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The Influence of Host Genotypes on the Endophytes in the Leaf Scar Tissues of Apple Trees and Correlation of the Endophytes with Apple Canker (*Neonectria ditissima*) Development

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ABSTRACT

Bacterial and fungal endophytes may help their host in terms of improved tolerance to abiotic and biotic stresses and enhanced growth. European apple canker, caused by *Neonectria ditissima*, is widespread in apple-growing regions. Infection by *N. ditissima* occurs through artificial or natural wounds, including leaf scars, picking wounds, and pruning cuts. Using F₁ progeny trees in an experimental orchard derived from a cross between a canker-susceptible genotype and a canker-tolerant or -resistant genotype, we assessed both bacterial and fungal endophyte communities in apple leaf scars, and determined correlations of endophytes with canker development. All trees were artificially inoculated with an *N. ditissima* isolate postplanting. Specific components of apple endophytes as well as a number of individual fungal or bacterial groups in leaf scars were

partially genetically controlled by host genotypes. Several bacterial groups were significantly correlated with canker-related traits, mostly positively associated with canker tolerance. A few fungal groups may facilitate canker development whereas others may compete with canker. However, most of these microbial groups could not be identified to the species level with confidence; even for those groups which could be assigned to the species level, there is insufficient knowledge about their ecological characteristics in relation to plants. The present results may be used to inform further research using biocontrol to manage *N. ditissima* and breeding for resistance.

Keywords: apple endophytes, crop, European apple canker, heritability, leaf scars, *Neonectria ditissima*, plant pathology

Many bacterial and fungal endophytes inhabit plant tissues without causing any adverse effect and may benefit their host in terms of improved tolerance to abiotic and biotic stresses (e.g., pests and pathogens) and enhanced growth (Dini-Andreote 2020; Khare et al. 2018; Omomowo and Babalola 2019). Due to increasing public concern about using pesticides, increased incidence of pesticide resistance, and decreasing numbers of commercial pesticides available (primarily due to regulation), there is an urgent need to exploit

beneficial microorganisms, including endophytes, and microbial-derived compounds for disease management.

Neonectria ditissima is a fungal pathogen that causes wood cankers in apple and other broad-leaved trees (Saville and Olivieri 2019; Weber 2014). Canker is widespread in apple-growing regions in most of the temperate areas of the world. *N. ditissima* infects trees through artificial or natural wounds; most frequently, through leaf scars, wounds caused by picking fruit, and pruning cuts. A most damaging phase of this disease is that latent infection established in nurseries can develop rapidly on the main stem postplanting, killing young trees. Due to withdrawal of several effective fungicides (e.g., carbendazim and copper-based products), current chemical control strategies only have a limited effect on the establishment and spread of *N. ditissima*. Thus, host resistance is an important component in managing canker disease. Unfortunately, many modern apple varieties are derived from a narrow genetic background with a high susceptibility to apple canker (Gómez-Cortecero et al. 2016). High-density orchards further exacerbate this problem because canker lesions on the main stem can easily girdle and subsequently kill small trees postplanting in such an orchard. Host resistance against *N. ditissima* is of a quantitative nature (Bus et al. 2019; Gómez-Cortecero et al. 2016). Therefore, it may take a long time to breed apple cultivars with effective

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resistance against the pathogen given the nature of perennial trees and quantitative resistance.

There is evidence of host-genotypic effects on endophyte communities in apple (Hirakue and Sugiyama 2018; Liu et al. 2018, 2020), influencing both species richness and composition. Although the endophytic microbe composition was only studied in a limited number of apple cultivars, the results indicate that cultivars with a higher degree of relatedness also share similarities in endophyte community (Liu et al. 2018). Apple resistance to leaf pathogens appears to correlate more with fungal endophytes than bacterial endophytes (Hirakue and Sugiyama 2018). In addition to cultivar, specific location and apple tissue type can also considerably influence endophyte composition (Liu et al. 2020; Olivieri et al. 2021a).

It has been demonstrated recently that a number of apple endophytes at leaf scars were associated with the host susceptibility to *N. ditissima* (Olivieri et al. 2021a). Specific endophytes from apple showed in vitro antagonistic effects against *N. ditissima* (Liu et al. 2020). Thus, manipulating apple endophyte communities may be a viable approach to manage canker disease. Apple endophyte composition could be altered through specific agronomic practices or augmented application of specific endophyte strains with biocontrol ability to improve host resistance or tolerance against *N. ditissima*. Another approach would be to include specific (desirable) endophytes as a selection criterion in breeding programs to breed cultivars with desirable endophyte composition or the ability to recruit these specific microbes for disease suppression or tolerance. To adopt this second approach, we need to assess the magnitude of the heritability of endophyte communities with respect to overall communities as well as specific endophytes. Such knowledge of the host genetic component of endophytes is lacking because the current research has been focusing on the determination of differences in endophytes between genotypes.

