ARTICLE IN PRESS

Fungal Ecology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Fungal endophytes and origins of decay in beech (*Fagus sylvatica*) sapwood

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ARTICLE INFO

Corresponding Editor: Petr Baldrian

Keywords:
HTS
Metabarcoding
Fungal guilds
Latent fungi
Wood saprotroph
Decay fungi
Mycobiome
Xylem

ABSTRACT

Sapwood comprises much above-ground forest biomass, but its mycobiome in living trees is largely unknown. Here, we characterize the endophytic fungal communities of the functional sapwood of young and mature living beech trees (*Fagus sylvatica)* at multiple scales, from within individual trees to woodland sites across the southern United Kingdom. Fungal community composition was determined using both culture-based and molecular approaches across two loci. Wood decay fungi, including those that cause heart rot, were detected in approximately 80% of all samples. Fungal community composition differed according to the survey approach (high throughput sequencing vs. isolation of fungi into culture) and between geographic location and individual trees, but no significant patterns were detected at different heights in individual trees or around their circumferences. ITS and LSU sequencing detected more distinct taxa than culturing. However, LSU primers yielded more OTUs than did ITS primers, though both identified unique OTUs. This highlights the importance of multiple survey approaches, including multiple primer pairs, for better characterisation of communities and confidence in results of endophyte studies.

1. Introduction

Sapwood is the water-conducting tissue of living trees and is their vital connection from roots to leaves. It mostly comprises long, non-living tube elements through which water moves, but it also contains living parenchyma cells capable of nutrient storage and mobilisation, and of defence responses (Pearce 1996; Morris et al. 2016). As with all plant tissues examined so far (Rodriguez et al. 2009; Vandenkoornhuyse et al. 2015), sapwood contains endophytic fungi, as well as bacteria and archaea. However, despite being the predominant feature of the world's three trillion trees (Crowther et al. 2015), sapwood is one of the least explored forest habitats (Baldrian 2017).

The presence of decay fungi latent in functional sapwood of twigs, branches and trunks was first demonstrated by allowing freshly harvested living material to dry at different rates and to different extents, and then to isolate fungi from chips of wood onto agar culture media (Chapela and Boddy 1988; Boddy and Griffith 1989; Griffith and Boddy 1990; Hirst 1995; Danby 2000; Hendry et al. 2002; Baum et al. 2003). A narrow range of basidiomycetes and ascomycetes in the Xylariaceae were revealed, plus a few heart rot species — *Fomes fomentarius* in *Betula* spp. and *Fagus sylvatica* (Danby 2000; Baum et al. 2003). Different decay communities emerged from the endophyte communities of freshly-cut branches and twigs depending on moisture, gaseous and temperature regimes they were incubated in (Chapela and Boddy 1988; Hendry et al. 2002). This suggested that subsets of species can develop from a larger, endophytic pool of decay fungi, depending on the prevailing environment. Indeed, subsequently, fungal species-specific PCR primers revealed their presence in the sapwood of a range of tree species. This includes tree species in which such fungi rarely form part of the actual decay community (Parfitt et al. 2010; Jusino et al. 2015).

Several more recent culture-based studies, in which small chips were excised from functional sapwood and placed on culture media, have documented endophytic fungi in a range of tree species. In general, these have revealed communities low in diversity, comprising mostly Ascomycota many of which are sterile and non-identifiable based on morphological characters. Basidiomycota, including wood decay species, comprise a small fraction, though Martin et al. (2015) found much higher diversity in the tropical host, *Hevea*. Endophytic communities of

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https://doi.org/10.1016/j.funeco.2022.101161
Received 1 July 2021; Received in revised form 20 March 2022; Accepted 20 April 2022

non-woody tissues seem to vary depending on tree species, tissue age, altitude (Siddique and Unterseher 2016), site, and height above ground (Harrison et al. 2016), and might be predicted to vary similarly in sapwood. Different approaches also reveal contrasting community composition, for example with culture-based approaches being heavily biased toward taxa that germinate and grow rapidly (Johnston et al. 2017; Siddique et al. 2017). High throughput sequencing (HTS) studies are also likely to reveal greater diversity in wood than other survey approaches, as demonstrated by studies of standing and fallen logs (Kubartová et al. 2012; Ovaskainen et al. 2013; Skelton et al. 2019).

Endophytic wood decay fungi are important as they begin the process of wood decomposition in standing, living trees (heart rot), and in the absence of wounding (Boddy and Griffith 1989; Song et al. 2016; Boddy et al. 2017). Wood decay fungi might pre-empt niche space in sapwood, in advance of suitable conditions developing there. As decay progresses, early colonists are replaced by more combative secondary colonisers, but their initial occupancy can determine the succession of the fungal community, i.e. they cause priority effects (Lindner et al. 2011; Hiscox et al. 2015; Schilling et al. 2015; Cline et al. 2018). Decades later, the influence of these primary colonisers may be reflected in the succession of wood decay fungi and rate of decay when a tree dies and falls to the ground (Van Der Wal et al., 2015).

This study aimed to characterize fungi in functional, water-conducting xylem in trunks of standing, living trees of the widespread European beech (*Fagus sylvatica*). This builds on the earlier latency studies (Chapela and Boddy 1988; Hendry et al. 2002; Baum et al. 2003) and that using PCR specific primers (Parfitt et al. 2010) in beech. Its novelty lies in being the first to: (1) use HTS to determine endophytes within functional sapwood of living beech trees, with measures to prevent possible aerial contaminants; (2) use a combination of two-locus HTS and isolation of fungi into culture; (3) compare communities at different geographical sites within the UK; and (4) on 2 sites to compare samples from different cardinal directions of trees and heights up tree trunks. We predicted that wood decay saprotrophs would be a large component of endophyte communities, but those whole communities would vary by site, tree, and cardinal directions or height on the trunk.

