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Fungi Inhabiting Stem Wounds of *Quercus robur* following Bark Stripping by Deer Animals

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Abstract: We investigated fungal communities in oak wounds to determine how fungal species richness and community composition changes depending on the age of wounds. The sampling of wood cores was carried out from 10-, 20-, 30-, 40-, and 50-year-old wounds. The fungal community was analyzed using high-throughput sequencing of ITS2 rDNA. Sequence analysis showed the presence of 534 fungal OTUs, which were 83.4% Ascomycota, 16.3% Basidiomycota, and 0.3% Mucoromycota. The fungal OTU richness changed over time: it increased as compared between 10- and 20-year-old wounds, remained similar in 20- to 40-year-old wounds, and decreased in 50-year-old wounds. The fungal community composition also changed over time with the largest differences detected between 10-year-old and older wounds ($p < 0.001$). The most common representatives of Basidiomycota were *Laetiporus sulphureus* (34.7%), *Mycena galericulata* (17.0%), and *Cylindrobasidium evolvens* (6.5%), and the most common of Ascomycota were *Aposphaeria corallinolutea* (13.6%), *Sclerostagonospora cycadis* (7.6%), and *Cadophora malorum* (5.8%). In conclusion, oak wounds of different ages were colonized by a high diversity of fungi including oak-associated species. Fungal communities in oak wounds underwent qualitative and quantitative changes over time, which led to the gradual shift from fungal generalists in young wounds to oak specialists in older wounds.

Keywords: common oak; fungal communities; deer damages; decay fungi

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1. Introduction

The common oak (*Quercus robur*) is a tree species with the highest diversity of associated species in Northern Europe [1,2]. Oaks are long-lived trees, and this longevity leads to many different micro-habitats being formed on and inside the trees, which promotes the establishment of vast biodiversity. According to some estimates, oaks in general can provide habitats to nearly 900 different species [3,4]. Among these, 252 species of phytophagous insects and mites have been identified as oak specialists. In the Nordic countries, about 770 oak-related species are included in the national red list, about 400 of which are in wood, including about 300 beetles and 50 fungi [5]. As oaks age, the activity of wood-decay fungi and wood-inhabiting insects results in decayed wood, which also provides a habitat for larger organisms such as small mammals (e.g., dormouse, bats) or birds [6]. However, the decline of oak trees over the last century in Europe has also resulted in the decline of many oak-associated species [7,8]. Although old oaks still exist in Europe, in many places, their populations are isolated, resulting in unfavorable conditions for the long-term survival of associated organisms [9–12]. In Lithuania, old oak trees make up a significant part of European oak habitats, where endangered and protected species of organisms are found. The starting point for the formation of valuable oak habitats is usually reached at about the age of 200 years [13]. In oaks older than 200 years, there is a rapid increase in both hollows [14] and colonization of trees by epiphytic lichens [15]. Hollows in trees younger than 200 years old are usually rare, which is likely due to factors such as a high tree vigor

and/or occasional colonization of wood by wood-decay fungi [16]. In many areas, there is a lack of oak trees older than 100 years [17]. In Lithuania for example, the average age of oak stands is around 80 years. Moreover, trees growing in managed forests are often harvested at the age of 120 years, i.e., long before the time when valuable habitats appear on and in these trees. Therefore, the limited availability of old-growth oaks calls for alternative solutions to maintain associated biodiversity.

Tree veteranization is one means used for conservation and maintenance of tree-associated biodiversity [18–20]. The purpose of veteranization is to shorten the long waiting period until habitats suitable for protected and rare species are naturally formed. To achieve this goal, trees are damaged using tools to mimic natural processes in nature by creating damages such as a woodpecker hole or a broken branch, thereby creating habitats with exposed wood suitable for the establishment of new species [18]. Such measures are usually used where the number of old trees is not sufficient to ensure the preservation of biodiversity. For this, younger trees are usually used due to both their availability and the lack of exposed wood habitats needed for the natural establishment of particular species [21]. However, tree veteranization should be limited in areas with a relatively large number of large animals causing natural damages to younger oak trees.

Large herbivores, especially several species of deer (red deer or moose), cause significant damages to forestry due to browsing and bark stripping [22–24]. These animals generally prefer deciduous trees, and oaks are particularly frequently browsed [25,26]. Most often, these animals damage the stem of trees at a relatively early age, when oaks reach the age of 15–30 years, and when the bark is still relatively young and has not begun to roughen. In Lithuania, damages caused by deer animals encompass up to 1000 hectares of different forest stands each year. About 10%–15% of these damages are in young oak stands, while the remaining are in *Picea abies* and *Pinus sylvestris* stands [27]. Although severely damaged oak trees do not recover, the vast majority remain viable and are able to close wounds over time (Figure 1).

Figure 1. Wounds of different ages made by deer animals on the stems of *Quercus robur*: (**A**) a 20-year-old oak with an eight-year-old wound with exposed wood; (**B**) a 50-year-old oak with a more than 30-year-old wound, which was closed.

Tree vigor is a phenotypic trait that refers to the ability of a tree to grow and remain healthy in the prevailing environment [28]. Trees may also have well-functioning defense mechanisms that allow them to effectively resist attacks by native agents of damage [29]. Living and healthy trees are often colonized by fungal endophytes, which are fungi with different functional roles that colonize and persist in different tissues asymptomatically until environmental conditions become favorable for their development [30–32], i.e., when they can begin to respond to changes in substrate quality [33]. These endophytes can influence many biotic processes in trees, and under certain conditions, can shift to a pathogenic or saprophytic lifestyle [34]. They can act as first decomposers, thereby influencing the spatial and temporal active layer of biodiversity in their hosts [35]. However, wounds caused by animals and the presence of exposed wood facilitate the establishment of many different fungi due to new colonization opportunities [36,37]. In young wounds, there are often appropriate conditions for plant pathogens and/or wood-decay fungi to establish. Most often, these fungi are divided into three groups: obligate pathogens that interact only with living tree cells, facultative pathogens that live in both living and dead tree tissue, and characteristic saprophytes that only affect dead tree tissue [38]. Exposed wood can be infected by pathogenic or wood-decay fungi through fungal spores that are abundant in the forest [39]. In a common case, the transition of wood pathogens from the wound surface to deeper wood layers is limited due to the activity of wood preservatives and the limited availability of oxygen. Bacterial wet wood or a slow type of rot called "soft-rot" can develop in such wounds in the presence of low oxygen levels [40]. Although a relatively rapid wound closure may prevent further infections, closed wounds can eventually open due to wood decay caused by brown rot fungi, thereby in young oaks creating suitable habitats for many oak-associated organisms. Fungi such as *Cadophora malorum*, *Panellus stipticus*, and various species of *Ophiostoma*, *Helotiales*, or *Cystofilabasidiales* were often found in newly produced oak wounds, while *Trametes versicolor*, *Peniophorella pubera*, *Phlebia radiata*, *Mollisia* sp., or *Stereum* sp. were reported from older wounds [24,41,42].

