Complex associations between cross-kingdom microbial endophytes and host genotype in ash dieback disease dynamics

Sarah M. Griffiths[1], Marciana Galambao[1], Jennifer Rowntree[1], Ian Goodhead[2], Jeanette Hall[3], David O'Brien[3], Nick Atkinson[4], Rachael E. Antwis[2 *]

1. Ecology and Environment Research Centre, Department of Natural Sciences, Manchester Metropolitan University, UK
2. School of Environment and Life Sciences, University of Salford, UK
3. Scottish Natural Heritage, Inverness, UK
4. The Woodland Trust, Grantham, Lincolnshire, UK

Address for Correspondence:

* Dr Rachael Antwis, University of Salford, Peel Building, The Crescent, Salford, M5 4WT, UK, 01612954641, r.e.antwis@salford.ac.uk

KEYWORDS: bacteria, fungi, microbiome, tree pathogen, *Hymenoscyphus fraxineus*, *Fraxinus excelsior*, amplicon sequencing, genetic diversity

ABSTRACT

1. Tree pathogens are a major threat to forest ecosystems. Conservation management strategies can exploit natural mechanisms of resistance, such as tree genotype and host-associated microbial communities. However, fungal and bacterial communities are rarely looked at in the same framework, particularly in conjunction with host genotype. Here, we explore these relationships and their influence on ash dieback disease, caused by the pathogen *Hymenoscyphus fraxineus*, in European common ash trees.
2. We collected leaves from UK ash trees and used microsatellite markers to genotype trees, qPCR to quantify *H. fraxineus* infection load, and ITS and 16S rRNA amplicon sequencing to identify fungal and bacterial communities, respectively.
3. There was a significant association between *H. fraxineus* infection intensity and ash leaf fungal and bacterial community composition. Higher infection levels were positively correlated with fungal community alpha diversity, and a number of fungal and bacterial genera were significantly associated with infection presence and intensity. Under higher infection loads, leaf microbial networks were characterised by stronger associations between fewer members than those associated with lower infection levels. Together these results suggest that *H. fraxineus* disrupts stable endophyte communities after a particular infection threshold is reached, and may enable proliferation of opportunistic microbes. We identified three microbial genera associated with an absence of infection, potentially indicating an antagonistic relationship with *H. fraxineus* that could be utilised in the development of anti-pathogen treatments.
4. Host genotype did not directly affect infection, but did significantly affect leaf fungal community composition. Thus, host genotype could have the potential to indirectly affect disease susceptibility through genotype x microbiome interactions, and should be considered when selectively breeding trees.
5. *Synthesis*. We show the diversity, composition and network structure of ash leaf microbial communities are associated with the severity of infection from ash dieback disease, with evidence of disease-induced dysbiosis. We also show that host genotype influences leaf fungal community composition, but does not directly influence tree infection. These findings help to elucidate relationships between host genetics, the microbiome, and a tree pathogen,

highlighting potential resistance mechanisms and possible co-infection concerns that could inform ash tree management.

INTRODUCTION

Invasive pathogens are an increasing threat to trees and forest ecosystems across the globe (Burdon, Thrall, & Ericson, 2005). This rise can be largely attributed to human activity. For example, the international trade in wood products and live plants has introduced pathogens to naïve tree populations with no evolved resistance mechanisms, whilst climate change has also rendered environments more conducive to tree infection and pathogen proliferation in many areas (Anderson et al., 2004; Linnakoski, Forbes, Wingfield, Pulkkinen, & Asiegbu, 2017; Roy et al., 2014). Large-scale mortalities in tree species endanger associated biodiversity, natural capital, and ecosystem service provision (Boyd, Freer-Smith, Gilligan, & Godfray, 2013; Freer-Smith & Webber, 2017), and are therefore a key priority area for natural resource management and conservation.

One pathogen of great concern is *Hymenoscyphus fraxineus* (Ascomycota; Leotiomycetes, Helotiales; Helotiaceae), which causes ash dieback disease in a number of ash species, including European ash (*Fraxinus excelsior*) - a highly abundant and ecologically, economically, and culturally important tree species. This fungal pathogen produces the toxic compound viridiol, which damages leaves, stems and eventually, the trunk, ultimately causing xylem necrosis and canopy loss (Grad, Kowalski, & Kraj, 2009). Ash dieback has caused up to 85% mortality in plantations within 20 years of exposure (Coker et al., 2019; McKinney et al., 2014), and is driving extensive declines across mainland Europe and the UK (Coker et al., 2019; Jepson & Arakelyan, 2017; McKinney et al., 2014; Mitchell et al., 2014). The disease is likely to have been introduced by trade and is largely spread by wind and water-borne ascospores at a rate of approximately 20-30 km per year (Gross, Zaffarano, Duo, & Grünig, 2012). Due to its severity and the lack of effective treatment or control methods, the import of ash trees is currently banned in the UK.

A range of silvicultural and arboricultural management practices have been suggested for ash dieback mitigation, such as increasing local tree species diversity, removing infected tissue and/or autumn leaf fall, reducing tree density, and applying fungicides (Hrabětová, Černý, Zahradník, & Havrdová, 2017; Skovsgaard et al., 2017). However, such methods may be expensive, labour-intensive, and damaging to the environment. Exploiting natural host resistance mechanisms offers a promising alternative, which may provide a more long-term solution whilst avoiding some of these disadvantages.

Ash dieback resistance has a strong host genetic component; nearly 50% of phenotypic variation in crown damage is based on host genotype (McKinney et al., 2014; McKinney, Nielsen, Hansen, & Kjær, 2011; Muñoz, Marçais, Dufour, & Dowkiw, 2016). Furthermore, progeny from low-susceptibility mother clones exhibit lower symptoms of disease, indicating a heritable basis for tolerance (Lobo, McKinney, Hansen, Kjær, & Nielsen, 2015). The specific genetic drivers of tolerance are still unclear, but may be linked to genetically-induced variation in phenology (McKinney et al., 2011; Stener, 2018). In addition, a suite of 20 gene expression markers associated with low susceptibility to *H. fraxineus* have been identified (Harper et al., 2016; Sollars et al., 2017), demonstrating that coding regions of the host genome are intrinsically involved in disease resistance.

Whilst selective breeding for tolerant genotypes may be desirable for timber production purposes, there are problems associated with this approach. Given the long generation time of trees, reduced genetic diversity could leave populations vulnerable to extinction through pathogen evolution as well as other emerging threats (e.g. emerald ash borer, *Agrilus planipennis*) (Jacobs, 2007). In addition, the proportion of trees tolerant to ash dieback are currently unknown but are likely to be very low, perhaps in the range of 1-5% (McKinney et al., 2014; McMullan et al., 2018). Furthermore, mortality occurs most rapidly at the sapling stage, meaning selection pressure is very high and the pool of genetic diversity to draw from may be low. Thus, a more holistic understanding of the mechanisms of tolerance may assist the development of management strategies to maximise the regeneration potential of trees and forests at a local and landscape level. This approach will allow managers to identify tolerant individuals in the wider landscape, which could then form the basis of tree breeding programmes.

The plant microbiome forms an important component of disease tolerance. Host-microbiome interactions encompass a range of types from antagonistic to mutualistic, however, the overwhelming benefits of a healthy microbiome are now clear, including protection from infectious diseases (Turner et al., 2013). In several tree species, changes in microbiome composition in response to pathogenic infection have been observed (Busby, Peay, & Newcombe, 2016; Cross et al., 2017; Koskella, Meaden, Crowther, Leimu, & Metcalf, 2017), suggesting an interaction between the host microbiome and invasive pathogens. As such, interest is growing in the potential to engineer host microbiomes to enhance or induce microbially-mediated traits (Foo, Ling, Lee, & Chang, 2017; Mueller & Sachs, 2015; Quiza, St-Arnaud, Yergeau, & Rey, 2015; Sheth, Cabral, Chen, & Wang, 2016; Yergeau et al.,

2015). Identifying particular leaf endophytes that limit *H. fraxineus* infection may allow us to manipulate the leaf microbiome (i.e. the phyllosphere) for tree resistance. This could be achieved through a number of mechanisms including; selection of individuals based on microbial communities associated with host tolerance (Becker et al., 2015); addition of microbial inoculants that inhibit pathogenic growth (Marcano, Díaz-Alcántara, Urbano, & González-Andrés, 2016); alteration of environmental conditions that promote a desirable microbiome (Bender, Wagg, & van der Heijden, 2016; Thijs, Sillen, Rineau, Weyens, & Vangronsveld, 2016); or genetic modification of trees that alters signalling or selection traits that determine microbial community composition and function (Beckers et al., 2016). Culturing studies have identified a number of endophytic fungi of ash trees that inhibit the growth or germination of *H. fraxineus* and thus could be used as potential micro-biocontrol agents (Haňáčková, Havrdová, Černý, Zahradník, & Koukol, 2017; Kosawang et al., 2018; Schlegel, Dubach, Buol, & Sieber, 2016; Schulz, Haas, Junker, Andrée, & Schobert, 2015).

In order to implement such strategies, we first need to characterise the phyllosphere community in response to infection. Cross et al. (2017) previously showed fungal community composition in ash leaves altered as *H. fraxineus* infection intensified over time, however it is not clear if this was driven by infection dynamics or temporal variation across the season. In addition, the role of cross-kingdom (e.g. bacterial and fungal) interactions in determining microbiome function is of growing interest (Menezes, Richardson, & Thrall, 2017). For example, cross-kingdom interactions may be important for biofilm production on leaf surfaces (Frey-Klett et al., 2011; van Overbeek & Saikkonen, 2016), and fungal communities can influence bacterial community colonisation via the modulation of carbon, nitrogen and environmental pH (Hassani, Durán, & Hacquard, 2018; Johnston, Hiscox, Savoury, Boddy, & Weightman, 2018). Thus, such interactions may be important for limiting pathogen invasion, although bacterial-fungal associations are not well characterised in this context (but see Jakuschkin et al., 2016). There are also complex interactions between host genotype and microbiome composition (Agler et al., 2016; Bálint et al., 2013; Griffiths et al., 2018; Smith, Snowberg, Gregory Caporaso, Knight, & Bolnick, 2015; Wagner et al., 2016) that can also alter pathogen susceptibility (Koch & Schmid-Hempel, 2012; Ritpitakphong et al., 2016). Understanding genetic influences on microbial community composition may allow us to use these two powerful determinants of disease susceptibility in combination to maximise disease tolerance across populations.