In this study, we estimated the broad-sense heritability of endophyte communities as well as individual endophytes in apple leaf scars, an important entry site for *N. ditissima*. Progeny derived from a cross between two genotypes (one canker-susceptible cultivar and the other a canker-tolerant cultivar) were evaluated under field conditions. Bacterial and fungal endophytes at the leaf scar tissues were profiled with amplicon sequencing and correlated with canker development. All sequences have been deposited in the European Nucleotide Archive under project reference PRJEB49633.

MATERIALS AND METHODS

Field design and layout. A cross between cultivars ‘Aroma’ and ‘Golden Delicious’ was made in 2015. Canker resistance is not well understood yet but it is quantitative in nature. The two parents were selected based on empirical evidence as well as limited lab artificial-inoculation data (Gómez-Cortecero et al. 2016), which classified the two parents as moderately susceptible and highly resistant or tolerant, respectively, to *N. ditissima*. All 70 F₁ genotypes in the family, including the parents, were grafted onto M9 EMLA rootstocks in January 2017 at East Malling, United Kingdom. The plants were maintained in pots in polytunnels until October 2017, when they were planted in a randomized block design in an orchard. There were four blocks, with one tree per genotype in each block.

Inoculation with *N. ditissima*. The trees were artificially inoculated with a single isolate of *N. ditissima* (Hg199) at leaf scars in November 2018 in order to eliminate the issue of inoculum heterogeneity in the orchard. Because the research objective was to compare resistance and susceptibility of genotypes to *N. ditissima*, we decided to eliminate the issue of field inoculum heterogeneity via artificial inoculation from complicating data interpretation.

The inoculum was prepared according to a published protocol (Gómez-Cortecero et al. 2016). Five artificial leaf scars were inoculated per tree, and each leaf scar (pseudoreplicate) was positioned on a separate branch. A droplet of 6 µl of spore suspension (10⁵ macroconidia/ml) was pipetted onto each artificial leaf scar wound, then covered with petroleum jelly (Vaseline) immediately after absorption. The petroleum jelly was removed 2 weeks after inoculation.

Each inoculated leaf scar was marked to allow repeated measurements over time. Canker lesion development was measured with a digital caliper at three time-points: 5, 8, and 11 months postinoculation (mpi). A final assessment was conducted at 20 mpi on the percent branch area with foliage, percent branches with cankers, and number of cankers. Average number of cankers per branch was then calculated for each tree.

Sampling leaf scars, DNA extraction, and sequencing.

Many trees died of canker before leaf scars were sampled in autumn 2019 before leaf fall. Only those genotypes with a minimum of three surviving trees were sampled. In total, we sampled 216 trees, including the two parents and 54 F₁ genotypes. Leaf scars were sampled for characterization of endophytes in leaf scar tissues, following a published protocol (Olivieri et al. 2021a). Briefly, sampling and subsequent sample processing consisted of the following steps. Four 20-cm-long, healthy (i.e., free of any disease symptoms on all leaf scars), 1-year-old extension shoots were sampled randomly from each tree, not including those inoculated shoots (because those already became 2-year-old shoots). Our previous study showed that *N. ditissima* is unlikely to be systemic but usually is confined within the vicinity of canker lesions (Olivieri et al. 2021b). Thus, *N. ditissima* is not expected to be present in these healthy leaf scars because new infections of leaf scars usually take place after leaf fall in autumn. Three to four freshly exposed leaf scars were sampled from each of the four shoots and pooled into one sample for a single tree. Samples were freeze dried and dry weight was recorded before homogenization with a Geno/Grinder 2010 (SPEX CertiPrep) for 2 min at 1,500 rpm using 2-ml tubes and two 5-mm steel ball bearings. Sterile 0.1 M phosphate-buffered saline (PBS) buffer was added to the homogenized samples at a 1:5 ratio (dry weight [milligrams]/volume [microliters]). DNA was extracted from 120 µl of PBS resuspended homogenate with the DNeasy Plant mini kit (Qiagen) according to the manufacturer's protocol. Pure DNA was eluted from the spin columns in two steps, each using 50 µl of molecular-grade water and 1 min of incubation before spinning at 14,000 × g. The purity and quantity of extracted DNA was assessed with NanoDrop1000 (Thermo Fisher Scientific). DNA extraction of samples was repeated if their concentration did not exceed 10 ng/µl. The samples were shipped on ice to Novogene UK (Cambridge, U.K.) where PCR amplification, library prep, and metabarcoding sequencing was done. The target regions were internal transcribed spacer (ITS)1-1F (ITS1-1F-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3', ITS1-1F-R: 5'-GCTGCGTTCCTTCATCGATGC-3') and 16S V5-V7 (799F: 5'-AACMGGATTAGATACCCCKG-3', 1193R: 5'-ACGTCATCCCCACCTTCC-3'). To reduce untargeted amplification of plant mitochondrial and plastid DNA with the 16S amplicon, we used mPNA (5'-ggc aagtgttcttcgga-3') and pPNA (5'-ggctcaaccttgacag-3') oligo blockers (PNA Bio) at 200 nM final concentration. Samples were sequenced on Illumina NovaSeq platform in the 250-nucleotide paired-end mode.