2. Materials and methods

2.1. Study design

66 living beech trees were sampled from ten sites across the southern United Kingdom (Table 1, Fig. S1), between July and September 2017. Trees selected were visibly healthy and with no obvious decaying regions, wounds, or fungal sporocarps. At three sites, smaller, young trees were selected, of a size between 10 and 30 cm diameter measured at breast height (DBH: 1.3 m above ground). At a further seven sites the trees were mature, between 50 and 90 cm DBH (Table 1). All trees were sampled at one point on the trunk, on the southern aspect at 1.3 m above the ground. A subset of five mature trees were sampled more intensively, at 13 points, including 4 points at different cardinal directions (N, S, E, W) at 1.3 m above the ground, and 9 points ascending the trunk and along large diameter branches at the lowest and highest positions in the crown (Fig. 1A and B).

Table 1
Locations and details of trees sampled, including trees sampled once at 1.3 m above ground and a subset of five trees (in brackets) that were also sampled a total of 13 times throughout the trunk and crown.

Location			Tree Size (Mean ± SD)	No. of Samples	
Site	Latitude	Longitude		Trees	Total
Buckfast	50.49353	−3.77390	21 ± 2.2	5	5
Savernake	51.38723	−1.66528	69 ± 8.8	10	10
Windsor	51.45407	−0.62268	69 ± 11.2	11 (3)	47
Melyn	51.58379	−3.01988	81 ± 3.7	5 (2)	29
Epping	51.66941	0.05682	70 ± 9.8	10	10
Wytham	51.77139	−1.34331	17 ± 2.2	5	5
Moccas	52.07526	−2.97338	63 ± 14.3	5	5
Wyre	52.40258	−2.37980	53 ± 2.3	5	5
Esgynfa	53.27520	−3.34795	17 ± 3.0	5	5
Clumber	53.28625	−1.03297	69 ± 10.6	5	5
			Total	66 (5)	126

2.2. Field sampling

Scrupulous aseptic technique is essential to be certain that fungi detected in samples were truly from within functional sapwood rather than from aerial or bark contamination. Our pilot studies indicated that there are several possible sources of contaminants if the practice of simply catching wood drillings falling from drill bits, commonly seen in decaying wood studies, is adopted. These include sample contact with tree bark, deposition of aerial spores, transfer from tools between trees, and from collectors. Most previous studies, where samples have been collected from standing trees in the field, do not provide evidence that sufficient rigour was adopted during sampling. Thus, a sampling strategy was developed to minimise introduction of contaminants into samples.

Sterile drill bits were preassembled with sterile microfunnels in the laboratory and were transported to the field in individual sterile falcon tubes (Fig. 1C). Drill bits were considered DNA-sterile since they were wiped with 10% bleach solution and flamed and later placed under a UV hood when in the falcon tubes. At each tree wood samples were collected by drilling into exposed sapwood. First, a 5 cm square of bark was aseptically removed with a hammer and chisel that were wiped with 10% bleach solution followed by 70% isopropanol (Fig. 1D). With a cordless drill and 4 mm diameter drill bit, sapwood was drilled to a depth of 4 cm. Bits and tubes were attached to the drill without handling or exposure to airborne contaminants and were changed between each tree and sample. The wood drillings were drawn into a sterile microfunnel held around the drill bit and against the sapwood (Fig. 1E). The contents of the microfunnel were then carefully emptied into a sterile, 1.5 ml microtube under cover of a sterilized plastic box (Fig. 1F). Drilling was repeated at each corner of the exposed sapwood to provide four subsamples in separate microtubes. These were placed into a cool bag containing freezer blocks and transported to the laboratory. To preserve DNA, 1 ml of filter-sterilized cell lysis solution (CLS: Lindner and Banik, 2009) was added to the first tube and frozen at −80 °C within 12 h. The remaining three subsamples were processed immediately for isolation of fungi. Field controls were taken by running the drill, in air, approximately 2 cm away from the bark surface of standing tree trunks, chosen at random. These field controls were otherwise treated as samples (i.e. CLS was added and they were included in downstream steps), although no DNA was detected from them.

2.3. Isolation of fungi into culture

Microtubes containing wood drillings were weighed and one third of contents from each of the three subsamples were pooled. Sterile distilled water was added to wood drillings to produce 1% (weight/volume) suspensions. Suspensions were then plated onto three media types in 9 cm diameter Petri dishes by spreading 1 ml onto: (1) 2% malt extract agar (MA; 20 g l^{-1} malt (Lab M) and 15 g l^{-1} agar (Lab M)); (2) MA amended with kanamycin (100 mg l^{-1} kanamycin monosulphate (Sigma-Aldrich); and (3) the base of a Petri dish and covering with MA. Petri dishes were sealed with film , incubated in darkness at 20 °C for 12 weeks and checked periodically. Developing mycelia were subcultured onto MA to give clean cultures.

