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Do all trees carry the seeds of their own destruction? PCR reveals numerous wood decay fungi latently present in sapwood of a wide range of angiosperm trees

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ABSTRACT

Primary colonising wood decay fungi develop rapidly in attached branches and standing trunks, forming extensive decay columns, suggesting they may be latently present. For a few, latency has been demonstrated by culturing. While there is apparent specificity of association of some primary colonisers for wood species, that might be due to host specificity among endophytic fungi, it might alternatively be due to abiotic requirements for development of the mycelium. We, therefore, developed specific PCR primers for 11 wood decay species (five Ascomycota, six Basidiomycota) and used PCR on DNA from functional sapwood to determine the prevalence of these fungi in 11 angiosperm tree species. We also analysed sequences from libraries of fungal ITS sequences from two tree species.
Fungal DNA was extracted from all 11 tree species. Analysis of sequences from DNA libraries revealed the presence of species not previously considered to be latently present. Our data support the suggestion that wood decay fungi are latently present in functional sapwood of all angiosperm trees and that overt development of particular species is regulated by environmental factors. This raises interesting questions about the time and mode of entry of fungal propagules, their maintenance in sapwood and the cues that trigger their development.

Introduction

Nutrient cycling is crucial to woodland ecosystem functioning, and wood decomposition is almost exclusively effected by fungi, notably basidiomycetes and xylariacious ascomycetes (Rayner & Boddy 1988). Fungal community development begins while branches are still in the canopy and trunks are still standing (Boddy 2001), but it remains unclear how this is regulated. Extensive decay columns develop in less than one growing season, and are often much longer than could be achieved by a fungus extending by mycelial growth from a single inoculum point (Boddy & Rayner 1982, 1983a, b; Hendry *et al.* 1998). To account for this rapid development it was hypothesised that fungal propagules are extensively but sparsely distributed throughout the sap stream in functional sapwood, but these fungi do not develop overtly because of the high water content (Boddy & Rayner 1983b). The lower water content (and probably higher O_2 levels) developing when sapwood becomes dysfunctional allows mycelia to develop from these propagules, which quickly meet and, if they are the same genotype, fuse and act as a single individual.

The presence of wood decay fungi within functional sapwood of trunks and branches of temperate angiosperm trees is only infrequently detected by placing chips of excised wood onto agar culture media, presumably because

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propagules have not received the appropriate germination cues (Chapela & Boddy 1988). The method was more successful for Chilean trees, and light and electron microscopy revealed spores within undecayed wood (Oses *et al.* 2008), perhaps indicating greater abundance of latent propagules. The latent presence of wood decay fungi has been revealed, usually by the development of decay columns within newly felled, apparently healthy temperate angiosperm branches and trunks cut into lengths and dried at different rates, in a number of species including alder (*Alnus* species) (Fisher & Petrini 1990), sycamore (*Acer pseudoplatanus*) (Hirst 1995), birch (*Betula* spp.) (Danby 2000), hazel (*Corylus avellana*) (Hirst 1995), beech (*Fagus sylvatica*) (Chapela & Boddy 1988; Hendry *et al.* 2002; Baum *et al.* 2003), American beech (*Fagus grandifolia*) (Chapela 1989), aspen (*Populus tremuloides*) (Chapela 1989) and oak (*Quercus robur*) (Hirst 1995). With some fungal species genetic differences in mycelia result in long decay columns containing several or sometimes many different fungal individuals.

In temperate woodland, there appears to be some degree of specificity of these primary colonising fungi for tree species (as evidenced by production of fruit bodies). For example, *Daldinia concentrica* fruit bodies are usually found on ash (*Fraxinus excelsior*) in the UK, though also occasionally on beech in the south and quite commonly on birch in the north (Whalley & Watling 1980). Similarly other species commonly fruit on certain hosts: *Eutypa spinosa* and *Hypoxylon fragiforme* on beech; *Fomes fomentarius* on birch and sometimes on beech; *Hypoxylon fuscum* and *Stereum rugosum* on hazel; and *Stereum gausapatum* and *Vuillemina comedens* on oak (*Quercus* spp.) and beech (Boddy & Heilmann-Clausen 2008). However, the absence of fruit bodies is not necessarily indicative of the absence of a fungus. Some fungal species are evidently latently present in tree species in which they rarely if ever develop overtly to produce decay columns or fruit bodies, e.g. *D. concentrica* has been isolated from functional oak (*Q. robur*) twigs (Griffith & Boddy 1990). On the other hand, other fungal species (e.g. *E. spinosa* on beech), whose distribution and ecology implies that they are latently present, have not been detected by the approaches applied so far (Hendry *et al.* 2002). Furthermore, incubation conditions, including rate of drying, temperature and gaseous regime affect which species develop overtly (Chapela & Boddy 1988; Hendry *et al.* 2002). Position in the canopy and geographical aspect may also have some effect (Baum *et al.* 2003).

