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Predicting the unpredictable: How host specific is the mycobiota of bark and ambrosia beetles?

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ABSTRACT

Bark and ambrosia beetles (Curculionidae: Scolytinae) are known for their symbioses with fungi and play a key role in the dispersal of phytopathogens. The scolytine community of eight pine stands along a latitudinal gradient in the UK was surveyed and beetle-associated fungal communities (mycobiota) were assessed using ITS2 metabarcoding (304 specimens, 12 species). Distribution patterns among 2,257 detected fungal Operational Taxonomic Units (OTUs) revealed that beetle species identity was an important predictor of mycobiotic richness and composition, while the effects of environmental and spatial variables were negligible. Network-based specificity analysis suggested that a relatively small subset of OTUs (75 in total) exhibit an affinity for a subset of beetle species and that these include many Microascales and Saccharomycetes. Notably though, of the OTUs belonging to the family Ophiostomataceae, relatively few display host specificity. Our results add to the complex picture of host-associated fungal communities and suggest that host range limits are unlikely to restrict the spread of economically important phytopathogens.

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1. Introduction

Fungi display a diverse range of functional roles within forest ecosystems as decomposers, facilitators and phytopathogens. Some associations between fungi and trees are facilitated by interactions with bark and ambrosia beetles (Curculionidae: Scolytinae) (Paine et al., 1997; Six, 2012). While the majority of these beetle species feed upon dead or dying trees, some favour the phloem of living trees and, in partnership with their fungal symbionts, can overcome plant defenses (Brasier, 1991; Paine et al., 1997). The associations between bark and ambrosia beetles and fungi are usually multipartite, range from mutualistic to parasitic, obligate to facultative, and also comprise transient, non-symbiotic associations (Six, 2012; Hulcr et al., 2015). Together, these insect-fungus partnerships can shape forest ecosystems by facilitating wood degradation and increasing tree disease prevalence and mortality (Kirisits, 2004). As such, fungal-beetle systems can lead to the devastation of huge stands of trees and entire arboreal species at continental scales. The fungal order Ophiostomatales includes some of the most destructive species, for example the blue stain *Grosmannia* attacking various pine species in North America and Europe (Krokene and Solheim, 1998), the *Ophiostoma novo-ulmi* pathogen causing Dutch Elm Disease (Brasier, 1991), or the more recent outbreak of the laurel wilt *Raffaelea* vectored by *Xyleborus glabratus* in southeastern North America (Fraedrich et al., 2015). Each of these epidemics has had significant impacts through the loss of unique ecosystems and associated species, large-scale carbon release, and effects on local economies.

Due to their small size and ease of transport, bark and ambrosia beetles comprise a high proportion of alien invasive species; over 70 bark and ambrosia beetle species have been reported as invasive for North America and Europe alone (Haack, 2006; Kirkendall and

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Faccoli, 2010). In some cases, fungal phytopathogens are able to switch between beetle hosts upon entering a non-native range, forming a new association with a different beetle species. This was observed during the spread of the Dutch Elm Disease fungus, *O. novo-ulmi* and as a result of host switching, this fungus can be carried by 11 species of *Scolytus*, allowing its rapid dissemination throughout both North America and Europe in the 1960s (Brasier, 1991; Webber, 2000). Given the propensity of bark and ambrosia beetles to invade new ranges, host switching is of particular concern in this system. Indeed, a recent study found evidence of host switching between fungal symbionts that were previously believed to be highly specific and that phylogenetic distance is likely to influence the probability of this phenomenon occurring (Skelton et al., 2019). The degree to which beetle host identity governs fungal communities is essential knowledge for predicting host switching events as well as projecting range expansion and epidemic outbreaks.

In addition to the dependence on their scolytine hosts, fungal communities respond to other biotic, abiotic and spatial variables. Dead-wood associated fungi show significant tree species preferences (Purahong et al., 2018), and investigations into arbuscular mycorrhizal fungi suggest that temperature, sunshine hours (Dumbrell et al., 2011) and rainfall (Öpik et al., 2006; Hazard et al., 2013) all have a significant influence on community composition. Further, a recent large-scale global analysis of soil mycobiota revealed that climatic variables, particularly precipitation, were the dominant determinants of community composition (Tedersoo et al., 2014). The local environment is also likely to influence the mycobiota of insects, even if the effects may be constrained by symbiotic interactions.

Many studies investigating scolytine-fungus interactions have focused upon economically important beetles with a defined taxonomic and geographic focus, or those that can be cultured (Cassier et al., 1996; Hulcr et al., 2007; Cardoza et al., 2009; Endoh et al., 2011; Biedermann et al., 2012). While shedding light on potentially harmful pest species, this approach has left us with a somewhat limited perspective of the community level beetle-fungal system, which has made it difficult to apply quantitative comparisons to separate drivers of these beetle mycobiota (Persson et al., 2009; Six, 2012).

Metabarcoding with fungal-specific ITS primers can assay fungal assemblages directly from the genomic DNA of an individual beetle or from the isolated mycangia, allowing rapid and relatively inexpensive molecular analysis of entire fungal communities associated with each specimen (Miller et al., 2016; Jacobsen et al., 2017; Malacrinò et al., 2017). Studies employing this approach have already revealed a much greater species diversity than expected from non-DNA based studies (Kostovcik et al., 2015; Miller et al., 2016). Here, we examine the mycobiota of bark and ambrosia beetles present in pine plantations along a north-south transect of the United Kingdom, to test for the partitioning of fungal communities within and between 12 scolytine species, in addition to the effects of biogeographic and environmental factors that may determine the turnover of fungal communities. The methodology permits the analysis of composition and host affinity for a large proportion of the fungal community, as well as a detailed study to examine which fungal taxonomic groups may be driving the community-level patterns. While it has been demonstrated that different beetle species display variation between their mycobiota, the relative importance of host identity and environmental conditions has not been previously explored in this system. We expect there to be an effect from each of these drivers and anticipate the variety of interactions as well as the diversity of fungal species to be reflected in the complexity of the communities uncovered.