We estimated fungal and bacterial community sizes with quantitative PCR (qPCR). Briefly, a pooled sample was prepared combining 2 µl of extracted DNA from each sample. Calibration curve (six steps, 10-fold serial dilutions) was prepared using the pooled sample, aliquoted, and stored at -20°C. Each block of samples was

run on a separate 384-well plate in at least two dilutions (5× and 20×), each dilution in duplicate. The standard curve was run in triplicate alongside samples and a nontemplate control on each plate. Reactions (10 µl each) were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 2 µl of template DNA. The same primers as in the metabarcoding sequencing were used (ITS1-1f and 16S V5-V7) at a final concentration of 100 nM. PNA primers (mPNA and pPNA) at 200 nM final concentration were used in combination with 16S V5-V7 to prevent amplification of mitochondrial and chloroplast contaminants. The cycling protocol was denaturation for 5 min at 94°C and 40 cycles of 60 s at 94°C, 10 s at 75°C, 30 s at 55°C, and 60 s at 72°C, followed by melt curve analysis.

Community size in terms of ITS or 16S copy number was estimated using a calibration curve for each plate separately. First, we calculated the efficiency of amplification for each sample and standard curve on each plate separately [$E = 10^{(-1/\text{slope})}$]. Efficiency of calibration curves and samples were set to 100% by correcting their raw quantification cycle (Cq) values with their respective efficiency using the following formula: $Cq_c = \log_2(E^{Cq})$. The corrected Cq (Cq_c) value of a sample was used to calculate its theoretical copy number present in each sample using the efficiency corrected calibration curve equation: $C = (Cq_c - I_c)/S_c$, where C represents estimated $\log_{10}$ copy number of ITS or 16S in the sample, Cq_c represents efficiency corrected Cq value of the sample, and I_c and S_c represent intercept and slope, respectively, of the efficiency corrected calibration curve.

The samples with efficiency below 0.8 and above 1.2 were repeated at lower DNA dilution (10× and 40× or 20× and 100×) to minimize the effect of PCR inhibitors. Samples that failed the efficacy threshold were removed from further analysis. The mean of four technical repeats on each sample was used.

Sequencing processing and taxonomy assignment. Paired-end amplicon sequence data were processed to produce operational taxonomic unit (OTU) representative sequences and frequency tables. Read pairs containing incorrect bases in primer regions or less than 250 bases in both pairs were discarded. Remaining read pairs were merged with a maximum difference in overlap of 5% with the UPARSE pipeline (version 10.0) (Edgar 2013). Merged reads of < 250 and 400 bp for ITS and 16S, respectively, were discarded. All remaining (unfiltered) reads were retained. Unfiltered reads were then filtered for quality, with a maximum expected error threshold of 0.5 per sequence (Edgar and Flyvbjerg 2015). Forward and reverse primers were removed from both filtered and unfiltered datasets. Filtered reads were dereplicated and singletons were discarded; then, representative OTUs at 97% similarity were constructed with the UPARSE pipeline. UPARSE also removes suspected chimeral sequences. Then, the unfiltered reads were aligned the OTU representative sequences at the level of 97% similarity to produce OTU frequency tables. Finally, the SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) was used to assign taxonomic ranks to the OTU representative sequences with the Unite V8.3 (2021-05-10) fungal database (Kõljalg et al. 2013) and the RDP training set 18 bacterial database (Cole et al. 2014). OTUs which were identified as chloroplast and mitochondria were then removed before statistical analysis.

Statistical data analysis. Only the most abundant OTUs that accounted for 99.0% of the total counts were retained for statistical data analysis. Rare taxa were excluded from statistical analysis for several reasons. First, the present objective was to assess the extent of differences between genotypes. However, genotypes would unlikely differ in the relative abundance of rare taxa (given their low counts). For the same reason, correlation of these rare taxa with canker variables also would not be expected to be significant.

Second, these rare taxa had very little effect on the main characteristics of microbiome composition, represented by principal components (PCs). Finally, the abundance of these rare taxa is more prone to sequencing errors than for the other taxa.

Before statistical analysis, the fungal and bacterial OTU tables were normalized by two methods: by the qPCR values of the generic ITS or 16S primers, or by the median-of-ratios (MR) method implemented in DESeq2 (Love et al. 2014). The former normalization incorporates the amount of microbial biomass between samples, estimated as the total number of DNA copies per sample, as well as relative proportion of individual OTUs within a sample. The latter only considers the relative proportions of individual OTUs within a sample. The MR method was used because of (i) nonsignificant differences between genotypes in qPCR values of 16S and ITS and (ii) large variability in qPCR values between replicates within each genotype.

Both α - and β -diversity indices were calculated. The former measures within-sample diversity in term of the number of species present and their corresponding abundance, whereas the latter measures between-sample diversity. Two α -diversity indices (Shannon and Simpson) were calculated with the R vegan 2.3-1 package (Dixon 2003). The ranks of α -diversity indices were subjected to analysis of variance to assess the differences between the two parents and between progeny genotypes via a permutation test for statistical significance. The β -diversity indices were calculated as Bray-Curtis indices, then subjected to permutational multivariate analysis of variance between parents and between progeny genotypes with a permutation test based on pseudo- F ratios (implemented as the Adonis function in the vegan package).