Detection of fungal DNA using PCR provides a way of analysing a range of species without an enormous number of different culturing regimes (Guglielmo *et al.* 2007, 2008). The latently present *Biscogniauxia mediterranea* was successfully detected in oak sapwood using this approach (Mazzaglia *et al.* 2001). Further, we have recently extracted and detected *Creolophus cirrhatus* DNA from Turkey oak (*Quercus cerris*) sapwood samples from which it was unculturable (Parfitt *et al.* 2005).

We therefore set out to clarify the relationship between fungal propagule or endophyte distribution in functional sapwood in relation to known development of decay fungi, testing whether species specificity of decay fungi can be related to the distribution of the endophytes. Target fungi were selected based on their ecological role as early colonising species and previous detection as fruit bodies on the target tree species in the UK (Boddy 2001).

We test the hypotheses that: (1) early colonising fungi that appear selective for certain tree taxa are more widely spread amongst hosts in their latent phase; and (2) many more fungal species are latently present than might be anticipated based on those seen overtly.

Materials and methods

Extraction of reference DNA

DNA was extracted from cultures of early colonising species from the Cardiff culture collection (Ascomycota – *D. concentrica* (×3 strains), *E. spinosa* (×5), *H. fragiforme*, *H. fuscum*, *Nemania serpens*; Basidiomycota – *F. fomentarius* (×2), *S. gausapatum* (×4), *S. rugosum* (×6) and *V. comedens* (×2)) by scraping surface mycelium from 20 cm^2 of agar into a 1.5 ml Eppendorf tube and then following the method of Cenis (1992), with the exception that an equal volume of dried skimmed milk (0.4 % w/v) was added to the extraction buffer. DNA was extracted from freshly collected fruit bodies of *D. concentrica* and *H. fuscum* using the same approach except that samples were surface sterilized with 5 % sodium hypochlorite (NaOCl), washed with water and 0.15 g of tissue excised.

Amplification and sequencing of reference DNA

Reference DNA extracts were amplified using the fungus specific ITS1F, ITS4 primer pair (White *et al.* 1990; Gardes & Bruns 1993). The reactions were carried out in a GeneAmp PCR System 2700 (Applied Biosystems) thermal cycler using 2 ng of template per 25 μl reaction at 96 °C for 15 min, 40 cycles of {94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min} 72 °C for 10 min. PCR products were isolated on a 1.5 % w/v agarose gel stained with ethidium bromide. Bands were visualised using UV and extracted from gel using a QIAquick Gel Extraction Kit (Qiagen Ltd., Crawley, UK) following the manufacturer's instructions. The amplified DNA extract concentrations were measured using an Ultraspec 2100 Pro spectrophotometer (Amersham Biosciences, Little Chalfont, UK) and sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, USA). New sequences were deposited with EMBL and accession numbers are listed in Supporting Information Table S1.

Design of specific primers and optimisation of PCR conditions

Primer pairs specific to each reference species were designed between 2 and 9 sequences per species from the Cardiff University culture collection and from sequences from the NCBI database, where available (Table 1, Supplementary Table S1). After design, primers were checked using the NCBI BLAST facility to ensure that there were no close matches to other species. Additionally, each species-specific primer pair was used in a series of PCR reactions with all reference DNA samples to determine conditions that amplified only DNA from the target species. DNA that had been extracted from

