

Biotic drivers of fungal communities associated with bark beetles and their host trees



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Abstract

Bark and ambrosia beetles (Coleoptera: Scolytinae) associate with pathogenic fungi to colonise new territories. Large scale forest pest and disease outbreaks are linked to this phenomenon, which remains still poorly understood. To prevent or mitigate these outbreaks is crucial to characterise beetle-fungi associations, understanding the underlying mechanisms behind these interactions. However, studying fungal communities has been challenging due to the cryptic nature and complex taxonomy of fungi. Thanks to molecular techniques like metabarcoding, it is possible to unveil many previously unnoticed fungi. In the present work, I first compare two widely used DNA markers (ITS and LSU) to evaluate their suitability for beetle-associated fungal community characterization (Chapter 1). I then compare the fungal communities associated with bark and ambrosia beetles on forests consisting of different host trees at the New Forest National Park (Chapter 2). This way, I evaluate the effect of the host tree identity as a driver of these fungal communities. Chapter 3 further takes this comparison at the regional level by assessing the impact of different forest compositions from 20 sites across the UK, ranging from monospecific conifers to mixed broadleaves. Finally, I used the same methodology to compare the fungi from these native beetle communities with fungi harboured by the first UK-born population of the invasive bark beetle *Ips typographus*. Results unveiled the potential invasive fungal associates that this species could be vectoring into the United Kingdom. I also evaluated the fungal communities harboured by *Ips typographus* specimens across different developmental stages and host trees. Further implications of the present work are discussed in Chapter 6. My research underlines the power of metabarcoding as a tool to better understand, evaluate, and compare fungal communities with a wide range of fungal disease and forest management-related implications.

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Declaration of Originality

I confirm that this thesis is my own work and that any assistance received is appropriately referenced along with this thesis.

Specimen collection and morphological identification were conducted in collaboration with Dr Daegan Inward at Forest Research. Along with Dr Inward, I collected the *Ips typographus* specimens (Chapter 5). Dr Inward collected the rest of the analysed beetle specimens during 2013-2017 and performed a first identification and a pre-sorting of these specimens. I conducted a second identification and selected the specimens to carry out the laboratory work. All the laboratory work, except library preparation and sequencing, were carried out by myself. This work included DNA extraction, PCR amplification, gel electrophoresis, purification and equimolar pooling. Upon receiving the sequencing results, I also carried out the bioinformatics and statistical analyses. Furthermore, I created the figures and tables and written each of the chapters.

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Chapter 1. General Introduction

1.1. Fungal community ecology

The kingdom of Fungi harbours an extraordinary diversity of taxa. Estimates suggest that there could be from 0.8 million to 5.1 million fungi species (Blackwell, 2011; Hawksworth & Lücking, 2017). Fungal communities have a pivotal role in ecosystems, underpinning a vast amount of crucial ecological functions. Despite their importance for ecosystems, only 3-8% of the fungi are described so far (Anon, 2017b).

Fungi represent a widely undescribed clade of eukaryotes mainly because of their difficult taxonomy and their cryptic life cycles. Over the years, different taxonomy-based classifications have been suggested, without reaching a general consensus (Tedersoo *et al.*, 2018; Wijayawardene *et al.*, 2020; Hibbett *et al.*, 2011). Fungi can also be classified according to their ecological guilds (Schimper, 1903). Fungal guilds are categories within the main trophic modes, which classify fungi according to their functional relationship with their hosts, i.e., symbiotrophs, saprotrophs or pathotrophs (Nguyen *et al.*, 2016a; Simberloff & Dayan, 1991; Blondel, 2003). Although a high proportion of fungi remains unclassified, recent studies have suggested that the different abiotic and biotic factors present in ecosystems could differently affect fungi depending on their guilds (Větrovský *et al.*, 2019; Tedersoo *et al.*, 2020a). Consequently, fungal guilds could be an excellent proxy to classify fungi into ecologically meaningful categories without relying on their complex taxonomies (Nguyen *et al.*, 2016a). However, the link between fungal taxonomy and fungal guilds is still under debate because of fungal interactions. For example, a recent study has shown that some of the most common bark beetle-associated fungi, such as *Ophiostoma ips* or *Raffaelea*, previously classified as saprotrophs, can actually compete with the wood decomposers already present in the tress, slowing decomposition (Skelton *et al.*, 2019b). Therefore, the essential role of these fungi could be contrary to what was previously thought. These authors also suggest that these inhibitory effects would be neutralised depending on the fungi inoculated along with these species, remarking on the high complexity exhibited by these kinds of microbial communities.

Fungal associates may range from beneficial, neutral or even detrimental to their plant hosts. The denominated “fungal endophytes” fall between these first two categories. Endophytes inhabit plants without causing any disease symptoms. De Bary first defined this term in the mid-19th century, and this concept has evolved until reaching the current consensus (Wilson, 1995).

Some fungal endophytes can significantly contribute to tree health by generating defence metabolites as well as growth-promoting phytohormones (Schulz & Boyle, 2005; Terhonen *et al.*, 2019). Certain species act as litter decomposers or contribute to the uptake and transfer of nutrients (Smith & Read, 2010), establishing mutualisms with their host plants. However, other fungal endophytes can remain neutral to plants through commensalisms. This way, endophytes coexist inside the plant for most of their life cycle (Saikkonen *et al.*, 1998). The extent to which plant-microbial interactions vary depends on the species or even the fungal strain and various abiotic and biotic factors (Porrás-Alfaro & Bayman, 2011).

The third category of fungal associates is the pathogenic fungi, which can act as commensal or parasites, alone or in combination with pests like bark and ambrosia beetles (Ferrell & Parmeter Jr, 1989; Six, 2020; Zhao *et al.*, 2019). As much as 20% of the fungi described so far are categorised as plant pathogens, causing 80% of plant diseases (Ray *et al.*, 2017). Only in agriculture, 68% of the average annual loss of crop production tonnage is a consequence of fungal pathogens (Oerke, 2006). In the forestry sector, the impact of these pathogens remains more challenging to quantify. Trees in their earliest stages of the disease do not necessarily show any symptoms, which underlines the importance of early detection mechanisms to prevent pest outbreaks. However, as the fungi progress through the tree vascular system, symptoms such as canopy wilt or cankers may become more apparent, indicating the mass colonisation of the tree that may eventually lead to its death.

Given that fungal diseases are a global phenomenon, new cases should not be assessed in isolation but under an ecological and evolutionary context (Hulcr & Dunn, 2011). Understanding the underlying mechanisms behind fungal diversity and biogeography remains crucial to anticipate fungal outbreaks and fungal invasions (Desprez-Loustau *et al.*, 2007). Under the climate change scenario and partially enhanced by the global trade in raw materials, fungal invasions have developed exponentially over the past decades (Desprez-Loustau *et al.*, 2007). Invasive fungi may not necessarily cause any disease outbreak, but consequences on ecosystem dynamics should not be neglected, given the many functions carried out by fungi.

The full role of fungal communities on ecosystem functioning is yet to be determined. The “insurance hypothesis” suggests that the more significant variance in species responses would ensure ecosystem balance and resilience, not only affecting the individual trees (McCann, 2000). For example, some fungi may not only enhance abiotic stress tolerance on single trees (Singh, Gill & Tuteja, 2011), but fungi may also help to mitigate the effects of climate change on forests

by limiting nutrient losses after severe rainfalls (Martínez-García *et al.*, 2017). Another essential factor to consider is that fungal communities interact with different kinds of forest biota. Besides plants, fungi establish links with microorganisms like those coexisting in the soil and, of course, with insects.

Insect-fungal associations are extraordinarily diverse and have been profusely described, ranging from mutualisms to commensalisms. Some mutualisms are highly specific, like in the case of fungi-growing ants, which have co-evolved with fungi for at least 23 million years (Chapela *et al.*, 1994). However, other insects like aphids may benefit from certain fungi like mycorrhizae but are negatively affected by others like plant endophytes (Hartley & Gange, 2009). Furthermore, most interactions among fungi and insects are extremely context-specific depending on temperature (Hofstetter *et al.*, 2007), humidity (Athanassiou *et al.*, 2017) or even fungal nutrition (Chiu *et al.*, 2019). This context-dependency affect fungal commensalisms like those from the genus *Beauveria* and *Metarhizium*, considered as biocontrol on herbivory insects (Mudrončėková *et al.*, 2021; McKinnon *et al.*, 2017). Therefore, these interactions are highly complex, to the point that the same fungal species may affect a wide range of organisms. Given this complexity, a holistic and context-specific approach is necessary to understand fungal dynamics. With this purpose, research should consider all the biotic and abiotic factors that may mediate those interactions. Although the effect of the fungi on the insect was more visible at first, recent techniques have contributed to revealing the benefits that fungi may obtain, such as dispersal, nutrition and protection (Biedermann & Vega, 2020). Likewise, recent techniques have allowed minimising the biases generated by past literature, focused on laboratory-based trials (Hartley & Gange, 2009).

Research on fungal community ecology faces, therefore, multiple challenges. These challenges could be better answered by using a multi-disciplinary approach based on the interface of traditional and new techniques (Nilsson *et al.*, 2019a). In particular, molecular techniques have contributed to underscore a high proportion of previously unknown fungi during the past decades, diminishing the share of the so-called “dark taxa” or cryptic fungi (Nilsson *et al.*, 2019b). Although the science behind the characterisation of fungal communities is still developing, the fast development of these techniques presents a prodigious opportunity to study fungal communities.

1.2. Bark and ambrosia beetles

Bark and ambrosia beetles belong to the subfamilies Scolytinae and Platypodinae, within the family Curculionidae. Within the UK, which is the geographical focus of the current study, coexist over 64 established species of bark and ambrosia beetles, plus four recently established invasive species (Inward, 2019). Beetle-fungal outbreaks contribute to substantial losses in every link in the forest-related value chain, causing critical ecological impacts (Hlásny *et al.*, 2019).

The Scolytinae life cycle could be summarised as follows. Upon finding a suitable host tree, beetles penetrate the tree bark, creating a mother gallery where they lay their eggs. After hatching, larvae excavate side galleries, where they finally pupate (**Figure 1-1**). This process may take from weeks to months, depending on factors like temperature, humidity or the ability of tree defences to repel attacks. The shape and the extension of the excavated gallery rely mainly on the beetle species feeding strategies (Ayres *et al.*, 2000) and the offspring performance (Sauvard, 2007). Parental care differs depending on the genus and even sometimes on the beetle species. For example, beetles from the genus *Trypodendron* place fungal mycelium in each side gallery to feed larvae, replacing it once depleted (Borden, 1988). After pupation, the new adults exit the tree to continue their life cycle.

Some Scolytinae species can complete their life cycle in one year, while others need two years. Under certain climatic conditions, beetles may reproduce several times per year, having plenty of overlapping generations at a single annual cycle. Moreover, beetles from the tribe Xyleborini exhibits a complementary reproduction strategy, which is termed *arrhenotokous inbreeding*. This sib-mating technique allows individuals from the same family to mate in the parental galleries without exiting the tree (Biedermann, 2007). Such a mating system would enable them to grow new galleries inside the same tree, thereby increasing in high numbers without any predatory risk (Smith & Hulcr, 2015).

Entire fungal communities are transported and shared during the whole tree colonisation process. Some beetle species have evolved particular body structures to hold fungi called -mycangia- (Batra, 1963); others store fungi in pores in their cuticle (Harrington, 2005; Six, 2003), or their guts (Biedermann, Klepzig & Taborsky, 2009). The mycangium can be defined as a “structure that consistently functions to transport specific fungi”, naming the most complex structures as “complex mycangium” or “true mycangium” as opposed to more primitive mycangia types (Six, 2003). While some fungi are merely incidental, others establish symbiotic relationships with the beetle. Through this symbiosis, fungi can benefit from transport and

protection, while beetles may obtain nutritional or defensive benefits from certain fungi (Ranger *et al.*, 2018). Fungal pathogens will access the tree at some point during the colonisation process, affecting different tree organs. For example, the Dutch Elm disease *Ophiostoma novo-ulmi* access elms through their branches since these beetles cause feeding wounds before maturation (Brasier, 1991). From there, the fungus progress through their vascular system, collapsing the xylem vessels, which causes death in most of the attacks. The bark beetle benefits from weak or dying trees to create their galleries and continue their life cycle.



*Figure 1-1 Gallery of the bark beetle *Ips typographus*, displaying the pupae in the pupal chambers and some adults ready to emerge from the tree. Beetle-associated fungi colonise the galleries and part of the wood—image taken at the Forest Research Centre at Alice Holt, UK.*

Bark and ambrosia beetles differ depending on their behaviour towards fungi, which determine their feeding style and the exact place in which galleries are built (Wood, 1982). Bark beetles do not get their primary nutrients from the fungi, but from the wood of the tree they inhabit. Consequently, they build their galleries on the more nutritious phloem tissues. In contrast, ambrosia beetles tend to construct their galleries on the sapwood of trees. These beetles actively promote the growth of some fungal species -called “ambrosia” fungi- to use them, mainly as food sources (Wood, 1982; Ranger *et al.*, 2016). Ambrosia beetles carry these fungal spores in their mycangia and actively promote their growth inside their galleries (Bleiker & Six, 2007; Six, 2003; Ranger *et al.*, 2016). Therefore, they feed directly on fungi rather than the trees themselves (Beaver, 1989; Wood, 1982), explaining their higher colonisation capacity and broader host diversity than bark beetles (Hulcr & Stelinski, 2017). The great majority of the

Scolytinae and Platypodinae found in the UK are bark beetles, therefore feeding directly on the wood.

Bark and ambrosia beetles could be further categorised depending on their behaviour towards tree invasion. This way, beetles could fall into two other categories, including tree-killing beetles that are primary pests and deadwood colonisers categorised as secondary pests (Paine, Raffa & Harrington, 1997). Secondary pests would represent a lower risk to healthy forests since they only attack weakened or dying trees. However, there have been cases of species normally acting as secondary pests that under certain conditions attack healthy trees, such as *Trypodendron domesticum* (Kühnholz, Borden & Uzunovic, 2001). Bark and ambrosia beetle populations tend to remain stable (endemic phase) until the ecosystem balance is affected by a natural phenomenon that enhances tree susceptibility, like severe droughts or windstorms (Raffa *et al.*, 2008). This situation may trigger an outbreak since beetle populations may exponentially grow and reach an epidemic phase on their population dynamics (Hlásny *et al.*, 2019).

1.3. Beetle-fungal associates and association specificity

The associations among Scolytinae and fungi have been studied for almost two centuries. The concept of “ambrosia” fungi was first developed by Schmidberger (1836). Since then, the different efforts to explore associations among fungi and Scolytinae have been summarised in qualitative and quantitative studies, which have evolved along with the methodologies used to detect and classify fungi (Anon, 2004). The peculiarities and limitations of the different methods are crucial when comparing the associated fungi recovered in each of these studies.

The association of Scolytinae beetles with fungi is a product of co-evolution. Both beetle and fungi have evolved through multiple independent lineages and across 60 Myr (Farrell *et al.*, 2001). A consequence of this co-evolution would be the different types of mycangia exhibited by bark and ambrosia beetles, associated with other fungal species (Skelton *et al.*, 2019a). Ambrosia beetles cultivate fungi inside tree carved galleries, forming fungal gardens. These gardens also contain other fungi, bacteria and yeasts (Haanstad & Norris, 1985). Earlier studies were carried out *in vitro* by culturing the fungi harboured by the beetles or in the galleries as part of breeding experiments (Batra, 1985). The conclusion of these earlier studies associated fungal symbionts mainly with ambrosia beetles and aggressive bark beetles like *Ips typographus*, *Ips sexdentatus* and *Tomicus piniperda* (Siemaszko, 1939). Some earlier studies were carried out under the hypothesis that, since the bark beetle had no specialised structures to transport fungi, there could be no evolutionary relationship such as those in fungus-farming beetles (Batra,

1963). Most of these studies have been focused on adults, although a small proportion has been focused on fungal associations across different developmental stages (Yearian, Gouger & Wilkinson, 1972). Unlike the bark beetles (Siemaszko, 1939), at first, each ambrosia species was thought to be associated with a single fungal species (Gadd & Loos, 1947)), but further studies revealed that those associations were more extensive than previously thought (Francke-Grosmann, 1967).

The fungi associated with the most abundant beetle species of this study, along with their mycangium and behaviour, are summarised in

Table 1-1. While earlier studies focused on single beetle species and a limited number of host trees, current techniques allow for higher-scale studies, including thousands of individuals from different beetle species across more expansive areas in the same analysis. These techniques had also facilitated the research of fungal communities associated with bark and ambrosia beetles classified as non-aggressive or secondary, which were not studied before because there were not causing significant outbreaks (Six, 2020). Consequently, in recent years, more and more bark beetles have been proved to harbour fungal associates.

Current techniques have also allowed investigations into fungal assemblages transmitted across beetle species and generations. The mechanisms by which beetles acquire or maintain their fungal associates can be summarised in two different evolutionary strategies: vertical and horizontal transmission. Vertical transmission occurs across generations of the same beetle species through the parental galleries. Mayers (2015) suggested a tight beetle-fungi co-evolution by associating each mycangium type with certain fungal species. Besides the vertical transmission, beetles may obtain fungal associates from other beetle species' galleries through horizontal transmission, in the so-called "crop theft" (Hulcr & Cognato, 2010).

Most fungal-associates of scolytine beetles are Ascomycetes (Li *et al.*, 2016), including the fungal order Ophiostomatales with some of the most destructive species (Bueno, Diez & Fernández, 2010; Kirisits, 2004; Linnakoski *et al.*, 2010; Wingfield *et al.*, 2017). Species include *Ophiostoma novo-ulmi* (the agent of Dutch Elm Disease) attacking elm trees throughout the Northern Hemisphere (Santini & Faccoli, 2015), the blue stain *Grosmannia* attacking various pine species in the American Northwest (Rice, Thormann & Langor, 2007, 2008), or the more recent outbreak of the laurel wilt *Raffaelea* vectored by an introduced bark beetle in South-eastern North America (Harrington *et al.*, 2011). Among the Basidiomycetes, the genus *Entomocorticium* establishes mutualisms with some bark beetles (Hsiau & Harrington, 2003). Despite their high

importance for the ambrosia beetles, ambrosia fungi have been studied in only 5% of the ambrosia beetles (Hulcr & Stelinski, 2017).

Table 1-1 Fungi associated with the most abundant bark and ambrosia beetles of this thesis

Beetle	Mycangium type	Host /Behaviour	Associated fungi	References
<i>Anisandrus dispar</i>	Prothoracic-pleural mycangia (Batra, 1963)	Polyphagous: broadleaves and conifers	<i>Ambrosiella hartigii</i> , <i>Ceratocystus sp.</i>	(Batra, 1963)
			<i>Fusarium eumartii</i> and <i>F. solani</i>	(Tiberi and Ragazzi, 1998)
			<i>Ophiostoma pluriannulatum</i> , <i>Diplodia pinea</i>	(Romón et al., 2007)
		Secondary, rarely primary species (hazelnut)	<i>A.hartigii</i>	(Mayers et al., 2015)
			<i>Candida fructus</i> (25%), <i>Fusarium spp</i> (25%specimens)	(Bezós et al., 2018)
			<i>Fusarium sp.</i> , <i>F. oxysporum</i> , <i>Acremonium spp</i>	(Tuncer, Kushiyevev & Erper, 2017)
<i>Dryocoetes spp</i>	Mandibular mycangia (<i>D.confusus</i> (Farris, 1969))	Oligophagous	<i>G. cucullata</i>	(Kirisits, 2004; Linnakoski et al., 2012b)
		<i>D.autographus</i> : Conifers	<i>O. piceae</i> <i>O. canum</i>	
		<i>D.villosus</i> : Broadleaves	<i>Ophiostoma sp.</i>	(Yamaoka et al., 2004)
		Secondary species	<i>Leptographium guttulatum</i> , <i>Ophiostoma ips</i> , <i>Ophiostoma olivaceum</i> , <i>Ophiostoma minus</i> , <i>Ophiostoma piliferum-like</i> , <i>Ophiostoma stenoceras</i> , <i>Ophiostoma rectangulosporium-like</i>	(Romón et al., 2007)

<i>Gnathotrichus materiarius</i>	Procoxal mycangia (Farris, 1963)	Oligophagous: conifers	<i>Ambrosiozyma monospora</i>	(Batra, 1963)
<i>Hylastes ater</i>	Pit-non-glandular mycangia: fungal repositories formed by shallow depressions of the tegument, mainly in the elytra (Klepzig & Six, 2004)	Monophagous: conifers	<i>Ophiostoma</i> spp, <i>Leptographium</i> spp	(Harrington, 1988; Jacobs & Wingfield, 2001)
		Secondary species	<i>Leptographium wingfieldii</i> , <i>Ophiostoma olivaceum</i> , <i>Ophiostoma pluriannulatum</i> , <i>Ophiostoma piliferum</i> -like, <i>Ophiostoma stenoceras</i> , <i>Ophiostoma quercus</i> , <i>Ophiostoma floccosum</i> , <i>Ophiostoma rectangulosporium</i> -like	(Romón et al., 2007)
			<i>Sphaeropsis sapinea</i> , <i>Grosmannia radiaticola</i> , <i>Leptographium lundbergii</i> , <i>Leptographium procerum</i> , <i>Grosmannia olivacea</i> , <i>Leptographium truncatum</i>	(Jankowiak & Bilański, 2013)
			<i>Graphium aureum</i> , <i>G. penicillata</i> , <i>L. lundbergii</i> , <i>L. serpens</i> , <i>O. floccosum</i> , <i>O. ips</i> , <i>O. minus</i> , <i>O. piceae</i> , <i>O. piliferum</i>	(Linnakoski et al., 2012b; Mathiesen, 1950)
<i>Hylastes brunneus</i>	Not known	Monophagous: conifers (<i>Picea abies</i> , <i>Pinus sylvestris</i>)	<i>Grosmannia galeiformis</i> , <i>O. pallidulum</i> ,	(Linnakoski et al., 2012a, 2010)

		Secondary species	<i>G. cucullata</i> , <i>L. chlamydatum</i> , <i>L. lundbergii</i> , <i>O. canum</i> , <i>O. piceae</i> , <i>O. tapionis</i>	
<i>Hylastes cunicularius</i>	Not known	Monophagous: Conifers (Spruce) Secondary species	<i>G. penicillata</i> , <i>G. olivacea</i> , <i>G. galeiformis</i> , <i>L. chlamydatum</i> , <i>L. curvisporum</i> , <i>O. piceae</i>	(Jacobs <i>et al.</i> , 2010; Mathiesen-Käärik, 1953)
<i>Hylastes attenuatus</i>	Pit-non-glandular mycangia: fungal repositories formed by shallow depressions of the tegument, mainly in the elytra (Klepzig & Six, 2004)	Monophagous: conifers	<i>Leptographium guttulatum</i> , <i>Ophiostoma stenoceras</i> , <i>Ophiostoma piceae</i> , <i>Sphaeropsis sapinea</i> (incidental)	(Romón <i>et al.</i> , 2007)
		Secondary species	<i>Candida fructus</i> (8%), <i>Fusarium spp</i> (32%)	(Bezós <i>et al.</i> , 2018)
<i>Hylastes opacus</i>		Oligophagous: conifers and broadleaves Secondary species	<i>Grosmannia radiaticola</i> , <i>Leptographium lundbergii</i> , <i>Leptographium procerum</i>	(Jankowiak & Bilański, 2013)
<i>Hylurgops palliatus</i>	Not known	Oligophagous: conifers	<i>Leptographium guttulatum</i> , <i>Ophiostoma pluriannulatum</i> , <i>Ophiostoma piceae</i> , <i>Leptographium wingfieldii</i>	(Romón <i>et al.</i> , 2007)
		Secondary species	<i>Grosmannia piceiperda</i> , <i>Ophiostoma ainoae</i> , <i>Ophiostoma piceae</i>	(Jankowiak, Kacprzyk & Młynarczyk, 2009)
			<i>Grosmannia galeiformis</i>	(Linnakoski <i>et al.</i> , 2012a)
			<i>Candida fructus</i> (11%), <i>Fusarium spp</i> (55%)	(Bezós <i>et al.</i> , 2018)

<i>Ips typographus</i>	Not known (Bentz <i>et al.</i> , 2019)	Oligophagous: Primary Norway spruce (<i>Picea abies</i>)	<i>Endomyces bisporus</i> (<i>Saccharomycetales</i>), and blue-staining fungi <i>Ophiostoma</i> <i>penicillatum</i> , <i>O.</i> <i>polonicum</i> n.sp., and <i>Graphium</i> <i>pycnocephalum</i>	(Siemaszko, 1939)
		Documented attacks on other conifers		
		Primary species	<i>Cop. minuta</i> , <i>C. norvegica</i> , <i>C.</i> <i>polonica</i> , <i>Gr. fimbriisporum</i> , <i>Gr.</i> <i>pseudormiticum</i> , <i>Gr.</i> <i>pycnocephalum</i> , <i>G. cucullata</i> , <i>G. olivacea</i> , <i>G. penicillata</i> , <i>G.</i> <i>piceiperda</i> , <i>L. chlamydatum</i> , <i>L. taigensis</i> nom. prov., <i>O.</i> <i>ainoae</i> , <i>O. bicolor</i> , <i>O.</i> <i>brunneo-ciliatum</i> , <i>O. canum</i> , <i>O. flexuosum</i> , <i>O. floccosum</i> , <i>O. fuscum</i> , <i>O. karelicum</i> , <i>O.</i> <i>minus</i> , <i>O. piceae</i> , <i>O.</i> <i>piliferum</i> , <i>O. pluriannulatum</i> , <i>O. saponiodorum</i> , <i>O.</i> <i>stenoceras</i> , <i>O. tapionis</i> , <i>O.</i> <i>tetropii</i> , <i>P. fragrans</i>	(Furniss, Solheim & Christiansen, 1990; Linnakoski <i>et al.</i> , 2012b; Mathiesen-Käärrik, 1953; Solheim, 1992a)
			<i>Ophiostoma bicolor</i> , <i>O.</i> <i>penicillatum</i> , <i>Ceratocystiopsis</i> <i>minuta</i> , <i>Ceratocystis polonica</i>	(Viiri & Lieutier, 2004)
			<i>Ophiostoma piceae</i> , <i>Ophiostoma bicolor</i> , <i>Ophiostoma piceaperdum</i> , <i>Ophiostoma ainoae</i> , <i>C. polonica</i>	(Jankowiak, 2005)
			<i>Ophiostoma piceae</i> , <i>Ophiostoma bicolor</i> , <i>Nectria</i> <i>sp</i> , <i>Grosmannia euophioides</i> <i>Graphium pseudormiticum</i> ,	(Persson <i>et al.</i> , 2009)

			<i>O. breviusculum</i> , <i>O. bicolor</i> , <i>O. polonicum</i>	(Giordano <i>et al.</i> , 2013)
<i>Tomicus piniperda</i> : * <i>Tomicus</i> sp	Not known	Oligophagous:	<i>Ophiostoma pini</i>	(Siemaszko, 1939)
		Primary Scots pine, but also other pines and occasionally spruce	<i>Ophiostoma minus</i> , <i>O. Piceae</i> , <i>Leptographium lundbergii</i> , <i>L. procerum</i> , <i>L. wingfieldii</i> , <i>Graphium pycnocephalum</i> and <i>Graphium</i> sp.	(Jankowiak, 2006)
		Secondary species	* <i>Leptographium guttulatum</i> , <i>Ophiostoma piceae</i>	(Romón <i>et al.</i> , 2007)
			<i>Cop. minuta</i> , <i>Gr. pseudormiticum</i> , <i>G. cucullata</i> , <i>G. olivacea</i> , <i>G. penicillata</i> , <i>H. tingens</i> , <i>L. chlamydatum</i> , <i>L. lundbergii</i> , <i>L. wingfieldii</i> , <i>O. brunneo-ciliatum</i> , <i>O. canum</i> , <i>O. clavatum</i> , <i>O. floccosum</i> , <i>O. ips</i> , <i>O. minus</i> , <i>O. piceae</i> , <i>O. piliferum</i>	(Linnakoski <i>et al.</i> , 2012b; Mathiesen-Käärrik, 1953)
			<i>Fusarium circinatum</i>	(Bezós <i>et al.</i> , 2015)
			* <i>Ophiostoma canum</i> , <i>Leptographium</i> spp	(Pan <i>et al.</i> , 2018)
<i>Trypodendron</i> spp	Prothoracic-pleural mycangia (paired tubes) (Batra, 1963; Francke-	Oligophagous	<i>Ambrosiella trypodendri</i> , <i>A. ferruginea</i> , <i>O. piceae</i> , <i>O. piceaperdum</i>	(Batra, 1967)
		(<i>T. domesticum</i> and <i>T. signatum</i> : broadleaves; <i>T.</i>	<i>G. penicillata</i> , <i>O. canum</i> , <i>O. minus</i> , <i>O. piceae</i> , <i>O. rachisporum</i>	(Linnakoski <i>et al.</i> , 2010)

	Grosmann, 1967)	<i>lineatum</i> : conifers)	<i>Grosmannia galeiformis</i>	(Linnakoski <i>et al.</i> , 2012a)
		Secondary species, rarely primary pests (Kühnholz, Borden & Uzunovic, 2001)	<i>Phialophoropsis ferruginea</i> , <i>Phialophoropsis spp.</i>	(Lehenberger, Biedermann & Benz, 2019)
<i>Xyleborinus saxesenii</i>	Elytral mycangia (Batra, 1963)	Polyphagous: conifers and broadleaves	<i>Raffaelea sulphurea</i> , <i>Nectria sp.</i> ,	(Grubbs <i>et al.</i> , 2020)
		Secondary species	<i>Fusicolla acetilerea</i> , <i>Paecilomyces variotii</i>	(Biedermann <i>et al.</i> , 2013)
			<i>Ramularia sp.</i> , <i>Alternaria sp.</i>	(Malacrinò <i>et al.</i> , 2017)
			<i>Candida fructus</i> , <i>Fusarium spp</i>	(Bezós <i>et al.</i> , 2018)
<i>Xylosandrus germanus</i>	Mesonotal mycangia (Hulcr <i>et al.</i> , 2012)	Polyphagous: conifers and broadleaves	<i>Ambrosiella hartigii</i>	(Batra, 1963)
		Primary species	<i>Ambrosiella grosmanniae</i> , <i>Ambrosiella spp.</i> , <i>Ceratocystis adiposa</i> , <i>Ceratocystis spp.</i> , <i>Meredithiella norrisi</i> , <i>Phialophoropsis ferruginea</i>	(Ito & Kajimura, 2017)
			<i>Fusarium proliferatum</i> , <i>F. solani</i> , <i>F. oxysporum</i> , <i>Acremonium kiliense</i>	(Tuncer, Kushiyevev & Erper, 2017)

Fungal symbionts may play diverse crucial roles to facilitate beetle development or colonisation, establishing various relationships with beetles. For symbionts such as *Ambrosiella* or *Ambrosiozyma*, associated with different ambrosia beetles, the primary role is nutrition (Batra, 1963). Other fungi that are very commonly associated with bark and ambrosia beetles are yeasts. The most prevalent associates from this group belong to the family Saccharomycetaceae, i.e. *Ogataea pini* or *Kuraishia capsulata* (Davis, 2015), which are also present in some of the beetles analysed in this thesis. The potential role of yeasts, however, is yet poorly defined. This gap in the literature could be linked to the few available studies on yeasts associated with bark beetles, which are primarily focused on primary pests like *Dendroctonus* or *Ips*. Although not

clearly defined, several studies point out a potential nutritious role of yeasts associated with bark and ambrosia beetles since they are primarily found in their digestive system. Moreover, yeasts may generate important chemicals to promote beetle attraction, or they could even modify tree chemistry (Davis, 2015). For the non-bark beetle genus *Carpophilus*, yeasts can contribute towards host selection and larval fitness (Baig *et al.*, 2020a). Therefore, the potential role of yeast on bark beetle fitness and invasion dynamics should not be disregarded.

Considerable efforts have also been made to gain insight into the underlying mechanisms of pathogenicity in fungal species (Schafer, 1994; Schuelke *et al.*, 2017) and the role Scolytinae beetles play in disease transmission (Carrillo *et al.*, 2014; Hulcr & Dunn, 2011; Yamaoka, 2017). Besides being more abundant and diverse than previously thought, fungal associates are also more promiscuous (Kostovcik *et al.*, 2015). The fact of being more promiscuous implies a higher number of beetle species sharing the same fungal associates. This promiscuity is directly connected with the types of symbiotic relationships that each beetle species may establish with their associated fungi. Six (2020) recently categorised the bark and ambrosia beetles by their attack behaviour, suggesting that different colonisation behaviours may be linked to a different symbiosis with fungi. This way, an “obligate symbiont”, a denomination for symbionts the beetle species rely on to survive, may be linked to aggressive tree killers, which possess complex mycangia to ensure the vertical transmission (Six, 2020) of such fungi. Bark beetles are considered “aggressive secondaries”, which have less reliance upon fungi and mainly feed on phloem. However, even if a fungal pathogen is not present in most beetle specimens, it may still cause significant disease epidemics, which is the case of *Endoconidiophora polonica*, associated with the bark beetle *Ips typographus* (Giordano *et al.*, 2013). This last author also suggests that fungal-beetle associations would differ depending on the phase of the epidemics, establishing a temporal succession among the Ophiostomatoid fungi.

1.4. The use of HTS to characterise beetle-associated fungal communities

The study of the fungal composition of Scolytinae communities has traditionally been constrained by culturing techniques, which can retrieve only a small proportion of the fungal species carried by the beetle. Besides, the lack of a simple taxonomy on fungi (*e.g.* in the genus *Fusarium*, belonging to an extremely-diverse clade that includes some tree-killing fungal species linked to bark beetles (Bezós *et al.*, 2015; Goldazarena, Romn & Lpez, 2012) represents a considerable challenge to this morphology-based approach.

Over the past decade, sequence-based taxonomic identification has become an essential technique when studying fungi (Bálint *et al.*, 2016; Hibbett *et al.*, 2016; Kulik, Biliska & Żelechowski, 2020; Matute & Sepúlveda, 2019; Miller *et al.*, 2016; Schmidt *et al.*, 2013). A fast, reliable and accurate species-level detection remains crucial for the early detection of pathogenic fungi since most of them could not be assessed through traditional techniques alone (Cai *et al.*, 2011; Luchi, Ios & Santini, 2020; Tremblay *et al.*, 2019). DNA-based studies are significantly enhancing our knowledge of beetle-fungal communities. Specifically, metabarcoding of entire fungal communities carried by these beetles can provide an unprecedented resolution, unveiling a vast number of cryptic fungi. This technique facilitates research on fungal composition and associations with beetle species, host trees, climatic conditions, or biogeography. Dissecting these various factors is the first step towards establishing the drivers of fungal associations.

To characterise fungal communities carried by bark and ambrosia beetles using metabarcoding, the fungal DNA present in the beetle specimens is extracted and amplified by PCR before sequencing it through High Throughput Sequencing (HTS). DNA barcodes are short standardised DNA sequences that work as unique identification markers for all species (Yu *et al.*, 2012). This way, from a single DNA extraction, thousands of sequences can be obtained. After being retrieved from the sequencing facility, sequences are processed by following different bioinformatics pipelines to obtain the fungal Operational Taxonomic Units (OTUs), representing fungal species. This process allows for achieving solid species identification before ecological analysis. Several steps of filtering and assembly are required to process the different sequences (**Figure 1-2**).

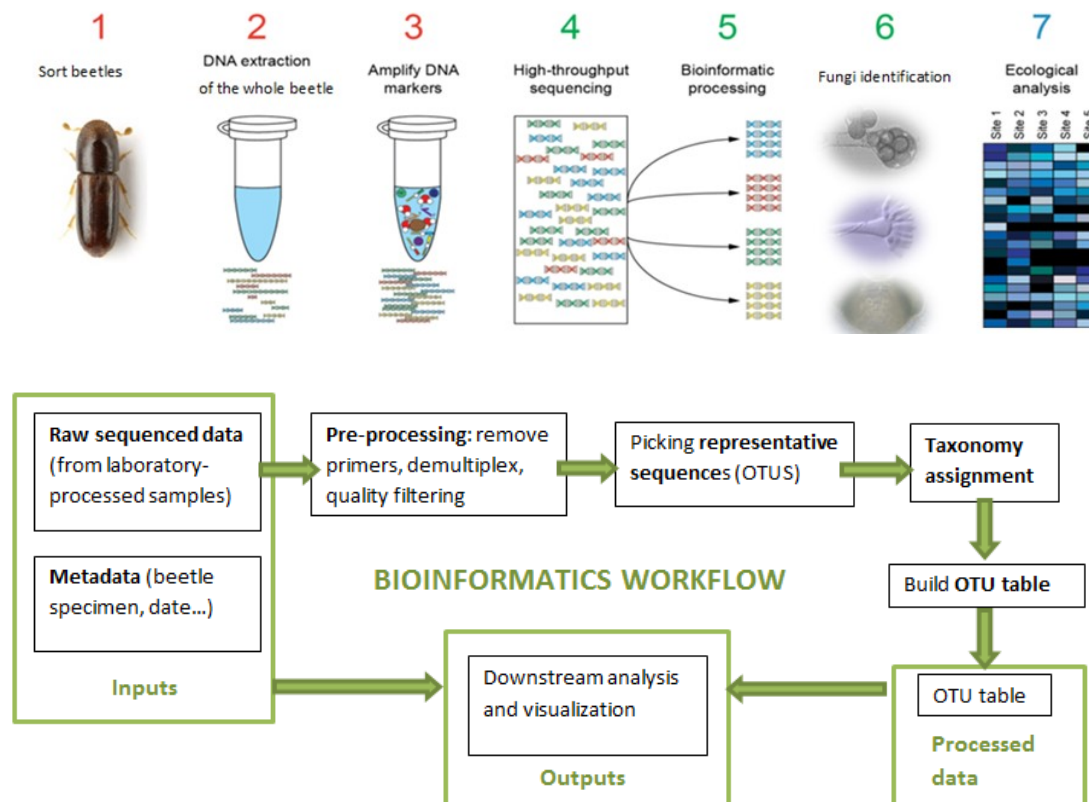


Figure 1-2 General workflow of metabarcoding assays (modified from <https://naturalis.github.io/mebioda/>) and general outline of the bioinformatics pipeline

High Throughput Sequencing (HTS) methods differ mainly in two features: the length of the amplicon and the target gene region amplified. Longer amplicons may lead to a more efficient fungal identification (Tedersoo *et al.*, 2020b). Although providing relatively short reads, “second-generation” HTS platforms like Illumina remain powerful tools to unveil fungal diversity and global patterns in ecological studies (Větrovský *et al.*, 2019).

Besides the technical peculiarities of each of the amplification techniques, there is also an ongoing debate about the most efficient process for fungal molecular data (Kandawatte Wedaralalage, Jayawardena & Hyde, 2020). The DNA extraction and amplification can generate biases, which, if not acknowledged, may condition further analysis (Frau *et al.*, 2019; Zinger *et al.*, 2019). The downstream procedure for raw reads processing has also been subjected to a long-term debate. This pipeline is usually a set of customised tools sequentially added to filter the raw data, removing the primers and dropping all those sequences that could be considered artefacts or because of their low quality, length or characteristics contaminations. These crucial decisions have also been summarised in several papers and reviews (Bálint *et al.*, 2014; Miller *et al.*, 2016; Pauvert *et al.*, 2019). Other works apply different pipelines to different data

extracted from artificial or natural mock communities to compare these techniques (Edgar, 2018; Somervuo *et al.*, 2016). This way, by calculating how many OTUs of each species are recovered, these studies aim to find the best set of bioinformatic decisions to take at each step of the pipeline (Pauvert *et al.*, 2019).

In recent years, molecular techniques have contributed enormously to clarifying the role of fungal communities associated with bark and ambrosia beetles (Hulcr *et al.*, 2020). An excellent example of this is the recent molecular-based study challenging the idea of the fungi associated with bark and ambrosia beetles acting as wood decomposers (Skelton *et al.*, 2019b), which was assumed by previous studies (Ulyshen, 2016). Furthermore, Li *et al.* (2016) pointed out that there are still thousands of symbioses yet to unveil. In this direction, a recent study suggested that symbiotic relationships among bark beetles and their fungal communities would be more common than previously thought (Six & Elser, 2020). Given that fungal databases remain incomplete due to the challenging and cryptic nature of fungi, the use of metabarcoding to characterise beetle-fungal communities has proven to be extremely useful in detecting new associations and ecological patterns. This way, the combination of culturing and molecular techniques facilitates the analysis of previously unknown fungal-beetle symbiosis (Li *et al.*, 2016), becoming essential to diagnosing and controlling pathogenic fungi (Jacobs *et al.*, 2010; Kulik, Bilska & Żelechowski, 2020; Romón *et al.*, 2007; Lücking *et al.*, 2020).

However, what remains clear is that both the experimental design and the methodology used may significantly affect the obtained results (Hulcr *et al.*, 2020; Nilsson *et al.*, 2019a; Pauvert *et al.*, 2019). A criticism faced by these studies is that they have been focused on aggressive Scolytinae and pathogenic fungi, leaving the secondary beetles almost unstudied and some potentially essential fungi such as yeasts neglected by the literature (Six, 2020). Moreover, for decades, there has been a considerable research bias towards easy-to-culture fungi, mainly the blue-stain fungi (Henriques, de Lurdes Inácio & Sousa, 2006). Therefore, these kinds of studies require a rigorous design to avoid generating further biases that could impact the results.

1.5. Invasive bark and ambrosia beetles

More than 6000 species in 247 genera of Scolytinae have been described to date, most of them found in tropical or subtropical areas (Grousset *et al.*, 2020). According to the Bark And Wood Boring Insects In Living Trees (BAWBILT) database, only 47 species are naturally present in Europe (Anon, 2004). In addition, there are at least 18 beetle species introduced into Europe, mainly through international trade (Kirkendall & Faccoli, 2010). This phenomenon is not new,

but the rate at which exotic bark and ambrosia beetles are being introduced into Europe has been accelerated by factors like globalisation and climate change (Bentz *et al.*, 2010; McDowell *et al.*, 2011). While some non-indigenous or exotic species remain in low numbers, the invasive species proliferate without control when finding so-called “naïve” host trees. This term is given to indigenous tree species, which have never encountered exotic species and have not evolved a natural resistance (Hulcr & Dunn, 2011). An example of this phenomenon is the invasion of the ambrosia beetle *Xyleborus glabratus*, which is associated with the fungus *Raffaelea lauricola*. This beetle is native from South East Asia but has colonised North America. While the beetle is generally affecting deadwood in their native range, in these new territories, the beetle is decimating living trees from the laurel family (Hlásny *et al.*, 2019). In the United Kingdom, the Dutch Elm Disease invasion was particularly damaging due to the poor management of the epidemics carried out by the institutions, based on a mix of inaccurate scientific information and slow reaction to the fast-spreading disease (Tomlinson & Potter, 2010). Also, in this country, the great spruce bark beetle (*Dendroctonus micans*) was accidentally introduced in the early 1970s and remained unnoticed until 1982 (Bevan & King, 1983; Fielding *et al.*, 1991). Despite the efforts, its eradication has not been possible since then, and this beetle species has become one of the most important invasive species in British forests. Current studies focus on studying the ecology of this beetle species in the United Kingdom to predict outbreak risks (Gent *et al.*, 2017). Therefore, invasive species proliferation generates negative outcomes like pest and diseases outbreaks, economic losses and a consequent loss of native species.

Knowing the ecology and introduction paths of non-indigenous species is essential to prevent them from accessing new territories (Desprez-Loustau *et al.*, 2007). Matthews (2017) *et al.* pointed out international trade as the primary source of introduction for plants and animals. Many of the most recent bark and ambrosia beetle introductions into Europe have probably used packing wood as a transport path into Europe (Inward, 2019). These species were not explicitly listed as quarantine pests since they were not expected, especially those not associated with coniferous wood. To summarise these introductions, the European and Mediterranean Plant Protection Organization (EPPO) has recently published an extensive report (2020). This report suggests that some risk factors such as beetle inbreeding, the ability to colonise live trees, polyphagy and fungus-farming may become an advantage when occupying new territories. Other factors like voltinism and the presence of natural predators also remain crucial for predicting the potential spread of the introduced pests. These risk factors set the ground for a method termed “horizon scanning” (Gallardo *et al.*, 2016; Matthews *et al.*, 2017; Roy *et al.*, 2014). “Horizon scanning” is a foresight method that evaluates the likelihood of

introduction of exotic species to identify potential hazards. Based on them, EPPO has pointed out 26 species and groups of bark and ambrosia beetles, posing a significant risk of invasion to Europe (Grousset *et al.*, 2020). Despite the increase of knowledge on the ecology of invasive species, active monitoring is the most powerful tool to identify new pests (Malacrinò *et al.*, 2017).

In the UK, there have been four invasive species of bark and ambrosia beetles detected in the past years (Inward, 2019). One of the most concerning pests is the beetle *Ips typographus*, which associates with several Ophiostomatoid fungi (Giordano *et al.*, 2013). These fungi help the beetle overcome tree defences by metabolising damaging substances (Zhao *et al.*, 2019). This beetle, which attacks spruce trees (*Picea* spp.), is native to Northern Europe and part of Asia, where it produces massive outbreaks in combination with these fungi (Hlásny *et al.*, 2019). Only very recently, its presence has been detected in the United Kingdom (Inward, 2019). The United Kingdom has not faced any significant bark-beetle related outbreak in the recent times, but the great spruce bark beetle (*Dendroctonus micans*) has caused considerable damage in the past (Grégoire, 1988). Therefore, the presence of *Ips typographus* (**Figure 1-3**) represents a substantial economic and ecological threat, as nearly half of the British conifer forests are inhabited only by spruce (Mason & Perks, 2011).



Figure 1-3 One of the analysed specimens of the bark beetle *Ips typographus*. The image was taken at the Imaging Analysis Centre, Natural History Museum, London.

Likewise, the introduction of fungi has had a substantial impact, mainly in agriculture (Rossman, 2008) and forestry (Loo, 2008). Since fungal associates are promiscuous, it is not straight forward to determine which fungal associates the invasive beetles may carry along when colonising a

new territory. This disparity of the mycobiota has been detected for *Ips typographus*, which exhibits a high fungal variation across countries and continents. While there are some frequent fungal associates, there are main quantitative and qualitative differences across *Ips typographus* European populations, especially for the blue-stain fungi (Giordano *et al.*, 2013, Anon, 2004).

1.6. Biotic factors shaping beetle-associated fungal communities

Potential biotic factors underlying the distribution and relative abundance of fungi within the fungal communities associated with bark and ambrosia beetles remain understudied. Kiristis (2004) compiled a list of associated fungi across beetle species and host trees, noticing that a large spectrum of fungi occurred on the same beetle species over different host trees. Using metabarcoding, Miller (2015; 2019) suggested that the beetle species would explain part of the variation on the fungal communities. Some of these fungi are associated with certain types of mycangia and thus associated with the beetle species (Skelton *et al.*, 2019a). However, fewer studies have shown how other biotic factors, apart from the beetle species, may affect fungal communities. Bach (2010) suggested an influence of individual trees on soil fungal communities. Differences linked to dominant host trees have also been detected in soil fungal communities (Scheibe *et al.*, 2015; Sun *et al.*, 2016; Urbanová, Šnajdr & Baldrian, 2015), including some ectomycorrhizal fungi for beech and spruce (Asplund *et al.*, 2019). Tree influence may indirectly affect fungal communities by modifying the environmental conditions at a local scale by affecting the biogeochemical functioning of the ecosystem (Augusto *et al.*, 2015).