Isolates were grouped by morphotype and representatives of each morphotype were selected for Sanger sequencing. Fresh mycelium from pure cultures was scraped into tubes and DNA extracted using a DNeasy Plant Mini Kit (Qiagen). The full ITS region was amplified using primer pair ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3’; Gardes & Bruns,

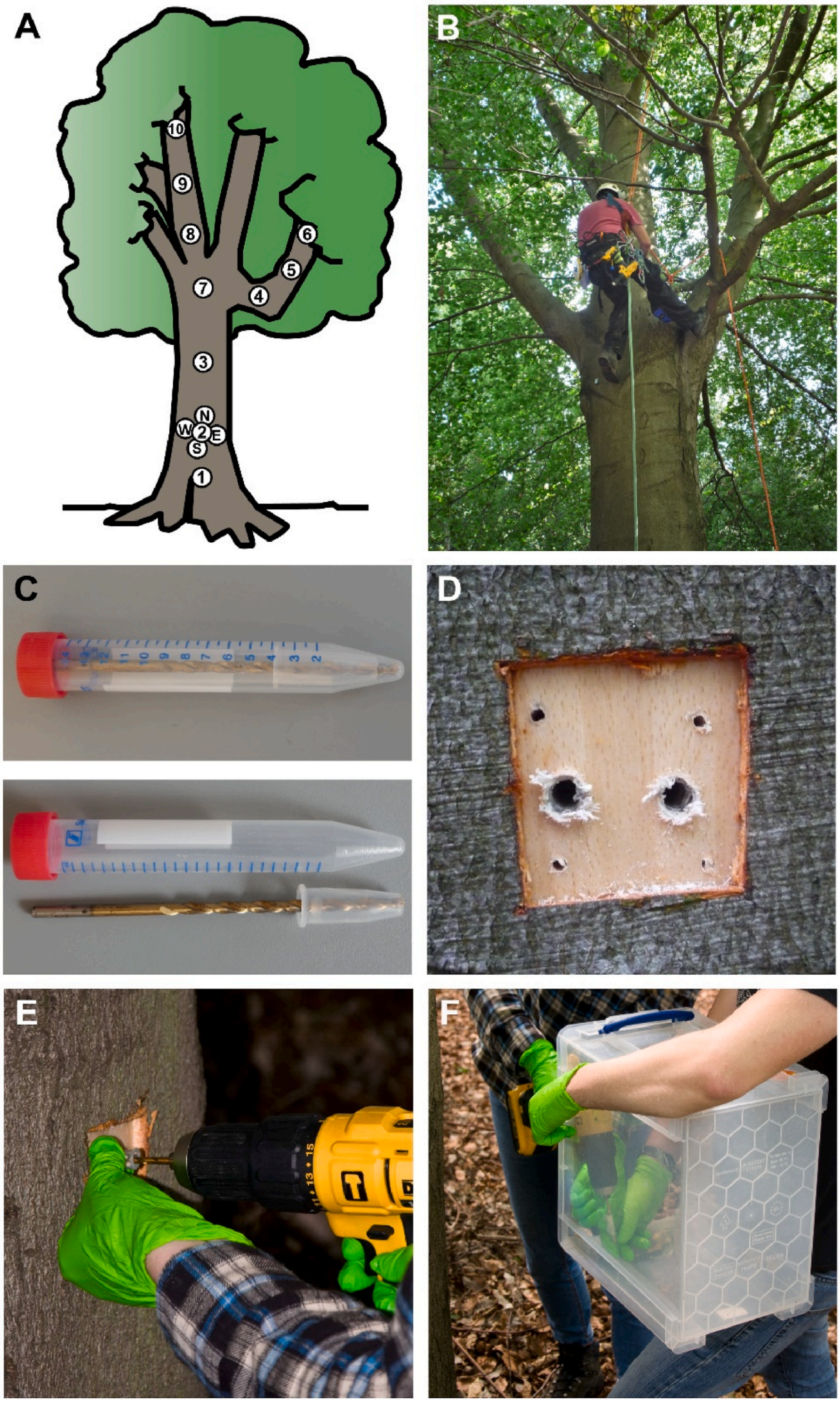

Fig. 1. Drill bit preparation and field sampling. A - Summary of thirteen sampling locations throughout the trunk and large branches of five trees. B - Roped climber sampling trees at height. C - Assembly of drill bits in sterile falcon tubes for transportation (top), and arrangement within the falcon tube of drill bit in relation to collecting microtubule (bottom). D - Tree trunk after bark square removal, showing location of four drilled holes in each corner, whilst in the centre two larger core holes from increment borer sampling for a different study. E – Collecting a sample of wood drillings into microtube held against exposed sapwood. F - Emptying of wood drillings into clean microtube under cover of sterile box.

1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3'; White et al., 1990). PCR was performed with program: 95 °C for 3 min, 35–37 cycles of: 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, followed by a final 10 min elongation at 72 °C. PCR products were visualised on 1.5% agarose gels stained with Sybr Safe (Invitrogen) and purified with a QIAquick Purification Kit (Qiagen). DNA was quantified on a Qubit 2.0 fluorometer (Invitrogen) and sequenced in one direction by Eurofins Genomics UK.

2.4. Processing of wood samples for HTS

Field samples and controls, in DNA-sterile CLS at −80 °C, were first thawed at room temperature and then heated at 65 °C for 3 h. 100 μl of supernatant was used for DNA extraction, using the protocol detailed in Lindner and Banik (2009) with modifications in Brazee and Lindner (2013). Two barcode regions were amplified separately from each sample to capture greater fungal diversity. These were part of the internal transcribed spacer gene (ITS2) –the universal fungal barcode– and the flanking large subunit (LSU). Barcoded HTS amplicons were generated in one PCR step. Mock fungal community controls for each barcode region –ITS2 (SynMock; Palmer et al. 2018) and LSU (LSU Mock; Skelton et al., 2019)– were used for bioinformatics parameterization, including clustering efficiency, estimation of index bleed and recovery of a known community (Palmer et al. 2018; Jusino et al. 2019).

