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Endophytes dominate fungal communities in six-year-old veteranisation wounds in living oak trunks

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ABSTRACT

Old trees are rare in the landscape, as are many of their associated species. Veteranisation is a method by which attempts are made to create microhabitats, otherwise found only in old trees, in younger trees at an earlier stage than would occur naturally. Here, we analysed the early fungal succession in 6 y-old veteranisation wounds in ca. 100 y old living oak trunks by DNA-barcoding of the wood at eight sites in Sweden and Norway. We hypothesised basidiomycetes would be most abundant, and exposed sapwood and heartwood would select for different communities. We identified 686 fungal taxa, mainly ascomycetes, with a large overlap in species composition and surprisingly similar species richness, i.e. 325 vs. 308–360, between intact and different types of damaged wood, respectively. Endophytes continued to be present and common in damaged wood. The results demonstrate that damage to sapwood and heartwood partly select for different fungi and that 6 y is too early to evaluate if veteranisation can positively favour fungi of conservation interest.

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1. Introduction

In central and northern Europe, oaks (*Quercus robur* and *Q. petraea*), especially old ones, provide unique habitats and support an exceptionally high biodiversity, and are thus often considered as biodiversity hotspots (Molder et al., 2019). Frequently living in closed forests for 300 y, 400–600 y in open woodlands, and more than 1000 y under optimal conditions (Niklasson and Nilsson 2005; Drobyshchev and Niklasson 2010), oaks have a higher species richness and associated organism diversity per tree than any other tree in Fennoscandia. This in the context of the species only constituting 0.3–1.3% of the tree biomass (Tingstad et al., 2018). A recent

compilation reports that almost 1800 species are associated with oaks in Sweden (Sundberg et al., 2019).

This is largely due to the longevity of oaks and the variety of microhabitats forming over time such as hollows with wood mould and coarse bark structure. As a result, many associated fungal and insect species are dependent on old oak trees (Nilsson 2006; Jansson et al., 2009). In total, about 770 oak-associated species are nationally red-listed in the Nordic countries; about 400 of these species are wood-inhabiting, of which about 300 are beetles and 50 are fungi (Tingstad et al., 2018).

Although Sweden, England and Romania in particular have relatively large concentrations of old oaks in Europe, these are declining (Farjon 2017; Hartel et al., 2018). Since the late 1800s, as much as 80% of the old oaks have been removed from the Swedish landscape alone (Eliasson and Nilsson 2002). A similar and more severe pattern has also occurred elsewhere in Europe and as a

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result, old oaks are rare or absent in many parts of Europe, with remaining trees in fragmented populations or as individuals (Pilskog et al., 2016). The remaining areas with old oaks are often lacking middle-aged oaks, putting the landscape continuity of old oaks and the associated biodiversity under threat.

As oaks age, the tree microhabitat diversity increases with dead branches, coarse bark and different types of decayed wood that may persist for centuries available for other organisms to exploit (Nordén et al., 2004a; 2004b). The amount of dead wood in the crowns of oaks, i.e. dead branches, start to become significant at the age of 150–200 y and increases as the trees get older. Oaks with hollows are normally older than 200 y. For example, when oak is 200–300 y old, ca. 50% of the oaks have hollows (Ranius et al., 2009). The non-functional heartwood of oak trees has a special chemical composition that generally limits fungal growth. The heartwood has also a more extensive gaseous phase than functional sapwood, allowing pathogens, general opportunists in wounds, and latent fungi already present in the sapwood to colonise and initiate decay in oaks while the tree is still standing (Boddy 2001). Indeed, Boddy (2001) demonstrated that for many tree species, latent propagules (yeast, mycelial fragments, spores) already present in the wood, but unable to germinate due to poor nutrient availability and high water content, may activate once drying of the sapwood begins. The decay in living standing oaks is a protracted process as the passive and active defence mechanisms of functional sapwood effectively prevent fungal activities, and fungi are restricted to the heartwood or sapwood exposed to the air and thus dry (Shortle and Dudzik 2012).

Dead wood provides habitats for numerous organisms including wood decay fungi (Siitonen 2001), the activity of which result in decaying wood. The abundance and richness of these fungi correlates with the amount of dead wood (Sandström et al., 2019). Studies comparing fungal communities in non-functional sapwood and heartwood within trees are quite scarce, but suggest they become more similar as decay progresses (Leonhardt et al., 2019). However, in living trees, most decay occurs in the heartwood, while in the fallen trees most rapid decay takes place in the sapwood (Boddy et al., 2017). There are five main colonisation strategies by wood-decay fungi in standing trees: heart-rot; opportunism – wound colonisation; special opportunism – latent invasion of functional sapwood; active pathogenesis and secondary colonisation (Boddy et al., 2017 and references therein). Wood decay fungi have been extensively studied in conifers (e.g. Kubartova et al., 2012; Ovaskainen et al., 2013; Ottosson et al., 2015) due to their wide distribution and great economic importance, but to a lesser extent in broadleaved trees such as oaks (*Quercus* spp.) (Heilmann-Clausen et al., 2005; Parfitt et al., 2010; van der Wal et al., 2016; Holec et al., 2019). Such studies may generate valuable information and better understanding of the biology, succession and dynamics of wood inhabiting fungi in living oaks, and provide guidelines for management of these trees and their associated biodiversity including red-listed species.

Exposed wood can potentially facilitate the establishment of a number of different fungi with various backgrounds due to more niche opportunities (Boddy 2001; Bässler et al., 2010). Ecological adaptations and enzymatic differences between fungi from Ascomycota and lignin-degrading Basidiomycota may influence the fungal community structure over time and thus affect overall habitat formation. Living trees are also commonly inhabited by fungal endophytes, which are fungi with different functional roles that colonise and persist in different tissues asymptotically until the environmental conditions become favourable for these fungi (Sieber 2007), when they may respond to changes in substrate quality (Cline et al., 2018). These endophytes possibly influence many biotic processes in trees and under certain conditions may

shift to pathogenic or saprophytic life styles, thereby acting as pioneer decomposers and influencing the spatial and temporal active layer of biodiversity in their hosts (Agostinelli et al., 2018; Terhonen et al., 2019).