The possibility of the dominant host trees driving a larger effect on fungal specificity and fungi speciation has already been discussed, mainly for ophiostomatoid fungi (Kirisits, 2004). Urbanová (2015) found a significant effect of the dominant tree species on local microbial communities, suggesting carrying out larger-scale analysis on forests with increasing plant diversity. This last author also suggested that fungal communities in mixed forests would be more diverse and complex. Based on the “insurance hypothesis”, more diverse forests could be more resistant and resilient against disturbances than monospecific forests (Jactel *et al.*, 2009). Bark beetles could also find mixed forests more challenging to colonise due to a more considerable distance among target host trees, the presence of non-target host tree species and a higher abundance of natural predators (Hlásny *et al.*, 2019). It is therefore necessary to further explore the extent to which biotic factors could have an effect on the fungal communities associated with bark and ambrosia beetles.

1.7. Outline of the thesis

My research investigates the factors shaping fungal communities associated with bark and ambrosia beetles, focusing on the influence of biotic drivers. For this purpose, I used metabarcoding to compare the fungal communities obtained from target bark and ambrosia beetle species collected on fieldwork using funnel traps. Metabarcoding allowed me to identify and compare the different fungi present in the individual beetles to find ecological patterns linked to the forest abiotic and biotic factors. I further applied this technique to characterise the fungal communities present in specimens of the invasive bark beetle *Ips typographus*, which has been recently introduced into the United Kingdom. Given the role of bark and ambrosia beetles as disease vectors and the increase in numbers of invasive species, it is paramount to understand the underlying mechanisms shaping these beetle-fungal associations.

Chapter 2: Comparing ITS and LSU primers to characterise bark and ambrosia beetle-associated fungal communities

At the moment, there is not any preferred identification method that applies to all the fungal metabarcoding studies (Lücking *et al.*, 2020; Pauvert *et al.*, 2019). It is, therefore, critical to ensure the adequate execution of the technique to accurately identify fungal associates. To adapt the current molecular techniques to the context-specific characteristics of these communities, the fungal communities of 80 beetle specimens were analysed through metabarcoding. This technique identifies entire fungal communities from each of the specimens through their DNA barcodes. For this experiment, a total of four beetle species - two of them invasive to the United Kingdom - were selected. These beetles were collected across three major British forest types (pine, spruce and oak). Their associated fungal communities were then characterised and compared using two different primer sets (ITS and LSU markers).

Chapter 3: The effect of host trees on the composition of fungal communities associated with bark and ambrosia beetles

To clarify the role of bark and ambrosia beetles in spreading tree-pathogenic fungi, it is necessary to understand which biotic factors mediate beetle-fungi interactions. Chapter 3 aims to assess the effect of the dominant host tree identity on the fungal communities associated with bark and ambrosia beetles. With this aim, beetle-associated fungal communities were explored for three major British forest types (pine, spruce and oak). For this experiment, a total of 160 beetle specimens from seven species - two of them invasive to the United Kingdom - were

selected. Fungal communities were then compared among the beetles, which were collected on these forest types using funnel traps. I studied the effect of the forest type on the fungal assemblages by collecting flying beetles in the same geographical area, the New Forest national park. This experimental design aimed minimising the potential effect of abiotic factors that could be more noticeable at a regional scale.

Chapter 4: Biodiversity Survey across the UK reveals the influence of the forest composition on the fungal communities associated with bark and ambrosia beetles

The fungal communities associated with bark and ambrosia beetles form diverse and highly complex assemblages. Most of the biological patterns behind this complexity still remain poorly understood, but forest composition is suspected of playing a role in fungal diversity. Chapter 4 aimed to study which abiotic and biotic factors mediate on these assemblages. With this aim, the fungal communities harboured by a total of 1064 beetles collected from 20 different sites across the United Kingdom were analysed. These beetles were trapped on forests consisting of different tree species composition, ranging from monospecific conifers to mixed broadleaves.

Chapter 5: The fungal communities associated with the first UK breeding population of the bark beetle *Ips typographus* include potentially invasive pathogens

As a consequence of climate change and globalisation, bark and ambrosia beetles are reaching new territories. With the recent detection of the invasive bark beetle *Ips typographus*, the United Kingdom risks suffering similar bark beetle outbreaks to Central Europe. However, the United has not yet encountered any significant bark beetle outbreak in recent years, which provides the opportunity of studying these beetles to investigate the factors mediating a potential outbreak. The potential effects of an outbreak on Sitka spruce, the most commonly-planted conifer in the UK, remain largely unknown. Several fungi establish symbiotic relationships with bark beetles, including some fungal pathogens, but fungal assemblages are highly variable. To report which fungi may be vectored into the UK and which fungal species formed associations with these beetles, we analysed fungal assemblages from 48 *Ips typographus* specimens. Chapter 5 explores the fungi associated with the first UK-born population of the bark beetle *Ips typographus* across developmental stages and, in the case of pupae, for the two different species of spruce broadly planted in the UK.

In Chapter 6 I further discuss my findings and their implications at a wider ecological context, including forest and invasive species management implications and suggesting future work.

Chapter 2. Comparing ITS and LSU primers to characterise bark and ambrosia beetle-associated fungal communities.

2.1. Abstract

Bark and ambrosia beetles (Coleoptera: Scolytinae) associate with pathogenic fungi. Large scale forest pest and disease outbreaks are linked to this phenomenon, which remains still poorly understood. To prevent or mitigate the impacts of these outbreaks is crucial to characterise beetle-fungi associations fully. However, the characterisation of fungal communities has been challenging due to the cryptic nature and complex taxonomy of fungi. Thanks to molecular techniques like metabarcoding, it is possible to unveil many previously unnoticed fungi. At the moment, there is not any preferred identification method that applies to all the fungal metabarcoding studies (Lücking *et al.*, 2020). It is, therefore, critical to ensure the adequate execution of the technique to accurately identify fungal associates. To adapt the current molecular techniques to the context-specific characteristics of these communities, the fungal communities of 80 beetle specimens were analysed through metabarcoding. This way, the fungal communities from each of the specimens were evaluated through their DNA barcodes, which are short standardised DNA sequences that work as unique species identification markers. For this experiment, a total of four beetle species - two of them invasive to the United Kingdom - were selected. These beetles were collected across three major British forest types (pine, spruce and oak). Their associated fungal communities were then characterised and compared using two different primer sets (ITS and LSU markers). Results included potential biases in both ITS and LSU amplifications, with generally higher amplification success and higher taxonomic resolution for the ITS primers. Both primer sets draw similar conclusions on the applied ecological analysis, but ITS have the advantage of more complete databases, which contributed to identifying target fungi, like fungal pathogens. Based on these premises, I found the ITS primers more informative than the LSU primers. However, we need to be careful when comparing the different studies since different primers may cause variations in the ecological analysis outcomes.

2.2. Introduction

Fungal communities associated with insects have been widely studied across the years to disentangle the nature of their interactions (Six, 2020; Malacrinò *et al.*, 2017; Li *et al.*, 2016; Raman, Wheatley & Popay, 2012; Ganter, 2006). For these kinds of studies to succeed, an accurate and reliable fungal identification remains fundamental. However, the cryptic nature and complex taxonomy of fungi difficult fungal identification. Consequently, only 3-8% of the fungi are described so far (Anon, 2017b).

Over the years, a wide variety of methods to characterise fungal communities have been developed. The studies of fungal communities were carried out first by culturing and isolating each fungus recovered from the specimens (Batra, 1963), but this generated biases towards the easy-to-culture fungi. To overcome this issue, molecular techniques (Bálint *et al.*, 2014; Miller, 2015) have become the new standard methodology (Hulcr *et al.*, 2020). Molecular techniques are based on short gene sequences or barcodes to identify species using high-throughput sequencing and subsequent bioinformatics analysis (Yu *et al.*, 2012). Among these techniques, metabarcoding has been demonstrated to be extremely useful to characterise fungal communities, unveiling a wide variety of cryptic fungi (Malacrinò *et al.*, 2017; Johnson *et al.*, 2018; Miller *et al.*, 2019). Metabarcoding generates fungal amplicons by amplifying specific DNA regions called metabarcodes using a set of customised markers. Amplicons are then sequenced, filtered through a set of pipelines and clustered into Operational Taxonomic Units (OTUs). OTUs can be used as a proxy of the fungal species, which facilitate the study of those fungal species that taxonomists have not yet officially named or those cryptic species not easily obtained via culturing (Blaxter *et al.*, 2005).

The rapidly-evolving molecular techniques have contributed to building a wide range of methodologies to characterise fungal communities. However, there is still a significant debate regarding the suitability of the methods used to generate and process high throughput sequencing (HTS) data to study fungi (Hulcr *et al.*, 2020; Nilsson *et al.*, 2019b; Bálint *et al.*, 2016). HTS sequencing methods differ from each other, mainly in two features: the length of the amplicon and the target gene region amplified. Longer amplicons may lead to a more efficient fungal identification since they can capture more variability to distinguish the fungus at a deeper taxonomic level (Liu *et al.*, 2012; Porras-Alfaro *et al.*, 2014; Tedersoo *et al.*, 2020b). The length of the amplicon is directly connected with the target gene region amplified, as different primer sets will amplify different segments of the rDNA (Bellemain *et al.*, 2010), significantly affecting

the diversity and composition of the obtained metabarcodes (Li *et al.*, 2020). Consequently, selecting a standard target genomic region to amplify has also been a long-term debate in HTS studies.

Although since 2012, the internal transcribed spacer (ITS) has officially become the target region to amplify fungal DNA (Schoch *et al.*, 2012), it is not possible to sequence the whole gene using second-generation HTS techniques, with the subregions ITS1 and ITS2 becoming widely used (Nilsson *et al.*, 2019b). However, some authors started to raise concerns about the potential biases generated by choosing a single marker like ITS1 or ITS2 (Bellemain *et al.*, 2010; Li *et al.*, 2020). To try to minimise that bias, the use of other regions like the large subunit (LSU) (**Figure 2-1**) or the small subunit(SSU) ribosomal RNA(rRNA) genes, or a combination of them has started to become more common (Skelton *et al.*, 2019c; Porras-Alfaro *et al.*, 2014; Lücking *et al.*, 2020). Despite this increasing diversity of choices, primer sets are often selected without any preliminary study (Op De Beeck *et al.*, 2014). As a result, the primers selected to amplify heterogeneous samples may not be the most appropriate to capture sample diversity (Hulcr *et al.*, 2020).

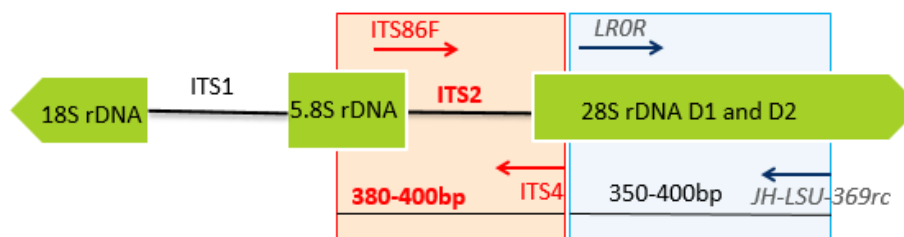


Figure 2-1. Overview of the ITS and the 28S rDNA region. The ITS and LSU regions amplified by each primer are marked in red and blue, respectively.

In practice, both ITS and LSU exhibit particularities that could be detrimental or beneficial depending on the aim and the scope of the target study (Porras-Alfaro *et al.*, 2014). While LSU is less variable, favouring alignment and tree-building-based methodologies, this decreases the taxonomic resolution at the species-level. In comparison, ITS holds a better species resolution than LSU, but it cannot achieve a species delimitation of particular lineages. Some studies showed that the ITS primers are biased towards the amplification of specific taxa (Harrington *et al.*, 2011; Dreaden *et al.*, 2014) and suggested the use of other regions to minimise the potential bias as a secondary barcode (Hulcr *et al.*, 2020; Taylor *et al.*, 2016; Lücking *et al.*, 2020). Moreover, ITS exhibits a high variability, which is often translated into poor alignments, making it challenging to apply to tree-based methods (Porras-Alfaro *et al.*, 2014; Vrålstad, 2011).

However, this intraspecific and intragenomic variation is still poorly documented in most regions, not only in ITS (Lücking *et al.*, 2020). Furthermore, the suitability of the primers used to amplify fungal communities is often tested by using mock communities (Bakker, 2018; Egan *et al.*, 2018; Frau *et al.*, 2019), but few studies have addressed these differences on highly-complex natural fungal communities (Parada, Needham & Fuhrman, 2016; Skelton *et al.*, 2019b; Li *et al.*, 2020).

On top of these potential biases, the lack of complete fungal databases makes fungal identification more challenging. While the most economically significant clades like those containing pathogenic fungi are broadly present in the databases, some clades are under-sampled (Lücking *et al.*, 2020), generating additional biases (Six, 2020; Liu *et al.*, 2012). To overcome these biases, a wide range of fungal classifiers have been developed and tested with mock communities (Nilsson *et al.*, 2019b; Murali, Bhargava & Wright, 2018; Deshpande *et al.*, 2016; Wang *et al.*, 2007). Nevertheless, there is still a general lack of consensus concerning the classification of the fungal OTUs (Porrás-Alfaro *et al.*, 2014; Lücking *et al.*, 2020; Liu *et al.*, 2012), which makes the fungal metabarcoding studies difficult to compare (Hulcr *et al.*, 2020). It is, therefore, necessary to ensure that the methodology chosen achieves both aims: to capture the highest fungal diversity and to classify the highest proportion of OTUs reliably.

Metabarcoding of fungal communities faces, therefore, two main challenges. First, it needs to ensure an overall amplification success to reliably amplify the wide variety of fungi present in the highly-complex fungal communities, which seems directly connected with the amplicon length (Bellemain *et al.*, 2010; Li *et al.*, 2020). Second, it needs to hold sufficient taxonomic resolution to accurately detect fungi at the species level, a factor that heavily relies on the completeness of the databases. Alternatively, it depends on the phylogenetic power of placing a sequence relative to a sparsely sampled database. While these challenges are still difficult to overcome, it is necessary to reach a consensus to facilitate the comparisons across fungal metabarcoding studies by, for example, using different databases and exploring their limitations for natural fungal communities.

Here I compared the performance of two primer sets (ITS and LSU) on natural and highly-complex fungal communities. To do this, I used metabarcoding to analyse the fungal communities from 80 bark and ambrosia beetle specimens, which were collected across three different forest types. More specifically, in the present chapter, I aimed to examine the effect of the primer election on 1) the amplification success and the choice of a fungal classifier on the

taxonomic resolution of the fungal communities 2) on the downstream ecological analysis of these complex natural fungal communities.

2.3. Materials and Methods

Samples were collected by Forest Research (Forestry Commission) during the years 2013 -2015 in the New Forest National park (50°50'52.08" N 1°35'33.51" W), in the South of the UK. A total of 80 beetle specimens were collected with Lindgren multiple-funnel funnel traps ((Lindgren, 1983), Phero Tech). These traps were located in oak (*Quercus robur* L.), spruce (*Picea abies* Karst.) and pine (*Pinus sylvestris* L.) forests (**Table 2-1**). Traps spaced 50m apart along 250m of the transect and baited with lures (100% ethanol, plus α - pinene) to mimic volatile chemicals emitted by dying trees, as shown in Inward (2019). Propylene glycol (65% concentration) was placed at the bottom of each trap to ensure the correct preservation of the beetles. Traps were emptied every two weeks between April and August. Captured specimens were then kept frozen in pure ethanol to preserve their DNA quality until being sorted and analysed. These specimens were first identified using standard identification keys (Duffy, 1953; Grüne, 1979). I randomly selected the beetle specimens among those identified as target species to obtain the same number of samples per beetle species and forest type.

Table 2-1. Overview of the beetle species and the number of the specimens per forest type included in the study, along with information related to their origin and primary structures for fungal transportation (Batra, 1963; Wood, 1982).

Forest type	Beetle species	Status	Adapted structures	Specimens
Spruce, oak	<i>Xylosandrus germanus</i>	Invasive	Mesonotal mycangia	20
	<i>Xyleborinus saxesenii</i>	Native	Elytral mycangia	20
Pine, spruce	<i>Gnathotrichus materiarius</i>	Invasive	Tubular structure opening near precoxal	20
	<i>Tomicus piniperda</i>	Native	Mycangia not known	20

2.3.1. Laboratory procedures

Selected specimens were rinsed with pure water to wash out external impurities and placed in individual 1.5ml tubes. Sterile pestles were used to completely break up the individual beetle specimens to ensure that all the fungi inside the specimens were captured. The DNA of the specimens was then individually extracted, purified and eluted in 200 μ l of AE buffer using the

QIAGEN DNA easy blood and tissue spin column extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Metabarcoding of associated fungi was performed using two different fungal segments (ITS and LSU), amplified using PCR. To do this, the ITS primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') /ITS4(5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck *et al.*, 2014) and the LSU primers LR0R (5'-ACCCGCTGAACTTAAGC-3') (Vilgalys & Hester, 1990) / JH-LSU-369rc (5'-CTTCCTTTCAACAATTTAC-3') (Li *et al.*, 2016)) were used at separate reactions. Unique six-letter indices were added to each primer pair to distinguish the specimens inside the different libraries. The PCR reaction mixture contained 2.5 µl of TaKaRa buffer™, 0.5 µl of dNTPs (Bioline), one µl of forward primer, one µl of reverse primer, 0.1 µl of TaKaRa Taq™ and 18µl of ddH₂O. 2 µl of DNA were added to every PCR reaction, making a final volume of 25 µl. Cycling conditions of the PCRs were: 94°C for 3 minutes followed by 34 cycles of 94°C for 45 seconds, 55°C for 55 seconds, 72°C for 1.5 minutes, and a final extension of 72°C for 10 minutes. For each of the analysed samples, PCRs were replicated with three annealing temperatures (54°C, 55°C and 56°C) to consider the stochastic nature of PCR reactions (Schmidt *et al.*, 2013). A minimum of one blank PCR reaction per plate was used as a negative control to control potential contaminations.

To corroborate the taxonomic identification of the beetle, the beetle COI region was also amplified using barcoding. Unique six-letter indices were also added to each primer pair to distinguish the specimens inside the different libraries. For the COI barcoding, the PCR reaction mixture contained 2,5 µl of Bioline buffer™, 0.75 µl of MgCl₂, 0.25 µl of dNTPs (Bioline), 0.75 µl of forward primer Fol-degen-for (5'-CCNGAYATRGCTTYCCNCG-3'), 0.75 µl of reverse primer Fol-degen-rev (5'-TANACYTCNGGRTGNCCRAARAAYCA-3') (Yu *et al.*, 2012), 0.1 µl of Bioline Taq™ and 18µl of ddH₂O. 2 µl of DNA was added to every PCR reaction, for a final volume of 25 µl. Cycling conditions of the PCRs were: 94°C for 4 minutes followed by 40 cycles of 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 45 seconds and a final extension of 72°C for 10 minutes.

Successful PCR amplification was confirmed in every case by visualising 3 µL of the PCR product using gel electrophoresis. For this visualisation, 2% agarose TAE gel, GelRed™ (Biotium) and Hyperladder IV™ (Bioline) were used. PCR products were then purified to eliminate fragments under 200bp, using the Agencourt® AMPure® XP purification kit, by following protocol 000387v001. The amplified DNA was pooled in equal proportions thereafter, forming 96 different pools of barcodes and metabarcodes. At the sequencing facility, the 96 libraries were

prepared, homogenised and indexed using Nextera® XT with a second PCR. Libraries were then sequenced on an Illumina® HiSeq® 2500 platform with version 2 chemistry (Rapid mode), using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads.

2.3.2. Bioinformatics

Raw sequence data were processed using bioinformatics to achieve solid species identification before its ecological analysis. The initial procedure was similar for both metabarcoding and barcoding samples, differing only in the last steps. The 96 libraries were firstly demultiplexed by using the Illumina software to remove their XT indices. The raw reads generated for these analyses are available as Bio-Project PRJNA727174 (Sequence Read Archives (SRA)) in the BioSample Submission Portal (Barrett *et al.*, 2012).

Samples were further demultiplexed with Cutadapt (Martin, 2011) scripts modified to account for bulk samples. During this step, the unique six-letter indices attached during the first round of PCR were trimmed from each sample file, which was then assigned a unique name related to every single beetle specimen. Also, at this step, singletons were removed, and Cutadapt was used to trim primers and zero-length sequences before read assemblage. Read quality was evaluated using FASTQC (Andrews, 2014). Forward and reverse reads were subsequently assembled using PEAR v. 0.9.8 (Zhang *et al.*, 2014). This step included a quality filtering of the paired-assembled reads (PEAR quality score threshold ≥ 30 , as recommended by Illumina® (https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf)). PEAR software tests all possible overlaps among reads due to its tolerance to variable read lengths (Zhang *et al.*, 2014). A further quality test was carried out using USEARCH v. 7.0 (Edgar, 2010), with the `--fastx_filter` command (`-fastq_maxee 1.0`).

Merged full-length barcoding reads averaged 418 bp in length, which targets the amplified COI region. To confirm beetle species identifications, the most abundant read for each COI sample was then selected and identified by BLAST (Altschul *et al.*, 1990) into GenBank (Benson *et al.*, 2009).

Merged full-length metabarcoding reads had an average length of 252 bp for the ITS samples. For the LSU samples, reads had an average length of 357 bp. Read clustering into Operational Taxonomic Units (OTUs) was subsequently performed using USEARCH (Edgar, 2010). After their dereplication, sequences were filtered by length, eliminating those sequences shorter than 100bp or longer than 500bp and removing those with less than two reads to avoid potential

sequencing errors or artefacts. Chimaeras were also detected and filtered out with UCHIME (Edgar *et al.*, 2011). Reads were then denoised using UNOISE (Edgar, 2016:p.2) and clustered using UCLUST of the USEARCH v. 9.2 pipelines (Edgar, 2010) at a 97% similarity threshold to delimit different OTU clusters. The average length of the OTUs was 270 bp for ITS and 347 bp for LSU (**Supplementary Figure APP I-1**). Since the ITSx tool (Bengtsson-Palme *et al.*, 2013) was not available for the LSU sequences, I did not use it for this analysis. Reads were then mapped to the OTU clusters, outputting an OTU table suitable for the ecological analysis of the samples.

2.3.3. Primer comparison

To compare the taxonomic resolution obtained by each of the primer sets, fungal OTUs were classified, following three complementary paths. The Ribosomal Database Project (RDP) Bayesian Classifier (Wang *et al.*, 2007) was used to assign the first classification for the OTUs. This classifier has been broadly used for fungal identification, using the Warcup fungal ITS (v2_March2018) and UNITE (v2020_February2020) training datasets to achieve both broad coverage and accurate OTU classification (Deshpande *et al.*, 2016; Edgar, 2018). Simultaneously, OTUs were processed through the PROTAXFUNGI pipeline (Abarenkov *et al.*, 2018), implemented in the PlutoF ('PlutoF Biodiversity Platform User Community (2017): PlutoF platform reference-based occurrences. v1.13. PlutoF. Dataset/Occurrence.', no date) platform and based on the UNITE fungal database (last update, February 2020). PROTAXFUNGI hierarchically assigns the OTU identities from the taxonomy root node through the species level (Nilsson *et al.*, 2019b). Since PROTAXFUNGI was not yet implemented for LSU, it was not possible to use this classifier for the LSU-amplified OTUs. The final classifier selected, IDTAXA, employs machine learning to reduce over-classification errors to obtain a higher accuracy (Murali, Bhargava & Wright, 2018). This classification was carried out separately on class, order, genus and species level. Combining these methods has the advantage of adding three different percentages of probability to every identification for ITS and two for LSU data. These percentages represent an estimation of how likely each OTU is currently identified at every level and can contribute to arriving at an informed consensus for correct identification. A minimum threshold of 70% probability was set, below which the OTUs were considered as "Unclassified"; along with those that, despite of having a higher probability, included the words "Unclassified", "Unidentified" and "*incertae sedis*". When the classifiers disagreed in their classification, the one with the highest probability was selected. To further complement this information, the ten most abundant fungal OTUs were blasted against the GenBank National Centre for Biotechnology

Information (NCBI) database (MEGA-BLAST). All this information was then combined with the data of the collected beetle specimen to perform the ecological analysis.

To minimise potential artefacts, I removed singletons-doubletons and performed rarefaction to 1000 reads to retain most of the samples while making them more comparable, losing five samples. Since few samples did not amplify well in either of the primers and removed by the rarefaction, I removed the same samples in both regions to keep the comparison balanced. A total of 146 samples (73 samples for each region) were retained for further analysis. To compare the amplification success obtained ITS and LSU at the class level and detected potential amplification biases, the proportions of OTUs amplified by each primer pair was then compared with a barplot using the function `ggplot2` (Wickham, 2016:p.2) in Rstudio (RStudio team, 2015).

To further explore the differences between ITS and LSU amplification and the accuracy of OTU classification, I constructed and compared phylogenetic trees for each of the regions, selecting those OTUs belonging to the class Sordariomycetes. This class hosts a wide range of fungi that play critical roles in bark and ambrosia beetle-associated fungal communities (Vanderpool, Bracewell & McCutcheon, 2018), including those belonging to the order Ophistomatales (*Ophiostoma* spp.) (Jankowiak, Kacprzyk & Młynarczyk, 2009; Yamaoka, 2017) and Microascales (*Ambrosiella* spp.). To do this, after removing singletons-doubletons and performed rarefaction, the OTUs belonging to this class were then aligned using MAFFT v 7 (Katoh, 2002) global alignment. Tree construction was implemented in the CIPRES Science Getaway platform (Miller, Pfeiffer & Schwartz, 2010) using RAXML v 8.2 (Stamatakis, 2014) with 1000 rapid bootstrap replicates. Three OTUs from the class Leotiomycetes were used as outgroup taxa, as seen in (Hongsanan *et al.*, 2017). Trees were visualised in FigTree (Rambaut, 2006) exported to Rstudio (RStudio team, 2015), where the identity of the individual tips was assigned by merging the OTU number belonging to the sequence with their taxonomic identification at genus/species level and classifier by using the `phyloseq` package (McMurdie & Holmes, 2013). To show incongruences among fungal classifiers at order and species level, the phylogenetic tree was displayed with different colours, showing also the classification at the order level.

2.3.4. Effects of the amplified sub-region on the ecological analysis

After generating the OTU table through the bioinformatics process and identifying the retrieved fungi, data analysis was split into three parts. While the first part aimed to estimate differences of richness, the second part evaluated the diversity of the fungal communities across different forest types. The third part aimed to determine how extended the presence of fungal associates

(including symbionts and pathogens) was through the whole set of samples. Statistical analyses on the remaining 73 samples from each region were carried out at the OTU level.

Species accumulation curves were built with Rstudio (vegan package (Oksanen, Jari, et al., 2018), “*speccacum*” function). These curves are frequently used to corroborate that the average sample size was sufficient for the biodiversity survey to be able to capture a reasonable amount of fungal OTU variability (Hughes et al., 2001). Since many of the curves did not approach an asymptote, the extrapolated OTU richness was chosen over the observed OTU richness to carry out the richness modelling. The extrapolated fungal OTU richness was estimated using the Chao1 index, which was calculated using the “*estimateR*” function from the vegan R package (Oksanen, Jari, et al., 2018). A set of generalised linear models (GLM) were built to compare the extrapolated fungal richness, using the R package lme4 (Bates et al., 2015:p.4). These models used the Chao1 richness as a response variable and beetle species and forest type as dependent variables. Each of the models was built under the assumption that beetle specimens originated from different galleries and trees because beetles were trapped at random from the forest. First, the model was tested with the Poisson distribution. However, as the data showed overdispersion (the residual deviance was greater than the degrees of freedom), two more models were tested, the Quasi-Poisson and the Negative Binomial models. Finally, the Negative Binomial model was chosen, as it is suitable for over-dispersed and showed a slightly better fit than the Quasi-Poisson. Model assumptions were corroborated using the DHARMA R package (Hartig, 2017).

To characterise the fungal communities across different forest types and beetle species, we calculated distance matrices based on different indexes. Sorensen and Jaccard indexes calculate the beta-diversity between sample pairs based on presence-absence data (richness), while the Bray-Curtis index focuses on the relative abundance of each OTU. Community dissimilarities were visualised in a metric multidimensional scaling MDS graph (Betapart R package (Baselga & Orme, 2012), Jaccard index). To quantify the variations shown in the MDS, a permutational multivariate analysis of variance (PERMANOVA) was performed. By doing this, a model for forest type, beetle species and their interaction were calculated by using the “Adonis” function of the vegan package (permutations=999)(Oksanen, Jari, et al., 2018). A permutational multivariate analysis of dispersion (PERMUTEST) was performed to evaluate the assumption of homogeneity of variance in the data by using the Betadisper function(Anderson & Walsh, 2013) (Vegan package). The non-euclidean distances between the group centroids were also calculated to assess the group dispersion (data available on request).

To evaluate the associated fungal OTUs for each assembly, a multilevel pattern analysis was carried out by calculating Pearson's phi coefficient of association ("p.g")(Chytrý *et al.*, 2002) between sample pairs, correcting this index to account for the differences in specimen numbers among the compared groups (function `multipatt`, `indicspecies` R package (De Cáceres *et al.*, 2011)). The identified OTUs for which the association values were significant were then selected and displayed as a heatmap (`Superheat` R package (Barter & Yu, 2018)).

2.4. Results

2.4.1. Primer comparison

The HTS run produced 2,436,075 quality-filtered and merged ITS reads, around 30% more than the 1,742,119 reads from the LSU data. After the bioinformatics filtering, a total of 1546 OTUs from ITS and 632 OTUs from LSU were retrieved after clustering. Since their reads abundance was low compared to the rest of the OTUs, the 12 OTUs found in the negative controls were subsequently excluded from further analysis. After removing OTUs present in negative controls, singletons-doubletons and performing rarefaction, the number of OTUs was 1157 and 548, respectively. After the combined use of `IDtaxa`, `PROTAXfungi` and `RDP` classifier at 70% or more of probability, 38% OTUs were classified to the genus level using the ITS markers, 13% more than those retrieved using the LSU markers. At the species level, most of the OTUs remained unidentified for both primer sets, which is not surprising when thinking that only 3-8% of the fungi are described so far (Anon, 2017b).

There was a higher consensus among the classifiers for ITS than for LSU since the classifiers reached the same classification for the genus in around 20% and 5% of the OTUs for ITS and LSU, respectively (**Figure 2-2**). Interestingly, at the order level, there were more OTUs only classified by `PROTAXfungi` or `RDP` than on the other taxons. In ITS at the genus level, `RDP` was the classifier that achieved the highest probabilities of identification, and thus the most used, followed by `IDtaxa`. However, for LSU at the genus level and ITS at the species level, `RDP` classification was less successful than the others. This fact illustrates the importance of combining different classifiers to obtain a complete characterisation of fungal communities. The species-level classification was only possible for the ITS-amplified data since this possibility was not included based on the LSU databases.

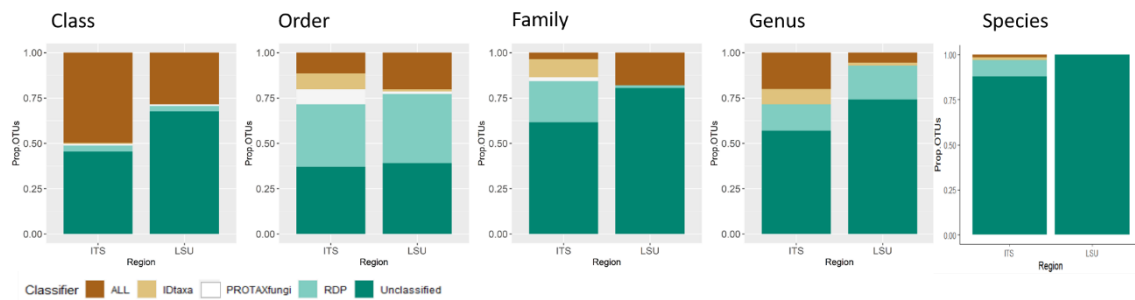


Figure 2-2. Overview of the proportion of fungi classified with IDtaxa, PROTAxfungi and RDP at different taxonomic levels. “ALL” refers to the proportion of OTUs for which the tested classifiers agreed in their classification.

Regarding the differences in fungal amplification success, Dothideomycetes and Sordariomycetes were the dominant classes for ITS and LSU, respectively (**Figure 2-3**). For most of the classes, the ITS primers accounted for a higher number of OTUs than LSU and even five times more in the Saccharomycetes, Leotiomyces and Tremellomycetes classes. Moreover, for the same number of reads, the ITS primers captured OTUs from six extra classes not recovered by LSU data, although only one or two OTUs were recovered from these classes. LSU also obtained a small proportion of OTUs from the class Atractiellomycetes, which was not recovered for ITS. The biases mentioned above may explain that ITS and LSU amplify different fungi proportions, but because the proportion of unclassified fungi is high, this extent is difficult to prove.

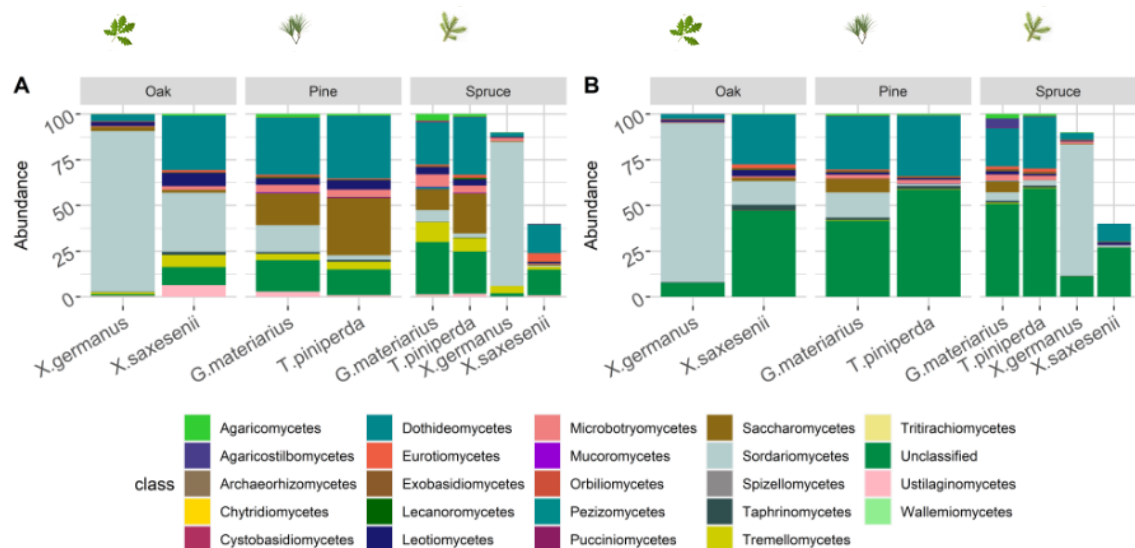


Figure 2-3. Proportion of OTUs belonging to the different classes detected by the ITS (A) or LSU (B) primers for each of the beetle species and forest composition. Samples were rarefied to 1000 reads to make them more comparable.

These results were affected by the disagreement among fungal classifiers, generating mismatches between class and species-level for certain fungi. For example, *Ambrosiella* spp.,

which were correctly assigned to class Microascales by ITS primers (IDtaxa classifier), were identified as Ambrosiella by RDP in the LSU dataset but included in the Sordariales order. However, there were also problems at connecting the results of the combination of classifiers across the different levels for ITS. For example, although one OTU was identified as *Ophiostoma angusticollis* by all classifiers for ITS and to the genus level by RDP in LSU, it was incorrectly assigned at the order level in both cases.

To corroborate the accuracy of the OTU classification, a phylogenetic tree of the class Sordariomycetes was built for each of the sets (**Figure 2-4, Figure 2-5**). A total of 79 and 45 OTUs were identified as Sordariomycetes by ITS and LSU, respectively. In this class, the ITS primers detected a total of eight orders, double than of those detected by LSU. Despite obtaining a higher proportion of OTUs at this class and despite having on average longer reads than the OTUS amplified with ITS, the LSU OTUs recovered fewer orders than ITS.

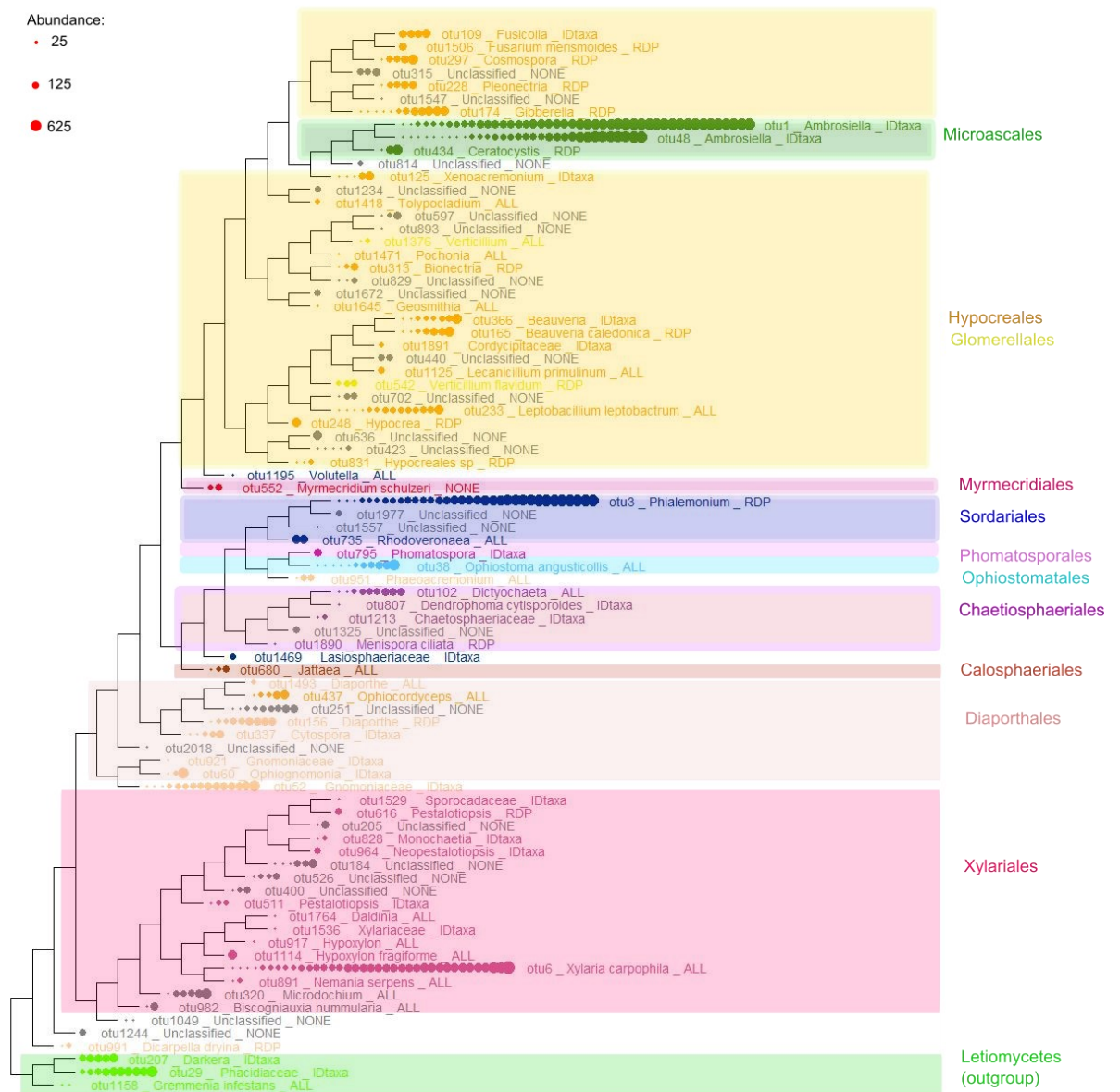


Figure 2-4. Phylogenetic placement of the OTUs generated from the ITS metabarcodes identified as Sordariomycetes by a combination of IDtaxa, RDP and PROTAXfungi with probability >70%. Different colours show the different orders. The genus or species and the classifier are detailed where the species-level identification succeeded. "ALL" means that all the 3 classifiers agreed with the classification of the fungi. The number of samples containing each OTU is shown with the dots, which represents the size of each dot the number of reads per sample of that OTU.

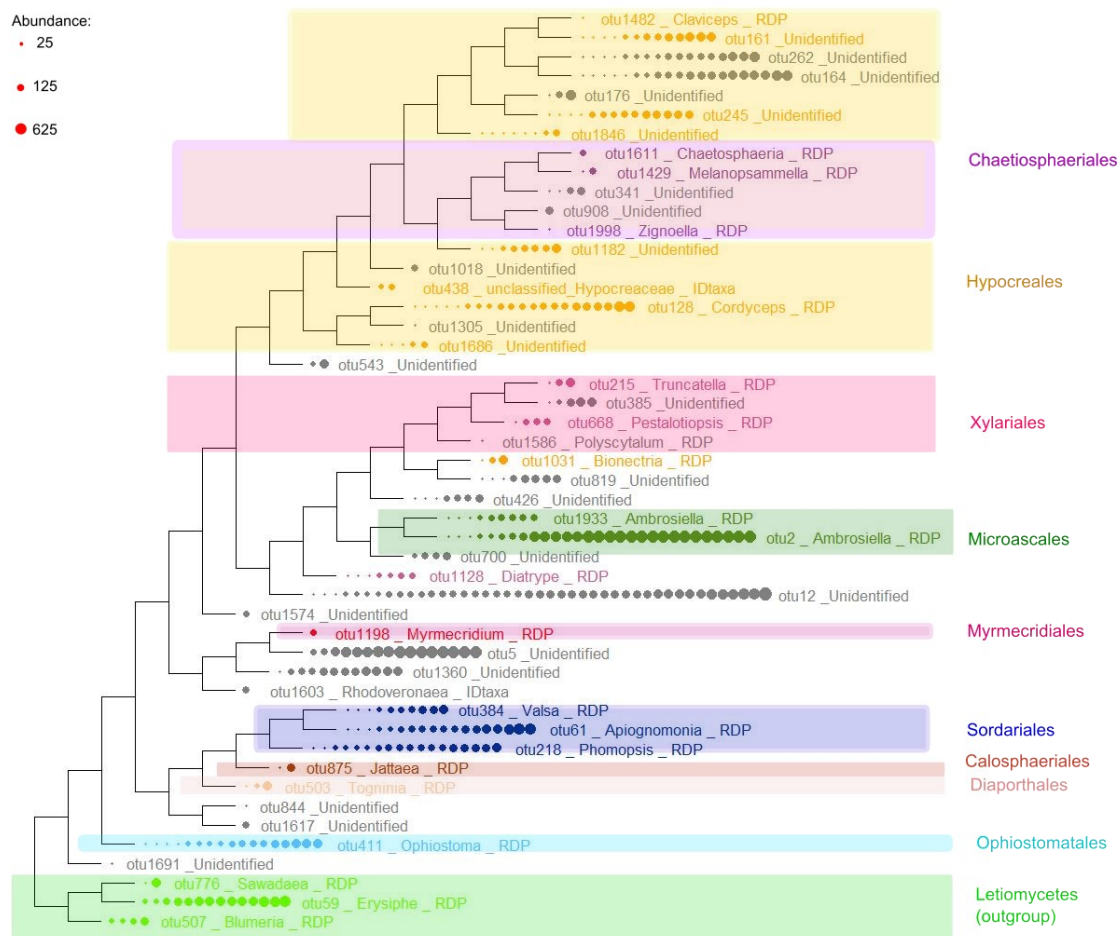


Figure 2-5. Phylogenetic placement of the OTUs generated from the LSU metabarcodes identified as Sordariomycetes by a combination of IDtaxa and RDP, with probability >70%. Different colours show the different orders. The genus or species and the classifier are detailed where the species-level identification succeeded. "ALL" means that all the 2 classifiers agreed with the classification of the fungi. The number of samples containing each OTU is shown with the dots, which represents the size of each dot the number of reads per sample of that OTU.

Both trees showed several major clades that broadly correspond to 11 different orders out of the 28 orders currently included in the class Sordariomycetes (Hyde, 2020). Both primers sets captured the same orders, except from the order Phomatosporales (Senanayake, 2016), which was only captured by ITS in a single specimen of *Xyleborinus saxesenii* and the order Glomerellales, retrieved from two beetle specimens. Moreover, the orders Diaporthales and Xylariales were remarkably more abundant in ITS than in LSU, although the phylogeny was not well resolved. Although all of these orders are polyphyletic, the order Xylariales could be well represented in the subclass Xylariomycetidae for the ITS data, along with one of the two proposed sister clades (Hyde, 2020). This order, which was considered monophyletic before

using molecular techniques, has been subjected to different outlines in recent years, recently acquiring its sister clades (Hongnanan *et al.*, 2017; Hyde, 2020).

Although there was a general lack of congruence between both trees, some OTUs seem to represent the identical fungus in both trees, like the monophyletic *Jattaea*, which belongs to the order Calosphaerales or *Myrmecridium*, which form the monotypic order Myrmecridiales (Crous *et al.*, 2015). These OTUs were relatively rare, but both were present in the same specimen of *Gnathotrichus materiarius*, highlighting the remarkable taxonomic resolution at which metabarcoding can unveil fungal communities.

Reliably capturing low-abundance OTUs is crucial for introduced vectors like *Gnathotrichus* since it can facilitate the early detection of fungal pathogens (Batovska *et al.*, 2018; Malacrino *et al.*, 2017). Among the identified fungi retrieved from the class Sordariomycetes, there were diverse plant pathogens (e.g., *Fusarium*, *Verticillium*, *Cytospora*, *Phaeoacremonium*, *Biscogniauxia*), with the potential to affect a wide variety of plant and organs, which stress the high capacity of these beetles to vector fungal diseases. The order Hypocreales, which was the most abundant, mainly was split into two clades in both ITS and LSU trees. In this order, there were mismatches among the different classifiers, primarily at the order level, mostly due to an outdated classification carried out by the RDP classifier. This is why, when mismatches were found, the species-level classification was used to correct them. A single species-level mismatch was detected in the case of *Lecanicillium flavidum* (Inderbitzin *et al.*, 2011; Zare & Gams, 2008), which was correctly classified by the three classifiers, but its final classification came out as *Verticillium flavidum* because RDP entry was outdated but presented higher classification probability values. Although the genus *Lecanicillium* was previously included in the *Verticillium* complex as part of the order Glomerellales, phylogeny has afterwards placed this genus into the Hypocreales (Hyde, 2020; Zhang *et al.*, 2006). Therefore, perhaps because these genera are taxonomically related and their taxonomy has been subjected to a long-term debate, the OTU classified as *Verticillium* appears to be part of the Hypocreales instead of splitting into the order Glomerellales.

2.4.2. Effects of the amplified sub-region on the ecological analysis

2.4.2.1. Species accumulation curves and fungal OTU richness

To assess the completeness of the 73 samples, the species accumulation curves were generated for the different forest types (**Figure 2-6, Supplementary Figure APP I-2**). The curves displayed a similar pattern across the different forest types, but more than double the number of OTUs was obtained from the ITS data than from LSU (0). The ITS data seemed closer to reach the asymptote in the oak forest, which would suggest that ITS captured more diversity than LSU in that particular forest type.