2. Methods

2.1. Study sites and collecting protocol

Beetle specimens were collected from eight localities within UK Forestry Commission plantation forests during the summer of 2013 (Supplementary Data S1 and S2a). All sites comprised mature Corsican (*Pinus nigra*) and Scots pine (*Pinus sylvestris*) or a mix of the two, planted between 1952 and 1977 without recent thinning, and were selected to be typical of pine production forests across the UK. To reduce the incidence of non-pine specialists within catches, all trapping localities were within pine stands of at least 65 ha and were surrounded by other pine stands.

For each site, five receding Lindgren multiple-funnel traps (Pherotec, Victoria, Canada) were set up at 50 m intervals along a 250 m transect (see Supplementary Data S2b and S3 for details). Traps were baited with alpha-pinene and 100% ethanol, both of which are effective attractants for saproxylic beetles (McIntosh et al., 2001). Ethanol was placed in collecting vials at the base of each trap, where it acted both as a lure and as a preservative for collected specimens. Traps were emptied once a week for 15 weeks between May and August. At each site all beetle specimens from all five traps were pooled together into fresh 100% ethanol and stored at −20 °C until processing.

2.2. DNA extraction and sequencing

Morphological identification of scolytines was conducted using standard identification keys (Duffy, 1953; Grüne, 1979) and confirmed using molecular sequence data. Specimens from the 12 most abundant beetle species were selected for mycobiome analysis. Between 6 and 70 individuals from between 4 and 8 sites (except *Xylosandrus germanus* from 1 site) were analysed from each species (Table 1).

To reduce the risk of cross-contamination between specimens within collection vials, we attempted to dislodge loose fungal spores by vortexing each beetle twice in fresh 70% ethanol for 30 s. Since we were interested in the composition of the fungi attached to the cuticle of these specimens we did not surface sterilise, but to assess the level of cross-contamination we tested for an effect of the collection vial on the similarity of beetle mycobiota. PERMANOVA analysis suggested that there was no such effect (see Supplementary Data S4 for results) so while cross-contamination cannot be ruled out entirely, we are confident that the effect on our results and conclusions was negligible.

Prior to DNA extraction, the thorax of each beetle specimen was separated from the abdomen using sterilised forceps and both body parts were placed into a single well of a 96-well plate. One well of each plate was left empty as a negative control. DNA extractions were carried out for each beetle specimen individually using the Biosprint 96 Blood and Tissue extraction protocol (Qiagen, Valencia, CA). The ITS2 region was amplified using the fungus-specific, tagged primers ITS3 and ITS4 (White et al., 1990), which have low affinity for the corresponding locus in insects. Three separate PCRs were conducted for each specimen, each at a different annealing temperature (50, 53, 56 °C), and resulting amplicons were combined for sequencing (see Supplementary Data S5 for PCR cycling conditions). Extraction blanks and PCR blanks were used as negative controls. Library preparation was conducted separately on all amplicons corresponding to a single specimen using KAPA library kits (Kapa Biosystems), although the initial cleaning steps were modified to retain large DNA fragments and reduce amplicon loss. Sequencing was conducted on an Illumina MiSeq v. 3 (2 x 300 bps, paired-end) at the Natural History Museum Sequencing Facility.

Table 1
Details of the beetle species and specimens included in fungal community analysis with information regarding the mycangia (cuticular invaginations used to carry associated fungal spores and mycelia). possessed by each species.

	Beetle Species	Description of Mycangial Structure	Number of specimens	Number of sites
Bark Beetles	*Hylastes ater*	Primative mycangia - secretion filled punctures in integument especially on the elytra, both sexes (Kirisits, 2004)	41	5
	Hylastes attenuatus	No known mycangia	39	4
	Hylastes opacus	No known mycangia	11	7
	Hylurgops palliatus	Primative mycangia - secretion filled punctures in integument especially on the elytra, both sexes (Kirisits, 2004)	27	8
	Pityogenes bidentatus	No known mycangia	11	7
	Pityophthorus pubescens	No known mycangia	12	6
	Tomicus piniperda	No known mycangia	51	8
Ambrosia Beetles	*Trypodendron domesticum*	Prothoracic-pleural mycangia (Batra, 1963)	6	4
	Trypodendron lineatum	Prothoracic-pleural mycangia (Batra, 1963)	12	6
	Anisandrus dispar	Prothoracic-pleural mycangia (Batra, 1963)	39	5
	Xylosandrus germanus	Mesonotal mycangia (Hulcr et al., 2012)	14	1
	Xyleborinus saxeseni	Elytral mycangia (Batra, 1963)	41	6

2.3. Bioinformatic processing

All libraries were quality checked using FastQC (Andrews, 2010) before merging with Fastq-join at a minimum similarity of 99% > 50 bp (Aronesty, 2011). PRINSEQ-lite 0.20.4 (Schmieder and Edwards, 2011) was then used to remove reads with an average Q score of <25 and to trim bases below Q25 from 3′ ends. No Ns were allowed, and only read lengths of over >150 bp were retained. Sequences were demultiplexed based on the unique tags and library indexes using Qiime's split_libraries.py script allowing for one-base mismatches (Caporaso et al., 2010). All sequences were then passed through the ITSx bioinformatics pipeline (Bengtsson-Palme et al., 2013) and only reads recognised as fungal ITS2 were retained. Operational taxonomic unit (OTU) clustering of ITS2 sequences was then done using the UPARSE pipeline (Edgar, 2013) at 97% similarity, and fungal taxonomic identification was mostly based upon BLASTn searches with a cut-off of $e = 10^{-9}$ against the UNITE database using the 01.12.2017 release version (Koljalg et al., 2005). However, due to some recent reclassifications of fungal taxa (Mayers et al., 2015), the identity of two fungal OTUs was modified, see Supplementary Data S6 for details.