The main objective of the present study was to determine whether there is significant genetic variability among F_1 progeny genotypes. This was achieved by a random effect model in which the total variability among F_1 genotypes was partitioned into environmental (V_E = between blocks + residual) and genetic (V_G = between F_1 genotypes) variability. The significance of genetic variability (component) was statistically tested by comparisons of two nest models with a χ^2 test with one degree of freedom: one with the genotypic component included and the other without. The broad-sense heritability was then estimated as $V_G/(V_E + V_G)$.

These variance components were estimated with the lmer function in the lme4 package (Bates et al. 2015). For each normalized set of counts data, two types of data were used for estimation of the genetic components: PC scores, representing the overall microbial composition, and the normalized counts data of those OTUs with highest count values (Table 1). Before PC analysis, the normalized counts data were logarithm transformed on the natural base, then standardized. Similarly, normalized OTU count values were logarithm transformed on the natural base before analysis. The random effect model was fitted to the data with the R lme4

TABLE 1
Number of the principal components (PCs) and operational taxonomic units (OTUs) with the highest counts for inclusion in estimation of differences between F_1 genotypes and correlation with canker variables

Group	Quantitative PCR normalized		Median of ratio normalized	
	PCs	OTUs	PCs	OTUs
Fungi (internal transcribed spacer)	30	100	80	100
Bacteria (16S)	30	200	50	200

package (Bates et al. 2015). Within the analysis of each data type (PC or OTU), the Benjamini-Hochberg (BH) adjustment (Benjamini and Hochberg 1995) was applied to correct for the false discovery rate associated with multiple testing. Statistical significance was determined at the 5% level (BH adjusted). In addition to the estimation of broad-sense heritability, correlation (both Pearson and Spearman) of canker-related variables with PC scores and normalized OTU counts were calculated.

All statistical analyses were carried out in R 4.0.3 (R Core Development Team 2019).

RESULTS

Overall sequencing, OTU generating, and qPCR results.

Fungi. In total, there were 4,268 fungal OTUs, most of which had few reads. The top 6 and 34 most abundant OTUs accounted for over 50 and 90% of the total reads, respectively (Fig. 1A). The top 206 OTUs accounted for 99.0% of total reads and were included in subsequent analysis.

The number of reads assigned to OTUs in each sample ranged from 51,780 to 134,300, with a median of 117,676; the number of reads for each OTU ranged from 1,706 to 4,289,841, with a median of 7,532. The top two were both identified as *Filobasidium* spp.: *F. wieringae* and *F. chernovii*, accounting for 18.0 and 12.0% of the total reads, respectively. The OTUs with the third and fourth most reads were both identified as *Vishniacozyma* spp., jointly accounting for 13.0% of the total reads. Of the 206 fungal OTUs, only 137, 118, 104, 79, 60, and 34 could be assigned to the taxonomic rank of phylum, class, order, family, genus, and species, respectively, with >80% confidence. Basidiomycota and Ascomycota accounted for 59.3 and 15.8%, respectively, of the total reads, whereas 24.9% of the reads could not be assigned to a phylum with >80% confidence and, hence, were designated as unknown (Fig. 2A).

For 13 of the 216 samples (54 F_1 genotypes and the two parents), we failed to obtain reliable qPCR ITS values. Of the remaining samples, there were large variabilities in the ITS qPCR values among replicates within a given genotype (Fig. 3A); genotypes did not differ significantly in the ITS qPCR values.

Bacteria. One sample failed to generate sequences. In total, there were 3,639 bacterial OTUs; the top 51 and 583 most abundant OTUs accounted for >50 and 90% of the total reads, respectively (Fig. 1B). Only the top 1,694 OTUs were included in subsequent analysis because all others jointly accounted for <1.0% of the total reads.

Of the 1,694 OTUs, the number of reads for each OTU ranged from 490 to 1,034,106, with a median of 2,654. The number of reads assigned to OTUs in each sample ranged from 14,180 to 127,718, with a median of 110,064. The top two OTUs by abundance were *Sphingomonas* sp. (4.6%) and *Methylobacterium* sp. (4.2%). Of the 1,694 bacterial OTUs, only 1,341, 1,091, 809, 605, and 388 could be assigned to the taxonomic rank of phylum, class, order, family, and genus, respectively, with >80% confidence. *Proteobacteria* and *Actinobacteria* accounted for 54.9 and 24.5% of the total reads, respectively, whereas 9.0% of the reads could not be assigned to a phylum (Fig. 2B).

Of the 216 samples, we failed to obtain reliable qPCR 16S values for 14 samples. For eight samples, qPCR data for both fungi and bacteria were not available. As for fungi, there were large variabilities in the 16S qPCR values among replicates within a given genotype (Fig. 3B), and genotypes did not differ significantly in the 16S qPCR values. However, there were significant ($P < 0.001$) differences in the 16S qPCR values among the four blocks.