Table 1 – Primers specific for reference species

Specific for	Primer	Sequence (5′–3′)	Annealing temp. (°C)
Fungi			
Stereum rugosum	SrugF	AATCGGCGCAAGTCGTAGTA	68
	SrugR	AGCAAAGCCGGCAAAGCCT	
Nemania serpens	NserF	GACGGCCCMCGAAACTCTG	68
	NserR	ACGTCTGGAGTGCGAACC	
Hypoxylon fuscum	HfusF	TGTGGCTGCCTGGTAATTG	66
	HfusR	GCGACCAGGTTACTGCAA	
Stereum gausapatum[a]	SgauF	GCGGGGGTCTCTTCGTTA	65
	ITS4	TCCTCCGCTTATTGATATGC	
Vuilleminia comedens	VcomF	GCTGGGGTTAACGCCTTG	68
	VcomR	GATCCGATGTAGTAGGCAG	
Daldinia concentrica	DconF	TACCGAATCTCTGAATGCTTC	60
	DconR	CCCTAACTCCGCCAATCAC	
Eutypa spinosa	EspiF	ATTTGACGGGGCGAACTAC	60
	EspiR	ACCGCTATAAAGCCAGCTA	
Hypoxylon fragiforme	HfraF	ATCCGAGCCTACCCTGTAG	60
	PbetR	CAAGTCCAAGCCTACAGAC	
Fomes fomentarius	FfomF	GGGTTGTAGCTGGCCTTC	60
	FfomR	CCAGCAAAAGCCTCCAATC	
Creolophus cirrhatus	HER2F	ATCTCATCCATCTTACACC	50
	HER3R	CATATGACAAGAGGATCGA	
Hericium spp.	HER2F	ATCTCATCCATCTTACACC	53
	HER2R	CTCATAACAAGAGGATTGA	
Trees			
Corylus avellana	HazF	GAGACACTCGTGCCTTCTT	68
	HazR	TCGTCTCTTGGAGAGGCGA	
Quercus robur	OakF	ACGCGCCAAGGAAATCTAAC	68
	OakR	TTTTGGGCTAACCGCGCG	
Fraxinus excelsior	AshF	GGGAGGACGTCGTGCGTG	68
	AshR	CGAGTTGAGTTCTTCAACCAC	

a These primers do not detect all strains of *S. gausapatum*.

C. cirrhatus, *Hericium erinaceus*, *Hericium coralloides* and *Hericium alpestre* in a previous study, together with previously designed primers specific for *C. cirrhatus* or for the genus *Hericium* (Parfitt *et al.* 2005) were included in the specificity checks.

DNA extraction from functional sapwood

DNA was extracted from the functional sapwood of 18 trees from 11 different species (Table 2, Supplementary Table S2) comprising apple (*Malus domestica*), ash (*F. excelsior*), beech (*F. sylvatica*), birch (*Betula* sp.), Japanese cherry (*Prunus serrulata*), elder (*Sambucus nigra*), hazel (*C. avellana*), cherry laurel (*Prunus laurocerasus*), field maple (*Acer campestris*), oak (*Q. robur*), and willow (*Salix* sp.). Bark was stripped, using a chisel, from freshly felled branches/trunks and the exposed surface sterilized by swabbing with sodium hypochlorite solution (5 %). After 10 min the branches were split along the grain by inserting a chisel at one end and then bending apart the two halves. Samples (2.0 × 0.4 × 0.4 cm) were excised with a sterile chisel, avoiding areas near wounds, branching points or which may have been touched during splitting. Samples (0.15 g) were cut into <1 mm cubes, wrapped in foil, immersed in liquid nitrogen for at least 5 min, transferred to a mortar and, using liquid nitrogen additions, ground to a fine dust in 300 μl of extraction buffer. Following thawing, the sample was transferred to a 1.5 ml Eppendorf tube. The mortar was washed with 300 μl of 0.4 % w/v dried skimmed milk and the washings transferred to the Eppendorf tube containing the powder. The extract was vortexed for 15 s, shaken for 30 min and allowed to stand for 24 hr. Vortexing and shaking were then repeated and the sample microcentrifuged (17 000 g for 5 min). The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and 3 M sodium acetate added (at a volume equivalent to half that of the supernatant). The solution was allowed to stand at −20 °C for 10 min, microcentrifuged (17 000 g for 5 min), and the supernatant transferred to a 14 ml Falcon tube. The DNA was purified using a QIAquick PCR Purification Kit (Qiagen Ltd., Crawley, UK) following the manufacturers instructions except that, because of the high volumes involved, repeated applications to the column of 0.8 ml aliquots of DNA in buffer PB interspersed with washings with pure buffer PB were necessary. A minimum of four independent DNA extractions were made from each branch/trunk. In addition for one hazel tree (*C. avellana* (i)) ten samples taken at approx. 35 cm intervals along a 3 m length plus fork, were extracted individually (Supplementary Fig S1). As a negative control, an empty Eppendorf tube was left open in the same area during excision, then closed and stored with excised samples as a check for airborne contaminants. This tube was washed with 300 μl of extraction buffer and the washing ground with liquid nitrogen. This was taken through all stages of extraction and amplification in an identical manner to tissue samples.

