coralloides* DNA to confirm that these primers were picking up only *H. cirrhatum* DNA; a reaction with water rather than template DNA for each set of primers to prove that there was no contamination of primers or other PCR reagents. In each PCR it was therefore checked that the primers were working, but specifically enough not to detect DNA of a closely related species (*H. cirrhatum*), and that the DNA of that species was detectable if present.

PCR products were electrophoresed on a 1.5% agarose gel (1.5% agarose w/v in 1X TAE buffer (50X TAE: 242 g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA pH 8)) containing ethidium bromide (10 µg ml) and the bands visualised under UV light with the Gene Genius Bio Imaging System (Syngene, Cambridge, UK). To allow comparison of fragment size, a 1 kb ladder (500 ng; Invitrogen, Paisley, UK) was also run on each gel.

If results of duplicate PCRs were not consistent a third PCR was performed, with two exceptions. First, if the two results comprised a very weak band (see Fig 5.4) and a negative, which could be caused by a low DNA concentration on the border of

detection. Second, if there was already a (duplicated) positive result at another concentration for that sample.

5.3 Results

Excepting small areas of dark staining surrounding the dowel, decay columns were not apparent in T8, 12 or 15 (e.g. Fig 5.1; Appendix IIa-d). Columns were visible in T4 when it was first cut into quarters, but these faded within hours, and were not very clear in photographs (Fig 5.2). It was also noted that calli had formed over dowels in T8 and T15 (Fig 5.3).

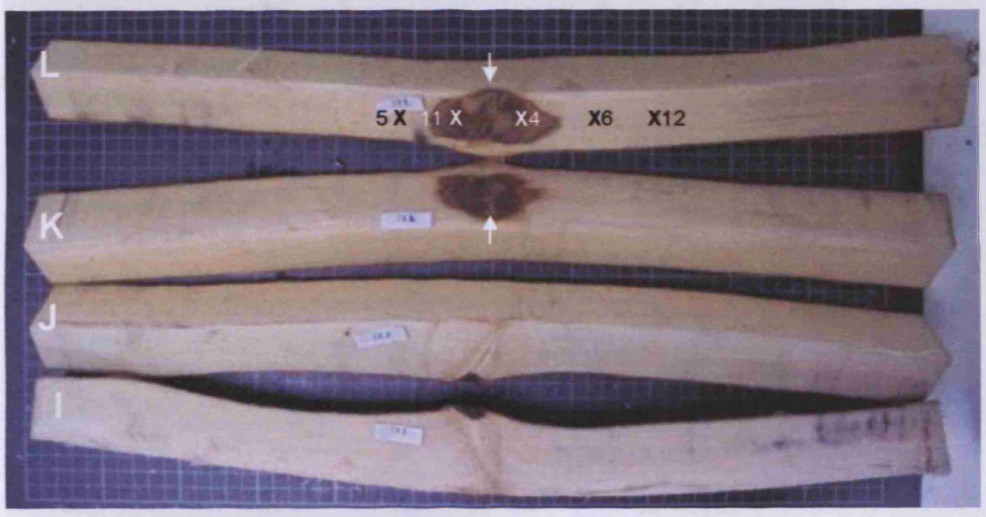


Figure 5.1 Cut section of tree T8 inoculated with *Hericium coralloides* showing inoculation and sampling locations
Length of each section is approximately 50 cm; inoculation height 1.5 m; X, sample location; arrow, dowel inoculum.

Of the 26 samples on agar from T4, eight were contaminated with bacteria or *Penicillium* type fungi, two yielded mycelium with clamp connections, i.e. basidiomycetes, and the remaining 16 had either no growth or growth of a non-basidiomycete (Table 5.1). The two clamped cultures became contaminated before it was possible to conduct further tests to ascertain if they were *H. coralloides*. One the corresponding samples of these clamped cultures was positive by PCR analysis; the other had not been sampled for molecular testing.

All samples were positive with PUV2/PUV4 primers, which showed that DNA had been extracted from the samples and was of sufficient quality for PCR amplification. Only four samples, all from around a single dowel in T4 yielded definite positive results with HER2F/HER2R primers (samples 1, 3, 6, 7; Fig 5.2).

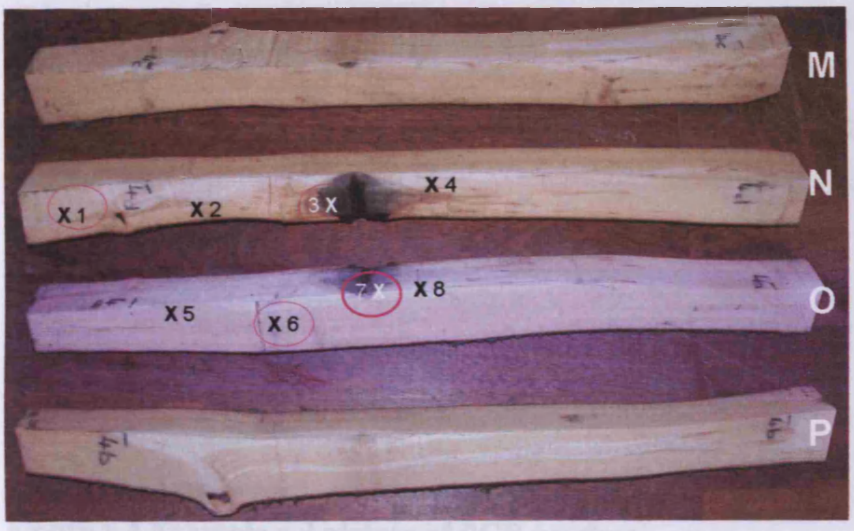


Figure 5.2 Cut section of tree T4 inoculated with *Hericium coralloides* showing inoculation and sampling locations
Length of each section is approximately 50 cm; inoculation height 2.0 m; X, sample location; arrow, dowel inoculum; solid red circle indicates a presence of *Hericium* (band present on gel).

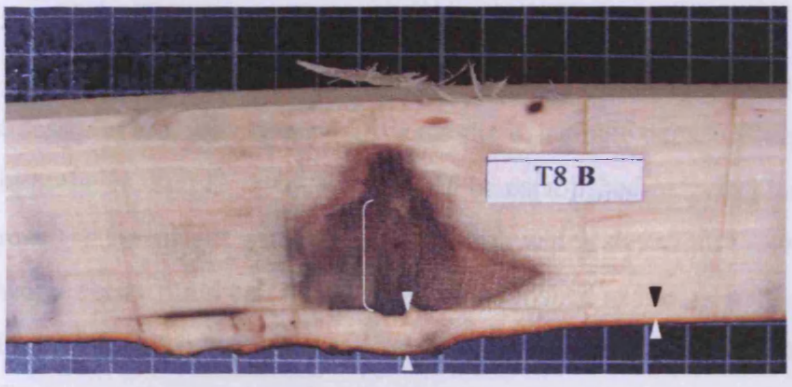


Figure 5.3 Callus formed in living beech over *Hericium coralloides*-colonised dowel inoculum
Bracket indicates dowel; arrowheads indicate increased growth over inoculum

The four positive samples were between 0.5 and 19 cm from the inoculum (Table 5.1; Fig 5.2); not all samples in this putative decay column were positive (Fig 5.2; Table 5.1), samples 2, 4 5 and 8 from the same area being negative by PCR analysis. A further three samples from T4 had weak positive results (18, 19 and 20). These were from sections G and H and were 8-17 cm from the inoculation point; however samples 16 and 17 which were closer to the inoculation point were negative. A result was classed as a strong positive when the band was clearly visible on the gel

in both repeats; a weak positive was an extremely faint band in one replicate of the PCR and no band in the repeat (Fig 5.4).

Reproducibility of the PCRs was 96%, although when weak bands that were not reproduced in the second PCR were excluded, this figure is increased to 99%.

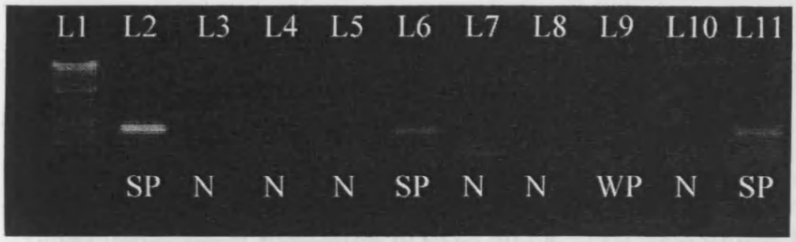


Figure 5.4 Example gel picture of PCR products
All samples are from T4, using primers HER2F/HER2R. L1, 1kb ladder; L2, sample 1 undiluted; L3, sample 1 diluted 1in 100; L4, sample 2 diluted 1 in 10; L5, sample 2 diluted 1 in 100; L6, sample 3 diluted 1 in 10; L7, sample 3 diluted 1 in 100; L8, sample 4 undiluted; L9, sample 4 diluted 1 in 10; L10, sample 6 undiluted; L11, sample 6 diluted 1 in 100. SP, strong positive; WP, weak positive; N, negative.

5.4 Discussion

Hericium coralloides successfully established in one (T4) of the three experimental trees sampled, with four positive results in a single decay column, proving the initial hypothesis, that *H. coralloides* could be established in living beech, to be correct. This result is consistent with existing research which shows that many, but no all, primary wood decay fungi can be artificially established in functional sapwood. For example, four canker-producing fungi (*Biscogniauxia nummularia, Hypoxylon fragiforme, Diatrype stigma* and *D. disciformis*) were consistently successfully established in living beech trunks from colonised dowel inocula (Hendry, 1993), but only three out of six white-rotting basidiomycetes inoculated into attached living oak branches established successfully (*Exidia glandulosa, Phellinus ferreus* and *Schizopora paradoxa* failed to establish; *Peniophora quercina*, *Stereum gausapatum* and *Vuilleminia comedens* successfully established; Boddy and Rayner, 1984).

The furthest positive result (19 cm) from an inoculation point shows that *H. coralloides* was well established. The length of this decay column is consistent with previous research: freshly felled beech trees artificially inoculated with *H. coralloides* developed decay columns to an average of 55 mm six months after inoculation (Boddy *et al.*, 2004). If columns continued extending at this rate, a length of 19 cm

would be much less than expected after five years, but in other inoculation experiments it was found that decay columns were well established at six months but subsequently failed to extend much further (Boddy and Rayner, 1984).

That establishment in living trees was poorer than had previously been found in felled wood (Boddy *et al.*, 2004) is not surprising, as the former type of habitat is generally inimical to fungal growth. This has been attributed to high water content in the functional sapwood, leading to poor aeration, and a lack of readily available nutrients (Boddy and Rayner, 1983b).

The regions of increased tree growth over inocula, i.e. callus production, are a typical response to wounding in trees and is attributed to this rather than presence of the fungus, the response being seen in control as well as experimental trees.

Consistency of results in repeated PCRs gives confidence in this method, as does the clustering of positive results around a single inoculation point. The high number of negative results, particularly those from the centre of the detected decay column, are deemed reliable due to the robust repetition of PCRs over a range of concentrations, and it having been proven by PCRs with general plant primers (PUV2/PUV4) that DNA of PCR quality had been successfully extracted. Running the PCRs with different concentrations of template DNA allowed for possible PCR inhibition due to poor DNA quality, which can be a problem when extracting directly from wood (D. Parfitt, pers. comm.). The negative results from within the detected decay column may be due to the amount of DNA present being below the limits of detection. Future work could investigate using nested PCR, which can detect much lower levels of DNA than traditional PCR (e.g. Lochman *et al.*, 2004; Parfitt *et al.*, in prep.).

Molecular methods are becoming more popular than traditional techniques, as they are often able to detect fungi that are unculturable or present at extremely low levels, and because results can be obtained more quickly (Guglielmo *et al.*, 2007). For example, *H. cirrhatum* (Parfitt *et al.*, 2005), *Biscogniauxia nummularia* (Mazzaglia *et al.*, 2001) and *Phlebia brevispora* (Suhara *et al.*, 2005) were all detected using PCR based methods where traditional isolation failed to find them. It was for this reason that traditional techniques were only used on one of the four trees. Unfortunately, since cultures with clamp connections were not confirmed as *H. coralloides,* it is difficult to draw any conclusions as to the extent that results from the two techniques

were in agreement. Perhaps this circumstance in itself shows the relative ease of molecular *vs.* traditional methods.

Throughout the above, it has been assumed that the inoculated *Hericium coralloides* was being detected, despite there being no proof of this: primers used were not species specific, and *H. coralloides* was not re-isolated from wood, which would have allowed somatic compatibility tests to confirm that what had been inoculated was still present. However, given the rarity of *H. coralloides* and other members of the genus, it seems unlikely that one of these would be coincidentally present in these trees. It is assumed that the more plausible scenario, that positive PCR results were finding the inoculated *H. coralloides*, is what occurred.