Reads were mapped against OTUs using UPARSE to create an OTU by sample matrix. Fungal OTUs with more than one read in negative control samples were removed from further analyses. Associations only supported by a single read were removed, samples containing fewer than 100 reads in total were also removed, and the dataset was normalized using the cumulative-sum scaling CSS approach (Paulson et al., 2013). Analyses were also run using a rarefied dataset to ensure results were not affected by normalisation method choice (see Supplementary Data S7 for a comparison of results).

2.4. Statistical analysis

2.4.1. The drivers of bark and ambrosia beetle mycobiota

To assess the relative contribution of beetle species, environmental conditions and spatial distribution to the variability of fungal communities, we applied a variance partitioning approach (Whittaker, 1984; Borcard et al., 1992). Models were built to examine the impact of these three predictor groups on both fungal OTU richness and on fungal OTU community composition.

For the environmental predictors, we conducted a principal component analysis (PCA) to reduce the dimensionality of rainfall and temperature variables taken from both the Worldclim dataset (Worldclim v. 1.4; Hijmans et al., 2005) and from the UK Met Office Land Surface Observation Stations Dataset (UK Met Office, 2012). The PCA comprised 20 initial variables (Supplementary Data S8), the three components accounting for 89% of the total variance were retained for further analysis (Supplementary Data S9). Spatial predictors comprised distance-based Moran eigenvector map (dbMEM) variables (Borcard and Legendre, 2002; Dray et al., 2006). This approach reduces the dimensionality of the spatial relationships between study sites into variables which are then used in further analyses to represent these relationships. A subset of four dbMEM variables were retained using the forward selection approach of Blanchet et al. (2008). Principal component analyses and dbMEM calculation and selection were performed using R (R Core Team, 2017).

Fungal OTU richness was determined using the Chao1 non-parametric richness estimator (Chao et al., 2009) which was used to generate extrapolated richness. Only beetle species found in two or more locations were used for this analysis, that is 11 species in total, as *X. germanus* was found only at Thetford. Generalised linear models were made with Poisson errors. Where overdispersion was detected, standard errors were corrected using a quasi-GLM model to reduce overestimating the significance of model predictors (Crawley, 2007; Zuur et al., 2009). All models were built using the *stats* package in R (R Core Team, 2017).

Permutational multivariate analyses (PERMANOVAs) were conducted to examine the impact of beetle species, environmental and spatial drivers on fungal community composition using the *vegan* package in R (Oksanen et al., 2013; R Core Team, 2017). We used the Sørensen index for incidence data. As with the extrapolated fungal OTU richness analysis, variance partitioning was conducted to assess the unique and combined contribution of individual predictors and predictor combinations.

To understand the relative contribution of species replacement (turnover) and species loss (nestedness) to the dissimilarity between fungal communities from different beetle species, an additive partitioning framework following that of Baselga (2010) was used. To achieve this, incidence-based compositional dissimilarity was split into Simpson dissimilarity (β_{sim}), accounting for turnover, and nestedness-resultant dissimilarity (β_{sne}) using the *betapart* package (Baselga and Orme, 2012) in R, which produced two pairwise community dissimilarity matrices. We then used Wilcoxon rank sum tests to compare both beta-diversity components between fungal communities derived from the same beetle species (intraspecific comparisons) and between those derived from different beetle species (interspecific comparisons).

The host specificity of each fungal OTU was calculated using a

bipartite network based approach similar to that of Toju et al. (2013); for this analysis all 12 beetle species were included. At this point we would like to clarify that this analysis is not able to categorically distinguish symbiotic fungi from non-symbionts, so any perceived host specificity could be due to microhabitat use, host behavioural traits or other non-random factors. The term 'host specificity' as used here is simply a way to indicate that a given fungal OTU is associated with a given subset of beetle species more often than one would expect by chance. The standardised Kullback-Leibler distance d' (Kullback and Leibler, 1951; Blüthgen et al., 2006) was calculated with the *bipartite* package in R (Dormann et al., 2008; R Core Team, 2017). This index uses frequency of association data to assess the 'specificity' of each OTU relative to others, which ranges from 0 (no specificity) to 1 (highly specific). To determine whether fungal OTUs were specific to a particular subset of beetle species, we compared observed d' values with null d' distributions generated from 1,000 randomised matrices (generated using the vaznull model) using a one-tailed permutation test with a 95% significance cut-off. The vaznull method was deemed the most appropriate because it retains the total number of beetle-fungal links for each species, but randomises the identity of the associates. This approach has been used to measure the specificity of pollinators and root-associated fungi (Blüthgen et al., 2006; Toju, 2015) and will indicate which fungal OTUs are restricted to a reduced set of beetle species. If the observed d' index of an OTU is significantly greater than expected by chance based upon comparisons with d' index distributions generated from random networks, it was classed as specific. The proportion of specific and non-specific fungal OTUs in each fungal order was then compared (Newcombe, 1998). Bonferroni adjustments were applied to each p-value to reduce the risk of Type I errors.