Here, we integrate these genetic and microbial factors within one framework by using microsatellite characterisation of host genotype, ITS rRNA and 16S rRNA sequencing to identify fungal and bacterial communities of leaves, qPCR to quantify *H. fraxineus* infection, and phenotypic scoring of tree infection levels across two sites (Manchester and Stirling, UK) to. We aimed to: i) identify differences in fungal and bacterial communities associated with ash leaves (i.e. the phyllosphere) according to *H. fraxineus* infection (at specific time points for multiple stands); ii) identify co-occurrence patterns between fungal and bacterial communities according to *H. fraxineus* infection; and iii) identify relationships between host genotype, phyllosphere composition, and *H. fraxineus* infection intensity.

MATERIALS AND METHODS

Tree Scoring, Leaf Sampling and DNA extraction

We conducted sampling and transport of ash material under Forestry Commission licence number FCPHS2/2016. We collected leaves from ash trees in semi-natural stands during the summer months from two areas. We sampled saplings from Balquhidderock Wood in Stirling, Scotland (25th July 2016) and mature trees from multiple sections of the off-road National Cycle Route 6 in Manchester, England (the Fallowfield Loop, River Irwell and Drinkwater Park; 19th – 25th August 2017). We sampled later in the season to maximise the potential for trees to have been exposed to *H. fraxineus*, and at both sites, widespread and epidemic levels of ash dieback were evident. We selected and scored trees displaying a range of ash dieback infection signs, from visibly clear of infection (infection score = 0) through to heavily infected with extensive signs of ash dieback (infection score = 5). We collected leaves that were visibly clear of infection from 25 trees in Stirling (three leaves per tree) and 63 trees in Manchester (one leaf per tree) in sterile bags and froze these immediately in the field using dry ice. We transferred samples to a -20°C freezer within 12 hours of collection, where they remained until DNA extraction. We weighed 50mg of leaf material and disrupted samples in a TissueLyser bead beater (Qiagen) for two minutes. We extracted DNA using the Qiagen DNeasy Plant MiniKit (along with two extraction blanks) according to the manufacturers protocol, and used this DNA for all downstream molecular analyses.

Hymenoscyphus fraxineus quantitative PCR

To quantify *H. fraxineus* infection, we conducted quantitative PCR (qPCR) on leaves according to a modified version of Ioos et al. (Ioos, Kowalski, Husson, & Holdenrieder, 2009) and Ioos & Fourrier (Ioos & Fourrier, 2011). Based on preliminary assessments of Ct values obtained during qPCRs (Cross et al., 2017), we diluted our DNA by a factor of 10. We conducted 10µl reactions using 0.4µl each of 10µM forward (5′-ATTATATTGTTGCTTTAGCAGGTC-3') and reverse (5′-TCCTCTAGCAGGCACAGTC- 3′) primers, 0.25µl of 8µM dual-labelled probe (5′-FAM-CTCTGGGCGTCGGCCTCG-MGBNFQ-3′), 5µl of QuantiNova PCR probe kit (Qiagen), 1.95µl of molecular grade water and 2µl of template DNA (~2ng). We used the following thermocycler conditions; initial denaturation of 95°C for 2 minutes followed by 50 cycles of 95°C for 10 seconds and 65°C for 30 seconds, using the green channel on a RotorGene Q real-time PCR machine (Qiagen). We included *H. fraxineus* standards ranging from 0.1 to 100 ng. We ran samples, standards and extraction blanks in duplicate and used the mean average of these for subsequent analyses. We multiplied the concentrations obtained from the qPCRs by the dilution factor of 10, and normalised the data for further analyses by calculating log concentrations using log(*H. fraxineus* infection)+1 (henceforth "log *H. fraxineus* infection"). Additionally, based on the distribution of *H. fraxineus* qPCR data (Figures S1a-c), we assigned samples with infection categories of "absent" for samples with 0 ng/µl; "low" for <200ng/µl; "medium" for 200 < 2000 ng/µl and "high" for > 2000 ng/µl.

Host genotype characterisation

To characterise tree genotype, we used 10 previously developed *F. excelsior* microsatellite markers (Brachet, Jubier, Richard, Jung-Muller, & Frascaria-Lacoste, 1998; Lefort, Brachet, Frascaria-Lacoste, Edwards, & Douglas, 1999; Harbourne, Douglas, Waldren & Hodkinson, 2005) (Table S1). We used a three-primer approach to fluorescently label PCR products (Neilan, Wilton, & Jacobs, 1997) using universal primers (Blacket, Robin, Good, Lee, & Miller, 2012; Culley et al., 2013) tagged with the fluorophores FAM, NED and PET (Table S1). We carried out PCRs in 10µl singleplex reactions using 5µl MyTaq Red Mix (Bioline), 1-10 ng DNA, 1µM of the 5' modified forward primer and 4µM each of the reverse primer and universal primer. PCR cycling conditions varied in annealing temperature among loci (Table S1), but otherwise consisted of an initial denaturation of 95°C for 3 minutes, 30 cycles of 95°C for 15 seconds, 46-60°C for 15 seconds and 72°C for 15 seconds, followed by a final extension step at 72°C for 5 minutes. PCR products for certain loci were then multiplexed for

automated capillary electrophoresis, and the remaining loci were analysed separately (Table S1). Capillary electrophoresis was carried out at the University of Manchester Genomic Technologies Core Facility using a 3730 DNA Sequencer (Thermo Fisher Scientific) with GeneScan 500 LIZ (Thermo Fisher Scientific).

We scored and binned alleles using GeneMapper v3.7 (ThermoFisher Scientific) and MsatAllele v1.05 (Alberto, 2009). One locus, CPFRAX6, was monomorphic and was therefore removed from subsequent analyses. We estimated null allele frequency using the Expectation Maximization algorithm (Dempster, Laird, & Rubin, 1977) as implemented in FreeNA (Chapuis & Estoup, 2007). We removed loci with null allele frequencies above 20% for each site for individual-level heterozygosity analyses to reduce bias associated with false homozygotes. We also removed locus CPFRAX5 from the Manchester data file as this was monomorphic. This made datasets of five loci and eight loci for Manchester and Stirling, respectively. Five measures of individual-level heterozygosity (proportion of heterozygous loci, observed heterozygosity, expected heterozygosity, internal relatedness and homozygosity by locus) were calculated using the *genhet* function (Coulon, 2010) in RStudio (v1.2.1335) (RStudio Team, 2016) for R (v3.4.1) (R Core Team, 2017).

Pairwise Euclidean genetic distances between trees were calculated for each site separately, and again together, using GenoDive v2.0b23 (Meirmans & Van Tiendener, 2004). As missing data can skew genetic distance calculations, we used GenoDive to impute missing data based on overall site allele frequencies prior to calculations. To investigate the presence of genetic differentiation in trees between sites, we estimated F_{ST} corrected for null alleles using ENA correction (Chapuis & Estoup, 2007) in FreeNA, and conducted an Analysis of Molecular Variance (AMOVA) in GenoDive using the least squares method. We also carried out a principle coordinates analysis (PCoA) in GenAlEx v6.503 (Peakall & Smouse, 2012) based on Euclidean distances using the standardised covariance method to visualise the variation in host genotype according to site.

ITS 1F-2 and 16S V4 rRNA amplicon sequencing

To identify leaf fungal communities, we amplified DNA for the ITS 1F-2 rRNA gene (White, Bruns, Lee, & Taylor, 1990) using single indexed reverse primers and a modified protocol of Smith & Peay (Smith & Peay, 2014) and Nguyen et al. (Nguyen, Smith, Peay, & Kennedy, 2014). Briefly, we ran PCRs in duplicate using Solis BioDyne 5x HOT FIREPol® Blend Master Mix, 2μM primers and 1.5μl of

sample DNA. Thermocycling conditions used an initial denaturation at 95°C for 10 minutes, with 30 cycles of 95°C for 30 seconds, 52°C for 20 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 8 minutes. We combined PCR replicates into a single PCR plate and cleaned products using HighPrep™ PCR clean up beads (MagBio) according to the manufacturers' instructions. We quality checked the PCR products using an Agilent TapeStation 2200. To quantify the number of sequencing reads per sample, we constructed a library pool using 1µl of each sample. We ran a titration sequencing run with this pool using an Illumina v2 nano cartridge (paired end reads; 2 x 150bp) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) on an Illumina MiSeq at the University of Salford. Based on the percentage of reads sequenced per library, we calculated the volume required for the full sequencing run and pooled these accordingly. Full ITS rRNA amplicon sequencing was conducted using paired-end reads with an Illumina v3 (2 x 300bp) cartridge on an Illumina MiSeq. We also included negative (extraction blanks) and positive (fungal mock community and *H. fraxineus*) controls.

To identify bacterial communities in leaves, we amplified DNA for the 16S rRNA V4 region using dual indexed forward and reverse primers according to Kozich et al. (Kozich et al., 2013) and Griffiths et al. (Griffiths et al., 2018). We ran PCRs in duplicate as described above, using thermocycling conditions of 95ºC for 15 minutes, followed by 28 cycles of 95ºC for 20 seconds, 50ºC for 60 seconds, and 72ºC for 60 seconds, with a final extension at 72ºC for 10 minutes. To quantify individual libraries, we again pooled 1µl of each library and sequenced this using an Illumina v2 nano cartridge as described above, then pooled samples according to read coverage and conducted a full paired-end sequencing run using Illumina v2 (2 x 250 bp) chemistry. We included extraction blanks and a mock bacterial community as negative and positive controls, respectively.

Pre-processing of amplicon sequencing data

We trimmed remaining adapters and primers for ITS rRNA sequencing data using cutadapt (Martin, 2011). This step was not required for 16S rRNA sequencing data. Unless otherwise stated, we conducted all subsequent data processing and analysis in RStudio (see supplementary files for full code).

A total of 6,346,506 raw sequence reads from 139 samples were generated during ITS sequencing. We conducted ITS rRNA gene amplicon sequence processing in DADA2 v1.5.0

(Callahan et al., 2016). Modal contig length was 181bp (range 75-315bp) once paired-end reads were merged. We did not conduct additional trimming based on sequence length as the ITS region is highly variable (Schoch et al., 2012). No contaminants were identified in the negative controls. We removed chimeras and assigned taxonomy using the UNITE v7.2 database (UNITE, 2017). We obtained a median of 29,043 reads per sample. We exported the final exact sequence variant (ESV) table, taxonomy table and sample metadata to the phyloseq package (McMurdie & Holmes, 2013). DADA2 identified 12 unique sequence variants in the sequenced mock community sample comprising 12 fungal isolates.