Microbial diversity indices. **Fungi.** For both the qPCR- and MR-normalized data, there were no significant differences in both Simpson and Shannon indices among the 54 F_1 genotypes. However, the blocks differed significantly ($P < 0.001$) in the two indices. For the qPCR-normalized data, F_1 genotypes did not differ in Bray-Curtis indices but differed ($P < 0.001$) in the indices for the MR-normalized data, accounting for approximately 25% of the total variability in the indices. The two parents did not differ in all indices for either normalized data set.

Bacteria. For both the qPCR- and MR-normalized data, 54 F_1 genotypes did not differ significantly in either Simpson or Shannon indices, and the blocks differed ($P < 0.001$) in the two indices. For the qPCR-normalized data, neither F_1 genotypes nor the two parents differed in Bray-Curtis indices. In contrast, F_1 genotypes ($P < 0.001$) as well as the two parents ($P < 0.05$) differed in Bray-Curtis indices for the MR-normalized data.

Fig. 1. Proportion of the cumulative sequence reads plotted against the number of **A**, fungal and **B**, bacterial operational taxonomic units (OTUs), where the OTUs were sorted in the descending order with respect to the number of their sequence reads.

Genetic components of PC scores. The summary of results for PC scores is shown in Table 2.

Fungi. For the qPCR-normalized data, the first two PCs explained approximately 50.9 and 4.0% of the total variability (Supplementary Fig. S1A). F₁ genotypes differed ($P < 0.001$) for PC5, with the corresponding estimated heritability value of 27.5%. For the MR-normalized data, the first two PCs explained only approximately

9.3 and 7.4% of the total variability (Supplementary Fig. S1B). F₁ genotypes differed for PC1, PC4, P9, PC12, P17, and P44, with corresponding heritability estimates of 5.5, 33.7, 20.8, 23.4, 19.4, and 21.9%.

Bacteria. For the qPCR-normalized data, the first two PCs explained approximately 45.3 and 3.8% of the total variability (Supplementary Fig. S2A). Only for 2 (PC3 and PC8) of the top 30 PCs

Fig. 2. Histogram of the number of the **A**, internal transcribed spacer and **B**, 16S sequences that were assigned to the phylum at the 80% confidence level; the “Unknown” group consists of those operational taxonomic units that cannot be assigned to a unique phylum.

Fig. 3. Quantitative PCR results of both the internal transcribed spacer and 16S of endophytes in apple leaf scar tissues of individual trees of F₁ and parental genotypes. The cross was between cultivars Aroma and Golden Delicious.

did the 54 F₁ genotypes differ significantly ($P < 0.05$), with estimated heritability of 29.5 and 30.0%, respectively. For the MR-normalized data, the first two PCs explained only approximately 9.2 and 8.0% of the total variability (Supplementary Fig. S2B). For 7 (PC2, PC7, PC8, PC16, PC17, and PC26) of the top 50 PCs, F₁ genotypes differed significantly ($P < 0.05$), with heritability estimates ranging from 14.5% (PC13) to 33.2% (PC7).

Genetic components of individual OTUs. The summary of results for individual OTUs is shown in Table 2.

Fungi. For the qPCR-normalized data, F₁ genotypes did not differ significantly in any of the top 100 OTUs. For the MR-normalized data, F₁ genotypes differed ($P < 0.05$) for 24 of the top 100 OTUs (Fig. 4A; Table 3). Only 7 of the 24 OTUs could be assigned to the

species level; 2 of these OTUs were both identified as *Tilletiopsis washingtonensis*. The estimate of broad-sense heritability ranged from 10.2 to 34.7%, with an average of 18.5%.

Bacteria. For the qPCR-normalized data, F₁ genotypes differed significantly in only 1 of the top 200 OTUs (OTU89, *Roseomonas* sp.), with an estimated broad-sense heritability of 27.9%. For the MR-normalized data, F₁ genotypes differed significantly for 47 of the top 200 OTUs (Fig. 4B; Table 4). The estimate of broad-sense heritability ranged from 8.1 to 37.3%, with an average of 18.5%. These 47 OTUs also included OTU89; indeed, there were other OTUs from *Roseomonas* spp.

Canker development. Average canker size over the first three measurements (month 5, 8, and 11) ranged from 4.6 to 23.5 cm,

TABLE 2 Number of cases where there was significant genetic variability in the principal components (PCs) and top fungal or bacterial operational taxonomic units (OTUs) or correlation of endophytes with canker variables^a								
Variables	Quantitative PCR normalized				Median of ratio normalized			
	Fungi		Bacteria		Fungi		Bacteria	
	PCs	OTUs	PCs	OTUs	PCs	OTUs	PCs	OTUs
Genetic variance	1	0	2	1	6	24	7	47
Correlation								
Individual based								
Canker size	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)
Canopy	1 (1)	2 (0)	1 (1)	0 (0)	1 (1)	7 (7)	1 (1)	6 (0)
Shoots cankered (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Family based								
Canker size	1 (1)	0 (2)	0 (0)	2 (2)	1 (1)	2 (2)	2 (2)	0 (0)
Canopy	1 (0)	5 (0)	0 (0)	0 (0)	1 (0)	8 (6)	1 (0)	4 (10)
Shoots cankered (%)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)

^a Values, shown as x (y), indicate the x and y number of significant Pearson and Spearman correlations, respectively.