3. Results

3.1. Sequencing, OTU generation and taxonomic composition

304 individual beetle specimens were processed for mycobiome analysis. Starting with a total of 17,239,168 paired-end reads, bioinformatic processing reduced the data to 5,416,312 bidirectional fungal ITS2 sequences. These were grouped into 2,284 non-chimeric OTUs. After removal of OTUs contained within the negative control samples (see Supplementary Data S10 for details) 2,257 OTUs were retained for further analysis. The LCA method in MEGAN indicated no non-fungal OTUs. Out of these, 2,094 OTUs were identified to phylum, 2,012 to class, 1,601 to family and 1,436 were identified to genus level using the UNITE database. Despite only considering those identified (~93% of the total number of OTUs found), the taxonomic composition of the fungal communities associated with UK bark and ambrosia beetles was highly diverse (Fig. 1); identified OTUs spanned 32 fungal classes, 214 families and 424 genera. Based on the assigned identity of OTUs, the most prevalent fungal phylum was Ascomycota (73.1%) and the most common class was Saccharomycetes (27.5%). Many of the detected taxa have been isolated previously from specimens and galleries of Scolytinae and Platypodinae, including the Microascales genera *Ceratocystis*, *Ambrosiella* and *Graphium*. Additionally, a number of plant pathogens such as *Chalara* and *Cladosporium* species were uncovered as well as 41 OTUs belonging to the family Ophiostomataceae.

3.2. Drivers of bark and ambrosia beetle mycobiota

When analysing OTU richness, the total amount of explained

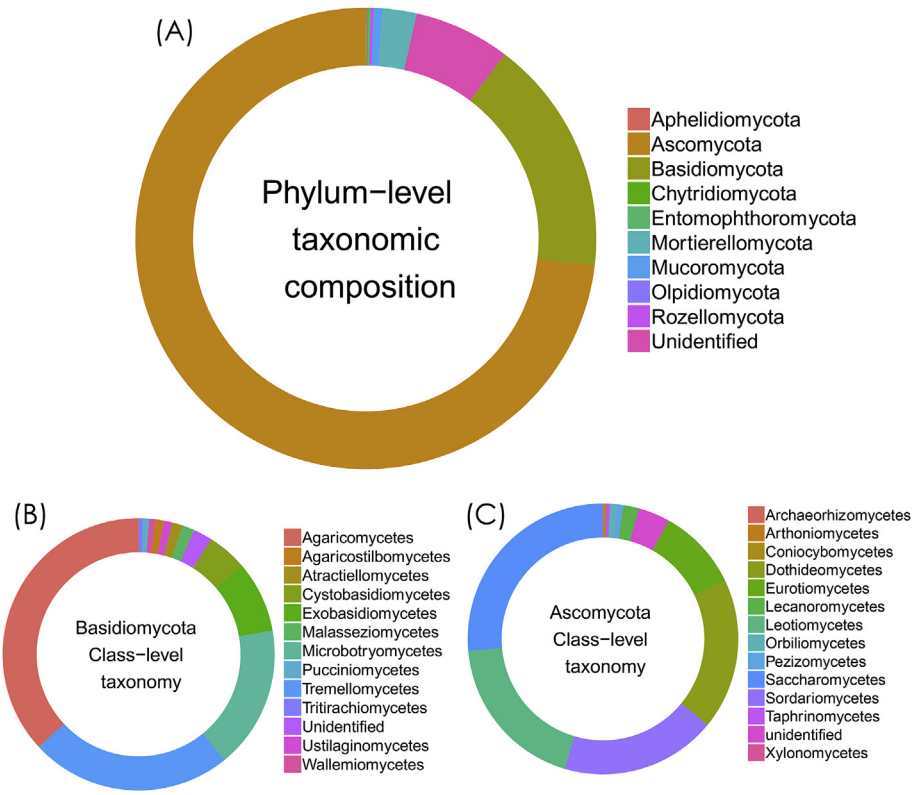

Fig. 1. Taxonomic composition based on number of fungal OTUs (A) composition of total fungal community obtained from all beetle samples at the level of phylum. (B) class-level taxonomy for Basidiomycetes. (C) class-level taxonomy for Ascomycetes. Identifications are based upon BLAST searches against the UNITE database (Altschul et al., 1990; Koljalg et al., 2005).

variance over the full model comprising beetle, environmental and spatial predictors was 21.2% ($F_{17, 273} = 5.9$, $p < 0.0001$). Beetle species identity uniquely explained 14.5% ($F_{10, 280} = 4.3$, $p < 0.0001$) of variation, while environmental and spatial predictors explained 5.3% ($F_{3, 287} = 9.36$, $p = < 0.001$) and 1.5% ($F_{4, 286} = 5.28$, $p = 0.02$) respectively.

For the fungal community analysis, beetle host identity also uniquely explained the greatest proportion of variation: 13.7%. Environmental and spatial variables accounted for little variation independently (between 0.91% and 2.60%), shared variance varied between 0.08% and 1.46%, and the full model explained 20.92% (Table 2). See Fig. 2 for an illustration of the dissimilarity between beetle species at each location.

Upon examining the contributions of fungal OTU turnover and nestedness to intraspecies and interspecies mycobiotic dissimilarities, OTU turnover accounted for pairwise dissimilarities between mycobiota to a much greater degree than nestedness for both types of dissimilarity ($W = 48599$, $p < 0.0001$) (Fig. 3a). However, nestedness-resultant dissimilarity was significantly higher for intraspecific comparisons ($W = 160550000$, $p < 0.0001$) whilst turnover was significantly lower ($W = 351770000$, $p < 0.0001$) (Fig. 3b).

3.3. The specificity of fungal associates

Based on the d' specificity metric, 75 fungal OTUs (6.96%) exhibited a higher association specificity than expected by chance (Fig. 4). The d' index for specific OTUs ranged from 0.041 to 0.498, and from 0 to 0.275 for non-specific OTUs (based on a randomisation test; see Methods). Of the specific OTUs, 23 (30.7%) were associated with a single beetle species. Upon examination of the proportion of specific and non-specific OTUs for each fungal order, a high degree of variation in association specificity between closely related OTUs was found. Ten fungal orders contained both specific and non-specific OTUs (Fig. 5). However, when the proportion of specific and non-specific OTUs within each fungal order were compared, two orders exhibited a high number of specific OTUs; the Saccharomycetales ($X = 22.86$, $df = 1$, $p = 1.75 \times 10^{-05}$) and the Microascales ($X = 25.59$, $df = 1$, $p = 4.23 \times 10^{-06}$).