A total of 4,055,595 raw sequence reads from 139 samples were generated during 16S rRNA sequencing. As with ITS rRNA amplicon data, we conducted 16S rRNA gene amplicon sequence processing in DADA2 v1.5.0. Modal contig length was 253bp once paired-end reads were merged. We removed ESVs with length >260bp (78 ESVs; 0.026% of total sequences) along with chimeras and two ESVs found in the negative controls. We assigned taxonomy using the SILVA v128 database (Quast et al., 2013; Yilmaz et al., 2014). We stripped out chloroplasts and mitochondria from ash leaf samples, and removed 31 samples for which no sequence data remained, leaving a median of 2930 reads per sample. We exported the final ESV table, taxonomy table and sample metadata to phyloseq. DADA2 identified 20 unique sequence variants in the sequenced mock community sample comprising 20 bacterial isolates.

Phyllosphere composition by site and H. fraxineus *infection*

We converted the ESV abundance data of individual samples to relative abundances for fungi and bacteria separately. We produced box plots visualising the variation in relative abundance of the top 10 most abundant classes according to site and *H. fraxineus* infection category as described above (i.e. “absent”, “low”, “medium”, or “high”). We conducted a permutational ANOVA (PERMANOVA; adonis) in the vegan package (Oksanen et al., 2018) to determine the variation in fungal and bacterial community composition according to site and *H. fraxineus* infection category, and produced PCoA plots using Bray-Curtis dissimilarity matrices in phyloseq. We calculated alpha-diversity measures (species richness and community evenness) for each sample by subsampling the raw ESV count table to a standardised number of reads (equal to the sample with the lowest number of reads) using an iterative approach (100 times), and averaged the diversity estimates from each trial. In addition, as

a measure of beta-diversity, we extracted PCoA scores for axes 1 and 2 obtained from ordinating relative abundance data for each sample, as described previously. To determine the relationship between these microbial community measures and *H. fraxineus* infection intensity and tree infection score, we used separate linear mixed models in the lme4 package (Bates, Mächler, Bolker, & Walker, 2014), with tree ID and site as random factors, and log *H. fraxineus* infection or tree score as the response variable. We used the *associate* function in the microbiome package (Lahti & Shetty, 2017) to identify cross-correlation between the centred log ratios of microbial genera and log *H. fraxineus* infection using Spearman's rank correlation. We constructed a heatmap in ggplot2 (Wickham, 2009) to visualise statistically-significant taxa (that were successfully identified to genus level) according to their correlation coefficients.

As samples from Manchester included both infected (n = 36) and uninfected (n = 27) leaves (whereas all samples from Stirling were infected; see Results), we subsetted the Manchester samples for further analyses that aimed to identify microbial genera associated with the presence or absence of infection. We agglomerated microbial data to genus level and calculated the relative abundance of each taxon, then conducted an indicator analysis using the *multipatt* function in the indicspecies package (Cáceres & Legendre, 2009) to identify microbial genera associated with the presence or absence of *H. fraxineus* in leaves. Finally, we conducted a DESeq2 analysis (Love, Huber, & Anders, 2014) to identify ESVs with significantly different abundances according to infection status of the leaves.

Functional analysis of fungal communities

To identify the trophic modes and functional guilds of fungal ESVs, we extracted the OTU table of all samples complete with taxonomic annotation, and uploaded this to the online FUNGuild tool (Nguyen et al., 2016). We plotted stacked bar charts to visualise the variation in relative abundance of trophic mode and guild representations according to *H. fraxineus* infection category.

Relationships between fungal and bacterial communities

To identify relationships between fungal and bacterial communities, we extracted Jensen-Shannon divergence matrices between all samples for both fungal and bacterial communities in the phyloseq

and vegan packages. We used Mantel tests to correlate fungal and bacterial community distances and visualised the relationship using a scatter plot.

To identify co-occurrence networks between taxa according to *H. fraxineus* infection category in the Manchester samples, we rarefied fungal communities to 14080 reads, and bacterial communities to 800 reads (resulting in the loss of three samples). We merged these rarefied phyloseq objects for bacterial and fungal communities and converted them to binary presence/absence data. We then calculated the co-occurrence between each pair of ESVs by constructing a Spearman's correlation coefficient matrix in the bioDist package (Ding, Gentleman, & Carey, 2018; Williams, Howe, & Hofmockel, 2014). We calculated the number of associations with $p < 0.05$ for each infection category (absent, low, medium and high), and those with $-0.50 > rho > 0.50$, and $-0.75 > rho > 0.75$. We visualised those with $rho > 0.75$ (positive associations only) using network plots for the four infection categories.

Relationships between tree genotype and phyllosphere composition, and tree genotype and H. fraxineus *infection*

For the Stirling samples, we used the *merge_samples* function in phyloseq to calculate the mean phyllosphere composition across the three leaf samples collected per tree, and converted the per-tree values to relative abundance (for the Manchester samples we only collected one leaf per tree and so this step was not necessary). To measure pairwise microbial community dissimilarities among trees, we extracted Jensen-Shannon divergence matrices between trees for both fungal and bacterial communities using phyloseq and vegan. We created separate datasets for each site, as well as a combined dataset. We then used Mantel tests to test for correlations between the microbial distance matrices and tree genetic distance matrices (as calculated above).

To identify relationships between *H. fraxineus* infection and host genotype, we used individual generalised linear mixed models in lme4 (with site as a random factor) to determine whether multiple measures of genetic diversity (proportion of heterozygous loci, observed heterozygosity, expected heterozygosity, internal relatedness and homozygosity by locus) influenced tree infection score and average log *H. fraxineus* infection intensity.

RESULTS

H. fraxineus *prevalence*

We found variable *H. fraxineus* infection prevalence between sites. All samples collected at Stirling were infected, including trees that showed no visible signs of infection (i.e. tree infection score of 0), whereas in Manchester, 20 out of the 33 (60.6 %) trees sampled were infected.

Phyllosphere composition by site and H. fraxineus *infection*

The most abundant fungal classes across all samples were Tremellomycetes, Dothideomycetes, Leotiomycetes, Eurotiomycetes, Taphrinomycetes and Cystobasidiomycetes (Figure 1a). The most abundant bacterial classes were Alphaproteobacteria, Cytophagia, Betaproteobacteria, Actinobacteria, Deltaproteobacteria, Sphingobacteriia, and Deinococci (Figure 1b). PERMANOVA (adonis) analysis showed a significant effect of site (i.e. Manchester or Stirling; $F_{1,136} = 34.615$, $R^2 = 0.204$, $p = 0.001$) but not *H. fraxineus* infection category (i.e. “absent”, “low”, “medium” or “high”) ($F_{3,136} = 1.061$, $R^2 = 0.019$, $p = 0.342$) (Figure 2a) on fungal community composition. Similarly, site had a significant effect on bacterial community composition ($F_{1,105} = 25.968$, $R^2 = 0.199$, $p = 0.001$) but *H. fraxineus* infection category did not ($F_{3,105} = 1.088$, $R^2 = 0.025$, $p = 0.301$) (Figure 2b). Site explained a similar proportion of the variation in fungal (20.4%) and bacterial (19.9%) communities, whereas *H. fraxineus* infection category explained only 1.9% and 2.5% of fungal and bacterial community composition, respectively. The relative abundance of the top 10 most abundant fungal (Figure 1a) and bacterial (Figure 1b) classes were considerably different between sites. Within sites, there were also differences in the relative abundance of different taxa according to *H. fraxineus* infection category, however, there were no clear patterns in how these groups varied between these categories, either within sites or across sites (Figures 1a and 1b).

In the linear mixed models, fungal community alpha-diversity significantly predicted *H. fraxineus* infection intensity in terms of both community richness ($X^2 = 4.560$, $p = 0.033$; Figure 3a) and evenness ($X^2 = 3.932$, $p = 0.047$; Figure 3b). In both cases, as fungal community alpha-diversity increased, so did *H. fraxineus* infection. Relationships were not statistically significant between log *H. fraxineus* infection and bacterial community alpha-diversity (richness, $X^2 = 0.787$, $p = 0.375$; evenness, $X^2 = 0.509$, $p = 0.475$). There was a significant relationship between log *H. fraxineus* infection and fungal community beta-diversity (PCoA axis 1 score, $X^2 = 39.528$, $p < 0.001$, Figure 3c;

PCoA axis 2 score, $X^2 = 5.511$, $p = 0.019$), and log *H. fraxineus* infection and bacterial community beta-diversity (PCoA axis 1 score; $X^2 = 5.4606$, $p = 0.019$; Figure 3d). However, tree infection score was not significantly predicted by any microbial diversity measure (all $p > 0.05$).

We identified 26 fungal genera (out of 390) and six bacterial genera (out of 255) with significant positive correlations with log *H. fraxineus* infection intensity (all $p < 0.05$; Figure 4). We also identified 217 fungal genera and four bacterial genera with a significant negative correlation with log *H. fraxineus* infection intensity (all $p < 0.05$; Figure 4). Indicator analysis only identified one fungal genus (*Neofabraea*, IndVal = 0.378, $p = 0.025$) and one bacterial genus (*Pedobacter*, IndVal = 0.643, $p = 0.005$) that were significantly associated with the absence of *H. fraxineus* infection (i.e. these genera were much more commonly found in the absence of infection). Association analysis identified two fungal genera significantly associated with the presence of *H. fraxineus* infection (*Hannaella*, IndVal = 0.525, $p = 0.050$; *Keissleriella*, IndVal = 0.450, $p = 0.020$). DESeq2 analysis did not identify any differentially abundant bacterial ESVs between infected and uninfected leaves (Figure S2), but did for fungal ESVs; *Phyllactinia fraxini* was significantly more abundant in uninfected leaves (log2FoldChange = -24.429, $p < 0.001$) and one *Genolevuria sp.* was significantly more abundant in infected leaves (log2FoldChange = 3.753, $p < 0.001$; Figure S3). For both fungi and bacteria, however, the DESeq2 analysis indicated there was no clear pattern of ESVs within genera showing particular patterns in abundance according to *H. fraxineus* infection. That is, genus is not an accurate indicator of anti-pathogen capabilities (Figures S2 and S3).

The genus *Hymenoscyphus* had a significant positive correlation with *H. fraxineus* infection intensity ($r = 0.375$, $p < 0.001$; Figure 4). Although six species of *Hymenoscyphus* were identified (*H. scutula, repandus, menthae, albidus, kathiae, caudatus*) to species level through ITS rRNA amplicon sequencing, as well as one other unidentified *Hymenoscyphus sp.* that was found at low prevalence and abundance, *H. fraxineus* itself was not found in our ITS rRNA dataset. However, the amplicon produced by ITS rRNA sequencing of DNA extracted from a pure *H. fraxineus* culture was not identified by UNITE (UNITE, 2017). Further NCBI BLAST searches of all the unidentified *Hymenoscyphus* and *Chalara* sequences in addition to unidentified sequences belonging to Fungi, Ascomycota, Leotiomycetes, Helotiales or Helotiaceae identified an additional 18 ESVs in our dataset as *H. fraxineus* (E value $< e^{-20}$, bit score > 80). However, five of these were removed during filtering of low read numbers, and the remainder did not sum up to more than 0.001% in any of the samples.