Veteranisation is the practice of damaging younger trees with the aim of primarily initiating wood decay earlier, in living trees, and which may support the development of valuable habitats usually only found on older trees (Read 2000; Lonsdale 2013; Hedin et al., 2018). Generally 150–200 y is needed for decay to develop in oaks, while veteranisation may help this to happen much earlier. This management strategy may be a promising method to diversify available habitats for species confined to specific niches, i.e. species of conservation interest, through the creation of cavities, dysfunctional sapwood or exposed heartwood, but needs to be scientifically evaluated. Bengtsson et al. (2013) have carried out veteranisation treatments on 700, 50–100 y-old oak trees (280 additional trees were left as controls) in 20 sites throughout Sweden, Norway and England between 2011 and 2012. The aim was to initiate the development of decay and thus the formation of hollows with wood mould, to benefit fungi and threatened beetles that prefer hollow oaks as their habitat, particularly on sites where there is a generation gap (Hedin et al., 2018).

At a subset of these veteranisation sites, we investigated the richness and species composition of wood-inhabiting fungi in veteranisation wounds of living oaks i.e. to what extent, at least initially, they may contribute to the fungal communities developing after wood damage and whether different types of damage may select for varying fungal communities. Our overall aim was to get a better understanding of the biology, succession and dynamics of wood inhabiting fungi in living oaks. In addition, the long-term aim was to see whether veteranisation may facilitate oak fungi of conservation interest to establish in younger trees than naturally observed. In comparison with intact oak trees, we hypothesised that wood damage (veteranisation) i.e. wood exposure would increase fungal species richness due to changes in wood physical and chemical properties thus favouring fungal colonisation. We also expected that there would be a higher abundance of wood-decomposing fungi, i.e. basidiomycetes and fewer ascomycetes, e.g. mostly endophytes. We expected these changes to be stronger with bark damage causing exposed sapwood than cavity damage exposing heartwood and that these types of damage partly select for different fungal species. We expected 6 y-old wood damage to largely be dominated by other species than those present as endophytes prior to the damage.

To test these hypotheses, we used a high-throughput sequencing method that is a sensitive and powerful tool to explore fungal diversity directly from environmental samples (Tedersoo et al., 2014). However, there are potential risks including methodological biases, limitations of markers and bioinformatics challenges (Lindahl et al., 2013). Among these, the abundance of genetic markers may not well reflect biomass in the samples, and therefore, results should be interpreted with caution. Therefore, we used both qualitative and abundance of genetic markers as semi-quantitative information in our analyses.

2. Materials and methods

2.1. Study sites

The sites with veteranised oaks (*Q. robur* and/or *Q. petraea*) represented a broad geographical distribution of oaks in the area (Fig. 1) (Bengtsson et al., 2013). These sites were originally selected to contain even-aged oak stands, not exceeding 120 y of age. Due to their age, the oaks did not display any veteran tree features; if they did, these trees were excluded from the trial. On each site, 49 oak



Fig. 1. Map of Northern Europe showing sampling sites denoted by triangles. Sites are numbered as in [Table 1](#). Oak distribution for the area is highlighted in grey ([Dumolin-Lapegue et al., 1997](#)).

trees 25–60 cm in girth at breast height, of which 35 (14 control trees) were subjected to different veteranisation treatments using a chainsaw as described in [Bengtsson et al. \(2013\)](#). Our sampling was carried out in 2018 i.e. up to 6 y after the establishment of veteranisation treatments, on eight such sites, seven in Sweden and one in Norway ([Fig. 1](#), [Table 1](#)).

2.2. Veteranisation treatments

At each site, we randomly selected non-treated control trees (C) and trees subjected to three different veteranisation treatments ([Fig. 2](#)). Additional information on these trees is in [Bengtsson et al. \(2013\)](#). Horse damage (H) treatment was created by removing the bark of the tree from 1/3 of the girth of the trunk from ground level up to 1 m height. Bark was also removed from aboveground roots where possible. Nest box (N) treatment consisted of a rectangular hole, 1/3 of the stem diameter wide and at least 10 cm deep and 50 cm long, at 4 m from the ground. From the extracted piece of wood, the top 10–15 cm was cut off, the heartwood was removed

and the remaining part was placed back into the rectangular hole leaving a gap at the top and creating a hollow behind. Woodpecker holes (W) were also sawn 4 m above the ground to create a 10 cm deep, 8 cm wide and 12 cm long oval opening, carved into the centre of the trunk (see photos in [Hedin et al., 2018](#)) ([Supplementary Figs. 1A–C](#)). The H treatment resulted in exposed non-functional sapwood and the N and W treatments in exposed sapwood and heartwood. Primarily sapwood and some heartwood was sampled from C and H, while only heartwood from N and W. Unfortunately, we did not specifically separate the sapwood and heartwood samples from C and H.

2.3. Sampling

Sampling included three random trees of each C, H, N and W treatments at each site, in total, 12 trees per site, and 96 trees across the eight sites. At each tree, four wood samples were taken by drilling into the wood using a 10 mm diameter wood drill and ca. 5 cm deep into the wood ([Fig. 2](#)). Drills were sterilised by gas

Table 1

A summary of the number of the high-quality ITS rDNA fungal sequences and detected richness of fungal taxa in oak trunks at the sampled sites from the control (C) and the three treatments: horse damage (H), nest box (N) and woodpecker hole (W). For C, N and W, the number of trees per site equals 3, in total 24 trees. For H, the number of trees per site 2 equals 2 and the remaining sites 3, in total 23 trees.

Site No. ^a	Treatment		Horse damage (H)		Nest box (N)		Woodpecker hole (W)	
	Control (C)							
	No. of sequences	No. of fungal taxa	No. of sequences	No. of fungal taxa	No. of sequences	No. of fungal taxa	No. of sequences	No. of fungal taxa
1	1301	56	6687	82	2831	91	3689	76
2	885	106	3211 ^c	60 ^c	2990	70	8920	136
3	879	32	3681	65	2754	67	3789	84
4	5 ^b	3 ^b	8336	67	2444	45	2869	79
5	868	93	6114	132	4358	90	4222	101
6	826	83	6388	97	7741	77	13,346	62
7	1492	108	10,027	80	8193	108	16,665	125
8	718	103	5507	106	3897	80	5033	81
All	6974	325	49,951	311	35,208	308	58,533	360

^a (1) Berg Fengsel, (2) Ekenäs, (3) Haga Ekbackar, (4) Hjälmshult, (5) Strömsholm, (6) Tinnerö, (7) Vanserum, (8) Västerby.

^b Sequencing gave a very low amount of high-quality reads.