4. Discussion

This community metabarcoding approach for assessing beetle and fungus assemblages gives us an insight into the stringency of symbiotic partnerships, while also revealing the variation in these beetle-fungal associations along environmental and spatial axes. As typical of recent metabarcoding analyses (e.g. Kostovcik et al., 2015; Miller et al., 2016; Malacrinò et al., 2017), we uncovered a large number of fungal OTUs associated with each individual beetle, greatly exceeding those from classical approaches based on culturing and morphology (see Linnakoski et al., 2008; Silva, 2011).

Table 2
Results from PERMANOVA modeling of incidence-based (Sørensen dissimilarity index) fungal community composition using all predictors, i.e. the full model. Bold type indicates significant p-values.

Predictor	Pseudo F	R^2	*p*
Beetle species	5.80	0.160	**0.001**
Environment component 1	3.22	0.009	**0.001**
Environment component 2	2.39	0.007	**0.001**
Environment component 3	2.72	0.008	**0.001**
dbMEM 2	3.47	0.010	**0.001**
dbMEM 4	1.30	0.004	0.066
dbMEM 5	3.57	0.010	**0.001**
dbMEM 6	1.05	0.003	0.388

Our findings demonstrate that the species identity of beetle hosts is an important determinant of both the richness and composition of the mycobiota, while, within pine plantation communities, the effects of abiotic and spatial factors are essentially negligible at the scale of the British Isles examined here. We then show that the compositional dissimilarity between the mycobiota of individual beetles is largely driven by the turnover of fungal OTUs, but that a larger portion of dissimilarity is attributed to richness differences when looking at beetles from within the same species than between species. Finally, we discovered that a high proportion of yeasts and Microascales are affected by beetle host identity, but that relatively few of the OTUs belonging to the family Ophiostomataceae show an affinity for a particular beetle species. Thus, we confirm that beetle host identity affects the fungal communities vectored between trees, but show that, for the fungal taxa uncovered here, the stringency of association as measured by standard specialisation indices remains low and is driven by a relatively small subset of fungal taxa.

4.1. The drivers of scolytine-associated fungal communities

There is a growing number of investigations which examine the fungal communities associated with bark and ambrosia beetles. The economic importance and abundance of these insects has made them an obvious target from an applied perspective and, given the complex history of their symbioses with fungi, they are also an interesting system from an co-evolutionary standpoint (Jordal et al., 2011). Studies have ranged from those focusing on individual beetle species, particularly pests such as *Ips typographus* (Yamaoka et al., 1997; Jankowiak and Hilszczanski, 2005; Linnakoski et al., 2016) to multiple species (Kostovcik et al., 2015; Skelton et al., 2018), and some have demonstrated variability between the mycobiota of different beetle species. However, to our knowledge none have attempted to quantify the relative importance of host species and environmental drivers such as done here. The results of this analysis show that while these fungal assemblages are difficult to predict based on the drivers we examine, host beetle species does explain more variation than climate and geographic location. The additive partitioning analysis, which revealed that fungal OTU turnover (β_{sim}) showed a greater contribution than nestedness (βsne) to differences between mycobiota, thus indicating that diversity patterns are driven by the substitution of fungal OTUs between beetle individuals rather than the loss of OTUs between them (Baselga, 2010, 2012). However, the fact that nestedness was significantly higher and turnover significantly lower for intraspecific comparisons suggests that more OTUs are shared between fungal communities derived from the same beetle species, but each individual only carries a certain portion of that community. This result is consistent with the significant but low level of variance explained in the OTU composition analysis and the relatively small proportion of host specific fungal taxa uncovered in the specificity analysis.

The relatively small effect of environmental and spatial model variables suggests that these factors only play a minor role in the composition and richness of bark and ambrosia beetle mycobiota in our system. Both temperature and precipitation have been shown to affect fungal community richness and composition across a wide range of habitats and functional groups (Rudgers et al., 2015), and environmental change can demonstrably remove microbial associates from a symbiosis (Kikuchi et al., 2016). However, climate is expected to have a reduced effect on the composition of host-associated rather than free-living communities, as hosts can be seen to represent 'islands' of suitable habitat and thus reduce habitat selection pressure (Peay et al., 2016). Similarly, spatial separation is a major determinant of microbial community