Fig. 4. Estimated genetic variance plotted against the total environmental variance for the top **A**, 100 fungal and **B**, 200 bacterial operational taxonomic units based on the median of ratio normalized counts data. The point color indicates whether the genetic variance is statistically significant or not.

with an average of 11.1 cm (Fig. 5A). In month 20, 35 trees had cankers in all shoots (Fig. 5C). The two parents did not differ in any of the three canker measurements. In contrast, F₁ genotypes differed significantly in average canker size ($P < 0.05$) and canopy coverage (Fig. 5B) and percent shoots with canker in month 20 ($P < 0.001$). The broad-sense heritability was 13.1, 23.8, and 31.9% for average canker size, percent canopy coverage, and percent shoots with canker at 20 mpi, respectively.

Correlation of microbial variables with canker variables.

The summary of correlation results is shown in Table 2, with detailed correlation results given in Tables 5 and 6.

Fungi. Among the top 30 fungal PCs of the qPCR-normalized data, only PC5 scores were correlated with percent canopy coverage in month 20 based on the individual tree data. At the F₁ genotypic level, PC5 scores were correlated with percent canopy coverage in month 20, PC7 with canker size, and PC23 with percent shoots cankered. For the MR-normalized data, of the top 80 PCs, only PC4 scores were correlated with percent canopy coverage based on individual trees whereas, based on F₁ genotypic means, PC3 and P9 were correlated with percent canopy coverage and canker size, respectively.

In total, there were 42 significant correlations of canker variables with 16 fungal OTUs; 33 of these 42 cases were for the MR-normalized data. In 26 of the 42 cases, correlation was at the level of F₁ genotypic means. In 35, 6, and 1 cases, it involved percent canopy coverage, average canker size, and percent shoots with cankers, respectively. Among these 42 significant correlations,

25 and 17 were of the Pearson and Spearman type, respectively. Correlation coefficient ranged from -0.56 to 0.51 . The magnitude of correlation was greater for genotypic means (from -0.56 to 0.51) than for individual trees (from -0.30 to 0.26) (Table 5). For several OTUs, the observed correlation was inconsistent; namely, different signs between qPCR- and MR-normalized data (e.g., OTU69) (Table 5), or between individual and genotypic levels (e.g., OTU49) (Table 5), or between Pearson and Spearman correlations (e.g., OTU1896) (Table 5). Nevertheless, several OTUs had consistent correlation patterns, including a group from *Entyloma* sp. (OTU35) and *Pseudoophiobolus rosae* (OTU57) (Table 5).

Bacteria. Among the top 30 PCs of the qPCR-normalized data, both Pearson and Spearman correlations of PC11 with percent canopy coverage were significant based on individual trees ($r = -0.24$, -0.32). For the MR-normalized data, PC7 was correlated with percent canopy coverage on individual trees (Pearson: -0.24 , and Spearman: -0.26) (Fig. 6), whereas PC29 was correlated with average canker size (Pearson: 0.27). At the F₁ genotypic level, both PC6 and PC10 were correlated with average canker size ($r = -0.50$, 0.47) and PC7 with percent canopy coverage (Pearson: -0.44) (Fig. 6).

In total, there were 24 significant correlations of canker variables with 16 bacterial OTUs; 20 of these 24 cases were for the MR-normalized data (Table 6), all with percent canopy cover. All four correlations of qPCR-normalized data were with canker size. In 18 of these 24 cases, correlation was at the F₁ genotypic level. Half of the correlation was of the Pearson type. Correlation

TABLE 3
Summary of those fungal operational taxonomic units (OTUs) within the top 100 highest counts in the median of ratio normalized counts for which the genetic differences between F₁ genotypes were statistically significant

OTU	Taxonomy (>80% confidence)	Total reads (%)	Heritability (%)
OTU29	Ascomycota	0.37	17.57
OTU3360	Basidiomycota	1.30	11.00
OTU143	Dothideomycetes	0.05	21.76
OTU35	<i>Entyloma</i>	0.19	17.26
OTU50	<i>Entyloma calendulae</i>	0.07	24.21
OTU12	Fungi	1.97	24.43
OTU1226	Fungi	0.04	11.64
OTU147	Fungi	0.07	15.67
OTU19	Fungi	0.94	24.31
OTU40	Fungi	0.65	10.20
OTU73	<i>Leptospora</i>	0.03	17.04
OTU17	<i>Neosetophoma</i>	1.51	15.79
OTU20	Phaeosphaeriaceae	0.83	23.39
OTU62	Phaeosphaeriaceae	0.05	13.31
OTU76	Pleosporales	0.04	16.57
OTU57	<i>Pseudoophiobolus rosae</i>	0.08	22.26
OTU21	<i>Sporobolomyces roseus</i>	0.62	18.66
OTU26	<i>Subplenodomus iridicola</i>	0.49	20.57
OTU24	<i>Symmetrospora</i>	0.71	10.20
OTU310	<i>Taphrina</i>	0.04	29.12
OTU71	<i>Taphrina</i>	0.05	13.02
OTU16	<i>Taphrina carpini</i>	2.04	34.73
OTU15	<i>Tilletiopsis washingtonensis</i>	1.40	16.54
OTU4179	<i>T. washingtonensis</i>	0.05	13.68