^c Data represents two trees; sequencing from the third tree was unsuccessful.

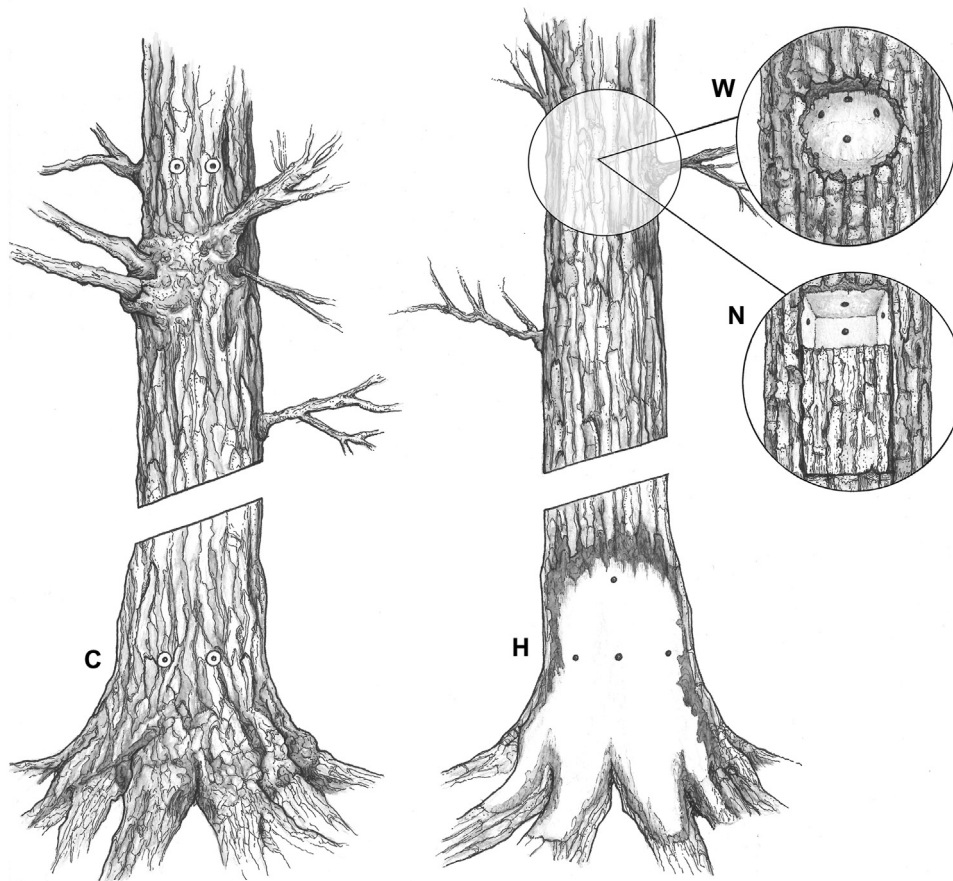


Fig. 2. Graphical representation of different treatments and sampling on oak trees. Control tree (C) with bark removed from surrounding drilling sites, drilled twice at 0.5 m from ground and twice at 4 m. Horse damage (H) treatment, with four drill sites around 0.5 m from ground, 5 cm away from damaged edge. Woodpecker hole (W) and nest box (N) treatments 4 m above ground, each drilled four times inside the upper area of the damage. Note that the treatments illustrated were all on separate trees.

flaming between each tree. Control trees were drilled at two heights, 1 m and 4 m from the ground to match the locations of the treatments; two holes were horizontally drilled ca. 12 cm apart at each height and these samples represented primarily functional sapwood (Fig. 2). To minimise contamination of fungi from the bark (often colonised by crustose lichens forming soredia), and prior to the drilling of control trees, the bark was removed using a 14 mm flat wood drill bit. The samples were then taken from the centre of

the area where the bark had been removed. The samples from the H, N and W treatment were taken with the wood surface intact. H treatment trees were drilled 1/2 m from the ground and 5 cm from the sides of the vertical edges, in the middle of the damage and 5 cm from the upper horizontal edge (Fig. 2). N treatment trees were drilled directly into the back of the cavity: ca. 5 cm from the box ceiling, ca. 5 cm to the left, right, and top of the initial hole (Fig. 2). W treatment trees were also drilled into the back of the

cavity, ca. 5 cm to the left, to the right, and at the top of the hole (Fig. 2). The sawdust samples were collected on sheets of aluminium foil, pooled for each tree and stored in Ziploc plastic bags. In treatment N and W, aluminium foil was placed on the bottom and unfolded along the cavity edges to avoid contact with any surfaces e.g. the bark or exposed wood. After drilling, the aluminium foil with sawdust was carefully folded before being removed (Supplementary Fig. 2). After removal, the sawdust was collected and tipped into Ziploc plastic bags. Collected sawdust samples were placed in -20°C for storage on the same day.

2.4. DNA work

Individual sawdust samples used for DNA extraction were weighed at 2-g increments and sealed in filter paper packages that were freeze-dried at -105°C for 72 h in a VirTis SP Scientific Freeze Dryer (SP Industries Inc., Suffolk, UK). Remaining sawdust was stored at -20°C . For DNA extraction, 0.4 g of freeze-dried sawdust from each tree was placed separately into a 2 mL screw cap centrifugation tube (resulting in 96 tubes) and homogenised with metal bolts in a 3% CTAB solution in a Precellys 24 homogeniser (Bertin Corp, Rockville, MD, USA), incubated for 1.5 h at 65°C and cleaned with chloroform. The resulting upper phase was then purified using the Techtum NucleoSpin soil kit (Macherey-Nagel, Düren, Germany) according to manufacturer's recommendations. The quality and quantity of resulting DNA products were evaluated with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and DNA concentration adjusted to 10 ng/mL.