For ITS2, the forward primer comprised the Ion A adapter sequence, followed by the Ion key signal sequence, a unique Ion Xpress Barcode sequence (10–12 bp), a single base-pair linker (A), and the fITS7 primer (5′-GTGARTCATCGAATCTTTG-3’: Ihrmark et al. 2012). The reverse primer comprised a trP1 adapter followed by ITS4 (White et al. 1990). Amplification was performed in 15 μl reactions with: 3 μl of (1x or 1:10 dilution) template DNA, 0.3 μl each of forward and reverse primers, 0.3 μl dNTPs, 0.1 μl GoTaq Polymerase (Promega Corporation, Madison, WI, USA), 3 μL reaction buffer, the additives BSA (ITS, 0.12 μl) or DMSO (LSU, 0.6 μl), and molecular-grade water. PCRs were performed with a program of: initial denaturation at 94 °C for 3 min, 11 cycles of 94 °C for 30 s, 60 °C for 30 s (−0.5 °C per cycle), and extension at 72 °C for 1 min, followed by 26 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

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

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

2.5. Bioinformatics

High throughput sequencing data were processed using AMPtk v1.1.3 (Palmer et al., 2018). ITS and LSU libraries were processed separately. Individually barcoded reads were first pre-processed using USEARCH (version 9.2.64), then reads with forward and reverse primers were retained and primer sequences removed. Reads shorter than 125 bp were discarded and those remaining were truncated to 300 bp. Clustering into OTUs was performed with UPARSE (Edgar 2013) at 97% similarity for ITS, but for LSU this was performed with DADA2 (Callahan et al. 2016) which performs better for this barcode (Skelton et al. 2019). Index bleed percentages in and out of the mock community samples were calculated, then read counts within the range of index bleed were filtered from the OTU table using the filter module within AMPtk.

2.6. Identification and determination of ecological roles

Taxonomy was assigned to OTUs using the AMPtk built-in ITS and LSU databases by means of a hybrid approach using global alignment, UTAX and SINTAX. All non-fungal OTUs were removed at this stage prior to further analysis. Distinct taxon assignments were compared with the open-source database FUNGuild (Nguyen et al. 2016) within the AMPtk pipeline. OTUs were also manually examined, and taxa known to be involved in wood decay were identified. To determine whether the same fungi were both isolated into culture and detected using HTS sequencing, the ITS2 region was extracted from full ITS culture sequences using ITSx (Bengtsson-Palme et al. 2013) and a local BLAST search, at 97% similarity, was performed for these sequences against sequences from the ITS2 HTS dataset.

2.7. Statistical analysis

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

Fungal community analyses were performed with the vegan package (Oksanen et al. 2018) in R (R Core Team 2020). Fungal community composition of samples was visualised using non-metric multidimensional scaling (NMDS), using the metaMDS function. Community dissimilarities were calculated on modified Raup-Crick distance matrices (Chase et al. 2011) implemented with the *raupcrick* function. Community dissimilarity PERMANOVA tests (Anderson 2001) were performed using the *adonis* function to test for significant community differences by site, tree, cardinal direction and sampling height. As PERMANOVA is sensitive to non-normality (Anderson et al. 2006), a further test for homogeneity of dispersion among groups was performed with the *betadisper* function. Mantel tests (function *mantel*) were used to further examine site variation by correlating fungal community distance matrices (ITS and LSU) first with a matrix of geographic distance between sites and then with a matrix generated from site-level scaled climatic factors (mean annual temperature, annual precipitation, seasonality of precipitation and isothermality). Site-level climatic factors were extracted from the WorldClim data set (worldclim.org) using the raster package (function *getData*). Partial mantel tests (function *mantel.partial*) were used to determine if site-level climate variables explained variation in ITS and LSU fungal community composition that was not explained by geographic distance between sites.

3. Results

3.1. Detection and identification of fungi in beech trees

In a total of 126 samples from 66 individual trees, 328 OTUs were detected by HTS using the ITS barcode (Ascomycota/Basidiomycota taxa; 67%/25%). For LSU, 412 OTUs were detected (72%/22%) (Fig. S2A). Other high-level clades detected were Zygomycota, Glomeromycota, Neocallimastigomycota and Chytridiomycota. Sanger sequencing of fungal cultures with the ITS barcode generated 76 OTUs (84%/13%).

The ITS and LSU sequencing datasets had more OTUs in common than either did to the culture dataset. The LSU barcode detected 23 fungal classes, whilst ITS detected 17 (Fig. 2A). Culturing detected 13 fungal classes and, of these, three – the Lobulomycetes, Schizosaccharomycetes and Ustilaginomycetes – were missed by HTS (Fig. 2B). Conversely, HTS detected three classes that were not found by the culture-based approach - the Exobasidiomycetes, Microbotryomycetes and Saccharomycetes. At family-level, 12 and at the genus-level, 5 OTUs were common to all approaches. The ITS and culture datasets both had the species *Clonostachys rosea* in common, whilst the LSU and culture datasets had *Aspergillus fumigatus*, *A. niger*, *Aureobasidium pullulans* and *Cladosporium langeronii* in common.

A local BLAST search of sequences from cultured fungi against sequences from the ITS dataset found 37 OTUs common to both approach and, of those, seven were detected from the same wood sample (Table S1).