The aim of this study was to investigate fungal communities in oak stem wounds caused by deer animals using high-throughput sequencing. We also aimed to determine how fungal species diversity changes depending on the wound age. We hypothesized that (a) wounds caused by deer animals in young oaks create conditions for the establishment of oak-associated fungal species, which can persist in colonized wood; and (b) the fungal species richness increases with the age of wounds.

2. Materials and Methods

2.1. Study Sites and Sampling

The study sites were at Kėdainiai (K), Prienai (P), and Telšiai (T), which were in three different regions of Lithuania (Figure 2). These regions have the highest density of *Q. robur* in forest stands. At each site, *Q. robur* trees, which were damaged by deer animals (Figure 1), were between 20 and 60 years old. The age of the trees and the age of the wounds were determined by drilling them with an increment borer (Haglöf, Sweden) and counting the annual tree rings. By comparing the difference between tree rings in the wound and in the intact wood, the age of the wound was determined. Wounds were often 15–20 cm long and 10 cm wide. Wounds that were 10 and 20 years old were not closed, but older wounds were either partially or completely closed. All 50-year-old wounds were closed. In each sampling site (K, P, or T), the distances between the individual oak stands, which were used for sampling, were up to 20 km. Different oak stands often differed in herbaceous vegetation, but other woody vegetation was often absent as pure stands of *Q. robur* prevailed.

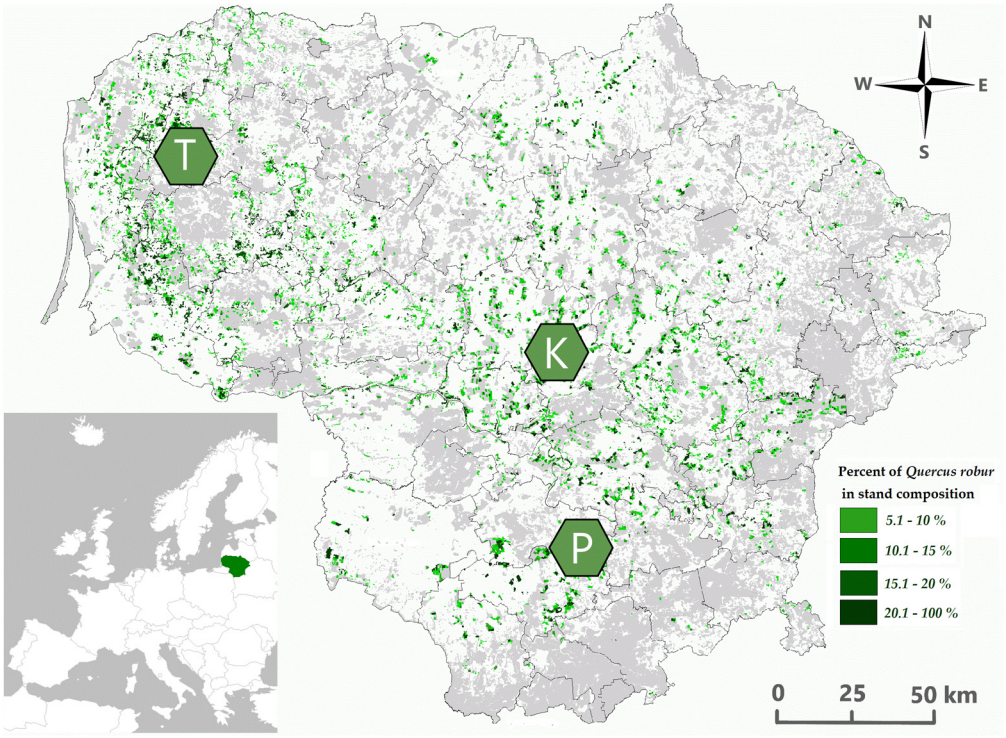

Figure 2. Map of Lithuania showing the distribution of common oak (*Quercus robur*) in forest stands (in green). The intensity of green color shows the percentage of *Q. robur* in the composition of forest stands. Sampling sites are denoted by K—Kėdainiai, P—Prienai, and T—Telšiai. The map was reproduced with permission from FORESTGEN, www.forestgen.mi.lt (accessed on 13 July 2023).

The sampling of oak wood was carried out in 2021. Wood cores were taken from wounds that were either 10 (1 to 10 y), 20 (11–20 y), 30 (21–30 y), 40 (31–40 y), or 50 (41–50 y) years old. In each site, wood samples from wounds of the same age were collected from five trees growing within the same stand. Wood samples were taken ca. 1.2 m from the ground, i.e., at the height where the deer damage prevails. Prior to sampling, the upper layer (2–4 cm thick) of the wood, or bark covering the wound, was removed using an axe, and three replicate samples (ca. 7 cm in depth) were taken from each tree by drilling into the wound using an increment borer. Wood sampling tools were thoroughly cleaned between individual trees using 96% ethanol. A total of 75 oak trees were sampled (3 regions × 5 age classes × 5 trees). The collected wood cores were individually placed in sterile plastic tubes, labelled, transported the same day to the laboratory, and stored at −20 °C before further processing.

2.2. DNA Isolation, Amplification, and Sequencing

Before the isolation of DNA, three wood cores from the same tree were put together. The DNA work was based on the study by Marčiulynas et al. [43]. Firstly, wood samples were grinded in liquid nitrogen and ca. 0.5 g of wood was placed into a 2 mL screw-cap tube with two (2 mm in diameter) metal beads. Then, samples were homogenized using a Fast prep shaker (Montigny-le-Bretonneux, France), and the DNA was extracted using CTAB buffer and incubated at 65 °C for 1 h (vortex every 15 min). The supernatant was mixed with an equal volume of chloroform and cleaned with 2-propanol. The pellet was washed in 500 µL 70% ethanol, dried, and dissolved in 30 µL sterile milli-Q water. The DNA was further purified using a NucleoSpin®Soil kit (Macherey-Nagel GmbH & Co. Duren, Germany) according to recommendations by the producer. Following the extraction and purification of the DNA, its concentration was measured using a NanoDrop™ One spectrophotometer (Thermo Scientific, Rodchester, NY, USA) and adjusted to 10 ng/mL.