Each sample was amplified separately using a primer pair gITS7 and ITS4 (Ihrmark et al., 2012) each containing sample identification barcodes resulting in 96 barcoded samples. PCR was performed in 15 μL reactions containing 1.2 μL of DNA template. Each reaction included 1% of Taq Polymerase (5 u/ μL) (DreamTaq Green, Thermo Scientific, Waltham, USA); 11% of 10 \times Buffer; 11% of dNTPs (10 mM); 1% of MgCl_2 (25 mM); 2% of each primer (200 nM) and 72% of Milli-Q water. Samples were placed in an Applied Biosystems 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) and ran for 35 cycles. Amplification consisted of a 2 min 95°C initial denaturation and 30 s 95°C denaturation, a 56°C annealing for 30 s, a 72°C extension for 1 min, and a final 72°C extension for 7 min. Resulting PCR products were loaded onto 1.5% agarose gels (Agarose D1, Conda, Madrid, Spain) and following electrophoresis at 300 V for 30 min scanned via QuantityOne software (Piovanelli, 2006). Concentration of PCR products was evaluated using a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Following evaluation, the equimolar mix of 96 PCR products was produced and purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA). The quality of PCR mix was assessed using both NanoDrop 3300 fluorospectrometer and an Invitrogen QUBIT fluorometer (Fisher Scientific, Loughborough, UK). High-throughput sequencing was completed at SciLifeLab using the Pacific Biosciences (PacBio RSII) platform (Uppsala Genome Centre, NGI Uppsala, Sweden).

Bioinformatics Principles of bioinformatics followed a study by Menkis et al. (2015). The sequences generated were subjected to quality control and clustering in the SCATA NGS sequencing pipeline (<http://scata.mykopat.slu.se>). Quality filtering of the sequences included the removal of short sequences (<200 bp), sequences with low read quality, primer dimers and homopolymers, which were collapsed to 3 bp before clustering. Sequences that were missing a barcode or primer were excluded. The primer and sample barcodes were then removed from the sequence, however information on the sequence association with the sample was stored as meta-data. The sequences were then clustered into different taxa using single-

linkage clustering based on 98% similarity. The most common genotype (real read) for clusters was used to represent each taxon. For clusters containing two sequences, a consensus sequence was produced. Singleton taxa were removed following standardised practices (Nilsson et al., 2011). The fungal taxa were taxonomically identified using GenBank (NCBI) database and the Blastn algorithm (Altschul et al., 1997). The criteria used for identification were: sequence coverage >80%; genus level 94–97% and species level $\geq 98\%$. Sequences not matching these criteria were considered unidentified and were given unique names (Table 2 and Supplementary Tables 1 and 2). Representative sequences of fungal non-singletons are available from GenBank under accession numbers MT265805 - MT266525. We excluded all 40 detected fungal taxa belonging to Lecanoromycetes as they mainly represent lichen mycobionts and most likely originate from contamination from the bark (Supplementary Table 2). The results should be interpreted with caution as the possibility for contamination with other fungi (e.g. from the bark or air-borne spores) cannot be excluded in any of the samples/treatments. However, in this case, such contamination is likely to constitute only a small proportion of the total community. Following taxonomic identification, an ecological role was assigned for the 100 most common fungi as saprotroph, parasite or unknown. We used a broad concept of ecological roles; saprotrophs included both generalist saprotrophs and wood-decay fungi, while parasites included different plant pathogenic fungi. The assignment of ecological roles was based on information acquired from Hallingbäck and Aronsson (1998) and other published literature complemented with searches of the Nordic Saproxylic Network database described in Stokland and Meyke (2008) and available upon request from the Swedish Species Information Centre (www.artdatabanken.slu.se/en/) and of FUNGuild (Nguyen et al., 2016). The assignment of ecological roles was based on information from several sources to become as complete as possible. Conservation status was assigned according to the Swedish National Red List (Artdatabanken, 2015).

Statistical analyses Rarefaction analysis was performed using Analytical Rarefaction v.1.3 available at <http://www.uga.edu/strata/software/index.html>. As both qualitative and quantitative data of high-throughput sequencing has been shown to be consistent and highly reproducible (Porazinska et al., 2010), the number of reads was used to estimate the relative abundance of fungal taxa in the samples. Differences in richness of fungal taxa in different veteranisation treatments and control (data pooled from all sites) was compared by nonparametric chi-square test (Magurran 1988). As each of the datasets were subjected to multiple comparisons, confidence limits for p-values of chi-square tests were reduced a corresponding number of times as required by the Bonferroni correction (Sokal and Rohlf 1995). The Shannon diversity index, qualitative Sørensen similarity index and nonmetric multidimensional scaling (NMDS) using Bray-Curtis distance in Canoco 5 (Shannon 1948; Magurran 1988; ter Braak and Smilauer, 1998) were used to characterise the diversity and composition of fungal communities. MANOVA in Minitab v. 18.1 (Minitab® Inc, 2003) was used to evaluate the degree of separation (along NMDS axis 1 and 2) between the fungal communities in different veteranisation treatments and the control. The nonparametric Mann-Whitney test in Minitab was used to test whether the Sørensen similarity index and the Shannon diversity index among different treatments and controls were statistically similar or not.

3. Results

High-throughput sequencing of ITS rDNA from 96 pooled amplicon samples was successful for 95 samples (one H sample from Ekenäs gave no sequences) resulting in 458,329 reads. Quality filtering showed the presence of 191,587 (41.8%) high quality reads,

Table 2

The 20 most frequently detected fungi from 95 oak tree trunks from control (C) trees and three types of veteranisation damage i.e. horse damage (H), nest box (N) and woodpecker hole (W). The taxa are sorted according to presence in the number of most frequent trees. The table shows the percentage of trees the taxon was detected from (n = 24 C, N and W, n = 23 H); different colours indicate the degree of relative abundance: green – high, pale – moderate and reddish – low. Within each row, values with the same letter do not differ significantly at $p > 0.05$.

Fungal taxa	Treatments (% of trees)				All
	C	H	N	W	
<i>Penicillium carneum</i>	75 a	87 a	100 a	96 a	89
<i>Moristroma quercinum</i>	42 a	83 b	96 b	96 b	79
<i>Cladosporium langeronii</i>	63 a	57 a	75 a	83 a	69
<i>Monocillium tenue</i>	21 a	70 b	54 ab	71 b	54
<i>Collophora</i> sp. 3991_5	38 a	30 a	79 b	58 ab	52
<i>Ochrocladosporium</i> sp. 3991_16	38 a	70 a	42 a	42 a	47
<i>Phialocephala compacta</i>	50 a	61 a	33 a	38 a	45
<i>Querciphoma carteri</i>	21 a	39 ab	63 b	50 ab	43
<i>Helotiales</i> sp. 3991_41	17 a	65 b	42 ab	42 ab	41
<i>Trichosporonales</i> sp. 3991_43	17 a	65 b	42 ab	42 ab	41
<i>Hypocreales</i> sp. 3991_15	21 a	43 ab	38 ab	63 b	41
<i>Cadophora</i> sp. 3991_34	0 a	87 c	29 b	25 ab	35
<i>Chaetothyriales</i> sp. 3991_94	46 a	35 a	29 a	29 a	35
<i>Stereum hirsutum</i>	29 ab	65 b	17 a	21 a	33
<i>Chaetothyriales</i> sp. 3991_77	17 a	57 b	29 ab	29 ab	33
<i>Rhinochadiella atrovirens</i>	8 a	26 a	33 ab	63 b	33
<i>Malassezia</i> sp. 3991_56	63 a	22 b	25 ab	21 b	33
<i>Ophiostoma</i> sp. 3991_22	8 a	70 b	29 a	21 a	32
<i>Kockovaella</i> sp. 3991_24	21 a	17 a	17 a	71 b	32
<i>Sydowia polyspora</i>	17 a	30 a	46 a	29 a	31