In summary, it has been shown that *H. coralloides* can be successfully artificially established in living trees, although not consistently; it has previously been discovered that it readily colonises freshly felled wood (Boddy *et al.*, 2004) and that it, or other members of the genus *Hericium*, are latently present in a variety of tree species including beech (Parfitt *et al.*, in prep.). Although establishment in living trees occurs less readily than in dead wood, if *H. coralloides* does manage to colonise living trees it is capable of significant growth; it is still unknown how *H. coralloides* establishes in its natural habitat.

Chapter 6: Population structure, spore germination and extension rates of *Piptoporus quercinus*

6.1 Introduction

Piptoporus quercinus is rare and protected under UK law primarily due to the rarity of its habitat, which is the exposed heartwood of veteran oak trees or fallen branches located in old growth wood pasture and parkland (Roberts, 2002). Little is known of the species beyond when and where it fruits (Chapter 1; Fig 6.1), and its combative ability and extension rates on agar under various conditions: on agar, *P. quercinus* is a poor combatant against other wood decay fungi, and grows slowly (1.9 – 3.15 mm d^{-1} depending on isolate) even under optimum conditions of 25 °C and pH 3.75 agar (Wald *et al.*, 2004a). The heartwood on which it fruits is inimical to growth of most fungi, having a low pH due to presence of tannins (Rayner and Boddy, 1988), which may explain how, despite being a poor combatant and slow grower, it can survive for long enough within a substrate to assimilate sufficient nutrients to produce sexual fruit bodies. *P. quercinus* is also known to produce chlamydospores (Stalpers, 1978), asexually produced thick-walled resting spores that may enable the mycelium to survive periods of environmental stress (Rayner and Boddy, 1988).

Once fruit bodies are produced, the dispersal, germination and establishment of spores may present a severe bottleneck in the lifecycle of a species with such specific habitat requirements, appropriate substrata being few and far between. In this situation inbreeding is particularly likely: most spores land within a few metres of basidiomycete fruit bodies (Kallio, 1970; Stenlid, 1994; Nordén and Larsson, 2000), thus making it likely that sibling spores will land near to each other. Given the specificity of habitats for fruiting, germination may also be limited to certain substrata, and if fruit bodies are scarce and far apart, there is an even greater likelihood that if spores do germinate they will encounter siblings rather than non-siblings. If *P. quercinus* has a unifactorial mating system (see Chapter 4), as does the only other species in the genus (*P. betulinus*; Cant, 1980), there is a 50% chance that sibling primary mycelia will be sexually compatible, allowing twice as much inbreeding as with a bifactorial system. Given these factors it is highly possible that there may be inbreeding in the UK population of *P. quercinus*.

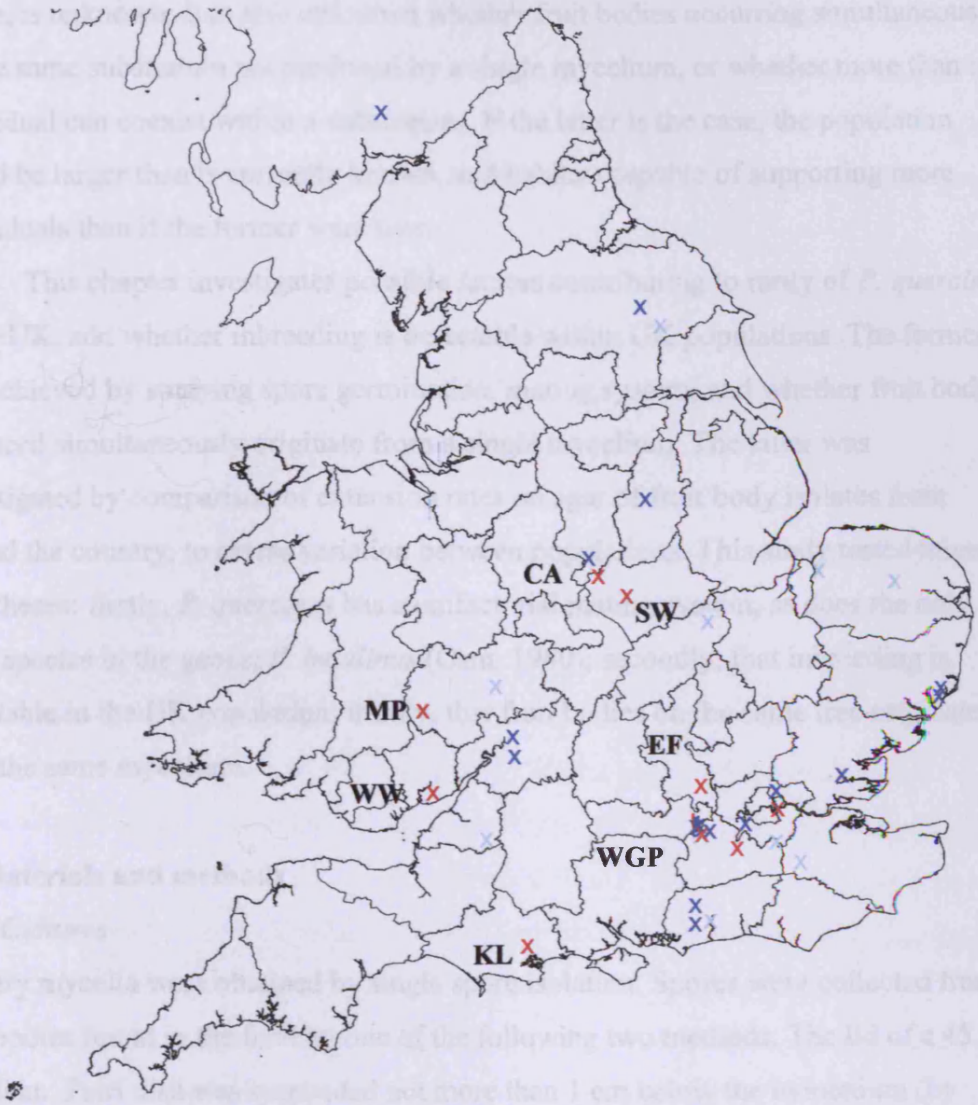


Figure 6.1 Distribution of *Piptoporus quercinus* in the UK: sampled locations and pre- / post-1970 records
Data from Roberts (2002), expanded to include subsequent findings of *P. quercinus*. Light blue dots, recorded pre-1970; dark blue dot, recorded post-1970; red dot, used in current study (Table 6.1). Location codes: CA, Calke Abbey; SW, Sherwood Forest; MP, Moccas Park; WW, Wentwood; EF, Epping Forest; WGP, Windsor Great Park; KL, Kingston Lacey.

P. quercinus populations are generally small (Roberts, 2002) and widely spread (Fig 6.1), occurring in on veteran oaks in old growth parkland or wood pasture, rare and fragmented habitats in the UK. Habitat fragmentation has been cited as a possible factor contributing to possible inbreeding in two rare boreal wood decay basidiomycetes (Edman *et al.*, 2004a). Whether inbreeding has occurred to any noticeable extent within UK populations of *P. quercinus*, due to reasons outlined

above, is unknown. It is also unknown whether fruit bodies occurring simultaneously on the same substratum are produced by a single mycelium, or whether more than one individual can coexist within a substratum. If the latter is the case, the population would be larger than is currently known, and habitats capable of supporting more individuals than if the former were true.

This chapter investigates possible factors contributing to rarity of *P. quercinus* in the UK, and whether inbreeding is detectable within UK populations. The former was achieved by studying spore germination, mating system, and whether fruit bodies produced simultaneously originate from a single mycelium. The latter was investigated by comparison of extension rates on agar of fruit body isolates from around the country, to assess variation between populations. This study tested three hypotheses: firstly, *P. quercinus* has a unifactorial mating system, as does the only other species in the genus, *P. betulinus* (Cant, 1980); secondly, that inbreeding is detectable in the UK population; thirdly, that fruit bodies on the same tree originate from the same mycelium.

6.2 Materials and methods

6.2.1 Cultures

Primary mycelia were obtained by single spore isolation. Spores were collected from fruit bodies found in the field by one of the following two methods. The lid of a 45 mm diam. Petri dish was suspended not more than 1 cm below the hymenium (by pinning directly to the fruit body, or arranging the dish by other means), which was left in position overnight. Alternatively, a small section of hymenium was cut from the fruit body and affixed to the lid of a 9 cm Petri dish with petroleum jelly. A glass slide was positioned directly under the hymenium and left overnight. Spores were washed from the Petri dish lid or slide and suspended in sterile distilled water, then spread on 2% malt agar (MA; 20g l^{-1} Lab M malt extract; 15 g l^{-1} Lab M agar no. 1), adjusted to pH 4 as follows. Potassium hydroxide (KOH; 0.1 M) was added dropwise to 250 ml phosphoric acid (H_3PO_4; 0.4 M) while continuously stirring until desired pH was reached (measured using a Gelplas flat tip combination pH electrode; BDH, Poole and a Delta 340 pH meter; Mettler-Toledo Ltd., Leicester, UK). As pH increased between this stage and the final poured plate, a calibration curve was constructed to enable the correct pH to be achieved. Thus at this stage the solution was adjusted to pH 3.3, reaching pH 4 on poured plates. This solution was then made

up to 500 ml, autoclaved, and mixed with 500 ml double strength MA (as for 2% MA, but made up to only 500 ml with distilled water). After pouring, pH was measured using a flat tip electrode. Plates with spores were incubated at 20 °C in darkness and checked regularly until germination was seen. Single, well spaced germinating spores were transferred to fresh agar plates as described in section 4.2.1.

Secondary mycelia (Table 6.1) were obtained by excising small pieces of tissue (avoiding the hymenium) from within fruit bodies collected from the field, which were surface sterilised by flaming before being placed onto 2% MA.

6.2.2 Spore germination

Spores of *P. quercinus* were collected and spread as described above, but at a higher density of 5-40 spores per field of view at x100 magnification. The effects of a variety of media, treatments and spore age on time to germination and the percentage germination were investigated. All spores were spread on pH 4 2% MA which was used as a comparison with the other treatments. Media were as follows: 2% MA adjusted to pH 3 and 4; 2% MA, pH unadjusted (ca. pH 5.6); pH 4 water agar (15 g l^{-1} agar); oak extract 2% MA: 15 g l^{-1} oak heartwood soaked overnight in distilled water, the filtrate of which was used in place of distilled water in normal 2% MA; charcoal agar: pH 4 2% MA with 0.28 g l^{-1} activated charcoal. Effect of a presence of a secondary mycelium was investigated by taping a plate well colonised with secondary mycelium above a plate of spores, allowing any volatiles produced to reach the spores. Plates were incubated at 20 °C in darkness and checked regularly until germination commenced. Some spores were incubated at 10 °C. Percent germination was obtained by scoring approximately 500 spores from three replicate plates as germinated or ungerminated at x400 magnification. Counting was continued every 2-3 d until colonies grown from previously germinated spores were large enough to inhibit accurate counting. Germination below 1% was not quantified, but recorded as <1%.