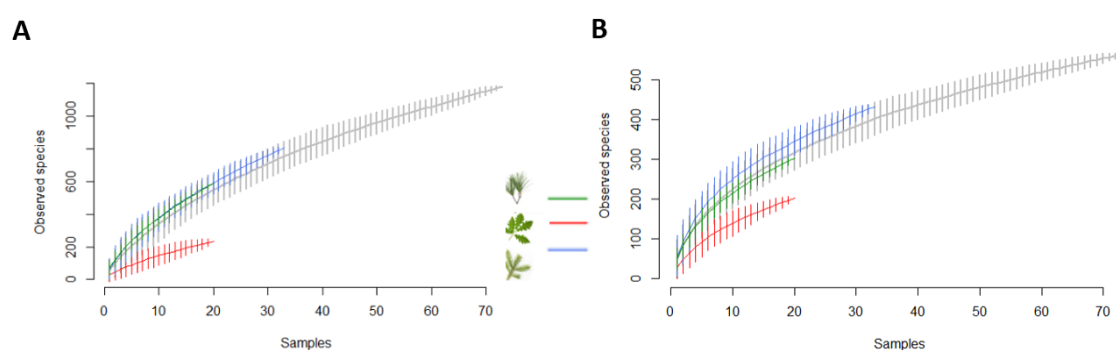


Figure 2-6. Species accumulation curves of the OTUs generated from the ITS (A) and LSU (B) metabarcodes. Different colours show the different forest types in which beetles were trapped (red: oak, green: pine, blue: spruce, grey: all)

OTU richness in LSU samples ranged from 11 to 109 OTUs, with an average of 48 OTUs, while ITS richness ranged from 9 to 140 OTUs per sample and an average of 56 OTUs (Figure 2-7).

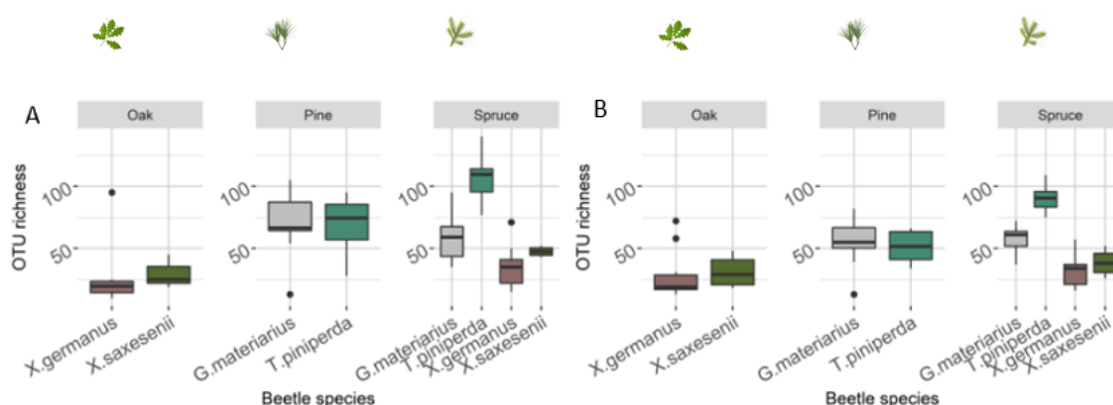


Figure 2-7. Effect of forest type (oak, pine and spruce) and beetle species on fungal OTU richness from rarefied data for ITS (A) and LSU (B).

The number of OTUs per sample was less variable for the LSU data, especially in the case of *Xylosandrus germanus* in oak and for *Xyleborinus saxesenii* in oak and spruce. A key finding is a close correlation in OTU richness obtained with both markers, indicating that both markers

revealed a similar set of fungal species. This correlation even holds at the individual level, representing the two outliers in each of the graphs to the same beetle specimens. Therefore, using any of the markers would lead us to similar outcomes, although a higher richness is found with ITS than with LSU.

When assessing the contribution of beetle species and forest type to explaining fungal variability across the beetle specimens, the differences between LSU and ITS data are more noticeable (**Table 2-2**). The amount of fungal richness variance induced by beetle species is remarkably higher in ITS (27.07%) than LSU (18.25%). Instead, the LSU data indicates that the forest type alone can explain the fungal variability (27.47% compared to 18.75 % from ITS). Therefore, even if the percentage of explained variance is similar, different regions point out additional factors to explain fungal variation. The interaction among forest type and beetle species was less variable across datasets, contributing to explain around 8% of the variation in both ITS and LSU.

Table 2-2. Overview of the GLM analysis results, showing the significant p-values, the percentage of explained variance and the F-statistic for every factor included in the model.

Factor	Explained variance		$F_{x,y}$		p	
	ITS	LSU	ITS	LSU	ITS	LSU
Beetle + forest type	8.46%	7.58%	$F_{6, 65} = 2.809$	$F_{6, 65} = 3.521$	<0.1 *	<0.05 *
Beetle	27.07%	18.25%	$F_{3, 69} = 30.265$	$F_{3, 69} = 29.888$	<0.001 ***	<0.001 ***
Forest type	18.75%	27.47%	$F_{2, 67} = 6.772$	$F_{2, 67} = 5.236$	<0.01 **	<0.01 **
Unexplained	45.72%	46.68 %				

2.4.2.2. Fungal community composition and diversity

The fungal community composition was also affected by the choice of the region to amplify. This MDS shows that the fungal communities of the beetle specimens trapped at each forest type clustered together (**Figure 2-8**). The amplified fungal communities seemed more diverse in pine for ITS and oak for LSU, while spruce communities were similar in both primer sets. Since these differences seem more noticeable for LSU than for ITS, this could point out biases towards the amplification of specific taxa on both regions.

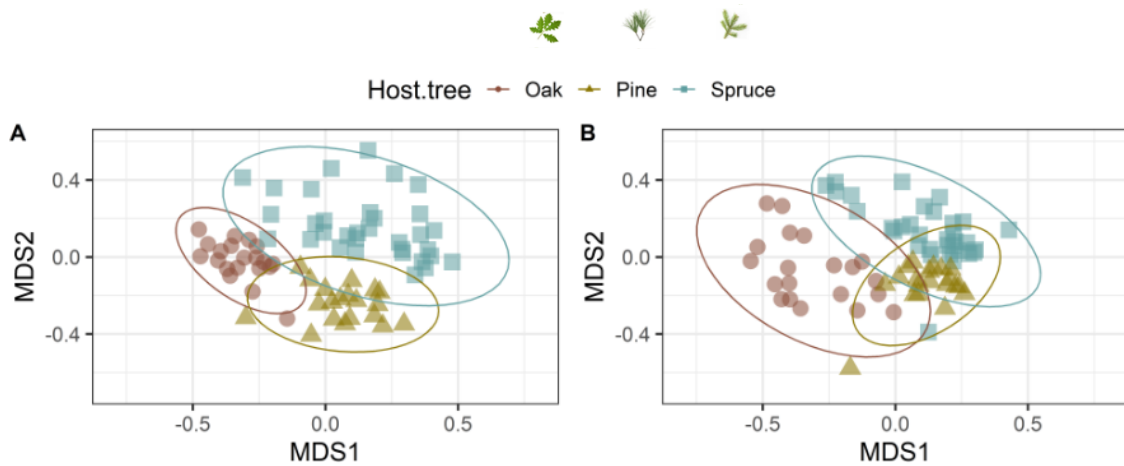


Figure 2-8. MDS ordination plot of all specimens sampled with ITS (A) or LSU (B) data, based on the fungal community composition of the individual beetles. Colours and shapes represent different forest types. Stress for both graphs fell within acceptable ranges (<0.2).

Results from the Bray-index-based PERMANOVA analysis revealed that the beetle species explain 34.58% and 33.59% of the total variation of the fungal communities amplified with ITS and LSU, respectively. The forest type accounted for 5.96% and 5.99% of the variation, while the interaction between beetle species and forest type would represent 3.36% and 2.80% of the total variation. Therefore, both primers sets would obtain similar results in the fungal composition analysis.

2.4.2.3. Beetle-associated fungi

Despite having obtained a smaller number of OTUs for the general analysis, the number of associated OTUs was slightly higher in LSU than ITS data, being 60 and 50, respectively (**Figure 2-9**). Interestingly, there is a higher specificity in fungi belonging to pine and spruce forests than in oak. The fungi associated with *Tomicus piniperda* were, in all cases, the most abundant. However, the fungal communities associated with this beetle were associated with pine forests in the ITS data. In contrast, in LSU data, the fungal communities of this bark beetle were associated with spruce. The contrary applies with *Gnathotrichus materiarius*, which had higher specificity on spruce only for ITS data.

Nevertheless, the proportion of associated fungi and strength of association remain similar across the OTUs from both amplified regions. Interestingly, *Ophiostoma angusticollis* was present for the ITS data, but none of the two Ophiostomatoid fungi recovered from LSU data was associated with the beetles. Among those fungi identified and shared by both datasets, there was a more noticeable presence of yeasts like *Yamadazyma* or *Candida*, although the

traditionally associated yeasts like *Ogataea pini* and *Sydowia polyspora* (Davis, 2015) were only identified in the ITS dataset.

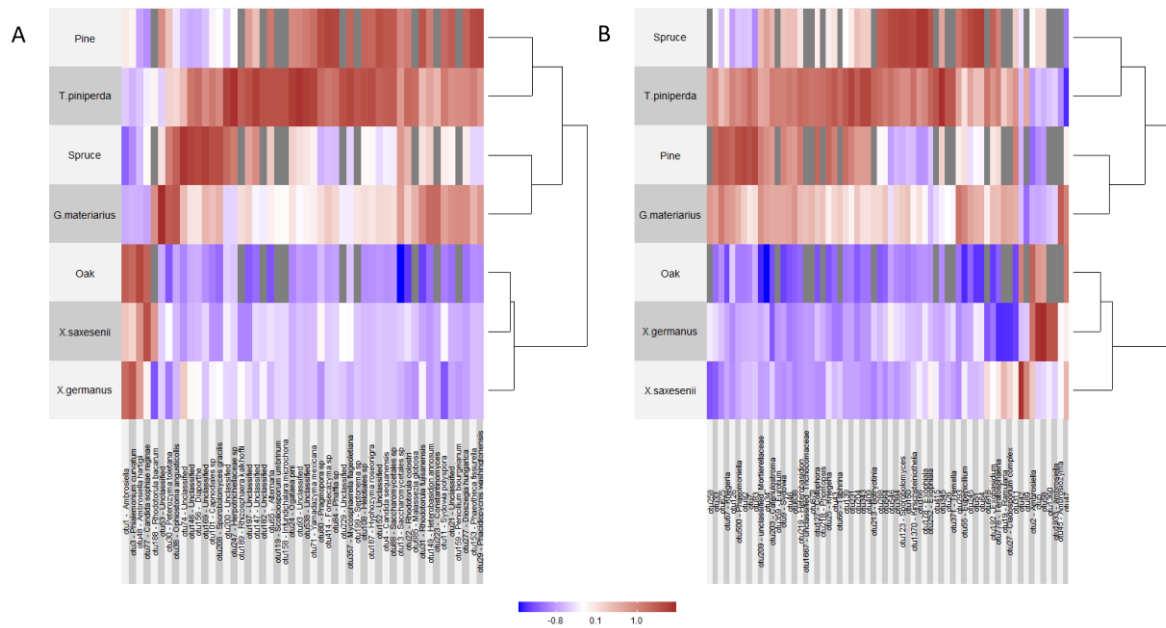


Figure 2-9. Specificity analysis of the OTUs generated from the ITS (A) and LSU (B) metabarcodes. Squares indicate the strength of association between OTU and beetle/forest (blue: no association found- red: strong association found). Fungal OTUs (on the x-axis) were classified to the genus or species level where possible.

2.5. Discussion

Two essential factors affecting the choice of loci on metabarcoding-based fungal studies were evaluated in the present chapter: the amplification success and the taxonomic resolution. To evaluate the amplification success of the primers, I compared the proportion of amplified OTUs primer set. By analysing the fungi present in the same beetle specimens with both ITS and LSU regions, I compared side-by-side the potential biases generated by each region. Results suggest that the ITS primer set was able to amplify more fungal classes than LSU. Besides, a higher proportion of fungal OTUs was retrieved by the ITS primers for most of the classes. To address how the taxonomic resolution differs between both primer sets, I combined three well-known fungal OTU classifiers. By comparing and merging the results of different classifiers, I was able to perform a more informed evaluation of how these classifiers were dealing with the OTUs for each of the primer pairs. Overall, the ITS primer set displayed a higher taxonomic resolution since it was able to reliably identify a higher proportion of the fungi to the genus and species level than the LSU. Then, I explored how the choice of the region to amplify, which has been a long-term debate, affects the results we can obtain from the analysis of complex natural fungal communities. When performing the downstream ecological analysis, these differences in the

amplification success were translated into differences in richness and specificity in the fungal communities. It is, therefore, necessary, according to these results, to be cautious when comparing and interpreting the results of ecological studies where different primer sets or different classifiers have been used.

When combining the different classifiers, a high-probability of taxonomic classification ($p > 0.7$) to the genus level was possible only in 38% of the OTUs for ITS and 25% for LSU. Metabarcoding of fungal communities often leads to a poor species-level identification (Hibbett, Ohman & Kirk, 2009), which explains the tendency of identifying fungal OTUs only at the order or class-level showed in previous studies (Miller, 2015; Skelton *et al.*, 2019b). However, ensuring the accuracy of this classification to the species level remains fundamental (Lücking *et al.*, 2020), especially for those studies, including the detection and monitoring of fungal diseases. Obtaining a more robust classification has been facilitated by the growing amount of classifiers applying probabilistic taxonomic placement methods to their classifications (Somervuo *et al.*, 2016). These methods can reliably quantify the certainty by distributing the probability of one among all possible classification results (Somervuo *et al.*, 2016). By combining different classifiers, I obtained an overview of the high percentage of agreement among them, and I was able to identify more sequences to the species level than using a single one.

Both RDP and IDTAXA use a naïve Bayes method. However, IDTAXA applies different weights on the *k-mers* in order to minimise the error rates across highly diverse taxa and variable sequence lengths. Therefore, IDTAXA classification is more stringent than RDP, as the latter tends to over-classify sequences and heavily relies on the quality and completeness of the databases. It is also important to note that, because these classifiers were not built in the same way, the meaning of their confidence levels may not be equivalent (Murali, Bhargava & Wright, 2018). This is why we need to be careful when interpreting the RDP classifier as the one classifying more sequences since we have seen already that IDTAXA is more stringent and has fewer errors. Knowing that one OTU classification is not congruent across classifiers may help to be cautious at assigning an identity. Even if RDP provides less-accurate classifications than IDTAXA, it has been broadly used for fungal metabarcoding studies. Therefore, I considered it essential to add it to the comparison to be more comparable with other studies.

PROTAXfungi, based on a probabilistic model using similarity-based predictions, is also known to provide more accurate predictions than RDP classifier (Abarenkov *et al.*, 2018; Somervuo *et al.*, 2016). Both IDTAXA and PROTAXfungi claim that their quantification of the uncertainty at

assigning fungal identities is accurate, but they are subjected to the completeness and accuracy of the fungal databases. Even if PROTAXfungi was not able to identify extra OTUs on top of those identified by the three classifiers, it is still useful to see how all the three classifiers agreed with the identification of most of the identified fungi.

The lack of taxonomic resolution of ITS has been previously assessed by multiple studies (Lücking *et al.*, 2020; Meyer *et al.*, 2019). However, previous studies tended to use tools like ITSx to extract the ITS2 region, which could have contributed to generate those results (Heeger *et al.*, 2019; Pauvert *et al.*, 2019). In the present study, which did not use ITSx, there is a lack of taxonomic resolution in the data obtained from the LSU region compared to ITS. At the genus level, 13% more of the ITS-based fungal species were identified compared to LSU. Therefore, I found the ITS-amplified data much more informative than those sequences obtained with LSU primers. Another particularity of this study is the stringency of the taxonomic identification, identifying only the fungi for which the classifiers were sure with a 70% or more probability. Plenty of previous studies have assigned a classification to a higher proportion of fungi, but that means that the probability of misclassification is more than 30%. In addition of a more stringent fungal classification, the method implemented to classify the OTUs is more informative than using a single classificatory alone, since it allows us to be able to detect potential incongruences across datasets that may be missed by single-classifier studies.

Although these results are difficult to compare due to the high proportion of unclassified OTUs, ITS and LSU could display different amplification biases towards specific taxa. This is particularly noticeable when comparing the OTUs at the class level, where the ITS primers accounted in some cases for a double proportion of OTUs than LSU and even five times more in some classes. The choice of ITS as a barcoding region has been challenged by a growing number of studies (Edgar, 2018; Bellemain *et al.*, 2010; Stadler *et al.*, 2020). To overcome this criticism in the field of the bark beetle fungal metabarcoding, the choice of LSU as secondary or even primary barcode have been suggested (Hulcr *et al.*, 2020; Asemaninejad *et al.*, 2016). However, in this case, it seems that also LSU may display some biases towards the amplification of specific taxa, which is also in agreement with recent findings showing that both ITS and LSU would exhibit such tendencies (Skelton *et al.*, 2019b). The amplification success is species-specific for many other regions like COI (Elbrecht & Leese, 2015), and therefore it would be realistic to think that neither ITS nor LSU nor even a combination of them would perfectly show a complete picture of the fungal communities. It has been suggested that ITS amplification could be more successful than others, obtaining a higher amount of fungal OTUs on the same sample (Schoch *et al.*, 2012).

Furthermore, rather than on the selected region, this amplification depends on the specific primers utilised to amplify the ITS sub-region. In this case, the ITS86-ITS4 primer pair was selected because of their outstanding amplification capacity compared to other ITS primers tested (Op De Beeck *et al.*, 2014). In this direction, recent studies still point out the necessity of documenting the variation of fungi detected among barcoding primers (Lücking *et al.*, 2020). This study contributes towards understanding the biases that both ITS and LSU primers may create on the characterisation of beetle-associated fungal communities. Once known the potential biases that each amplification may originate for each primer set, it will become easier to draw conclusions that make the growing number of studies comparable.

Both amplification success and taxonomic coverage are also influenced by the variable fragment length (Skelton *et al.*, 2019c; Frau *et al.*, 2019). Fragment lengths of more than 300 bp could enhance fungal identification at the genus level (Porrás-Alfaro *et al.*, 2014). However, in this case, even if reads were on average 77 bp longer in LSU than in ITS, this did not contribute to making LSU-amplification more efficient than ITS. Even if the sequences are longer, because the LSU region is less variable, it seems to capture less fungal diversity than ITS. Rather than utilising secondary primers, the use of third-generation sequencing could be more useful to provide species-level classification since longer reads that capture the whole ITS region may improve fungal identification (Tedersoo *et al.*, 2020b). However, the suitability of the primers or even the chosen bioinformatics to analyse the molecular data (Pauvert *et al.*, 2019) depend on the scope of the study. In this direction, the use of ITS primers could provide sufficient taxonomic resolution for ecology studies, especially since fungal identification still relies on the implementation and completeness of the databases.

The last factor to consider when comparing the primer performance is the suitability of the fungal databases. More specifically, it is necessary to evaluate both their completeness and homogeneity. Neither ITS nor LSU databases are complete; all of them display a wide range of biases, for example, towards the easy-to-culture or the pathogenic fungi (Nilsson *et al.*, 2014; Ohsowski *et al.*, 2014). Classifiers like PROTAX or IDTAXA were built to try to minimise this bias by accounting for missing branches in the taxonomy. Still, in the case of RDP this kind of correction was not implemented, generating further biases. While a more complete ITS database will increase the identification accuracy (Heeger *et al.*, 2019; Nilsson *et al.*, 2008), the existing biases may influence subsequent ecological studies (Porrás-Alfaro *et al.*, 2014). ITS data holds an essential advantage compared with LSU because, because it has been denominated as the “official” barcoding region since 2011. Consequently, the ITS databases are more

comprehensive, and more OTUs can be identified to detect and acknowledge both amplification and sequencing biases. However, there are still so many missing fungi on the databases that it is impossible to arrive to the species-level identification in many cases, especially when working with environmental data, which is the case. This fact hampers to conclude which are the biases across databases and their origin, highlighting the importance of including mock communities on these kinds of studies.

2.5.1. Effects of the amplified sub-region on the ecological analysis

Following the differences in fungal taxa retrieved by both primer sets, the ecological analysis showed differences in fungal richness. The impact on the fungal richness of both forest type and beetle species oscillated between 18-26% depending on the amplification region. However, even if the primers capture different amounts of fungal diversity, the differences in terms of the number of OTUs were preserved across some of the samples. Therefore, depending on which primers we use, we could partially reach different conclusions based on the same initial DNA. The effect of the beetle species on their fungal richness has been previously documented utilising similar ITS primers (ITS3-ITS4), explaining 14.5% of the variation for pine forests (Miller *et al.*, 2019). My results suggest that the influence of the beetle species could be more robust than previously thought. Results also suggest that this impact would be higher on fungal richness than on fungal community composition.

The fungal composition analysis generated similar results among the ITS and LSU markers, showing that beetle species explained a higher proportion of the variation (33%) than the forest type (6%) for both markers. A similar analysis by Miller (2019), using ITS primers (ITS3-ITS4), pointed out the beetle species explaining only 13.7% of the variation for pine forests. This study was carried at a single forest type, but at a regional scale and using more beetle species, which could have influenced the results by adding other factors that could minimise the impact of the beetle species. The impact of the forest type on the fungal communities has been previously documented by using metabarcoding (Asplund *et al.*, 2019).

In terms of fungal specificity, there is a more substantial fungal-host specificity in pine and spruce forests than in oak. A similar trend was present in both ITS and LSU. Interestingly, despite the lower fungal diversity recovered from LSU samples, the LSU primers recovered ten more associated fungi than the ITS primers. This result may support the idea of certain fungal associates better amplified by LSU (Hulcr *et al.*, 2020). However, since the taxonomic resolution

of the LSU primers is so low, it would be difficult to use this particular primer set to investigate this issue further.

Although some discrepancies were found depending on which primer set was used, the general conclusions of the study remain the same independently of the utilised primer. Here we can also see that *Tomicus piniperda*, despite of not having mycangia exhibits similar number of associated fungi than the ambrosia beetles, which are also preserved across forest types. Although it is well known that certain fungal associates with specific beetle species, most of the work has been focused on ambrosia beetles, neglecting the bark beetles. Therefore, rather than focusing on the structures that the beetle may display to hold the specific fungi, it is essential to look at the context surrounding the insect, like the forest type in which it is captured. In this direction, the number of associated fungi is surprisingly higher in the two conifer species compared with oak. This result is in agreement with a previous study finding a higher specificity in Norway spruce compared with broad-leaf forests (Lindhe, Åsenblad & Toresson, 2004). A similar result was also found by Küffer and Senn-Irlet (2005), which suggested that the host tree diversity would affect the variety of decaying fungi. This last result is also in agreement with my finding of a strong influence of the forest type on the fungal communities associated with the beetles. If the host tree has an effect on the decaying fungi, it may well have also an impact on the fungal communities associated with the beetle. These results may have implications for future forest management, which, in line with previous studies (Knops *et al.*, 1999; Tedersoo *et al.*, 2020a; Yachi & Loreau, 1999), may promote more diverse forests to ensure fungal diversity and avoid fungal outbreaks.

2.5.2. Future improvements

These results point out the importance of choosing the amplification region when comparing different studies since the same DNA could be interpreted differently depending on the amplification region selected. The differences between ITS and LSU amplification were more remarkable when addressing the questions concerning fungal richness and fungal specificity. More reads and, consequently, fungal OTUs were detected by ITS than by LSU, which may be due to the 100bp length difference of the amplicons since shorter reads tend to be favoured by HTS (Zhou & Rokas, 2014). This fact was taken into account at the time of pooling the samples together, but still, there was impossible to fully control. Even if the rarefaction was used to make the samples more comparable in terms of abundance, it is complicated to quantify to what extent the initial conditions were the same for both primer sets. Moreover, when choosing to rarefy we are also risking to lose some rare fungal OTUs, which is one of the most notable

arguments against this practice (Cameron *et al.*, 2020). Despite of the difficulties, this is one of the first studies to show the differences among the amplifying regions on natural communities rather than on mock communities. The use of mock communities remains a powerful tool to improve the metabarcoding pipelines, but it is somehow unrealistic compared with the great deal of abundance that the real metabarcoding data naturally exhibits.

On metabarcoding studies, there is a natural variation due to the nature of the DNA template, to the stochasticity of the PCR reactions (Best *et al.*, 2015; Suzuki & Giovannoni, 1996) or even to the taq used to amplify the DNA (Li *et al.*, 2020). These differences are translated into variability in terms of OTU numbers. Thus the identification of some fungi retrieved from the same DNA extractions may vary (Op De Beeck *et al.*, 2014). To minimise this stochasticity, it is a standard practice to replicate the PCR reaction at slightly different temperatures and pool the replicates together. Consequently, to minimise these biases, three replicates of PCR product were pooled together for every specimen. However, given that those fungal communities could be considered environmental DNA, they could be retrieved in different proportions from the same DNA extraction (Piñol, Senar & Symondson, 2019). The study considered the inter-variability across beetle specimens by adding ten replicates per beetle species and forest type. However, it would have been better to add replicates of the DNA extractions for some of the specimens to account for this variability.

To quantify the lack of taxonomic accuracy of ITS or LSU, it would help generate more data to compare the generated sequences from second and third-generation techniques, combining the complex-natural communities with other samples belonging to mock communities to minimise the uncertainty of the fungal classifications. In this direction, it would also be helpful to join more classifiers to reach a higher consensus among sequence identities. Finally, it would be essential to map which parts of the fungal tree are not sufficiently characterised on the databases to keep minimising the amount of unknown fungal clades.

This study broadens previous studies, showing that fungal communities associated with bark and ambrosia beetles also rely on the forest type in which the beetle coexist (Buée *et al.*, 2011; Tedersoo *et al.*, 2020a), and the same results are supported by the analysis of two different fungal regions. Consequently, fungal communities associated with these beetles would be more preserved under a single forest type since the associated fungi would more easily pass through the beetle generations. These results could therefore contribute towards the hypothesis of

mixed forests being more fungal-diverse than monospecific forests. Such diversity could dilute fungal pathogens in mixed forests, which could help avoid fungal outbreaks.

2.6. Conclusion

The study of fungal communities associated with bark and ambrosia beetles needs to achieve the double objective of obtaining a high species-identification accuracy while sufficiently capturing fungal diversity. At the moment, there is not any preferred identification method that applies to all the fungal metabarcoding studies (Lücking *et al.*, 2020). It is still necessary to find the most suitable methodology for every kind of analysis.

Overall, I found potential biases in ITS and LSU amplifications, with higher amplification success and more profound taxonomic resolution for ITS primers. Both primer sets draw similar conclusions on the applied ecological analysis in fungal community composition but differ when assessing fungal richness. However, ITS have the advantage of more complete databases, which contributed to identifying target fungi, like fungal pathogens.

Because of their more complete databases, their higher amplification success and the more profound taxonomic resolution achieved using them, I found the ITS primers more informative than the LSU primers for these kinds of ecological analyses. Therefore, in light of these findings, subsequent analyses will be carried out using the ITS2 barcode only.

Chapter 3. The effect of host trees on the composition of fungal communities associated with bark and ambrosia beetles

3.1. Abstract

Bark and ambrosia beetles (Coleoptera: Scolytinae) establish symbiotic relationships with fungi. These beetles become, in some cases, tree-killing pests with severe economic and ecological consequences. Scolytinae beetles can host entire fungal communities, but the underlying mechanisms by which certain fungal-beetle relationships can become a threat to forests are still poorly understood. In this chapter, I investigate two biotic factors potentially shaping fungal communities coexisting inside bark and ambrosia beetles: the dominant host tree (here denoted as “forest type”) and the beetle species identity. With this purpose, the fungal communities associated with a total of 160 beetle specimens belonging to seven different beetle species were captured across three different forest types. These forests belonged to the same locality, the New Forest national park, aiming to minimise abiotic factors like temperature and rainfall that affect fungal communities at larger scales. Fungal communities were assessed using metabarcoding. Fungal richness, diversity and degree of association with the beetle were subsequently compared. Results show that these fungal communities are affected by the forest type from which the bark and ambrosia beetles were collected. Bark beetle identity played an essential role regarding fungal OTU richness, especially in the bark beetle *Tomicus piniperda*. Fungal communities from the same beetle species were more similar than those found in the same forest type. However, in some cases, the effect of the forest type was more apparent. An example of this is the significant proportion of ambrosia fungi associated with the beetles *Anisandrus dispar* and *Xylosandrus germanus* unexpectedly found in *Xyleborinus saxesenii*, but only in the oak forest. Therefore, both forest type and beetle species identity could significantly affect fungi vectored by bark beetles.

3.2. Introduction

Bark and ambrosia beetles (Coleoptera: Scolytinae) establish diverse associations with fungi, including symbioses (Bracewell & Six, 2015; Farrell *et al.*, 2001). Beetle-fungal associates are sometimes tree pathogens, attacking entire forests, often over broad areas (Anon, 2009; Edburg *et al.*, 2012), killing even healthy trees (Wood, 1982; Krokene, 2015). These outbreaks are especially worrying in invasive Scolytinae, which would not share an evolutionary history with the host trees and local fungal communities (Hulcr & Dunn, 2011). In addition to killing the host trees, a recent study suggests that fungi could slow down wood decay, affecting the carbon fluxes of the forest (Skelton *et al.*, 2019c). Despite these findings, the ecology of beetle-fungal associations is still poorly understood to the point that these outbreaks remain highly unpredictable (Lausch *et al.*, 2013). Due to climate change and global transport, bark beetles species commonly colonise new areas (McDowell *et al.*, 2011; Waring *et al.*, 2009; Bentz *et al.*, 2010), forming new associations (Wingfield *et al.*, 2017; Kerchev *et al.*, 2017). Consequently, the frequency and virulence of disease outbreaks are likely to increase (Marini *et al.*, 2012). Therefore, it is crucial to develop new strategies to prevent or mitigate the economic and ecological impacts of these disease outbreaks (Kausrud *et al.*, 2012).

In the UK, which is the focus of the current study, there are 64 established species of Scolytinae, of which the great majority are bark beetles feeding directly on the wood. However, three invasive ambrosia species recently arrived in the UK (Inward, 2019), two of them being part of this study. Ambrosia beetles feed directly on fungi rather than depending on trees themselves (Beaver, 1989; Wood, 1982), which would explain their higher colonisation capacity and broader host diversity compared to bark beetles (Hulcr & Stelinski, 2017). Most fungal-associates of scolytine beetles are Ascomycetes (Li *et al.*, 2016), which includes the fungal order Ophiostomatales with some of the most destructive species (Bueno, Diez & Fernández, 2010; Kirisits, 2004; Linnakoski *et al.*, 2010; Wingfield *et al.*, 2017). Species include *Ophiostoma novo-ulmi* (the agent of Dutch Elm Disease) attacking elm trees throughout the Northern Hemisphere (Santini & Faccoli, 2015), the blue stain *Grosmannia* attacking various pine species in the American Northwest (Rice, Thormann & Langor, 2007, 2008), or the more recent outbreak of the laurel wilt *Raffaelea* vectored by an introduced bark beetle in South-eastern North America (Harrington *et al.*, 2011). Despite their high importance for the ambrosia beetles, ambrosia fungi have been studied in a small number of species only (Hulcr & Stelinski, 2017).

The study of the fungal composition of Scolytinae communities has traditionally been constrained by the use of culturing techniques, which are able to retrieve only a small amount of the fungi actually carried by the beetle. In addition, the lack of a simple taxonomy on fungi (e.g. in the genus *Fusarium*, an extremely-diverse clade that includes some tree-killing fungal species linked to bark beetles (Bezoes *et al.*, 2015; Goldazarena, Romn & Lpez, 2012)) represents a considerable challenge to this morphology-based approach. However, recent DNA-based studies are greatly enhancing the knowledge about beetle-fungal communities. Specifically, metabarcoding of entire fungal communities carried by these beetles can provide an unprecedented resolution of their composition and associations with beetle species, host trees, climatic conditions or biogeography. Dissecting these various factors is the first step towards establishing the drivers of fungal associations.

A recent study of Scolytinae of the British Isles revealed only limited structuring of fungal communities along a latitudinal gradient of nearly 1000 km, showing only negligible effects of environmental and spatial predictors (Miller, 2015; Miller *et al.*, 2019). This finding would be in agreement with existing studies suggesting that most fungal associates might be incidental, and relations with bark beetle hosts are non-permanent (Six, 2012). However, the identity of beetle species from which these fungi were isolated did predict a certain proportion (~15%) of the variation in fungal communities. The study was based on plantation forests of only a single tree species, Scots Pine (*Pinus sylvestris*), and thus the effect of host tree on the community composition was not tested, despite the fact that fungal communities in soil microbiota and decaying wood have been shown to be affected by the dominant tree species of the forest (Asplund *et al.*, 2019; Yuan *et al.*, 2017). Thus, the relationship between a beetle and fungal communities could partially be mediated by the forest type in which beetles are trapped

Here, I evaluated the role of the dominant tree species on fungal communities vectored by bark beetles. To do this, I compared the effect of the dominant tree species on the fungal communities of 160 beetles belonging to seven beetle species across three different forest types. I studied the beetle-forest type assemblies by trapping beetles across three different forests coexisting in the same area to minimise abiotic factors like temperature and rainfall.

3.3. Material and methods

Samples were collected by Forest Research (Forestry Commission) during the years 2013, 2014 and 2015 in the New Forest National park (50°50'52.08" N 1°35'33.51" W), in the South of the UK. Three locations consisting on planted mature oak (*Quercus robur* L.), Norway spruce (*Picea*

abies Karst.) and pine (*Pinus sylvestris* L.) stands were selected and five Lindgren multiple-funnel traps ((Lindgren, 1983), Phero Tech) were set up at each location. Traps were spaced 50m apart along 250m of the transect and baited with lures (100% ethanol, plus α - pinene in conifers) to mimic volatile chemicals emitted by dying trees, as shown in Inward (2019). Propylene glycol (65% concentration) was placed at the bottom of each trap to ensure the correct preservation of the beetles. Traps were emptied every two weeks between April and August. Captured specimens were then kept frozen in pure ethanol to preserve their DNA quality until being sorted and analysed.

3.3.1. Laboratory procedures

At first, the specimens were morphologically identified using standard identification keys (Duffy, 1953; Grüne, 1979) and a total of 160 specimens from seven beetle species were randomly selected to obtain –when possible- the same number of specimens per beetle species and forest type (**Table 3-1**). Subsequently, selected specimens were rinsed with pure water to wash out external impurities and placed in individual 1.5ml tubes. Sterile pestles were used to completely break up the individual beetle specimens to ensure that all the fungi inside the specimens were captured. The DNA of the 160 specimens was then individually extracted, purified and eluted in 200 μ l of AE buffer using the QIAGEN DNA easy blood and tissue spin column extraction kit (Qiagen, Valencia, CA).

Table 3-1. Overview of the beetle species and number of the specimens per forest type included in the study along with information related to their origin and primary structures for fungal transportation (Batra, 1963; Wood, 1982).

Forest type	Beetle species	Status	Adapted structures
Pine, spruce, oak	<i>Xylosandrus germanus</i>	Invasive	Mesonotal mycangia (Hulcr <i>et al.</i> , 2012)
	<i>Xyleborinus saxesenii</i>	Native	Elytral mycangia (Batra, 1963)
Spruce, oak	<i>Anisandrus dispar</i>	Native	Prothoracic pleural mycangia (Batra, 1963)
Pine, spruce	<i>Gnathotrichus materiarius</i>	Invasive	Tubular structure opening near precoxal (Batra, 1963; Farris, 1963)
	<i>Hylastes attenuatus</i>	Native	Mycangia not known
	<i>Tomicus piniperda</i>	Native	Mycangia not known
	<i>Hylurgops palliatus</i>	Native	Secretion-filled punctures (Francke-Grosmann, 1956)

Two different target segments were amplified using PCR. While the CO1 region was amplified to generate the barcodes to ensure the correct beetle species identification, the ITS region was amplified using the metabarcoding technique to allow the fungal identification. Unique six-letter indices were added to every primer pair in order to distinguish the specimens across the different libraries.

Barcoding using COI primers (5'-CCNGAYATRGCTTYCCNCG-3') was carried out on every specimen to corroborate its taxonomic identification. The PCR reaction mixture contained 2,5 µl of Bioline buffer™, 0.75 µl of MgCl₂, 0.25 µl of dNTPs (Bioline), 0.75 µl of forward primer, 0.75 µl of reverse primer, 0.1 µl of Bioline Taq™ and 18µl of ddH₂O. 2 µl of DNA were added to every PCR reaction, for a final volume of 25 µl. Cycling conditions of the PCRs were: 94°C for 4 minutes followed by 40 cycles of 94°C for 30 seconds, 48°C for 30 seconds 72°C for 45 seconds and a final extension of 72°C for 10 minutes.

Metabarcoding of associated fungi was performed using the ITS primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') /ITS4(5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck *et al.*, 2014), following the same procedure detailed in Chapter 2. Successful PCR amplification was confirmed in every case by visualising 3 µL of the PCR product using gel electrophoresis. For this visualisation, 2% agarose TAE gel, GelRed™ (Biotium) and Hyperladder IV™ (Bioline) were used. PCR products were then purified to eliminate fragments under 200bp using the Agencourt® AMPure® XP purification kit, by following the protocol 000387v001. The 96 libraries were then sequenced on an Illumina® HiSeq® 2500 platform with version 2 chemistry (Rapid mode), using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads.

3.3.2. Bioinformatics

Raw sequence data were processed using bioinformatics to achieve solid species identification prior to its ecological analysis. The initial procedure was similar for both metabarcoding and barcoding samples, differing only in the last steps. The 96 libraries were firstly demultiplexed by using the Illumina software to remove their XT indices. Samples were further demultiplexed with Cutadapt (Martin, 2011) scripts modified to account for bulk samples. During this step, the unique six-letter indices attached during the first round of PCR were trimmed from each sample file, which in turn was then assigned a unique name related to every single beetle specimen. Also at this step, singletons were removed and Cutadapt was used to trim primers and zero-length sequences before read assemblage. Read quality was evaluated using FASTQC (Andrews, 2014). Forward and reverse reads were subsequently assembled using PEAR v. 0.9.8 (Zhang *et*

et al., 2014). This step included a quality filtering of the paired-assembled reads (PEAR quality score threshold ≥ 30 , as recommended by Illumina® (https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf)). PEAR software tests all possible overlaps among reads, due to its tolerance to variable read lengths (Zhang *et al.*, 2014). A further quality test was carried out using USEARCH v. 7.0 (Edgar, 2010), with the `--fastx_filter` command (`-fastq_maxee 1.0`). The ITSx software (Bengtsson-Palme *et al.*, 2013) was used on the metabarcoding samples to filter out non-fungal reads, selecting and extracting target ITS2 regions for each full-length read. This step was performed to ensure the best results on their further identification. Merged full-length metabarcoding reads had an average length of 271 bp, but after the ITSx step, reads had an average length of 169 bp. Merged full-length barcoding reads averaged 418 bp in length, which targets the amplified COI region.

Read clustering into Operational Taxonomic Units (OTUs) was subsequently performed using USEARCH. After dereplication, assemblies were filtered by length, eliminating those sequences shorter than 200bp or longer than 350bp and removing also those with less than two reads to avoid potential sequencing errors or artefacts. Chimaeras were also detected and filtered out with UCHIME (Edgar *et al.*, 2011). Reads were then denoised using UNOISE (Edgar, 2016:p.2) and clustered using UCLUST of the USEARCH v. 9.2 pipelines (Edgar, 2010) at a 97% of similarity threshold to delimit different OTU clusters. Reads were then mapped to the OTU clusters, outputting an OTU table suitable for the ecological analysis of the samples.

3.3.3. Fungal OTUs taxonomic identification

To ensure the maximum species-level identification, three complementary paths were used. The Ribosomal Database Project (RDP) Bayesian Classifier (Wang *et al.*, 2007) was used to assign a first classification for the OTUs. This classifier has been broadly used for fungal identification, using the Warcup fungal ITS (v2_March2018) and UNITE (v2020_February2020) training datasets to achieve both broad coverage and accurate OTU classification (Deshpande *et al.*, 2016; Edgar, 2018). Simultaneously, OTUs were processed through the PROTAXFUNGI pipeline (Abarenkov *et al.*, 2018), implemented in the PlutoF (Anon, 2017a) platform and based on the UNITE fungal database. PROTAX hierarchically assigns the OTU identities from the root node of the taxonomy through the species level (Nilsson *et al.*, 2019b). The last classifier selected, IDTAXA, employs machine learning to reduce over classification errors to obtain a higher accuracy (Murali, Bhargava & Wright, 2018). Combining these three methods has the advantage of adding three different percentages of probability to every identification. These

percentages represent an estimation of how likely each OTU is currently identified at every level and can contribute arriving at an informed consensus for a correct identification. To further complement this information, some sequences of particular interest were blasted against the GenBank National Centre for Biotechnology Information (NCBI) database (MEGA-BLAST). All this information was then combined with the data of the collected beetle specimen to perform the ecological analysis.

3.3.4. Ecological Analysis

After generating the OTU table through the bioinformatics process and identifying the retrieved fungi, data analysis was split into three parts. The first part aimed to estimate differences of richness. The second part evaluated the diversity of the fungal communities across different forest types and the last one aimed to determine how extended the presence of fungal symbionts and pathogens was through the whole set of samples.

Statistical analyses were carried out at the OTU level. As the difference of fungal reads across the beetle specimens was considerable, rarefaction was necessary. Increasing rarefaction was observed to have a more dramatic effect on sample numbers than on OTU numbers. Therefore, samples were rarefied to 2500 reads to retain the majority of the samples while making them more comparable. Samples that did not have enough reads were removed from the downstream analysis, leaving 151 samples left for the analysis.

Species accumulation curves were built with Rstudio (vegan package (Oksanen, Jari, et al., 2018), “speccacum” function). These curves are frequently used to corroborate that the average sample size was sufficient for the biodiversity survey to be able to capture a reasonable amount of fungal OTU variability (Hughes *et al.*, 2001). Since many of the curves did not approach an asymptote, the extrapolated OTU richness was chosen over the observed OTU richness to carry out the modelling. The extrapolated fungal OTU richness was estimated by using the Chao1 index, which was calculated using the “estimator” function from the vegan R package (Oksanen, Jari, et al., 2018). To compare the extrapolated fungal richness a set of generalised linear models (GLM) were built, using the R package lme4 (Bates *et al.*, 2015:p.4). These models used the Chao1 richness as a response variable and beetle species and forest type as dependent variables. Each of the models under the assumption that beetle specimens originated from different galleries and trees because beetles were trapped at random from the forest. First, the model was tested with the Poisson distribution. However, as the data showed overdispersion (the residual deviance was greater than the degrees of freedom) two more models were tested, the

Quasi-Poisson and the Negative Binomial models. Finally, the Negative Binomial model was chosen, as it is suitable for over-dispersed and similar to the Quasi-Poisson and showed a slightly better fit. Model assumptions were corroborated using the DHARMA R package (Hartig, 2017).

To characterise the fungal communities across different forest types and beetle species, I calculated distance matrices based on different indexes. Sorensen and Jaccard indexes calculate the beta-diversity between sample pairs based on presence-absence data (richness), while the Bray-Curtis index focuses on the relative abundance of each OTU (Borcard, Gillet & Legendre, 2018). Community dissimilarities were visualised in a non-metric multidimensional scaling NMDS graph (Betapart R package, Jaccard index). To quantify the variations shown in the graphs, a permutational multivariate analysis of variance (PERMANOVA) was performed. By doing this, a model for forest type, beetle species and their interaction were calculated by using the “Adonis” function of the “vegan” package (permutations=999). A permutational multivariate analysis of dispersion (PERMUTEST) was performed to evaluate the assumption of homogeneity of variance in the data by using the Betadisper function (Anderson & Walsh, 2013) (Vegan package). The non-euclidean distances between the group centroids were also calculated to assess the group dispersion. Moreover, the total beta-diversity was split between its nestedness and turnover components with the Betapart R package (Baselga & Orme, 2012). To quantify which component of the beta-diversity was the dominant, these components were compared using a Wilcoxon rank sum test, as seen in Miller (Miller *et al.*, 2016, 2019).

To evaluate the associated fungal OTUs for each assembly, a multilevel pattern analysis was carried out by calculating Pearson’s phi coefficient of association (“p.g”)(Chytrý *et al.*, 2002) between sample pairs, correcting this index to account for the differences in specimen numbers among the compared groups (function `multipatt`, `indicspecies` R package (De Cáceres *et al.*, 2011)). The identified OTUs for which the association values were significant were then selected and displayed as a heatmap (Superheat R package (Barter & Yu, 2018)).

3.4. Results

The HTS run produced a total of 22 048 332 reads, which were then filtered to obtain 10 782 465 unique reads that clustered into 2508 OTUs. Since their reads abundance was low compared to the rest of the OTUs, the 30 OTUs found in the negative controls were subsequently excluded from further analysis. After the rarefaction step, the sum of the reads per sample was 2500, leaving 2482 fungal OTUs for the ecological analysis. As a consequence of this rarefaction, from

the 160 initial samples, 9 samples were lost, leaving a total of 151 samples for the ecological analysis.

3.4.1. Species accumulation curves and fungal OTU richness

The species accumulation curves of the fungal OTUs retrieved from the bark and ambrosia beetles showed a steeper gradient in pine and spruce forests than in oak forests (**Figure 3-1, Supplementary Figure APP II-1**). This result suggests a considerably lower variation of fungal composition in beetle specimens trapped in oak forests in comparison with those from pine and spruce forests.

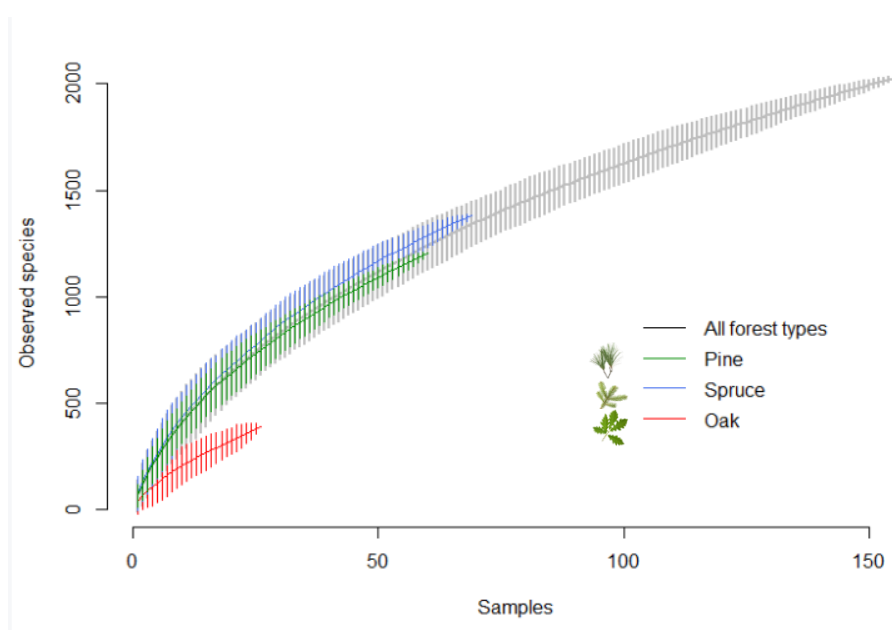


Figure 3-1. Species accumulation curves of compiled fungal OTU richness for each forest type. Rarefaction was conducted at 2500 reads. The 'specaccum' function ('Vegan' R package, 'random' method) was applied, displaying the confident intervals as bars.