Table 2 – Latent presence of fungi within functional sapwood of 11 angiosperms detected by PCR specific primers on sapwood DNA pre-amplified with ITS1F/ITS4

Tree species		Target fungal species and primer identity										
		Hericium sp.	*C. cirrhatus*	*S. rugosum*	*N. serpens*	*D. concentrica*	*S. gausapatum*	*E. spinosa*	*H. fragiforme*	*F. fomentarius*	*H. fuscum*	*V. comedens*
		HER2F/HER2R	HER2F/HER3R	SrugF/R	NserF/R	DconF/R	SgauF/ITS4	EspiF/R	HfraF/R	FfomF/R	HfusF/R	VcomF/R
Apple	*Malus domestica*	ND	ND	+	−	+	−	+	−	+	−	−
Ash	*Fraxinus excelsior* (i)	−	−	+	+	+	+	+	+	+	+	+
	Fraxinus excelsior (ii)	+	+	+	+	+	+	+	+	−	+	+
Beech	*Fagus sylvatica* (i)	+	+	+	+	+	+	+	+	−	+	+
	Fagus sylvatica (ii)	−	+	+	+	+	+	+	+	+	+	+
	Fagus sylvatica (iii)	+	+	+	+	+	+	+	+	+	+	+
Birch	*Betula* sp.	ND	ND	+	−	+	−	+	−	+	−	−
Japanese cherry	*Prunus serrulata*	ND	ND	+	−	+	−	−	−	−	−	−
Elder	*Sambucus nigra* (i)	−	+	+	+	+	+	+	+	−	−	+
	Sambucus nigra (ii)	ND	ND	+	+	+	+	+	+	+	+	+
Hazel	*Corylus avellana* (i)	+	+	+	+	+	+	+	+	+	+	+
	Corylus avellana (ii)	ND	ND	−	−	+	−	+	−	+	−	−
Cherry laurel	*Prunus laurocerasus*	ND		+	−	+	−	+	−	+	−	−
Field maple	*Acer campestris* (i)	+	+	+	+	+	+	+	+	−	+	+
	Acer campestris (ii)	+	+	+	+	+	+	+	+	+	+	+
Oak	*Quercus robur* (i)	−	−	+	+	+	+	+	+	+	−	+
	Quercus robur (ii)	ND	ND	+	−	+	−	+	−	+	−	−
Willow	*Salix* sp.	ND	ND	+	+	+	+	+	+	+	+	+
Sterile distilled water		−	−	−	−	−	−	−	−	−	−	−
Contamination check from open tube at excision		−	−	−	−	−	−	−	−	−	−	−

ND, not determined.

Amplification, with specific primers, of DNA extracted from hosts

Primer pairs specific to the ITS1–ITS2 sequences of three host species: hazel, oak and ash were designed from sequences from the NCBI database. The conditions for PCRs with species-specific primers were as above, except that the number of cycles and the annealing temperatures differed between primer pairs (Table 1). Genomic DNA extracts from hazel, oak and ash sapwood were used as an additional control for specificity of the newly designed fungal specific primers. PCRs were carried out on these samples using the hazel, oak and ash specific primers hazF/R, oakF/R and ashF/R to confirm that the extracts were of PCR quality and to get an indication of the proportions of host and fungal DNA present. PCR products were examined on agarose gel as detailed above.