Therefore, despite high infection loads quantified through targeted qPCR, *H. fraxineus* did not appear to be present in our ITS rRNA amplicon sequencing data to any substantial degree.

Functional analysis of fungal communities

We obtained functional hypotheses for 65% of ITS rRNA ESVs. Functional analysis of fungal communities indicated that the relative abundance of pathotrophs (fungi causing disease and receiving nutrients at the expense of host cells; Nguyen et al., 2016; Tedersoo et al., 2014) increased as *H. fraxineus* infection intensity increased (Figure 5 and S4). However, the proportion of fungal species with unidentified trophic modes were higher in the absent and low infection categories (Figure 5). Despite this, the most abundant pathogen, *Phyllactinia fraxini,* had a relatively high abundance in leaves absent of infection (7.0%) and with low infection levels (6.2%), compared to medium (0.1%) and high (1.7%) infection levels. The genus *Phyllactinia* also had a significant negative correlation with log *H. fraxineus* infection intensity ($r = -0.378$, $p < 0.001$) although the negative relationship between log *H. fraxineus* (+1) and log *P. fraxini* (+1) was only approaching significance ($r = -0.15$, $p = 0.077$). Overall, *P. fraxini* was the most abundant pathogen and the fifth most abundant fungus across all samples (*Vishniacozyma foliicola, V. victoriae* and two species of Venturiales were more abundant; Table S2). The second most abundant pathogen was the yeast *Itersonilia pannonica* (formerly *Udeniomyces pannonicus*; Niwata, Takashima, Tornai-Lehoczki, Deak, & Nakase, 2002), which in contrast to *P. fraxini*, had lower abundance in leaves with absent (0.2%) and low (1.2%) *H. fraxineus* infection then in those with medium (6.5%) and high (8.1%) infection levels. Correlation analysis indicated a significant positive relationship between log *H. fraxineus* (+1) and log *I. pannonica* (+1) ($r = 0.49$, $p < 0.001$). In addition, the relative abundance of symbiotrophs (which receive nutrients through exchange with host cells), primarily lichens, also increased on infection by *H. fraxineus* (Figure 5; Figure S4).

Relationships between fungal and bacterial communities

Mantel tests identified significant correlations between fungal and bacterial communities of leaves across both sites ($r = 0.552$, $p = 0.001$; Figure 6a). Co-occurrence analysis indicated that leaves highly infected with *H. fraxineus* had fewer statistically significant ($p < 0.05$) cross-kingdom microbial connections than the other infection categories (Table 1). The majority of microbial associations in the

uninfected categories were of medium strength (-0.50 > rho and rho > 0.50) rather than strong (-0.75 > rho and rho > 0.75), and were characterised by sprawling, less-well-connected hubs with a considerable number of members (Table 1; Figure 7). The proportion of strong microbial connections increased as *H. fraxineus* infection increased, and in highly infected leaves, 100% of associations were strong and positive (rho > 0.75), but characterised by very few, strongly associated larger hubs involving relatively few members (Table 1; Figure 7; Table S3).

Effects of host genotype on phyllosphere composition and H. fraxineus *infection*

We found very little genetic differentiation between trees in Stirling and Manchester; F_{ST} between the sites was 0.034, while an AMOVA showed that only 2.4% of total genetic variation was found between sites (Table S3), with little clustering of sites in the PCoA (Figure 2c).

Across sites, there was a significant correlation between genetic distance and fungal community composition (r = 0.106, p = 0.005; Figure 6b), but no significant relationship between genetic distance and bacterial community composition (r = 0.013, p = 0.365). Within sites, the correlation between tree genetic distance and fungal community composition was statistically significant for Manchester (r = 0.155, p = 0.002) but not Stirling (r = 0.042, p = 0.372). Genetic distance was not significantly correlated with bacterial community composition at either site (Manchester: r = -0.065, p = 0.749; Stirling: r = 0.151, p = 0.091).

No heterozygosity measures significantly predicted *H. fraxineus* infection intensity or tree infection score (all p > 0.05).

DISCUSSION

Our results show that both fungal and bacterial community composition, as well a considerable number of microbial genera, are significantly correlated with *H. fraxineus* infection intensity. Cross et al. (2017) previously demonstrated that fungal community composition altered as the season progressed and *H. fraxineus* infection intensified, although it was not clear whether these changes resulted from seasonal effects or infection intensity. We extend this work to show that at a given time point, differences in both fungal and bacterial phyllosphere communities relate to *H. fraxineus* infection, even in the absence of phenotypic signs of infection. These effects were apparent in our

mixed model analysis, but not significant in the PERMANOVA analysis; this may be due to a loss of statistical power from the use of infection categories (i.e., “absent”, “low”, “medium” or “high”) in the PERMANOVA rather than the continuous log *H. fraxineus* data used in the linear mixed model analysis. Changes in microbiome composition that correlate with pathogenic infection have also been identified in other tree species. For example, the bacterial microbiome of horse chestnut bark was altered by bleeding canker disease caused by the bacterium *Pseudomonas syringae* pv *aesculi* (Koskella et al., 2017). Similarly, Jakuschkin et al. (2016) found evidence of cross-kingdom endophytic dysbiosis in pedunculate oak (*Quercus robur* L.) on infection by *Erysiphe alphitoides*, the causal agent of oak powdery mildew.

Fungal alpha-diversity was positively correlated with *H. fraxineus* infection intensity, although bacterial alpha-diversity was not. Although it may be expected that higher microbiome diversity would increase microbiome-mediated resistance to invasive pathogens through competitive exclusion, the relationship between microbiome diversity and pathogen susceptibility actually varies considerably among host taxa (e.g. Bates et al., 2018; Dillon, Vennard, Buckling, & Charnley, 2005; Johnson & Hoverman, 2012; Näpflin & Schmid-Hempel, 2018; Upreti & Thomas, 2015; Wehner, Antunes, Powell, Mazukatow, & Rillig, 2010). Our results suggest that low diversity may reflect a stable and resilient microbiome that resists infection, or that *H. fraxineus* infection is associated with dysbiosis that allows for the proliferation of many new members in the microbiome. Indeed, co-occurrence analysis showed that medium-strength, minimally-connected networks in leaves with absent or low *H. fraxineus* infection become a few, high-strength, highly-connected hubs under medium or high infection. The co-occurrence analysis indicates that although *H. fraxineus* infection is associated with strong microbial networks, these are relatively depauperate in members and so the stability of phyllosphere communities in infected leaves may be compromised. Conversely, leaves with absent or low infection rates have more complex co-occurrence hubs with more medium-strength connections involving more members. Together with the higher fungal diversity observed as *H. fraxineus* infection intensity increased, these results suggest *H. fraxineus* infection is associated with dysbiosis in ash leaves that allows for the proliferation of microbial phyllosphere endophytes. Furthermore, Cross et al. (2017) suggested that phyllosphere communities are not significantly altered by *H. fraxineus* until a particular infection density is reached, and our findings support this. We also show that even leaves with high infection intensities can appear asymptotic but exhibit evidence of phyllosphere dysbiosis, although it

is not clear whether such dysbiosis is a result of infection, or in fact facilitates infection. Although causality can be hard to identify without explicit infection trials, leaves in this study were collected late in the season in areas of epidemic infection. This suggests leaves that were clear of infection at the time of sampling may have been able to resist infection up to that point, and so patterns identified here may be representative of real-world infection trials.

There is other evidence that associations between plants and microbes become stronger when the host is stressed, with positive effects for the host (Mendes et al., 2011; Pineda, Dicke, Pieterse, & Pozo, 2013). For example, plants can exploit beneficial microbes when under water or nutrient stress, with positive effects on plant growth and insect attack (Pineda, Dicke, Pieterse, & Pozo, 2013). How the networks identified in our data influence the host to improve resistance to *H. fraxineus* remains to be explored. We also identify considerable co-variation between bacterial and fungal communities, and extensive cross-kingdom associations in the leaves of ash trees. Syntrophy (i.e. cross-feeding between microbial species) is phylogenetically and environmentally widespread throughout microbial taxa and leads to high connectedness between members of the microbiome (Hassani et al., 2018; Kouzuma, Kato, & Watanabe, 2015; McInerney et al., 2008). Furthermore, nutrient and pH modulation by fungal communities can influence bacterial colonisation (Hassani et al., 2018; Johnston et al., 2018). Thus, such interactions between these two kingdoms may be expected, and the importance of these in the context of disease resistance warrants considerable attention.

The functional analysis identified an overall increase in fungal pathogens as *H. fraxineus* infection increased. Disruption to the natural endosymbiont community by *H. fraxineus* infection may break up previously filled niches, thus allowing co- or secondary infections. Alternatively, prior infection by other pathogens may allow *H. fraxineus* to proliferate. In particular, we found convincing evidence of co-infection by *Itersonilia pannonica,* a likely yeast pathogen (Nguyen et al., 2016). Other secondary infections have previously been documented in ash dieback outbreaks, including *Alternaria alternata, Armillaria spp., Cytospora pruinosa, Diaporthe eres, Diplodia mutila, Fusarium avenaceum, Fusarium lateritium, Fusarium solani, Phoma exigua, Phytophthora spp.* and *Valsa ambiens* (Kowalski, Kraj, & Bednarz, 2016; Marçais, Husson, Godart, & Caël, 2016; Orlikowski et al., 2011). Co-infection can have considerable implications for host fitness and the evolution of pathogens (Tollenaere, Susi, & Laine, 2016), and may well contribute to the progression of ash dieback. Similar findings have been shown in other study systems, whereby disruption of the resident microbiome

allows other microbial groups to proliferate (Antwis, Garcia, Fidgett, & Preziosi, 2014; Erkosar & Leulier, 2014; Kamada, Chen, Inohara, & Núñez, 2013; Liu, Liu, Ran, Hu, & Zhou, 2016). *Hymenoscyphus fraxineus* infection also appeared to be associated with the growth of fungal symbiotrophs, particularly lichens. Mitchell et al. (2014) identified 548 lichen species associated with *F. excelsior*, indicating such associations are common for this host. Converse to these positive associations between *H. fraxineus* and other microbes, we saw a reduction in the pathogen *Phyllactinia fraxini* as *H. fraxineus* increased, suggesting the latter may displace the former. *Phyllactinia fraxini* is an ecto-parasitic fungus that causes powdery mildew in ash trees (Takamatsu et al., 2008). The rapid outcompeting of one pathogen by another has been termed a 'selective sweep' and is well-documented in plant hosts, particularly crops (Zhan & McDonald, 2013). These results are contrary to Cross et al. (2017), who found *Phyllactinia* positively correlated with *H. fraxineus* infection, indicating that further research is required to improve our understanding of the interactions between *H. fraxineus* and other pathogens.