The Chao1 estimate of the fungal OTU richness analysis ranged from 7 to 274 fungal OTUs per beetle specimen, with an average of 82. An average of 47 OTUs were recovered from the specimens trapped on the oak forest, 86 for pine and 91 for spruce (**Figure 3-2**). The ambrosia beetles *Xylosandrus germanus* and *Xyleborinus saxesenii* showed a significantly lower richness in oak forests compared with pine, which was not different from spruce. Compared to the ambrosia beetle species, fungal richness was significantly higher in the bark beetles *Hylurgops palliatus* and *Tomicus piniperda*. However, *Tomicus piniperda* specimens harboured fewer OTUs in pine than in spruce, which did not happen for *Hylurgops*.

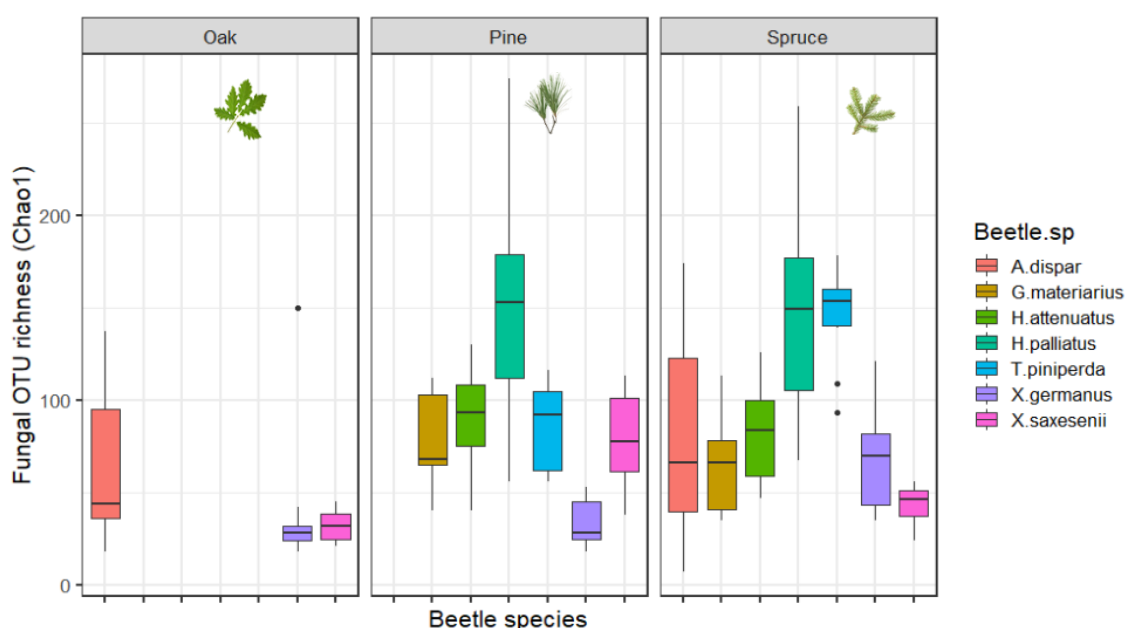


Figure 3-2. Fungal OTU richness across forest types (oak, pine and spruce) and beetle species. Rarefied data. Richness was estimated using Chao1 index.

According to the generalised linear model (Negative binomial) fungal richness was significantly different across beetle species and forest types. However, it is important to be cautious when turning these differences into numbers, since these differences were exacerbated due to the small sample size and the unbalanced design of the experiment. When considering only the tree beetle species present in oak and comparing them across forest types, the effect of the beetle species was only 9.81% and the forest type accounted for only 6.1% of the variation. What seems clear is that the beetle species would play the most important role explaining fungal richness, followed by the forest type (Table 3-2).

Table 3-2. Overview of the GLM analysis results, showing the significant *p*-values, the percentage of explained variance and the *F* parameter for every factor included in the model.

Factor	Explained variance	$F_{x,y}$	<i>p</i>
Beetle + forest type	4.70 %	$F_{8, 151} = 4.781$	<0.001 ***
Beetle	51.00 %	$F_{6, 153} = 20.772$	<0.001 ***
Forest type	19.30 %	$F_{2, 157} = 4.646$	<0.001 ***
Unexplained	25.00 %		

3.4.2. Taxonomic coverage and fungal diversity

From the 2482 fungal OTUs, 2006 were classified to phylum, 1695 to class, 1692 to order, 1323 to family and 663 to species. The OTUs were distributed over four different phyla, most abundant being the phylum Ascomycota (55.8% OTUs) followed by Basidiomycota (21.2% OTUs). Taxonomic coverage was further explored across beetles and forest types, showing 25

different classes (**Figure 3-3**), 100 different orders, 257 different families, 572 genera and 592 species. The fungal composition was highly diverse, representing the “Helotiales”, “Tremellales” and “Capnodiales” the most abundant orders.

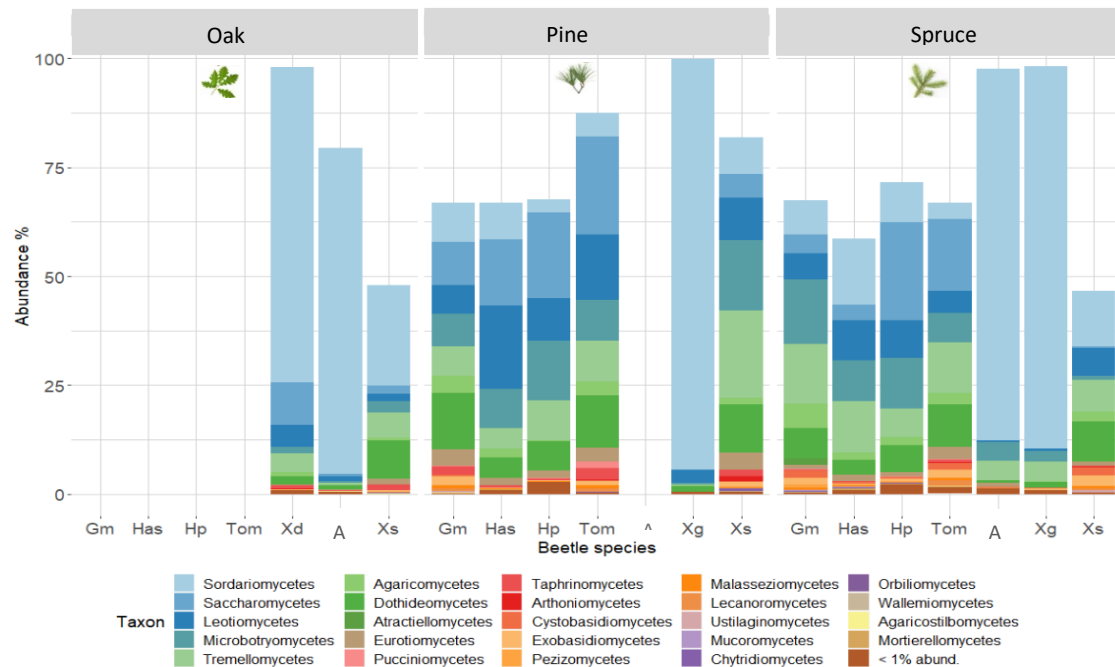


Figure 3-3. Relative abundances for rarefied read counts for fungal classes detected across the different beetle species and forest types. The least abundant fungal OTUs (relative abundance is <0.1) are clustered together. Beetle species: *Hylastes attenuates* (Has), *Gnathotrichus materiarius* (Gm), *Hylurgops palliatus* (Hp), *Tomicus piniperda* (Tom), *Anisandrus dispar*(Ad), *Xyleborinus saxesenii*(Xs), *Xylosandrus germanus* (Xg)

The fungi detected at species level presented a whole range of ecological guilds, from pathogens (*Ophiostoma* spp., *Lophodermium* spp., *Pachyramichloridium pini*, *Heterobasidion annosum*) to endophytes (*Hamamotoa lignophila*) or saprotrophs (*Daldinia vanderghuchtae*, *Sympodiella acicola*). In addition, two entomopathogenic fungi, *Beauveria bassiana* and *Beauveria caledonica* were detected, plus other species associated with bark and ambrosia beetles like the ambrosia fungi *Ambrosiella hartigii* and yeasts of the genera *Saccharomycopsis* and *Ogataea*.

The effect of the beetle species and forest type on the fungal diversity was visualised through an NMDs graph, which showed how different beetle specimens cluster together in terms of their fungal community similarities (**Figure 3-4**). Interestingly, phylogenetically close beetle species like those belonging to the Xyleborini tribe (*Xylosandrus*, *Anisandrus* and *Xyleborinus*) exhibited

more similar fungi than those from the tribe Hylastini (*Hylastes* and *Hylurgops*), while *Tomicus piniperda* fungal communities are the most clearly split among forest types.

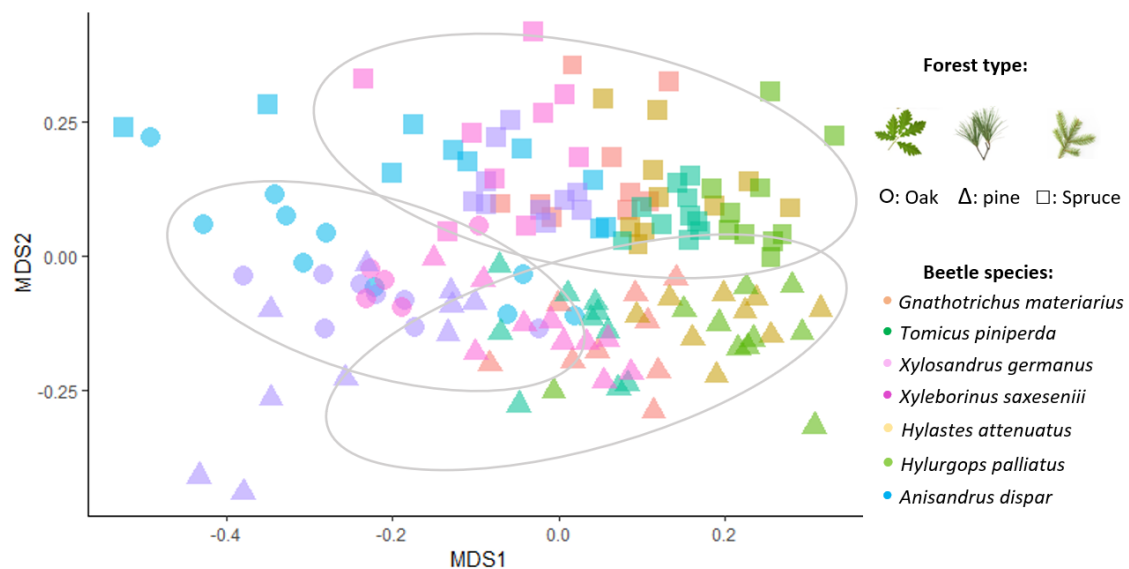


Figure 3-4. NMDS ordination plot of all specimens sampled, based on the fungal community composition of the individual beetles. Colours represent different beetle species and shapes represent forest types. Stress for this graph fell within acceptable ranges (<0.2).

Fungal communities were turnover-dominated ($W=141772928$, $p\text{-value}<0.0001$) (**Figure 3-5 A-B-C**). The two conifer sites were more similar in turnover and nestedness than the oak forest. At the oak forest, *Xylosandrus germanus* and *Anisandrus dispar* fungal communities presented a lower Sorensen index, indicating a higher turnover than the same species at a different forest type (Schmera, Podani & Legendre, 2020). This would illustrate a higher fungal species replacement between conifers and broadleaves for those beetle species. Results from the PERMANOVA analysis using the Sorensen index revealed that the forest type and the beetle species explain a 10.32% and a 12.44% respectively of the total fungal community variation. The Bray index (based on abundance data) provides very different results, attributing only a 5.93% of the variation to the forest type and 26.69% to the beetle species. Both indexes suggest an effect of the interaction among beetle species and forest type that would account for 5.8 % of the total variation. The analyses of the dispersion showed significantly different distances to the group centroids for both the forest type and the beetle species (**Figure 3-5 D-E**).

(a)

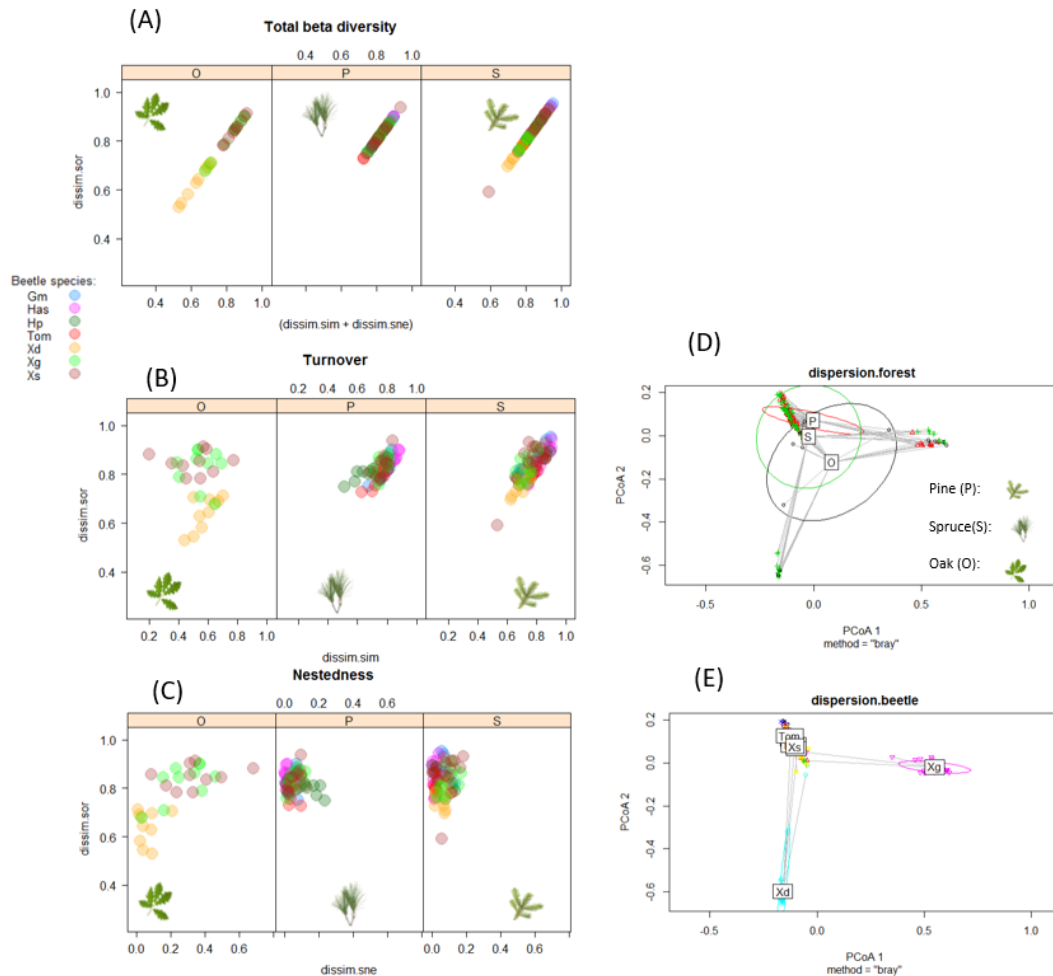


Figure 3-5. (A) Total beta-diversity and its partition into (B) turnover and (C) nestedness across forest types. Colours represent different beetle species. Dispersion across forest types (D) and beetle species (E), shown as the distance to centroids. Beetle species: *Hylastes attenuates* (Has), *Gnathotrichus materiarius* (Gm), *Hylurgops palliatus* (Hp), *Tomicus piniperda* (Tom), *Anisandrus dispar* (Xd), *Xyleborinus saxesenii* (Xs), *Xylosandrus germanus* (Xg)

3.4.3. Associated fungi across forest types and beetle species

From the 1482 OTUs generated in the clustering step of the bioinformatics analysis, the multilevel species analysis results suggest that 239 were significantly correlated ($p < 0.05$) with the beetle species and 176 fungal OTUs were associated with the forest type from which beetles were collected (**Figure 3-6**). The beetle species clustered together, denoting their phylogenetic links, forming a group with the Xyleborini tribe separated from the Hylestini tribe. Interestingly, *Tomicus piniperda* collected from the pine forest shared more similarities with the *Gnathotrichus* specimens collected in the same forest type than with other bark or ambrosia beetles. *X. saxesenii* associated fungi is more similar between oak and pine than between the conifers, exhibiting the lowest number of fungal associates in spruce and the highest in pine.

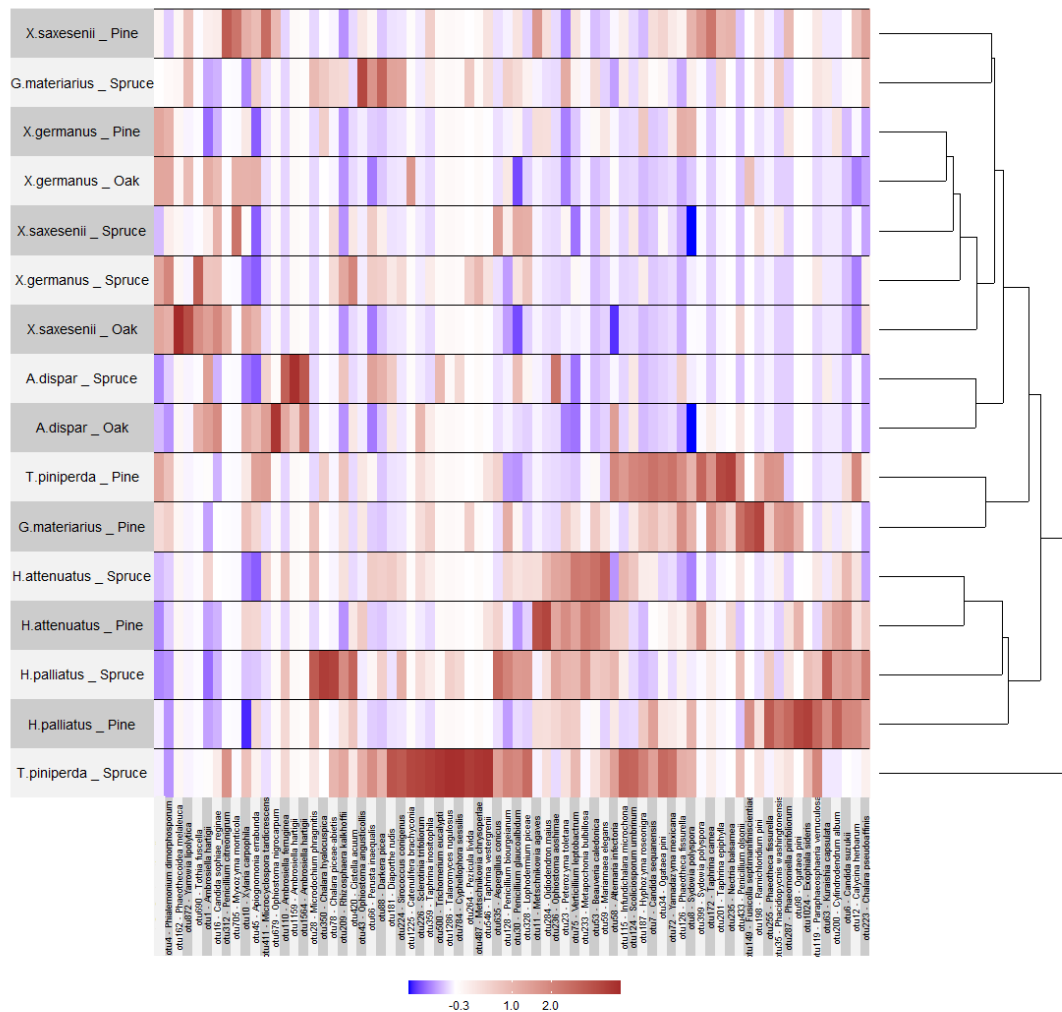


Figure 3-6. Frequency of association of fungal OTUs (p.g index) across the different beetle species and forest types. Only the identified OTUs from the phylum Ascomycota are displayed.

Beetle-associated fungi included three different species of the genera *Ophiostoma* (*O.angusticollis*, *O.nigrocarpum* and *O.aoshimae*), three species of *Ambrosiella* (*A. ferruginea*, *Ambrosiella hartigii* and *Ambrosiella grosmanniae*) and one species of *Ceratocystis* (*C. adiposa*). It is important to clarify that at the end of the bioinformatic pipeline *A. hartigii* was clustered into three different OTUs, which differed from each other in 3% of their nucleotides. Since there were all classified as the same species, I considered them as fungal strains. *Ambrosiella hartigii* and *Ambrosiella grosmanniae* were present only in a moderate proportion in half of the specimens of the invasive beetle *Gnathotricus materiarius*, thus not being associated with it at any particular forest type. However, the fungal symbiont *Ambrosyozyma monospora* was present in the majority of the specimens of this beetle species, especially in pine forests, where

it was highly abundant for some of the specimens (**Figure 3-7**). When considering fungal pathogens; *Ophiostoma angusticollis*, which attacks tree wood, was present in eight out of ten specimens of *Gnathotrichus* in spruce, but only in one specimen trapped in the pine location. This could also suggest an effect of the forest type in the fungal species turnover for the conifers.

Significantly associated fungi belonging to the phylum Basidiomycota were fewer than those belonging to Ascomycota (**Supplementary Figure APP II-2**). The majority were classified as fungal saprotrophs, and the most frequent genera were *Exobasidium* and *Cryptococcus*.

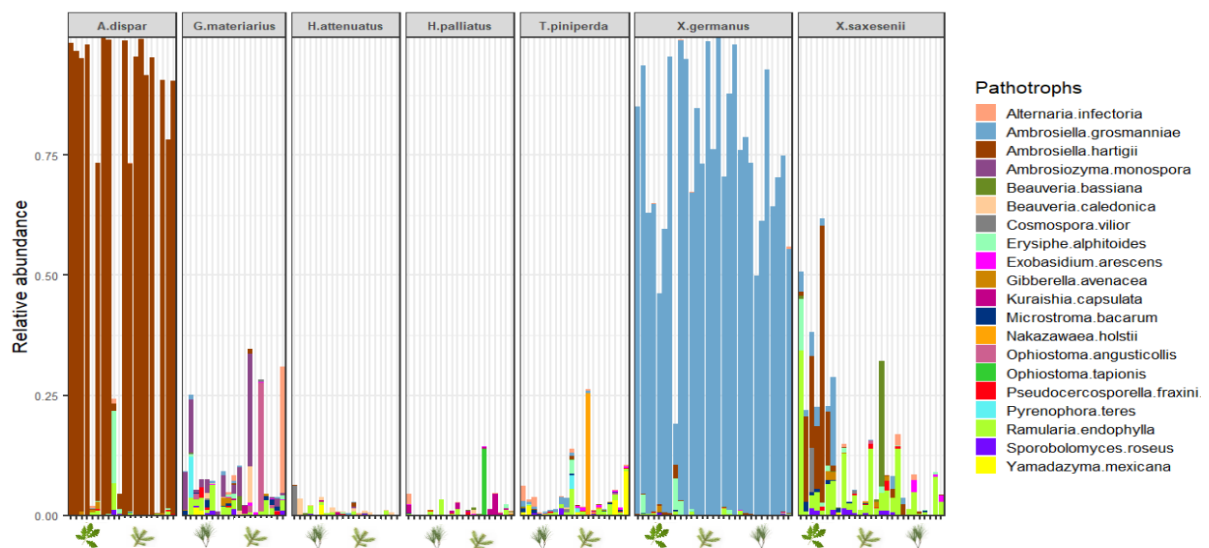


Figure 3-7. Bar plot showing the different relative abundance of fungal pathotrophs across beetle specimens. The fungal symbionts *Ambrosiella Hartigii* (brown) and *Ambrosiella grosmanniae* (blue) on the beetle species *Anisandrus dispar*, *Xylosandrus germanus* and *Xyleborinus saxesenii* across the different forest types. The symbiont *Monosporium monosporum* is associated only with *Gnathotrichus materiarius*

3.5. Discussion

The present chapter assesses the influence of the dominant tree species (here denoted as “forest type”) and the beetle species identity on the fungal communities associated with bark and ambrosia beetles at the local level. I used high throughput amplicon sequencing to compare fungal community richness and community across these biotic factors. Previous studies have shown beetle-associated fungal communities shaped by abiotic factors such as rainfall and temperature (Six, 2012). Miller (2016, 2019) suggested that the fungal communities associated with bark and ambrosia beetles could rely primarily on the beetle species identity. By focusing on the local level, this study rules out the potential effect of abiotic factors across forest types to focus on the biotic factors shaping these communities.

My results show that fungal community richness differed across beetle species, and the extent of that influence sometimes rely on the dominant tree species. The forest type would play a more critical role in the fungal community composition, explaining a non-neglectable amount of fungal variation. To my knowledge, this is one of the first studies assessing the effect of the dominant host trees on the beetle-associated fungal communities, with important forest management implications.

3.5.1. Drivers of fungal community richness

The species accumulation curves did not reach asymptotes in any case, indicating that more fungal species are likely to coexist on these beetle species, which is a typical result in microbial community studies (Ugland, Gray & Ellingsen, 2003). A slower rate of new fungal species in an oak forest could perhaps reflect also the lower diversity of beetle species collected at this forest type.

The beetle species identity was the main driver of the fungal OTU richness in the New Forest. This pattern could be partially explained using phylogeny. Fungal communities have already been described as specific for certain Scolytinae species but promiscuous for others (Kostovcik *et al.*, 2015). The ambrosia beetles *Xylosandrus germanus* and *Xyleborinus saxesenii* harboured the lowest fungal richness. A significant proportion of the fungi carried by each of these ambrosia species corresponded with its putative fungal symbiont.

In contrast, the highest richness was detected in the bark beetles *Tomicus piniperda* and *Hylurgops palliatus*. Since fungal symbionts tend to compete with other fungi (Skelton *et al.*, 2019a), a lower fungal richness could be expected compared with bark beetles. Therefore, it could be expected that the beetle species identity could drive the fungal community richness for certain beetle species more than for others. In previous studies of British beetle communities conducted by Miller (Miller, 2015; Miller *et al.*, 2019), beetle species identity explained approximately 15% of the fungal community richness variation. This result is in agreement with past works, showing beetles species identity as having an effect on the fungal communities, especially in the case of the ambrosia beetle species (Biedermann, Klepzig & Taborsky, 2009; Batra, 1963; Beaver, 1989). My results suggest that the beetle species could play a more significant role in fungal community richness, accounting for a higher fungal variation. However, since the present work has only been carried out for seven beetle species, not all of them being present in all the forest types, I need to be careful when interpreting these numbers. What

remains clear is that the beetle species identity is the primary driver of fungal community richness.

Besides the beetle species identity, variations corresponding with the forest type shaped fungal richness. Both *Xyleborinus saxesenii* and *Gnathotrichus materiarius* exhibited a looser fungal association than the other ambrosia beetles. However, *Xyleborinus saxesenii* hosts a much higher proportion of ambrosia fungi in oak forests than in the conifer spots. Given that oak species are more phylogenetically distant than conifers, this could explain why the fungal communities on pine and spruce were more similar than in oak. Asplund (2019) documented differences among soil fungal communities on forests dominated by beech and spruce, detecting a lower OTU richness on beech than on spruce or mixed forests. Variable response of fungal communities to the dominant host trees has also been documented in a previous study, which linked tree diversity to fungal diversity (Urbanová, Šnajdr & Baldrian, 2015). Tedersoo found that soil fungal patterns would be generally unrelated to plant diversity (Tedersoo *et al.*, 2014). Still, a more recent study has found a regional-scale effect of certain tree species on fungal richness (Tedersoo *et al.*, 2020a). This author also suggested that biotic and abiotic effects could be guild-specific rather than relying on certain fungal species.

3.5.2. Taxonomic coverage and fungal community composition

The fungal composition retrieved from the beetle species was highly diverse. This finding agrees with previous results, which showed a high diversity of fungal OTUs on bark and ambrosia beetles (Miller *et al.*, 2016; Skelton *et al.*, 2019b; Malacrinò *et al.*, 2017). However, it differs from the fungal OTU diversity obtained using traditional methods (such as fungi culture) in the same beetle species (Li *et al.*, 2016). This confirms that metabarcoding techniques are extremely useful to identify fungi that otherwise would have remained cryptic.

As detailed in the introduction, fungal species identification through molecular techniques faces critical challenges (e.g., the uncomplete databases), making fungal taxonomic identification often extremely difficult to achieve. Accordingly, even though 1482 fungal OTUs were clustered in the present preliminary data, I managed to identify only 100 of them to the species level. Despite the above difficulties, some important fungal associates were identified, and their relative abundance inside the beetles was investigated. These results are in agreement with recent studies pointing out that fungal communities are much more diverse and less species-specific than previously thought (Kostovcik *et al.*, 2015; Hulcr & Stelinski, 2017).

The forest type had a higher effect on the fungal community composition than the beetle species. Six (2012) suggests that some fungal associates might be incidental, creating non-permanent relations with bark beetle hosts. Our findings indicate that some of the fungi vectored by these beetles would be associated with the dominant tree species rather than being incidental. A previous study has documented that tree bark could act as an environmental filter, contributing to endophyte selection (Pellitier, Zak & Salley, 2019). Therefore, different tree species could harbour and exchange diverse fungal endophytes with beetles since they spend an essential proportion of their life cycle under the tree bark.

Since most fungal community variation could not be explained, there is an essential proportion of unexplained fungal variability that relies on the context in which beetles develop. These results align with the idea of fungal community drivers being context-specific (Alzarhani *et al.*, 2019). This context could also include other biotic factors like forest composition, forest structure and understory. What remains clear is that the dominant host trees play a role in explaining part of the variability on fungal community composition in the case of bark and ambrosia fungal associates.

3.5.3. Associated fungi across forest types and beetle species

Three *Ambrosiella* species were detected as fungal associates: *A. grosmanniae*, *A. ferruginea* and *A. hartigii*. Besides, three different fungal strains were detected from *A. hartigii*, showing different patterns across forest types. *Ambrosiella hartigii* and *Ambrosiella grosmanniae* were particularly abundant, especially on the ambrosia beetles. Due to their obligate symbiosis with these fungal species (French & Roeper, 1972; Mayers & Harrington, 2015), *Anisandrus dispar* and *Xylosandrus germanus* showed a high proportion of these fungi in their bodies, as expected from the literature.

The influence of the dominant host trees affected the frequency and strength of fungal associations. In the exotic beetle *Xylosandrus germanus*, *A.grosmanniae* was significantly correlated in the three forest types. It is known that ambrosia and bark beetle fitness rely upon the quality of their symbionts since they are their primary nutritional source (Bleiker & Six, 2007; Harrington, 2005). Given that *Xylosandrus germanus* is exotic, it could rely more on its fungal symbiont, and this could explain why this fungus is so abundant and widespread across the beetle specimens. However, one strain of *A. hartigii* was positively correlated with *Xylosandrus*, but not in pine forests. This could indicate that this polyphagous beetle could harbour two different fungal associates in oak and spruce but only one in pine. Given that *A. grosmanniae*

and *A. hartigii* are closely related, both fungi could be carried by the beetle (Mayers *et al.*, 2020). However, a previous study suggested that *X. germanus* would be unable to exchange fungal symbionts among clades at its native range (Ito & Kajimura, 2017). Given that the beetle has recently arrived in the UK, it is not likely that more than one clade has been established, especially at the New Forest local level. Therefore, the beetle could have acquired another fungal symbiont horizontal transmission while getting established in the UK.

Xyleborinus saxesenii specimens were also found to be hosting a high proportion of both *Ambrosiella hartigii* (one of the strains) and *Ambrosiella grosmaniae* only in oak forests. This is a shocking result, which to our knowledge has not been previously shown by any literature. Despite being also an ambrosia beetle, *Xyleborinus saxesenii* has not yet been associated with any fungus from the *Ambrosiella* clade. My results suggest that this beetle could form more loose associations than other beetles from the same tribe but still being able to preserve these ambrosia fungi once obtained. These fungi could have been obtained using mycoceptism or crop-theft, a relatively common method of horizontal transmission of fungi among ambrosia beetle clades (Hulcr & Cognato, 2010). Interestingly, *Ramularia endophylla*, which has been previously associated with *Xyleborinus saxesenii* (Malacrinò *et al.*, 2017), was present in nearly all the specimens independently of the dominant tree species. The genera *Ramularia* harbours phytopathogens responsible for leaf spots (Videira *et al.*, 2016). These findings indicate the importance of researching fungal associations by sampling beetle specimens across different forest types to detect the presence of fungal pathogens fully.

Anisandrus dispar associated with the three *Ambrosiella* species but exhibited a higher correlation in spruce than in oak for *A.ferruginea* and one of the *A.hartigii* strains. In the pine forest, the symbiont *Ambrosiozyma monospora* was present in 80% of the specimens of *G.materiarius*. However, the presence of this symbiont was not correlated with the beetle at a significant level ($p < 0.05$) since it was rare in spruce, and its number of reads was relatively low in some of the pine specimens. A recent study has documented the selective effect of ethanol on certain beetle-associated fungal lineages since beetles would rely on fungal partners that could metabolise this substance (Lehenberger, Benkert & Biedermann, 2021). This could contribute to explain the differences in fungal associations found between conifers and broadleaves.

The three analysed bark beetles have been previously associated with several ophiostomatoid fungi, including *Ophiostoma minus* and *Ophostoma piceae* ((Jankowiak, Kacprzyk & Młynarczyk,

2009; Romón *et al.*, 2007)). However, in this analysis, the ophiostomatoid fungi were significantly associated with the ambrosia beetles. Two of the ophiostomatoid fungi, *Ophiostoma nigrocarpum* and *Ophiostoma aoshimae* were substantially more abundant in *Anisandrus dispar* but differing across forest types: While the first was more abundant in *A. dispar* collected from oak forests, the second was more abundant in spruce. The third ophiostomatoid fungi, *Ophiostoma angusticollis*, was correlated with the exotic beetle *Gnathotrichus materiarius* in spruce but not in the pine forest. This fungus has been previously linked to decline in pine forests in combination with other Ophiostomatoid fungi (Álvarez-Baz, Fernández & Diez Casero, 2015; Pérez Vera *et al.*, 2019), but to our knowledge has never been linked to spruce forests. The analysis of the fungal communities for individual beetles can give us an idea of how widespread certain key-fungi are within the beetle community and how this could also be related to the forest type.

There was a high proportion of yeasts, mainly associated with the bark beetles, which is in agreement with previous studies (Miller *et al.*, 2016, 2019; Giordano *et al.*, 2013; Bezos *et al.*, 2018, Anon, 2015). Although most of the functions carried out by the associated yeast species are still unknown, yeasts play an important role for bark and ambrosia beetles (Davis, 2015). The turnover of associated yeasts across forest composition was more clearly noticeable for *Tomicus* specimens, showing that the associated yeasts differed from pine to spruce. Interestingly, different strains of *Ogataea pini* were associated with *Tomicus piniperda* and *Hylurgops palliatus*. This yeast has been previously associated with several *Ips* species (Davis, 2015), and it is thought to alter the phytochemicals of tree vascular tissues during beetle development (Davis & Hofstetter, 2011). Along with *Kuraishia capsulata* in the *H. palliatus* specimens, this yeast is thought to generate volatile chemicals that may help beetle colonisation and tree selection (Hunt & Borden, 1990).

3.6. Conclusion and research implications

As a whole, the results of the present study support my prediction of a potential tree-mediated relationship among fungal communities and bark and ambrosia beetles. These results are promising and should be validated in further studies with more forest locations and beetle species. In future, it is also advised to explore the fungal communities present in the trees at the period in which beetles get collected. This way, the proportion of the different fungal species carried by the beetles could be compared with the ratio of fungi naturally present in the living

or dying trees. This would greatly help to understand better how fungal communities coexist inside bark and ambrosia beetles and on which conditions they may vector fungal diseases.

To study fungal communities associated with certain beetle species, it would be necessary to explore the forest types each beetle species may colonise. This way, we could also explore the question of the extent bark beetle fungal community could become a proxy to forest fungal community composition.

Chapter 4. Biodiversity Survey across the UK

reveals the influence of the forest composition on the fungal communities associated with bark and ambrosia beetles

4.1. Abstract

Bark and ambrosia beetles significantly contribute to the dispersal of fungi. Fungi play critical roles in several essential forest functions. However, bark and ambrosia beetles may also vector pathogens, facilitating fungal outbreaks. Despite the efforts made to study the biotic and abiotic drivers of fungal communities associated with bark and ambrosia beetles, most of the fungal variation associated with them remains unexplained. The present chapter aimed to explore the differences in fungal communities associated with bark and ambrosia beetles across four different forest compositions to study the extent to which they are interdependent. To do this, I sequenced fungal communities belonging to 1064 specimens from 25 species of bark and ambrosia beetles. Beetles were collected in forests across the UK, ranging from monospecific conifers to mixed broadleaves. Results revealed that the effect of the forest composition on the fungal communities associated with bark and ambrosia beetle relies on the tree species composition. To complement these results, I compared 177 specimens from *Hylurgops palliatus* and 88 specimens of *Xyleborinus saxesenii* present in the four different forest compositions. Results showed how the forest composition affected richness only in *Xyleborinus saxesenii*, while fungal community composition alone drove 8.3% and 12.1% of the fungal composition in *Hylurgops palliatus* and *Xyleborinus saxesenii*, respectively. When comparing monospecific forests of different pine species, the host tree identity also played a role in the fungal community composition. My work contributes to explaining the extent to which the fungal communities associated with bark and ambrosia beetles are driven by forest composition, with significant management implications.

4.2. Introduction

Fungi play crucial roles in forest ecosystems by contributing to several essential functions, including decomposition or the uptake and transfer of nutrients (Smith & Read, 2010). Some fungi may even help mitigate climate change effects on forests (Martínez-García *et al.*, 2017). However, fungal communities may also represent a threat to forest health. Pathogenic fungi can negatively affect the ecosystem balance, alone or in interaction with pests like bark and ambrosia beetles (Ferrell & Parmeter Jr, 1989; Six, 2020; Zhao *et al.*, 2019). The risk of pathogenic fungi is exacerbated by climate change and globalisation, which may enhance their amount of hosts (Gange *et al.*, 2011; Holec, 2016) and their scope of impacts (Marini *et al.*, 2012; Pautasso *et al.*, 2012). Although fungal communities widely affect forest performance, little is known about the factors continuously shaping fungal communities at different geographical scales.

Fungal species exhibit different ecological patterns, forming very diverse and complex communities. The complexity of these drivers can be disentangled with molecular techniques, which have become the most widely used approach to study fungal communities (Kulik, Bilska & Żelechowski, 2020; Miller *et al.*, 2016; Schmidt *et al.*, 2013). With these techniques, several studies have been able to point out the main drivers of fungal community composition at global scales (Davison *et al.*, 2015; Kivlin, Hawkes & Treseder, 2011; Tedersoo *et al.*, 2014). While the main identified abiotic drivers of fungal communities at a global level include climate and geography, the anthropogenic activity has been suggested as one of the most influential biotic drivers (Al-Yasiri *et al.*, 2017; Moora *et al.*, 2014; Öpik *et al.*, 2006). Climate has a global impact on fungal community composition due to the variation in factors like temperature or rainfall (Větrovský *et al.*, 2019). Climate change may also affect fungal diversity within the communities (Newsham *et al.*, 2016; Větrovský *et al.*, 2019). The use of these molecular techniques has also helped to disentangle some previously unknown associations among beetle and fungi (Hulcr *et al.*, 2020; Six, 2012; Zhao *et al.*, 2019) as well as to establish surveillance mechanisms to deal with introduced beetle-associated fungal diseases (Malacrinò *et al.*, 2017). Furthermore, recent studies point out that these differences could be guild-mediated, depending on the functional group rather than the fungal identity (Alzarhani *et al.*, 2019). For example, pathogenic fungi may display broader climatic tolerances than mycorrhiza (Větrovský *et al.*, 2019). Although our knowledge of fungal diversity and biogeography has increased in recent years, it is still constrained. Therefore, it is necessary to monitor fungal communities to understand better the biotic and abiotic drivers of their changes and effects on forests.

For fungal species associated with bark and ambrosia beetles, the beetle species seems to play a significant role in fungal community composition (Miller *et al.*, 2019; Six, 2012). Earlier studies thought that this role was only reduced to the ambrosia fungi since they can culture the beetle species and carry them with specialised structures called mycangia. Another limiting factor is that most of the published studies focused on single beetle species, mainly the most aggressive ones, neglecting the fungal communities associated with less aggressive or secondary bark beetles (Six, 2020). However, recent studies show that there is no significant difference between bark and ambrosia beetles in terms of associated species (Six & Elser, 2020). In the UK, Miller *et al.* (2019) studied fungal communities associated with bark and ambrosia beetles on pine forests, finding that the beetle species accounted for 14.5% of the fungal variation, while environmental and spatial predictors accounted for 5.3% and 1.5% of the variation respectively. Despite the great efforts in determining which factors influence fungal communities, these factors only explain a small part of the fungal species variation of beetle-associated fungi.

Forest structure and composition modifies the local climatic conditions, contributing to creating micro-climates (Chen *et al.*, 1999). Consequently, both forest structure and composition may also affect fungal communities associated with bark beetles, with important forest management implications. Likewise, forest management may also influence bark beetle populations (De Groot, Diaci & Ogris, 2019; Toivanen, Liikanen & Kotiaho, 2009; Wallin *et al.*, 2008) since it modifies factors like forest composition and structure. From a regional-scale analysis, Tedersoo suggested that the tree species identity affects fungal communities in *Pinus sylvestris*, *Picea* spp and *Populus*, especially for biotrophic fungi (Tedersoo *et al.*, 2020a). For example, the study showed that the different proportion of *Picea* spp affected the composition of fungal guilds. In a recent review, Tomao (2020) observed the positive correlation of fungal diversity with canopy cover, basal area and tree species diversity. However, the long-term forest management effect on fungal communities remains incomplete due to the lack of agreement among the few studies evaluating it (Castaño *et al.*, 2018; Parlade *et al.*, 2019; Sterkenburg *et al.*, 2018). Consequently, there is still a debate about which factors linked to forests may affect fungal communities associated with bark beetles and under which conditions.

The purpose of the present chapter was to evaluate the influence of abiotic and biotic drivers on fungal communities associated with bark and ambrosia beetles collected from forests consisting of different tree species compositions, which ranged from monospecific conifers to mixed broadleaf forests. More specifically, we hypothesised that: (1) The forest composition affects the beetle-associated fungal communities (2) A higher tree diversity is correlated with a

higher fungal diversity (3) A higher tree diversity is linked with a lower fungal-beetle association specificity (4) Abiotic drivers have a lower effect on beetle fungal richness and fungal diversity than biotic drivers.

4.3. Material and Methods

Samples were collected by Forest Research (Forestry Commission) during the year 2017 in 20 different sampling sites across the UK. A total of 1064 specimens from 25 species of bark and ambrosia beetles were collected with Lindgren multiple-funnel funnel traps ((Lindgren, 1983), Phero Tech). These traps were located in forests consisting of different compositions, which were classified into four categories (**Figure 4-1, Supplementary Table APP III-1**).

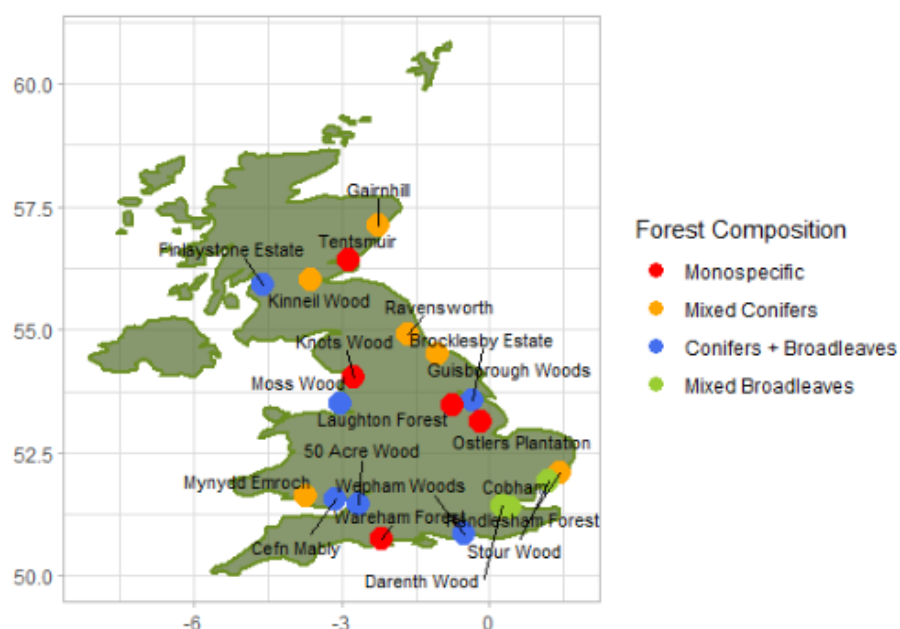


Figure 4-1. Location and forest composition of the sampling sites where the beetle specimens were collected from. Monospecific forests (in red) consisted of only Scots (*Pinus sylvestris*) or Corsican (*Pinus nigra*) pine. Whether one of these species was mixed with Sitka spruce (*Picea sitchensis*), the forest composition was denominated a “Mixed Conifers” (yellow). Where a conifer or mix of a single species of conifer was mixed with a smaller proportion of broadleaves, the forest was classified as “Conifers + Broadleaves” (Blue). If the forest consisted of a mix of broadleaves, it was classified as “Mixed Broadleaves” (Green).

Monospecific forests consisted of only Scots (*Pinus sylvestris* L.) or Corsican (*Pinus nigra* Arnold) pine. Whether one of these species was mixed with Sitka spruce (*Picea sitchensis* (Bong.) Carr.), the forest composition was denominated a “Mixed Conifers”. Where a conifer or mix of a single species of conifer was mixed with a smaller proportion of broadleaves, the forest was classified as “Conifers + Broadleaves”, and if the forest consisted of a mix of broadleaves, it was classified as “Mixed Broadleaves”. Traps spaced 50m apart along 250m of the transect and baited with lures (100% ethanol, plus α - pinene) to mimic volatile chemicals emitted by dying trees, as

shown in Inward (2019). Propylene glycol (65% concentration) was placed at the bottom of each trap to ensure the correct preservation of the beetles. Traps were emptied every two weeks between April and August. Captured specimens were then kept frozen in pure ethanol to preserve their DNA quality until being sorted and analysed. Captured specimens were morphologically identified and target beetles randomly selected. When the number of beetle specimens per species and site was higher than eight, a minimum of eight and a maximum of 12 beetle specimens was randomly selected. For those beetles that were rare (<8 specimens per species and site), only one specimen per species and site was selected (**Table 4-1**).

Table 4-1. Overview of the beetle species and number of the specimens per forest composition included in the study.