Construction of clone libraries

Clone libraries were created using ITS1F/ITS4 amplified DNA from beech and oak sapwood samples. PCR products were visualised on agarose gel stained with ethidium bromide (1 % wt/vol). Bands were excised and DNA purified using a Qiaquick gel extraction kit (Qiagen Ltd., Crawley, UK). Products from three separate amplifications were pooled prior to cloning to minimise PCR drift. Purified amplification products were cloned into the pGEM-easy T vector (Promega UK Ltd., Southampton, UK) and ligations were transformed into *Escherichia coli* XL1-Blue sub-cloning grade competent cells in accordance with the manufacturer's directions (Stratagene Ltd., Cambridge, UK). White colonies were screened directly for inserts by performing colony PCR with ITS1F/ITS4 and plasmids containing the correct inserts were purified using the QIAprep spin miniprep kit (Qiagen Ltd., Crawley, UK). Nine clones derived from oak and 11 from beech were sequenced on an ABI Prism 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Nested PCRs on DNA extracted from hosts

PCR amplifications were carried out using sapwood derived DNA samples from each individual tree and the fungus specific primer pair ITS1F/ITS4. The products were gel extracted as above and all four or more extracts from each individual were pooled. Each pooled sample was used as the template in further PCRs with each of the fungal species-specific primers, under the optimised conditions (Table 1). The pooled ITS1F/ITS4 products from the hazel, oak and ash samples were also used as templates in nested PCRs with the tree species-specific primers hazF/R, oakF/R and ashF/R to confirm that little host DNA was amplified. To confirm identity of the PCR products from the nested PCR a sample of the recovered PCR products were purified and sequenced (data not shown). Furthermore, the ten samples extracted from the hazel, *C. avellana* (i) tree were analysed separately as above. To confirm reproducibility, PCR reactions were repeated at least twice, and in some cases three times with consistent results.

Sequence analysis

Sequences from the cloned ITS1/4 PCR products were compared with similar rDNA sequences retrieved from the GenBank databases. Sequences were aligned using Clustal X (Higgins *et al.* 1992) and the alignment was corrected manually. A Neighbour-Joining tree was constructed from a distance matrix calculated using Kimura's two-parameter model (Kimura 1980) in the MEGA program (Tamura *et al.* 2007).

Results

Primers were designed to nine wood decay fungal species and tested for species specificity

Using the optimised PCR conditions, eight of the nine specific primer pairs detected all reference isolates of their target species (Table 1). For the other (*S. gausapatum*) the forward specific primer, paired with ITS4, detected 3 out of 4 reference isolates of the target species. Previously designed primers to *C. cirrhatus* and *Hericium* spp. (Parfitt *et al.* 2005) were also included. None of the specific primers amplified products from any of the other ten, non-target, reference species (Supplementary Table S2). BLAST checks with the primers resulted in the closest hits being the target species or there were no close hits (where no relevant sequences were present in the database).

Nested PCR revealed a broad distribution of all the saprotrophic fungal species

In total, 18 individual trees, representing 11 tree species, were tested with all nine species-specific primer sets, and ten tree samples (representing six tree species) were also tested with primers to *Hericium* sp. and *C. cirratus*, both rare tooth fungi for which species-specific primers had already been developed (Parfitt *et al.* 2005). The PCR analysis revealed widespread presence of fungal DNA (Table 2). Overall, all the trees tested were positive by PCR for at least two of the fungal species, and in twelve out of the 18 more than half of the target fungi were detected. There were some differences in the prevalence of the different fungal species: *D. concentrica* was the most widespread, found in all samples of all tree species tested, followed by *S. rugosum* that was detected in all species of trees tested, but was not detected in one of the hazel trunks. *E. spinosa* was also very widespread, found in all the tree species tested except Japanese cherry. However, *H. fuscum* and *Hericium* spp. were detected in only half of the tree species tested. Field maple had the heaviest loading of fungal species: all the fungal species tested were present in both samples tested.

The numbers of different fungal species detected in a single, tree species also varied. All 11 fungal species were detected in two tree species – beech and field maple. In a further two species, elder and willow, only nine of the fungal species were tested and all were present. However, in apple, birch and cherry laurel only four of the nine fungal species were detected. In the hazel stem from which ten samples were taken *C. cirrhatus*, *D. concentrica*, *E. spinosa*, *H. fragiforme* and *V. comedens* were detected in all samples. The other fungal species were present in 7–9 of the samples.

For six of the tree species two or more individuals from the same species were tested. Although there was good agreement in the prevalence of the fungi detected between different trees of the same species for three of the species – ash, beech, and field maple, agreement was less good for the other three species – elder, hazel and oak, where prevalence of the fungi varied widely (Table 2). Thus there was as much variation between individual trees of different species as there was between individuals of the same tree species.