Based on a combination of analyses, we identified *Neofabraea* fungi and *Pedobacter* bacteria as potential antagonists of *H. fraxineus* infection, which may have potential for development of anti-pathogenic inoculants or probiotics. *Neofabraea* has previously been shown to inhibit *H. fraxineus in vitro* (Schlegel et al., 2016). Given the large number of microbial genera present in the leaves, it is surprising that only three genera showed significant association with the absence of *H. fraxineus*, despite widespread and heavy infection in the study sites. This finding may reflect the propensity for wide variation within genera for anti-pathogen capabilities (Antwis & Harrison, 2018; Becker et al., 2015), as indicated by the DESeq2 analysis, in which ESVs within a genus did not necessarily show the same type of response (i.e. positive or negative) to *H. fraxineus*. Thus, although we identify potential genera of interest, a genus-by-genus approach may not be the best method for identifying potential probiotics. *In vitro* studies have identified over 70 species of fungi that inhibit the growth of *H. fraxineus* (Kosawang et al., 2018; Schlegel et al., 2016; Schulz et al., 2015). In addition, secondary metabolite production by endophytes is generally down-regulated when cultured individually but activated in response to other microbes (Schroeckh et al., 2009; Suryanarayanan, 2013), indicating complex and bi-directional interactions between members of the phyllosphere microbiome. Thus, co-culturing such microbes, potentially identified through co-occurrence hubs, may help guide the development of consortium-based approaches to probiotic development, which may be more effective

than single-species probiotics (Antwis & Harrison, 2018; Kaminsky, Trexler, Malik, Hockett, & Bell, 2018; Schulz et al., 2015).

We did not find evidence of host genotype influencing tree infection score or *H. fraxineus* pathogen loads. Host genetic variation has previously been found to influence ash dieback susceptibility (Harper et al., 2016; Sollars et al., 2017). However, these studies used genomic and transcriptomic approaches that give finer resolution than microsatellite markers allow. Furthermore, microsatellites cover non-coding regions of DNA and so may be less likely to directly affect pathogen susceptibility, although they are often physically linked to genes that code for functional traits (Santucci et al. 2007; Gemayel et al. 2010; Tollenaere et al. 2012). Host genetic distance did, however, predict variation in fungal community composition (both across sites and within Manchester, but not within Stirling alone). Thus, microsatellites used in this study may be linked to functional traits that influence phyllosphere fungal communities. As such, host genetic influence on phyllosphere fungal communities could indirectly influence *H. fraxineus* susceptibility. The expression of a number of MADS box genes varies between susceptible and tolerant genotypes of ash trees, which may influence secondary metabolite production (Gantet & Memelink, 2002; Sollars et al., 2017) and thus, influence microbial community diversity on the leaf. Furthermore, higher iridoid glycoside concentrations were identified from biochemical profiles of leaves from susceptible ash trees, which may alter fungal growth (Sollars et al., 2017; Whitehead, Tiramani, & Bowers, 2016). Identifying genes associated microbiome composition in ash trees will allow us to determine whether these can be used along with host genetic markers to improve selection of tolerant trees and thus increase the pool from which selective breeding could occur.

Sampling site was the main predictor of total community composition for both fungal and bacterial communities of ash leaves. Considerable variation in phyllosphere composition still existed between the sites despite the Stirling and Manchester trees being genetically similar, indicating that site-level variation was not due to population differentiation. Both fine- and broad-scale geographic variation affects microbiome composition in many study organisms (Antwis, Lea, Unwin, & Shultz, 2018; Griffiths et al., 2018; Yatsunenko et al., 2012) including plants (Edwards et al., 2015; Peiffer et al., 2013; Wagner et al., 2016). The site-level differences observed in this study may reflect a range of differences in abiotic conditions, given that environmental variables, such as temperature, and rainfall are considerable determinants of both microbiome composition and pathogen activity (Barge,

589 Leopold, Peay, Newcombe, & Busby, 2019; Busby, Newcombe, Dirzo, & Whitham, 2014; Busby,
590 Ridout, & Newcombe, 2016; Dal Maso & Montecchio, 2014; Laforest-Lapointe, Messier, & Kembel,
591 2016; Zimmerman & Vitousek, 2012). Methodological differences could also be responsible – in
592 Stirling, we sampled saplings whereas in Manchester we sampled mature trees. Tree and leaf age
593 both significantly affect phyllosphere microbiome structure, possibly due to microbial community
594 succession patterns, as well as niche variation associated with age-related physiological changes in
595 leaves (Redford & Fierer 2009, Meaden, Metcalf, & Koskella, 2016). Thus, site-level patterns in our
596 data may reflect these considerable drivers of microbiome composition. Alternatively, there may well
597 have been different isolates of *H. fraxineus* at the two sites, which may have differentially affected leaf
598 microbial communities through isolate variation in enzyme profiles and growth rates (Junker, de Vries,
599 Eickhorst, & Schulz, 2017). We also observed variation in the strength of genotype x microbiome
600 interactions between sites. This may be due to environmental differences, and thus could indicate the
601 presence of genotype by microbiome by environment (G x M x E) interactions (Smith et al., 2015). G x
602 M x E interactions may be particularly important for disease susceptibility and mitigation as
603 environment plays a considerable role in pathogenicity. Thus, microbially-derived resistance to *H.*
604 *fraxineus*, in addition to the effectiveness of any microbial treatments, may be population, age, or site
605 specific, and may vary between sites based on environmental and biological variables, including
606 abiotic factors as well as pollution, tree density, species mix, and herbivore activity. Much more work
607 is required to determine how environmental factors and pathogen strain variation affect microbially-
608 derived resilience to *H. fraxineus* infection, and identifying cross-population and cross-isolate
609 microbial signatures of resistance will be key to the success of a microbial-based approach to disease
610 management.

611 It is worth noting that we did not identify *H. fraxineus* itself to species level using ITS rRNA
612 amplicon sequencing (or through additional BLAST searches), despite qPCR demonstrating
613 widespread and high infection rates. Cross et al. (2017) found similar results when using ITS-1. This
614 may be because *H. fraxineus* (or its many strains) is not fully represented in the UNITE database, or
615 because *H. fraxineus* has a long fragment length (~550bp) for the primer combination we used, which
616 would be less readily sequenced than shorter reads on the Illumina MiSeq platform (Lindahl et al.,
617 2013). As with all amplicon sequencing, there are limitations to the taxa that can be identified based

on the primers used, and wider analysis using multiple markers will identify further genera involved in *H. fraxineus* infection dynamics on ash leaves (Cross et al., 2017; Lindahl et al., 2013).

In conclusion, we show that bacterial and fungal communities of ash leaves are strongly associated with one another, and the composition of both are associated with *H. fraxineus* infection dynamics. Leaves with absent or low infection rates have more complex microbial co-occurrence hubs characterised by medium-strength connections involving many members, whereas under medium to high infection levels, microbial networks were characterised by stronger associations between fewer members and with fewer hubs. This suggests after a particular infection pressure is reached, phyllosphere communities become disrupted. Although host genotype did not affect *H. fraxineus* infection directly, it did have a significant effect on fungal community composition and thus, may have indirect consequences for pathogen susceptibility. Identifying host genes that determine microbiome composition in ash trees may improve selection of trees with more resistant microbiomes, which in combination with host genetic markers of tolerance, may increase the proportion of ash trees from which selective breeding could occur.

ACKNOWLEDGEMENTS

We would like to thank City of Trees (particularly Sam Bolton) and SusTrans (particularly Mary Seaton) for access to sampling sites. We are very grateful to Elizabeth Harriet Meade, Bryony Unwin and Jade Newton-Youens for assistance with field and lab work, and to Dr Andrea Harper for comments on a draft of this manuscript. Our thanks to BEI Resources, the US Forest Service, and the University of Wisconsin-Madison for provision of bacterial and fungal mock communities, as well as The John Innes Centre and Renaud Ioos for providing *H. fraxineus* standards. This research was funded by The Tree Research and Education Endowment Fund, Scottish Natural Heritage, and The Woodland Trust.

AUTHOR CONTRIBUTIONS

RA, SMG, JH and DO'B conceived the study. RA, SMG, MG and JR produced the data. RA, SMG and IG analysed the data. All authors wrote and approved the manuscript.

DATA ACCESSIBILITY

Sequence data for this project are available from the NCBI Sequence Read Archive (project numbers PRJNA515030 and PRJNA515031) and microsatellite genotypes are available at doi:10.6084/m9.figshare.7599902. All analysis code has been provided as RMarkdown files.