3.2. Ecological roles and wood decay fungi

For ITS and LSU sequencing respectively, 166 and 145 distinct taxa were assigned to 18 functional guilds. From cultures, 43 taxa were assigned to 13 guilds. In total, 19 functional guilds were detected, and the relative proportions of guilds across the three approaches were

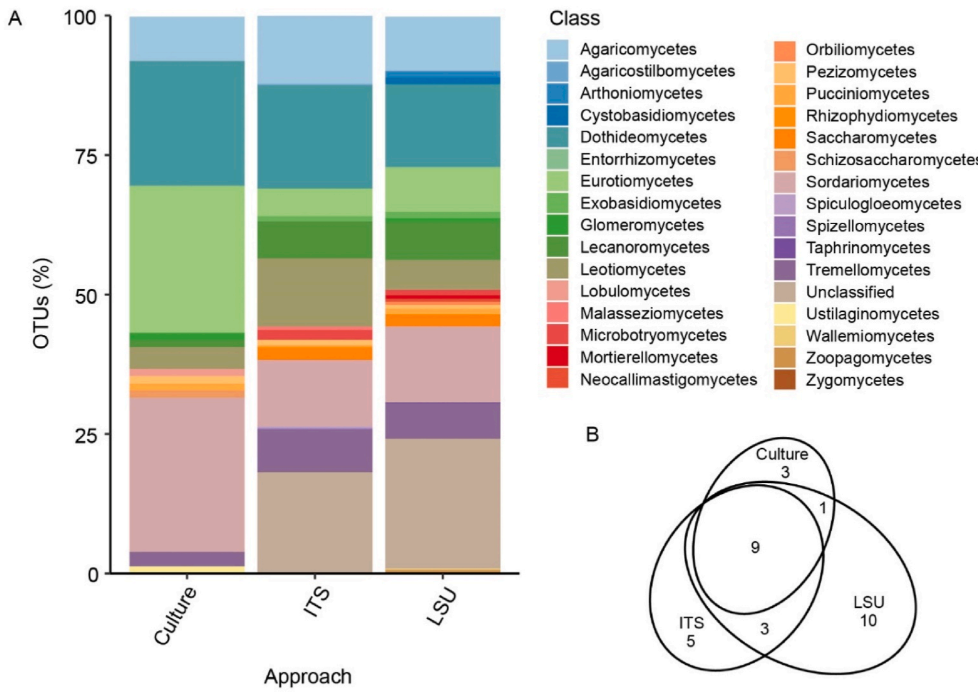

Fig. 2. Comparison of distinct fungal taxa (OTUs), defined at class-level, found by the different survey approaches. A - Percentage of classes within each dataset. B - Number of classes unique or in common to each dataset.

broadly similar (Fig. 3). 'Undefined saprotroph' and 'plant pathogen' were the most assigned guilds across all approaches. 'Animal pathogen' and 'endophyte' guilds occurred as a greater proportion of assigned taxa in the culture dataset than in the HTS sequencing datasets.

Wood decay fungi were detected in 101 (80%) of 126 samples by HTS (ITS: 44% and LSU: 71%) but relatively few were detected from two or more samples (Table 2). Whilst most decay fungi were identified via the FUNGuild database, some (ITS: 8 and LSU: 11) were also identified during manual inspection of the OTU table. Overall, more wood decay fungi were detected by ITS sequencing (37 OTUs) than by LSU sequencing (32 OTUs) and culturing (12 OTUs). The wood decay genera *Ceriporiopsis*, *Inonotus*, *Meripilus*, *Mycena*, *Phallus* and *Xylaria* were found by both ITS and LSU sequencing, but not culturing. *Ganoderma* was found by both ITS sequencing and culturing. Those that cause white rot were most frequently identified overall, although brown rot taxa and soft rot ascomycetes were also identified.

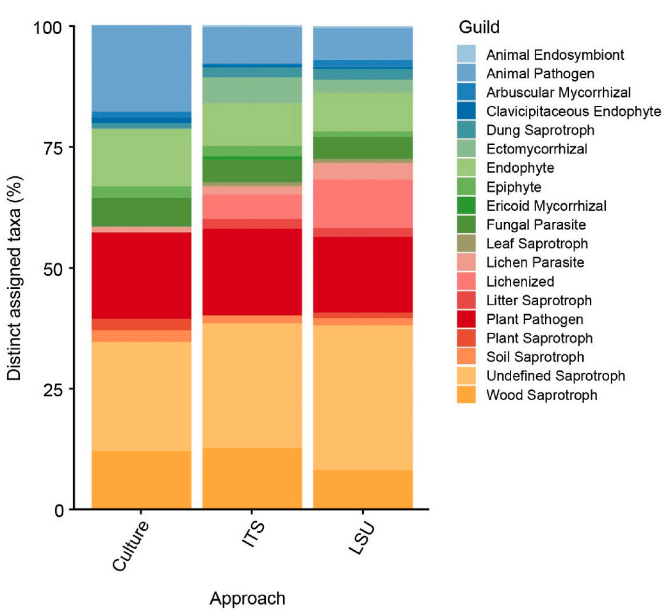

Fig. 3. Functional guild assignments as a percentage of distinct taxa by ITS or LSU sequencing and culture-based approaches.

3.3. Fungal diversity and community composition between trees

Considering all 66 trees on a one sample per tree basis (at 1.3 m above ground level), a total of 169 and 261 OTUs were detected (ITS and LSU, respectively; Fig. S2B). Most OTUs were detected once or twice, with 82 and 77% of OTUs detected in one or two different trees. 98 and 96% of OTUs were detected in ten different trees or fewer. The two most frequently detected OTUs were, for ITS, from the Dothideomycetes (41% of trees) and Malasseziomycetes (27%; Table S2). For LSU, the two most frequent OTUs were from the Eurotiomycetes (42%) and Pezizomycetes (26%; Table S3).