When DNA from hazel, oak and ash was amplified with the ITS1F/4 primers and used as a template for a second round of PCR with the tree host species-specific primers a product could not be detected unless template concentrations were increased by at least a factor of 10^3. This demonstrates that although most genomic DNA extracted from these trees was host DNA, after amplification with ITS1F/ITS4 primers most DNA was fungal and hence positive results were not due to non-specific amplification of host sequences.

Sequencing of products from nested PCRs further confirmed: (1) that these were sequences from the appropriate fungi; and (2) the specificity of the primers in the context of this study. Products amplified from hazel, oak and ash with SrugF/R (*S. rugosum* specific) and NserF/R (*N. serpens* specific) primers were almost identical (>99 %) to the corresponding *S. rugosum* and *N. serpens* reference sequences. The product from hazel (*C. avellana* (i)) DNA amplified with DconF/R (*D. concentrica* specific) primers was almost identical (>99 %) to the corresponding *D. concentrica* reference sequence.

Presence of fungal DNA was not associated with branch diameter

For all the samples tested (Table 2), equal amounts of DNA extracts from at least four separate blocks of functional sapwood were pooled to ensure that local variations in fungal prevalence did not bias the results. However, we also wondered whether presence of the fungal propagules might be associated with the size of the branch. We thus took ten independent samples from one of the hazel trees and analysed them independently (Supplementary Fig S1 and Table 3). Again the vast majority of the fungal species were detected in all the samples suggesting that the distribution within the tree is relatively uniform.

Unexpected fungal species detected in oak and beech functional sapwood

To explore whether functional sapwood might also contain fungal propagules from species other than those tested for above, we cloned the primary PCR product from the ITS primers derived from two tree species – oak and beech – and sequenced clones from each library (nine from oak and 11 from beech). All the clones from the oak and beech libraries showed high levels of similarity to the ITS1 region of fungal rRNA sequences from the NCBI database (Fig 1). The sequences identified as basidiomycetes and ascomycetes separated from each other, however, the beech-derived and oak-derived clones did not separate into different clusters indicating again that there is no clear specificity in the association between fungal and tree species from these data.

Three of the clones: Beech1, Beech9 and Oak10 matched database sequences with 100 % identity. No clone was identical to any other, although some clones matched the same database sequence. Surprisingly, many of the primary wood decay species for which we developed specific primers were not detected in these libraries. However, two of the clones – Beech14 and Oak5 – showed high levels of similarity with *H. fuscum* and another two – Oak11 and Oak16 – were related to a *Stereum* species, both of which were targets in our work with the fungal species-specific PCR primers. In all cases, the nearest matching NCBI sequence for each of the clones was from either a soil or plant sample. However, many of these sequences were derived directly from environmental samples and, were therefore, unfortunately unidentified.

Discussion

Using specific primers and a nested-PCR approach we have successfully surveyed for the presence of 11 fungal species in the sapwood of 11 tree species. This method is vastly superior to the previously used approaches of direct isolation from freshly felled wood, or slow drying of wood followed by isolation onto agar media. The latter are limited in the species that they detect under any particular set of abiotic conditions and do not allow small-scale resolution of the location of propagules from which decay columns develop. However the PCR approach does not generate cultures for further study. Note also that although the specificity of the PCR primers was tested against all the target species in this study, and against database accessions, this is not exhaustive and it is still possible that other non-target taxonomically related species could be detected.

The fact that fungal DNA was extracted from functional sapwood of all angiosperm trees tested, including field maple, apple, willow, elder, cherry laurel and Japanese cherry, that have not previously been examined for latently present fungi, gives support to the suggestion that decay fungi are latently present in functional sapwood of all angiosperm trees. The presence of DNA is highly unlikely to be due to contamination as ''no sample'' negative controls, used from tissue excision onwards, always gave negative results. Furthermore, the fact that most fungal taxa, for which specific primers had been developed, were detected in the wood of most tree species confirms the hypothesis that host specificity of fungi that develop overtly is not directly linked to the presence of latent propagules in functional sapwood. Rather, apparent specificity may reflect microclimatic conditions when full mycelial development begins (Hendry *et al.* 2002). Given the limits of detection even of the very sensitive PCR methods employed, the possibility that all the fungal species tested are present in all the tree species surveyed cannot be excluded, as negative results may just mean that the fungi are present but below the levels detectable. It is also possible that intra-specific divergence may result in negative PCR results using the primer sets designed and the PCR conditions used. Moreover, latently present fungi seem to be distributed extensively within branches, as evidenced by the presence of fungal DNA in the vast majority of samples when multiple samples were taken from a single hazel stem.