REFERENCES

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S. T., Weigel, D., & Kemen, E. M. (2016). Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. *PLoS Biology*, *14*(1), 1–31. doi: 10.1371/journal.pbio.1002352

Alberto, F. (2009). MsatAllele_1.0: An R package to visualize the binning of microsatellite alleles. *Journal of Heredity*, *100*(3), 394–397. doi: 10.1093/jhered/esn110

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution*, *19*(10), 535–544. doi: 10.1016/j.tree.2004.07.021

Antwis, R.E., Garcia, G., Fidgett, A. L., & Preziosi, R. F. (2014). Tagging frogs with passive integrated transponders causes disruption of the cutaneous bacterial community and proliferation of opportunistic fungi. *Applied and Environmental Microbiology*, *80*(15). doi: 10.1128/AEM.01175-14

Antwis, R.E., & Harrison, X. A. (2018). Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates. *Molecular Ecology*, *27*(2). doi: 10.1111/mec.14456

Antwis, Rachael E., Lea, J. M. D., Unwin, B., & Shultz, S. (2018). Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. *Microbiome*, *6*(1), 207. doi: 10.1186/s40168-018-0593-2

Bálint, M., Tiffin, P., Hallström, B., O'Hara, R. B., Olson, M. S., Fankhauser, J. D., … Schmitt, I. (2013). Host Genotype Shapes the Foliar Fungal Microbiome of Balsam Poplar (Populus balsamifera). *PLoS ONE*, *8*(1). doi: 10.1371/journal.pone.0053987

Barge, E. G., Leopold, D. R., Peay, K. G., Newcombe, G., & Busby, P. E. (2019). Differentiating spatial from environmental effects on foliar fungal communities of Populus trichocarpa. *Journal*

678 *of Biogeography*, (January), 2001–2011. doi: 10.1111/jbi.13641
679 Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). *Fitting Linear Mixed-Effects Models using*
680 *lme4*. (1). doi: 10.18637/jss.v067.i01
681 Bates, K. A., Clare, F. C., O'Hanlon, S., Bosch, J., Brookes, L., Hopkins, K., … Harrison, X. A. (2018).
682 Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community
683 structure. *Nature Communications*, *9*(1), 1–11. doi: 10.1038/s41467-018-02967-w
684 Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., … Becker, M.
685 H. (2015). *Composition of symbiotic bacteria predicts survival in Panamanian golden frogs*
686 *infected with a lethal fungus*.
687 Beckers, B., Op De Beeck, M., Weyens, N., Van Acker, R., Van Montagu, M., Boerjan, W., &
688 Vangronsveld, J. (2016). Lignin engineering in field-grown poplar trees affects the endosphere
689 bacterial microbiome. *Proceedings of the National Academy of Sciences*, *113*(8), 2312–2317.
690 doi: 10.1073/pnas.1523264113
691 Bender, S. F., Wagg, C., & van der Heijden, M. G. A. (2016). An Underground Revolution: Biodiversity
692 and Soil Ecological Engineering for Agricultural Sustainability. *Trends in Ecology and Evolution*,
693 *31*(6), 440–452. doi: 10.1016/j.tree.2016.02.016
694 Blacket, M. J., Robin, C., Good, R. T., Lee, S. F., & Miller, A. D. (2012). *Universal primers for*
695 *fluorescent labelling of PCR fragments — an efficient and cost-effective approach to genotyping*
696 *by fluorescence*. 456–463. doi: 10.1111/j.1755-0998.2011.03104.x
697 Boyd, I. L., Freer-Smith, P. H., Gilligan, C. A., & Godfray, H. C. J. (2013). The consequence of tree
698 pests and diseases for ecosystem services. *Science*, *342*(6160). doi: 10.1126/science.1235773
699 Brachet, S., Jubier, M. F., Richard, M., Jung-Muller, B., & Frascaria-Lacoste, N. (1998). *Rapid*
700 *identification of micro satellite loci using 5 ' anchored PCR in the common ash Fraxinus*
701 *excelsior*. (June).
702 Burdon, J. J., Thrall, P. H., & Ericson, and L. (2005). The Current and Future Dynamics of Disease in
703 Plant Communities. *Annual Review of Phytopathology*, *44*(1), 19–39. doi:
704 10.1146/annurev.phyto.43.040204.140238
705 Busby, P. E., Newcombe, G., Dirzo, R., & Whitham, T. G. (2014). Differentiating genetic and
706 environmental drivers of plant-pathogen community interactions. *Journal of Ecology*, *102*(5),
707 1300–1309. doi: 10.1111/1365-2745.12270

Busby, P. E., Peay, K. G., & Newcombe, G. (2016). Common foliar fungi of Populus trichocarpa modify Melampsora rust disease severity. *New Phytologist*, *209*(4), 1681–1692. doi: 10.1111/nph.13742

Busby, P. E., Ridout, M., & Newcombe, G. (2016). Fungal endophytes: modifiers of plant disease. *Plant Molecular Biology*, *90*(6), 645–655. doi: 10.1007/s11103-015-0412-0

Cáceres, M. De, & Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. *Ecology*, *90*(12), 3566–3574. doi: 10.1890/08-1823.1

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. doi: 10.1038/nmeth.3869

Chapuis, M.-P., & Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, *24*(3), 621–631. doi: 10.1093/molbev/msl191

Coker, T. L. R., Rozsypálek, J., Edwards, A., Harwood, T. P., Butfoy, L., & Buggs, R. J. A. (2019). Estimating mortality rates of European ash (*Fraxinus excelsior*) under the ash dieback (*Hymenoscyphus fraxineus*) epidemic. *Plants, People, Planet*, *1*(1), 48–58. doi: 10.1002/ppp3.11

Coulon, A. (2010). *GENHET : an easy-to-use R function to estimate individual heterozygosity*. 167–169. doi: 10.1111/j.1755-0998.2009.02731.x

Cross, H., Sï¿½nstebï¿½, J. H., Nagy, N. E., Timmermann, V., Solheim, H., Bï¿½rja, I., … Hietala, A. M. (2017). Fungal diversity and seasonal succession in ash leaves infected by the invasive ascomycete Hymenoscyphus fraxineus. *New Phytologist*, *213*(3), 1405–1417. doi: 10.1111/nph.14204

Culley, T. M., Stamper, T. I., Stokes, R. L., Brzyski, J. R., Nicole, A., Klooster, M. R., & Merritt, B. J. (2013). *An Efficient Technique for Primer Development and Application that Integrates Fluorescent Labeling and Multiplex PCR A N EFFICIENT TECHNIQUE FOR PRIMER DEVELOPMENT AND APPLICATION THAT INTEGRATES FLUORESCENT LABELING AND MULTIPLEX*. *1*(10). doi: 10.3732/apps.1300027

Dal Maso, E., & Montecchio, L. (2014). Risk of natural spread of Hymenoscyphus fraxineus with environmental niche modelling and ensemble forecasting technique. *Forest Research*, *3*(4), 131. doi: 10.4172/21689776.1000131

Dempster, A. P., Laird, N. M., & Rubin, D. B. (1977). Maximum likelihood from incomplete data via the

738 EM algorithm. *Journal of the Royal Statistical Society, Series B*, *39*(1), 1–38.
739 Dillon, R. J., Vennard, C. T., Buckling, A., & Charnley, A. K. (2005). Diversity of locust gut bacteria
740 protects against pathogen invasion. *Ecology Letters*, *8*(12), 1291–1298. doi: 10.1111/j.1461-
741 0248.2005.00828.x
742 Ding, B., Gentleman, R., & Carey, V. (2018). *bioDist: Different distance measures. R package version*
743 *1.54.0.*
744 Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., …
745 Sundaresan, V. (2015). Structure, variation, and assembly of the root-associated microbiomes of
746 rice. *Proceedings of the National Academy of Sciences*, *112*(8), E911–E920. doi:
747 10.1073/pnas.1414592112
748 Erkosar, B., & Leulier, F. (2014). Transient adult microbiota, gut homeostasis and longevity: novel
749 insights from the Drosophila model. *FEBS Letters*, *588*(June), 4250–4257. doi:
750 10.1016/j.febslet.2014.06.041
751 Foo, J. L., Ling, H., Lee, Y. S., & Chang, M. W. (2017). Microbiome engineering: Current applications
752 and its future. *Biotechnology Journal*, *12*(3), 1–11. doi: 10.1002/biot.201600099
753 Freer-Smith, P. H., & Webber, J. F. (2017). Tree pests and diseases: the threat to biodiversity and the
754 delivery of ecosystem services. *Biodiversity and Conservation*, *26*(13), 3167–3181. doi:
755 10.1007/s10531-015-1019-0
756 Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., & Sarniguet, A. (2011). Bacterial-
757 Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food
758 Microbiologists. *Microbiology and Molecular Biology Reviews*, *75*(4), 583–609. doi:
759 10.1128/MMBR.00020-11
760 Gantet, P., & Memelink, J. (2002). Transcription factors: Tools to engineer the production of
761 pharmacologically active plant metabolites. *Trends in Pharmacological Sciences*, *23*(12), 563–
762 569. doi: 10.1016/S0165-6147(02)02098-9
763 Grad, B., Kowalski, T., & Kraj, W. (2009). Studies on Secondary Metabolite Produced By Chalara
764 Fraxinea and Its Phytotoxic Influence on Fraxinus Excelsior. *Phytopathologia*, *54*, 61–69.
765 Griffiths, S. M., Harrison, X. A., Weldon, C., Wood, M. D., Pretorius, A., Hopkins, K., … Antwis, R. E.
766 (2018). Genetic variability and ontogeny predict microbiome structure in a disease-challenged
767 montane amphibian. *The ISME Journal*, 1. doi: 10.1038/s41396-018-0167-0

Gross, A., Zaffarano, P. L., Duo, A., & Grünig, C. R. (2012). Reproductive mode and life cycle of the ash dieback pathogen Hymenoscyphus pseudoalbidus. *Fungal Genetics and Biology*, *49*(12), 977–986. doi: 10.1016/j.fgb.2012.08.008

Haňáčková, Z., Havrdová, L., Černý, K., Zahradník, D., & Koukol, O. (2017). Fungal endophytes in ash shoots – Diversity and inhibition of Hymenoscyphus fraxineus. *Baltic Forestry*, *23*(1), 89–106.

Harper, A. L., McKinney, L. V., Nielsen, L. R., Havlickova, L., Li, Y., Trick, M., … Bancroft, I. (2016). Molecular markers for tolerance of European ash (Fraxinus excelsior) to dieback disease identified using Associative Transcriptomics. *Scientific Reports*, *6*(January). doi: 10.1038/srep19335

Hassani, M. A., Durán, P., & Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome*, *6*(1), 58. doi: 10.1186/s40168-018-0445-0

Hrabětová, M., Černý, K., Zahradník, D., & Havrdová, L. (2017). Efficacy of fungicides on Hymenoscyphus fraxineus and their potential for control of ash dieback in forest nurseries. *Forest Pathology*, *47*(2), 1–9. doi: 10.1111/efp.12311

Ioos, R., & Fourrier, C. (2011). Validation and accreditation of a duplex real-time PCR test for reliable in planta detection of Chalara fraxinea. *EPPO Bulletin*, *41*(1), 21–26. doi: 10.1111/j.1365-2338.2010.02430.x

Ioos, Renaud, Kowalski, T., Husson, C., & Holdenrieder, O. (2009). Rapid in planta detection of Chalara fraxinea by a real-time PCR assay using a dual-labelled probe. *European Journal of Plant Pathology*, *125*(2), 329–335. doi: 10.1007/s10658-009-9471-x

Jacobs, D. F. (2007). *Toward development of silvical strategies for forest restoration of American chestnut (Castanea dentata) using blight-resistant hybrids*. *7*, 1–10. doi: 10.1016/j.biocon.2007.03.013

Jakuschkin, B., Fievet, V., Schwaller, L., Fort, T., Robin, C., & Vacher, C. (2016). Deciphering the Pathobiome: Intra- and Interkingdom Interactions Involving the Pathogen Erysiphe alphitoides. *Microbial Ecology*, *72*(4), 870–880. doi: 10.1007/s00248-016-0777-x