which were retained, while the remaining 266,742 (58.2%) low quality reads were excluded from further analyses. Clustering of high quality reads showed the presence of 929 non-singleton contigs at 98% similarity representing different taxa. In addition, there were 1418 singletons, which were excluded. Taxonomic identification showed that 727 (78.3%) taxa were fungal and 202 (21.7%) were identified as non-fungal, and the latter were excluded. Among fungal non-singletons, taxa from the class Lecanoromycetes that include lichen-forming fungi, were considered as possible contaminants and were excluded, resulting in 686 fungal taxa (Supplementary Table 1). Information on fungal taxa from the class Lecanoromycetes is in Supplementary Table 2.

3.1. Richness of fungal taxa

In total, there were 325 fungal taxa in C, 311 in H, 308 in N and 360 in W, but the number of sequences obtained in the C was up to 8 times lower than in any of the three veteranisation treatments (Table 1). Rarefaction showed that the fungal taxa detected in the C and in the three different veteranisation treatments did not reach the species saturation (Fig. 3). When the same number of sequences had been taken from the control and each of the three treatments, species richness was significantly higher in the control than in any of the veteranisation treatments ($p < 0.0001$). A similar comparison, when the same number of sequences had been taken, among the three veteranisation treatments showed that these did not differ significantly from each other ($p > 0.05$) (Fig. 3).

3.2. Identified fungal taxa

In total, 67.9% of the detected fungal taxa were ascomycetes, 29.8% basidiomycetes, 1.9% mucoromycetes and 0.4% chytridiomycetes (Fig. 4, Supplementary Table 1). Of these, 25.3% were

identified to species level, 24.1% to genus level and remaining 50.6% to higher orders only. The 20 most frequently detected fungi from 95 oak trees represented 58.9% of the fungal sequences and occurred in all veteranisation treatments and controls (Table 2). The most common fungi were *Penicillium carneum* (89.4% of trees colonised/12.6% of all high-quality fungal sequences), *Moristroma quercinum* (79.0%/16.7%), *Cladosporium langeronii* (69.6%/2.8%), *Monocillium tenue* (53.8%/2.9%), *Collophora* sp. 3991_5 (51.4%/5.6%), *Ochrocladosporium* sp. 3991_16 (47.6%/0.9%), *Phialocephala compacta* (45.4%/0.8%) and *Querciphoma carteri* (43.1%/1.9%) (Table 2). Among the 20 most frequent fungi, the occurrence of *P. carneum*, *C. langeronii*, *Ochrocladosporium* sp. 3991_16, *P. compacta*, *Chaetothyriales* sp. 3991_94 and *Sydowia polyspora* did not differ significantly in the control vs. different treatments (Table 2). By contrast, the occurrence of *M. quercinum* was significantly lower in the control than in all veteranisation treatments ($p < 0.03$). The occurrence of the remaining fungi in the control vs. different veteranisation treatments was either significantly lower or similar (Table 2). This showed a certain habitat preference of particular fungi; e.g. as compared to control, the occurrence of *M. tenue* was significantly higher in H and W, *Q. carteri* in N, *Stereum hirsutum* in H, *Rhinochadiella atrovirens* in W, while the occurrence of *Malassezia* sp. 3991_56 was significantly lower in H and W (Table 2). The remaining 666 fungal taxa of the study were relatively rare (Supplementary Table 1).

3.3. Fungal community composition

Analyses were carried out both with and without Lecanoromycetes, showing that this class of fungi had no or negligible effect on the overall fungal community composition. Despite this, Lecanoromycetes were excluded (see above). Ascomycetes dominated fungal communities in C (72.1%), N (85.6%) and W (81.5%), but their

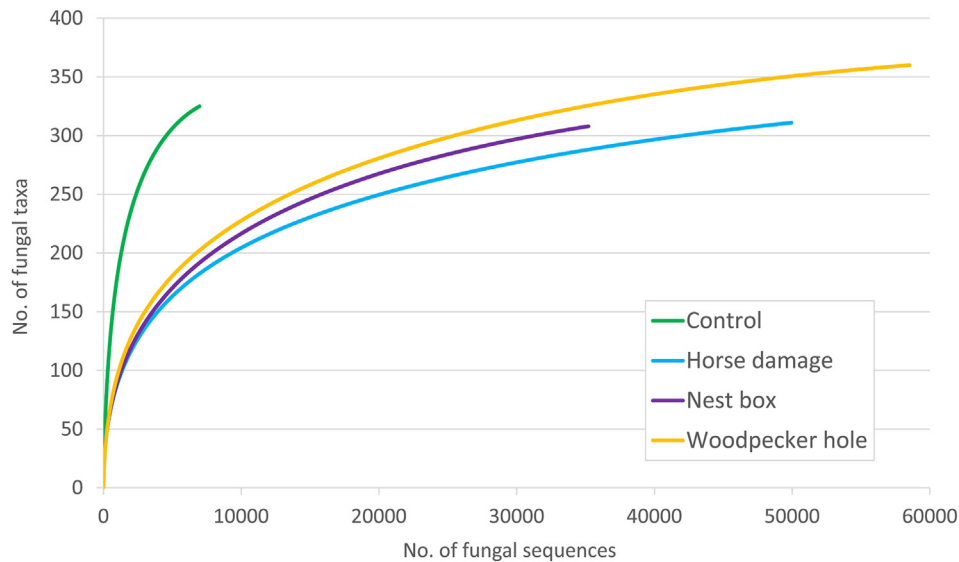


Fig. 3. Rarefaction curves showing the relationship between the cumulative number of fungal taxa and the number of ITS rDNA sequences from control and the three veteranisation treatments of oak trees.