TABLE 4
Summary of those bacterial operational taxonomic units (OTUs) within the 200 highest counts (median of ratio normalized) for which the genetic differences between F₁ genotypes were statistically significant

OTU	Taxonomy (>80% confidence)	Total reads (%)	Heritability (%)
OTU68	<i>Acetobacteraceae</i>	0.18	17.87
OTU16	<i>Actinobacteria</i>	1.21	8.61
OTU644	<i>Actinobacteria</i>	0.19	26.27
OTU383	<i>Alphaproteobacteria</i>	0.16	23.45
OTU105	<i>Arthrobacter</i>	0.10	21.01
OTU27	<i>Aurantimonadaceae</i>	0.67	10.49
OTU38	<i>Aureimonas</i>	0.48	21.04
OTU128	<i>Bacteria</i>	0.10	14.91
OTU59	<i>Bacteria</i>	0.22	19.06
OTU813	<i>Bacteria</i>	0.11	17.52
OTU950	<i>Bacteria</i>	0.21	18.97
OTU52	<i>Comamonadaceae</i>	0.26	13.76
OTU135	<i>Deinococcus</i>	0.08	25.53
OTU18	<i>Deinococcus</i>	1.15	19.88
OTU124	<i>Deltaproteobacteria</i>	0.08	16.79
OTU170	<i>Dyadobacter</i>	0.10	17.86
OTU57	<i>Enterococcus</i>	0.20	17.64
OTU46	<i>Erythrobacteraceae</i>	0.28	12.13
OTU2048	<i>Firmicutes</i>	0.07	19.93
OTU5	<i>Kineococcus</i>	2.74	14.17
OTU53	<i>Marmoricola</i>	0.23	14.46
OTU13	<i>Methylobacteriaceae</i>	1.28	12.11
OTU3	<i>Methylobacterium</i>	4.27	9.34
OTU462	<i>Microbacteriaceae</i>	0.09	17.65
OTU504	<i>Microbacteriaceae</i>	0.13	14.23
OTU37	<i>Nakamurellales</i>	0.54	17.83
OTU123	<i>Nocardioides</i>	0.09	18.42
OTU42	<i>Nocardioides</i>	0.35	10.78
OTU7	<i>Novosphingobium</i>	1.86	22.73
OTU2625	<i>Proteobacteria</i>	0.09	21.81
OTU73	<i>Proteobacteria</i>	0.19	21.27
OTU922	<i>Proteobacteria</i>	0.14	25.14
OTU85	<i>Pseudokineococcus</i>	0.14	20.44
OTU100	<i>Rhodobacteraceae</i>	0.10	17.66
OTU30	<i>Rhodococcus</i>	0.70	8.12
OTU3169	<i>Rhodospirillales</i>	0.08	23.89
OTU126	<i>Roseomonas</i>	0.08	22.19
OTU47	<i>Roseomonas</i>	0.32	21.44
OTU84	<i>Roseomonas</i>	0.14	17.45
OTU89	<i>Roseomonas</i>	0.13	37.34
OTU20	<i>Sphingomonadaceae</i>	0.93	20.67
OTU114	<i>Sphingomonas</i>	0.08	15.86
OTU149	<i>Sphingomonas</i>	0.08	19.25
OTU8	<i>Sphingomonas</i>	1.76	18.97
OTU49	<i>Spirosoma</i>	0.32	27.32
OTU99	<i>Terracoccus</i>	0.10	16.25
OTU60	<i>Williamsia</i>	0.24	19.93

coefficients ranged from −0.54 to 0.51, and were greater for genotypic means (from −0.54 to 0.51) than for individual trees (from 0.22 to 0.25) (Table 6). For the qPCR-normalized data, only two OTUs (*Pseudokineococcus* sp. and order *Propionibacteriales*) were significantly correlated with average canker size at the genotypic level (Table 6). For the MR-normalized data, several OTUs had multiple high and consistent correlations with canopy size, including *Actinobacteria* (OTU93), *Armatimonadetes_gp5* (OTU107), *Sphingomonas* (OTU72), and *Dyadobacter* (OTU170) (Table 6).

DISCUSSION

The present study showed that specific components of apple endophytes as well as some individual fungal or bacterial groups in tissues around leaf scars in autumn are partially controlled genetically by host genotypes in an F₁ segregating population. Furthermore, there are specific microbial groups that are significantly correlated with canker-related plant traits. However, such correlations are often inconsistent for a given microbial group in terms of whether it is based on individual trees or genotypes or whether it is based on the Pearson or Spearman correlation.