The amplification of the ITS2 rDNA region was achieved using a primer pair gITS7 [44] and ITS4 [45], both containing sample identification barcodes. The polymerase chain reaction (PCR) was performed in 50 µL reactions and consisted of the following final concentrations: 200 µM dNTPs, 750 µM $MgCl_2$, 0.025 µM DreamTaq Green polymerase (5 U/µL) (Thermo Scientific, Waltham, MA, USA), 0.02 ng/µL template DNA, and 200 nM of each primer; sterile milli-Q water to a final volume of 50 µL. The amplifications were carried out using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The following thermocycling pattern was used: 95 °C for 5 min; 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min (30 cycles); and 72 °C for 7 min. The PCR products were examined using gel electrophoresis on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, USA). The PCR products were cleaned using a 96% ethanol and 3 M sodium acetate (pH 5.2) (Applichem GmbH, Darmstadt, Germany) mixture (20:1). After the quantification of PCR products using a Qubit fluorometer 4.0 (Life Technologies, Stockholm, Sweden), an equimolar mix was produced, which was sequenced using a PacBio platform and one Sequel II SMRT cell at a SciLifeLab facility in Uppsala, Sweden.

2.3. Bioinformatics

Obtained sequence reads were quality filtered in the Sequence Clustering and Analysis of Tagged Amplicons (SCATA) bioinformatics tool available at http://scata.mykopat.slu.se/ (accessed on 5 July 2023). Short sequences (<200 pb), and those with low read quality (Q < 20), and primer dimers were removed. Sequences without a tag or primer were also excluded. High-quality sequences were clustered into different OTUs using single linkage clustering based on 98.5% similarity. The GenBank (NCBI) database and Blastn algorithm were used to determine taxonomic identities of different OTUs. Taxon-delimiting ITS homology was 98%–100% at the species level, 94%–97% at the genus level, and 80%–94% at the higher level, corresponding to at least 90% of the sequence length [46]. Representative sequences of fungal OTUs were deposited in GenBank under accession numbers OR481117–OR481666. Fungal functional groups were assigned using the FUNGuild fungal database [47].

2.4. Statistical Analysis

Rarefaction analysis was implemented using Analytical Rarefaction v.1.3, (http://www.uga.edu/strata/software/index.html) (accessed on 9 July 2023). Differences in fungal OTU richness (the number of different OTUs) in *Q. robur* wounds of different ages were compared using the non-parametric chi-square test [43]. The Shannon diversity index (α-diversity) and Sørensen qualitative similarity (β-diversity) index were calculated using SAS v. 9.4 (Cary, NC, USA) [48,49]. The non-parametric Mann–Whitney test in SAS was used to assess whether the Shannon diversity index differed among wounds of different ages. The composition of fungal communities in wounds of different ages was analyzed using nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis similarity index (β-diversity). One-way ANOSIM analysis was carried out to assess for significant differences between different samples. These analyses were carried out using Vegan 2.5.7 and Stats 3.6.2 in R 4.1.1 (https://www.r-project.org, accessed on 19 July 2023) [50,51].

3. Results

PacBio sequencing and quality filtering revealed the presence of 141,671 high-quality sequences, representing 629 OTUs. Taxonomic identification showed that 534 OTUs were fungal (Table S1) and 95 were non-fungal, which were removed from further analyses. The detected fungi were 83.4% Ascomycota, 16.3% Basidiomycota, and 0.3% Mucoromycota. Although 10-year-old wounds had the highest absolute richness of fungal OTUs (all samples taken together, Table 1), when the number of fungal sequences had been taken into consideration, the OTU richness was lower as compared to older wounds (e.g., 10 y wounds had 213 OTUs among 62,406 sequences vs. 20 y wounds had 159 OTUs among 10,427 sequences) ($p < 0.05$) (Table 1). The OTU richness in 50-year-old wounds was sig-

nificantly lower than in 20-, 30-, or 40-year-old wounds ($p < 0.05$), which did not differ significantly from each other ($p < 0.05$). Differences in OTU richness were also demonstrated by species accumulation curves (Figure 3). The species accumulation curve from 10-year-old wounds was approaching the asymptote, while those from 20–50-year-old wounds did not reach the asymptote, showing that in these wounds, a higher OTU richness could be detected with deeper sequencing (Figure 3). The Shannon diversity index (α-diversity) was moderate in wounds of different ages and ranged between 2.51 and 2.93 (Table 1). Consequently, no significant differences in this respect were found between wounds of different ages ($p > 0.05$).

Table 1. Generated high-quality fungal sequences and detected diversity of fungal OTUs in oak wounds of different ages in three sampling sites in Lithuania.

Site	Sequences/fungal OTUs in oak wounds of different ages				
	10 y	20 y	30 y	40 y	50 y
Prienai	118/31	738/31	68/24	4/3	1261/21
	564/15	4/4	1714/17	179/20	1092/24
	1759/25	8/8	208/20	50/18	128/20
	1542/27	2633/16	16/11	37/27	5154/18
	4731/24	87/26	17/12	26/13	654/18
Prienai total	8714/71	3450/59	2023/57	296/54	8289/73
Telšiai	88/13	1/1	400/10	64/6	1283/31
	2/2	621/25	202/14	36/6	4008/60
	243/14	2179/10	22/4	477/6	55/9
	20/9	1011/40	24/8	352/8	139/6
	6832/46	3/3	536/17	3162/9	-/-
Telšiai total	7185/72	3815/65	1184/44	4091/26	5485/92
Kėdainiai	8565/62	2338/39	3514/8	124/48	3300/8
	8527/68	55/24	60/14	372/12	161/8
	10,894/78	16/11	1571/59	7848/26	1067/8
	8965/58	662/26	3789/51	5770/74	5105/36
	9556/59	91/23	3804/33	488/33	380/16
Kėdainiai total	46,507/152	3162/83	12,738/128	14,602/141	10,013/56
All total	62,406/213	10,427/159	15,945/179	18,989/193	23,787/179
Shannon diversity index (H)	2.74	2.89	2.90	2.51	2.93