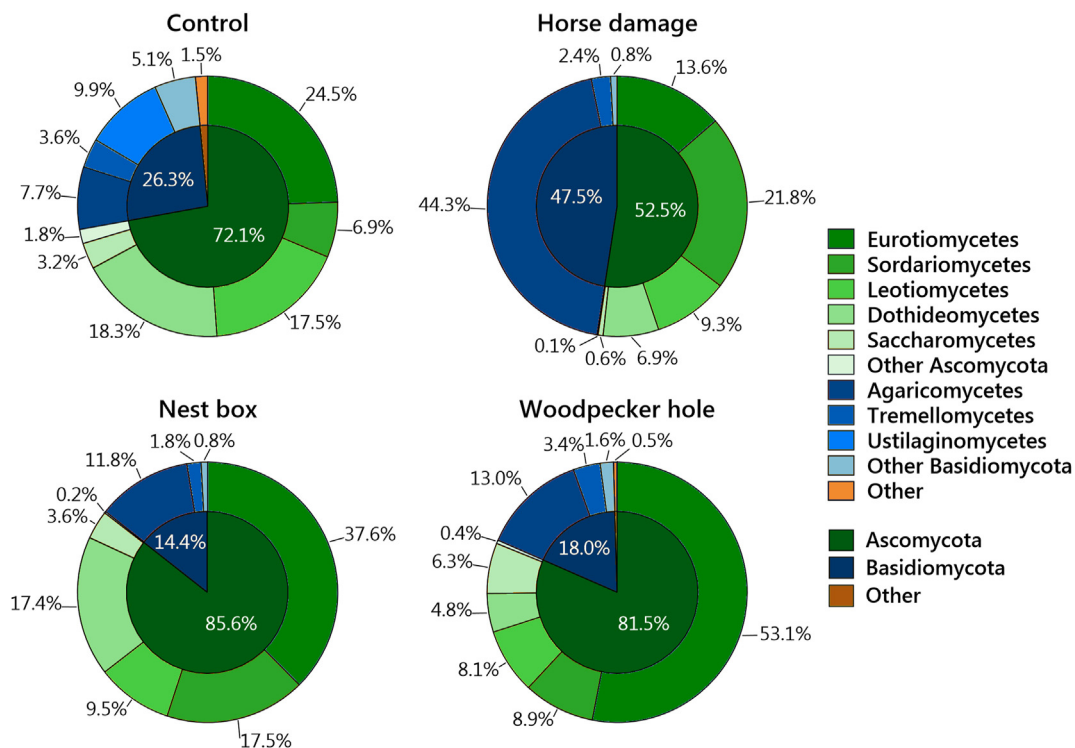


Fig. 4. Relative abundance of the fungal classes (outer circle) and phyla (inner circle) from control and veteranisation treatments (horse damage, nest box and woodpecker hole) of oak trees. Data from different sites is pooled. Classes and phyla comprising <1.0% of relative abundance for a given set of sequences were combined and shown as *Other*.

occurrence was lower in H (52.5%) (Fig. 4). Among ascomycetes, fungi from the class Eurotiomycetes were most common in C, N and W, while fungi from the class Sordariomycetes were most common in H (Fig. 4). Among basidiomycetes, fungi from the class Agaricomycetes were most common in H, N and W, while fungi from the class Ustilaginomycetes in control (Fig. 4). Nonmetric multidimensional scaling of fungal communities showed that fungal communities from the three veteranisation treatments were intermingled and largely separated from the control along the diagonal (Fig. 5).

MANOVA showed that this separation was statistically significant between the control and veteranisation treatments ($p < 0.0001$), but not when the three veteranisation treatments were compared with each other ($p = 0.196$). The Sørensen similarity index of fungal communities among the different treatments and control was moderate: C vs. H – 0.42, C vs. N – 0.45, C vs. W – 0.42, H vs. N – 0.54, H vs. W – 0.54 and N vs. W – 0.58. The Shannon diversity index of fungal communities at different sites was: 0.54–3.54 in C, 0.68–2.70 in H, 0.72–2.56 in N and 0.94–2.88 in W. Mann-Whitney

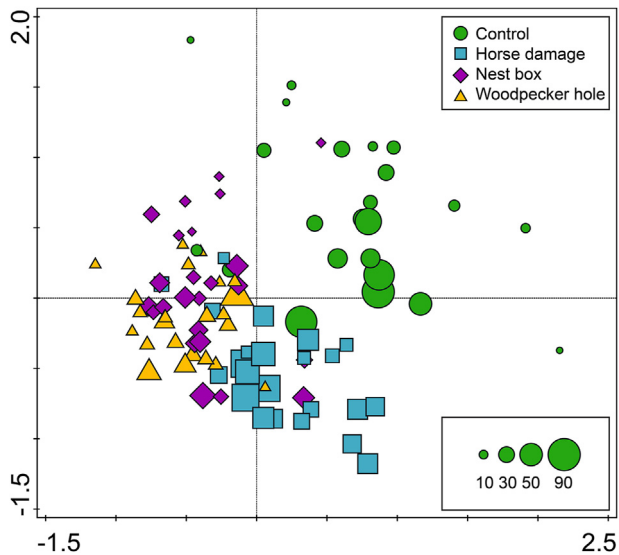


Fig. 5. Ordination diagram based on nonmetric multidimensional scaling of fungal communities in control and three veteranisation treatments (horse damage, nest box and woodpecker hole) of oak trees, with 40.8% variation on Axis 1 and 34.7% on Axis 2. Each point in the diagram represents a single tree and their size reflects richness of fungal taxa as indicated in the lower right corner.

test showed that both the Sørensen similarity index and the Shannon diversity index were statistically similar among all treatments and control ($p > 0.05$).

3.4. Fungal ecological roles

Fungal ecological roles were assigned to the 100 most abundant fungal taxa, representing 95.2% of fungal sequences (Fig. 6). In the C, H, N and W, the majority of ascomycetes taxa were unidentified (45.1–48.2%), followed by saprotrophs (21.2–23.8%) and the least common were parasites (4.7–7.3%) (Fig. 6). For basidiomycetes, the majority of taxa were saprotrophs (18.8–20.0%), followed by parasites (2.4–4.0%) and the least common were unidentified taxa

(2.4–3.6%) (Fig. 6). In the C and all treatments, the assessed saprotrophic basidiomycetes included eight (66.6%) taxa of wood-decay fungi, three (25%) of which were heart-rot fungi (*Laetiporus sulphureus*, *Xylobolus frustulatus* and *Inocutis dryophila*), two (16.7%) being oak specialists (*X. frustulatus* and *I. dryophila*). Another heart-rot fungus and oak specialist (in northern Europe) was *Fistulina hepatica*, but it was not among the 100 most abundant fungal taxa (Supplementary Table 1).