Scientific_name	Specimens	Forest Composition
<i>Cryphalus asperatus</i>	28	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Dryocoetes autographus</i>	89	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves
<i>Dryocoetes villosus</i>	4	Monospecific, Mixed broadleaves
<i>Gnathotrichus materiarius</i>	1	Monospecific
<i>Hylastes ater</i>	65	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Hylastes attenuatus</i>	61	Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed Broadleaves
<i>Hylastes brunneus</i>	75	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Hylastes cunicularius</i>	19	Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Hylastes opacus</i>	51	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves
<i>Hylesinus varius</i>	33	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Hylurgops palliatus</i>	178	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves
<i>Orthotomicus laricis</i>	13	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Pityogenes bidentatus</i>	5	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Pityogenes lineatum</i>	1	Monospecific
<i>Pityophthorus pubescens</i>	5	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Polygraphus polygraphus</i>	14	Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Tomicus minor</i>	12	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Tomicus piniperda</i>	159	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Trypodendron domesticum</i>	29	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves
<i>Trypodendron lineatum</i>	82	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves
<i>Trypodendron signatum</i>	2	Monospecific, Mixed Conifers + Mixed Broadleaves
<i>Xyleborinus saxesenii</i>	91	Monospecific, Mixed Conifers, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves

<i>Anisandrus dispar</i>	23	Monospecific, Mixed Conifers + Mixed Broadleaves, Mixed broadleaves
<i>Xyleborus monographus</i>	1	Mixed Conifers + Mixed Broadleaves
<i>Xylosandrus germanus</i>	20	Mixed Broadleaves

4.3.1. Laboratory procedures

Selected specimens were rinsed with pure water to wash out external impurities and placed in individual 1.5ml tubes. Sterile pestles were used to completely break up the individual beetle specimens to ensure that all the fungi inside the specimens were captured. The DNA of specimens was then individually extracted, purified and eluted in 200µl of AE buffer using the QIAGEN DNA easy blood and tissue spin column extraction kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Metabarcoding of associated fungi was performed using the ITS primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') /ITS4(5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck *et al.*, 2014) for a first PCR reaction. Unique six-letter indices were added to each primer pair to distinguish the specimens inside the different libraries. The PCR reaction mixture contained 2.5 µl of TaKaRa buffer™, 0.5 µl of dNTPs (Bioline), 1 µl of forward primer, 1 µl of reverse primer, 0.1 µl of TaKaRa Taq™ and 18µl of ddH₂O. 2 µl of DNA were added to every PCR reaction, making a final volume of 25 µl. Cycling conditions of the PCRs were: 94°C for 3 minutes followed by 34 cycles of 94°C for 45 seconds, 55°C for 55 seconds 72°C for 1.5 minutes, and a final extension of 72°C for 10 minutes. For each of the analysed samples, PCRs were replicated with three annealing temperatures (54°C, 55°C and 56°C) to take into account the stochastic nature of PCR reactions (Schmidt *et al.*, 2013). A minimum of one blank PCR reaction per plate was used as a negative control to control for potential contaminations.

Successful PCR amplification was confirmed in every case by visualising 3 µL of the PCR product using gel electrophoresis. For this visualisation, 2% agarose TAE gel, GelRed™ (Biotium) and Hyperladder IV™ (Bioline) were used. PCR products were then purified to eliminate fragments under 200bp, using the Agencourt® AMPure® XP purification kit, by following protocol 000387v001. The amplified DNA was pooled in equal proportions thereafter, forming 96 different pools of barcodes and metabarcodes. At the sequencing facility, the 96 libraries were prepared, homogenised and indexed using Nextera® XT with a second PCR. Libraries were then sequenced on an Illumina® HiSeq® 2500 platform with version 2 chemistry (Rapid mode), using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads.

4.3.2. Bioinformatics

Raw sequence data were processed using bioinformatics to achieve solid species identification before its ecological analysis. The initial procedure was similar for both metabarcoding and barcoding samples, differing only in the last steps. The 96 libraries were firstly demultiplexed by using the Illumina software to remove their XT indices. Samples were further demultiplexed with Cutadapt (Martin, 2011) scripts modified to account for bulk samples. During this step, the unique six-letter indices attached during the first round of PCR were trimmed from each sample file, which in turn was then assigned a unique name related to every single beetle specimen. Also, singletons were removed at this step, and Cutadapt was used to trim primers and zero-length sequences before read assemblage. Read quality was evaluated using FASTQC (Andrews, 2014). Forward and reverse reads were subsequently assembled using PEAR v. 0.9.8 (Zhang *et al.*, 2014). This step included a quality filtering of the paired-assembled reads (PEAR quality score threshold ≥ 30 , as recommended by Illumina® (https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf). PEAR software tests all possible overlaps among reads due to its tolerance to variable read lengths (Zhang *et al.*, 2014). A further quality test was carried out using USEARCH v. 7.0 (Edgar, 2010), with the `--fastx_filter` command (`-fastq_maxee 1.0`). Merged full-length metabarcoding reads had an average length of 220 bp.

Read clustering into Operational Taxonomic Units (OTUs) was subsequently performed using USEARCH (Edgar, 2010). After their dereplication, sequences were filtered by length, eliminating those sequences shorter than 200 bp or longer than 350bp and removing those with less than two reads to avoid potential sequencing errors or artefacts. Chimaeras were also detected and filtered out with UCHIME (Edgar *et al.*, 2011), remaining 96.5% of the reads. Reads were then denoised using UNOISE (Edgar, 2016:p.2). The ITSx software (Bengtsson-Palme *et al.*, 2013) was used to filter out non-fungal reads, selecting and extracting target ITS2 regions for each full-length read. This step was performed to ensure the best results on their further identification. After using ITSx, the average length of the 8528 remaining sequences was 171 bp. The remaining reads were then clustered using UCLUST from the USEARCH v. 9.2 pipelines (Edgar, 2010) at a 97% similarity threshold to delimit different OTU clusters. The average length of the OTUs was 171 bp. Reads were then mapped to the OTU clusters, outputting 5546 OTUs in the shape of an OTU table suitable for the ecological analysis of the samples.

Fungal OTUs were classified following three complementary paths. The Ribosomal Database Project (RDP) Bayesian Classifier(Wang *et al.*, 2007) was used to assign the first classification for the OTUs. This classifier has been broadly used for fungal identification, using the Warcup fungal ITS (v2_March2018) and UNITE (v2020_February2020) training datasets to achieve both broad coverage and accurate OTU classification (Deshpande *et al.*, 2016; Edgar, 2018). Simultaneously, OTUs were processed through the PROTAXFUNGI pipeline(Abarenkov *et al.*, 2018), implemented in the PlutoF (Anon, 2017a) platform and based on the UNITE fungal database (last update, February 2020). PROTAXFUNGI hierarchically assigns the OTU identities from the root node of the taxonomy through the species level (Nilsson *et al.*, 2019b). The last classifier selected, IDTAXA, employs machine learning to reduce over-classification errors to obtain a higher accuracy (Murali, Bhargava & Wright, 2018). This classification was carried out separately on class, order, genus and species level. Combining these methods has the advantage of adding three different percentages of probability to every identification. These percentages represent an estimation of how likely each OTU is currently identified at every level and can contribute to reaching an informed consensus for correct identification. A minimum threshold of 70% probability was set, below which the OTUs were considered as “Unclassified”; along with those that, despite of having a higher probability, included the words “Unclassified”, “Unidentified” and “*incertae sedis*”. When the classifiers disagreed in their classification, the one with the highest probability was selected. To further complement this information, the ten most abundant fungal OTUs were blasted against the GenBank National Centre for Biotechnology Information (NCBI) database (MEGA-BLAST). All this information was then combined with the data of the collected beetle specimen to perform the ecological analysis.

4.3.3. Ecological analysis

After generating the OTU table through the bioinformatics process and identifying the retrieved fungi, data analysis was split into three parts. While the first part aimed to estimate differences of richness, the second part evaluated the diversity of the fungal communities across different forest types. The third part aimed to determine how extended the presence of fungal symbionts and pathogens was through the whole set of samples. Statistical analyses were carried out at the OTU level. To remove potential artefacts, singletons and doubletons were firstly eliminated from the databases. To carry out the ecological analysis, samples were subsequently rarefied to 2000 reads to retain the majority of the samples while making them more comparable, following Cameron *et al.* (2020). Samples that did not have enough reads were removed for the downstream analysis, leaving 1035 samples.

Individual and grouped species accumulation curves were built with Rstudio (vegan package (Oksanen, Jari, et al., 2018), “speccacum” function) (**Supplementary Figure APP III-1**). These curves are frequently used to corroborate that the average sample size was sufficient for the biodiversity survey to be able to capture a reasonable amount of fungal OTU variability (Hughes et al., 2001). Since many curves did not reach the asymptote, the fungal OTU richness was extrapolated to simulate the response variable for the Generalised Linear Models (GLMs), which is advised when there is a noticeable variation across the sampling sizes (Hughes et al., 2001). In addition, to ensure the correct sample representativity, only beetle species with 25 or more specimens were included for assessing both fungal richness and community composition, leaving 921 beetle specimens for the richness and fungal community composition analysis.

The extrapolated fungal OTU richness was estimated by using the Chao1 index, which was calculated using the “estimateR” function from the vegan R package (Oksanen, Jari, et al., 2018). To compare the extrapolated fungal richness, a set of generalised linear models (GLM) were built, using the R package lme4 (Bates et al., 2015:p.4). Each of the GLMs was set with the extrapolated fungal OTU richness as a response variable. As dependent variables, I tested a set of abiotic (latitude, longitude, altitude, mean annual precipitation and an average temperature of the summer season) and biotic (beetle species and forest composition) variables. The bioclimatic data was obtained from the UK Met Office as 5 km gridded Met Office long term averages for 1981-2010. First, the model was tested with the Poisson distribution. However, as the data showed overdispersion (since the variance was larger than the mean in the dependent variables), two more models were tested, the Quasi-Poisson and the Negative Binomial models. Finally, the Negative Binomial model was chosen, as it is suitable for over-dispersed and provided a slightly better fit than the Quasi-Poisson according to the modified Akaike information criterion (QAIC). Model assumptions were corroborated, and the amount of variation in the independent variable explained by each of the factors was estimated using the “performance” R package (Daniel et al., 2020). This package calculates the Nagelkerke's R² (Nagelkerke, 1991), which approaches R-squared as an improvement from the null model to the fitted model. Model results were reported according to the protocol from Zuur and Ieno (2016).

To evaluate the factors affecting fungal beta diversity, dissimilarity matrices were first calculated based on three different indexes to estimate the beta-diversity between sample pairs. The Bray-Curtis index focuses on the relative abundance of each OTU, while Jaccard and Sorensen indexes are based on presence-absence data (Borcard, Gillet & Legendre, 2018). Based on Jaccard index, community dissimilarities were visualised in a multidimensional scaling MDS graph (Betapart R

package (Baselga & Orme, 2012), which is a robust unconstrained ordination method useful to visualise general dispersion patterns across the entire dataset (Anderson & Willis, 2003). A further permutational multivariate analysis of dispersion (PERMUTEST) was performed to evaluate the assumption of homogeneity of variance in the data by using the Betadisper function (Anderson & Walsh, 2013) (Vegan package).

Subsequently, to quantify the variations shown in the MDS, a permutational multivariate analysis of variance (PERMANOVA) was performed, comprising abiotic (latitude, longitude, mean annual precipitation) and biotic (beetle species and forest composition) grouping factors. A PERMANOVA is useful to determine if the centroids of the clusters of samples for the different predictors are significantly different from each other. This PERMANOVA was based on the Bray Curtis dissimilarity matrix by using the “Adonis” function of the vegan package (permutations=999)(Oksanen, Jari, et al., 2018). Since the Bray-Curtis index is not affected by the number of null values between samples (Ramette, 2007), it was preferred to perform this analysis, but also the presence-absence data was assessed through the Jaccard index. To further illustrate this variation, a Canonical Analysis of Principal coordinates (CAP (Anderson & Willis, 2003)) was carried out for all the samples and for the beetle *Hylurgops palliatus*, which was the most abundant beetle species. This constrained ordination was selected for its flexible assumptions and because it can uncover patterns not previously shown in an unconstrained ordination like the MDS, and it is therefore very useful to complement it (Anderson & Willis, 2003). The total beta-diversity was then split between its nestedness and turnover components by using an additive partitioning framework (Baselga, 2010) with the Betapart R package (Baselga & Orme, 2012). This way, we can estimate the dissimilarity due to species loss (nestedness) and the dissimilarity due to species replacement (turnover) and extend these two indices to the multiple beetle species for a general comparison. To complement these results, unique OTUs belonging to each forest composition were displayed using a Venn diagram (Oliveros, 2007).

To evaluate the associated fungal OTUs for each beetle species and forest composition, a multilevel pattern analysis was carried out by calculating Pearson’s phi coefficient of association (“p.g”)(Chytrý *et al.*, 2002) between sample pairs, correcting this index to account for the differences in specimen numbers among the compared groups (*multipatt* function, *indicspecies* R package (De Cáceres *et al.*, 2011)). To facilitate these comparisons, beetles were grouped first only according to their phylogeny, and then according to the forest composition from which they were trapped. The identified OTUs for which the association values were significant ($p < 0.001$) were then selected and displayed as a heatmap (Superheat R package (Barter & Yu, 2018)).

4.4. Results

After processing the 1064 samples plus the negative controls, an average of 12076 reads per sample were obtained. At the end of the bioinformatics pipeline, the remaining 8528 unique sequences clustered into 5546 fungal OTUs. Since their reads abundance was low compared to the rest of the OTUs, the 65 OTUs found in the negative controls were subsequently excluded from further analysis. After classification, 3855 of these OTUs remained unclassified at the species level. After removing the negative controls and performing sample rarefaction, 1035 samples and 5410 OTUs remained for the subsequent ecological analysis.

4.4.1. Taxonomic assignment of the fungi

The 1064 samples spanned a total of 27 classes and 109 orders. The most abundant OTUs were present in the majority of the beetle species and, where present, accounted for a great proportion of the fungal reads (**Figure 4-2**). The three most abundant OTUs belonged to fungal symbionts of the genus *Ambrosiella*. The fungal pathogen *Cladosporium perangustum* was also highly abundant. The remaining most abundant fungal OTUs were identified as yeasts, seven of which belonged to the genera *Candida*. Some of the recovered yeasts were known as beetle-associated yeasts, like *Candida oregonensis* (Rivera *et al.*, 2009), *Candida tenuis* (Davis, 2015) or *Candida trypodendri*. Others, like *Sydowia polyspora* or *Peterozyma toletana* have also shown some association with beetle species in other analysis of the present thesis.

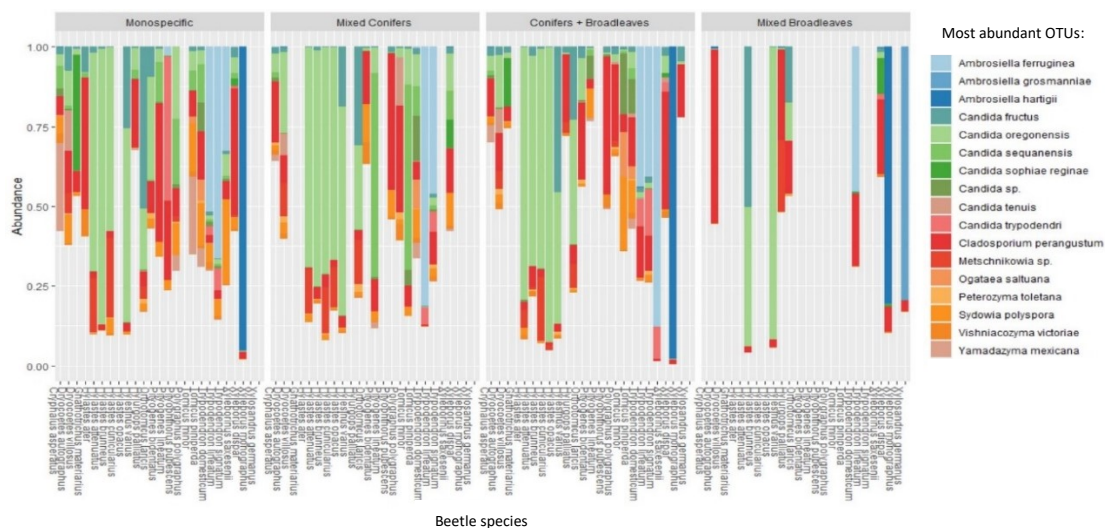


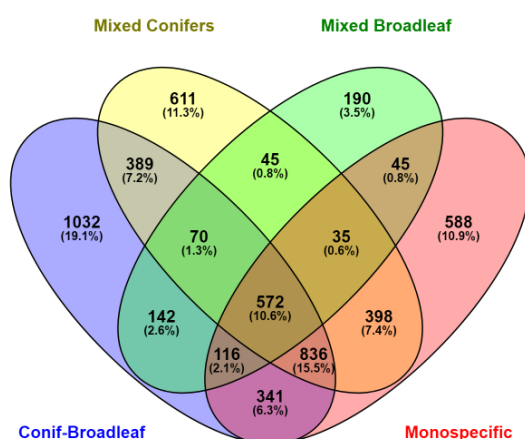
Figure 4-2. Relative abundance of the most abundant OTUs present in the whole beetle dataset, comprising 1035 beetle specimens.

4.4.2. Richness and diversity of fungi across forest compositions

Fungal richness, which was estimated using the Chao1 non-parametric richness estimator, ranged from 4 to 191 OTUs, with an average of 87 OTUs per beetle specimen. By the use of generalised linear models, we detected an effect of several abiotic and biotic factors on the fungal richness (**Supplementary Table APP III-2**). The fungal richness was significantly lower in the case of forests consisting of a mix of conifers and broadleaves or a mix of broadleaves and significantly higher for the bark beetles *Hylesinus varius*, *Dryocoetes autographus*, *Hylurgops palliatus* and *Tomicus piniperda*. While *Hylesinus varius* is generalist, attacking both conifers and broadleaves, the rest of them are conifer-specialists. The total amount of explained variance was 40.38%, with the beetle species identity contributing to explain 26.02% of the variation, followed by the Forest composition (6.19%) and spatial (longitude: 4.94%) and environmental (Mean annual precipitation + Mean temperature Apr-Aug=4.9%).

To explore differences in fungal communities among the beetle specimens, Venn diagrams were generated based on the unique OTUs present at each forest composition (**Figure 4-3 A**). Of a total of 5517 OTUs, the highest number of unique OTUs (1032) was detected in the Conifer-Broadleaf mix, followed by Mixed conifers and Monospecific with 611 and 588 OTUs, respectively. Therefore, with a similar number of beetle specimens analysed, the forest consisting of a mix of broadleaves and conifers hosted almost a double number of unique OTUs than those forests consisting of conifers only (single species or mixed). Mixed broadleaves accounted for the lowest number of unique OTUs. However, this was somehow expected since the number of beetles analysed for this forest composition was approximately one-third than for the other groups. Differences of fungal composition across forest compositions were also displayed using a MDS showing higher similarities on monospecific and mixed conifers (**Figure 4-3 B**).

(A)



(B)

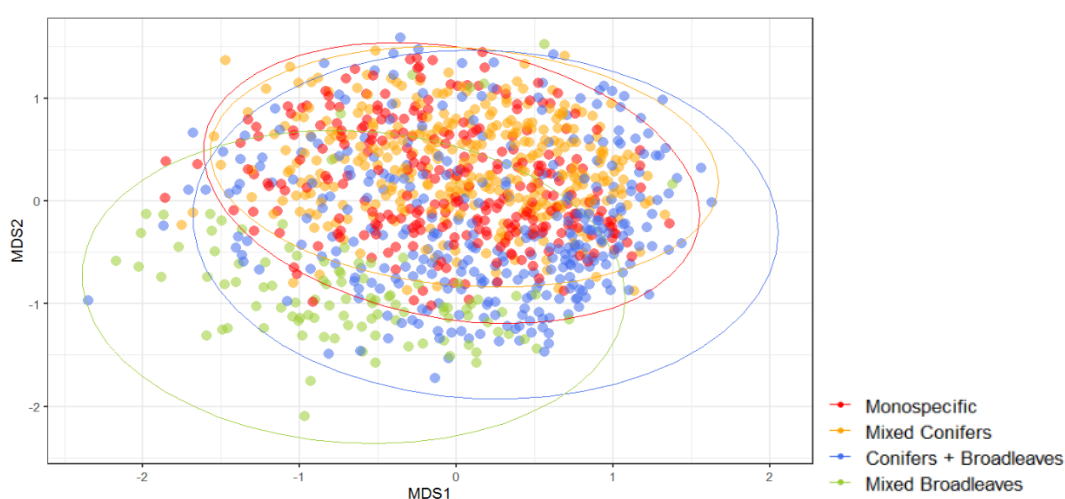


Figure 4-3. (A) Venn diagram showing the number of unique fungal OTUs in the four different forest compositions. (B) MDS based on Jaccard dissimilarity displaying the differences in fungal communities across forest compositions

The PERMANOVA based on the Bray Curtis index showed significant differences in fungal abundance among groups of beetles according to the different biotic and abiotic factors considered for the study (**Table 4-2**). Among the biotic factors, the insect identity the variable that explained the most, accounting for 31% of the variability. The forest composition explained a 3,7% of the variation, while the proportion of conifers in the forest mix accounted only for 0.2% of the variation. Therefore, the biotic factors explained a higher proportion of the variation than the abiotic factors, which accounted for 1.8% of the fungal variation altogether. The results from the PERMANOVA based on Jaccard dissimilarities are available in the supplementary data (**Supplementary Table APP III-3**).

Table 4-2. Results from PERMANOVA modelling fungal community composition using the Bray Curtis dissimilarities. P-values were obtained using 999 permutations under a reduced model. Beetle species identity (Insect_id) had the largest effect on fungal community abundance.

	Df	SumOfSqs	R2	PseudoF	Pr(>F)
Forest.comp	3	12.698	0.037	17.743	0.001
Insect_id	11	105.032	0.310	40.026	0.001
Longitude	1	1.668	0.005	6.992	0.001
Latitude	1	1.292	0.004	5.416	0.001
Altitude	1	0.706	0.002	2.961	<0.01
Mean.Annual.P	1	1.318	0.004	5.526	0.001
Mean.FS.T	1	0.995	0.003	4.172	0.001
Per.Conifers	1	0.651	0.002	2.733	<0.01
Unexplained			0.63		

To further assess the drivers of the fungal community composition, the Canonical Analysis of Principal Coordinates (CAP) displayed the variation axis for the different abiotic factors plus the percentage of conifers in the forest mix (**Figure 4-4 A**). When applying this ordination method to the fungal communities associated with the 177 specimens of *Hylurgops palliatus*, which was the most abundant in numbers and present in all the forest compositions, the first axis explained more than three times the variation than for the whole dataset and the second axis accounted for almost double variation than considering all the beetle species altogether (**Figure 4-4 B**).

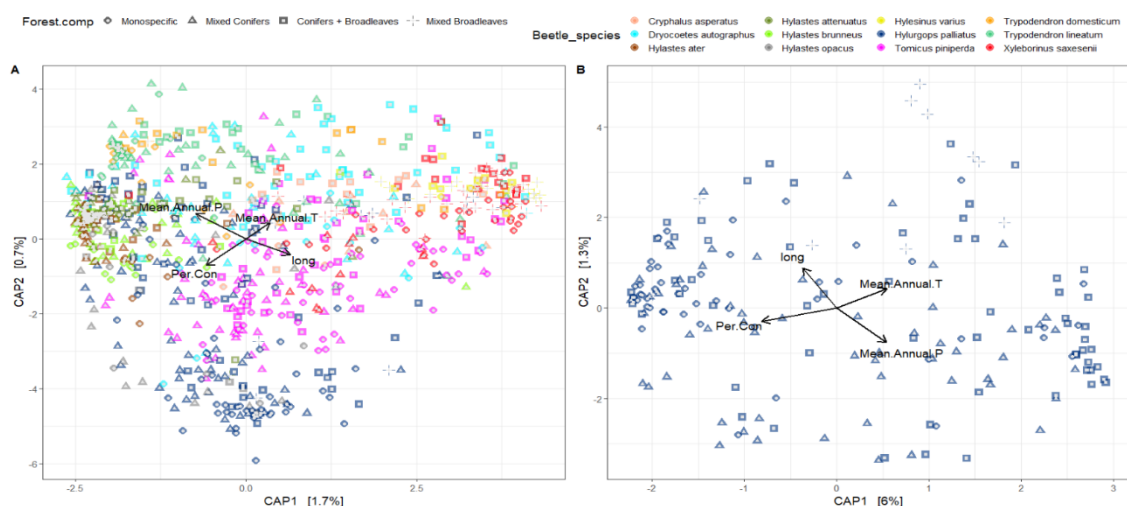


Figure 4-4. Canonical Analysis of Principal coordinates displaying the variations of the different abiotic variables plus the percentage of conifers. Different colours represent different beetle species, while different shapes represent different forest compositions in (A) the whole sample (beetle specimens >25 beetles per species) (B) Only *Hylurgops palliatus* specimens. (Per.Con: Percentage of conifers in the mix, long: longitude)

4.4.3. Richness and diversity of fungi within forest compositions and beetle species

To further explore the relative contribution of the forest composition on the fungal communities associated with bark and ambrosia beetles, the samples were split into groups belonging to each of the different forest compositions (**Table 4-3, Supplementary Table APP III-4**). Subsequently, independent models were generated for each of them to evaluate fungal richness and fungal community composition. For the different forest compositions except the mixed broadleaves, we selected only beetle species present in more than two sites ($n > 25$). For the mixed broadleaves, since the number of sites and specimens was lower, we included the beetle species consisting of more than ten specimens, selecting a total of 97 specimens from five different beetle species.

The fungal richness variation associated with the beetle species identity ranged from 15% in forests consisting of mixed conifers to 58.91% in mixed broadleaves. Results also show the strongest influence of beetle species on forest composition in mixed broadleaves (49.3%), followed by monospecific (48.2%), mixed conifers (34.5%) and forests consisting of a mix of conifers and broadleaves (31%). The abiotic factors were significantly affecting fungal community composition only for monospecific (1.64%) and conifers-broadleaf mixtures (2.3%). For the environmental variables, the mean annual precipitation (Mean.Annual.P) had a significant effect on the forest composition in all cases except mixed broadleaves, while the temperature (Mean.FS.T) only affected the conifer-broadleaf mixtures.

Testing whether the host identity had an effect at tree species level was only feasible for monospecific forests, where 120 beetle specimens were trapped in pure Corsican pine (*Pinus nigra*) and 128 specimens in pure Scots Pine (*Pinus sylvestris*). The effect of the forest type was significant on the fungal community composition, where the tree host identity explained 0.7% of the variation.

Therefore, the fungal communities coexisting with bark and ambrosia beetles in the UK are influenced mainly by biotic factors such as the beetle species and, to a lower extent, the composition of the forest in which both beetle and fungal community inhabit. Abiotic factors like longitude, mean annual temperature, and precipitation also explains some of the variability across fungal communities, especially in a forest consisting of mixed conifers.

Table 4-3. Results from Chao1 modelling richness and PERMANOVA modelling fungal community composition on the whole dataset (All) on each of the forest compositions (MO: Monospecific, MC: MixedConifers, CB: Conifers+Broadleaves, MB: MixedBroadleaves) and on two beetle species (HP: *Hylurgops palliatus* and XS: *Xyleborinus saxesenii*). Chao1 drivers were estimated using a negative binomial model Nagelkerke's R². PERMANOVA used Bray Curtis dissimilarities, and P-values were obtained using 999 permutations under a reduced model ("n.s." stands for "not significant"). Biotic (B), Geographical (G) and Environmental (E) variables were modelled, having the beetle species identity (Insect_id) the most considerable effect on fungal communities.

	Chao1 (Nagelkerke's R2)								Bray-Curtis (PERMANOVA)						
	Groups		Forest compositions				IndBeetles			Forest compositions				IndBeetles	
	Data	All	MO	MC	CB	MB	HP	XS	All	MO	MC	CB	MB	HP	XS
B	Insect_id	0.260	0.356	0.157	0.381	0.589	-	-	0.31	0.480	0.345	0.310	0.493	-	-
	Forest.comp	0.619	-	-	-	-	n.s.	0.670	0.037	-	-	-	-	0.093	0.121
	Prop.conif	n.s.	-	-	-	-	-	-	0.020	-	-	-	-	n.s.	n.s.
G	Long	0.494	n.s.	0.0276	n.s.	n.s.	n.s.	n.s.	0.050	0.009	n.s.	0.010	n.s.	0.013	0.018
	Lat	n.s.	n.s.	n.s.	0.0315	n.s.	n.s.	n.s.	0.040	n.s.	n.s.	0.007		0.027	0.015
	Alt	n.s.	n.s.	n.s.	n.s.	0.0374	n.s.	n.s.	0.020	n.s.	n.s.	0.006	n.s.	n.s.	n.s.
E	Mean.Ann.P	0.048	0.059	0.070	n.s.	n.s.	n.s.	n.s.	0.040	0.007	0.015	0.013	n.s.	n.s.	n.s.
	Mean.FS.T	0.001	n.s.	0.045	n.s.	n.s.	n.s.	n.s.	0.030	n.s.	n.s.	0.060	n.s.	n.s.	0.015

When evaluating the relative effect of the forest composition compared to climatic and environmental variables for a single beetle species, the effect of the forest composition was even more noticeable (**Table 4-3**, IndBeetles). The forest composition drove 9.3% of the variability on fungal community composition for *Hylurgops palliatus* and 12.1% for *Xyleborinus saxesenii*. Apart from the forest composition, both longitude and latitude had an effect on both beetle species, while the climatic variables affected *Xyleborinus* but not *Hylurgops* fungal communities. Interestingly, the forest composition greatly affected *Xyleborinus saxesenii* fungal richness, while the effect on *Hylurgops palliatus* was non-significant. Therefore, for specific beetle species, these variables may explain a different proportion of the total variance. Although the multiple variables affecting the fungal communities altogether are unable to explain most of the variability, the effect of the forest composition should not be neglected.

4.4.4. Turnover and nestedness

By splitting the total beta diversity (Figure 4-5 A), I was allowed to study the relative contribution of each of the beetle species into the turnover (Figure 4-5 B) and nestedness (Figure 4-5 C) components. Furthermore, the Wilcoxon test ($W=1.7942e+11$, $p<0.0001$) corroborated that the beta-diversity patterns across the whole sample set were turnover-dominated. The fungal communities harboured by *Xyleborinus saxesenii* showed a higher turnover than those present in *Hylurgops palliatus*, which exhibited a higher nestedness. When comparing across different

forest compositions, the variability of the mixed conifers was slightly lower than for the other forest compositions.

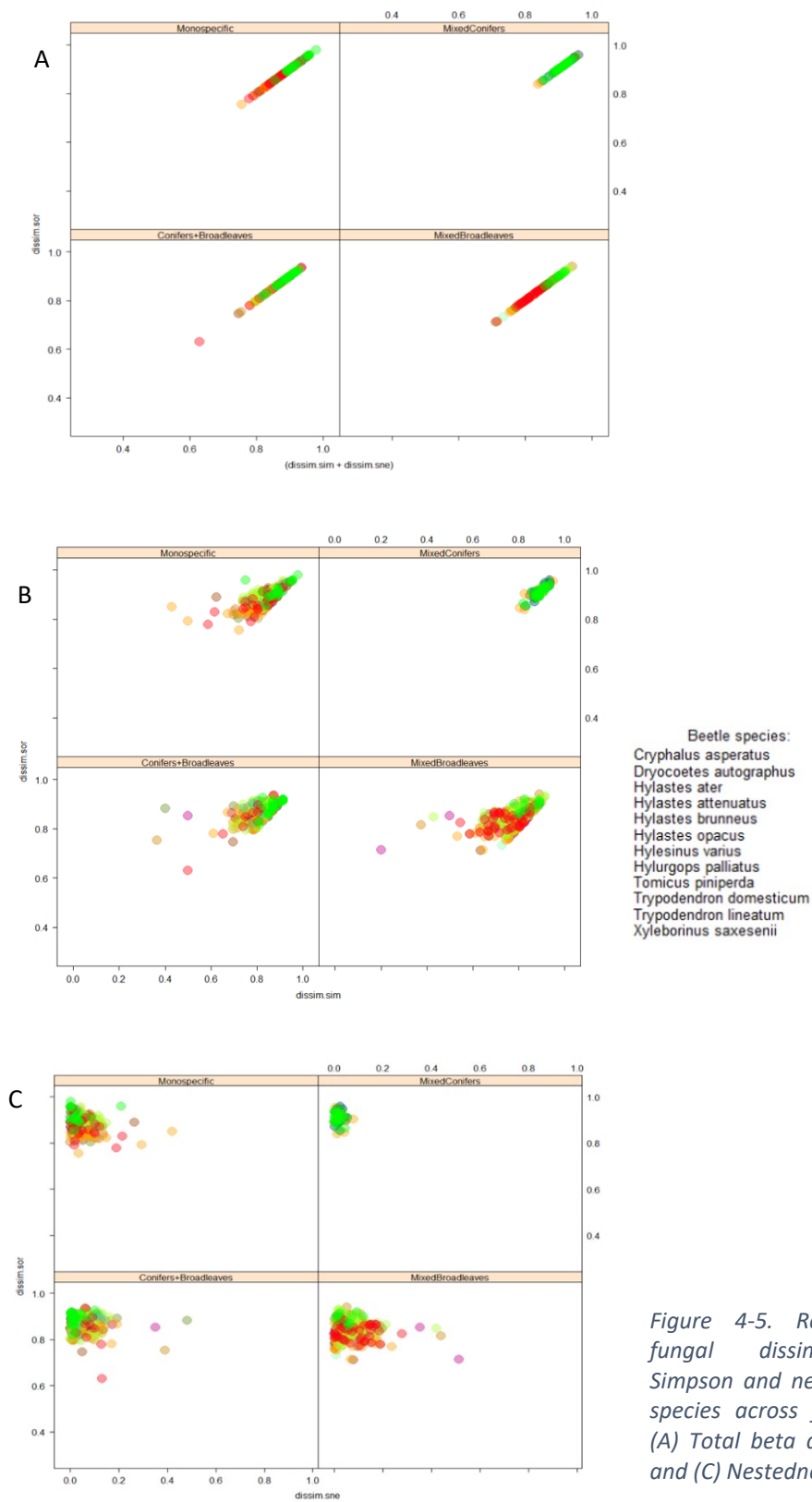


Figure 4-5. Relationship between fungal dissimilarity (Sorensen, Simpson and nestedness) and beetle species across fungal compositions. (A) Total beta diversity (B) Turnover and (C) Nestedness

Taken together, results suggest that, although the beetle species is the main driver of fungal community richness and composition, the forest composition affects these communities. Turnover is the most frequent pattern observed across the communities, although the extent seems to depend on the beetle-species rather than on the forest composition.

4.4.5. Fungal guilds and specificity across forest compositions

The dominant fungal guilds were pathotrophs and pathotrophs-saprotrophs-symbiotrophs (**Figure 4-6**). Saprotrophic fungi, which are usually the dominant guild in forests (Nguyen *et al.*, 2016b), accounted for less than 30% of the guild proportion. The proportion of saprotrophic fungi was particularly low in the beetles trapped at mixed broadleaves. *Hylastes spp.* had the lowest proportion of identified fungal guilds.

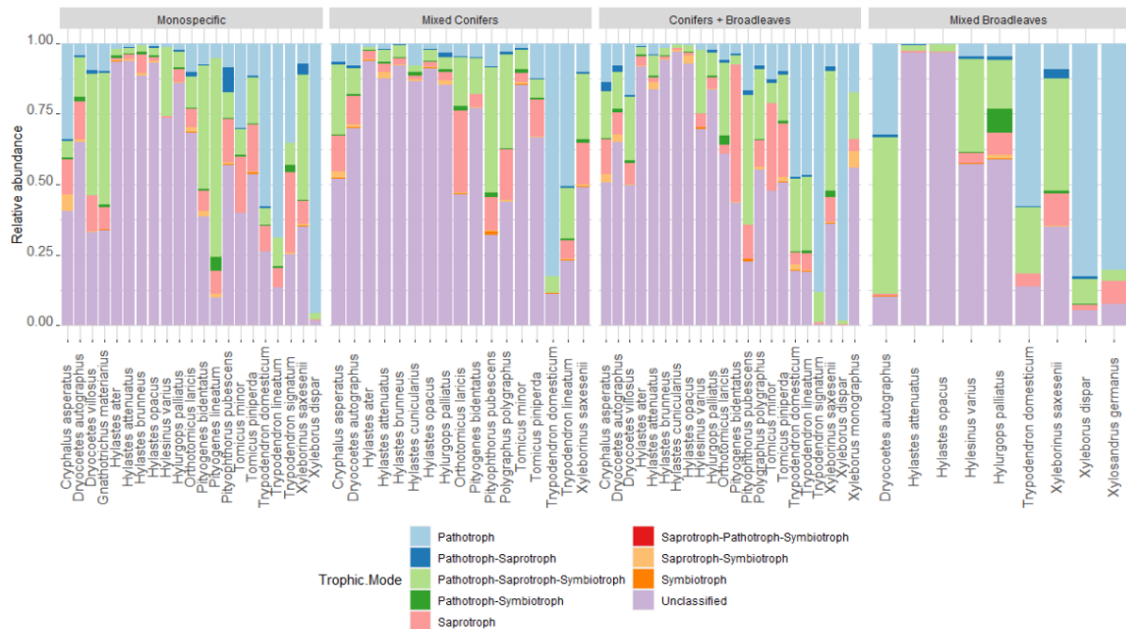


Figure 4-6. Relative abundance of the identified fungal guilds on the overall rarefied dataset, comprising 1035 beetle specimens.

Statistically significant differences in the strength of association across forest compositions were observed through the multilevel pattern analysis. These differences were displayed through a clustered heatmap. A dendrogram was created along with the clustered heatmap. The beetles that harboured similar fungal associates were included in the same cluster of the dendrogram.

When performing the analysis across beetle species without having into account the different forest compositions, there was a remarkable effect of the beetle species shaping fungal associations for both bark (Tribe Hylurgini) or ambrosia (Tribe Xyleborini) beetles (**Figure 4-7**).

There we can also corroborate that the strength of association is higher for obligate symbionts like *Ambrosiella hartigii* with *Anisandrus dispar* or *Ambrosiella grosmanniae* with *Xylosandrus germanus*.

However, when splitting these beetles according to their phylogeny and forest composition, a significant effect of the forest composition was clearly notable. On the ambrosia beetles (**Figure 4-9**), the beetle species from forests consisting on mixed broadleaves had more similar fungal associates than those from the same species but a different forest composition. The effect of the forest composition was even more remarkable for the bark beetles (**Figure 4-10**), with the exception of *Hylastes opacus* and *Hylastes cunicularius*.

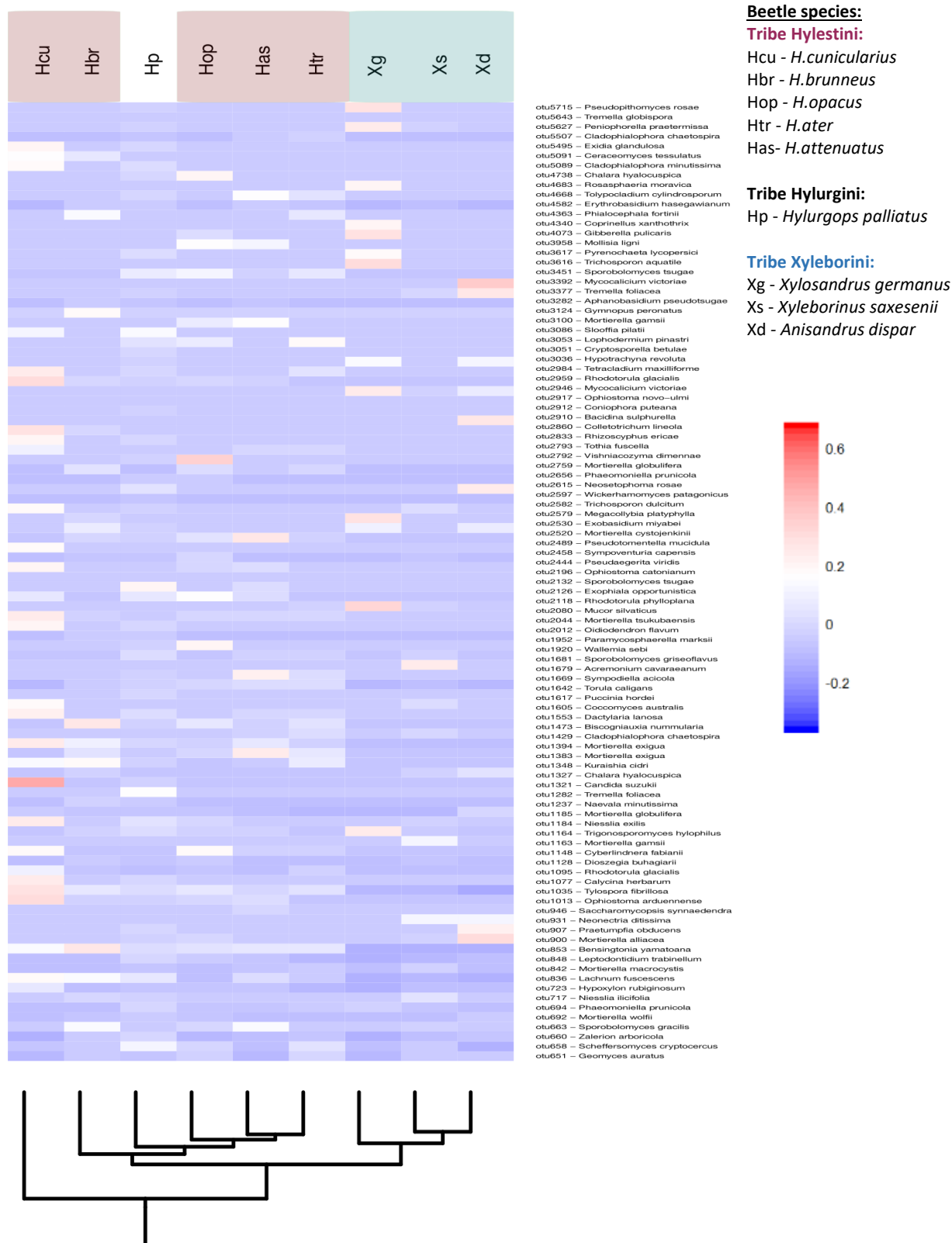


Figure 4-7. Multilevel pattern analysis showing the distribution of the specific OTUs across beetle species. Red or blue squares indicates positive or negative correlations, respectively. The dendrogram shows the clustering of similar beetle-fungal associates (Continue in next page).

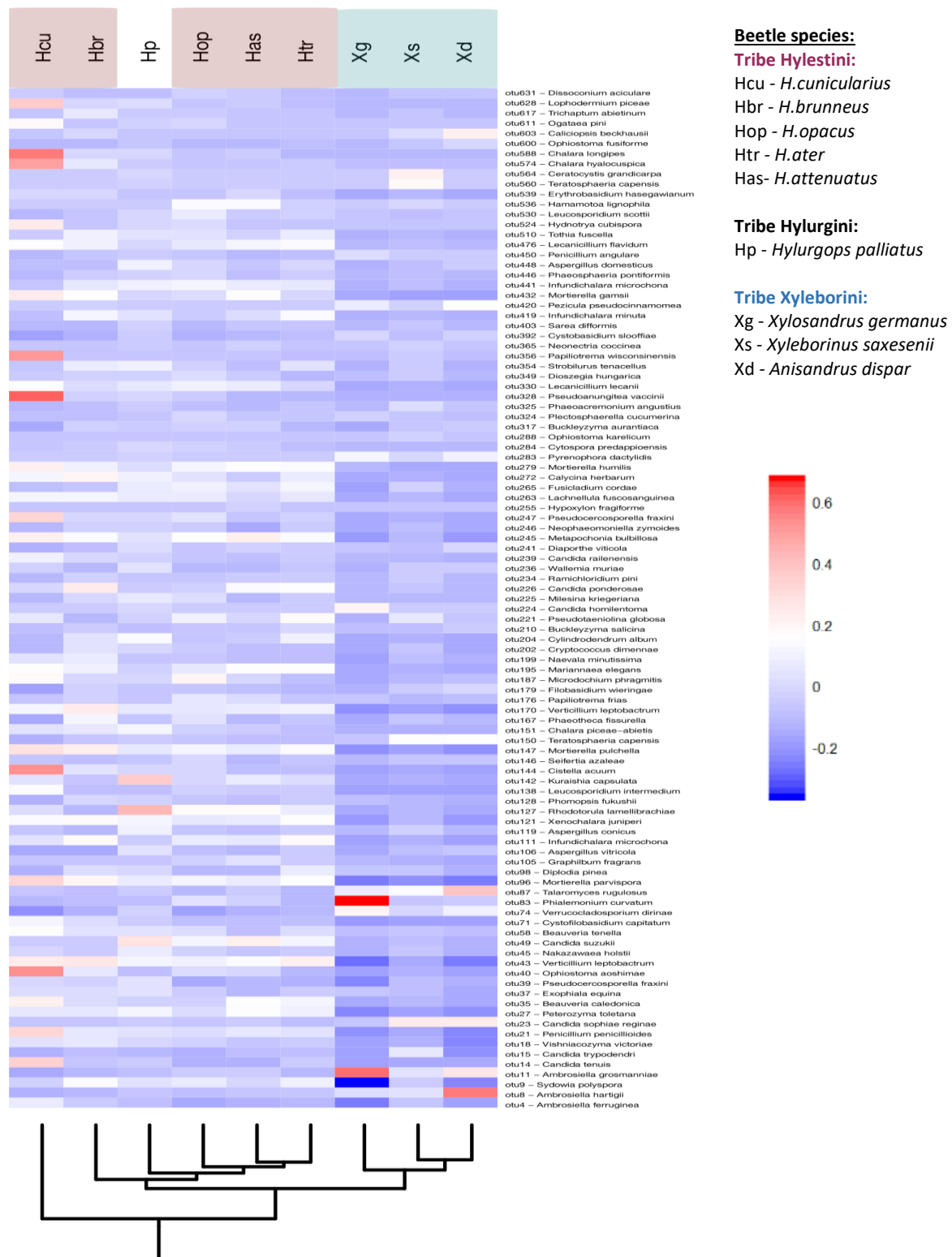


Figure 4-8 (cont). Multilevel pattern analysis showing the distribution of the specific OTUs across beetle species. Red or blue squares indicates positive or negative correlations, respectively. The dendrogram shows the clustering of similar beetle-fungal associates

Beetle species

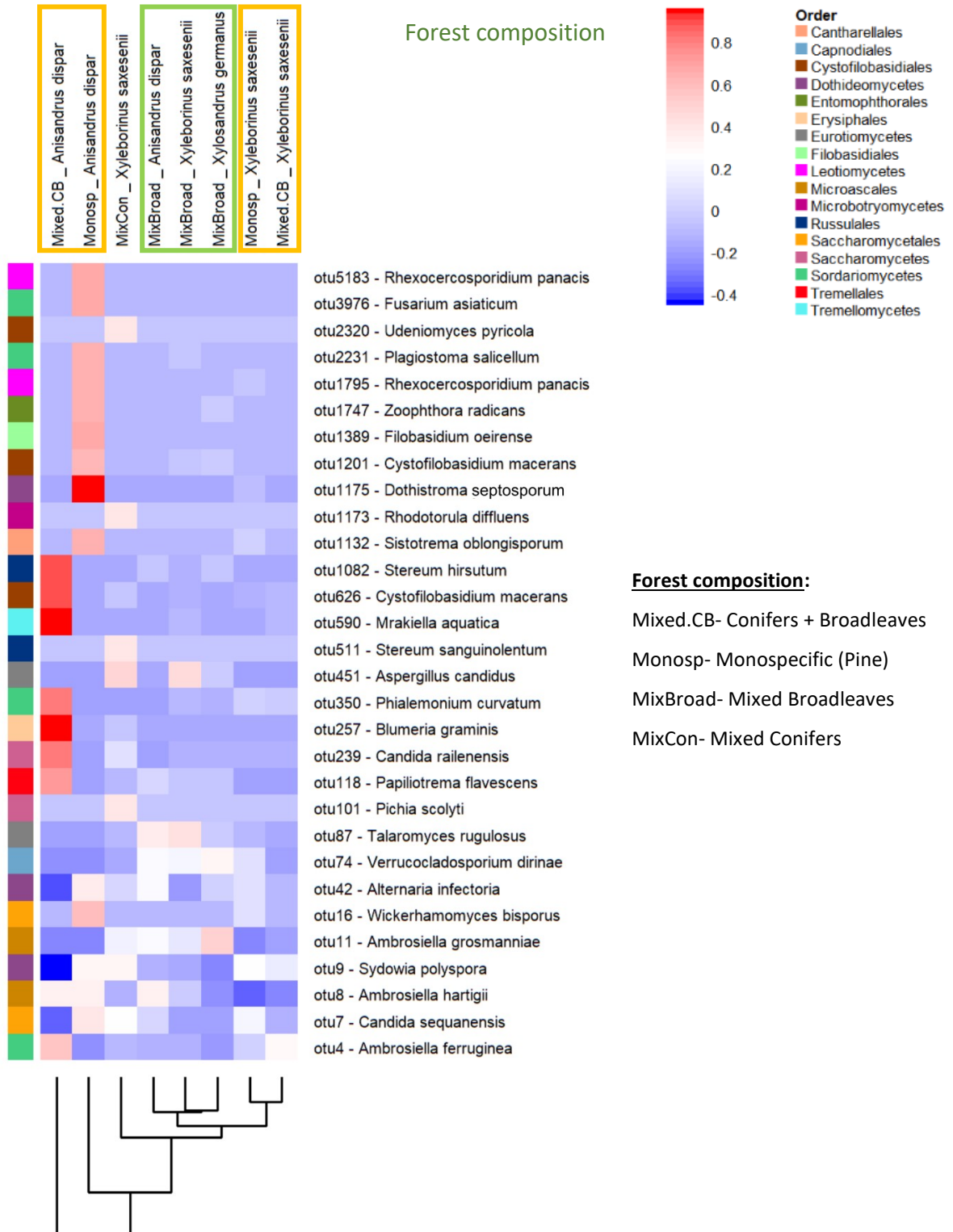


Figure 4-9. Multilevel pattern analysis showing the distribution of the specific OTUs across beetle species and forest compositions. Red or blue squares indicates positive or negative correlations, respectively. The dendrogram shows the clustering of similar beetle-fungal associates.