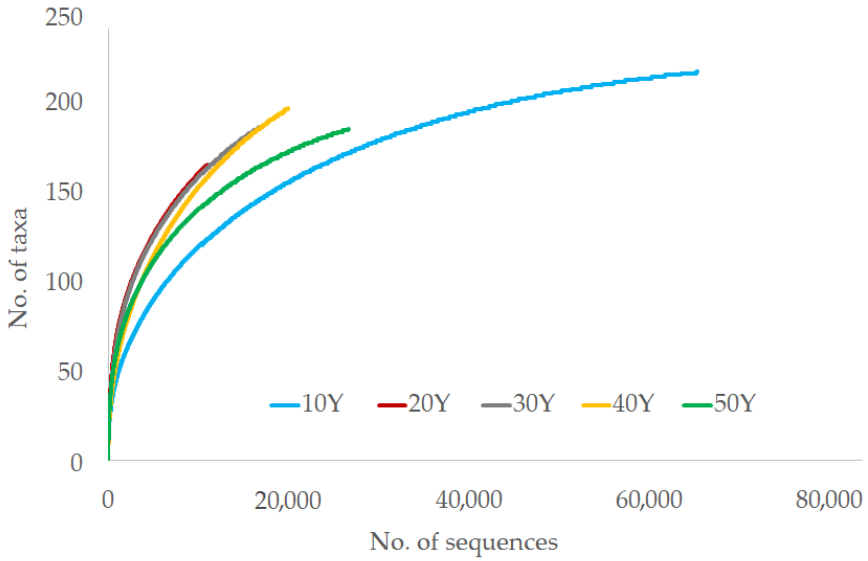

Figure 3. Rarefaction curves showing the relationship between the accumulated number of fungal OTUs and the number of ITS2 rDNA sequences from oak stem wounds of different ages (10 y, 20 y, 30 y, 40 y, and 50 y). Data from different sites are combined.

There were certain variations in OTU richness among different sampling sites and wounds of different ages (Figure 4). In general, the highest richness of fungal OTUs was in wounds from Kėdainiai, while the lowest was in wounds from Prienai. Regarding the age of wounds, the highest variation in OTU richness was in 30- and 40-year-old wounds from Kėdainiai and in 10-, 20-, and 50-year-old wounds from Telšiai (Figure 4).

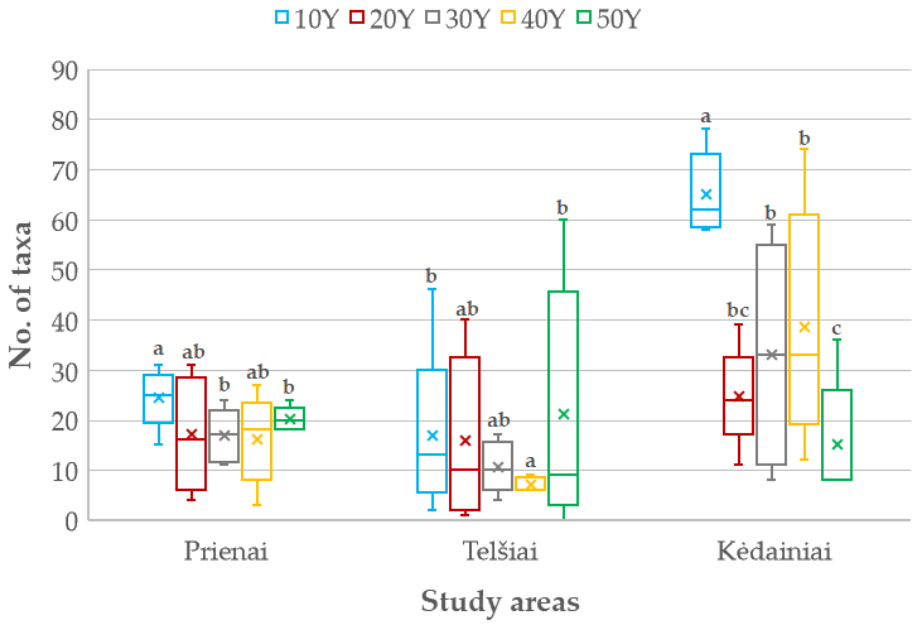

Figure 4. The richness of fungal OTUs in oak wounds of different ages (10 y, 20 y, 30 y, 40 y, and 50 y) at three different sites (Prienai, Telšiai, and Kėdainiai) in Lithuania. Within each site, columns followed by the same letter do not differ significantly at $p > 0.05$.

The most common fungi in 10-year-old wounds were *Aposphaeria corallinolutea* (23.7%), *Sclerostagonospora cycadis* (13.3%), and *Diatrype stigma* (9.6%); in 20-year-old wounds, these were *Cadophora malorum* (22.3%), *Diaporthe eres* (15.6%), and Unidentified sp. 5631_30 (15.4%); in 30-year-old wounds, these were *Laetiporus sulphureus* (22.0%), *Menispora* sp. 5631_29 (12.2%), and *Phialemonium inflatum* (10.6%); in 40-year-old wounds, these were *Fimetariella rabenhorstii* (30.7%), *Leptosillia macrospora* (18.3%), and *Mycena galericulata* (16.5%); and in 50-year-old, these were *Phaeostalagmus cyclosporus* (20.1%), *L. sulphureus* (16.6%), and *C. malorum* (10.3%) (Table 2).

Table 2. Occurrence and relative abundance of the 30 most common fungal OTUs (shown as a proportion of all high-quality fungal sequences) in *Quercus robur* stem wounds of different ages (10–50 years old). Data from different sites are combined.

Phylum *	OTU	Genbank Reference	Similarity, (%)	Age of Wound					Total, %
				10 y, %	20 y, %	30 y, %	40 y, %	50 y, %	
A	*Aposphaeria corallinolutea*	MT177916	245/245 (100)	23.74	1.38	-	0.04	-	11.38
A	*Sclerostagonospora cycadis*	KR611890	248/248 (100)	13.29	0.33	0.04	0.01	-	6.34
B	*Laetiporus sulphureus*	MH321898	296/296 (100)	-	-	22.04	0.01	16.62	5.68
A	*Cadophora malorum*	MT561395	241/241 (100)	0.01	22.28	8.13	1.60	10.25	4.84
A	*Diatrype stigma*	KT004563	251/252 (99)	9.57	0.13	0.01	-	-	4.55
A	*Fimetariella rabenhorstii*	MN547388	248/250 (99)	-	0.09	0.47	30.69	-	4.49
A	*Neosetophoma italica*	LC206635	249/249 (100)	8.98	0.07	-	0.01	-	4.27
A	*Phaeostalagmus cyclosporus*	ON989633	233/238 (98)	-	-	0.78	0.39	20.98	3.94
A	*Neocucurbitaria quercina*	OP896095	249/249 (100)	4.37	4.63	0.03	5.40	0.16	3.25
B	*Mycena galericulata*	KJ705178	314/314 (100)	-	-	2.05	16.54	0.78	2.78