Jepson, P., & Arakelyan, I. (2017). Exploring public perceptions of solutions to tree diseases in the UK: Implications for policy-makers. *Environmental Science and Policy*, *76*(February), 70–77. doi: 10.1016/j.envsci.2017.06.008

798 Johnson, P. T. J., & Hoverman, J. T. (2012). Parasite diversity and coinfection determine pathogen
799 infection success and host fitness. *Proceedings of the National Academy of Sciences*, *109*(23),
800 9006–9011. doi: 10.1073/pnas.1201790109
801 Johnston, S. R., Hiscox, J., Savoury, M., Boddy, L., & Weightman, A. J. (2018). Highly competitive
802 fungi manipulate bacterial communities in decomposing beech wood (*Fagus sylvativa*). *FEMS*
803 *Microbiology Ecology*. doi: 10.1093/femsec/fiy225
804 Junker, C., de Vries, J., Eickhorst, C., & Schulz, B. (2017). Each isolate of Hymenoscyphus fraxineus
805 is unique as shown by exoenzyme and growth rate profiles. *Baltic Forestry*, *23*(1), 25–40.
806 Kamada, N., Chen, G. Y., Inohara, N., & Núñez, G. (2013). Control of pathogens and pathobionts by
807 the gut microbiota. *Nature Immunology*, *14*(7), 685–690. doi: 10.1038/ni.2608
808 Kaminsky, L. M., Trexler, R. V., Malik, R. J., Hockett, K. L., & Bell, T. H. (2018). The Inherent Conflicts
809 in Developing Soil Microbial Inoculants. *Trends in Biotechnology*, 1–12. doi:
810 10.1016/j.tibtech.2018.11.011
811 Koch, H., & Schmid-Hempel, P. (2012). Gut microbiota instead of host genotype drive the specificity
812 in the interaction of a natural host-parasite system. *Ecology Letters*, *15*(10), 1095–1103. doi:
813 10.1111/j.1461-0248.2012.01831.x
814 Kosawang, C., Amby, D. B., Bussaban, B., McKinney, L. V., Xu, J., Kjær, E. D., … Nielsen, L. R.
815 (2017). Fungal communities associated with species of Fraxinus tolerant to ash dieback, and
816 their potential for biological control. *Fungal Biology*, *122*, 110–120. doi:
817 10.1016/j.funbio.2017.11.002
818 Kosawang, C., Buchvaldt, D., Bussaban, B., Vig, L., Xu, J., Kjær, E. D., … Rostgaard, L. (2018).
819 *Fungal communities associated with species of Fraxinus tolerant to ash dieback , and their*
820 *potential for biological control*. *122*, 110–120.
821 Koskella, B., Meaden, S., Crowther, W. J., Leimu, R., & Metcalf, C. J. E. (2017). A signature of tree
822 health? Shifts in the microbiome and the ecological drivers of horse chestnut bleeding canker
823 disease. *New Phytologist*, *215*(2), 737–746. doi: 10.1111/nph.14560
824 Kouzuma, A., Kato, S., & Watanabe, K. (2015). Microbial interspecies interactions: Recent findings in
825 syntrophic consortia. *Frontiers in Microbiology*, *6*(MAY), 1–8. doi: 10.3389/fmicb.2015.00477
826 Kowalski, T., Kraj, W., & Bednarz, B. (2016). Fungi on stems and twigs in initial and advanced stages
827 of dieback of European ash (Fraxinus excelsior) in Poland. *European Journal of Forest*

Research, *135*(3), 565–579. doi: 10.1007/s10342-016-0955-x

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Applied and Environmental Microbiology*, *79*(17), 5112–5120. doi: 10.1128/AEM.01043-13

Laforest-Lapointe, I., Messier, C., & Kembel, S. W. (2016). Host species identity, site and time drive temperate tree phyllosphere bacterial community structure. *Microbiome*, *4*, 1–10. doi: 10.1186/s40168-016-0174-1

Lahti, L., & Shetty, S. (2017). *Tools for microbiome analysis in R. Microbiome package version 1.1.10013.* (p. http://microbiome.github.com/microbiome.). p. http://microbiome.github.com/microbiome.

Lefort, F., Brachet, S., Frascaria-Lacoste, N., Edwards, K. J., & Douglas, G. C. (1999). *Identification and characterization of microsatellite loci in ash (Fraxinus excelsior L.) and their conservation in the olive family (Oleaceae)*. *288*, 1088–1089.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., … Kauserud, H. (2013). Methods Fungal community analysis by high-throughput sequencing of amplified markers – a user ' s guide. *New Phytologist*, *199*, 288–299.

Linnakoski, R., Forbes, K. M., Wingfield, M. J., Pulkkinen, P., & Asiegbu, F. O. (2017). Testing Projected Climate Change Conditions on the Endoconidiophora polonica / Norway spruce Pathosystem Shows Fungal Strain Specific Effects. *Frontiers in Plant Science*, *8*(May), 1–9. doi: 10.3389/fpls.2017.00883

Liu, Z., Liu, W., Ran, C., Hu, J., & Zhou, Z. (2016). Abrupt suspension of probiotics administration may increase host pathogen susceptibility by inducing gut dysbiosis. *Scientific Reports*, *6*, 1–12. doi: 10.1038/srep23214

Lobo, A., Mckinney, L. V., Hansen, J. K., Kjær, E. D., & Nielsen, L. R. (2015). Genetic variation in dieback resistance in Fraxinus excelsior confirmed by progeny inoculation assay. *Forest Pathology*, *45*(5), 379–387. doi: 10.1111/efp.12179

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. doi: 10.1186/s13059-014-0550-8

Marçais, B., Husson, C., Godart, L., & Caël, O. (2016). Influence of site and stand factors on

Hymenoscyphus fraxineus-induced basal lesions. *Plant Pathology*, *65*(9), 1452–1461. doi: 10.1111/ppa.12542

Marcano, I. E., Díaz-Alcántara, C. A., Urbano, B., & González-Andrés, F. (2016). Assessment of bacterial populations associated with banana tree roots and development of successful plant probiotics for banana crop. *Soil Biology and Biochemistry*, *99*, 1–20. doi: 10.1016/j.soilbio.2016.04.013

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet*, *17*(1), 10. doi: 10.14806/ej.17.1.200

McInerney, M. J., Struchtemeyer, C. G., Sieber, J., Mouttaki, H., Stams, A. J. M., Schink, B., … Gunsalus, R. P. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Annals of the New York Academy of Sciences*, *1125*, 58–72. doi: 10.1196/annals.1419.005

Mckinney, L. V., Nielsen, L. R., Collinge, D. B., Thomsen, I. M., Hansen, J. K., & Kjær, E. D. (2014). The ash dieback crisis: Genetic variation in resistance can prove a long-term solution. *Plant Pathology*, *63*(3), 485–499. doi: 10.1111/ppa.12196

McKinney, L. V., Nielsen, L. R., Hansen, J. K., & Kjær, E. D. (2011). Presence of natural genetic resistance in Fraxinus excelsior (Oleraceae) to Chalara fraxinea (Ascomycota): An emerging infectious disease. *Heredity*, *106*(5), 788–797. doi: 10.1038/hdy.2010.119

McMullan, M., Rafiqi, M., Kaithakottil, G., Clavijo, B. J., Bilham, L., Orton, E., … Clark, M. D. (2018). The ash dieback invasion of Europe was founded by two genetically divergent individuals. *Nature Ecology and Evolution*.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4). doi: 10.1371/journal.pone.0061217

Meirmans, P. G., & Van Tiendener, P. H. (2004). Genotype and Genodive: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes*, *4*(4), 792–794. doi: 10.1111/j.1471-8286.2004.00770.x

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., Van Der Voort, M., Schneider, J. H. M., … Raaijmakers, J. M. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, *332*(6033), 1097–1100. doi: 10.1126/science.1203980

Menezes, A. B. De, Richardson, A. E., & Thrall, P. H. (2017). Linking fungal – bacterial co-

occurrences to soil ecosystem function ScienceDirect Linking fungal – bacterial co-occurrences to soil ecosystem function. *Current Opinion in Microbiology*, *37*(July), 135–141. doi: 10.1016/j.mib.2017.06.006

Mitchell, R. J., Bailey, S., Beaton, J. K., Bellamy, P. E., Brooker, R. W., Broome, A., … Woodward, S. 2014. (2014). The potential ecological impact of ash dieback in the UK. *Joint Nature Conservation Committee*, *175*(483), Report No. 483. Retrieved from http://jncc.defra.gov.uk/pdf/JNCC483_web.pdf

Mueller, U. G., & Sachs, J. L. (2015). Engineering Microbiomes to Improve Plant and Animal Health. *Trends in Microbiology*, *23*(10), 1–12. doi: 10.1016/j.tim.2015.07.009

Muñoz, F., Marçais, B., Dufour, J., & Dowkiw, A. (2016). Rising Out of the Ashes: Additive Genetic Variation for Crown and Collar Resistance to *Hymenoscyphus fraxineus* in *Fraxinus excelsior*. *Phytopathology*, *106*(12), 1535–1543. doi: 10.1094/PHYTO-11-15-0284-R

Näpflin, K., & Schmid-Hempel, P. (2018). High Gut Microbiota Diversity Provides Lower Resistance against Infection by an Intestinal Parasite in Bumblebees. *The American Naturalist*, *192*(2), 000–000. doi: 10.1086/698013

Neilan, B. A., Wilton, A. N., & Jacobs, D. (1997). *A universal procedure for primer labelling of amplicons*. *25*(14), 2938–2939.