Fungal communities in trees at the same geographic location (site) appeared to be more similar than to trees at different sites (Fig. 4). There was a significant effect of site on community composition for both barcodes (PERMANOVA. ITS: *pseudo-F* $= 1.46$, $r^2 = 0.24$, $p = 0.05$. LSU: *pseudo-F* $= 1.66$, $r^2 = 0.22$, $p < 0.05$). This effect was not due to very different dispersions between site groupings (ANOVA. ITS: $F = 0.99$, $p = 0.46$. LSU: $F = 0.26$, $p = 0.98$). Correlations between geographic distance between sites and fungal communities were not significant for ITS (Mantel r = 0.27, p = 0.07), but were significant for LSU (Mantel r = 0.52, p = 0.001). The correlation between scaled climatic variables and fungal communities was also significant for LSU (Mantel r = 0.27, p = 0.04), but not for ITS (Mantel r = 0.10, p = 0.26). Partial mantel tests were not significant suggesting that spatial relationships are more important than climatic variables between our sites (ITS: p = 0.70; LSU: p = 0.69).

There was no significant effect of trunk diameter on community composition (PERMANOVA. ITS: *pseudo-F* $= 1.78$, $r^2 = 0.07$, $p = 0.08$. LSU: *pseudo-F* $= 1.53$, $r^2 = 0.05$, $p = 0.18$) (Fig. S3).

Table 2
The most frequently occurring wood decay fungi detected in all wood drilling samples (n = 126), according to their detection by ITS and LSU barcodes. These basidiomycete and ascomycete taxa include those identified as wood saprotrophs and their known rot type according to FUNGuild, unless otherwise indicated.

Taxon	Barcode		Rot Type[a]
	ITS	LSU	
Basidiomycota			
Antrodia	7		Brown
Collybia[b]		2	White[c]
Coriolaceae[b]		7	White
Cylindrobasidium[b]		2	White[d]
Fomitopsis	3		Brown
Ganoderma	10		White
Hyphodontia[b]		2	White
Inonotus	2	2	White
Lagarobasidium[b]		2	White
Meripilus	3	2	White
Phallaceae	1	3	White[d]
Pseudoinonotus[b]		2	White
Sistotrema	2		White
Stereaceae		3	White[e]
Trametes	2		White
Ascomycota			
Alternaria	10		Soft
Biscogniauxia	2		Soft[d]
Chaetosphaeriaceae		9	
Cladosporium	7	39	
Coniothyrium	2		Soft
Discula		3	Soft
Eutypa	5		Soft[d]
Fusarium		10	
Helotiaceae	3	4	
Penicillium		43	Soft
Periconia	2		Soft
Phialophora	3	13	
Phomopsis		3	Soft
Pseudeurotiaceae	4		
Pyrenochaeta		17	Soft
Xylaria	4	7	Soft

[a] Note that fungi are often able to operate different decay types under different scenarios, and even in different locations in the same piece of wood.
[b] Decay fungus not identified in FUNGuild but included by authors.
[c] Many *Collybia* species are litter decayers, *Gymnopus* (=*Collybia*) *fusipes* causes white rot of woody roots.
[d] From published sources other than FUNGuild.
[e] Most common rot type seems to be white rot but *Stereum sanguinolentum* can also cause brown rot.

3.4. *Fungal diversity and community composition within trees*

Of the five trees sampled at different heights and aspects around the trunk circumference (13 samples each), an average of 68 and 97 OTUs (ITS and LSU, respectively) were detected per tree (Fig. S4). Fungal communities in these samples appeared to cluster at the level of the individual trees (PERMANOVA. ITS: *pseudo-F* = 2.96, $r^2 = 0.24$, $p < 0.01$. LSU: *pseudo-F* = 2.47, $r^2 = 0.18$, $p < 0.01$) (Fig. 5). This effect was not due to very different dispersions between site groupings (ANOVA. ITS: $F = 1.65$, $p = 0.18$. LSU: $F = 1.74$, $p = 0.16$). There was no effect of height on fungal community composition (PERMANOVA. ITS: *pseudo-F* = 1.25, $r^2 = 0.09$, $p = 0.31$. LSU: *pseudo-F* = 1.66, $r^2 = 0.10$, $p = 0.12$) (Fig. S5). Fungal communities in samples taken at the same height above ground but on different sides of trunks did not differ significantly (PERMANOVA. ITS: *pseudo-F* = 0.53, $r^2 = 0.10$, $p = 0.79$. LSU: *pseudo-F* = 0.24, $r^2 = 0.04$, $p = 0.98$) (Fig. S6).

Wood decay fungi were detected throughout the trunks and branches sampled (Table 3). These were mostly in the orders Agaricales, Hymenochaetales and Polyporales.

4. Discussion

4.1. *Wood decay fungi*

In this study, we demonstrated that wood decay fungi are present in functional sapwood of beech trees; they were detected in most (80%) wood samples collected. Wood decay fungi were detected throughout trees sampled at multiple points on the trunk and limbs. These results show that living trees already contain taxa capable of causing significant decay of all wood components, potentially many years before the sapwood becomes dysfunctional and available for overt colonisation. Of the wood decay fungi found, ascomycetes and basidiomycetes causing white, brown and soft rot were all represented. These included generalists, such as *Trametes,* and those more usually recorded with other tree hosts, such as *Pseudoinonotus*. The presence of known beech decayers and heart rot species supports the notion that our datasets realistically reflect fungal communities in beech trees.