Table 2. *Cont.*

Phylum *	OTU	Genbank Reference	Similarity, (%)	Age of Wound					Total, %
				10 y, %	20 y, %	30 y, %	40 y, %	50 y, %	
A	*Leptosillia macrospora*	NR164064	265/265 (100)	0.14	0.65	-	18.32	0.01	2.76
A	*Cytospora predappioensis*	MT595364	260/260 (100)	5.34	0.09	0.03	0.01	-	2.54
A	*Cladosporium cladosporioides*	OP963820	243/243 (100)	5.17	0.19	0.09	0.17	0.03	2.51
A	*Paraphaeosphaeria sporulosa*	KX664338	244/245 (99)	5.17	0.47	-	-	-	2.49
A	*Lophiostoma corticola*	KU712227	244/244 (100)	3.76	0.29	0.03	0.04	-	1.81
A	*Neoleptosphaeria rubefaciens*	KT804116	250/250 (100)	3.35	0.01	0.31	-	-	1.63
A	*Sporocadus trimorphus*	MT223846	248/248 (100)	3.35	0.01	-	-	-	1.59
A	*Ascocoryne sarcoides*	MH857562	240/240 (100)	0.003	0.70	0.24	0.06	8.04	1.55
A	*Menispora* sp. 5631_29	MH859920	222/233 (95)	-	-	12.20	0.06	-	1.49
A	*Phialocephala compacta*	MH862480	234/239 (98)	0.003	7.36	4.95	1.38	0.35	1.45
A	*Diaporthe eres*	MT111115	255/255 (100)	0.31	15.63	-	-	-	1.39
A	*Phialemonium inflatum*	MT221573	242/246 (98)	-	0.02	10.57	0.04	0.004	1.29
A	Unidentified sp. 5631_30	MN096587	254/265 (96)	-	15.38	0.03	-	-	1.22
A	*Phlyctema vagabunda*	MK174720	239/239 (100)	2.50	0.03	0.19	-	-	1.21
B	*Cylindrobasidium evolvens*	MN947592	387/387 (100)	2.24	-	-	-	-	1.06
A	*Hyaloscypha fuckelii*	MT231692	238/238 (100)	-	-	-	0.01	5.76	1.04
B	*Armillaria gallica*	KY474051	476/477 (99)	-	-	0.01	-	5.25	0.95
B	*Phlebia acerina*	MN945144	289/289 (100)	-	-	7.46	-	-	0.90
B	*Pleurotus dryinus*	MK169240	284/284 (100)	-	0.40	-	0.24	3.66	0.73
A	*Lophiostoma* sp. 5631_40	MH178565	240/252 (95)	-	-	-	-	3.96	0.72
Total				91.28	70.14	69.64	75.02	75.84	81.84

* A—Ascomycota, B—Basidiomycota.

The most common Basidiomycota fungi were *L. sulphureus* (34.7%), *M. galericulata* (17.0%), and *Cylindrobasidium evolvens* (6.5%), and the most common Ascomycota fungi were *A. corallinolutea* (13.6%), *S. cycadis* (7.6%), and *C. malorum* (5.8%) (Figure 5).

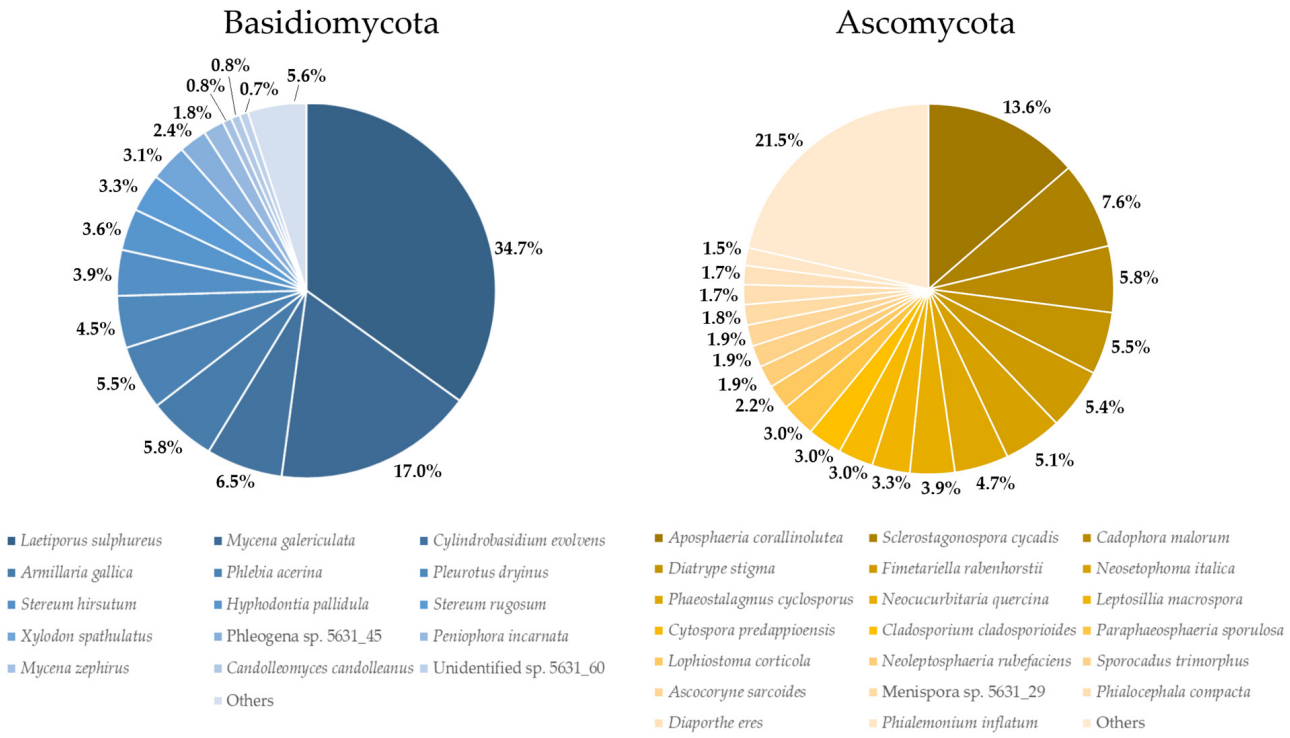

Figure 5. Relative abundance (%) of most common fungal OTUs in *Quercus robur* wounds of different ages (10 y–50 y). Others include fungal OTUs with a relative abundance of <0.7% for Basidiomycota and of <1.5% for Ascomycota.