Nguyen, N. H., Smith, D., Peay, K., & Kennedy, P. (2014). *Parsing ecological signal from noise in next generation amplicon sequencing*.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., … Kennedy, P. G. (2016). FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, *20*, 241–248. doi: 10.1016/j.funeco.2015.06.006

Niwata, Y., Takashima, M., Tornai-Lehoczki, J., Deak, T., & Nakase, T. (2002). Udeniomyces pannonicus sp. nov., a ballistoconidium-forming yeast isolated from leaves of plants in Hungary. *International Journal of Systematic and Evolutionary Microbiology*, *52*(5), 1887–1892. doi: 10.1099/ijs.0.02209-0

Oksanen, J., Blanchet, B., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Wagner, H. (2018). *vegan: Community Ecology Package.*

Orlikowski, B. L. B., Ptaszek, M., Rodziewicz, A., Nechwatal, J., Thinggaard, K., & Jung, T. (2011). *Phytophthora root and collar rot of mature Fraxinus excelsior in forest stands in Poland and*

Denmark. *41*, 510–519. doi: 10.1111/j.1439-0329.2011.00714.x

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research- an update. *Bioinformatics*, *28*(19), 2537–2539. doi: 10.1093/bioinformatics/bts460

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., … Ley, R. E. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences*, *110*(16), 6548–6553. doi: 10.1073/pnas.1302837110

Pineda, A., Dicke, M., Pieterse, C. M. J., & Pozo, M. J. (2013). Beneficial microbes in a changing environment: Are they always helping plants to deal with insects? *Functional Ecology*, *27*(3), 574–586. doi: 10.1111/1365-2435.12050

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. doi: 10.1093/nar/gks1219

Quiza, L., St-arnaud, M., Yergeau, E., & Rey, T. R. (2015). *Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering*. *6*(July), 1–11. doi: 10.3389/fpls.2015.00507

R Core Team. (2017). *R: A language and environment for statistical computing. R Foundation for Statistical Computing.* (p. Vienna, Austria. URL https://www.R-project.org/.). p. Vienna, Austria. URL https://www.R-project.org/.

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Métraux, J. P., & L'Haridon, F. (2016). The microbiome of the leaf surface of Arabidopsis protects against a fungal pathogen. *New Phytologist*, *210*(3), 1033–1043. doi: 10.1111/nph.13808

Roy, B. A., Alexander, H. M., Davidson, J., Campbell, F. T., Burdon, J. J., Sniezko, R., & Brasier, C. (2014). Increasing forest loss worldwide from invasive pests requires new trade regulations. *Frontiers in Ecology and the Environment*, *12*(8), 457–465. doi: 10.1890/130240

RStudio Team. (2016). *RStudio: Integrated Development for R.* (p. RStudio, Inc., Boston, MA URL http://www.rstudio.c). p. RStudio, Inc., Boston, MA URL http://www.rstudio.c.

Schlegel, M., Dubach, V., Buol, L. Von, & Sieber, T. N. (2018). *Effects of endophytic fungi on the ash dieback pathogen*. (February 2016), 1–8. doi: 10.1093/femsec/

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode

948 marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. doi:
949 10.1073/pnas.1117018109
950 Schroeckh, V., Scherlach, K., Nutzmann, H.-W., Shelest, E., Schmidt-Heck, W., Schuemann, J., …
951 Brakhage, A. A. (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal
952 polyketides in Aspergillus nidulans. *Proceedings of the National Academy of Sciences*, *106*(34),
953 14558–14563. doi: 10.1073/pnas.0901870106
954 Schulz, B., Haas, S., Junker, C., Andrée, N., & Schobert, M. (2015). Fungal endophytes are involved
955 in multiple balanced antagonisms. *Current Science*, *109*(1), 39–45.
956 Sheth, R. U., Cabral, V., Chen, S. P., & Wang, H. H. (2016). Manipulating Bacterial Communities by
957 in situ Microbiome Engineering. *Trends in Genetics*, *32*(4), 189–200. doi:
958 10.1016/j.tig.2016.01.005
959 Skovsgaard, J. P., Wilhelm, G. J., Thomsen, I. M., Metzler, B., Kirisits, T., Havrdová, L., … Clark, J.
960 (2017). Silvicultural strategies for Fraxinus excelsior in response to dieback caused by
961 Hymenoscyphus fraxineus. *Forestry*, *90*(4), 455–472. doi: 10.1093/forestry/cpx012
962 Smith, C. C. R., Snowberg, L. K., Gregory Caporaso, J., Knight, R., & Bolnick, D. I. (2015). Dietary
963 input of microbes and host genetic variation shape among-population differences in stickleback
964 gut microbiota. *ISME Journal*, *9*(11), 2515–2526. doi: 10.1038/ismej.2015.64
965 Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological
966 inference from next generation DNA sequencing. *PLoS ONE*, *9*(2). doi:
967 10.1371/journal.pone.0090234
968 Sollars, E. S. A., Harper, A. L., Kelly, L. J., Sambles, C. M., Ramirez-Gonzalez, R. H., Swarbreck, D.,
969 … Buggs, R. J. A. (2017). Genome sequence and genetic diversity of European ash trees.
970 *Nature*, *541*(7636), 212–216. doi: 10.1038/nature20786
971 Stener, L. G. (2018). Genetic evaluation of damage caused by ash dieback with emphasis on
972 selection stability over time. *Forest Ecology and Management*, *409*(October), 584–592. doi:
973 10.1016/j.foreco.2017.11.049
974 Suryanarayanan, T. (2013). Endophyte research: going beyond isolation and metabolite
975 documentation. *Fungal Ecology*, *6*, 561–568.
976 Takamatsu, S., Inagaki, M., Niinomi, S., Khodaparast, S. A., Shin, H. D., Grigaliunaite, B., &
977 Havrylenko, M. (2008). Comprehensive molecular phylogenetic analysis and evolution of the

genus Phyllactinia (Ascomycota: Erysiphales) and its allied genera. *Mycological Research*, *112*(3), 299–315. doi: 10.1016/j.mycres.2007.11.014

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., … Abarenkov, K. (2014). Global diversity and geography of soil fungi. *Science*, *346*, 1052–1053.

Thijs, S., Sillen, W., Rineau, F., Weyens, N., & Vangronsveld, J. (2016). Towards an enhanced understanding of plant-microbiome interactions to improve phytoremediation: Engineering the metaorganism. *Frontiers in Microbiology*, *7*(MAR), 1–15. doi: 10.3389/fmicb.2016.00341

Thomas R Turner, 1, Euan K James, 2, and Philip S Poole, & 1. (2013). The Plant Microbiome. *Genome Biology*, *14*(209), 1–10.

Tollenaere, C., Susi, H., & Laine, A. L. (2016). Evolutionary and Epidemiological Implications of Multiple Infection in Plants. *Trends in Plant Science*, *21*(1), 80–90. doi: 10.1016/j.tplants.2015.10.014

UNITE. (2017). *UNITE general FASTA release. Version 01.12.2017.*

Upreti, R., & Thomas, P. (2015). Root-associated bacterial endophytes from Ralstonia solanacearum resistant and susceptible tomato cultivars and their pathogen antagonistic effects. *Frontiers in Microbiology*, *6*(MAR), 1–12. doi: 10.3389/fmicb.2015.00255

van Overbeek, L. S., & Saikkonen, K. (2016). Impact of Bacterial-Fungal Interactions on the Colonization of the Endosphere. *Trends in Plant Science*, *21*(3), 230–242. doi: 10.1016/j.tplants.2016.01.003

Wagner, M. R., Lundberg, D. S., Del Rio, T. G., Tringe, S. G., Dangl, J. L., & Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature Communications*, *7*. doi: 10.1038/ncomms12151

Waldren, M. E. H. G. C. D. S., & Hodkinson, T. R. (2005). *Characterization and primer development for amplification of chloroplast microsatellite regions of Fraxinus excelsior*. *10*, 339–341.

Wehner, J., Antunes, P. M., Powell, J. R., Mazukatow, J., & Rillig, M. C. (2010). Plant pathogen protection by arbuscular mycorrhizas: A role for fungal diversity? *Pedobiologia*, *53*(3), 197–201. doi: 10.1016/j.pedobi.2009.10.002

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. In *Innis MA, Gefland DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to method and applications. San Diego, Academic Press.* (pp.

315–322). doi: 10.1016/b978-0-12-372180-8.50042-1

Whitehead, S. R., Tiramani, J., & Bowers, M. D. (2016). Iridoid glycosides from fruits reduce the growth of fungi associated with fruit rot. *Journal of Plant Ecology*, *9*(3), 357–366. doi: 10.1093/jpe/rtv063

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag New York.

Williams, R. J., Howe, A., & Hofmockel, K. S. (2014). Demonstrating microbial co-occurrence pattern analyses within and between ecosystems. *Frontiers in Microbiology*, *5*(JULY), 1–10. doi: 10.3389/fmicb.2014.00358

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. doi: 10.1038/nature11053

Yergeau, E., Bell, T. H., Champagne, J., Maynard, C., Tardif, S., Tremblay, J., & Greer, C. W. (2015). Transplanting soil microbiomes leads to lasting effects on willow growth, but not on the rhizosphere microbiome. *Frontiers in Microbiology*, *6*(DEC), 1–14. doi: 10.3389/fmicb.2015.01436

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., … Glöckner, F. O. (2014). The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Research*, *42*(D1), 643–648. doi: 10.1093/nar/gkt1209

Zhan, J., & McDonald, B. A. (2013). Experimental Measures of Pathogen Competition and Relative Fitness. *Annual Review of Phytopathology*, *51*(1), 131–153. doi: 10.1146/annurev-phyto-082712-102302

Zimmerman, N. B., & Vitousek, P. M. (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proceedings of the National Academy of Sciences*, *109*, 13022–13027.

FIGURE LEGENDS

Figure 1

Relative abundance of (a) fungal classes and (b) bacterial classes in ash tree leaves from Manchester (red) and Stirling (blue) across four different *Hymenoscyphus fraxineus* infection categories.

Figure 2

PCoA plots for Bray-Curtis distances among (a) fungal communities and (b) bacterial communities of ash tree leaves collected from Manchester (circles) and Stirling (triangles) with either absent (red), low (blue), medium (purple) or high (green) *Hymenoscyphus fraxineus* infection; (c) PCoA plot of Euclidean genetic distances between ash trees in Manchester (circles) and Stirling (triangles), calculated using multilocus microsatellite genotypes.

Figure 3

Relationship between *Hymenoscyphus fraxineus* infection and (a) fungal community richness, (b) fungal community evenness (Inverse Shannon), (c) fungal community beta-diversity, and (d) bacterial community beta-diversity.

Figure 4

Heatmap of fungal (black text) and bacterial (red text) genera significantly associated with *Hymenoscyphus fraxineus* infection intensity in ash tree leaves.

Figure 5

Functional analysis of trophic modes of fungal ESVs associated with ash leaves with varying degrees of *H. fraxineus* infection.

Figure 6

Relationship between (a) Jensen-Shannon divergence values of fungal communities and bacterial communities associated with ash tree leaves, and (b) Jensen-Shannon divergence values of fungal communities and Euclidean genetic distance of ash trees.

Figure 7

Co-occurrence networks between fungi and bacteria in the leaves of ash trees with varying degrees of *Hymenoscyphus fraxineus* infection; (a) absent; (b) low; (c) medium and (d) high. Edges coloured orange indicate fungi-fungi associations, those coloured green indicate bacteria-bacteria associations, and those in blue are fungi-bacteria associations.