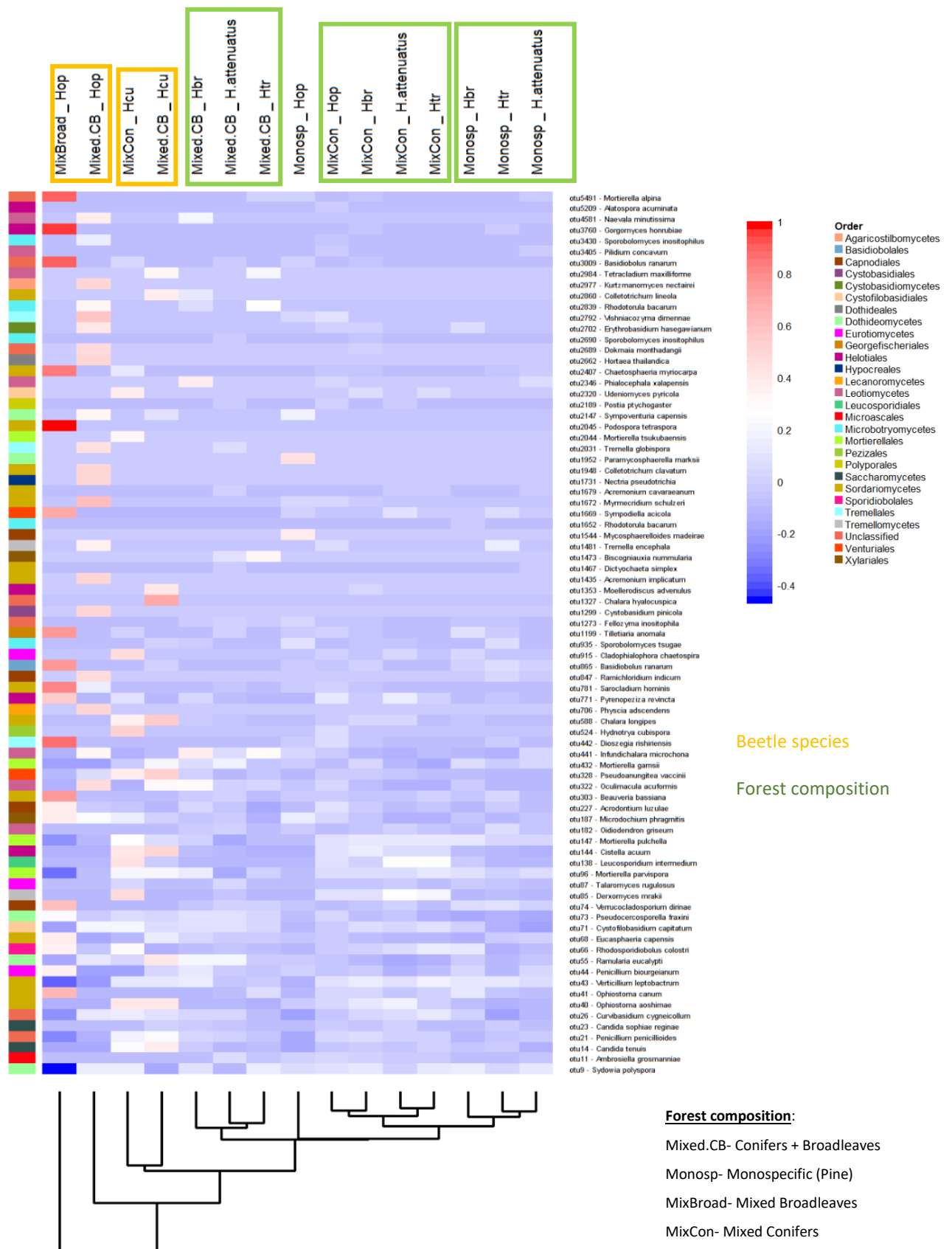


Figure 4-10. Multilevel pattern analysis showing the distribution of the specific OTUs across beetle species and forest compositions. Red or blue squares indicates positive or negative correlations, respectively. The dendrogram shows the clustering of similar beetle-fungal associates.

A substantial proportion of the associated fungi were yeasts, including some known beetle associates like *Sydowia polispora*, *Pichia scolyti*, or *Candida sequeanensis*. Among the fungal associates there were also fungal pathogens like *Ramularia eucalypti*, *Stereum sanguinolentum*, *Stereum hirsutum* and *Mycosphaerella pini* (Dothistroma blight). Other fungal associates included *Ophiostoma canum* and *Ambrosiella ferruginea*.

4.5. Discussion

Trees may affect forest microorganisms through a multitude of processes. Prior work has documented the effects of forest management (Tedersoo *et al.*, 2020a; Tomao *et al.*, 2020; Persiani *et al.*, 2010) and plant species composition on forest fungal communities (Asplund *et al.*, 2019; Nguyen *et al.*, 2016b; Tedersoo *et al.*, 2020a; Urbanová, Šnajdr & Baldrian, 2015). However, the roles of forest composition and host trees on beetle-fungal symbiosis remain poorly understood (Hulcr *et al.*, 2020). The present chapter aimed to explore the differences in fungal communities associated with bark and ambrosia beetles across four different forest compositions to study the extent to which they are interdependent.

4.5.1. Fungal community richness and composition across forest compositions

I found a significant effect of the forest composition on the fungal richness and fungal community composition. Forest composition directly drove 6% of the fungal community richness and 3.7% of the fungal community composition. My results agree with recent findings, which include the plant species composition as one of the main drivers of the fungal communities in Northern Europe (Tedersoo *et al.*, 2020a). The impact of the tree species identity was here tested only for monospecific forests, which were divided into *Pinus sylvestris* and *Pinus nigra*. Additional analysis carried out only with monospecific pine forests showed that 0.7% of fungal community composition variation accounted for the host tree species identity. Therefore, even phylogenetically close species like these pines may contribute to the differences among beetle-associated fungal communities.

My results also suggest that the role of the beetle species contributing to fungal community richness and composition could be more significant than previously thought. According to my results, the contribution of each beetle species would fluctuate across forest compositions, reaching its maximum for the mixed broadleaf forests. According to my results, the beetle species accounted for 34.5% of the variation on forest consisting of mixed conifers and broadleaves and 49.3% of the variation in mixed broadleaves. However, Miller (2016, 2019)

suggested that the role of the beetle species on the fungal community composition was less critical, accounting only for 12% of the variation. The present work indicates that the beetle species would account for a higher variation for fungal community composition. When assessing the role of beetle species on fungal richness, my results agreed with those from Miller, accounting for 15.7% of the richness variation for the beetle species. However, there was a much higher influence of the beetle species on fungal richness for other fungal compositions. Recent studies also suggest that the strength of the forest composition effect may be different depending on the tree species identity or the fungal functional group (Weißbecker *et al.*, 2018; Tedersoo *et al.*, 2020a). This could explain the different results found across forest compositions.

Surprisingly, the forest composition alone accounted for a higher variation than the studied abiotic factors altogether, except for the forests consisting of mixed conifers. In mixed forests, I found a more substantial influence of temperature and rainfall, which was consistent with the abiotic factors highlighted by Miller (2016, 2019).

I applied the general model to the two individual beetle species present in the four forest compositions to complement these results. Forest composition contributed to explaining a high proportion of fungal community composition variation for both beetle species, while fungal richness was only significantly affected in *Xyleborinus saxesenii*. My results suggest that *Xyleborinus saxesenii* would rely more on environmental drivers than *Hylurgops palliatus*. This dependence on environmental drivers is unexpected for an ambrosia beetle, which depends less on other factors because it relies more on its fungi. Since *Hylurgops palliatus* was able to thrive in such a vast amount of forest compositions and geographical locations, it seems that the adaptive capacity of this beetle species could be able to compete with those that ambrosia beetles exhibit.

4.5.2. Fungal guilds and fungal associates across forest compositions

By comparing fungal communities across beetle species and including different forest compositions, this study distinguished among beetle and tree-dependant fungal associations.

When considering fungal guilds, past literature suggests that forest-associated fungi tend to be dominated by saprotrophs in the case of ectomycorrhizal fungi (Nguyen *et al.*, 2016b). The dominant fungal guilds on the bark and ambrosia beetles of this study were pathotrophs and pathotrophs-saprotrophs-symbiotrophs. Only very recently there have been studies trying to disentangle the effect of forests on fungal guilds. On the one hand, Weißbecker (2018)

suggested that tree community composition could primarily affect pathogens. However, my analysis could not detect any specific change in pathogenic fungi compared with other fungal guilds. On the other hand, the effect of the tree species for widespread trees like *Pinus sylvestris* or *Picea abies* has been documented to be stronger on fungi feeding on living plants than on saprotrophic fungi (Tedersoo *et al.*, 2020a). Therefore, since beetle-fungal associations mainly belong to the first category, the forest composition would affect them more. Nevertheless, there was no clear pattern on fungal guilds across the different forest compositions, keeping a similar proportion across the beetles.

Among these associated fungi, there were also some fungal pathogens displaying very different patterns. *Alternaria infectoria*, previously associated with bark beetles like *Ips typographus* in Norway spruce forests (Giordano *et al.*, 2013), was positively correlated with *Anisandrus dispar* on monospecific forests. However, a stronger correlation was found in the same forest composition-beetle species combination for the pathogen *Dothistroma (Mycosphaerella) pini*, which is the causal agent of the Dothistroma blight. *Ophiostoma canum*, a pathogenic fungus previously associated with *Tomicus piniperda* in China (Pan *et al.*, 2019), was associated with *Hylastes opacus*. Interestingly, this beetle species also harboured an important proportion of the entomopathogenic fungi *Beauveria bassiana*, suitable for the biological control of bark beetles (Mudrončková *et al.*, 2021). The species of the genera *Ramularia*, which includes numerous phytopathogenic fungi and have been associated with the bark beetle *Xyleborinus saxesenii* (Malacrinò *et al.*, 2017). Nevertheless, my results show how this fungus could also be associated with the bark beetle *Hylastes cunicularius*. My results support the previous findings suggesting that fungal associates are highly promiscuous (Kostovcik *et al.*, 2015). When considering different fungal compositions, beetle-fungal associations become even looser. Consequently, it would be necessary to monitor the same beetle species across different forest compositions to disentangle the underlying mechanisms behind these interactions.

Most yeasts were not explicitly correlated with any beetle species or forest compositions but were present across the different samples. An example of this was *Sydowia polyspora*, which have been previously associated with the bark beetles of the genera *Dendroctonus* (Rivera *et al.*, 2009). Rivera (2009) suggested that beetle-associated yeast species composition relies on both geography and host tree species, although yeasts tend to be widespread across ecosystems. Besides this yeast, different yeasts belonging to the genus *Candida* scattered across the beetle species and forest compositions. Lou *et al.* suggested that yeasts of the genus *Candida* could be vertically transmitted across beetle generations (2014), facilitating its propagation across

different ecological conditions. These authors also pointed out the capacity of these *yeasts* to assimilate nutrients like nitrate or xylose, which can benefit the beetles.

The comparison of closely-related beetle species across forest compositions revealed some interesting patterns. The pattern shown by *Hylastes cunicularius* and *Hylastes opacus* is surprising, given that they seem to exhibit different fungi compared with the other species of the same genus. In particular, *Hylastes opacus* showed more substantial associated fungi on mixed broadleaf forests. The few studies assessing the fungal associations present in these beetles suggest that they form associations with *Grosmanniae* spp, *Lephtographium* spp and *Ophiostoma* spp (Jankowiak & Bilański, 2013; Jacobs *et al.*, 2010). However, none of these fungi was found to form associations with these beetles at the present analysis. Since these bark beetles have no known mycangia, they could not keep permanent fungal associates, forming more promiscuous associations than other beetles from the same tribe.

4.6. Conclusions

According to my results, the role of the beetle species in structuring its associated fungal communities could potentially be more critical than previously thought. Using molecular techniques, I was able to partially disentangle the effect of forest composition on the fungal communities associated with bark and ambrosia beetles. My results suggest that the influence of forest composition contribute to shaping beetle-fungal associates, but this influence would fluctuate depending on the beetle species. Future work should include a more extensive amount of beetle species, sites and trapping methods to be able to achieve a more balanced design that allows more precise results.

Chapter 5. The fungal communities associated with the first UK breeding population of the bark beetle *Ips typographus* include potentially invasive pathogens

5.1 Abstract

A breeding population of *Ips typographus* has recently been encountered for the first time in the UK. This bark beetle kills millions of Norway spruce trees each year across continental Europe and, benefited by climate change, it is undergoing even more severe outbreaks (Bentz *et al.*, 2019). However, the potential effects of an outbreak on Sitka spruce, the most commonly-planted conifer in the UK, remain largely unknown. Many species of fungi establish symbiotic relationships with bark beetles, including some fungal pathogens, but fungal assemblages are highly variable. To investigate which fungi may be vectored into the UK and which fungal species formed associations with these beetles, we analysed fungal assemblages from 48 *Ips typographus* specimens. To do this, we used metabarcoding of the ITS2 region of individual beetle specimens to recover a total of 416 fungal OTUs from both developing and flying beetles. Results revealed 18 potentially invasive fungi, not present in previously surveyed British bark and ambrosia beetle populations. Among these potentially non-native OTUs, there are two Ophiostomatoid fungi: *O. bicolor*, which is frequently associated with *Ips typographus* in their native area, and *O. floccosum*. Results also suggest that at least 26 of these 416 OTUs may preferentially associate with specific developmental stages of the beetle. Fungal communities in specimens collected from Norway spruce logs hosted more fungi categorised as symbionts, while saprotrophs were more abundant in Sitka spruce logs. Taken together, these results suggest that these bark-beetles are vectoring certain fungi into the UK and probably reintroducing others that were significantly correlated with the beetle specimens but already present in the country. These results provide a comprehensive overview of the fungal

communities associated with *Ips typographus* in the UK, which may be useful to inform future management decisions.

5.2 Introduction

Biological invasions represent a global threat to our ecosystems. Benefited by the changing climate (Bentz *et al.*, 2019; Netherer *et al.*, 2019), the eight-toothed spruce bark beetle (*Ips typographus* L. (Coleoptera: Scolytinae)) is the cause of the most significant tree mortality in temperate forests across continental Europe (Zahradník & Zahradníková, 2019; Seidl *et al.*, 2016). This bark beetle species has recently arrived in the southern part of the UK, establishing a breeding population for the first time, recently discovered and undergoing eradication. This bark beetle species poses a major threat to the economy and ecology of British forests.

In the UK, spruce forests (*Picea* spp.; Pinales: Pinaceae) account for more than a quarter of the woodland area, with Sitka spruce (*Picea sitchensis* L.) covering more than 1 million ha (Ward, 2019; Mason & Perks, 2011). Sitka spruce, introduced from North America more than two centuries ago, has been profusely planted in the UK and Ireland (Lee, Thompson & Hansen, 2013). In these countries, Norway spruce (*Picea abies* L.) is less abundant due to its lower productivity and poorer wind resistance. However, Norway spruce is extremely abundant in the north and west of Europe, where it is widely attacked by *Ips typographus*, which has resulted in the death of millions of mature trees during the long-term and large-scale outbreaks (Christiansen & Bakke, 1988). Despite its preference for Norway spruce, the beetle may also attack other species of spruce including Sitka spruce (Økland *et al.*, 2011) and occasionally other conifers including several species of pines (Schroeder & Cokoş, 2018). Furthermore, as tree defences are shaped by bark beetles, previously unencountered beetles constitute a higher threat to tree survival than native beetle species (Burke, Bohlmann & Carroll, 2017; Raffa, Powell & Townsend, 2013). Therefore, the potential damages that *Ips typographus* may produce on extensive areas of Sitka spruce are not yet known, as the UK and Ireland have remained free from the beetle until now.

Ips typographus has been comprehensively investigated over several decades, with special attention paid to the positive influence of abiotic factors such as higher temperatures, drought and storm damage on its brood development (Bentz *et al.*, 2019; Wermelinger *et al.*, 2012; Marini *et al.*, 2017). This beetle breeds subcortically in trees, by excavating galleries in the phloem (Wood, 1982). For successful colonisation, the beetles need to be able to overcome the tree defences, which is achieved by attacking in sufficient numbers thanks to their highly

efficient population aggregation pheromones (Berryman *et al.*, 1989; Christiansen, Waring & Berryman, 1987). However, tree mortality is also affected by biotic factors like the beetle-associated fungi (Paine, Raffa & Harrington, 1997; Lieutier *et al.*, 2004; Lieutier, Yart & Salle, 2009), which are mainly carried on the external parts of the beetle and inoculated into the tree through the breeding galleries (Furniss, Solheim & Christiansen, 1990). For example, the most studied *Ips typographus* fungal associates, namely *Endoconidiophora polonica*, *Grosmannia europhioides* and *Ophiostoma bicolor*, have been recently proved to contribute to degrading Norway spruce phloem metabolites, likely affecting host defensive capacity (Zhao *et al.*, 2019; DiGuistini *et al.*, 2011; Wadke *et al.*, 2016). Furthermore, *Ips*-associated fungi release volatile organic compounds (VOCs) that may induce in the immature beetle a preference for more aggressive fungi, improving their fitness (Zhao *et al.*, 2019; Kandasamy *et al.*, 2019). Despite these findings, the role of fungal communities associated with bark beetles during these attacks remains largely unknown, to the point that there is still a significant debate around determining to what extent fungi are necessary for successful beetle colonisation (Six & Wingfield, 2011).

Similarly, there is a lack of information regarding the biogeographic diversity of fungal communities (Lieutier *et al.*, 2004; Giordano *et al.*, 2013), which makes it difficult to determine which fungi may be vectored by the beetle when invading a new area. This issue is aggravated by the fact that the beetle may vector invasive fungi, never encountered before by the British trees. Invasive fungal species vectored by bark beetles have already caused major damage in elm and laurel trees, profusely attacked by the fungi *Ophiostoma novo-ulmi* and *Raffaella lauricola* respectively (Harrington, Fraedrich & Aghayeva, 2008; Santini & Faccoli, 2015; Wingfield *et al.*, 2017). It is, therefore, necessary to further investigate which fungi are being vectored into the UK by *Ips typographus* and how they may affect spruce trees, including their inner fungal communities.

Although previous works have studied the effect of certain fungi, few studies have comprehensively addressed how fungal communities may affect beetle populations. Part of the problem is that fungal studies are 'pathogen-centric' (Six, 2020), mainly focusing on the few aggressive fungi and neglecting the rest of the community, which may also interact with the beetle (Biedermann *et al.*, 2019; Skelton *et al.*, 2019c). However, there is a wide range of fungi that may benefit beetle nutrition by metabolising defensive substances naturally present in the woody tissues (Davis, 2015; Hofstetter *et al.*, 2015; Péter *et al.*, 2011) potentially affecting beetle developmental stages (Durand *et al.*, 2017). Recently, the use of high throughput techniques like metabarcoding has become a powerful method for understanding how fungi interact as a whole

with the individual beetles, unveiling a higher proportion of cryptic fungi than pre-molecular studies (Miller *et al.*, 2016, 2019; Malacrinò *et al.*, 2017). Metabarcoding has also been applied to characterise the fungi present in the inner bark of the host trees, where the role of endophytic fungi remain still largely unknown (Pellitier, Zak & Salley, 2019).

The present investigation builds on an experiment started by Forest Research. In summer of 2018, adult *Ips typographus* specimens were captured in Kent using pheromone traps as part of Forest Research 's annual survey for the pest. This detection was followed by the finding of breeding galleries on windblown trees in Packing Wood, Kent in December 2018. This 0.5 Ha pure stand of Norway spruce (*Picea abies* L.), became the first known breeding area of *Ips typographus* in the UK. The whole spruce stand was then felled and chipped/burned following the forest pest and disease eradication procedures. However, it is known that this beetle may overwinter in the soil litter, being likely to emerge again the following year (Biermann, 1977; Wermelinger *et al.*, 2012). For this reason, an experimental set up was placed on 6 March 2019 in the same area where the first *Ips typographus* breeding population was detected. The general experiment aimed to study the preferences of this species towards the three different host tree species and the developmental capacity of their brood at each of them. To do this, these logs would be left on the field during three months and afterwards evaluated to find out whether they were colonised by *Ips typographus* and the extension of their galleries. My role in this investigation involved designing a complementary line of research based on the sampling and analysing *Ips typographus* specimens and wood samples through metabarcoding. I aimed to test my improved methodology for the characterisation of the fungal communities present on these beetles, with a special focus on detecting invasive fungal pathogens. To do so, I compared fungal communities across different beetle developmental stages and locations, including Norway and Sitka spruce host trees.

5.3 Materials and Methods

5.3.1 Field collection of beetle specimens and woody tissues

This experiment consisted of a set of freshly-cut logs of Norway spruce (*Picea abies*), Sitka spruce (*Picea sitchensis*) and Scots pine (*Pinus sylvestris*) ranging from 15 to 24 centimetres in diameter and one-meter in length. 48 of these logs were placed standing up and 20 meters apart, following a Latin Square design. Additional logs of Norway spruce were stocked in 4 piles of eight separated 100 meters apart. These piles were affixed with specific *Ips typographus* pheromone lures, aiming to attract as many adult *Ips* as possible.

On the 10th and 11th of June 2019, these logs were assessed in a small site-based unit to try to minimise sample contamination. The bark of every log was carefully separated from the phloem to unveil the galleries, collecting wood and target beetle samples. This way, a total of eight logs from Norway and one from Sitka spruce containing *Ips typographus* galleries were recovered (Pine logs didn't attract any target beetles and were hence discarded). The four samples collected from Sitka spruce were pupae obtained from the same tree specimen, but samples from Norway spruce belonged to different tree specimens and developmental stages (**Supplementary Table APP IV-1, Figure 5-1**). Parental beetles, which were the specimens that created the parental galleries, were considered here as a different developmental category, here denoted as "parental adults". Parental adults were easily distinguished from the "filial adults" (new generation adults) due to their melanised dark brown colour (Merker & Wild, 1954). Samples were collected by using a sterilised forceps and placed into Eppendorf tubes containing 100% ethanol and kept cold to preserve their DNA quality.

In addition to the beetles collected from logs, nine beetle specimens (here denoted as "flying beetles") were collected using funnel traps (Lindgren, 1983). Five beetles were collected from the traps scattered among the log piles at the establishment site, while four other beetles were collected from traps that were part of a wider surveillance network, away from the establishment site.

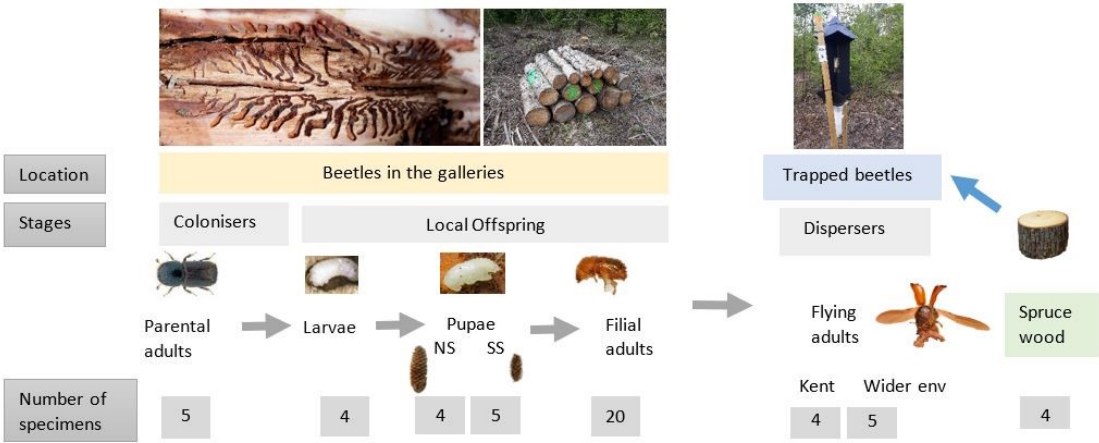


Figure 5-1. Experimental design showing the number of *Ips typographus* specimens belonging to the different developmental stages analysed. Pupae specimens were recovered from both Norway (NS) and Sitka (SS) spruce. In addition to beetle samples, four uncolonized wood samples belonging to Norway and Sitka spruce were analysed as a control.

In addition to the *Ips typographus* specimens, woody tissues were obtained from different tree specimens. The non-colonised spruce trees from which the wood samples were obtained were cut in logs from the same dimensions than the ones detailed above. Subsequently, these logs were kept separately to ensure that no beetle could colonise them. Pieces of wood were collected from beneath the tree bark with sterile forceps and were kept frozen to preserve their fungal DNA.

5.3.2 Laboratory procedures

At first, the specimens were randomly selected to obtain –when possible- the same number of specimens per developmental stage from each log. Subsequently, selected specimens were rinsed with pure water and placed in individual 1.5ml tubes. Sterile pestles were used to completely split up the individual beetle specimens to ensure that all the fungi inside the specimens were released. The DNA of the 52 specimens was then individually extracted, purified and eluted in 200µl of AE buffer using the QIAGEN DNA easy blood and tissue spin column extraction kit (Quiagen, Valencia, CA).

Because of the spruce wood characteristics, it was very challenging to extract DNA and there were no guarantees of success in sequencing its inner fungal communities with the current primers. It became therefore necessary to test different methodologies to extract the DNA. To completely break up the woody samples into powder it was necessary to use liquid nitrogen, which was poured on the samples before pulverising them with a mortar. Different quantities of this powder were then selected for their DNA extraction, which was carried out with two different DNA extraction kits (**Supplementary Table APP IV-2**).

The ITS region of both beetle and woody samples was amplified using PCR to allow the fungal identification via metabarcoding. Metabarcoding was performed using the ITS primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') /ITS4(5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck *et al.*, 2014). Unique six-letter indices were added to every primer pair to distinguish the specimens across the different libraries. The PCR reaction mixture contained 2.5 µl of TaKaRa buffer™, 0.5 µl of dNTPs (Bioline), 1 µl of forward primer, 1 µl of reverse primer, 0.1 µl of TaKaRa Taq™ and 18µl of ddH2O. 2 µl of DNA were added to every PCR reaction, making a final volume of 25 µl. Cycling conditions of the PCRs were: 94°C for 3 minutes followed by 34 cycles of 94°C for 45 seconds, 55°C for 55 seconds 72°C for 1.5 minutes, and a final extension of 72°C for 10 minutes. For each of the analysed samples, PCRs were replicated with three annealing temperatures (54°C, 55°C and 56°C) to take into account the stochastic nature of PCR reactions (Schmidt *et al.*,

2013). A minimum of one blank PCR reaction per plate was used as a negative control to control for potential contaminations. Successful PCR amplification was confirmed in every case by visualising 3 µL of the PCR product using gel electrophoresis. For this visualisation, 1% agarose TAE gel, GelRed™ (Biotium) and Hyperladder IV™ (Bioline) were used. PCR products were then purified to eliminate fragments under 300bp using the Agencourt® AMPure® XP purification kit, by following the protocol 000387v001. The amplified DNA was measured with a Qubit® compact fluorimeter (Qbit 3.0) and pooled in equal proportions thereafter. Samples were sharing the same Miseq with other samples detailed on the third chapter of this document, forming a total of 94 libraries. The 94 libraries were then sequenced on an Illumina® MiSeq® 2500 platform with version 2 chemistry (Rapid mode), using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads.

5.3.3 Bioinformatics

Raw sequenced data were processed through bioinformatics to achieve solid species identification before its ecological analysis. The 94 libraries were firstly demultiplexed by using the Illumina software to remove their XT indices. Samples were further demultiplexed with Cutadapt (Martin, 2011) scripts modified to account for bulk samples. During this step, the unique six-letter indices attached during the first round of PCR were trimmed from each sample file, which in turn was then assigned a unique name related to every single beetle specimen. Also, at this step, singletons were removed and Cutadapt was used to trim primers and zero-length sequences before read assemblage. Read quality was evaluated using FASTQC (Andrews, 2014). Forward and reverse reads were subsequently assembled using PEAR v. 0.9.8 (Zhang *et al.*, 2014). This step included a quality filtering of the paired-assembled reads (PEAR quality score threshold ≥ 30 , as recommended by Illumina® (https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf)). PEAR software tests all possible overlaps among reads, due to its tolerance to variable read lengths (Zhang *et al.*, 2014).

Sequence dereplication was carried out with VSEARCH (Rognes *et al.*, 2016), which was also used to filter the dereplicated sequences to minimise potential sequencing errors or artefacts and enhance species detection, as recommended by Pauvert (2019). To do this, the `--fastx_filter` command was used (`-fastq_maxee 1.0`). The remaining reads were denoised (`--minsize 4 --unoise_alpha 2`) and length-filtered (250-320bp). The ITSx software (Bengtsson-Palme *et al.*, 2013) was then employed to filter out non-fungal reads, selecting and extracting the ITS2 region

for each full-length read. Merged full-length metabarcoding reads had an average length of 266 bp, but after the ITSx step, reads had an average length of 171 bp.

Read clustering into Operational Taxonomic Units (OTUs) was performed with the VSEARCH clustering algorithm. Chimaeras were firstly detected and filtered out. Reads were then clustered at 97% of similarity threshold to obtain 5546 OTU clusters and mapped to these clusters, outputting an OTU table suitable for the ecological analysis of the samples.

5.3.4 Fungal OTUs taxonomic identification

To ensure a comprehensively accurate species-level identification, three complementary taxonomic classification approaches were used to assign taxonomic identity of the 5546 OTUs. The Ribosomal Database Project (RDP) Bayesian Classifier (Wang *et al.*, 2007) was used to assign a first classification for the OTUs. This classifier has been broadly used for fungal identification, using the Warcup fungal ITS (v2_March2018) and UNITE (v2020_February2020) training datasets to achieve both broad coverage and accurate OTU classification (Deshpande *et al.*, 2016; Edgar, 2018). Simultaneously, OTUs were processed through the PROTAXFUNGI pipeline (Abarenkov *et al.*, 2018), implemented in the PlutoF (Anon, 2017a) platform and based on the UNITE fungal database. PROTAX hierarchically assigns the OTU identities from the root node of the taxonomy through the species level (Nilsson *et al.*, 2019b). The last classifier selected, IDTAXA, employs machine learning to reduce over classification errors to obtain a higher accuracy (Murali, Bhargava & Wright, 2018). Combining these three methods has the advantage of adding three different percentages of probability to every identification. These percentages represent an estimation of how likely each OTU is currently identified at every level and can contribute to arrive to an informed consensus for a correct identification. Complementing this methodology, target OTUs which were of particular ecological interest for the analysis, were individually identified through the GenBank National Centre for Biotechnology Information (NCBI) database. All this information was then combined with the data of the collected beetle specimen to perform the ecological analysis.

5.3.5 Ecological Analysis

After generating the OTU table through the bioinformatics process and identifying the retrieved fungi, samples were split into the 1064 samples corresponding to the Biodiversity Survey (Chapter 4) and the 52 samples corresponding to *Ips typographus* beetles and spruce wood samples to continue with the analysis. This analysis consisted of four parts: The first part aimed to estimate differences across developmental stages of the *Ips typographus* specimens.

Secondly, fungal communities were compared for pupae collected from Sitka and Norway spruce galleries. Differences of fungal community composition were then evaluated across the trapped beetles (here named as “flying beetles”), comparing them with the developing beetles (inside tree galleries). Finally, the fungal OTUs obtained from *Ips typographus* were compared with those retrieved from the biodiversity survey carried out for Chapter 4 to unveil potential invasive fungi. Potentially invasive OTUs were then identified and displayed using a Venn Diagram (Oliveros, 2007) and a heatmap (Superheat R package (Barter & Yu, 2018)), as seen in Miller (Miller *et al.*, 2019).

All statistical analyses were conducted at the OTU level using RStudio (RStudio team, 2015). As the difference of fungal reads across the beetle specimens was considerable, rarefaction was necessary. Increasing rarefaction was observed to have a more dramatic effect on sample numbers than on OTU numbers. Therefore, samples were rarefied to 1200 reads to retain the majority of the samples while making them more comparable. Samples that did not have enough reads were removed for the downstream analysis, leaving 51 samples.

To compare fungal richness, data were analysed using the ManyGLM procedure from the Mvabund R package (RStudio team, 2015) (“anovamanyglm” function). The model was set with the fungal OTU richness as a response variable and beetle species and forest type as dependent variables. First, the model was tested with the Poisson distribution. However, as the data showed overdispersion (the residual deviance was greater than the degrees of freedom) two more models were tested, the Quasi-Poisson and the Negative Binomial models. Finally, the Negative Binomial model was chosen, as it is suitable for over-dispersed and fitted this particular data better than the Quasi-Poisson. Univariate test statistics and accompanying p-values were generated using 1000-iteration bootstrapping corrected for multiple testing for each species. To complement these results, species accumulation curves were also built with Vegan R package (Oksanen, Jari, *et al.*, 2018). These curves contributed also to corroborate that an average sample size necessary was reached to be able to capture a reasonable amount of fungal OTU variability.

To characterise the fungal communities across different forest types and beetle species, we calculated distance matrixes based on Jaccard and Bray-Curtis indexes, which calculate the beta-diversity between sample pairs based on presence-absence data (richness), while the Bray-Curtis index focuses on the relative abundance of each OTU. To illustrate these variations a metric multidimensional scaling MDS graph (“vegan” package (Oksanen, Jari, *et al.*, 2018),

Jaccard index). To quantify the variations shown in the graphs, a ManyGLM analysis was performed with Mvabund (Wang *et al.*, 2012). This method was preferred to a permutational multivariate analysis of variance (PERMANOVA) because it includes an assumption of a mean-variance relationship, improving power across samples with different variances (Warton, Wright & Wang, 2012; Warton, 2018). By this mean, models for fungal community structure as a function of beetle developmental stages or host tree (for pupae only) were calculated. As overdispersion was detected, negative binomial distributions were implemented, and the univariate test procedure implemented in ManyGLM was then used to obtain the pairwise comparisons.

To evaluate the fungal species more strongly correlated for each assembly, a Fourth Corner analysis was carried out by calculating the (Mvabund package). The mvabund package simultaneously fits multispecies GLMs, partitions the effects of the predictors and computes test statistics that additionally account for correlations among OTUs (Bálint *et al.*, 2016). This approach allows modelling the relative abundance of the fungi across different traits such as beetle developmental stage or location. Lasso penalty uses a regression analysis method that performs both variable selection and regularization to enhance the prediction accuracy and interpretability of the statistical model it produces (Brown *et al.*, 2014).

5.4 Results

This section summarises the main findings of this chapter, which aims to test an improved methodology for the characterisation of the fungal communities present on these beetles at different developmental stages and host trees, with a special focus on detecting the potential non-native fungi harboured by these *Ips typographus* specimens.

As the metabarcoding data concerning this chapter was sequenced along with the data from chapter 4, the general results corresponding to the number of reads per library and filtering processes are detailed in that chapter. After the bioinformatics process, the 52 samples consisting in *Ips* specimens (48) or spruce wood (4) clustered into 567 Operational Taxonomic Units (OTUs), 375 in the 51 samples corresponding to the rarefied dataset. As in previous analyses, the OTUs found in the negative controls were subsequently excluded from further analysis. The individual sequence-based species accumulation curves of observed OTU richness for each sample, rarefied using 1200 sequences, are nearly asymptotic in most of the cases (**Figure 5-2 A-D**). This indicates a satisfactory sequencing depth, sufficient to capture the fungal diversity present in the vast majority of the samples.

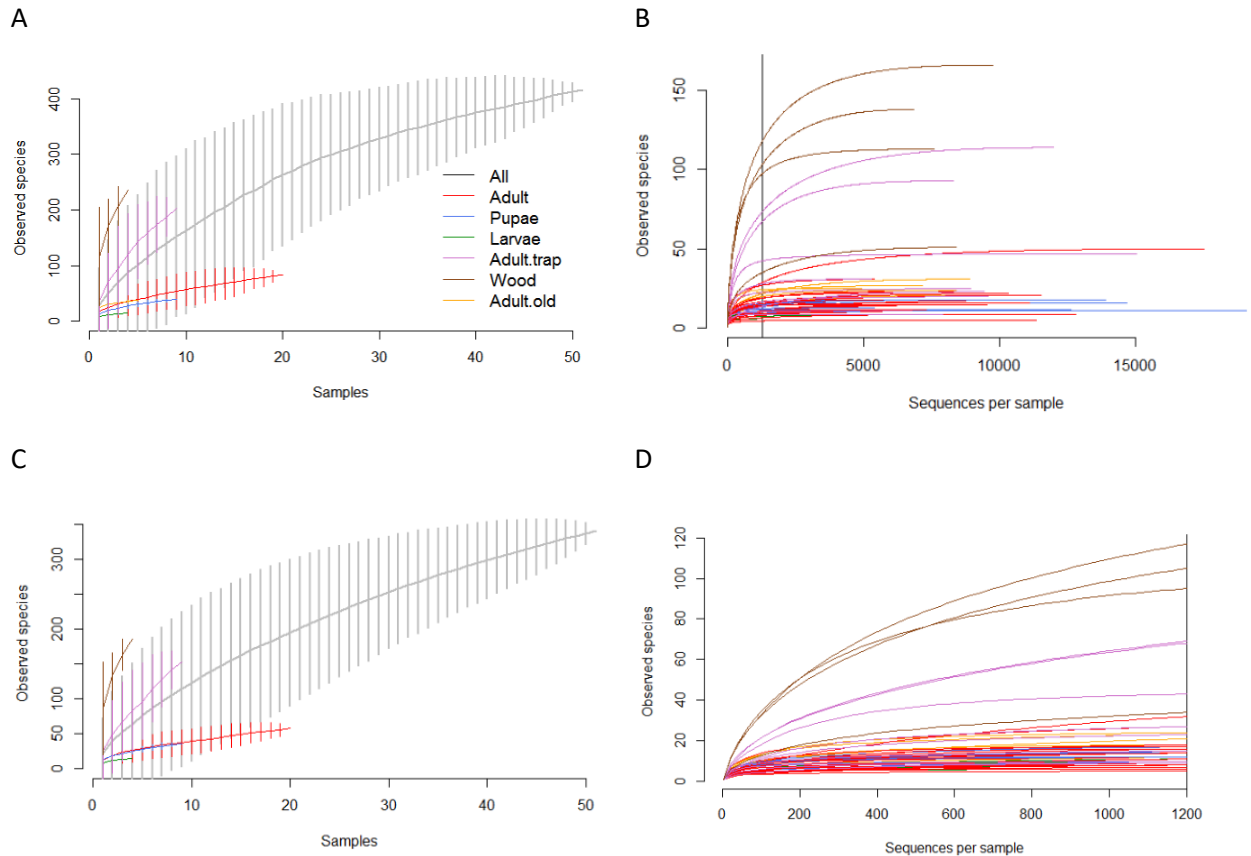


Figure 5-2. Species accumulation (A) and rarefaction curves (C) of compiled fungal OTU richness for each beetle stage level (the grey bar represents the whole community). Sequence-based rarefaction curves of raw (B) and rarefied (D) data. Rarefaction was conducted at 1200 reads.

5.4.1 Fungal communities at different beetle developmental stages

In the beetles collected from galleries, there were some differences in fungal species richness across the developmental stages for parental beetles and larvae (Dev=54.68, $P=0.001$), which harboured the highest and lowest fungal richness respectively (**Table 5-1, Figure 5-3A**). Fungal richness ranged from five to 50 OTUs, with an average of 17 fungal OTUs per beetle specimen.

Table 5-1. Analysis of deviance table generated from the ManyGLM procedure, multivariate and pairwise comparisons between beetle developmental stages and locations for fungal richness (** $P < 0.01$, * $P < 0.05$, "." $P < 0.1$; mvabund package. anova.manyglm test). Bill6, Bill4 and E4 are different logs from which samples were collected (see supplementary material).

Multivariate test			
Df.	Variable	Dev.	P-value
34	Stage	54.68	0.001 **
30	Location	19.13	0.002 **
Pairwise comparisons			
	Variables	Dev.	P-value
	Larvae vs Parental.adult	18.706	0.004 **
	Pupae vs Parental.adult	17.199	0.004 **
	Larvae vs Pupae	6.140	0.088 *
	Larvae vs Filial.adult	5.657	0.097 *
	Bill6 vs E4	8.368	0.053 *
	Bill4 vs E4	7.289	0.069 *

Fungal communities were structured with a different assembly in the case of parental adults (Table 5-2, Figure 5-3 B). This difference seems to be partly driven by a lower relative abundance of saprotrophs (Figure 5-3 C). Interestingly, pupae and filial adults showed a higher percentage of symbiotrophs and pathotrophs-saprotrophs fungi than larvae and parental adults.

Table 5-2. Analysis of deviance table generated from the ManyGLM procedure, multivariate and pairwise comparisons between beetle developmental stages and locations for fungal community structure (** $P < 0.01$, * $P < 0.05$, "." $P < 0.1$; mvabund package. anova.manyglm test). Bill6, Bill5, Bill4, E6 and E4 are the different logs from which samples were collected (see supplementary material).

Multivariate test			
Df.	Variable	Dev.	P-value
34	Stage	335.4	0.003 **
30	Location	439.0	0.001 **
Pairwise comparisons			
	Variables	Dev.	P-value
	Larvae vs Parental.adult	146.69	0.037 *
	Parental.adult vs Pupae	175.57	0.016 *
	Filial.adult vs Parental.Adult	129.69	0.0861 .
	Bill5 vs E4	151.29	0.060 .
	Bill6 vs E4	142.95	0.065 .
	Bill4 vs E4	140.81	0.068 .
	Bill5 vs E6	130.07	0.087 .

Results corresponding with the correlations of fungal species across beetle developmental stages are displayed in the **Figure 5-3.D**. The numerical results from the multilevel analysis are summarised as supplementary materials (**Supplementary Table APP IV-3**). *Grosmannia penicillata* was correlated with all the developmental stages of the beetles present in the galleries (**Figure 5-3 D**), is also the most abundant fungal pathogen harboured by the beetles (**Figure 5-7**).

Some yeasts, including *Kuraishia capsulata*, *Wickerhamomyces bisporus* and *Saccharomycopsis lassenensis* were also consistently carried by the beetles through all their developmental stages (**Figure 5-3-D**). Five OTUs were associated with the old and filial adults with the remarkable presence of *Ophiostoma canum* in all the five beetles belonging to both adult stages, but absent from the larvae and very rare in the filial adults that coexisted in the same log. Pupae displayed a high positive correlation with the yeast identified as *Cyberlindnera fabianii*, which is closely related with the bark beetle symbiont *Cyberlindnera americana* (Soto-Robles *et al.*, 2019).

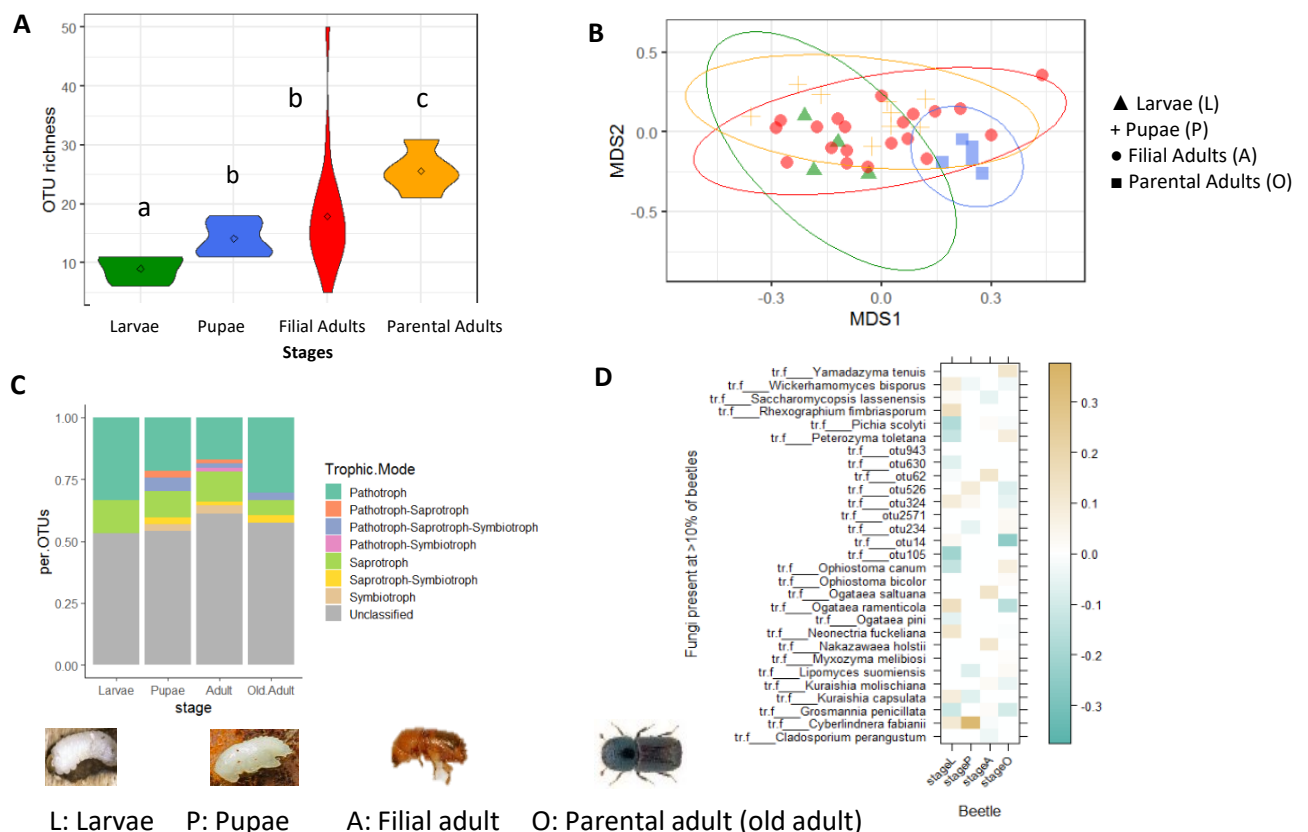


Figure 5-3. A. Fungal OTU richness according to *Ips typographus* developmental stage. The average richness of each group is represented by a black rhomboid. B. MDS plot of fungal communities on different stages (rarefied data, stress=0.1878). C. The relative abundance of the different fungal guilds across beetle stages. D. Frequency of common fungal OTUs detected across different stages and locations. Only the identified OTUs present in at least 10% of the specimens are shown.