6.2.3 Mating system

Initially primary mycelia were only available from five fruit bodies, all from Windsor Great Park (Table 6.1). Up to five siblings from each fruit body were selected and paired against each other in all combinations, thus investigating sibling and non-

Table 6.1 *Piptoporus quercinus* isolates used in experiments

Fruit body	Collector	Source	Nat Grid Ref	Date collected	Spore germ'n	Mating system	Somatic compatibility	Extension rate
A2	AMA	AC	TQ17736055	09.07.07	Y	Y		
AL2	AL	NF	SU37310436	02.08.05	Y			
BR1a [04]	AMA	WGP	SU977730	14.10.04			Y	
BR1b [04]	AMA	WGP	SU977730	14.10.04			Y	
BR4[06]	AMA	WGP	SU97927291	11.07.06	Y			
BR6[06]	AMA	WGP	SU97907288	11.07.06	Y			
BR7[05]	AMA	WGP	SU97977299	12.07.05	Y			
C5[06]	AMA	WGP	SU94657292	08.07.06	Y			
C8[06]	AMA	WGP	SU94117338	08.07.06	Y			
CA1	MEC	CA	SK363224	23.08.07	Y	Y	Y	
CA2	MEC	CA	SK363224	23.08.07	Y			
CBS 858.72		Germany		1975			Y	Y
E1 [05]	AMA	WGP	TQ397944	26.07.05				Y
E2[05]	AMA	EF	TQ41079551	04.08.05	Y			
E3 [05]	AMA	EF	TQ41409352	04.08.05			Y	
E11[07]	AMA	EF	TQ41459313	19.07.07	Y	Y		
EW1	AMA	AC	TQ17576034	23.07.06	Y			
HSH2 [05]	AMA	WGP	SU93577416	13.07.05	Y	Y		
HSH5 [05]	AMA	WGP	SU936741	13.07.05	Y	Y	Y	Y
HSH10	AMA	WGP	SU93557398	28.06.06	Y	Y		
HSH12a [05]	AMA	WGP	SU935741	13.07.05	Y		Y	Y
HSH12b [05]	AMA	WGP	SU935741	13.07.05	Y	Y	Y	Y
KC1627*	PL	BP	SK526101	09.01			Y	Y
KL1	MEC	KL	ST96670375	12.07.07	Y	Y		
KL2	MEC	KL	ST96670375	12.07.07	Y			
KL3	MEC	KL	ST96860394	12.07.07	Y			
MC17 [04]	AMA	MP	SO3442	14.10.04			Y	Y
POWP1 [05]	AMA	WGP	SU966737	12.07.05	Y	Y		
POWP2[06]	AMA	WGP	SU96617363	11.07.06	Y			
POWP3[06]	AMA	WGP	SU96577361	11.07.06	Y			
POWP3a [05]	AMA	WGP	SU965736	12.07.05	Y		Y	Y
POWP3b [05]	AMA	WGP	SU965736	12.07.05	Y	Y	Y	Y
POWP5[05]	AMA	WGP	SU96517344	08.08.05	Y			
POWP6[06]	AMA	WGP	SU96557366		Y			
PS1	PS	KL	ST9670403796	05.07.05	Y			
PS2	PS	KL	ST9667603756	05.07.05	Y			
PS3	PS	KL	ST96620375	21.07.06	Y			
PS4	PS	KL	ST96670375	14.07.06	Y	Y		
PS5	PS	KL	ST96670375	14.07.06	Y			
SF15[06]	AMA	WGP	SU94267214	01.08.06	Y			
SF6[06]	AMA	WGP	SU94057113	01.08.06	Y			
SG1a[06]	AMA	WGP	SU95137181	08.07.06	Y			
SG1b[06]	AMA	WGP	SU95137181	08.0.06	Y			
SMa1ii[06]	AMA	WGP	SU95747397	13.07.06	Y			
SMa2[05]	AMA	WGP	SU95697408	07.08.05	Y			
SS1	SS	NF	SU3739904499	26.07.06	Y			
SW1	HW	SWF	SK46213680	26.08.07	Y			
WW1	MEC	WW	ST40379368	25.07.06			Y	Y
WW2	MEC	WW	ST40379368	06.08.07	Y	Y		

AMA, Martyn Ainsworth; AL, Alan Lucas; HW, Howard Williams; MEC, Martha Crockatt; PL, Peter Long; PS, Peter Samson; SS, Stuart Skeates; for contact details: Prof L Boddy (boddyl@cf.ac.uk); AC, Ashtead Common; BP, Bradgate Park; CA, Calke Abbey; EF, Epping Forest; KL, Kingston Lacey; MP, Moccas Park; SWF, Sherwood Forest; NF, New Forest; WGP, Windsor Great Park; WW, Wentwood Forest. *in Kew Culture Collection as K(M) 88407.

sibling sexual compatibility at the same time. If more than ten primary mycelia were available from a single fruit body a separate mating matrix was performed by pairing them in all combinations. Controls were self pairings. Plugs (6 mm diam) from the growing margin of the colony were inoculated 1.5 cm apart on 9 cm non-vented Petri dishes (Greiner Bio-One, Austria) of 2% MA adjusted to pH 4 and incubated at 20 °C in darkness until colonies had met. Cultures were observed microscopically (x400 magnification) for presence or absence of clamp connections, indicating successful or unsuccessful mating, respectively. Successful matings were subcultured from the interaction zone and far sides of each colony and re-examined for clamp connections following outgrowth from inoculum plugs to check that a stable secondary mycelia had formed. As further primary mycelia from other fruit bodies were obtained, these were paired against two tester strains of each mating type selected at random from the initial matrices (HSH12b primary mycelium 5 / HSH5 primary mycelium 1 and HSH12b primary mycelium 6 / POWP1 primary mycelium 12).

6.2.4 Extension rate

Ten secondary mycelia (Table 6.1) were selected to compare extension rate over a range of temperatures. Plugs (6 mm diam) from the actively growing margin of the colony were inoculated centrally onto non-vented 9 cm Petri dishes of 20 ml 2% MA adjusted to pH 4. Five replicate plates of each isolate were incubated in darkness at 10, 20 and 30 °C. Colony diameter was measured in two dimensions perpendicular to each other at intervals of 3-14 d as appropriate according to extension rate, using Vernier dial callipers accurate to 0.1 mm (Fisher Scientific, UK), ensuring that the exponential growth phase was captured.

6.2.5 Somatic compatibility

Secondary mycelia obtained by tissue isolation from fruit bodies occurring simultaneously on a substratum were paired against each other and six isolates from as geographically diverse locations as possible (Table 6.1). Further pairings were conducted when isolates from new localities were available. Plugs (6 mm diam) from the growing margin of the colony were placed 3 cm apart on non-vented 9 cm Petri dishes of 2% MA adjusted to pH 4, with four replicates of each pairing; controls were self-pairings. Plates were incubated at 20 °C in darkness and observed regularly over at least 10 weeks for signs of rejection or antagonism, such as pigment production,

growth inhibition or invasive fronts. Once they had ceased changing, each interaction was given a score of strong somatic incompatibility, weak somatic incompatibility or no somatic incompatibility.

6.2.6 Statistical analyses

Mycelial extension rates (mm d^{-1}) were determined by linear regression analysis and mean extension rates of the 11 isolates compared at each temperature with ANOVA, using the Minitab statistical package. Homogeneity of variance and normal distribution of residuals were tested with Bartlett's and Anderson-Darling tests, respectively. If the variance was homogenous and the residuals normally distributed, ANOVA was performed, followed by Tukey's pairwise comparison of means if significant differences were found. If data could not be transformed to have homogenous variance and or normally distributed residuals, then a Kruskal-Wallis test the non parametric equivalent of ANOVA, followed by Mann-Whitney tests to compare pairs of medians, were performed.

6.3 Results

6.3.1 Spore germination

Of the 41 spore prints received over the three years, eight were entirely contaminated and six failed to germinate on any media or under any treatment (Fig 6.2; Appendix III). Percentage of spores germinating was always below 1% under all treatments, with the exception of PS4, which reached 1% germination on pH 4 2% MA. Average time for spores to germinate on each medium was consistently *ca.* 40 d with the exception of water agar, on which the single spore print that germinated (PS4) took 105 d (Fig 6.3).

The most successful medium was pH 4 2% MA, on which 23 of 29 spore prints germinated (Fig 6.2). In no instance did spores that failed to germinate on this medium germinate on any other media or under any other treatments. Spores also germinated on agar with unaltered pH and pH 3 agar, although in one case spores spread on all three media only germinated at pH 4.

In the presence of a secondary mycelium or with the addition of charcoal some spores from some fruit bodies failed to germinate, despite having done so on other media (SS1 and HSH10[06], respectively). Six of the seven prints spread on pH 4

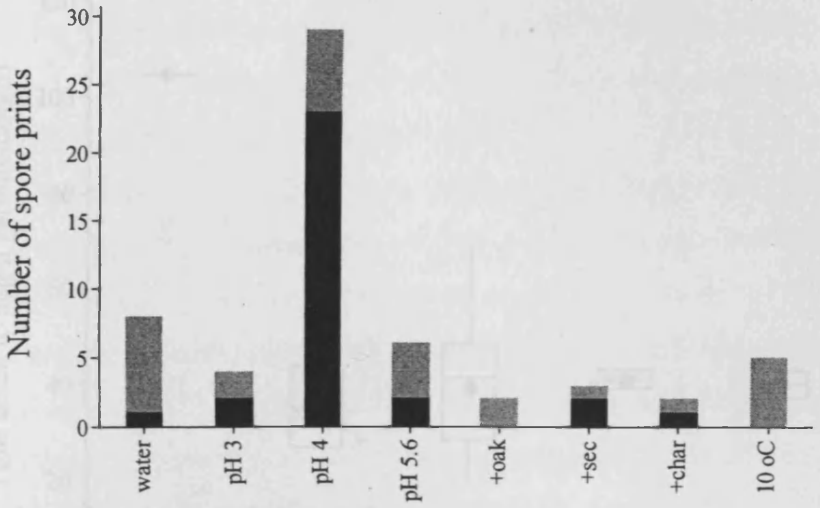


Figure 6.2 Number of *Piptoporus quercinus* spore prints that germinated under different conditions
Black bars, spore prints that germinated; grey bars, spore prints that did not germinate. "spore print" refers to spores collected from a single fruit body; not all spore prints from a single fruit body were spread on all media. Unless stated otherwise, all spores incubated at 20 °C. Water, pH 4 water agar; pH 3/4/5.6, 2% MA adjusted to pH 3, 4 and unadjusted (respectively); + oak, oak extract 2% MA; + sec, with secondary mycelium present; + char, 2% MA with activated charcoal; 10 °C, spores incubated at 10 °C; for full details see text.

water agar failed to germinate: one also failed to germinate under any other conditions (SF6), but five germinated on pH 4 2% MA (BR6[06], PS5, SMa1ii, SF15[06], POWP6[06]); the single print that germinated took 105 d (PS4). Spores never germinated on oak extract agar or when incubated at 10 °C, although all spores spread on the former medium failed to germinate under any conditions (PS1, PS2); those that failed to germinate at 10 °C had germinated under other conditions (HSH10[06], BR6[06], SMa1ii).

Days taken to germinate increased with age of spore print (Table 6.2), but not predictably. For example, spores from POWP1[05] and HSH2[05] took 49 and 19 d (respectively) to germinate when fresh, but 57 d when re-spread at 13 weeks. The single print spread at 24 weeks (HSH10[06]) failed to germinate. The exception to the positive correlation between spore age and days to germinate was PS4, which germinated at 35 d when fresh and 26 d when re-spread 30 weeks later.

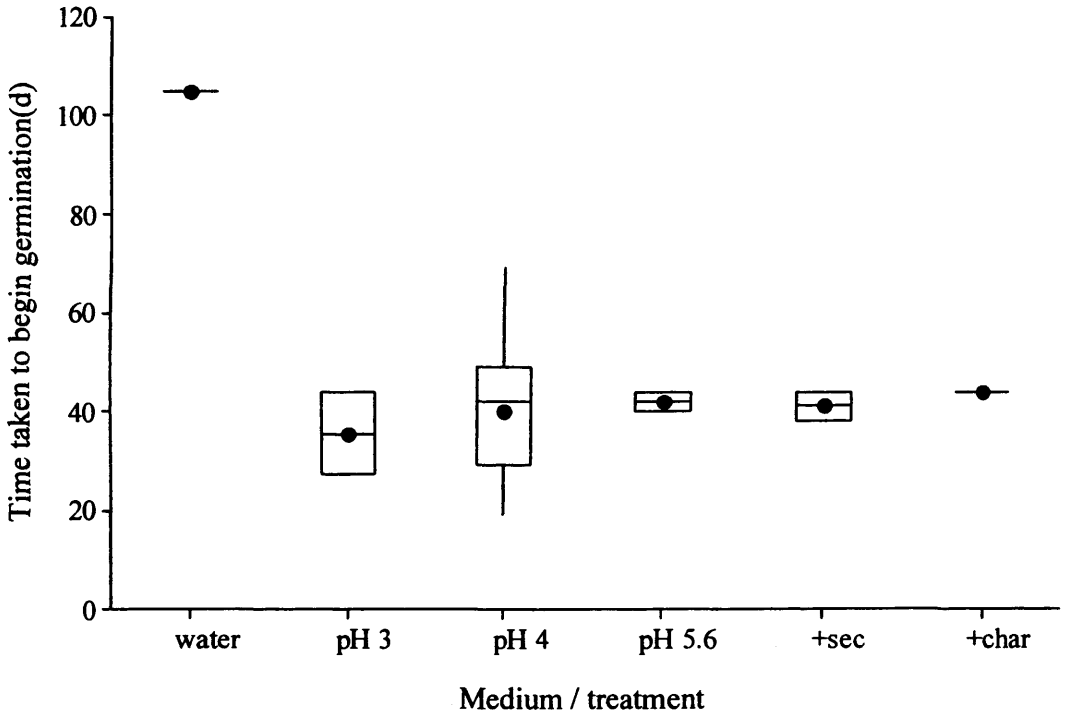


Figure 6.3 Germination time (d) for spore prints of *Piptoporus quercinus*
Whiskers = data range; boxes = 1st quartile, median, 4th quartile; • = median; details of media / treatments: see legend for Fig 6.2. Categories without boxes had only one spore print that germinated on that media, therefore the line represents the single result.