The number of unique OTUs (found only in a particular age class) varied substantially in stem wounds of different ages (Table S1). The most frequent unique OTUs in 10-year-old wounds were *Cylindrobasidium evolvens* (2.2%), *Peniophora incarnata* (0.6%), and *Hormodochis aggregata* (0.4%); in 20-year-old wounds, these were *Candida norvegica* (1.2%), Unidentified sp. 5631_140 (0.4%), and *Hyphodiscus luxurians* (0.3%); in 30-year-old wounds, these were *Phlebia acerina* (7.5%), Unidentified sp. 5631_39 (5.8%), and *Stereum hirsutum* (5.3%); in 40-year-old wounds, these were *Toniniopsis subincompta* (2.6%), *Zygosaccharomyces parabailii* (2.1%), and Unidentified sp. 5631_56 (2.0%); and in 50-year-old wounds, these were *Lophiostoma* sp. 5631_40 (4.0%), *Phleogena* sp. 5631_45 (2.2%), and *Mortierella hypsicladia* (1.2%) (Table S1). Among all fungi, unique OTUs in wounds of different ages constituted between 24.0% and 52.3% of all OTUs. The highest number of shared OTUs was between 30- and 40-year-old (86 OTUs) wounds, while the least number was between 10- and 50-year-old (37 OTUs) wounds. Most of the unique OTUs were in 10-year-old (112) and 50-year-old (71) wounds (Figure 6).

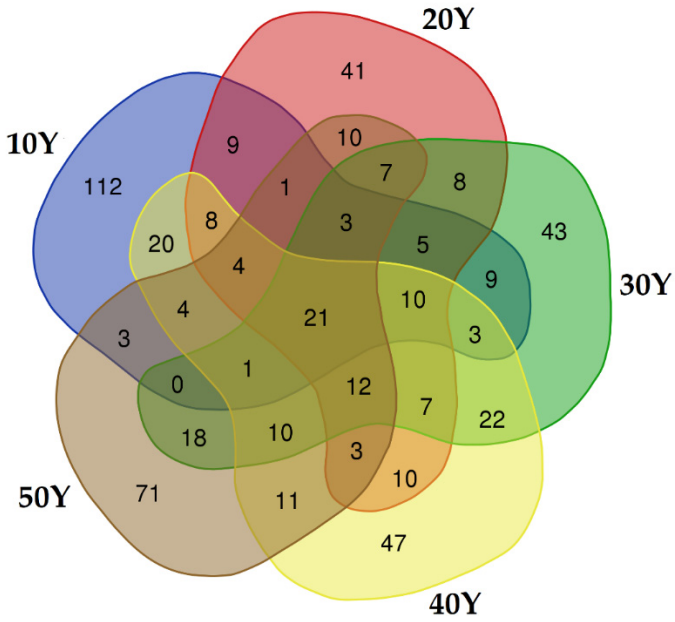

Figure 6. Venn diagrams showing the diversity and overlap of fungal OTUs in oak wounds of different ages (10 y–50 y). Data from different sites are combined.

The Sørensen similarity index (β-diversity) of fungal communities between wounds of different ages was moderate to low. The lowest Sørensen similarity index of fungal communities was between 10- and 50-year-old (0.19) wounds, while the highest was between 30- and 40-year-old (0.46) wounds (Table 3).

Table 3. The Sørensen similarity index (β-diversity) of fungal communities among wounds of different ages (10–50 years old). Data from different sites are combined.

Wound Age	10 y	20 y	30 y	40 y	50 y
10 y	-	0.32	0.26	0.35	0.19
20 y	0.32	-	0.43	0.42	0.36
30 y	0.26	0.43	-	0.46	0.40
40 y	0.35	0.42	0.46	-	0.35
50 y	0.19	0.36	0.40	0.35	-

Fungal functional groups were determined for 91.4% of fungal sequences, and their relative abundance in wounds of different ages is shown in Figure 7. In all samples, the most abundant fungal functional groups were undefined saprotrophs (37.9%), wood sapro-

trophs (24.2%), plant pathogens (14.2%), and endophytes (12.7%) (Figure 7). Undefined saprotrophs showed the highest relative abundance in 50-year-old (55.8%), 10-year-old (37.8%), and 30-year-old (37.7%) wounds, and their relative abundance differed significantly from those in 20-year-old wounds ($p < 0.05$). The highest relative abundance of wood saprotrophs was in 30-year-old (32.7%), 10-year-old (27.8%), and 50-year-old (23.2%) wounds, and their relative abundance differed significantly from those in 20-year-old wounds ($p < 0.05$) (Figure 7). The relative abundance of plant pathogens was highest in 10-year-old (24.6%) and 20-year-old (22.7%) wounds, which was significantly higher than in wounds of other ages ($p < 0.05$) (Figure 7). The relative abundance of endophytes varied substantially in wounds of different ages, with the highest difference being between 10-year-old (4.5%) and 20-year-old (38.3%) wounds ($p < 0.05$).