5.4.2 Differences in fungal communities on beetles from Norway vs Sitka spruce

On pupal stage, fungal richness ranged from 11 to 18 species, being slightly smaller in the beetles retrieved from the Sitka spruce billet (Dev=2.317, $P=0.083$) than those present on Norway spruce (**Figure 5-4 A**). The manyGLM analysis of fungal community structure showed also a modest variability in abundances between Norway and Sitka spruce (Dev=83.76, $P=0.072$) (**Figure 5-4 B**). A greater proportion of symbiotrophs were present in Norway spruce, while saprotrophs and pathotroph-saprotroph-symbiotrophs were more abundant in Sitka beetles (**Figure 5-4 C**). In terms of fungal OTU identity, only 40% of the total number of OTUs harboured by the pupae specimens were shared across both host trees, being the proportion of *Grossmannia penicillata* higher in Sitka than in Norway spruce larvae (**Figure 5-4 D**).

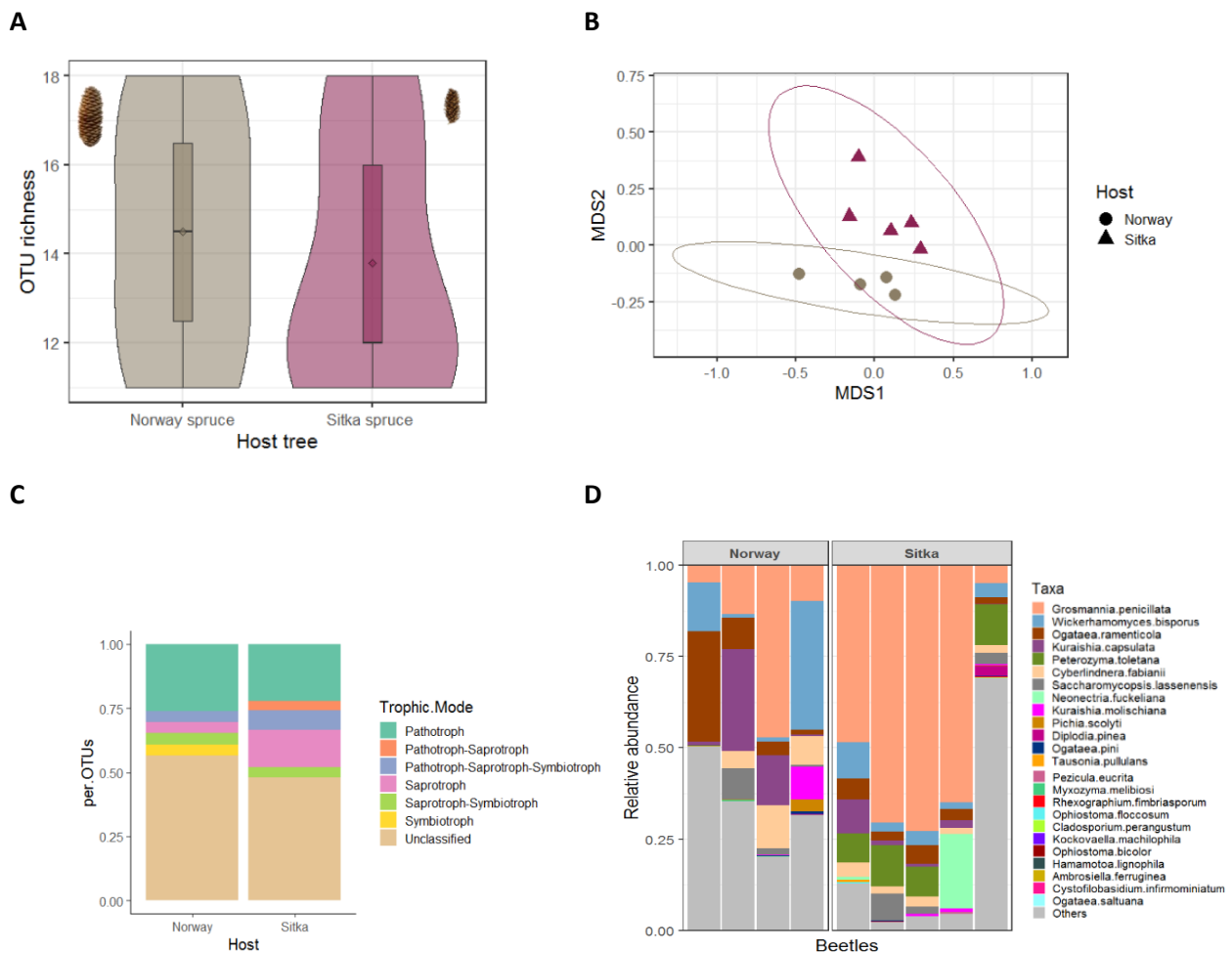


Figure 5-4. A. Fungal OTU richness according to *Ips typographus* host tree. The average richness of each group is represented by a black rhomboid. B. MDS plot of fungal communities on different host trees (rarefied data). C. The relative abundance of the different fungal guilds on each host tree. D. The relative abundance of fungal OTUs detected in Sitka and Norway spruce beetles (Only the 20 most abundant are shown).

5.4.3 Fungal communities in flying beetles

When comparing the flying beetles obtained from the traps with those filial and parental adults coexisting in the galleries, the number of OTUs ranged from 5 to 114, being significantly higher on beetles trapped on the Packing Wood forest (Kent) compared to the traps located in the wider environment and the galleries inside the spruce logs (**Figure 5-5 A, Table 5-3**).

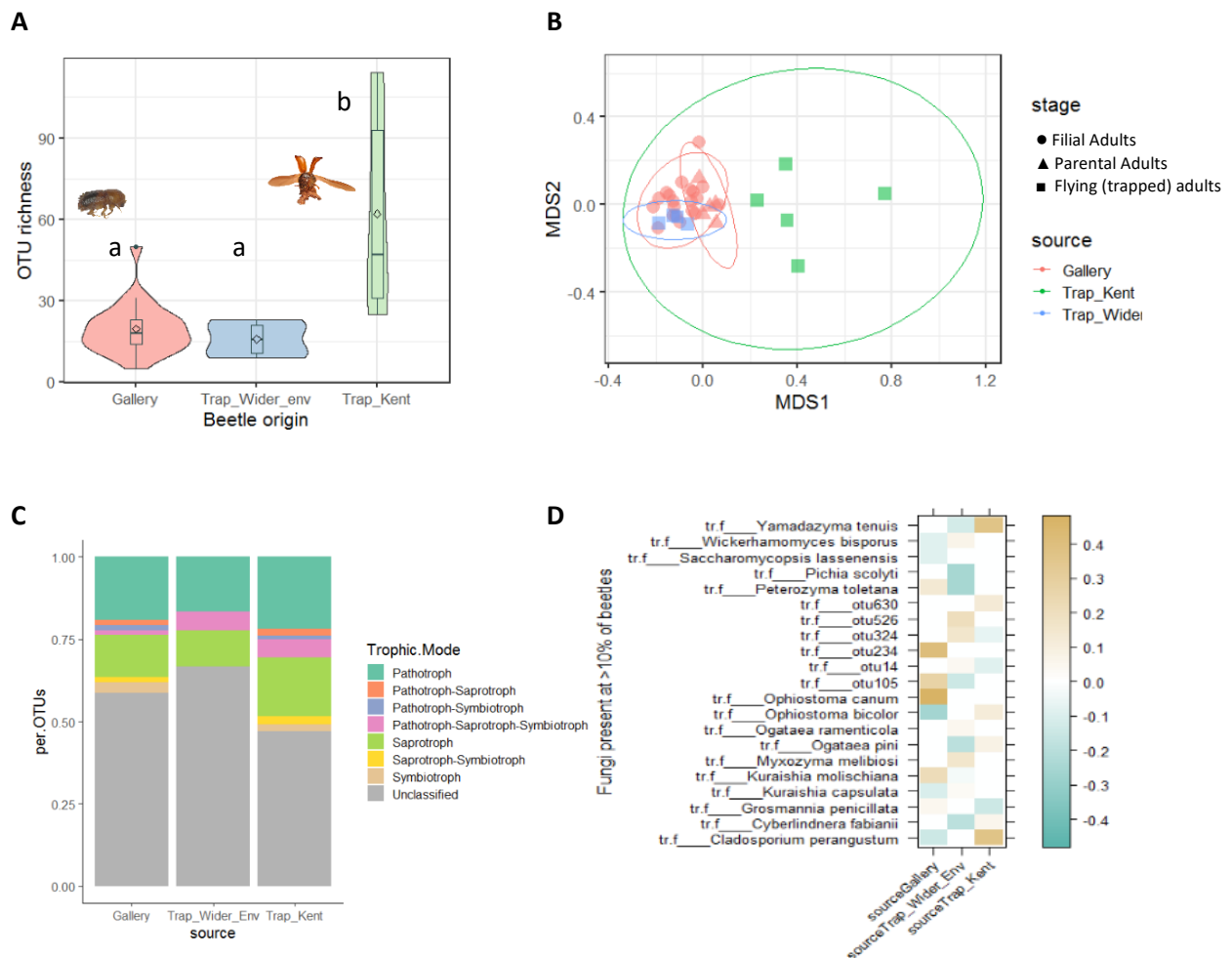


Figure 5-5. A. Fungal OTU richness according to *Ips typographus* source. The average richness of each group is represented by a black rhomboid. B. MDS plot of fungal communities from different sources (rarefied data). C. Relative abundance of the different fungal guilds on each beetle source. D. Frequency of common fungal OTUs detected in adult beetles (Filial and parental). Only identified OTUs present in more than 10% of the beetles are displayed.

Surprisingly, the diversity of fungi encountered in the Packing Wood -flying beetles was remarkably higher compared with the rest of the samples, to the point that fungal communities of beetles recovered from logs in the same location were more similar the flying beetles present in the wider environment (**Figure 5-5 B, Table 5-4**). These beetles were also more compositionally different in terms of fungal guilds, harbouring higher proportions of saprotrophs and pathotrophs (**Figure 5-5 C**). A total of ten OTUs were correlated only with flying beetles,

including several yeasts, but their association was weaker than those OTUs found also in beetles trapped in the galleries (**Figure 5-5 D**). The pathogen *Ophiostoma bicolor* was present only in parental and flying adults from Packing Wood, being much more abundant in this second group. Likewise, the xylose-fermenting yeast *Yamadazyma tenuis*, which has been frequently isolated from a wide range of wood-boring beetles, was more frequently encountered in flying and parental adults.

Table 5-3. Analysis of deviance table generated from the ManyGLM procedure, multivariate and pairwise comparisons between the source from which beetles were collected (Beetle source) for fungal richness (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, "." $P < 0.1$; mvabund package. anova.manyglm test).

Multivariate test			
Df.	Variable	Dev.	P-value
31	Beetle source	74.08	0.001 ***
Pairwise comparisons			
Variables		Dev.	P-value
Gallery vs Trap_ PackingWood		22.319	0.001 ***
Trap_Wider_Env vs Trap_ PackingWood		8.856	5.30

Table 5-4. Analysis of deviance table generated from the ManyGLM procedure, multivariate and pairwise comparisons between the source from which beetles were collected (Beetle source) for fungal community structure (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, "." $P < 0.1$; mvabund package. anova.manyglm test).

Multivariate test			
Df.	Variable	Dev.	P-value
31	Beetle source	950	0.001 ***
Pairwise comparisons			
Variables		Dev.	P-value
Gallery vs Trap_ PackingWood		804.6	0.001 ***
Trap_Wider_Env vs Trap_ PackingWood		371.68	0.024 *
Gallery vs Trap_Wider_Env		99.63	0.058 .

5.4.4 Potentially invasive fungi, beetle associates and fungal pathogens

From the 416 fungal OTUs obtained from *Ips typographus* and wood specimens, 341 fungal OTUs persisted after rarefaction. Remaining OTUs were then compared across beetle stages and sources at a multilevel analysis, revealing 26 fungi associated with the beetles and 90 associated with the wood (**Figure 5-6 A**).

To point out any non-native or potential invasive fungi, the 416 fungal OTUs obtained from *Ips typographus* specimens were compared with the 5116 fungal OTUs (5553 fungal OTUs before rarefaction) retrieved from the 1064 beetles trapped in 2017 in 20 different locations across the United Kingdom and four wood samples from Norway and one from Sitka spruce (Figure 5-6 A).

Results show that most of the OTUs were shared with those belonging to the surveyed UK beetles, but 18 OTUs were unique for the *Ips typographus* specimens (Figure 5-6 B). These OTUs, which were mainly encountered in the Packing Wood traps and included four fungal pathogens, namely *Ophiostoma floccosum*, *Ophiostoma bicolor*, *Cyberlindnera jadinii* and *Trichosporon aquatile*, which were present in three, 12, one and two samples respectively (Figure 5-6 C).

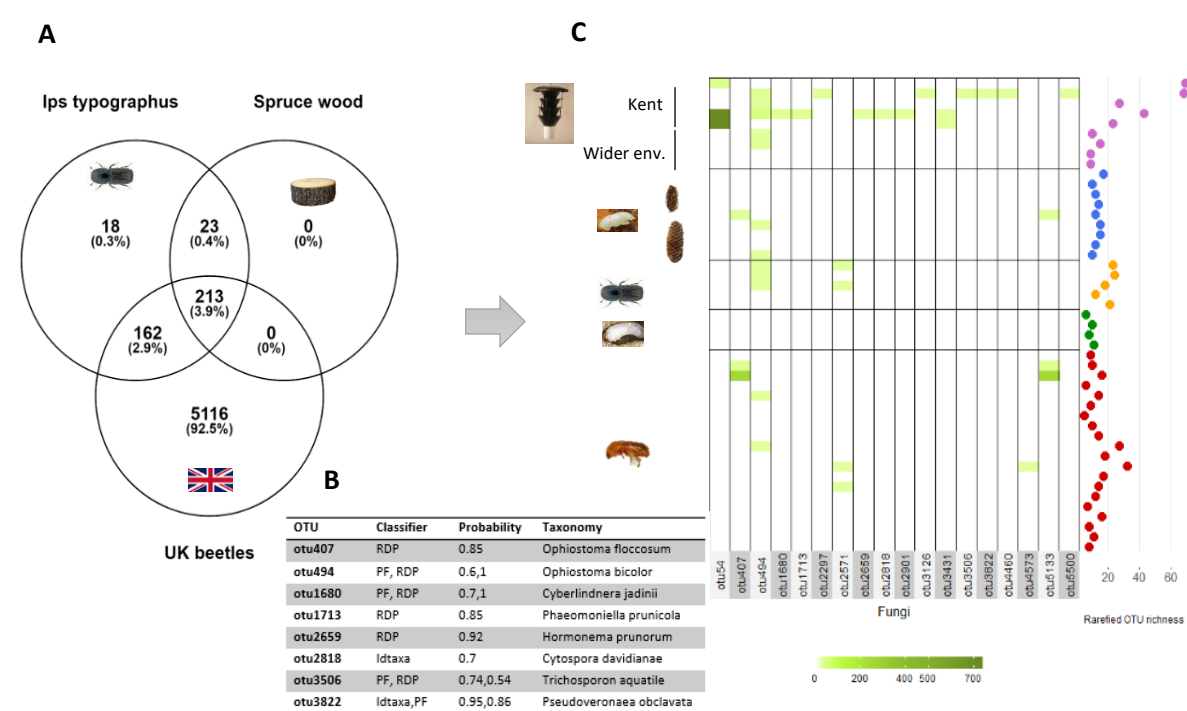


Figure 5-6. Venn-diagrams showing the intersection of fungal OTUs on *Ips typographus*, other bark and ambrosia beetles trapped across the UK and wood samples from non-colonised Sitka and Norway spruce from the same area from which most of the *Ips* beetles were collected. The attached table shows the identity of the identified OTUs along with the classification tool and associated probability (PF= ProtaxFungi). The remanent fungal OTUs could not be identified to the species level.

The Ophiostomatoid fungi *Grosmannia penicillata* dominated in most of the samples found in the gallery, except for the larvae, which carried a higher proportion of the yeast *Kuraishia capsulata*. Although pathogenic fungi were more abundant in the in the beetles retrieved from the galleries, the flying beetles belonging to the Kent area displayed a higher diversity of pathogens (Figure 5-7). The fungal pathogens encountered in wood samples were different than

those from the beetles, and also in were in a lower numbers and proportion than in beetles belonging to galleries

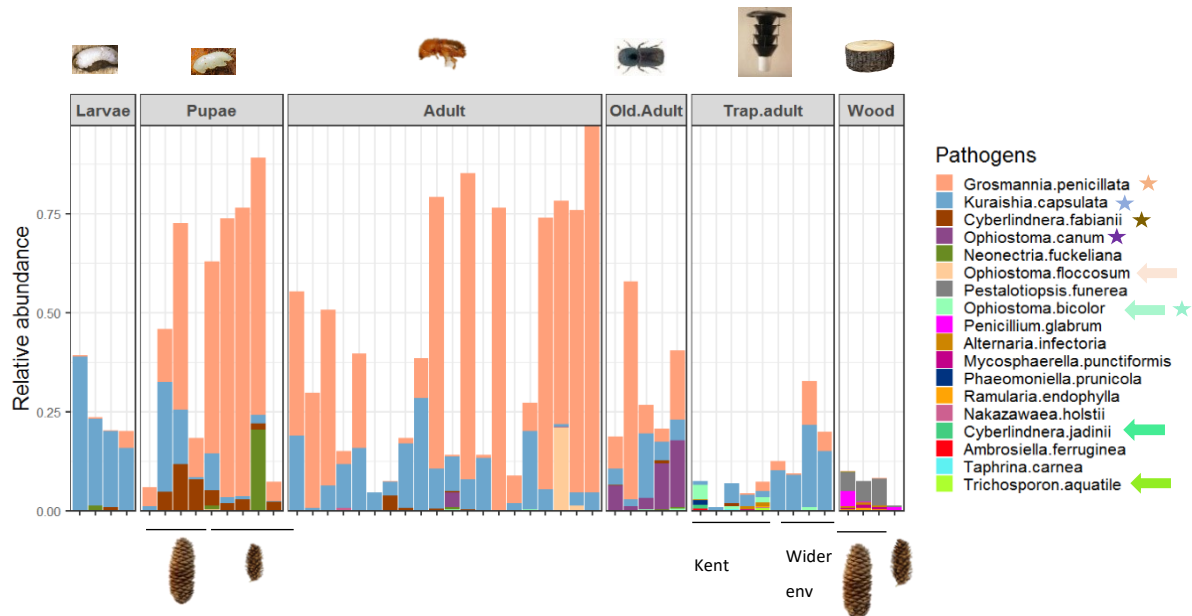


Figure 5-7. The relative abundance of the most abundant fungal pathogens on each beetle source, beetle stage and Norway/Sitka spruce woody tissue. The fungal pathogens not previously encountered in the UK-level biodiversity survey are marked with an arrow and those associated with the beetles are marked with a star.

The 375 fungal OTUs present in the *Ips typographus* specimens (rarefied dataset) were then compared across the gallery and flying beetles at a multilevel pattern analysis. These analyses revealed the presence of 26 fungal OTUs associated with the beetles at different developmental stages and 90 fungal OTUs associated with the wood (**Supplementary Table APP IV-3**). The analysis showed that only four fungi, namely *Kuraishia capsulata*, *Wickerhamomyces bisporus*, *Saccharomycopsis lassenensis* and *Ogataea ramenticola* were consistently carried by the beetles during their whole life cycle. These four yeasts which have been encountered in previous *Ips typographus* studies, and are therefore likely to play an important role across all the developmental stages of this beetle species.

5.5 Discussion

The present chapter explores the richness and community composition of the fungi associated with the first known UK breeding population of the bark beetle *Ips typographus*, aiming to answer several fundamental questions concerning the differences of fungal communities across

beetle developmental stages and host trees. These kinds of comparisons are not always straight forward, as there are usually dozens of fungi coexisting in every beetle, and this study was no exception. However, it is crucial to understand which fungi may be preferentially carried and perhaps even be transmitted into the UK forests by these non-native beetles. In this sense, there is still little literature addressing such questions, this being one of the first studies to explore them.

Results show how fungal assemblages differed the most in parental and flying beetles, which harboured the highest fungal richness and the most diverse fungal composition. Interestingly, a higher number of associated fungi were retrieved from beetles belonging to these categories, most being of the yeasts and Ophiostomatoid fungi. In contrast, larvae had the lowest fungal richness and no clearly associated fungi. The fungal assemblages from pupae collected in Norway and Sitka spruce logs tended to differ, with a higher proportion of saprotrophs and a lower number of symbiotrophs in Sitka spruce. Despite these differences, the yeast *Cibberlinidnera fabianii* was preferentially associated with pupae in both host species. Importantly, we observed that 18 of the 416 OTUs harboured by these beetles were not present in the 1076 other bark and ambrosia beetles surveyed across the UK. Among these newly encountered OTUs, there is a remarkable presence of two Ophiostomatoid fungi, *Ophiostoma bicolor* and *Ophiostoma floccosum*. These results have exciting implications on fungal transmission and detection of non-native fungal species, providing an insight into the different fungal communities carried by these bark beetles during this crucial colonisation step.

5.5.1 Fungal communities at different beetle developmental stages

It is known that a temporal succession of fungi takes place in the phloem and sapwood from the moment the parental adult penetrates the bark and throughout the different developmental steps of the offspring beetles (Durand *et al.*, 2017; Jankowiak, Kacprzyk & Młynarczyk, 2009; Solheim, 1992a, 1992b; Leufvén & Nehls, 1986; Nève Repe, Groot & Jurc, 2018). Most of these studies have focused more on Ophiostomatoid fungi, neglecting the yeasts, which may help beetles in many different ways (Davis, 2015). This is why the present study is focused on the entire beetle holobiome rather than studying target groups, investigating fungal communities across the whole beetle life cycle.

From the abundant number of yeasts retrieved, a significant amount of those significantly correlated with the beetle was also found in previous research on *Ips typographus*-associated

mycobiota (Giordano *et al.*, 2013; Davis, 2015; Leufvén & Nehls, 1986). This is the case of the yeasts *Kuraishia capsulata*, *Wickerhamomyces bisporus* and *Saccharomycopsis lasenensis*, which the beetles consistently carried during the whole life cycle. These yeasts were extremely widespread, which might indicate their importance for the beetle through the entire life cycle. These findings are in line with past literature since *Kuraishia capsulata* has also been detected through the different developmental stages of the bark beetle *Dendroctonus micans*, also in Norway spruce (Menkis *et al.*, 2017). The potential roles of beetle-associated yeasts are often disregarded, but may include effects on tree chemistry (Davis & Hofstetter, 2011), contribute towards the metabolisation of toxic tree exudates, improve beetle nutrition or affect host selection and larval fitness (Baig *et al.*, 2020a; Davis, 2015). Interestingly, no other fungus apart from these three was significantly correlated with the larval stage.

Myxozyma melibiosi and *Peterozyma toletana* were linked to parental adults, being surprisingly absent from the larvae and other specimens coexisting in the same log. *Myxozyma melibiosi* may improve nutrient processing and tolerate specific inhibitors (Sitepu *et al.*, 2014), while *Peterozyma toletana* would limit tree responses towards beetle aggregation pheromones (Leufvén & Nehls, 1986). Both *Myxozyma* and *Peterozyma* have been previously isolated from bark beetles of the genus *Dendroctonus*, but a previous study showed that *Peterozyma toletana* was present in all the beetle developmental stages (Menkis *et al.*, 2017). *Peterozyma toletana* has also been linked with *Ips typographus specimens* (Leufvén & Nehls, 1986). A potential explanation for yeast succession suggested by these authors is the nutrient depletion at the latest stages of the beetle invasion affecting the number and abundance of yeasts. These authors also suggest that since the male beetles of *Ips typographus* are more mobile than females, they may capture a higher yeast diversity. After mating, *Ips typographus* males remain at the gallery entrance while the females excavate the parental galleries. This way, males are exposed to potential interactions with other beetles or with endophytes from the bark. Therefore, based on the hypothesis of Leufvén & Nehls, parental adults could carry more fungi because of those extra interactions.

The yeast *Cyberlindnera fabianii* was significantly correlated with the pupae for both Norway and Sitka spruce host trees. This yeast is closely related to *Cyberlindnera americana*, which is suspected of supporting nutritional processes like the hydrolysis of starch in the gut of the bark beetle *Dendroctonus rhyzophagus* (Briones-Roblero *et al.*, 2017). The presence of *Cyberlindnera fabianii* might serve a similar function. A recent study has linked the genus *Cyberlindnera* with beetles from the genera *Ips* (Chakraborty *et al.*, 2020). However, to our knowledge, this is the

first study that finds this yeast associated with *Ips typographus*. The fact that yeasts play significant but often indeterminate functional roles in bark beetle ecology (Davis, 2015) makes these findings difficult to evaluate. However, linking them to certain beetle developmental stages may clarify these roles (Durand *et al.*, 2017). For example, a recent study explains how gut-associated yeasts can influence larval fitness in beetles of the genera *Carpophilus*, leading to larger pupae and more successful offspring (Baig *et al.*, 2020a). The presence of yeasts across the whole beetle life cycle forming a fungal succession has been recently shown also for the ambrosia beetle *Xyleborus affinis* (Ibarra-Juarez *et al.*, 2020). Although these authors could not determine which specific roles these yeasts could play, they suggest that they could contribute to degrading plant tissues, fixing nitrogen from the atmosphere or improving beetle nutrition by supplementing vitamins or amino acids. Therefore, even if we cannot yet know the exact mechanisms by which these fungi can help their host beetle, by knowing beetle preferences, we may be able to identify at least which yeasts may be more critical for beetle development or host colonisation. This may help prioritise which yeast species to focus upon in potential *in-vitro* studies to fill this knowledge gap.

To date, there has been a greater emphasis upon the study of associated Ophiostomatoid fungi compared to yeasts. Past studies have associated the presence of certain ophiostomatoid fungi with certain developmental stages. For example, Biedermann (2013) associated the fungus *Raffaelea sulfurea* with positive effects on egg and larval numbers in ambrosia gardens. However, the mechanisms explaining fungal succession remain largely unexplained. At a recent review, Netherer (2021) suggested a mechanism of fungal succession by which beetle-associated ophiostomatoid fungi would trigger extensive host defence reactions at the beginning of the colonisation, also causing tree reserves depletion. The same fungal associates would, later on, mobilise tree nutrients back to the phloem, which would help bark beetle nutrition and subsequent development. These fungi would play an essential role in beetle nutrition regardless of their aggressivity, as suggested in previous studies (Six & Elser, 2020; Six & Wingfield, 2011). *Grosmannia penicillata* was the most abundant Ophiostomatoid fungi found in the beetles recovered from the galleries, except for the larvae, in which it was quite rare. This fungus can degrade tree-defensive phenolic compounds, which helps beetles to persist colonisation over more extended periods (Zhao *et al.*, 2019). *Grosmannia penicillata* also seems to synthesise aggregation pheromone components, which may act as communication signals with other beetle specimens (Netherer *et al.*, 2021; Kandasamy *et al.*, 2019). I hypothesise that *Grosmannia* could become more abundant at pupae and adult stages to help to synchronise the beetle specimens from exiting the galleries. Beetles exiting galleries at the same time would give

them an advantage of their high numbers to avoid being captured by predators. Along with *Ophiostoma bicolor*, which was more abundant on flying beetles, *Grosmannia penicillata* has been the most common and most dominant *Ips typographus* fungal associate recorded by the literature (Giordano *et al.*, 2013; Jankowiak, Kacprzyk & Młynarczyk, 2009; Solheim, 1992a, 1992b; Netherer *et al.*, 2021).

In contrast, *Ophiostoma canum* was not previously encountered in any *Ips typographus* specimen, except for few isolates in non-outbreak areas (Linnakoski *et al.*, 2012b). This fungus was correlated here and relatively abundant in the parental beetles, being also present in two beetles from inside the galleries but absent from flying beetles. *Ophiostoma canum* is a mild pathogen of Scots pine in Europe and Asia, is associated with several species of *Tomicus* (Pan *et al.*, 2019; Mathiesen, 1950; HuiMin *et al.*, 2019). This fungus is present mainly in the parental beetles and not in the trapped beetles, indicating that the fungus may not be easily transmitted to a new brood. This fungus could also have been transmitted horizontally from the galleries of the native bark beetles since it has been detected associated with the bark beetle *Hylastes opacus* in the UK-based biodiversity survey (Chapter 4). Another possibility could be that the beetles could be picking up spores dispersed from the nearby forests, which consisted of Scots pine, forming new associations.

The lower fungal richness present in larvae compared to parental and filial adults from the same galleries suggests parallelisms to the fungal succession suggested by Netherer (2021). Beetle-fungal associates could first get transmitted into the inner tree tissues at the beginning of the colonisation stage, explaining the lower richness in larvae. Fungi could then develop parallel with the beetle and emerge into the galleries to be retrieved by the beetles at specific developmental stages. This could explain why certain developmental stages can be linked to particular fungal associates, while others are associated with the beetle specimens through their whole life cycle. When the beetles are pupating and undergoing metamorphosis, they regenerate their bodies almost completely. Particular fungal associates are linked with this crucial moment on beetle development, to the point that the absence of certain fungi can limit or stop beetle development (Strongman, 1987; Klepzig & Six, 2004). Fungal associates are thought not only to co-evolve with the beetle species but also to change across the developmental stages of the beetle in a temporal succession (Jankowiak, Kacprzyk & Młynarczyk, 2009; Solheim, 1992a). This co-evolution could produce the synchronisation of fungal and beetle development so that beetle metamorphosis coincides with fungal sporulation.

In line with my results, Zhao (2019) found using *in-vitro* essays that, when generating the side galleries, *Ips typographus* prefers tunnelling into medium colonised by particular fungal associates, including *O.bicolor* and *G.penicillata*. Furthermore, Nève Repe *et al.* (2018) found differences in fungal distribution and abundance over developmental stages of *Ips amitinus*. More specifically, they found a higher number of Ophiostomatoid fungi on adult beetles compared to larvae. These authors suggest that larvae naturally harbour a small proportion of fungal associates. When creating their pupping chambers, the Ophiostomatoid fungi would emerge from the inner woody tissues to develop their fruiting bodies on those cavities. This way, when the beetle is ready to emerge from the galleries, it carries whole fungal assemblages. It is interesting to note that both Nève Repe *et al.* and I have reached similar conclusions about fungal successions on *Ips* beetles, but we have used different approaches. While they have used culturing techniques and have been focused only on Ophiostomatoid fungi, my research has focused on a more complex approach by including the different kinds of fungal associates present in the *Ips typographus* fungal assemblies.

As a whole, our results support the idea of fungal assemblages being dynamic and changing over time. The parental beetles displayed a higher fungal richness than the rest of the developmental stages, supporting our suggestions of different transmission methods. By establishing further interactions with tree bark and other beetles, the parental beetles would have acquired fungi from the horizontal transmission while defending their galleries, as suggested by Leufvén (1986). It seems only a tiny proportion of fungi would be transmitted to their progeny and remain during the whole beetle life cycle, including the three yeasts mentioned earlier. The mutual benefits of these associations are slowly being disentangled by the literature, shedding some light on the complex nature of bark beetle-fungal interactions

5.5.2 Differences in fungal communities on beetles from Norway vs Sitka spruce

Fungal assemblages present in the same beetle species may differ from each other because of factors such as location (Giordano *et al.*, 2013; Miller *et al.*, 2016), colonisation stage (Nève Repe, Groot & Jurc, 2018; Romón *et al.*, 2007) or host tree identity (Wingfield *et al.*, 2017; Asplund *et al.*, 2019). However, the role of the host tree on the fungal communities associated with bark and ambrosia beetles remains largely unknown. *Ips typographus* is known to be able to attack different tree species and even different genera (Zhao *et al.*, 2019). However, the present study is the first to compare the fungal communities present in beetles recovered from galleries of Sitka spruce with those retrieved from Norway spruce. This comparison is made in the exact location to avoid other external influences or contaminations.

The analysis shows how fungal communities recovered from beetles in Sitka tend to differ from those collected in Norway spruce galleries. *Grosmannia penicillata* was substantially more abundant in Sitka spruce than in Norway spruce pupae, which could induce a different colonising behaviour from the beetle based, perhaps on a different amount of aggregation pheromones (Netherer *et al.*, 2021). Interestingly, all the beetles collected from Sitka spruce harboured the yeast *Peterozyma toletana*, indicating an adaptation by the beetle to breed in this previously unencountered host. It is known that the beetles can actively select certain fungi to obtain metabolic advantages or favour the detoxification of tree defence chemicals (Davis, 2015; Briones-Roblero *et al.*, 2017; Adams *et al.*, 2009). In the case of the beetles colonising Sitka spruce, the higher abundance of *Grosmannia* - which synthesises aggregation pheromones- and the higher frequency of *Peterozyma* -allegedly limiting tree response to these pheromones- suggest a more aggressive attack of this beetle on this particular host tree compared with Norway spruce. Therefore, we could argue that given that *Ips typographus* is adapted to attack Norway spruce, it could perhaps favour slightly more aggressive fungal assemblages to ensure successful colonisation in Sitka spruce.

Alternatively, the fungal composition could be influenced by different phytochemicals in Norway compared to Sitka spruce tissues. Lehenberger *et al.* (2021) suggest that the ethanol content on tree tissues can contribute to different fungal assemblages. This recent study indicates that *G. penicillata* seems to be neutral, while *Ophiostoma bicolor* would be negatively affected by a high proportion of ethanol on the host living tissues. Following this alternative hypothesis, non-adapted fungal associates like *O. bicolor* would not succeed at this new host. Other beetle associates could benefit from lower competition levels to increase their abundance and frequency on the beetle-fungal assemblages. Given the small proportion of *Ophiostoma bicolor* in pupae from both tree species, this extent would be challenging to compare. However, since the ratio of unclassified fungi is lower in Sitka spruce, other fungi could have been negatively affected by different chemicals on Sitka spruce tissues. A recent study from Zhao (Zhao *et al.*, 2019) found significantly higher levels of defensive compounds when inoculating *Ips typographus* fungal associates like *G. penicillata* or *O. bicolor* compared to other fungi. Based on these findings, a higher relative proportion of *Grosmannia penicillata* in Sitka spruce could point out a lower number of defensive compounds synthesised by Sitka than Norway spruce. In this direction, it would be interesting to compare the proportion of fungal associates found in the phloem tissues of both host trees to try to find a correlation with those found in the beetle specimens.

It is important to note that we need to be cautious when interpreting these results, as the number of samples is very limited, and only those from pupae stages were analysed. It is, therefore, necessary to study these differences at a more extensive study, comprising a higher number of samples and a broader diversity of beetle life stages.

5.5.3 Fungal communities in flying beetles

Fungal communities recovered from flying beetles (those collected using traps) at Packing Wood were more abundant and diverse than those recovered from the developing beetles present in the galleries. Some of the fungi retrieved from these beetles were also present in the samples from uncolonised spruce wood. Therefore, when beetles came out of the galleries or fed on the trees, they are likely to have captured some of the fungi naturally present in the wood. A recent study showed that bark structures could act as an environmental filter, selecting specific fungal endophytes that would develop behind tree bark (Pellitier, Zak & Salley, 2019). Since fungal endophytes present in the spruce wood were also present in the beetle specimens, these are likely to be acquired from the tree. Moreover, the beetles may accidentally capture spores from fungi floating in the air, which may or may not be introduced into the wood when boring the galleries (Six, 2003).

Fungal communities also differed depending on the trap location, being much more diverse in Packing Wood than in the wider environment. Packing Wood was heavily affected by the eradication works carried out to destroy *Ips typographus* initial breeding galleries. Past studies have shown that anthropogenic degradation could produce more diverse fungal communities than those coexisting under natural conditions (Tolkkinen *et al.*, 2015). Therefore, the number of incidental fungi could be higher in Packing Wood, contributing towards richer and more diverse fungal communities captured by the beetles. If that is the case, the potential consequences of this pest management on fungal communities are highly unpredictable, considering that they may also affect fungal dissemination. Alternatively, these beetles could be capturing more diverse fungal communities because they could be crossing directly from Continental Europe. *Ips typographus* may disperse over several kilometres, even more under suitable wind conditions (Byers, 2000; Gries, 1985).

5.5.4 Potentially invasive fungi, beetle associates and fungal pathogens

Fungal communities recovered from flying beetles also harboured some fungi not previously encountered in the 1064 bark and ambrosia beetles previously analysed across the United Kingdom. These fungi included some generally known fungal associates of *Ips typographus*,

namely *Ophiostoma bicolor* and *Ophiostoma floccosum* (Jankowiak, Kacprzyk & Młynarczyk, 2009; Solheim, 1992a; Zhao *et al.*, 2019). Therefore, while some of the newly encountered fungi might be incidental, the most abundant are very likely to be associated with these beetles and vectored by them.

Ophiostoma bicolor is one of the most abundant fungal pathogens associated with *Ips typographus* (Giordano *et al.*, 2013; Sallé *et al.*, 2005; Zhao *et al.*, 2019). Although this fungus seems to be less aggressive than *Grosmannia* (Zhao *et al.*, 2019), significant associations among the presence of *O. bicolor* and beetle-damage levels have been found by past literature (Sallé *et al.*, 2005). Interestingly, *O. floccosum* has been tested as biocontrol against sap stain fungi (Held *et al.*, 2003). *Cyberlindnera jadinii* is a cosmopolite yeast that has been associated with nutritious functions in insect guts (Peng *et al.*, 1984), wood decomposition (Sibirny, 2019) and chemical cues useful for pest biocontrol (Leblanc, Vargas & Rubinoff, 2010). Therefore, this yeast could play different roles when associated with *Ips typographus*. Nevertheless, since this species has been found only in one specimen, this association could be merely incidental. This study did not find any beetle specimen harbouring *Endoconidiophora polonica*, which is considered one of the most abundant and aggressive *Ips typographus* fungal associates. However, I found considerable levels of a *Grosmannia penicillata* strain, which according to a recent study, could be more aggressive than *E. polonica* (Zhao *et al.*, 2019). This last author study suggested that *G. penicillata* could play a similar role to *E. polonica* attacking host tree tissues on beetle-fungal outbreaks.

Given that I have not previously analysed fungi present in any of the two other *Ips* species that are already present in the UK, *Ips sexdentatus* and *Ips cembrae*, I cannot state that these fungal species are non-native to the UK. Therefore, it would be advisable to analyse the fungal communities present in these other two *Ips* species to investigate whether they carry the same fungal species. It is also essential to consider that even if most of the fungal species harboured by *Ips typographus* may be native to the UK, the fungal strains may be non-native. This would be the case if there would be an exchange of fungal communities between the UK and France, given that the beetles could potentially fly across the English Channel. Previously unencountered pathogenic fungi may cause more robust defensive responses on the host trees than those previously co-evolved with the trees (Giordano *et al.*, 2013; Malacrinò *et al.*, 2017). Extreme defensive responses may favour beetle tree colonisation (Lieutier, Yart & Salle, 2009). Therefore, it is crucial to characterise these fungal communities and try to disentangle the origin of the *Ips typographus* that has arrived in the UK.

5.6. Conclusion and future directions

Molecular techniques represent a powerful tool to detect cryptic species of fungal pathogens that otherwise could not have been detected before causing a critical outbreak. In this case, the Ophiostomatoid fungi *Ophiostoma bicolor* and *Ophiostoma floccosum* were detected for the first time, along with the first breeding population of the bark beetle *Ips typographus* in the UK. These findings are very significant, as they point out the potential damage that this beetle can cause not only in Norway but also in Sitka spruce.

Therefore, our findings suggest that if this beetle population spreads across the UK, it can produce significant economic and ecological damage to British forests. It is important to keep studying how the associated fungal communities may affect the breeding success of the beetle and if this varies depending on the host tree. My work will inform future management decisions that will help to minimise the ecological and economic losses.

Future work will concentrate on using metabarcoding on further *Ips typographus* specimens, which were trapped in 2019 across the Southern part of the United Kingdom. By characterising the fungal communities on beetles trapped in these different forests, I aim to investigate whether these beetles came from a single origin and whether they carry any other potentially invasive fungi. I will compare *Ips typographus* fungal associates with those from twenty *Ips sexdentatus* specimens from the United Kingdom. I will also compare these fungal communities with those coexisting on *Ips typographus* samples collected from other European countries like Romania and Belgium. Likewise, I will keep characterising Ips fungal communities across different developmental stages for both spruce species since I understand that the analysis carried out in the present chapter is limited by the number of specimens analysed. Therefore, I aim to compensate for these limitations by increasing the number of analysed beetles, beetle specimens and locations.

Chapter 6. General Discussion

6.1. Thesis overview

The importance of metabarcoding as a tool to study beetle-associated fungal communities has been visible throughout this thesis. I have used this molecular technique to study the effects of different dominant host trees and forest compositions on fungal communities associated with native and non-native species. Using this cost-effective technique, a vast number of beetle-fungal interactions were unveiled. A deep fungal community characterisation was possible thanks to a refined methodology and the extensive fungal database of beetle associates I gathered, to the point that I was able to unveil potentially invasive fungi carried by *Ips typographus* into the United Kingdom. Applying this technique to invasive beetle species have the advantage of early detection of exotic or re-introduced fungi vectored by the beetle (Malacrinò *et al.*, 2017). Exotic fungi may become aggressive when reaching naïve host trees or establish new symbiosis with the local insect vectors (Desprez-Loustau *et al.*, 2007). Therefore, early detection becomes crucial to stop diseases from spreading before becoming pandemic. Thanks to my research, using bark beetles as fungal disease sentinels in healthier forests is now closer to become a reality. To carry out these kinds of surveys, it will be necessary to sample across different forest types and compositions to capture the greatest amount of variability in beetle-fungal communities. My results also have implications regarding forest management, which should be adapted in the face of climate change to ensure balanced and resilient fungal communities.

6.2. The role of forests on beetle-fungal associations

In chapters 2-4, I have carried out a range of experiments to disentangle the effect of forests on beetle-fungal associations. My thesis digs into the mechanisms underlying the correlation of fungal communities harboured by different bark beetle species across two different forest metrics: the dominant host tree identity and the forest composition. Overall, results show that temperate forests play a substantial role in shaping the fungal communities associated with bark and ambrosia beetles. Although fungal associates would primarily rely on the beetle species identity, the effect of dominant host trees and forest composition should not be disregarded.

My findings support the idea that forest composition is a proxy of forest biodiversity. Also, in line with this idea, Ćosović *et al.* (2020) found that the structural characteristics of a forest

strongly contribute to determining its biodiversity. My results suggest that different forest compositions contribute to fungal turnover in the same beetle species. Consequently, more diverse forests patterns could contribute to diversification in beetle-fungal associates. However, the underlying mechanisms explaining this trend remain elusive. Pellitier *et al.* (2019) have suggested that forests could act as environmental filters of fungal communities, harbouring a vast amount of endophytes. This filtering could be possible thanks to the different chemicals coexisting in tree tissues, contributing to fungi selection. A recent study has shown that phloem ethanol content may contribute to the beetle selecting certain fungi since certain ambrosia fungi would exhibit a higher ethanol-tolerance (Lehenberger, Benkert & Biedermann, 2021). These fungi could more efficiently process ethanol compared to others, benefiting beetle nutrition and performance. Tree endophytes may also be shared with beetles since I showed in Chapter 5 that an essential proportion of the fungi obtained from the wood of healthy spruce trees was also present in the bark beetles.

Simultaneously, bark and ambrosia beetles could distribute a vast number of fungi across the different tree species, both in living trees and deadwood. Consequently, these beetles could play an essential role in enhancing and re-distributing forest fungal biodiversity across the trees. The different mycangia exhibited by beetle species would also contribute to fungal selection (Mayers *et al.*, 2020; Mayers & Harrington, 2015). However, some studies suggested that fungal associations would be more promiscuous than previously thought (Kostovcik *et al.*, 2015; Skelton *et al.*, 2019a). A forest mediation could partially explain fungal promiscuity on beetle-fungal interactions. For example, pheromones emitted from specific yeasts could also act as chemical cues to attract or repel beetle species towards certain trees, linking certain fungi with certain tree species (Baig *et al.*, 2020b; Davis, 2015). Therefore, mycangia could not be as critical for symbiosis as previously thought, but instead, future studies should also focus on disentangling forest effects on each of the beetle species. These studies could help to adapt forest management to minimise the risk of specific beetle-fungal outbreaks.

According to the *Insurance Hypothesis* (McCann, 2000), more diverse forests would contribute towards the balance of the fungal communities carried by bark and ambrosia beetles. More diverse fungal communities coexisting with bark and ambrosia beetles could also enhance fungal competition and even contribute towards diluting the presence of fungal pathogens in beetle populations. After an extreme phenomenon like drought or wind storms, tree-fungal communities could also be disturbed along with the trees, contributing to modifying beetle-fungal communities. This way, the ecosystem balance could be broken, facilitating both beetle

proliferation and their attack on stressed trees with lower chemical and physical defences (McDowell *et al.*, 2011). This alteration on forest fungal communities could explain that bark and ambrosia beetles exhibit different fungal associates in healthy *versus* attacked forests (Giordano *et al.*, 2013).

6.3. Invasive species

Chapter 5 applies the methodology refined in previous chapters to an emerging threat: the invasion of the bark beetle *Ips typographus*. This beetle is causing massive outbreaks in central Europe, significantly affecting forest management perspectives and carbon sequestration (Jaakkola *et al.*, 2021; Hlásny *et al.*, 2021). My research showed that *Ips*-associated fungi formed complex communities, including 18 fungal OTUs not previously detected in other bark and ambrosia beetle species in the United Kingdom. As mentioned before, these communities also included endophytic fungi that were present in the nearby trees, suggesting a fungal acquisition carried out by this species. In agreement with my results, two other studies have revealed a fungal acquisition by beetles colonising new territories (Morales-Rodríguez *et al.*, 2021; Carrillo *et al.*, 2014). These studies suggested that such fungi could be acquired directly from the trees or laterally from other beetle species galleries.

The consequences of a widespread presence of *Ips typographus* in the United Kingdom would be more challenging to predict since this beetle harbours complex and potentially invasive fungal communities. It would also be challenging to determine whether the fungi carried by the beetle is native to the UK. Malacrino (2017) suggested that certain fungal strains could be reintroduced in the countries by exotic beetles. When new fungal species colonise new territories, it may affect the ecological niche of other species (Desprez-Loustau *et al.*, 2007). Given that UK trees could be naïve hosts to both the beetle and its fungal associates, there are even more reasons to monitor the beetles that access the country (Hulcr & Dunn, 2011; Wingfield *et al.*, 2017). By comparing *Ips* fungal communities with native fungi, we can obtain the OTUs belonging to potential exotic fungi, which could become invasive under certain conditions. Furthermore, we would probably be able to determine whether new strains reach the country since we have already seen that I was able to detect different OTUs belonging to the same fungus in the case of symbionts like *Ambrosiella hartigii*. This level of detail could allow us to trace whether new fungal strains get developed and transmitted. In the case of fungal pathogens, this could be an excellent opportunity to isolate and study the virulence of these exotic strains on native trees.