Table 6.2 Germination of *Piptoporus quercinus* spores after storage for 0-30 weeks

	Isolate				
Spore age (wk)	POWP1[05]	HSH2[05]	POWP6[06]	HSH10[06]	PS4
0	49	19	61	29	35
13	57	57			
20			88		
24				-	
30					26

Figures in table 6.2 are days taken to reach germination following spreading at given number of weeks; -, no germination.

6.3.2 Mating system

Sexual compatibility or incompatibility could not be determined by examining cultures without the aid of a microscope to check for clamp connections, i.e. there

were no macroscopic signs of rejection, such as barrages or pigment production, between incompatible primary mycelia. Unstable secondary mycelia were never found, i.e. initial observations of clamp connections were always positively confirmed when rechecked following subculturing.

Almost all pairings between Windsor Great Park (WGP) primary mycelia had 50% compatibility, between both siblings and non siblings (Table 6.3). The exception to this was HSH10 [05] primary mycelia (Table 6.4), which had 17% compatibility between the eight sibling primary mycelia, secondary mycelia being formed only between sibling 27 and other siblings. Compatibility with tester strains was not completely as expected, sibling 27 being incompatible with all four tester strains and sibling 29 being compatible with only one of the four strains.

Table 6.3 Sexual compatibility of *Piptoporus quercinus* sibling and non sibling primary mycelia from Windsor Great Park

	1	2	2	24	20	10	11	5	1	6	5	6	7	8	5	12	2	18	20
20	+	+	+	+	+	+	+	+											\
18	+	+	+	+	+	+	+	+										\	
2		+	+	+		+	+	+									\		
12	+	+	+	+		+	+	+								\			
5		+	+		+		+	+							\				
8	+	+	+	+	+	+	+	+						\					
7	+	+	+	+	+	+	+	+					\						
6	+	+	+	+	+	+	+	+				\							
5	+	+	+	+	+	+	+	+			\								
6	+	+	+	+	+	+	+	+		\									
1	+	+	+	+	+	+	+	+	\										
5	+							\											
11							\												
10						\													
20					\														
24				\															
2		+	\																
2		\																	
1	\																		

Key:
Fruit body HSH12b POWP3b HSH5 POWP1 HSH2

Colour indicates from which fruit body primary mycelia originated; number is arbitrarily assigned number for that primary mycelium. +, stable secondary mycelium found; blank, no clamp connections found; \, control self pairing. Results have been re-arranged to group primary mycelia with the same mating alleles, indicated by black boxes.

Almost all pairings between primary mycelia from outside WGP and the WGP tester strains had 50% compatibility regardless of parental origin (Table 6.5). The exceptions were the two primary mycelia from WW2, which were compatible with

100% of non sibling primary mycelia, including the WGP tester strains. The WW2 primary mycelia were also compatible with each other.

Table 6.4 Sexual compatibility of *Piptoporus quercinus* HSH10[05] primary mycelia against each other and tester strains

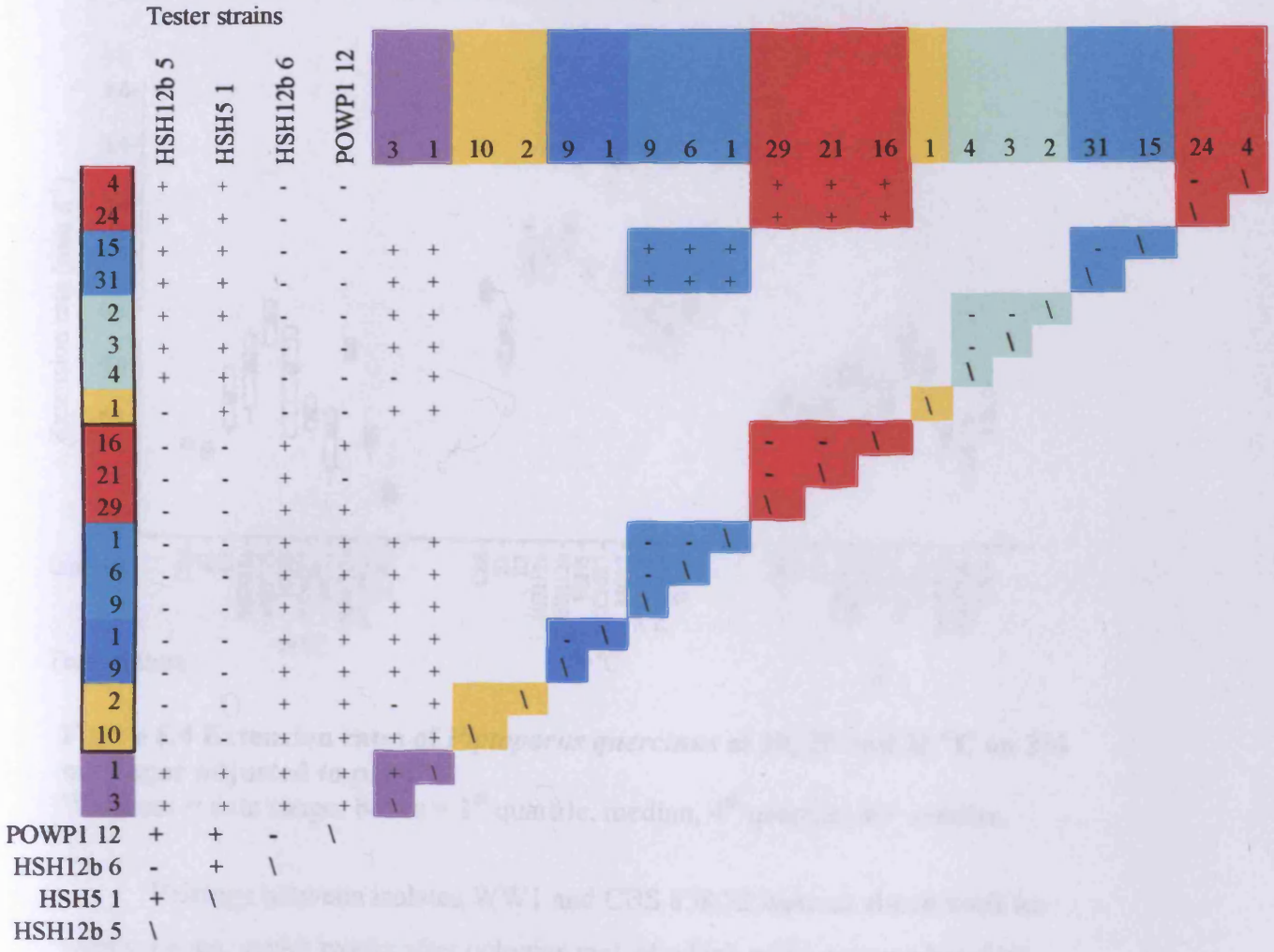
	Tester strains													
	HSH12b 5	HSH5 1	HSH12b 6	POWP1 12	8	9	10	17	19	22	24	25	27	29
29	-	-	+	-									+	\
27	-	-	-	-			+	+	+	+		+	\	
25	-	-	+	+								\		
24											\			
22										\				
19									\					
17	-	-	+	+				\						
10							\							
9						\								
8					\									
POWP1 12				\										
HSH12b 6			\											
HSH5 1		\												
HSH12b 5	\													

+, stable clamp connections found; -, no clamp connections found; \ , control self pairings; blanks, pairing not attempted.

6.3.3 Extension rate

There were significant differences in extension rate between the 11 isolates at all three temperatures (Fig 6.4) (10 °C: Kruskal-Wallis, $p<0.001$; 20 °C, one-way ANOVA, $p<0.001$; 30 °C, one-way ANOVA, $p<0.001$). However, pairwise comparisons of means (at 20 and 30 °C) and medians (10 °C) showed that these significant differences were never between HSH12a and HSH12b or between POWP3a and POWP3b at any temperature. The pairwise comparisons of mean extension rates also showed that there was a greater degree of statistically significant variation between pairs of growth rates at 20 than 30 °C: of the 50 comparisons, 23 were significantly different to each other at 20 °C, compared to just 13 at 30 °C (Appendix IV). At 10 °C only the medians of HSH12a/b and POWP3a/b were compared, as the greater the number of comparisons made the greater the possibility of type I error, i.e. false rejection of the null hypothesis (Bowker and Randerson, 2006). Extension rates of isolates relative to each other were not consistent across the three temperatures. For example, KC1627 was the fastest at 20 and 30 °C, but fifth fastest at 10 °C.

Table 6.5 Sexual compatibility of *Piptoporus quercinus* non-WGP primary mycelia against sibling mycelia and tester strains



Key: PS4 E11 KL1 A2 CA1 WW2

Colour indicates from which fruit body primary mycelia originated; number is arbitrarily assigned number for that primary mycelium. +, stable secondary mycelium found; -, no clamp connections found; \ , control self pairings; blanks, pairing not attempted. Results have been re-arranged so that primary mycelia with the same mating allele are grouped together, indicated by black boxes. Note that WW2 primary mycelia are compatible with all four tester strains as well as almost all other primary mycelia, whereas primary mycelia from other fruit bodies are generally compatible with only two of the four tester strains.

6.3.4 Somatic compatibility

Initial pairings between isolates from the same substrate and the six other isolates showed no signs of non-contact inhibition and met in the centre of the plate approximately three weeks after inoculation. No indications of somatic incompatibility developed over the following seven weeks, experimental and control

plates being indistinguishable from each other beyond slight variations in mycelial morphology, plates were thus discarded.

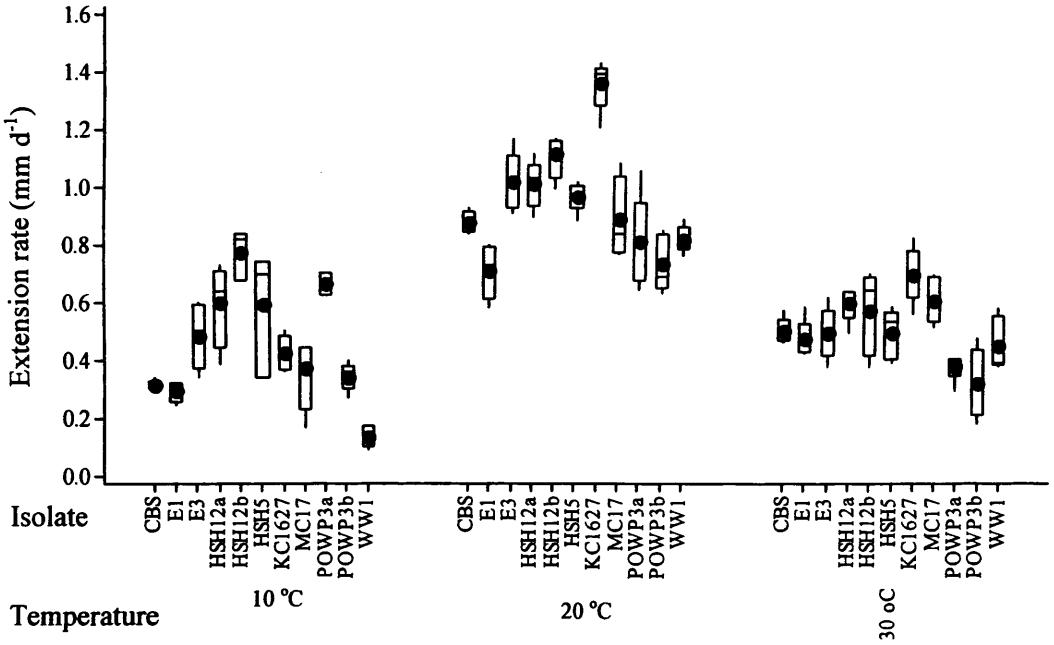


Figure 6.4 Extension rates of *Piptoporus quercinus* at 10, 20 and 30 °C on 2% malt agar adjusted to pH 4
Whiskers = data range; boxes = 1st quartile, median, 4th quartile; • = median.

Pairings between isolates WW1 and CBS 858.72 were as above until ten weeks, i.e. ca. seven weeks after colonies met, at which point a strong band of pigment accompanied by a barrage developed at the interaction zone in all experimental pairings with WW1 (strong somatic incompatibility reaction; Fig 6.5a); there was no pigment or barrage in WW1 control pairings or any pairings with CBS 858.72. The experiment was extended, and at 14 weeks a very narrow band of denser mycelium formed at the interaction zone on experimental pairings between CBS 858.72 and all other isolates except E3 (weak somatic incompatibility reaction; Fig 6.5d). Although the weak reaction became slightly more pronounced by 26 weeks, it did not approach the level of pigmentation seen in reactions with WW1.