Heart rot fungi are thought to have strong associations with particular tree hosts. For example, the species *Ganoderma adspersum*, *Kretzschmaria deusta* and *Meripilus giganteus*, are among the most important in beech in the UK (Cartwright and Findlay 1946; Rayner and Boddy 1988; Gilmartin 2020). *Eutypa spinosa* and *Biscogniauxia nummularia* are other known beech associates, causing extensive strip cankers on trunks following drought conditions (Hendry et al. 1998). These species were all detected in functional sapwood in the present study and, though they may colonise trees via other routes, are now also confirmed as latently present. The presence of *Fomitopsis*, both in trunks and branches, is notable. *Fomitopsis pinicola* is an exceptionally common heart rotter of beech in continental Europe, but rare in Britain (Abrego et al. 2017). Many other wood decay species, more commonly associated with other tree taxa or beech at later stages of decay, were also detected, reflecting the non-selective ease of entry. However, while these species are not currently associated with standing beech trees, they may be

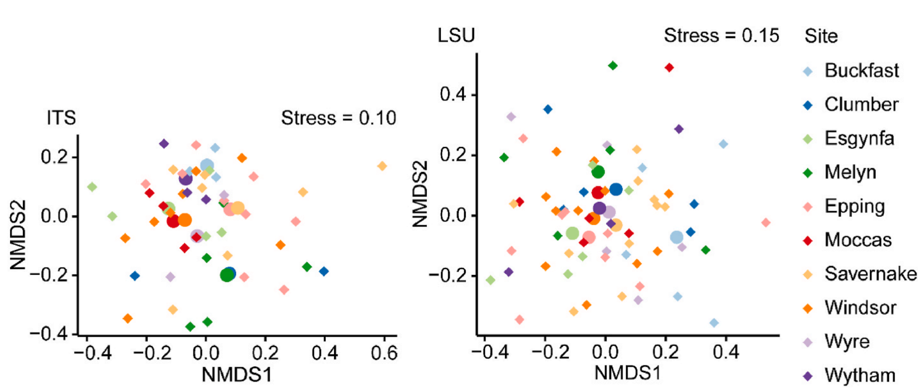

Fig. 4. NMDS ordinations of fungal communities for all trees sampled once at breast height (n = 66), according to ITS and LSU barcodes. Plots are based on Raup-Crick distance matrices, k = 3, dimensions 1 and 2 are displayed. Individual points are coloured by site, whilst the larger points represent centroids for each.

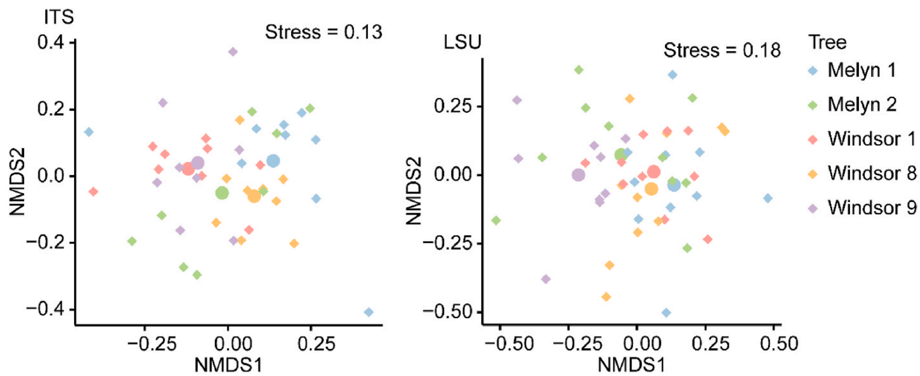

Fig. 5. NMDS ordinations of fungal communities for samples (n = 65) taken from five trees (n = 13 drill holes per tree), according to ITS and LSU barcodes. Plots are based on Raup-Crick distance matrices, k = 3, dimensions 1 and 2 are displayed. Individual points are coloured by tree, whilst the larger points represent centroids for each.

Table 3
The wood decay basidiomycetes and ascomycetes (only Xylariaceae) detected in the five trees subset (n = 65), according to detection by ITS and LSU barcodes.

Sample			Barcode	
Type	Number	Height (m)	ITS	LSU
High branch	10	18.0	*Antrodia, Inonotus, Serpula*	*Inonotus*
	9	14.1	*Fomitopsis, Ganoderma*	*Radulomyces*
	8	10.5	*Ganoderma, Sistotrema, Trichaptum*	
Low branch	6	11.2	*Biscogniauxia*	*Cylindrobasidium, Xylaria*
	5	8.8	*Antrodia, Meripilus*	*Lagarobasidium*
	4	6.7	*Antrodia, Eutypa, Meripilus*	
Trunk	7	7.8		*Hyphodontia, Pseudoinonotus*
	3	4.4	*Biscogniauxia, Ceriporiopsis, Eutypa, Mycena, Ganoderma, Sistotrema, Sistotremastrum, Xylodon*	
	2N	1.3	*Amyloporia, Ganoderma, Lentinus, Trametes*	*Collybia, Xylaria*
	2E	1.3	*Antrodia*	
	2S	1.3	*Kretzschmaria*	*Xylaria*
	2W	1.3	*Coniophora*	*Xylaria*
	1	0.5		*Meripilus*

under future climate scenarios, as which latent propagules develop as mycelia depends on drying regime, temperature etc. (Chapela and Boddy 1988; Hendry et al. 2002). Indeed, there is already evidence that early colonisers of standing beech wood are changing (Gange et al. 2011).