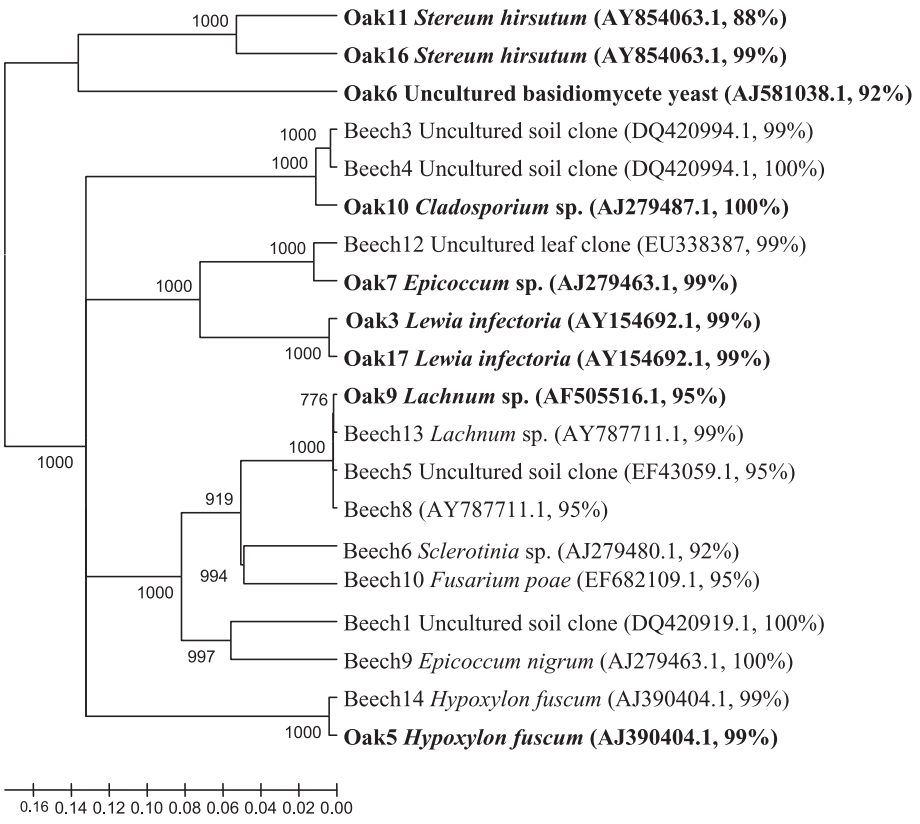

Fig 1 – Relationship between the sequenced clones derived from the ITS sequences amplified directly from functional sapwood of *Quercus robur* (oak in bold) and *Fagus sylvatica* (beech), and their homology to database sequences based on BLAST searches. The bootstrap values are the result of 1 000 replicates.

One of the species for which we designed specific primers had previously only been suspected to be latently present in functional sapwood; *E. spinosa* was demonstrated to be latently present in beech, a tree species in which it forms extensive (many metres) decay columns behind strip cankers on the trunk following drought (Hendry *et al.* 1998). Moreover, it is also latent in a wide range of tree species, though it does not appear to be a dominant early coloniser of other angiosperms in Britain (Boddy 2001).

The suspicion that there are many other wood decay species latently present in functional sapwood was confirmed by the detection of *Hericium* species and *C. cirrhatus* in several tree species. These fungi had hitherto not been suspected as developing from latent propagules. *H. coralloides*, *H. erinaceus* and *C. cirrhatus* are all considered rare in the UK, based on occurrence of fruit bodies (Boddy & Wald 2003). When they colonise wood was unknown, but it now looks as if they may be present before the tree dies. Further, they may be less rare

Table 3 – Latent presence of fungi in ten positions in a single hazel tree detected by PCR specific primers on sapwood DNA pre-amplified with ITS1F/ITS4