3.5. Species of conservation interest

Three red-listed fungi were recorded; *X. frustulatus* (Swedish red-list status NT) in two C and two N trees, *I. dryophila* (VU) in one N and one W tree and *F. hepatica* (NT) in one C tree (Supplementary Table 1). All records were with a low number of reads except for one record of *I. dryophila* in a W tree (0.4% of all reads) and one of *X. frustulatus* in a N tree (1.9% of all reads). In addition, *L. sulphureus* (LC) that is known to form hollows, was detected in five trees including two in C and one in each H, N and W. All records were with a low number of reads except a single record in the W (7.4% of all reads) (Supplementary Table 1).

4. Discussion

The results demonstrated that intact oak wood (C) was inhabited by a surprisingly species-rich (325) community of fungi, largely composed of unidentified taxa that occurred at low abundances (Fig. 3, Tables 1 and 2). In comparison, fungal culturing from 768 twig samples of 15 y-old *Q. robur* resulted in 28 fungal taxa (Agostinelli et al., 2018). Our results corroborate previous studies that in intact wood, fungi to a large extent occur latently, as e.g. propagules (Wilson 1995; Parfitt et al., 2010) as indicated by a low number of sequences obtained from the C (Table 1). The exposure of both sapwood (H) and heartwood (N and W) had only a minor effect on the absolute richness of fungal taxa, as the number of these 6 y after veteranisation treatments, remained similar to that in intact wood (C) (Table 1), thereby rejecting the hypothesis of an increase in species richness. Contrary to our expectations, the damaged wood areas on the veteranised trees were largely

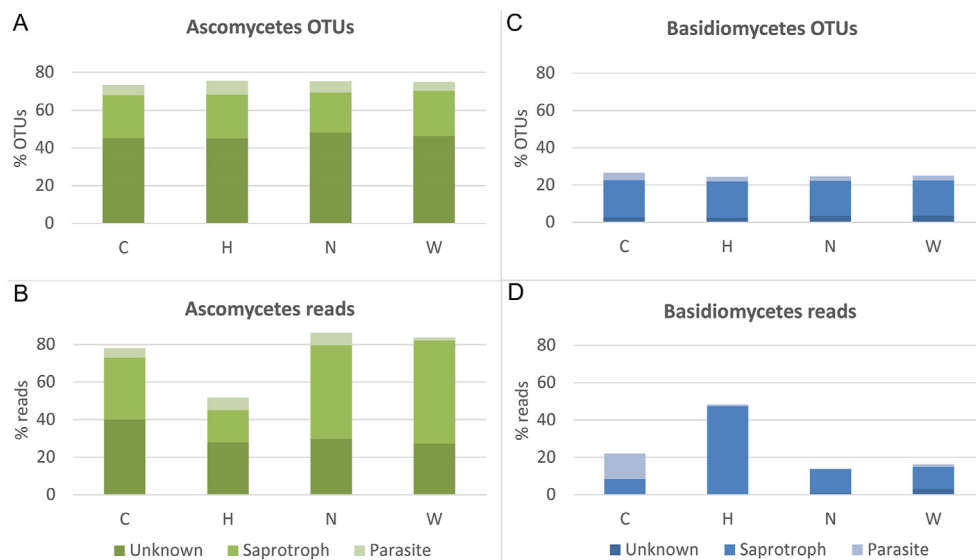


Fig. 6. Ecological roles of the 100 most frequent fungal taxa (OTUs) detected in oak wood at the control (C), and the three veteranisation treatments: horse damage (H), nest box (N) and woodpecker hole (W). Parameters are shown separately for Ascomycota OTUs (A) and reads (B), and Basidiomycota OTUs (C) and reads (D). Saprotrophs refers to wood decaying fungi and other saprotrophs.

dominated by the fungi that were present prior to the damage with only a few newly established fungal taxa. The latter may suggest that contamination of wood samples in the C by other fungi (e.g. from the bark or with airborne spores) was very low or absent. Contamination cannot, however, be ruled out, and there was some evidence of contamination by lichens. Similarly to our results, a slow development of fungal communities was also reported from 1 to 2 y-old decaying oak logs (van der Wal et al., 2016). However, oak veteranisation had in many cases a positive effect on the abundance of these fungi (Tables 1 and 2), indicating a higher activity and build-up of fungal biomass. The increased biomass was indicated by a much higher number of sequences obtained from wood of different treatments vs. the C trees (Table 1), thereby affecting the fungal community structure in wood from different treatments (Figs. 4 and 5). This complies with the fact that saprotrophic fungi that were present originally in lower abundances as endophytes have been shown to be responsible for initial decomposition of wood (Parfitt et al., 2010; Song et al., 2017). However, our data did not allow us to distinguish to what extent the increased abundance of fungal taxa originates from established endophytes and to what extent it originates from new external colonisations.