15 weeks after pairing, i.e. ca. 12 weeks after having met, there were no signs of somatic incompatibility between CA1 and isolates against it was paired, or between isolates originating from the same substrate, the latter experiments having been repeated in light of delayed somatic incompatibility reactions of CBS 858.72. Strong somatic incompatibility, as described above, was seen on WW1 vs CA1 at ten weeks.

Table 6.6 Outcomes of somatic compatibility experiments between isolates of *Piptoporus quercinus*

Isolate	BR1a [04]	BR1b [04]	CA1	CBS 858.72	E3 [05]	HSH5 [05]	HSH12a [05]	HSH12b [05]	KC1627	MC17 [04]	POWP3a [05]	POWP3b [05]	WW1
WW1	++	++	++	++	++	++	++	++	++	++	++	++	-
POWP3b [05]	-[a]	-[a]		+	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[b]	-	
POWP3a [05]	-[a]	-[a]		+	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-		
MC17 [04]	-[a]	-[a]		+	-[a]	-[a]	-[a]	-[a]	-[a]	-			
KC1627	-[a]	-[a]		+	-[a]	-[a]	-[a]	-[a]	-				
HSH12b [05]	-[a]	-[a]	-[b]	+	-[a]	-[a]	-[b]	-					
HSH12a [05]	-[a]	-[a]	-[b]	+	-[a]	-[a]	-						
HSH5 [05]	-[a]	-[a]		+	-[a]	-							
E3 [05]	-[a]	-[a]		-	-								
CBS 858.72	+	+	-[b]	-									
CA1	-[b]	-[b]	-										
BR1b [04]	-[b]	-											
BR1a [04]	-												

[a]results at 10 weeks; [b]results at 15 weeks; all other results 26 weeks. -, no evidence of somatic incompatibility; +, weak somatic incompatibility reaction; ++, strong somatic incompatibility reaction; see Figs 6.5 a-d.

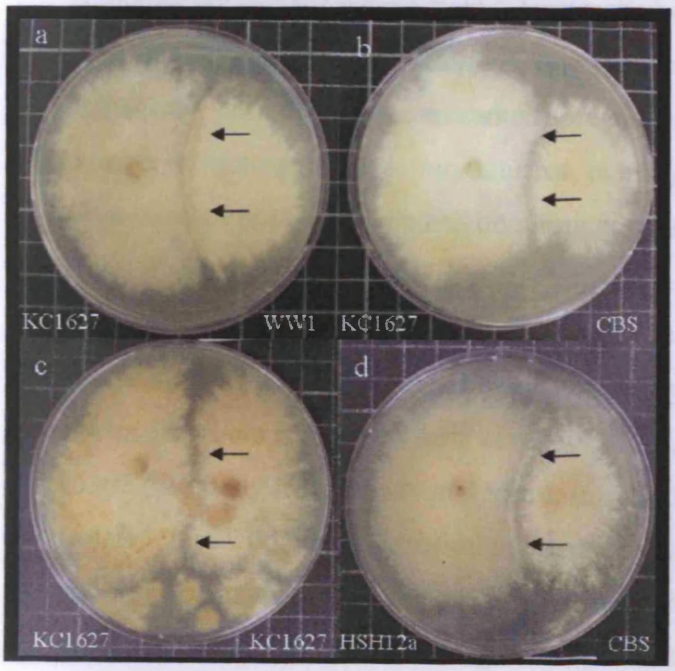


Figure 6.5 Examples of somatic incompatibility reactions between isolates of *Piptoporus quercinus* at 14 and 26 weeks
a, strong somatic incompatibility (14 weeks); b, no somatic incompatibility reaction (14 weeks); c, control, no somatic incompatibility reaction (26 weeks); d, weak somatic incompatibility (26 weeks). Arrows indicate interaction zone where indications of somatic incompatibility or the lack of it can be seen. Note that at 14 weeks pigment is clearly visible at the interaction zone between KC1627 and WW1, but no such indications of somatic incompatibility exist between KC1627 and CBS 858.72.

6.4 Discussion

Piptoporus quercinus has a unifactorial mating system, but only four mating alleles in the UK. The population is not clonal, as there are significant differences in extension rate between isolates, and somatic incompatibility was seen between 12 of the 13 FBT isolates tested. Lack of significant differences in extension rate between pairs of FBT isolates from the same tree implies that they are genetically identical, i.e. originate from single mycelia, but at present, somatic compatibility experiments which would support this are inconclusive.

The first hypothesis, that *P. quercinus* has a unifactorial mating system, is therefore accepted. This is the second most common mating system in homobasidiomycetes, being found in approximately 25% of species (Esser, 1967, based on analysis of mating systems of 335 species, cited in Hibbett and Donoghue, 2001), including the only other species in the genus, *P. betulinus* (Cant, 1980; Adams, 1982). As a brown rot species with a unifactorial mating system, *P. quercinus* fits the trend noted that these two features frequently occur together (Hibbett and Donoghue, 2001).

The second hypothesis, that inbreeding would be detectable in the UK population of *P. quercinus*, is accepted with reservations. The extremely low number of mating alleles (four) discovered implies inbreeding, but a degree of variability exists, as evidenced by the significant differences in extension rate at all three temperatures, and the somatic incompatibility between isolates from around the UK. It is interesting that the isolate from Germany had similar somatic incompatibility reactions against all UK isolates, whereas the Wentwood FBT isolate behaved differently, with a much more marked somatic incompatibility reaction. The low number of mating alleles is very unusual, more typical numbers being 81-83 for *Ganoderma boninense* from neighbouring populations in Papua New Guinea (Pilotti *et al.*, 2003), 33 in sampled UK populations of *P. betulinus* (Cant, 1980), and ca. 1000 for the entire species of *Heterobasidion annosum* (Ullrich and Raper, 1974). The distribution of the four mating alleles is intriguing: two alleles were ubiquitous in twelve fruit bodies sampled from seven locations, a further two being found in the single fruit body from Wentwood forest. In summary, there are a number of small, isolated populations with identical alleles at the single locus examined, but that are not clonal, and another single individual that has different alleles. Generally, small, isolated populations will be homogenous, with genetic variation detectable at a

regional scale, while large, continuous populations have greater variation within, but less between populations (Stenlid and Gustafsson, 2001). Clearly *P. quercinus* does not easily fit either of these scenarios.

The third hypothesis, that fruit bodies on the same tree are produced by the same mycelium cannot be accepted or rejected based on current results. Although pairs of FBT isolates never had significantly different extension rates, there were other isolates for which this was also the case. There were no signs of somatic incompatibility between isolates from the same substratum by 15 weeks, but neither were there clear somatic incompatibility reactions between CA1 and other isolates. At present, insufficient pairings have been made to decide whether weak, strong, or no somatic incompatibility reaction is "normal", hence conclusions cannot be drawn as to whether fruit bodies on the same tree are produced by a single mycelium. Further experiments pairing more isolates for longer periods of time are urgently required.

There is a trend for early colonisers to have few individuals within a substrate, compared to more combative secondary colonisers (L. Boddy, pers. comm.). For example, in general only one individual of the primary colonisers *P. betulinus* (Adams, 1982), *Stereum gausapatum* (Boddy and Rayner, 1982), or *Heterobasidion annosum* (Redfern *et al.*, 2001) was isolated from a substratum, whereas secondary colonisers such as *Trametes versicolor* and *Stereum hirsutum* often exist in populations with many individuals in a single substrate (Rayner and Todd, 1978). Evidence implies that *P. quercinus* is one of the former, a stress tolerant early coloniser (Wald *et al.*, 2004a), so would be expected to have few genetically different individuals within a single substrate. Although the present findings do not definitely support this, neither do they disprove it. It should be noted that this is based purely on fruit body occurrence, and true mycelial distribution has to date not been investigated.

As mentioned previously, the population structure of *P. quercinus* as shown here is difficult to account for. There is a consistent lack of diversity in mating alleles between the sampled populations (excluding Wentwood), and although such homogeneity usually results from gene flow between populations, this is not always the case (Stenlid and Gustafsson, 2001). This study has investigated population diversity using indirect measures of gene flow, i.e. a picture of historical, rather than present gene flow has been created. At present nothing is known of direct gene flow in the species, which could be measured by spore dispersal (Stenlid and Gustafsson,

2001). A combination of direct and indirect measures would allow a clearer picture of historical and potential gene flow between populations.

It is highly likely that some *P. quercinus* spores would be dispersed over the distances between the populations sampled (maximum is ca. 220 km, between Calke Abbey and Kingston Lacey); although few spores travel further than 100 m from the fruit body (Kallio, 1970; Stenlid, 1994; Nordén and Larsson, 2000), they can travel up to 1000 km (Hallenberg and Küffer, 2001). However, spore dispersal must be followed by germination and mating in order to be translated into gene flow. In contrast to *P. betulinus*, spores of which germinate within 72 hr (Cant, 1980), *P. quercinus* has very poor germination, the range of conditions assayed never increasing percentage germination nor decreasing time taken to germination. That spores from four out of five fruit bodies germinated following storage implies that even if conditions are not appropriate immediately upon arrival at a new substrate, germination could occur at a later date. It is unclear whether the extremely low percentage of spores germinating is due to low spore viability, or to favourable conditions not having been found. Condition and age of the fruit body when spores were sampled could also be a factor (Schmidt and French, 1983), which it was not possible to investigate in the present study. Interestingly, fruit bodies of the rare polypore *Fomitopsis rosea* from areas with a long history of forest fragmentation generally released sterile spores, whereas those from large old-growth forests, which also had higher levels of heterozygosity, were viable (Högberg, 1998, cited in Högberg and Stenlid, 1999).

The isolate from Wentwood is of particular interest, both FBT isolate and primary mycelia exhibiting considerable differences to all other fruit bodies sampled. Its mating alleles were unique to this location, and the FBT isolate had a markedly different somatic incompatibility reaction to all other FBT isolates, including the single non-UK isolate, which was widely separated from all other samples both geographically (being from Germany) and temporally (isolated in 1975). The Curley Oak, host to Wentwood's *P. quercinus* population, is one of the few remaining deciduous trees from what was once an ancient forest, that was converted to conifer plantation between late 19th and mid 20th century. This is an unfortunate location for what could be an extremely important reservoir of genetic diversity, as not only will long distance spore dispersal be hampered by the densely surrounding conifers, but there are virtually no suitable substrates in the immediate neighbourhood. It is hoped

that the diversity encapsulated within the Wentwood *P. quercinus* is not in a genetic blind alley.

Unfortunately, this study is based on a very small number of samples, particularly of primary mycelia. Due to the species' rarity, there has been a dearth of material, and obtaining primary mycelia from these few samples has been additionally hampered by lack of, or extremely low, spore germination, making finding well-spaced germinating spores an extremely labour intensive activity. Germlings transferred to fresh media often failed to grow, further diminishing sample sizes. It is hoped that studies into spore viability will show whether it is worth searching for a better medium for spore germination, and that molecular studies using micro satellite markers will be able to continue the investigations into population structure and variability using the existing culture collection that will hopefully continue its expansion. Tantalising facts have been discovered about the UK population of *P. quercinus*, and this is very much the beginning of a study rather than the end.

Chapter 7: Synthesis

This is one of the few studies to focus on rare wood decay basidiomycetes, and has substantially increased knowledge of the ecology and lifecycle of *Hericium cirrhatum, H. coralloides, H. erinaceus* and *Piptoporus quercinus*. Discussed below are the extent to which the studies have met objectives outlined in Chapter 1, whether any of the aspects investigated can account for the species' rarity, implications for conservation strategies and future research priorities.

Basidiospore dispersal of *Hericium* spp. (Objective 1)

Spore dispersal of *Hericium* spp. was typical of other wood decay basidiomycetes previously investigated, spore deposition decreasing logarithmically at distances up to 100 m from the fruit body (Kallio, 1970; Stenlid, 1994; Nordén and Larsson, 2000; Chapter 2). Evidence of spore deposition was not found at distances greater than 100 m, but given the relatively short time for which traps were exposed, this is perhaps unsurprising. If spore dispersal over longer distances is also typical of other wood decay basidiomycetes, then dispersal ability considered in isolation can be discarded as a cause for their rarity.

Assuming *Hericium* spp. to have typical spore dispersal patterns over long as well as short distances, they are likely to face the challenges outlined in Chapter 1 for this stage of the lifecycle. The first challenge is the haphazard nature of wind dispersal, meaning that a spore is unable to regulate its landing, which can be a particular problem if a spore has specific requirements for germination. A second challenge relates to short distance dispersal: as most spores fall within a small distance of the fruit body, inbreeding is likely to be an issue for rare species with small, widely spread populations of fruit bodies, in which there will be few non-sibling spores in the neighbourhood of a fruit body. Inbreeding was not directly investigated for *Hericium* spp., but unlike *P. quercinus*, no repetition of mating alleles was found for any species except possibly in *H. coralloides* and *H. erinaceus* fruit bodies from the same tree. Over greater distances such as tens or hundreds of miles, it is probable that only an extremely small fraction of spores will reach these distances and germinate successfully. However, even an extremely low amount of spore dispersal between populations may be sufficient to maintain gene flow between

populations and prevent inbreeding (Stenlid and Gustafsson, 2001), as just one immigrant per generation is sufficient to counteract the effects of inbreeding (Slatkin, 1987).