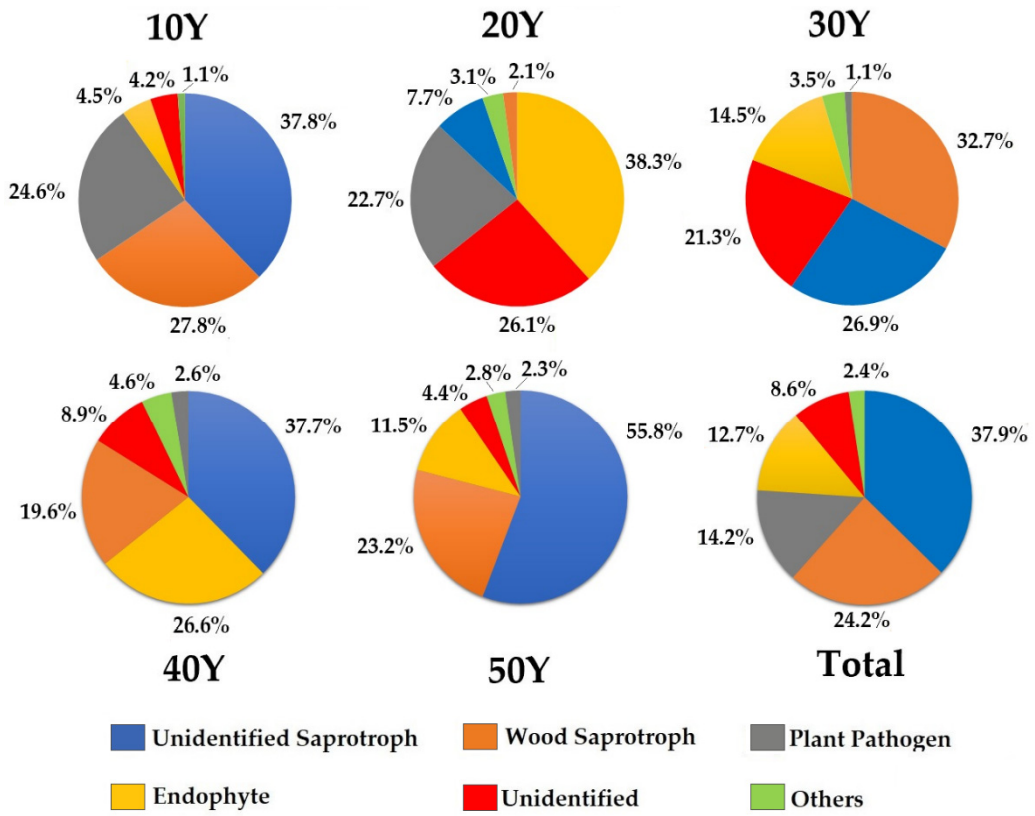

Figure 7. Relative abundance (%) of fungal functional groups in *Q. robur* stem wounds of different ages, estimated based on fungal sequences. Others represent fungi, which are not associated with plants (e.g., animal pathogens).

The detected wood-decay fungi were *L. sulphureus* (5.7%) (detected in ≥30-year-old wounds), *Armillaria gallica* (0.9%) (only in 50-year-old wounds), *Phlebia acerina* (0.9%) (only in 30-year-old wounds), *Stereum hirstum* (0.6%) (in ≥30-year-old wounds), *Stereum rugosum* (0.5%) (in ≥30-year-old wounds), and *Trametes versicolor* (0.03%) (in ≥40-year-old wounds). Fungi strongly associated with oaks such as *Phlebia acerina* (1.2%) (only in 30-year-old wounds) and *Pleurotus dryinus* (0.7%) (mostly in 50-year-old wounds) were also detected (Table S1).

NMDS analysis revealed a partial overlap of fungal communities in oak wounds of different ages (Figure 8). The permutation test showed that fungal communities in 10-year-old wounds differed significantly from those in older wounds (20 y, 30 y, 40 y, and 50 y) ($p < 0.001$). There was also a significant difference between fungal communities in 20-year-old and 50-year-old wounds ($p < 0.01$), and in 40-year-old and 50-year-old wounds ($p < 0.05$), while in wounds of other ages, fungal communities were similar ($p > 0.05$) (Figure 8).

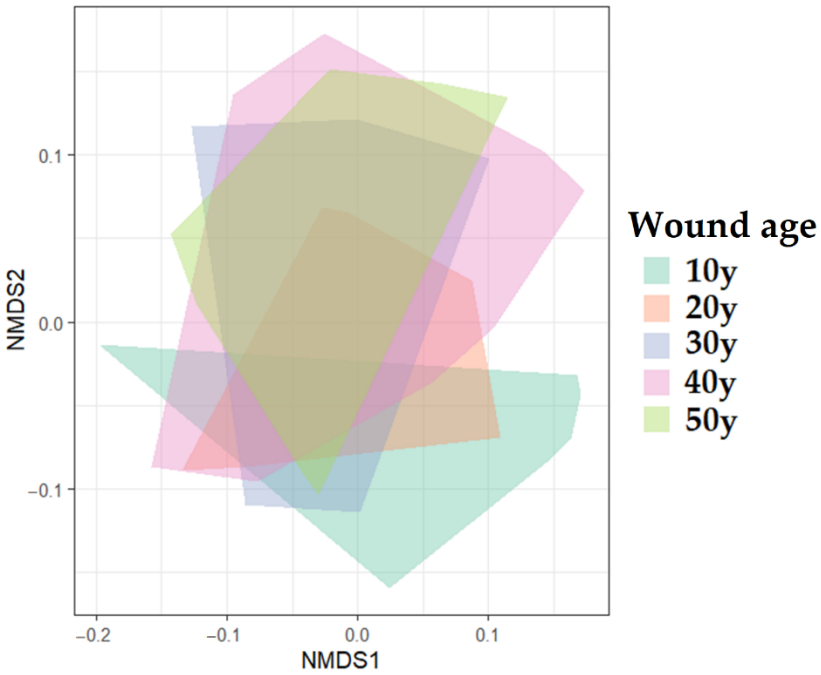

Figure 8. Non-metric multidimensional scaling (NMDS) of fungal communities associated with *Quercus robur* stem wounds of different ages (10, 20, 30, 40, or 50 years old).

4. Discussion

In most cases, living tree tissues beneath the intact bark represent an unfavorable environment for colonization by many fungi, but these tissues become readily available for colonization when the tree is injured, or in the case of bark dieback. Post-damage fungal colonization in angiosperms was extensively studied in North America [43,52], showing that fungal colonization is often determined by both the nature of the wound and its location on the stem. If the wound is only superficial, i.e., only the bark is removed in a small area, then conditions are often limited for fungal colonization. However, if the wound is relatively large, and especially if it penetrates several or more annual rings, the decay can spread more rapidly into the inner wood. The primary colonization of wounds starts with latent fungi that were already in the wood, but it may also include opportunistic pathogens, or fungi whose spores were on the damaged surface [13]. Following the expansion of these fungi in wounds of living trees, complex fungal communities are formed. In addition to latent fungi, wounds are also colonized by those with ruderal characteristics, even though their abundance is usually not high [42].