As only five trees were sampled at different heights, our first conclusions are tentative. Some known beech associates were detected high in the crown branches, contrary to general perceptions about their ecology i.e. that they cause basal decays or 'butt rots' (Cartwright and Findlay 1946; Schwarze et al. 2000; Boddy & Rayner 1988). *Ganoderma* species, for example, are usually considered to be butt rotters and *Meripilus* to rot woody roots. Their detection in branches may be entirely incidental, or it may reflect the limitations of assigning ecological strategies based historically on sporocarp observations.

Different sites inevitably have different fungal species pools, in the air, soil, and other substrates from which trees can be colonised. Differences in these species pools among sites can cause spatial variation in fungal community structure and influence later successional communities via priority effects (Hiscox et al. 2016). In the present study, the endophyte communities seemed to cluster according to site, whilst also varying substantially between trees and within-tree samples. The site differences detected were more correlated with geographic distance between sites than with site-level climatic variables. Realised endophyte and subsequent decay communities are thus the result of an interplay between localised species pools and random spatial and temporal colonisation. More intensive surveys, comparison of other plant tissues and of the air and soil spora between sites is necessary for further elucidation.

4.2. Functional guilds

The plant-endophyte symbiosis has been described as a continuum from mutualism to parasitism (Schulz and Boyle 2005; Sieber 2007). Even within functional sapwood, taxa from a wide range of functional guilds were detected, including both wood decay and non-wood decay species. Unlike in leaf tissue, it is unclear what role, if any, fungal taxa may have in xylem elements when the sapwood is functional in conduction of water, though there could be intimate associations with living parenchyma cells. It is more likely that these endophytes have passively accumulated in xylem, which is a 'dead end' for all except some of the wood decay species. The classification as 'endophyte' by FUNGuild, provides little insight to their role within functional sapwood — simply meaning that they have been detected in plants previously.

The diversity and number of sequences detected implies that entry into functional sapwood is relatively easy and common. Despite the diversity of fungi detected, these taxa are likely to be truly present, rather than contaminants from other sources. Entry to xylem could be via small wounds to roots or aerial tissues, made by physical factors or feeding invertebrates. It could also be through natural openings and via other tissues, e.g. leaves or by growth through the cambium from bark.

4.3. Comparability of approaches

HTS generated more OTUs than did the culture-based approach and can thus be recommended for general characterisation of fungal communities in functional sapwood. Taking all samples together, LSU detected more OTUs than did ITS. However, each approach, even at the class level, detected OTUs that the other approaches missed. All survey approaches suggest that detection of fungi was highly variable at single sampling points, such that multiple samples of a tree or across a site yielded the most species.

Primers and primer sets are known to have biases, with ecologists cautioned to interpret patterns carefully (Schadt and Rosling 2015). The ITS universal fungal barcode is reported as not optimal for use with samples containing low fungal biomass (Nilsson et al. 2018), and also for many Ascomycota (Skelton et al. 2019) and early-diverging fungi (Reynolds et al., 2022). We counteracted this potential issue by additionally using a separate LSU primer set which amplified less plant host DNA and a higher diversity of fungi. However, inclusion of the ITS approach was ultimately justified as we amplified more Agaricomycetes and wood decay taxa with the ITS primers.

Cultures yielded fewer numbers of OTUs, lower diversity and detected proportionately different functional guilds, as is also noted in studies of foliar endophytes (Unterseher et al. 2013). Culturing methodologies vary, and can impact the number and identity of fungi isolated from wood (Unterseher and Schnittler 2009). Wood drillings are highly comminuted samples, providing a markedly different microenvironment compared with larger samples, like wood chips. Many fungal hyphae present in the wood will inevitably be broken during the sampling process, probably reducing the number of isolates obtained. Thus, multiple culture methods are ideally needed, but culturing in general is labour-intensive and of declining overall use in fungal studies. Overall, the approach supported the interpretation of molecular-based detection methods and it revealed additional taxa to the HTS approach used, perhaps due to PCR biases, primer choice, and our plating of very diluted samples onto agar media. Further, cultures can add value to studies as they can be used for future assays and experimentation.

5. Conclusions

This study presents a fungal mycobiome of sapwood from healthy beech trees. Together, ITS and LSU barcodes recovered a wide diversity of fungi and generated more total OTUs than Sanger sequencing of fungal cultures, including species which are, as yet, unculturable. Along with adopting many of the recommended practices for HTS sequencing (Nilsson et al. 2019; Zinger et al. 2019) to ensure robustness of results, the additional fungal cultures validate the true presence of these taxa in sapwood, demonstrating the continued need to corroborate results in microbial ecology. The identity and number of fungi detected suggests that entry into functional sapwood is a regular event, though propagules seem sparsely distributed in wood. Endophyte communities differed between sites, trees and locations within trees. White, brown and soft rot fungi were detected, including those that cause heart rot, demonstrating that the origins of decay are present in the functional sapwood of healthy trees.

Data availability statement

Raw Ion Torrent sequence data are deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (accession PRJNA803183).

Declaration of competing interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

This work was funded by Cardiff University, City of London Corporation, The Crown Estate, Natural England and Fund4Trees. Thanks to Jonathan Palmer for AMPtk support and Jennifer Hiscox for pilot work which led to this study. Our sincerest gratitude is owed to the many field assistants and landowners who enabled the sampling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101161.

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