Spore dispersal over longer distances should be investigated to ascertain the potential for gene flow between populations, which in the UK are generally separated by distances in the region of hundreds of miles. Such experiments have previously used wood discs colonised with primary mycelium placed at distances 500 m to 100 km from known spore sources for up to two weeks (Edman and Gustafsson, 2003; Edman *et al.*, 2004a). Traps must be exposed for longer periods of time when investigating long distance dispersal, as only a very few spores are expected to travel distances greater than 1 km (Stenlid, 1994). Wood discs are preferred to malt agar as the former is a much less rich medium, therefore it has the advantage of being less prone to contamination and can be left *in situ* for the longer periods of time appropriate for studies into long distance spore dispersal (Edman and Gustafsson, 2003).

Until such experiments have been conducted full conclusions cannot be drawn, but it is expected from results on short distance spore dispersal that this stage of the lifecycle does not cause their rarity. However, as a bottleneck in the lifecycle of any basidiomycete, it may exacerbate their rarity, fewer fruit bodies implying fewer spores in the air, thus fewer opportunities for colonisation of new habitats or gene flow between existing populations. Similar conclusions have been reached for the *Phlebia centrifuga* and *Fomitopsis rosea,* rare wood decay basidiomycetes found in old growth boreal forest, for which low spore deposition and low germinability are cited as factors that may be a threat to the long-term persistence of the species in isolated populations (Edman *et al.*, 2004a).

Spore germination of *Hericium* spp. and *P. quercinus* (Objective 2)

The consistently low spore germination (less than 1%) of *Hericium* spp. (Chapter 2) and *P. quercinus* (Chapter 6) is not without precedent for basidiomycetes (see refs in Merrill, 1970). However, as percentage germination of spores from one fruit body of *H. erinaceus* increased five fold in the presence of a yeast contaminant, it may be that viability is higher than germination, and the appropriate medium or trigger has not yet been found. This hypothesis is supported by the often high percentages of spore trap wells with clamp connections for all three *Hericium* spp., implying that spore

germination was higher in these traps than under the laboratory treatments. There are no clues as to the spore viability of *P. quercinus*, as no treatments had any effect on percentage germination and spore trapping was not attempted. An investigation into the viability of all four species is essential before conclusions can be drawn as to whether spore germination is a contributing factor in their rarity.

Inbreeding as a result of habitat fragmentation has been cited as a possible reason for decreased germinability of spores of *F. rosea* and *Ph. centrifuga* in fragmented compared to continuous forests (Edman *et al.*, 2004a). It is possible that such factors could account for low germinability of both *P. quercinus* and *Hericium* spp. spores, but in the absence of populations in continuous habitat as a comparison, this cannot be proven.

That spores of *H. coralloides, H. erinaceus* and *P. quercinus* maintained germinability over timescales of up to 24 and 30 weeks, respectively, indicates that if an unsuitable habitat was reached spores could remain dormant, germinating subsequently if conditions improved. Chlamydospores produced by *P. quercinus* consistently had germination above 10%, and germinated readily following exposure to high and low temperatures and desiccation for up to 14 d, although percentage germination decreased and time taken to germinate increased compared to controls (A. Campbell, unpublished). The substrata colonised by *P. quercinus* are often subject to such extremes of temperature and desiccation (Roberts, 2002), under which mycelia of *P. quercinus* would be unable to survive (Wald *et al.*, 2004a). Production of chlamydospores, able to germinate following such extremes, may therefore enable *P. quercinus* to survive adverse conditions and re-colonise the substratum when microclimatic conditions become favourable. *Schizopora paradoxa* also produces chlamydospores (Rayner and Boddy, 1988) and is stress-tolerant but is a poor combatant (Boddy and Rayner, 1983c), chlamydospores perhaps enabling it to survive adverse conditions (L. Boddy, pers. comm.), as is hypothesised for *P. quercinus*. Chlamydospores may play a substantial role in the lifecycle of *P. quercinus*, enhancing its stress-tolerant characteristics and enabling it to inhabit substrata in which other fungi are unable to survive.

Relative significance of primary mycelium in the lifecycle of *H. coralloides* (Objective 3)

Primary mycelia had extension rates at least equal to those of secondary mycelia, and were more successful combatants against a range of wood decay species (Chapter 3). This agrees with the theory that primary mycelia may play a particularly significant role in the lifecycle of rare species compared to common species, as it is likely that encountering a sexually compatible mate will take longer for the former. Relative fitness of primary mycelium is discarded as a contributing factor to the rarity of *H. coralloides*. A primary mycelium able to successfully survive is important to a rare species, as the longer it survives, the greater the chance it has of encountering a mate, thus becoming a secondary mycelium with associated possibility of sexual reproduction.

This study is one of the first to examine relative fitness of primary and secondary mycelia in an ecological context, therefore it is difficult to draw comparisons, or extrapolate to say whether primary mycelia of the other *Hericium* spp. and *P. quercinus* might behave in a similar fashion. As one of the first such studies, it is hoped that the ecology of primary mycelia, particularly for rare species, will in the future receive greater attention.

Although it has been shown that primary mycelia of *H. coralloides* have the potential to survive as well as secondary mycelia, the persistence and relative abundance of the two under natural conditions is unknown. A survey of substrata appropriate as *Hericium* spp. habitats using species specific primers could potentially yield this information, if it could be ascertained from extracted DNA whether the individual was a primary or secondary mycelium. This can be done by investigating the degree of heterozygosity at known polymorphic loci (e.g. de Fine Licht *et al.*, 2005). At present, this survey would only be feasible for *H. cirrhatum*, as primers that distinguish between *H. coralloides* and *H. erinaceus* are yet to be developed. A survey as described combined with studies on long distance spore dispersal of these species (see above) is essential to an understanding of potential population sizes and locations of these species, but can only be achieved if species specific primers for *H. coralloides* and *H. erinaceus* are developed.

Mating systems of *Hericium* spp. and *P. quercinus* (Objective 4)

H. coralloides and *H. erinaceus* had bifactorial mating systems (Chapter 4), confirming previous research in North America (Hallenberg, 1983; Ginns, 1985). The mating system of *H. cirrhatum* could not be elucidated, due to extreme variation in frequency of clamp connections of highly variable appearance. Incidental discoveries associated with discovery of mating system are perhaps of greater significance to their conservation, particularly the fact that *H. coralloides* from ash and beech can interbreed. If the populations were inter-sterile this would present difficult challenges for conservationists; as it is, conservation efforts do not have to be divided between two groups, but can be focussed on the species as a whole.

The mating system of *P. quercinus* is unifactorial (Chapter 6), as is that of *P. betulinus*, the only other species in the genus (Cant, 1980; Adams, 1981). Unexpected discoveries again proved extremely interesting, there being only four mating alleles in the seven *P. quercinus* populations sampled, two of which were unique to a single fruit body (WW1). The Wentwood isolate also had an unusual reaction in somatic compatibility experiments: in pairings with all other FBT isolates the somatic incompatibility reaction occurred sooner and was more marked than in pairings of any other FBT isolates. Possessing unique mating alleles and having such a different somatic compatibility reaction, the isolate from Wentwood is clearly very different to the rest of the population. Although an unusual population structure clearly exists in the UK, given the extremely small number of samples it would be unwise to draw conclusions as to the entire UK population at present. Further research is required in order to understand the population structure of *P. quercinus*, and whether the unusual distribution and low number of mating alleles so far encountered is representative of the diversity or lack of it over the entire genome. This could be achieved by a survey of genetic diversity within UK populations using microsatellites, which have been successfully used with fungi for similar applications (e.g. Bergemann *et al.*, 2006). A detailed knowledge of population structure within the UK would enable development of informed strategies as to which habitats and locations should be protected to best conserve the species.

Are fruit bodies of *Hericium* spp. or *P. quercinus* occurring simultaneously on the same tree are produced by a single mycelium? (Objective 5)

From somatic compatibility experiments which highlighted differences and similarities in colony morphology, it appears that *H. coralloides* can produce multiple fruit bodies on the same tree (Chapter 4). This was supported by fruit bodies on the same tree sharing mating alleles. *H. erinaceus* somatic compatibility experiments were inconclusive, but fruit bodies on the same tree may share mating alleles; it has not been disproved that *H. erinaceus* can produce more than one fruit body from a single mycelium. These experiments were not relevant to *H. cirrhatum*, as fruit bodies always occurred singly.

P. quercinus may also produce more than one fruit body on the same substratum (Chapter 6), but evidence for this is sparse, and further investigations are required in order to accept or reject the hypothesis that a single mycelium can produce multiple fruit bodies. That extension rates of fruit body isolates from the same substrate were never significantly different provides some proof that they were produced by a single mycelium, but unfortunately somatic compatibility experiments were inconclusive. Portions of the latter experiments urgently require repeating: initial pairings that were discarded after ten weeks must be repeated and the experiment allowed to run for the full 26 weeks to ascertain whether a delayed somatic incompatibility reaction occurs, as was noted in later experiments.

Although fruit bodies of these species on the same tree are likely to be produced by a single mycelium, simultaneous occupancy of a substratum by conspecific individuals cannot be ruled out for *Hericium* spp. or *P. quercinus*. This is an important area of study for at least two reasons: firstly, if more than one conspecific individual can exist in a substratum then the potential number of individuals a habitat can support is increased; and secondly, the relationship between size of vegetative mycelium and fruiting is unknown. However, ascertaining the volume of wood occupied by an individual would be extremely difficult to investigate without using destructive methods, which would be undesirable with such rare species.

Objective 6: Investigate artificial establishment of *H. coralloides* in living beech

H. coralloides was successfully established in one of four trees sampled, found up to 19 cm from the inoculation point. This compares poorly with its establishment in

freshly felled logs (Boddy *et al.*, 2004), which is to be expected, the functional wood of living trees presenting a much harsher environment for fungal growth than non-functional wood (Boddy and Rayner, 1983b). Given the relatively poor establishment of *H. coralloides* in living trees, if additional populations were created for conservation purposes it might be more effective to colonise dead wood than living trees.

Establishment is an important stage in the lifecycle, without which spore dispersal and germination cannot result in ultimate success. These experiments have shown that within living trees establishment is uncertain even from a relatively large inoculum. It seems doubtful whether a spore, which has much smaller nutritional reserves, would be able to colonise such a substratum. However, *H. coralloides / H. erinaceus* has been found latently present in several host species using the primers described in Chapter 5 (Parfitt *et al.*, in prep.), and it is possible that development from latent propagules when microclimatic conditions are appropriate is one of the ways in which these species become established in new substrata.

Research priorities

Objectives of the study have been met, and several possibilities excluded as reasons for these species rarity, but in the light of this knowledge new questions have arisen that must be answered. However, it is felt that continuing investigations should be addressed from a different angle. Rather than asking why these species are rare, the focus should be on whether they are actually rare, i.e. are mycelia as rare as fruit bodies, what causes fruit bodies initiation, and what genetic diversity exists within and between UK populations. Genetic diversity of *P. quercinus* is of particular interest given the unusual distribution of mating alleles in the population studied so far.

Mycelial distribution of these species remains entirely unknown, thus rarity of all four is based solely on fruit body occurrence. Although only individuals that produce fruit bodies will contribute to the effective population size, mycelial distribution represents the potential effective population (Burnett, 2003). This leads to the question of what causes fruiting, which again has not been investigated, except for *H. erinaceus* in commercial settings, which can be induced to fruit by a sudden alteration in gaseous regime (Stamets, 2000). Such alterations in gaseous regime could occur in nature as a result of circumstances such as wounding or a branch

falling, suddenly exposing the inner wood to the atmosphere, and may lead to fruit body production if a mycelium is present. There is some evidence from conservation efforts in Epping Forest that haloing oak trees, i.e. clearing undergrowth from around the base of trees, promotes fruiting of *P. quercinus* (Ainsworth, 2006). Removal of undergrowth decreases shading and allows rainfall and dewfall to reach the tree, leading to increased warmth and moisture, thought to be required for initiation of fruiting (Ainsworth, 2006).