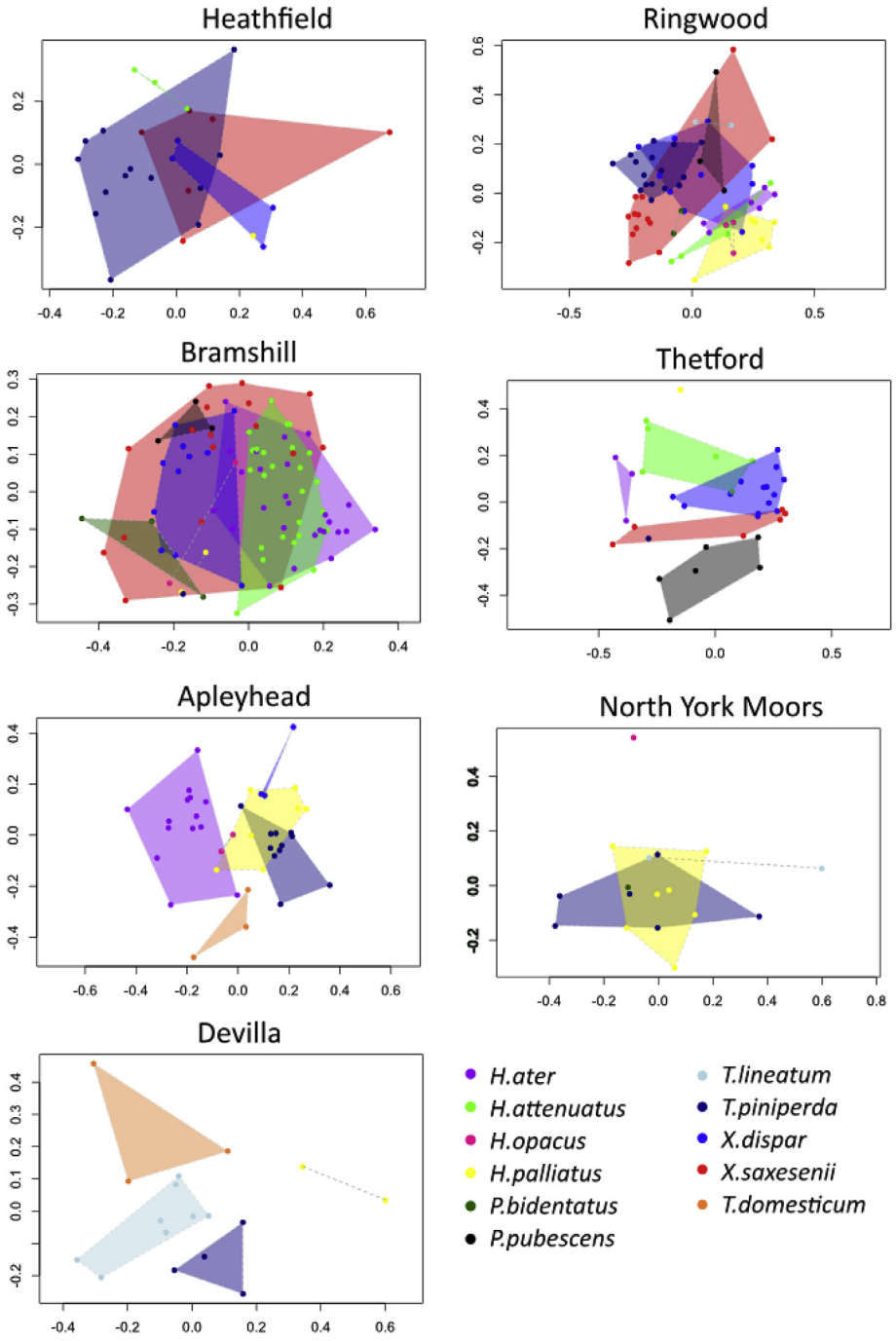

Fig. 2. Illustration of the fungal communities associated with beetle species using nonmetric multidimensional scaling (NMDS) based on Sørensen dissimilarity. Each point represents the mycobiota of an individual beetle specimen. Colours represent beetle species. Culbin was left out of this figure as it only contained two beetle species.

similarity in some systems (Córdova-Kreylos et al., 2006; Langenheder and Székely, 2011) but is seemingly unimportant in others (Van der Gucht et al., 2007; Winter et al., 2013). Our results mirror the latter studies, and a possible explanation for this rests on the dispersal abilities of scolytines. Whilst many European studies suggest that bark beetles generally migrate less than 2 km during their lifetime (Zumr, 1992; Duelli et al., 1997; Costa et al., 2013), under outbreak conditions passive wind dispersal at high altitudes can enable beetles to migrate much further (Jackson et al., 2008). Should this occur, it is likely to promote convergence between the mycobiota of beetle individuals from different populations. Additionally, the climatic gradient used here may not have been large enough to constrain fungal species distributions, and the fact that this study was restricted to pine forest would have further constrained variability in fungal communities (Giordano et al., 2012; Purahong et al., 2018).

4.2. Richness and composition of beetle-associated fungi

The deeper level of sequencing, higher resolution of species level identifications, and the removal of media selection bias can enable metabarcoding approaches to show a more complete

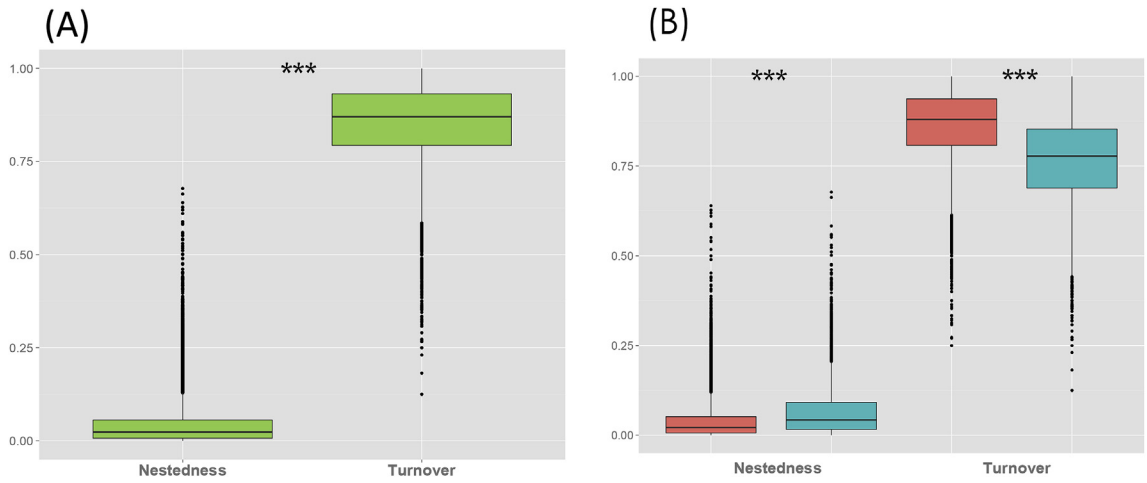

Fig. 3. (A) Pairwise nestedness and turnover resultant dissimilarities for all mycobiome comparisons. (B) Intraspecific pairwise dissimilarities (blue) and interspecific pairwise dissimilarities (red) between mycobiota. Stars represent significant differences between levels ($p < 0.05$).