Less than 12% of the United Kingdom is covered by forests, a much lower proportion than Central Europe. Thus, bark beetle outbreaks could contribute to higher habitat fragmentation in this country compared to others. Despite not suffering from any recent attack, there have been significant bark beetle eruptions in the United Kingdom. Examples of this are the Dutch-Elm-Disease (*Ophiostoma novo-ulmi*), which is vectored by the bark beetle of the genus *Scolytus* (Swinton & Gilligan, 1996) and the great spruce bark beetle (*Dendroctonus micans*) (Grégoire, 1988; Fielding *et al.*, 1991). Elms have been decimated by the Dutch-Elm-Disease showing that the effects of a beetle-fungal outbreak can be massive also with scattered and isolated trees and that a weak and slow disease management is highly detrimental in the case of invasive species outbreaks (Tomlinson & Potter, 2010; Potter *et al.*, 2011). Invasive species could pose a higher risk of biodiversity loss than native species, including the loss of native bark and ambrosia beetles less frequently encountered in forests. For example, the ambrosia beetle *Platypus cylindrus*, which has been catalogued as rare (Hyman, Parsons, & Joint Nature Conservation Committee (Great Britain), 1994), rely on old oak forests for its development. Bark and ambrosia beetle outbreaks could decimate ancient forests, contributing to the loss of such rare species.

Independent of what vectors them, broad host range fungal pathogens represent a high threat, since their ability to “host jumping” makes their population dynamics highly unpredictable (Slippers, Stenlid & Wingfield, 2005). A remarkable example of a fungus able to colonise different host trees is sudden oak/larch death (SOD) *Phytophthora ramorum* (Webber, 2008). This fungus became a threat in the mid-1990s by attacking to oak in the United States and rododhendron in Europe, but in the United Kingdom, it is currently attacking larch trees (Grünwald, LeBoldus & Hamelin, 2019). Even if not directly transmitting the disease, bark and ambrosia beetles may benefit from the presence of weakened or dying trees to increase their populations (Jung *et al.*, 2013; Jung, 2009; McPherson *et al.*, 2008). The current surveys carried out to detect invasive fungi focus on present threats or the potentially invasive species suggested by horizon scanning techniques (Sutherland *et al.*, 2008). However, thousands of fungi remain unnoticed in their native range and may quickly get introduced to a new country through nurseries or timber trade (Desprez-Loustau *et al.*, 2007; Anderson *et al.*, 2004). For example, while surveying rhododendron plants searching for *Phytophthora ramorum*, another invasive species of *Phytophthora* was also detected (Schlenzig, Purser & Perez-Sierra, 2016). This species has not caused any previous disease outbreak, but it has been detected in the United Kingdom only because of the risk posed by *Phytophthora ramorum*. Therefore, there is a high need to find novel ways of fungal monitoring to ensure that the introduction of invasive fungi is noticed before their expansion in the new territory.

6.4. Management implications

Management implications suggested by the present study are clear: forest management should promote tree species diversity and mosaic forests, which would increase both beetle and fungi diversity and promote a healthier ecosystem balance. A previous study has suggested a correlation among monospecific forests and *Ips typographus* outbreaks in Central Europe (De Groot, Diaci & Ogris, 2019). However, in the United Kingdom the threat could become even worse, given that there are two spruce species broadly planted, forming extensive even-aged forests.

Recent findings showing that *Ips typographus* can develop on Sitka spruce are concerning, given the important proportion of spruce planted in the United Kingdom. This is another reason why I suggest that mixing these tree species with broadleaves could diminish the impact of a potential *Ips* outbreak. In past decades there has been a tendency of planting monospecific or mixed-conifer forests, which are traditionally more economically valuable for forest owners. In such forests, management and wood extraction become easier with even-aged and highly-valuable timber since fewer interventions are necessary to ensure proper tree development (Wilson, 2013). Since rotation lengths are typical of 35–45 years for both spruce species, a monospecific spruce forest planted now would remain the same for the next decades. Given the risk that spruce monocultures pose in the face of a potential outbreak, other conifers have been suggested to be planted to increase the conifer species mix without losing productivity (Savill *et al.*, 2017).

However, more and more studies suggest that mixed forests consisting of conifers and broadleaves are more resilient, especially in the face of climate change. Consequently, silvicultural designs now aim to generate mixtures consisting of conifers and broadleaf mixtures that increase forest biodiversity and resilience while maintaining productivity, for example, mixtures of spruce with oak or birch (Delphine *et al.*, 2021; Holmström *et al.*, 2021). While past studies focused only on crop productivity, now the focus also covers plant and insect biodiversity. By using molecular techniques, we could go beyond these superficial diagnostics to study the microbial communities on the same insects trapped at the surveys. This way, we could better control the biotic factors affecting forest health and determine whether this monitoring could help predict future outbreaks.

Disease management implications include the necessity of creating a more comprehensive collaboration framework across the countries to trace and monitor fungal diseases associated

with insect vectors using metabarcoding. With a shared public fungal database based on OTU identity besides traditional taxonomy, it would be easier to unveil new beetle-fungal associations and compare those present across the different countries. A cohesive and synchronised methodology could help to achieve a more cost-effective fungal community characterisation to reveal ecological patterns at larger scales. For example, upon the arrival of *Ips typographus* specimens in the UK, it has not been possible yet to determine whether the beetles were able to fly from France or they were transported along with raw materials like wood or stone shipments. Determining the origin of the invasive species becomes key to help horizon-scanning for techniques that predict further introductions or the spread of current pests (Matthews *et al.*, 2017). If we could know which fungi are currently being detected in French forests and compare them with those caught in the UK, we could more easily determine the origin of these beetles.

6.5. Broader implications

Bark beetle outbreaks cause immense disturbances in Central Europe. Negative outcomes of these disturbances are the loss of wood production and the lower carbon fixation rate. Forests usually act as carbon sinks, contributing to fixing carbon dioxide from the atmosphere. However, when an outbreak occurs, they may become carbon sources (Kautz *et al.*, 2017; Ghimire *et al.*, 2015). Recent findings suggested that the fungi associated with bark and ambrosia beetles would not act as wood decomposers but could even contribute towards the opposite by slowing wood decomposition (Skelton *et al.*, 2019b). Therefore, the role played by bark beetles in carbon sequestration could be influenced by the fungal communities they carry. Since beetle-fungi relations seem to be context-dependent and mediated by forests, my results suggest that there would be necessary to tests these hypotheses across different forest types and compositions to fully disentangle these interactions.

On the positive side, recent studies show that bark and ambrosia beetle outbreaks in temperate forests seem to benefit both insectivorous birds and arthropod biomass (Kortmann *et al.*, 2021; Przepióra, Loch & Ciach, 2020). Arthropod biomass appears to increase after a bark beetle outbreak, even for red-listed species (Beudert *et al.*, 2015), including saproxylic beetles (Müller *et al.*, 2010). Nevertheless, another study suggests that bark and ambrosia beetle outbreaks could be detrimental for saproxylic beetles since they rely on large trees to survive (Ranius & Jansson, 2000; Kozák *et al.*, 2021). Therefore, there are still significant gaps in knowledge regarding the effect of these outbreaks on forests. One of the reasons explaining this gap could

be the lack of long-term studies. Few researchers have addressed fungal dynamics before and after bark beetle outbreaks.

Moreover, there is a clear bias towards studying insects or vertebrates compared with microbial communities. Given that the role of microbial communities is crucial for ecosystems, I would advise carrying out long-term studies based on the monitoring of microbial communities using molecular techniques. The results of the fungal biodiversity surveys carried out in the United Kingdom can provide an insight into how fungal communities coexisting in healthy forests look like. This starting point to evaluate fungal dynamics should not be disregarded, but it should be taken as an opportunity to start monitoring fungal communities on a periodic basis. At least it would be advisable to carry out surveys in the same sampling sites in the case of an outbreak or an adverse climatic phenomenon to disentangle the ecological patterns behind the differences on those communities associated with the disease outbreak.

Previous findings had connected forests and bark beetles with extreme climatic events in the face of climate change, which could facilitate beetle outbreaks (Seidl *et al.*, 2016). However, the factors shaping this inter-connection remain poorly understood. Since climate change is a global phenomenon, there are even more reasons to study these kinds of phenomenon from a worldwide perspective or at least taking into account temperate forests as a whole, comparing the effects of the different management approaches carried by the other countries to come up with combined strategies for forest management.

6.6. Future directions

Future studies should include more profound research of the role of forests on beetle-fungal associations, with long-term monitoring over more expansive areas that include more abiotic variability and beetle species. To do so, I would suggest including different trapping methods and lures to obtain a broader scope of beetle species. Future works should also look at different beetle developmental stages, since fungal successions may play a key role to be able to understand disease epidemics (Nève Repe, Groot & Jurc, 2018; Linnakoski *et al.*, 2016; Solheim, 1992a). At the same time, I would advise extending this study to saproxylic beetles to ensure the proper management of the rare species before they disappear from our forests.

I hope that my research will serve as a base for future studies on detecting and monitoring invasive fungi. Such studies should evaluate the potential use of fungal metabarcoding in routine checks for detecting exotic bark and ambrosia beetles in imported wood samples. These

analyses could facilitate fungal risk assessments or even horizon scanning by generating a fungi database carried by each alien beetle species detected. This way, there will be more information available about the natural niche of the invasive beetle and fungi to predict and prevent future outbreaks.

The role of host tree identity should also be subjected to further research. My work is based on natural populations, where long-term and complex interactions may difficult to reach clear conclusions. In this direction, *in-vitro* could help study the effect of host trees on the fungal communities associated with bark and ambrosia beetles by minimising the number of interactions. These studies could use artificial galleries or sterilised logs to study the effect of host tissues on beetles with a narrowed number of inoculated fungal associates. Studies could also focus on the abundance and distribution of fungal associates across beetle populations and the effect of the different host trees on fungal associates' vertical transmission. Further studies could also investigate how differences in host trees could affect beetle gallery extension and how this could be translated into beetle survival and performance. The effect of forest structure and composition would be more difficult to disentangle using *in-vitro* studies. Still, it could be better assessed using the available long-term monitoring sites to monitor and further study beetle-associated fungal populations (Forestry Commission, 2004).

Future studies should also investigate whether the most aggressive invasive fungal pathogens could be horizontally transmitted across beetle species since this could determine the management of the local or invasive beetle populations once the fungal pathogen is detected in new territories. Additionally, broader surveys for fungal species could be carried out based on environmental DNA, which has proved to help detect microbes and pollen using air samples (Creer *et al.*, 2016; Ruppert, Kline & Rahman, 2019). It remains clear that multiple paths can be followed better to understand the underlying mechanisms of beetle-associated fungal communities and improve invasive species detection.

Potential shortfalls of the used methods include the lack of mock communities/ positive controls at laboratory stage, the use of OTUs instead of ASVs at the bioinformatic pipeline and the use of broader classification tools to identify the OTUs prior to the ecological analysis. Mock communities have been proved to be extremely efficient when dealing with potential PCR errors and artifacts generated at the sequencing step (Bakker, 2018). Furthermore, the use of these kinds of standardised positive controls could help to homogenise the read numbers to be able to better link read abundances with relative abundances within and across the samples and the

libraries (Egan *et al.*, 2018). When dealing with OTUs instead of ASVs there is a risk of missing out a number of crucial species that could have been revealed otherwise (Callahan, McMurdie & Holmes, 2017; Glassman & Martiny, 2018; Tipton *et al.*, 2021). The use of mock communities could have been preferable in combination with the ASVs to unveil some of the ecological patterns, or at least it could have been tested in Chapter 2 along with the different primer sets to corroborate its efficiency for these kinds of fungal metabarcoding data. Last, but not least, the combination of classifiers could have been more thoroughly implemented to ensure the correct fungal identification and even enhanced by using a custom curated database. This database could have been complemented with cultured fungi from bark and ambrosia beetles trapped across the UK, which could have been processed using sanger sequencing.

6.7. Concluding remarks

The field of forestry has been traditionally dominated by a short-term approach based on maximising timber profits rather than forest sustainability. However, in the past decades, this focus has switched towards improving forest resiliency in the face of uncertain events like those associated with climate change. Forests are now studied as ecosystems formed by interconnected organisms, where forest biodiversity goes beyond tree or plant diversity. This ecological approach represents the perfect ground for applying metabarcoding to characterise microbial communities.

My results corroborate that forest composition and the dominant tree species play a role in the fungal communities associated with bark and ambrosia beetles. These findings are essential in the face of climate change to prevent or mitigate future pest and disease outbreaks by designing more resilient forests. Despite the increasing research efforts, there are still many unsolved questions regarding the underlying mechanisms of beetle-fungal interactions, which may be better addressed by including every term in the equation. My thesis provides some answers and postulates the forest-related terms of this equation, but further studies will be necessary to complement my results. Last but not least, my work with *Ips typographus* marks a new path to detect invasive fungi by using metabarcoding on exotic bark beetles.

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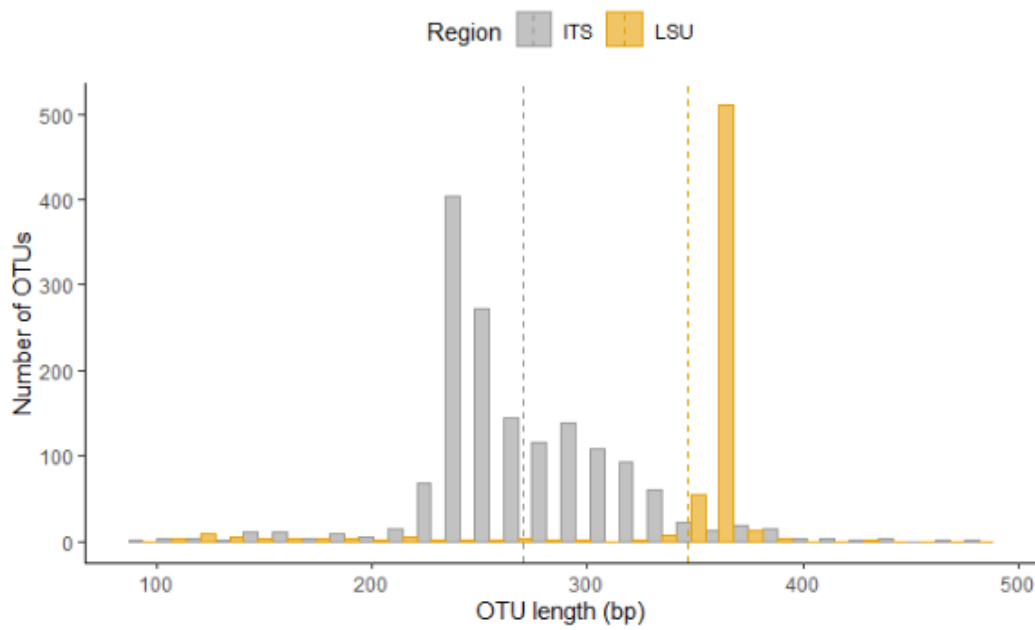
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APPENDICES

APPENDIX I. CHAPTER 2 SUPPLEMENTARY MATERIALS

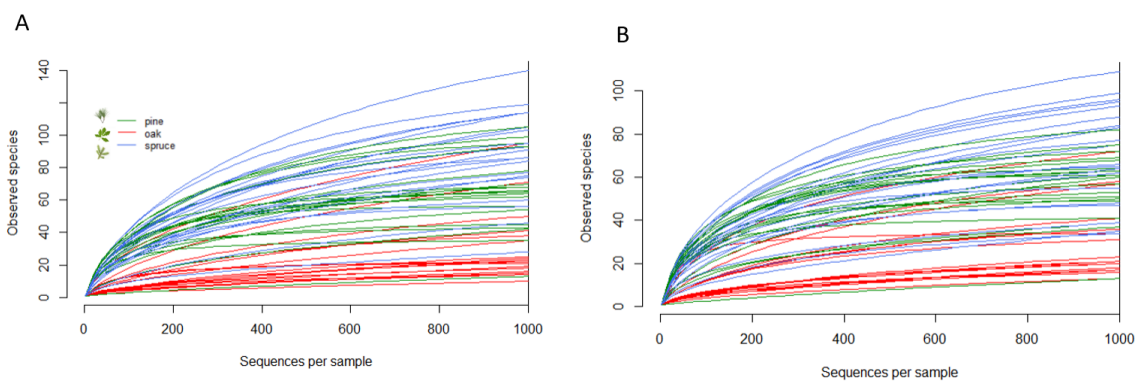


Supplementary Figure APP I-1. Length distribution of the OTUs generated from the ITS (grey) and LSU (orange) metabarcodes. The average length for each amplified region is indicated with a dashed line

Supplementary Table APP I-1. Number and proportion of OTUs belonging to the different classes detected by the ITS or LSU primers for the 73 beetles. Samples were rarefied to 1000 reads to make samples comparable.

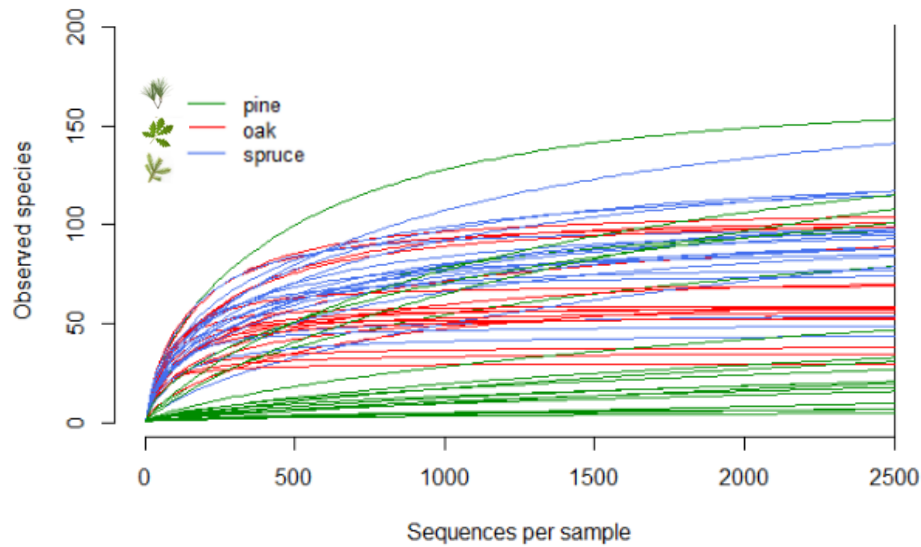
Class	Prop.OTUs.ITS	n.OTUs.ITS	Prop.OTUs.LSU	n.OTUs.LSU
Agaricomycetes	4.07	48	6.15	34
Agaricostilbomycetes	0.17	2	0.18	1
Archaeorhizomycetes	0.08	1	0	0
Chytridiomycetes	0.08	1	0	0
Cystobasidiomycetes	0.51	6	0.36	2
Dothideomycetes	10.08	119	5.61	31
Eurotiomycetes	4.24	50	2.17	12
Exobasidiomycetes	1.36	16	0.54	3
Lecanoromycetes	1.36	16	0.72	4
Leotiomycetes	7.88	93	1.99	11

Microbotryomycetes	1.61	19	0.36	2
Orbiliomycetes	0.17	2	0	0
Pezizomycetes	0.08	1	0.18	1
Pucciniomycetes	0.25	3	0.54	3
Saccharomycetes	3.14	37	1.08	6
Sordariomycetes	6.69	79	8.14	45
Spizellomycetes	0.08	1	0	0
Taphrinomycetes	0.76	9	0.18	1
Tremellomycetes	6.1	72	1.27	7
Tritirachiomycetes	0.08	1	0	0
Ustilaginomycetes	0.17	2	0	0
Wallemiomycetes	0.34	4	0.18	1
Atractiellomycetes	0	0	0.18	1
Unclassified	49.32	582	70.16	388
Total	100%	1546	100%	632

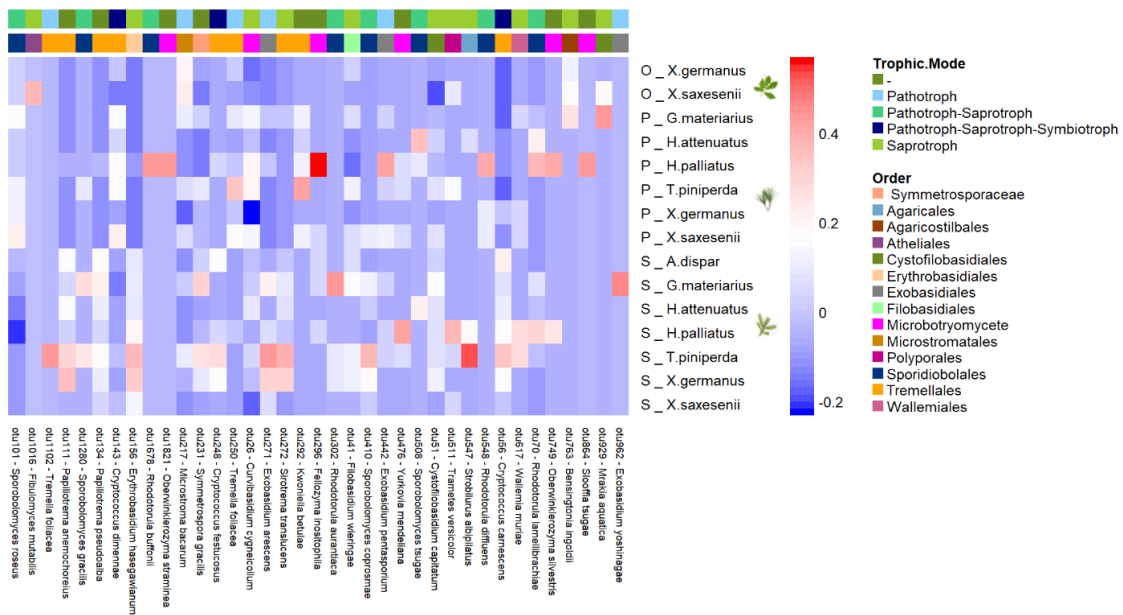


Supplementary Figure APP I-2. Individual-based species accumulation curves for ITS (A) and LSU (B) samples

APPENDIX II. CHAPTER 3 SUPPLEMENTARY MATERIALS



Supplementary Figure APP II-1. Rarefaction curves of compiled fungal OTU richness for each forest type. Rarefaction was conducted at 2500 reads. The 'specaccum' function ('Vegan' R package, 'random' method) was applied

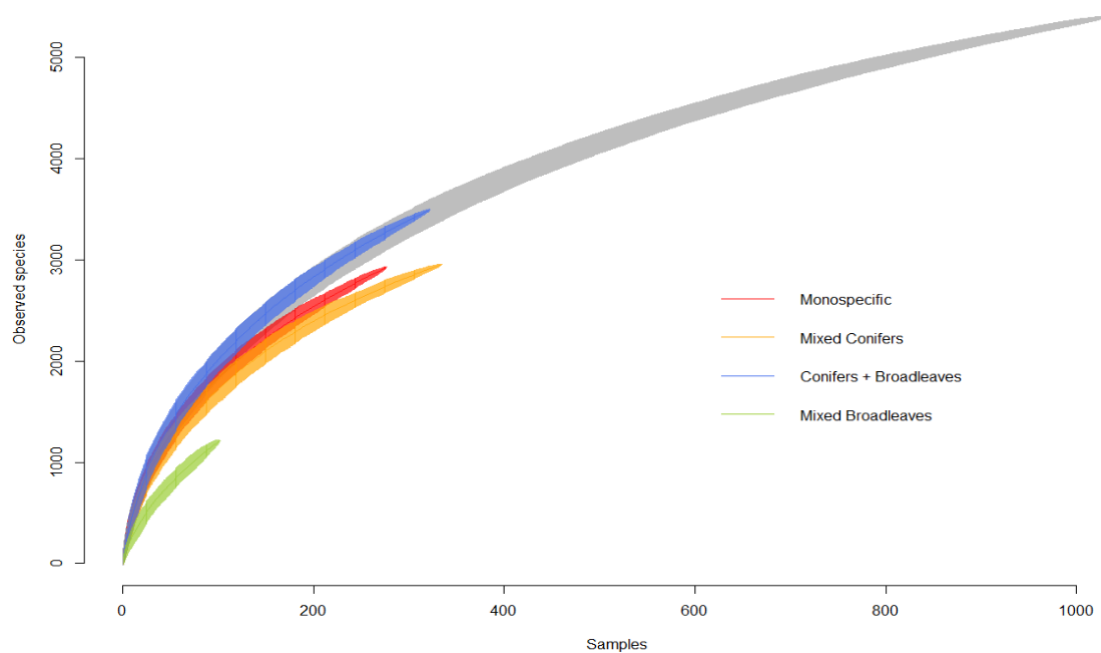


Supplementary Figure APP II-2. Frequency of association of fungal OTUs (p.g index) across the different beetle species and forest types. Only the identified OTUs from the phylum Basidiomycota are displayed

APPENDIX III. CHAPTER 4 SUPPLEMENTARY MATERIALS

Supplementary Table APP III-1. Sampling locations, including forest compositions and environmental variables of each location. Prim.tree/Sec.Tree: primary or secondary tree species. SP: Scots pine, CP: Corsican pine, SS: Sitka spruce, NS: Norway spruce, MB: Mixed broadleaves, MC: Mixed conifers, SC: Sweet Chesnut, BI: Birch. Mean.Annual.P: Mean annual precipitation, Mean.FS.T: Mean flying season temperature (°C), Mean.Annual.T: Mean annual temperature (°C), Growing.Season.Length: Length of the growing season (days), Median.FS.T: Median temperature of the flying season (°C), Per.Sec.Forest: Percentage of secondary forest. Per.Con: Percentage of conifers in the mix

Location	Latitude	Longitude	Forest type	Forest comp	Prim tree	Sec tree	Alt	Planting date	Mean Annual P	Mean FS.T	Mean Annual T	Growing Season Length	Median FS.T	Per Sec Forest	Per Con
Gairnhill	57.13121	-2.2411	SP / MC	MixCon	SP	MC	170	1958	851,87	11,6	8,33	282,13	12,15	40	100
Tentsmuir	56.42724	-2.84369	SP	Monosp	SP	NS	7	1927	694,17	12,43	9,09	298,07	13,04	5	100
Kinnell Wood	56.00143	-3.62945	SP / SS	MixCon	SP	SS	65	1950	925,84	12,66	9,21	305,63	13,21	50	100
Finlaystone Estate	55.92608	-4.60862	MC / MB	Mixed.CB	MC	MB	50	1960	1708,66	11,64	8,31	282,93	12,15	30	70
Ravensthorpe	54.91676	-1.64591	CP / MC	MixCon	MC	MB	165	1956	736,27	12,02	8,61	290,47	12,64	25	100
Gusborough Woods	54.51616	-1.04947	SP / SS	MixCon	SP	SS	300	1956	866,44	11,42	7,93	267,2	12,06	30	100
Knots Wood	54.05303	-2.75096	SP	Monosp	SP	MB	145	1961	1234,31	12,91	9,37	311,87	13,5	5	100
Mass Wood	53.51373	-3.01909	SP / MB	Mixed.CB	MB	SP	10	1950	818,14	13,67	10,19	324,5	14,3	50	50
Brooklands Estate	53.55456	-0.34178	SP / MB	Mixed.CB	SP	MB	75	1960	657,73	12,97	9,26	298	13,61	50	50
Laughton Forest	53.46781	-0.74263	SP	Monosp	CP	BI	5	1978	590,66	13,57	9,82	311,3	14,19	29	100
Ostlers Plantation	53.14526	-0.17525	CP	Monosp	CP	CP	20	1972	616,33	13,69	9,82	309,43	14,32	0	100
Mynydd Ennorch	51.611153	-3.7445237	CP / SS	MixCon	CP	SS	200	1970	1390,3	13,93	10,55	334,43	14,6	40	100
Cefn Mabry	51.55244	-3.14239	CP / MB	Mixed.CB	CP	MB	110	1970	1167,16	14,44	10,78	330	15,07	40	60
50 Acre Wood	51.44539	-2.66704	NS / MB	Mixed.CB	NS	MB	145	1957	951,68	13,64	10,17	326,07	14,33	35	65
Rendlesham Forest	52.07726	1.427016	CP / SP	MixCon	CP	SP	20	1969	619,23	14,12	10,3	320,7	14,81	15	100
Stour Wood	51.93777	1.187046	SC / MB	MixBroad	SC	MB	25	1970	572,84	14,24	10,38	318,93	14,9	35	0
Cobham	51.39115	0.435578	MB	MixBroad	MB	MB	110	1800	658,78	14,31	10,5	323,23	14,93	50	0
Darenth Wood	51.42633	0.263779	MB	MixBroad	MB	MB	85	1975	605,16	14,35	10,54	325,17	14,96	25	0
Wepham Woods	50.85348	-0.50245	MB / NS	Mixed.CB	MB	MC	50	1950	840,75	13,97	10,36	320,27	14,64	20	20
Wareham Forest	50.74152	-2.17453	CP	Monosp	CP	SP	30	1971	878	13,97	10,53	331,07	14,63	15	100



Supplementary Figure APP III-1 Species accumulation curves of compiled fungal OTU richness for each forest type. Rarefaction was conducted at 2500 reads. The 'specaccum' function ('Vegan' R package, 'random' method) was applied, displaying the confident intervals as bars. The grey bar represent the whole community.

Supplementary Table APP III-2. Estimated regression parameters, standard errors, z-values and P-values for the Negative binomial GLM, which had the Chao1 estimate as a response variable and several biotic and abiotic factors as dependent variables. Only the significant variables ($p < 0.05$) are displayed

	Estimate	Std.error	z-value	p-value
Intercept	3.082	0.293	10.506	<0.001
Beetle species:				
<i>Dryocoetes autographus</i>	0.592	0.107	5.541	<0.001
<i>Hylurgops palliatus</i>	0.490	0.1007182	4.868	<0.001
<i>Hylesinus varius</i>	0.494	0.137	3.583	<0.001
<i>Tomicus piniperda</i>	0.615	0.102	6.067	<0.001
Forest composition:				
Conifers + Broadleaves	-0.123	0.044	-2.804	<0.01
Mixed Broadleaves	-0.499	0.084	-5.929	<0.001
Longitud	0.040 *	0.018	2.234	<0.05
Mean annual precipitation	0.001	0.001	4.420	<0.001
Mean temperature of the summer season	0.060	0.021	2.879	<0.01

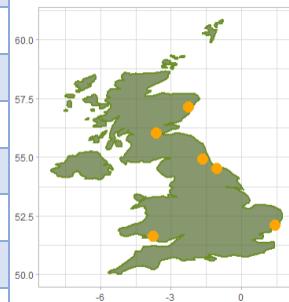
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Supplementary Table APP III-3. Results from PERMANOVA modelling fungal community composition using the Jaccard dissimilarities. P-values were obtained using 999 permutations under a reduced model. Beetle species identity (*Insect_id*) had the largest effect on fungal community abundance

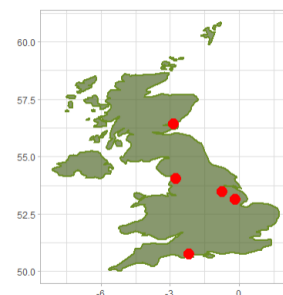
Jaccard index	Df	SumOfSqs	R2	F	Pr(>F)
Forest.comp	3	9.655	0.025	8.257	0.001
Insect_id	11	20.807	0.053	4.853	0.001
Longitude	1	3.027	0.008	7.768	0.001
Latitude	1	2.655	0.007	6.811	0.001
Altitude	1	1.719	0.004	4.411	0.001
Mean.Annual.P	1	1.387	0.004	3.559	0.001
Mean.FS.T	1	1.861	0.005	4.774	0.001
Per.Conifers	1		0.003	3.318	0.001
Unexplained			0.892		

Supplementary Table APP III-4. Results from Chao1 modelling richness and PERMANOVA modelling fungal community composition on each of the forest compositions. PERMANOVA used Bray Curtis dissimilarities and P-values were obtained using 999 permutations under a reduced model ("n.s." stands for "not significant"). Beetle species identity (*Insect_id*) had the largest effect on fungal communities. Sites distribution for each of the forest compositions is shown in each of the maps

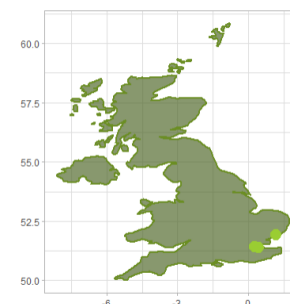
Monospecific (n=248)	Chao1 richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Insect_id	12	0.356	41.9	0.482	21.03	0.001
Longitude	1	n.s.	0.7	0.009	4.14	<0.01
Latitude	1	n.s.	0.5	0.006	2.70	<0.1
Altitude	1	n.s.	1.2	0.0014	6.74	0.001
Mean.Annual.P	1	0.0594	0.6	0.007	3.16	<0.01
Mean.FS.T	1	n.s.	-	-	-	n.s.
Forest type	1		0.6	0.007	3.16	<0.01
Unexplained			42	0.483		



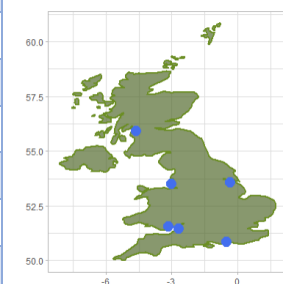
Mixed Conifers (n=319)	Chao1 richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Insect_id	10	0.1571	37.9	0.345	16.55	0.001
Longitude	1	0.0276	-	-	-	n.s.
Latitude	1	n.s.	-	-	-	n.s.
Altitude	1	n.s.	-	-	-	n.s.
Mean.Annual.P	1	0.0702	1.7	0.015	7.23	0.001
Mean.FS.T	1	0.045	-	-	-	n.s.
Unexplained			70.4	0.64		



Conifers + Broadleaves (n=290)	Chao1 richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Insect_id	11	0.3814	33.5	0.310	11.85	0.001
Longitude	1	n.s.	1.1	0.01	4.33	0.001
Latitude	1	n.s.	0.8	0.007	2.93	<0.01
Altitude	1	0.0374	0.6	0.006	2.53	0.01
Mean.Annual.P	1	n.s.	1.4	0.013	5.18	0.001
Mean.FS.T	1	n.s.	0.7	0.006	2.56	<0.01
Unexplained			70.1	0.650		

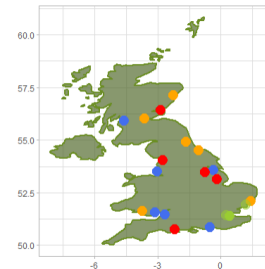


Mixed Broadleaves (n=97)	Community richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Insect_id	4	0.5891	18.0	0.493	22.4	0.001
Longitude	1	n.s.	-	-	-	n.s.
Latitude	1	0.0350	-	-	-	n.s.
Altitude	1	n.s.	-	-	-	n.s.
Mean.Annual.P	1	n.s.	-	-	-	n.s.
Mean.FS.T	1	n.s.	-	-	-	n.s.
Unexplained			18.5	0.507		

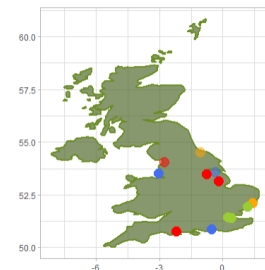


Supplementary Table APP III-5. Results from Chao1 modelling richness and PERMANOVA modelling fungal community composition on specimens of *Hylurgops palliatus* and *Xyleborinus saxesenii*. PERMANOVA used Bray Curtis dissimilarities and p-values were obtained using 999 permutations under a reduced model ("n.s." stands for "not significant"). The forest composition had the largest effect on fungal communities in both species. Site distribution for each of the beetle species is shown in each of the maps

<i>Hylurgops palliatus</i> (n=177)	Community richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Forest composition	3	n.s.	4.5	0.093	6.12	0.001
Longitude	1	n.s.	0.6	0.013	2.53	<0.1
Latitude	1	n.s.	1.3	0.027	5.32	<0.01
Altitude	1	n.s.	-	-	-	n.s.
Mean.Annual.P	1	n.s.	-	-	-	n.s.
Mean.FS.T	1	n.s.	-	-	-	n.s.
Unexplained			41.7	0.867		



<i>Xyleborinus saxesenii</i> (n=88)	Community richness		Community composition			
	Df	Nagelkerke's R2	SumOfSqs	R2	PseudoF	Pr(>F)
Forest composition	3	0.670	3.13	0.121	3.98	0.001
Longitude	1	n.s.	0.46	0.018	1.75	<0.1
Latitude	1	n.s.	0.39	0.015	1.49	<0.1
Altitude	1	n.s.	-	-	-	n.s.
Mean.Annual.P	1	n.s.	0.53	0.021	2.03	<0.01
Mean.FS.T	1	0.103	0.39	0.015	1.48	<0.1
Unexplained			21.01	0.811		



APPENDIX IV. CHAPTER 5 SUPPLEMENTARY MATERIALS

Supplementary Table APP IV-1. Collected Ips typographus specimens from each category and log/trap

Collection date	Collected from	Category	Log/Trap ID	Specimens
10/06/2019	Norway Spruce	Larvae	Billet 5	4
10/06/2019	Norway Spruce	Adult	Billet 5	5
10/06/2019	Norway Spruce	Mature Adult	Billet 5	5
10/06/2019	Norway Spruce	Pupae	Billet 4	5
10/06/2019	Norway Spruce	Adult	Billet 4	5
10/06/2019	Sitka Spruce	Pupae	E6	5
10/06/2019	Norway Spruce	Adult	E4	5
10/06/2019	Norway Spruce	Adult	Billet 6	5
03/06/2019	Funnel trap	Flying adult	TK1	5
10/06/2019	Funnel trap	Flying adult	WE10	1
10/06/2019	Funnel trap	Flying adult	WE09	1
10/06/2019	Funnel trap	Flying adult	WE35	1
10/06/2019	Funnel trap	Flying adult	WE08	1
			Total:	48

Supplementary Table APP IV-2. DNA extraction methods and for the DNA extraction from spruce wood along with the DNA quantification. NS= Norway spruce, SS=Sitka spruce

Sample	Extraction method	Tree sp.	Wet/Dry	Tissue(mg)	Qbit	Nanodrop	260/230
NS_A_W_60	QUIAGEN Plant mini kit	NS	W	60	5.04	5.409	-0.59
NS_A_D_EZ	EZNA plant DS kit	NS	D	10	6.66	6.071	0.09
NS_A_W_EZ	EZNA plant DS kit	NS	W	30	13.8	16.2	0.04
SS_A_W_EZ	EZNA plant DS kit	SS	W	40	5.4	8.154	0.03

Supplementary Table APP IV-3. Results for significant indicator OTUs from multi-level pattern analysis (Speciesind package. r.g statistic). "Association" refers to sample type fungi were correlated with. "Trophic Mode" correspond to the FUNguild classification.

Association	Trophic.Mode	OTU.ID	Taxonomy	r.g .stat	p.value
Pupae	-	otu174	<i>Cyberlindnera fabianii</i>	0.514	0.05 *
Mature adult	-	otu32	<i>Ophiostoma canum</i>	0.944	0.005 **
	-	otu857	<i>Myxozyma melibiosi</i>	0.62	0.010 **
	-	otu22	<i>Peterozyma toletana</i>	0.606	0.020 *
	-	otu105	-	0.702	0.005 **
	Symbiotroph	otu630	-	0.538	0.025 *
Filial adult + Pupae + Mature adult	Pathotroph	otu234	-	0.522	0.045 *
Larvae + Pupae + Filial adult + Mature adult	-	otu7	<i>Grosmannia penicillata</i>	0.538	0.03 *
Mature adult + Flying adult	-	otu9	<i>Yamadazyma tenuis</i>	0.641	0.005 **
	Pathotroph	otu494	<i>Ophiostoma bicolor</i>	0.544	0.010 **
Larvae + Filial adult + Mature adult + Flying adult	Pathotroph	otu234	-	0.553	0.010 **
Larvae + Pupae + Filial adult + Flying adult	-	otu48	<i>Ogataea ramenticola</i>	0.642	0.010 **
Larvae + Pupae + Filial adult + Mature adult + Flying adult	Saprotroph-Symbiotroph	otu26	<i>Kuraishia capsulata</i>	0.938	0.005 **
	Saprotroph	otu11	<i>Wickerhamomyces bisporus</i>	0.845	0.005 **
	Pathotroph	otu14	-	0.705	0.005 **
	Saprotroph	otu84	<i>Saccharomycopsis lassenensis</i>	0.542	0.025 *
Flying beetles	-	otu172	<i>Filobasidium wieringae</i>	0.542	0.030 *
	-	otu54	-	0.543	0.025 *
	-	otu1346	<i>Filobasidium oeirensae</i>	0.542	0.025 *
	-	otu282	-	0.439	0.025 *
	-	otu310	-	0.439	0.025 *
	-	otu331	<i>Dioszegia hungarica</i>	0.439	0.025 *
	-	otu560	<i>Cytospora ribis</i>	0.439	0.025 *
	Pathotroph-Saprotroph	otu563	<i>Selenophoma eucalypti</i>	0.439	0.025 *
	-	otu64	<i>Penicillium citreonigrum</i>	0.439	0.025 *
	-	otu79	-	0.439	0.025 *
Flying beetles + Wood	-	otu16	<i>Penicillium glaucoalbidum</i>	0.796	0.005 **
	Pathotroph	otu13	<i>Vishniacozyma victoriae</i>	0.746	0.005 **
	-	otu34	<i>Alternaria infectoria</i>	0.664	0.005 **
	-	otu2	<i>Cladosporium perangustum</i>	0.568	0.010 *

	Pathotroph	otu147	<i>Taphrina carnea</i>	0.557	0.020 *
	Pathotroph	otu17	<i>Aureobasidium pullulans</i>	0.557	0.020 *
	-	otu21	<i>Curvibasidium cygneicollum</i>	0.557	0.015 *
	Symbiotroph	otu86	<i>Sporobolomyces roseus</i>	0.557	0.020 *
Wood	-	otu1195	-	1	0.005 **
	-	otu1308	-	1	0.006 **
	-	otu199	-	1	0.007 **
	-	otu216	<i>Penicillium decaturense</i>	1	0.008 **
	-	otu334	<i>Pestalotiopsis funerea</i>	1	0.009 **
	-	otu521	<i>Zalerion arboricola</i>	1	0.010 **
	-	otu594	<i>Umbelopsis isabellina</i>	1	0.011 **
	-	otu862	-	1	0.012 **
	-	otu947	<i>Lepraria incana</i>	1	0.013 **
	-	otu635	<i>Zalerion arboricola</i>	0.971	0.014 **
	-	otu122	-	0.938	0.015 **
	-	otu30	<i>Penicillium virgatum</i>	0.938	0.016 **
	-	otu36	<i>Penicillium bialowiezense</i>	0.913	0.017 **
	-	otu1011	-	0.845	0.018 **
	-	otu110	<i>Penicillium glabrum</i>	0.845	0.019 **
	-	otu1158	-	0.845	0.020 **
	-	otu1177	-	0.845	0.021 **
	-	otu1448	<i>Wilcoxina mikolae</i>	0.846	0.022 **
	-	otu1581	-	0.845	0.005 **
	-	otu1590	-	0.845	0.005 **
	-	otu1626	<i>Umbelopsis isabellina</i>	0.845	0.005 **
	-	otu1643	-	0.845	0.005 **
	-	otu1743	<i>Phialemonium inflatum</i>	0.845	0.005 **
	-	otu178	-	0.845	0.005 **
	-	otu1809	-	0.845	0.005 **
	-	otu2495	<i>Leucosporidium fellii</i>	0.845	0.005 **
	-	otu335	-	0.845	0.005 **
	-	otu3411	-	0.845	0.005 **
	-	otu3812	-	0.845	0.005 **
	-	otu414	<i>Angustimassarina acerina</i>	0.845	0.005 **
	-	otu442	<i>Exobasidium bisporum</i>	0.845	0.005 **
	Pathotroph	otu473	<i>Rhodotorula phylloplana</i>	0.845	0.005 **

	-	otu532	<i>Hysterobrevium constrictum</i>	0.845	0.005 **
	-	otu5368	-	0.845	0.005 **
	-	otu546	<i>Pseudogymnoascus pannorum</i>	0.845	0.005 **
	-	otu562	-	0.845	0.005 **
	-	otu674	-	0.845	0.005 **
	-	otu898	-	0.845	0.005 **
	Pathotroph-Saprotroph-Symbiotroph	otu1691	<i>Sarea resinae</i>	0.811	0.005 **
	-	otu81	-	0.78	0.005 **
	-	otu104	<i>Mycosphaerella punctiformis</i>	0.774	0.005 **
	-	otu1450	<i>Umbelopsis ramanniana</i>	0.774	0.005 **
	-	otu163	-	0.774	0.005 **
	-	otu183	<i>Cryptococcus tephrensensis</i>	0.774	0.005 **
	-	otu19	<i>Ramularia endophylla</i>	0.774	0.005 **
	-	otu38	<i>Botrytis caroliniana</i>	0.774	0.005 **
	-	otu4	<i>Sydowia polyspora</i>	0.774	0.005 **
	-	otu80	<i>Tausonia pullulans</i>	0.774	0.005 **
	-	otu354	<i>Selenophoma eucalypti</i>	0.745	0.005 **
	-	otu1026	<i>Phialocephala scopiformis</i>	0.674	0.010 **
	-	otu1178	-	0.674	0.010 **
	-	otu1183	-	0.674	0.020 *
	-	otu1261	<i>Cystobasidium pinicola</i>	0.674	0.010 **
	-	otu1333	-	0.674	0.020 *
	-	otu1387	<i>Ciliolarina pinicola</i>	0.674	0.010 **
	-	otu1459	-	0.674	0.015 *
	-	otu1537	-	0.674	0.020 *
	-	otu1575	-	0.674	0.020 *
	-	otu1598	<i>Proliferodiscus dingleyae</i>	0.674	0.020 *
	-	otu1650	-	0.674	0.010 **
	-	otu1718	-	0.674	0.020 *
	Pathotroph	otu1785	<i>Orbilia euonymi</i>	0.674	0.020 *
	-	otu1802	-	0.674	0.015 *
	-	otu1834	-	0.674	0.020 *
	-	otu1956	-	0.674	0.020 *
	-	otu2095	-	0.674	0.010 **
	-	otu2115	-	0.674	0.015 *

	-	otu2205	<i>Cyphellophora sessilis</i>	0.674	0.020 *
	-	otu2403	-	0.674	0.010 **
	-	otu2538	<i>Bensingtonia yuccicola</i>	0.674	0.020 **
	-	otu2578	<i>Micarea czarnotae</i>	0.674	0.010 **
	-	otu2618	-	0.674	0.015 *
	-	otu266	-	0.674	0.020 *
	Pathotroph-Symbiotroph	otu2733	-	0.674	0.020 *
	-	otu3315	<i>Micarea micrococca</i>	0.674	0.020 *
	-	otu3423	<i>Lecanora expallens</i>	0.674	0.020 *
	-	otu357	<i>Penicillium brevicompactum</i>	0.674	0.020 *
	-	otu3627	-	0.674	0.020 *
	-	otu3888	-	0.674	0.015 *
	-	otu4185	-	0.674	0.015 *
	-	otu431	<i>Penicillium angulare</i>	0.674	0.015 *
	-	otu4590	-	0.674	0.020 *
	-	otu513	<i>Exobasidium arescens</i>	0.674	0.020 *
	-	otu543	-	0.674	0.020 *
	-	otu570	-	0.674	0.020 *
	-	otu690	<i>Niesslia ilicifolia</i>	0.674	0.020 *
	-	otu784	-	0.674	0.010 **
	-	otu1234	-	0.633	0.025 *
	-	otu281	-	0.589	0.040 *
	Pathotroph-Saprotroph	otu33	<i>Pseudocercospora fraxini</i>	0.589	0.050 *