The frequency of fruiting for the four species is intriguing, and presents challenges population surveyors. *H. erinaceus*, which commonly fruits regularly and over a long period of time on the same substrate (Marren and Dickson, 2000), will be easier to survey for in known habitats than *H. cirrhatum*, which generally appears only once on a substrate (Marren and Dickson, 2000; Ainsworth, 2008). How long *H. cirrhatum* inhabits a substrate before fruiting, what causes it to fruit, and what happens to the mycelium following fruiting are important questions. *P. quercinus* presents slightly different challenges: as it fruits earlier in the year than most species, and is of unspectacular appearance, it may be missed on fungal forays and thus under recorded.

Given that *P. quercinus* fruits on veteran oak trees, timescale is particularly interesting for the species. It is a suspected primary coloniser exhibiting stress-tolerant characteristics (Wald *et al.*, 2004a), and could be growing, or surviving as chlamydospores, within oak for long periods of time, potentially up to hundreds of years. Pentillä *et al.* (2006) highlight the lag that may exist for long-lived species, such as wood decay basidiomycetes, between current actions and a noticeable effect on the populations. This has particular relevance to *P. quercinus* whose hosts (veteran oaks) may have a lifespan of several hundred years. Hence future studies on the population of *P. quercinus* should take historical distribution of woodland and oaks into account when interpreting findings.

This brings us to the important question of whether *P. quercinus* is found in oaks of all ages, but merely fruits on older trees. Species specific primers have been designed with this end in mind (D. Parfitt, unpublished), and work is currently underway to isolate DNA of sufficient quality for PCR from oak heartwood, a difficult task due to extractives present within the wood (H. Rogers, pers. comm.). When protocols have been optimised, a survey of known host trees, likely host trees within and outside known populations and younger oaks would reveal the mycelial

distribution of *P. quercinus* geographically and in terms of host preference. The exposed heartwood of veteran oaks on which *P. quercinus* fruits could be easily sampled without damage to the tree; samples from younger trees could be obtained during routine management operations.

Similar investigations would also be appropriate for *Hericium* spp., although unless primers can be developed that distinguish between *H. coralloides* and *H. erinaceus* it would not be possible for these species.

Conservation strategies

If the research priorities outlined above were addressed, informed decisions could be made regarding where conservation efforts should be focussed in terms of what specific locations and habitat types to protect in order to benefit the UK population of each species as a whole.

An integrative approach to conservation is essential. Given present knowledge, the best conservation strategy may be simply to protect existing and potential habitats, and increase awareness and knowledge of the general public with regard to fungi as a whole, and these rare species in particular.

Protection of the habitats in which these species occur, wood pasture or parkland for *P. quercinus*, and old-growth woodland for *Hericium* spp., which are in themselves of conservation interest, would protect suitable substrata for *Hericium* spp. and *P. quercinus*. Existing and potential habitats for these species are under the ownership / management of a range of people and organisations, including charities such as the National Trust, private landowners, the Crown Estate, and local councils. In order to effectively protect suitable habitats, and thus the species that rely on them, it is essential to ensure that stakeholders have easy access to information regarding location and ecology of these species. In order to lift the profile of these species in particular and rare fungi in general, *Hericium* spp. (being more charismatic than *P. quercinus*) could become a flagship species for conservation of wood decay fungi, as have waxcaps (*Hygrocybe* spp.) for grassland fungi. A greater awareness of fungi would hopefully engender interest in, and a wish to preserve and protect, what at present is unfortunately associated by the general public with "darkness, decay and death" (Heilmann-Clausen and Vesterholt, 2008).

Specifically, land managers should be encouraged to: protect substrata on which these species are known to fruit; check known substrata during the fruiting

season for presence or absence of fruit bodies; wherever practical leave CWD *in situ* in order to protect possible current and / or future habitats; create haloes (see above) around veteran oak trees, snags or fallen limbs to encourage fruiting of *P. quercinus* (Ainsworth, 2006). By encouraging landowners to take these relatively simple measures, it is hoped that populations of *Hericium* spp. and *P. quercinus* can at least be maintained at current sizes until further research can enable more targeted conservation.

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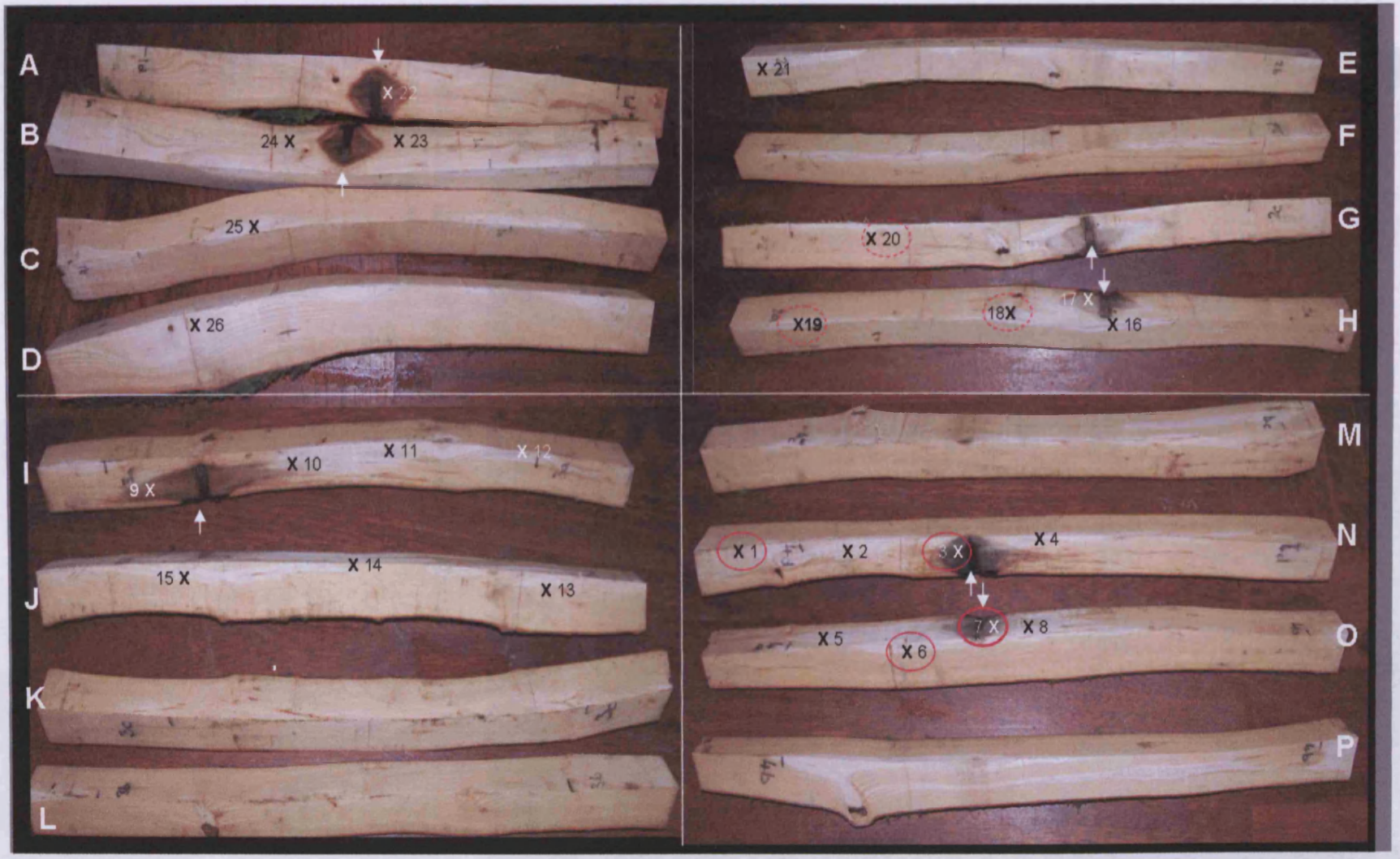
Appendix I: *Hericium erinaceus* fruit body references with corresponding substratum codes from Natural England report

In order to avoid future confusion a list has been compiled of the references of *H. erinaceus* fruit bodies used in the thesis, together with the code assigned to that substratum in the most recent survey of the species, conducted by Natural England (NE) in 2007 (Ainsworth, 2008). The NE report assigns a reference to a substratum rather than fruit body; National grid references sometimes vary slightly between those in the thesis and those in the NE report, due to slight variation in GPS systems or recorder estimates. That the thesis and report are referring to the same substratum / fruit body has been carefully confirmed through personal communication and comparison of site photographs. For details of collectors and collection dates see Tables 2.1 and 4.1.

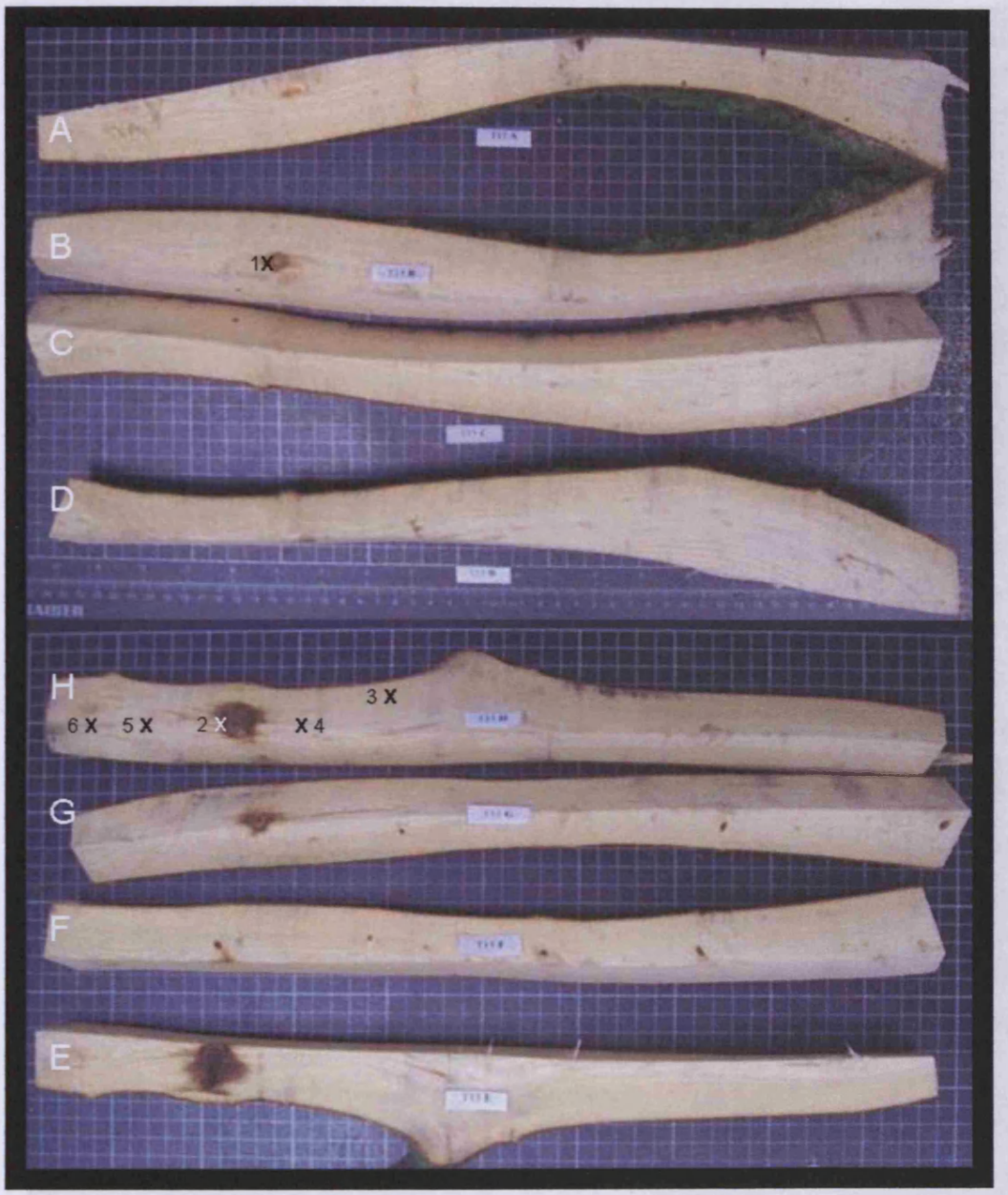
Thesis FB ref	Thesis NG Ref	NE substratum ref	NE NG Ref
AL3	SU2534106669	Wooson's Hill 1	SU25340766
AL4	SU3394305706	Unknown[b]	
Eye1, AL5 AL7[a]	SU227154	Eyeworth 1	SU22521518
Eye2	SU22731493	Eyeworth 2	SU22731492
OckA/B/C	SU246115	Bolderwood Walk	SU24541154
AL6, SW2[a]	SU28921180	Clay Hill 1	SU28971181
SW1a	SU28801193	Clay Hill 2	SU28901188

Thesis FB ref, reference assigned to fruit body in thesis; NG ref (thesis), national grid reference recorded during field work; NE tree reference, Natural England host substratum reference; NG Ref (NE), Natural England national grid reference of host substratum. [a]fruit bodies produced in different years. [b]this isolate cannot be definitely linked to a single NE code, as Denny Wood, the location in which this grid reference falls, has a large number of *H. erinaceus* substrates.