picture of microbial communities (O'Brien et al., 2005; Jackson et al., 2013; Sinclair et al., 2015). Yet, methodological issues must be considered. The ITS2 marker often comprises just under 300 bp, limiting its power of species resolution. It is also present in multiple copies, which may vary within an individual genome potentially resulting in artificially inflated OTU richness. Conversely, clustering at 97% could potentially combine closely related fungal species into the same OTU, artificially reducing OTU richness. Both of these outcomes would impact the network-based specificity analysis, the former could conceivably lead to an exaggeration of specificity levels while the latter would lead to an underestimation of specificity. However, the fact that we were able to reconstruct known associations (*Ambrosiella grosmanniae*, *Phialophoropsis ferruginea*, and *Ambrosiella hartigii* were all found to demonstrate significant specificity to their expected hosts: *X. germanus*, the two *Trypodendron* species, and *Anisandrus dispar* respectively) gives us confidence that these methodological issues, despite creating 'noise' in the dataset, have not masked clear ecological patterns. We believe that our treatment of the sequence data is conservative due to stringent parameters for sequence error removal, the elimination of low-abundance OTUs, and the use of HMM profiles to identify ITS2 sequences. In addition to high OTU diversity, the composite mycobiome of all beetle species was taxonomically diverse, represented by a wide range of taxa including many ophiostomatoid fungi. This finding is consistent with previous investigations into scolytine-associated fungi and known ecological associations of these groups (Harrington, 2005; Silva, 2011; Giordano et al., 2012). Members of the well represented class Dothideomycetes are usually saprotrophs associated with decaying plant material, while species in the equally highly represented class Sordariomycetes, which includes the harmful Microascales, often dominate communities in forests (O'Brien et al., 2005; Lauber et al., 2008; Meiser et al., 2013). Having said this, the primers utilised for this study are known to have a low affinity for ophiostomatoid fungi in the genus *Raffaelea* (Kostovcik et al., 2015), and this is reflected by the absence of any *Raffaelea* species amplified. Many *Raffaelea* are phytopathogens and some are believed to display high affinities for particular beetle hosts (Rabern Simmons et al., 2016). As such, we are careful to avoid generalising our results to suggest that all ophiotomatoid fungi have low host affinity, but rather that this is true for the 41 uncovered here.

Members of the Microascales, including known bark beetle symbionts in the genera *Graphium*, *Ceratocystis* and *Ambrosiella* (Klepzig et al., 2001), were recognised against the well-curated UNITE database and were among the OTUs classified as host-specific. Three OTUs in particular closely matched the barcode accessions of described fungal symbionts allowing species-level identifications that were also supported by known host distributions. Close database matches to the well-studied obligate fungal symbionts *A. hartigii*, *A. grosmanniae sp. nov.* and *P. ferruginea* were supported by recovery from their known hosts, *A. dispar*, *X. germanus*, and both *Trypodendron domesticum* and *Trypodendron lineatum* respectively (Batra, 1963; French and Roeper, 1972a,b; Skelton et al., 2018). These fungi are obligate symbionts and are 'farmed' and fed upon by these ambrosia beetles. Additionally, an OTU matched database accessions of the Hypocreales *Geosmithia langdonii*, which was only found in *Pityogenes bidentatus* individuals. While known to be associated with bark beetles, the ecological role of this apparent symbiont is not well understood.

Among the class Saccharomycetes we detected a large number of OTUs. Ascomycetous yeasts have been found with all developmental stages of bark and ambrosia beetles (Callaham and Shifrine, 1960; Francke-Grosmann, 1967; Davis, 2015), but their ubiquitous distribution has led to suggestions that they do not exhibit affiliations with particular beetle species (Six, 2003). Our finding calls this assertion into question. For example, we found the genus *Metschnikowia* to be overrepresented among the host-specific OTUs. *Metschnikowia* is a genus of insect-associated fungi that have been isolated from various bark beetles, including *I. typographus* and *Dendroctonus micans* (Weiser et al., 2003; Yaman and Radek, 2008), but here it was almost exclusively found in non-ambrosial bark beetles, specifically *Hylastes ater* and *Hylurgops palliatus*, which have no known obligate fungal associations (Reay et al., 2001; Romón et al., 2007). Although little is known about their life history, *Metschnikowia* species comprise insect pathogens (Weiser et al., 2003) suggesting that the associations observed here may reflect a specific parasitic relationship with a narrow range of beetle hosts. Other cases include the yeasts *Ogataea pini* and *Kuraishia capsulata*, which were only found in individuals of *P. bidentatus*. These fungal species are associates of the scolytine genera *Ips*, *Dendroctonus* and *Scolytus* (Yamada et al., 1994; Kolarík et al., 2007; Davis, 2015). *O. pini* produces volatiles that reduce the growth of entomopathogenic fungi (Davis et al., 2011). We are not currently aware of any studies that have expressly investigated the specificity of host-associated yeast, but

Fig. 4. Association specificity analysis showing the distribution of the specific OTUs across beetle species. Red squares indicate the strength of association between each OTU and beetle as a proportion of the total number of occurrences for each OTU ranging between 0 (the OTU was not found to associate with any specimens of a beetle species) and 1 (the OTU was only found to associate with a single beetle species).