Fungal species	H1	H2	Position on hazel tree			H6	H7	H8	H9	H10
			H3	H4	H5					
Creolophus cirrhatus	+	+	+	+	+	+	+	+	+	+
Daldinia concentrica	+	+	+	+	+	+	+	+	+	+
Eutypa spinosa	+	+	+	+	+	+	+	+	+	+
Fomes fomentarius	+	+	+	+	−	+	+	+	+	+
Hericium spp.	−	+	−	+	+	+	+	+	−	+
Hypoxylon fragiforme	+	+	+	+	+	+	+	+	+	+
Hypoxylon fuscum	+	+	+	+	+	−	−	+	−	+
Nemania serpens	−	+	+	+	+	+	+	+	+	+
Stereum gausapatum	+	+	+	+	+	+	+	−	−	+
Stereum rugosum	+	+	+	+	+	+	+	−	+	+
Vuilleminia comedens	+	+	+	+	+	+	+	+	+	+

than hitherto realized, perhaps being present as mycelia in many trees on which they have not yet fruited. However, latent presence does not necessarily mean that they will develop overtly when sapwood becomes non-functional (Table 2). There is evidence of restricted host range from fruit body records, over 80 % of each species being recorded on *F. sylvatica*, with a further 14 % of *H. coralloides* fruiting on *F. excelsior* (Boddy & Wald 2003). There may also be specificity with regard to latent presence, since they were not detected in several tree species (Table 2) nor were they detected in all hazel branches (Table 3).

Like *Hericium* spp. and *C. cirrhatus*, both *H. fuscum* and *F. fomentarius* were not detected in all branches/trunks. The latter commonly fruits on birch in northern Britain, although it is not very common in southern UK, but does fruit on beech and sycamore. It has been detected, by traditional approaches, as latently present in birch from several locations in Britain (Danby 2000), and from beech in Germany (Baum *et al.* 2003). In the present study it was detected in several tree species, but not in all beech branches. Perhaps, it is actually rare in beech. Alternatively, since higher concentrations of pre-amplified DNA were necessary to detect fungi in beech, its presence may have been missed. The reasons for less sensitivity with beech need to be explored further in the future.

Many of the sequences retrieved from the oak and beech clone libraries matched database sequences from fungi which are not regarded as wood decayers and were hitherto not suspected to be latently present in sapwood, though many have been found in close association with other plant parts (Wirsel *et al.* 2001). This suggests that a variety of fungal propagules find their way into sapwood and probably remain there with no likelihood of further development. This has some parallel with endophytes that remain latent within living cells of non-woody tissues (Sieber 2007), as opposed to the non-living xylem in wood. It is also possible that sapwood provides a route for infection for leaf endophytes and could serve to assist leaf endophytes over-wintering in deciduous trees. Differences in isolation frequencies of endophytes from leaf blade and petiole segments, have been observed for endophyte distributions in many species (Caroll 1995), suggesting that some endophytes enter from the woody tissue and some *via* infection of leaf surfaces. Endophytes belonging to the Xylariaceae (in the present study *E. spinosa*, *N. serpens*, *D. concentrica*, *H. fragiforme* and *H. fuscum*) have been isolated from a diverse array of tree species (Petrini *et al.* 1995; Sieber 2007), not only from stems and twigs but also from leaves. Infection of wood may be from leaves or *vice versa*. Hata & Sone (2008) in a study of the lauraceous tree species *Neolitsea sericea*, isolated a *Phomopsis* spp. at high frequencies from leaves and, again, species in this genus have been reported as common endophytes in twigs and leaves, suggesting that they infect leaves from twigs (Sahashi *et al.* 1999; Hata *et al.* 2002).

Thus, in summary our approach detected: (1) wood decay fungal species present in functional xylem of branches and trunks of a wide range of tree taxa, including species in which they are not usually overtly manifest; (2) fungal species that had not hitherto been suspected as being latent, namely the rare (based on fruit body occurrence) *C. cirrhatus* and *Hericium* spp.; (3) fungal species hitherto only suspected as being latently present (*E. spinosa* in beech); (4) fungal species latently present in sapwood where they had previously only been known as leaf endophytes (e.g. *Epicoccum nigrum*). Also, since fungal DNA can be detected in small ($2.0 \times 0.4 \times 0.4$ cm) samples we now have the tools to be able to look at smaller scale distribution of fungi latently present within different locations within standing trees, and to try to determine when, where and how these fungi effect entry into their host. These results emphasise the need to determine the time and mode of entry of fungal propagules, their maintenance in sapwood and the cues that trigger their development.

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Supplementary material

Supplementary material associated with this article can be found in online version at doi:10.1016/j.funeco.2010.02.001.

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