The fungal community analysis showed that different types of veteranisation partly selected for different groups of fungi (Fig. 4). Basidiomycetes were promoted in the H treatment and Ascomycetes were promoted in the N and W treatments, demonstrating that the choice of veteranisation treatment may directly affect the speed and magnitude of wood decay, and thus the formation of decayed wood habitats which are mainly created by basidiomycetes. The observed overlap of taxa, specifically between sapwood (H) and heartwood (N and W), could be, at least partially, due to the sampling strategy i.e. drilling 5 cm deep in exposed wood. This may have resulted in sampling of both sapwood and possibly some heartwood in the H and C, but this needs to be further investigated. Furthermore, the knowledge of exposed oak wood properties and the changes over time (e.g. how fast and deep with time such wood dries, the oxygen content changes and the wood decay proliferates) is scarce (Deflorio et al., 2008). Thus, the possibility should not be excluded that sampling in all veteranisation treatments may have resulted in some wood (from deeper in the tree trunks) that was similar to that in the C, thereby affecting the abundance and composition of fungal communities in all treatments (Figs. 4 and 5). On the other hand, the desiccation of veteranised sapwood over 6 y may have resulted in similar wood properties as in the heartwood, creating similar conditions for fungal colonisation. Furthermore, as we unfortunately lack a control for the heartwood, the comparison of fungal communities in the intact and veteranised heartwood is limited. However, these fungal communities might differ owing to possible differences in heartwood physical and chemical properties, and more fungal colonisation opportunities of exposed heartwood following veteranisation treatments. Nevertheless, the exposed heartwood (N and W) resulted in the development of similar fungal communities, particularly benefiting the fungi from the class Eurotiomycetes, which represents the species-richest endophyte class (Chen et al., 2015). Among those, *M. quercinum*, which was one of the most widespread and abundant fungi, showed a significantly higher relative abundance in all veteranisation treatments than in intact wood (Table 2). *Moristroma quercinum* was described in 2005 and is mainly recorded on the heartwood of attached and shed oak branches, and also from old stumps, suggesting that it occupies a distinctive niche of hard and dry dead wood (Nordén et al., 2005). This study expands the current knowledge, showing that *M. quercinum* is an endophyte in living oaks and an early coloniser of exposed sapwood and heartwood of ca. 100 y-old oak trunks. *Monocillium tenue* (Sordariomycetes) was another dominant fungus, which increased

significantly in abundance following veteranisation treatments, particularly in H and W treatments (Table 2). *Monocillium tenue* has been shown to be occasionally associated with the bark surface of oak (*Q. robur*) roots (Mischiati et al., 1992), and we found it to be a common endophyte in oak trunks. *Querciphoma carteri* (prev. *Pyrenochaeta minuta*) (Dothideomycetes) also increased in relative abundance in all veteranisation treatments, but especially in N (Table 2). This fungus is associated with dieback of oaks (*Quercus* spp.) (Carter 1941) and also on leaves and twigs of *Q. robur* (Crous and Groenewald 2017). *Rhinocladiella atrovirens* (Eurotiomycetes) showed a preference for W (Table 2), which could be due to a specific microclimate in this particular habitat compared to other treatments (Griffiths et al., 2018). *Rhinocladiella atrovirens* is a generalist associated with different coniferous and deciduous tree species (Lumley et al., 2001; Szewczyk et al., 2017). The horse damage (H) contained the highest percentage of the wood decomposing basidiomycetes, likely due to larger areas of exposed sapwood that selected for early colonisers such as e.g. *S. hirsutum* (Table 2). *Stereum hirsutum* has been shown to be the dominant fungus colonising oak wounds caused by elk 8–25 y previously (Vasiliauskas 1998). Among fruit bodies that were rare and only searched for on H (data not shown), we have also observed *S. hirsutum*, showing that in exposed sapwood this fungus was able to expand significantly and reproduce. *Peniophora incarnata* was another basidiomycete producing fruit bodies, which showed a strong preference for H (Supplementary Table 1). It is a generalist fungus as it commonly grows on a range of deciduous trees and less often on conifers (Boddy et al., 2008).

The assessment of fungal ecological roles implies that veteranisation treatments had little effect on functional diversity of fungal taxa in oak wood, which following the treatments remained similar to intact wood (Fig. 6). Although the majority of ascomycetes could not be identified to species or genus level, thereby limiting the interpretation of the results, in intact wood, sapwood and heartwood saprotrophs constituted a similar proportion of fungal taxa among both ascomycetes and basidiomycetes, while parasites were relatively rare. Larger differences were seen for sequence reads; ascomycete saprotrophs showed a reduction in sapwood and an expansion in heartwood as compared to intact wood, while basidiomycete saprotrophs expanded in all treatments, especially in sapwood. The latter suggests that activity of fungal saprotrophs depends on the substrate quality i.e. sapwood vs. heartwood. Interestingly, sequence reads of basidiomycetes in intact wood were dominated by parasites (Fig. 6), namely taxa representing Ustilaginomycotina that are known to have a dimorphic life cycle consisting of a saprobic haploid phase and a parasitic dikaryotic phase. The initial fungal community represented by the control trees displayed a higher portion of Lecanoromycetes than other sampled trees, probably due to contamination from the bark while sampling, and thus, were excluded from analyses. In support for doing this, there is little evidence that lichen mycobionts can exist as free-living fungi (Tuovinen et al., 2015).

The succession of fungal communities in oak wood and the development of decayed wood habitats appears to be a slower process compared to other tree species (van der Wal et al., 2016; Leonhardt et al., 2019), which is important to consider when managing oak trees and their associated biodiversity. Thus, it was too early to identify if any of the types of damage will be more advantageous regarding fungi of conservation interest or for other fungi forming favourable decay conditions for desired biodiversity. Nevertheless, the presence of at least three fungal taxa of conservation interest, i.e. *F. hepatica*, *I. dryophila*, and *X. frustulatus*, suggests that the wood of ca. 100 y-old oaks is a potentially suitable habitat for colonisation. While *F. hepatica* was only found in intact wood, both *X. frustulatus* and *I. dryophila* occurred in heartwood

(Supplementary Table 1). Similarly as *F. hepatica*, *X. frustulatus* and *I. dryophila* are wood decomposers that specifically contribute to hollow formation; both are threatened and mostly found in old oaks, thus their numbers are declining as suitable habitats are removed from the landscape (Sunhede and Vasiliauskas 1996; Artdatabanken 2015). Another detected wood decomposer was *L. sulphureus*, which contributes to oak hollow formation, thereby forming pivotal habitats for threatened beetles. Although the present study was done relatively early i.e. only 6 y following the treatments, the results as expected suggest that veteranisation has, in general, a positive effect on the establishment of wood decay fungi (Fig. 6 C, D) and that with time are likely to speed up wood decay and the formation of hollows. For example pollarding, that is a form of active tree management where branches are removed regularly, creates several different microhabitats, including hollowing more quickly (Sebek et al., 2013) and the establishment of a species-rich fungal community including species of conservation interest (Nordén et al., 2018).

In summary, oak wood was found to be exceptionally rich in fungal endophytes that continued to dominate fungal communities 6 y following veteranisation of oak trunks. The succession of fungal communities appeared to be a slow process, but the exposure of sapwood and heartwood favour, and partly selected for, different communities of fungi. To provide conservation guidelines for the management of younger oak trees, will require follow up studies to clarify how different types of veteranisation will affect species of conservation interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2020.101020>.

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