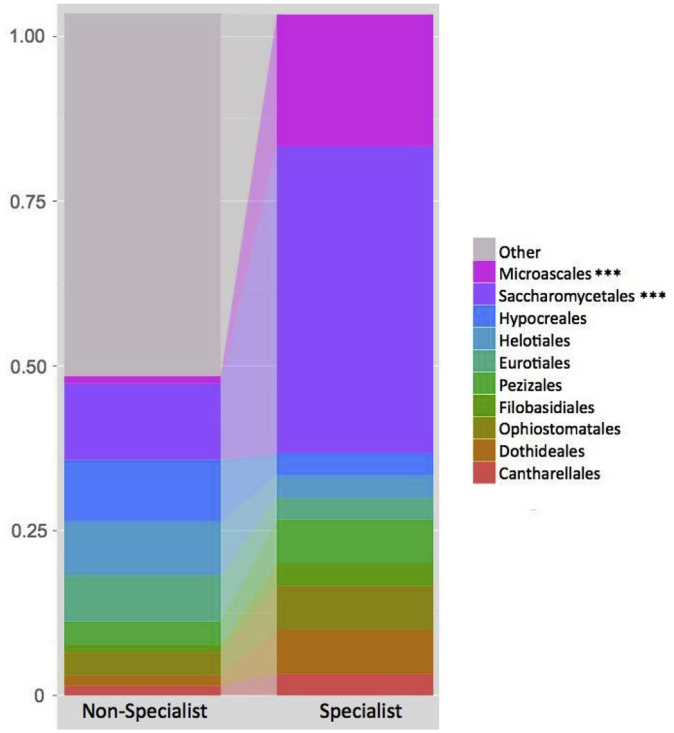

Fig. 5. Proportion of non-specific and specific OTUs for ten fungal orders. Stars indicate a significant difference in the proportions ($p < 0.005$). Classification of OTUs is based upon bipartite network analysis.

suggest that an analysis of this kind may reveal many more mutualistic and antagonistic relationships.

As touched upon previously, 'host specific' OTUs demonstrate an affinity to a subset of beetle species, this may be due to a symbiotic relationship or to another reason. For example, certain beetle species may come into contact with spores from a particular fungus due to behavioural preferences like overwintering in leaf litter as opposed to under bark, and it may be that there is no symbiosis occurring between the two taxa. It should also be noted, that an absence of specificity does not necessarily confirm a lack of symbiosis. Symbioses are increasingly understood to comprise a broad range of association types and current paradigms are moving away from traditional classifications (Leung and Poulin, 2008). Traditional ecological theory posits that specialisation reduces competition and is, therefore, the normal consequence of coevolution (Futuyma and Moreno, 1988). Because specialist symbionts typically exhibit lower fitness when interacting with sub-optimal host species, it has been argued that specialisation is irreversible and can lead to an 'evolutionary dead end' (Huxley, 1942; Poulin, 2007). However, recent studies suggest that generalism can also be adaptive and there is now empirical evidence supporting the assertion that transitions from specialist to generalist life histories can indeed occur (Nosil, 2002; Poulin et al., 2006; Johnson et al., 2009; Skelton et al., 2019). Further, it is becoming increasingly clear that facultative microbial symbionts play important roles in the structuring of whole communities (Ferrari and Vavre, 2011; Frago et al., 2012), although these taxa can be distributed across multiple host species and occurrence can be contingent upon external factors (Mitsuhashi et al., 2002; Chiel et al., 2009). In sum, our focus on specialisation as an indication of an association is the result of using a whole community, exploratory approach and is a useful first step in gaining a better understanding of such systems. Further work is certainly needed to clarify the nature of these relationships, such as the isolation and culture of these fungal species to elucidate identities and experimental work to assess the effects of manipulating the incidence of putative symbionts.

5. Conclusions

Earlier studies of mycobiota associated with bark and ambrosia beetles suggested that particular beetle species form close, specific associations with a small number of fungal taxa (Beaver, 1989; Cardoza et al., 2009). However, recent molecular-based analyses show that these fungal communities are much more diverse and promiscuous than originally thought (Kostovcik et al., 2015), potentially indicating the presence of facultative symbiosis and many non-specific associations. Our results are partially consistent with both of these conclusions: we demonstrate that although beetle host identity has a stronger explanatory power than environmental or spatial factors, it still explains less than 20% of the total variance in composition, and only a small subset of fungal taxa appear to be driving the trend. This pattern could be explained by the presence of a diverse array of fungal taxa exhibiting associations spanning the entire specificity spectrum, as well as incidental or transient associates not partaking in interactions at all. Our network analysis allowed us to identify 'host specific' taxa from this large diversity of fungi, some of which were expected symbionts, while others were poorly known yeasts whose tight associations may have been previously overlooked. However, these understudied taxa may well be important players within scolytine-driven dynamic forest systems. The fact that some fungal taxa were associated with novel insect species highlights the utility of adopting modern molecular approaches to re-examine established associations. Finally, despite identifying 41 fungal OTUs in the family Ophiostomatacea, only two showed host specificity indicating that the vectoring of these taxa is not restricted to particular beetle species. This finding has clear implications for the monitoring and management of an important group of forest pathogens. For instance, the low levels of specificity shown here could indicate a high probability of disassociation and host switching indicating that the environmental conditions that limit the distribution of a host may not limit the spread of an associated pathogen. This picture would be improved by investigating the distribution of *Raffaelea* species within this community as these may prove to buck the trend observed here for ophiostomatoid taxa. The role of biotic interactions in shaping fungal biogeography is relatively unexplored, but there is mounting evidence that it plays a crucial role in determining community composition. Only with more examples of natural host-fungus community assemblages can we assess its influence under different scenarios and across divergent fungal taxa.

Data accessibility statement

Sequence-based fungal community data: European Nucleotide Archive, Accession Number: PRJEB33668.

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Conflicts of interest

APV is a co-founder of NatureMetrics, a company delivering commercial services in molecular biomonitoring.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2019.07.008.

KEM, DJGI and APV designed the study; KEM and DJGI conducted fieldwork; KEM designed and conducted labwork and bioinformatics; KEM conducted analysis under the supervision of CGR, AB and APV, KEM and APV wrote the paper with contribution from all authors.

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