Fungal communities colonizing living tree stems were shown to persist in deadwood for many years after the tree injury, but they are also constantly changing both temporally and spatially [42,53]. In agreement, the results of the present study showed that the richness and composition of fungal communities changed over time. Consequently, certain trends in richness of fungal OTUs in oak wounds of different ages (10 y–50 y) were revealed, showing that there was an increase in OTU richness between 10- and 20-year-old wounds, the OTU richness remained similar between 20- and 40-year-old wounds, and the OTU richness decreased in 50-year-old wounds (Table 1, Figure 3), thereby only partly supporting the hypothesis that the species richness increases with the age of wounds. Although it was shown that the diversity of fungal species changes strongly during different stages of wood decomposition [54], it may be different in wounds. For example, the increase in richness of fungal OTUs in relatively young wounds can be associated with the availability of new substrate for colonization, while competition among fungi already established in oak wood and the ongoing process of wound closure could contribute to minor changes in the richness of fungal OTUs in mid-age (20- to 40-year-old) wounds. Furthermore, the complete wound closure in old wounds can drive the decline of fungal OTU richness due to

both the lack of access to exposed wood and reduced supply of oxygen. However, variation in richness of fungal OTUs was high at different study sites (Figure 4), showing a strong impact of local environmental conditions.

Differences in fungal community composition in wounds of different ages were also notable (Figure 8), and these differences were further supported by low values of the Sørensen similarity index (Table 3) and a low overall number of shared OTUs (21) (Figure 6). These changes, which were likely driven by changes in habitat availability and quality, led to the establishment and persistence of oak-associated fungi, thereby supporting the hypothesis that wounds in young oaks create conditions for the establishment and long-term survival of such fungi. Among these, there were fungi such as *L. sulphureus*, which causes a heart rot in living oak trees [55,56]; a widespread fungus *P. dryinus*, which causes a white rot [57]; and the wood-decay fungi *S. hirstum*, *S. rogosum*, and *Peniophora incarnata* [58–60]. A tree pathogen *A. gallica* was also detected in old (especially in 50-year-old) wounds, showing that it is a secondary plant parasite that usually causes infections only after the defense of host trees is weakened, e.g., after the insect defoliation, drought, or infection by other fungi. *Ophiostoma quercus*, which was suggested to be associated with the decline of oaks in Central Europe [61,62], was also detected. All these oak-associated wood-decay and/or pathogenic fungi were found to be more abundant in older wounds ($\geq$30 years old) (Tables 2 and S1). By contrast, endophytic and/or generalist pathogenic fungi were more common in 10-year-old wounds (Table 2). For example, in 10-year-old wounds, there were fungal endophytes such as *S. cycadis* and *Paraphaeosphaeria sporulosa* [63–65], and plant pathogens *Neoleptosphaeria rubefaciens* [66–68] and *Sporocadus trimorphus* [69] (Table 2), but their functional role in colonized oak wood is obscure.

The abundance and composition of fungal functional groups has also changed over time (Figure 7), as tree wounding enabled latent colonizers of intact wood to switch to saprophytic or pathogenic lifestyles while at the same time having a competitive advantage over other later arriving fungi due to readily available wood resources [70,71]. For example, *Lophiostoma corticola*, which is a widely distributed saprotroph, showed a high relative abundance in 10-year-old wounds [72,73]. Although it is not common in oaks, it is well characterized from leaves, petioles, or branches of *Fraxinus excelsior* [73–75]. *Diatrype stigma* was also found among the most common fungi associated with 10-year-old wounds. It is a saprotrophic species that is commonly associated with the genus *Quercus* but can also grow on various plants within *Rosaceae* and *Betulaceae* [76]. *Cytospora predappioensis* was also discovered in relatively young wounds. Some *Cytospora* species are known to cause canker diseases in woody plants [77,78]. *Cylindrobasidium evolvens* is a widespread saprotroph that is common in young wounds of hardwood tree species [79]. Such a composition of fungi in 10-year-old wounds indicates that newly created wounds provide favorable conditions for the establishment and expansion of primary colonizers, which are widely distributed in the environment [32]. As a possible result of expansion of such fungi, the relative abundance of endophytes has peaked in 20-year-old wounds (Figure 7) with *Diaporthe eres* and Unidentified sp. 5631_30 being the most common and unique fungi (Table 2). Although *D. eres* can cause stem canker, stem necrosis, dead branch, shoot blight, fruit rot, leaf spot, leaf necrosis, and crown browning [80–82], in Mediterranean oaks it was characterized as an endophyte [83]. In 30-year-old and older wounds, there was a notable change from endophytic to saprotrophic fungi (Figure 7), which can probably be attributed to the activity of secondary wood decomposers [84,85]. For example, *M. galericulata* and *Ascocoryne sarcoides* were commonly detected in older wounds (Table 2). *Mycena galericulata* is a saprotrophic wood-decay fungus, which occurs at later stages of wood decomposition, as it requires a substrate prepared by other fungi [86,87]. *Ascocoryne sarcoides* is commonly found in high humidity temperate forests, which are rich in beech and oak [88,89]. Although the fungus usually occurs on fallen trees or large pieces of wood, suggesting that it can be a saprotroph, the precise trophic mode is obscure [90].

5. Conclusions

Oak wounds of different ages are inhabited by a high diversity of fungi including oak-associated species such as *L. sulphureus*, *P. dryinus*, and *P. incarnata*, which often persist in old wounds after their closure, demonstrating that wounds in young oaks create conditions for the establishment and long-term survival of oak-associated fungi. The fungal OTU richness changed over time: it increased between 10- and 20-year-old wounds, remained similar between 20- and 40-year-old wounds, and decreased in 50-year-old wounds. Consequently, fungal communities in oak wounds undergo qualitative and quantitative changes over time, leading to the gradual shift from fungal generalists in young wounds to oak specialists in older wounds.

Supplementary Materials: The following Supporting Information can be downloaded at: https://www.mdpi.com/article/10.3390/f14102077/s1, Table S1: Distribution and relative abundance of fungal OTUs (shown as a proportion of all high-quality fungal sequences) in *Quercus robur* wounds of different ages (10–50 years old). The data from different study sites are combined.

Author Contributions: Conceptualization, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); methodology, A.M. (Adas Marčiulynas), V.S.-Š. and A.M. (Audrius Menkis); software, A.M. (Adas Marčiulynas), V.S.-Š. and A.M. (Audrius Menkis); validation, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); formal analysis, A.M. (Adas Marčiulynas), V.S.-Š. and A.M. (Audrius Menkis); investigation, A.M. (Adas Marčiulynas) and V.S.-Š.; resources, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); data curation, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); writing—original draft preparation, A.M. (Adas Marčiulynas) and V.S.-Š.; writing—review and editing, A.M. (Audrius Menkis); visualization, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); supervision, A.M. (Audrius Menkis). All authors have read and agreed to the published version of the manuscript.

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