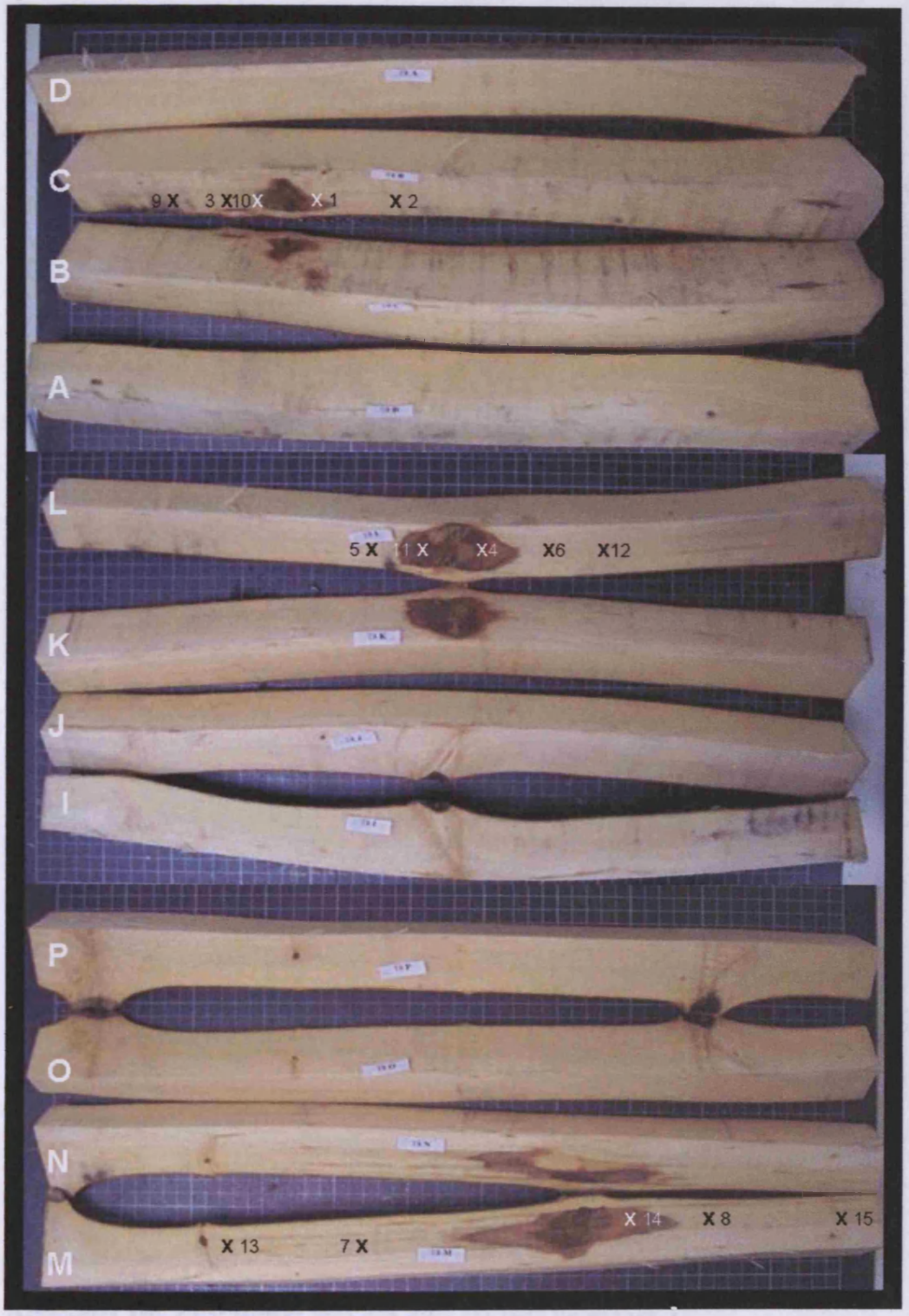


Appendix IIa T4: dowel inocula and sample locations

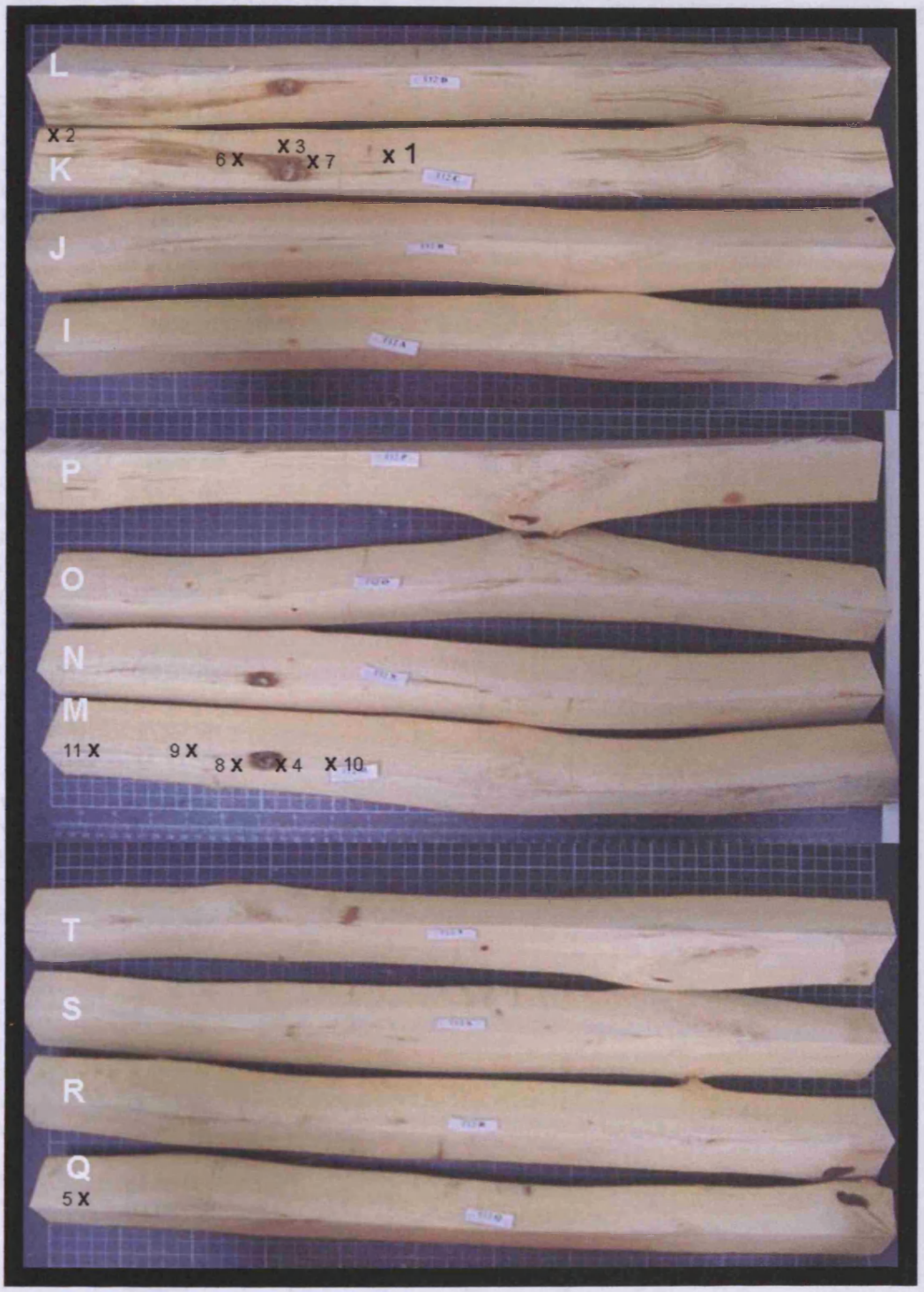
End closer to the ground is on the left; A-D are the lowest sections, M-P the highest; length of each section is approximately 50 cm; X, sample location; arrow, dowel inoculum – if dowel has been cut in half both portions are indicated; solid red circle indicates a presence of *Hericium* (strong band on gel in both PCRs with at least one dilution); dashed red circle indicates possible presence of *Hericium* (weak band on gel in one PCR, none in repeat or at any other dilution).



Appendix IIb T8: dowel inocula and sample locations
Legend as Appendix IIa.



Appendix IIc T12: dowel inocula and sample locations
Legend as Appendix IIa.



Appendix IId T15: dowel inocula and sample locations
Legend as Appendix IIa.

Appendix III: Days taken to reach germination for *Piptoporus quercinus* spores spread on different media

FB	Spread date	water	pH 3	pH 4	pH 5.6	+oak	+ sec	+ char	10 °C
A2	18.07.07			44					
AL2[a]	02.08.05			x					
BR4[06][a]	17.07.06		x	x	x				
BR6[06]	17.07.06	-	44	44	44				-
BR7[05]	19.07.05			37					
C5[06][a]	12.07.06	x	x	x	x				x
C8[06]	04.09.06			x					
CA1	29.08.07			23					
CA2	29.08.07			28					
E11	25.07.07			34					
E2[05]	11.08.05			42			-		
EW1[a]	04.08.06			x					
HSH10[06]	05.07.06		x	29				-	-
HSH12a[05]	19.07.05			49					
HSH12b[05]	19.07.05			49					
HSH2[05]	19.07.05			19					
HSH5[05]	20.07.05			23					
HW1[a]	04.09.06			x					
KL1	14.07.07			45					
KL2	14.07.07			22					
KL3	14.07.07			69					
POWP1[05]	19.07.05			49					
POWP2[06][b]	17.07.06			-					
POWP3[06]	17.07.06	-		45					-
POWP3a[05]	19.07.05								
POWP3b[05]	19.07.05			41					
POWP5[05][a]	11.08.05				x				
POWP6[06]	01.09.06	-	-	61	-				
PS1[b]	05.07.05			-	-	-			
PS2[b]	05.07.05			-	-	-			
PS3[b]	21.07.06		-	-	-				
PS4	21.07.06	105		35					-
PS5	21.07.06	-		54					
SF15[06]	01.08.06	-		43			38		
SF6[06][b]	01.08.06	-		-					
SG1a[06][a]	12.07.06	x	x	x	x				x
SG1b[06][a]	12.07.06	x	x	x	x				x
SMa1ii[06]	26.07.06	-		x			44	44	-
SMa2[05]	11.08.05			x					
SS1	04.08.06		27	x	40				
SW1[b]	04.09.07			-					
WW2	07.08.07			37					
	average	105	36	40	42		41	44	
	SD		12	13	3		4		
	n germ	1	2	23	2	0	2	1	0
	n ungerm	7	2	6	4	2	1	1	5
	n contam	3	5	11	5	0	0	0	3
	total	11	9	40	11	2	3	2	8
	fastest	105	27	19	40		38	44	
	slowest	105	44	69	44		44	44	

Figures in table are days to reach germination; x, all plates contaminated; -, spores failed to germinate; blanks, spores not spread on that media. [a]entire spore print contaminate; [b]spores from fruit body never germinated;. SD, standard deviation; germ, germinated; ungerm, ungerminated. For details of fruit body origins see Table 6.1. For details of media see Ch 6 Materials and methods.

Appendix IV: Significant differences in extension rate of *Piptoporus quercinus* isolates at 20 and 30 °C using Tukey-Kramer *a posteriori* pairwise comparison of means

20 °C

Isolate	CBS	E1	E3	HSH12a	HSH12b	HSH5	KC1627	MC17	POWP3a	POWP3b	WW1
WW1					X		X				\
POWP3b			X	X	X	X	X			\	
POWP3a			X		X		X		\		
MC17					X		X	\			
KC1627	X	X	X	X	X	X	\				
HSH5		X				\					
HSH12b		X			\						
HSH12a	X	X		\							
E3		X	\								
E1		\									
CBS	\										

30 °C

Isolate	CBS	E1	E3	HSH12a	HSH12b	HSH5	KC1627	MC17	POWP3a	POWP3b	WW1
WW1							X				\
POWP3b				X	X		X	X		\	
POWP3a				X	X		X	X	\		
MC17								\			
KC1627	X	X	X			X	\				
HSH5						\					
HSH12b					\						
HSH12a				\							
E3			\								
E1		\									
CBS	\										

Statistical analyses were carried out using Minitab 13. X, significant difference between isolates at the given temperature at p=0.05; blank, no significant difference at p=0.05; \ , comparison against self not conducted.