Sequence data were normalized by two methods, qPCR data of generic 16S or ITS primers and MR. In general, genotypic differences were less profound for the qPCR-normalized data than for the MR-normalized data. Moreover, there were far more cases of significant correlations of endophytes with canker-related variables for the MR-normalized data than for the qPCR-normalized data. In theory, qPCR-normalized data should provide more informative data than the MR-normalized data. Although there were often a couple of magnitude differences in qPCR values between trees, F₁ genotypes did not differ significantly in the qPCR values. We also observed a large variability in PCR efficiencies between samples (data not shown) and, hence, repeated qPCR was necessary for many samples. Our experience suggested that conducting qPCR analysis of fungal and bacterial DNA extracted from woody tissues with generic 16S and ITS primers is more problematic than other types of samples such as rhizosphere soils. Nevertheless, qPCR is valuable for pathogen diagnosis such as confirming latent infection of *N. ditissima* in leaf scar tissues (Olivieri et al. 2021b). Further studies are needed to improve quantification of overall microbial biomass in plant (particularly woody) tissues. As an alternative to the qPCR method, each sample may be spiked with a known amount of a synthetic DNA fragment to estimate absolute abundance (Tkacz et al. 2018).

The present study showed that the overall plant genetic component was similar for bacterial and fungal endophytes. For many microbial groups, albeit still a small proportion of the entire microbiome, the variability among F₁ offspring is greater than random variability, indicating existence of host genetic control. There is a significant host genetic component in several PCs, which are jointly determined by many microbial groups. There have been many reports demonstrating that plant genotypes differ in their phytobiomes associated with the rhizosphere, endosphere, and phyllosphere (Liu et al. 2020; Olivieri et al. 2021a; Peiffer et al. 2013; Wagner et al. 2016, 2020; Wei et al. 2019). We found significant host genetic components affecting specific endophyte components of apple leaf scars, represented by PCs, often with a moderate level of genetic variance relative to the environmental variance. However, these microbial components are only a minor proportion of the entire endophytes in the leaf scar tissues, which can be seen from the small number of PCs or OTUs with significant host genetic components. Similarly, maize inbred lines differed significantly in their rhizosphere microbiome but the heritability level was low and the genetic relationship among the inbred lines was not

correlated with the diversity characteristics of the rhizosphere microbiome (Peiffer et al. 2013). The present research is based on an F₁ population from a specific cross, hence representing genetic variance between the two specific parental genotypes only. The overall low heritability of the phytobiome may be due to strong environmental effects (Clouse and Wagner 2021), including spatial

and temporal variation. For instance, we recently demonstrated that, although cultivars differed in their endophytes in leaf scars, orchard locations accounted for much greater variability in endophyte composition (Olivieri et al. 2021a). Plants are only able to recruit those microbial organisms present at a specific site with the recruitment outcome, likely depending on frequencies of available taxa. This

Fig. 5. Histogram of three canker variables assessed on individual trees. **A**, Average canker size measured 5, 8, and 11 months postinoculation; **B**, percent canopy cover; and **C**, percent shoots with cankers assessed 20 months postinoculation. Blue and red lines indicate the range of values for the female and male parents, respectively.

TABLE 5								
Significant Pearson and Spearman (in parentheses) correlation coefficients of canker size, canopy cover and percent shoots with canker 20 months postinoculation with fungal operational taxonomic units (OTUs), with the highest counts based on individual trees or F ₁ genotypic means								
OTU ID	Taxonomy	Quantitative PCR normalized data			Median of ratio normalized data			
		Individual	Genotypic		Individual	Genotypic		
		Canopy	Canker size	Canopy	Canopy	Canker size	Canopy	Shoots (%)
OTU143	Dothideomycetes	–	–	–0.43	–	–	–	–
OTU35	Entyloma	–	(–0.47)	–	–	–0.50 (–0.55)	–	–
OTU59	Exobasidiomycetes	–	–	–	–	–	–0.45 (–0.42)	–
OTU36	Fungi	–0.25	–	–	–0.29 (–0.27)	–	–	–
OTU43	Fungi	–	(–0.52)	–	–	–0.53 (–0.56)	–	–
OTU6	Fungi	–0.28	–	–	–0.24 (–0.22)	–	–	–
OTU69	Fungi	–	–	–0.50	–	–	0.47	–
OTU17	Neosetophoma	–	–	–	0.24 (0.23)	–	–	–
OTU20	Phaeosphaeriaceae	–	–	–0.41	–	–	–0.40	–
OTU247	Phaeosphaeriaceae	–	–	–	–0.25	–	–	–
OTU49	Phaeosphaeriaceae	–	–	–0.41	–0.27 (–0.25)	–	0.43 (0.45)	–
OTU57	Pseudoophiobolus rosae	–	–	–0.45	–	–	–0.48 (–0.47)	–
OTU21	Sporobolomyces roseus	–	–	–	–0.21 (0.26)	–	0.51 (0.50)	–
OTU1896	Vishniacozyma	–	–	–	(–0.28)	–	–0.41 (0.44)	–0.48
OTU3	Vishniacozyma	–	–	–	–	–	0.40 (0.42)	–
OTU18	Vishniacozyma carnescens	–	–	–	0.26 (0.21)	–	–	–

potential large difference in aerosol microbiome over distance may also partially explain the block effects on endophytes observed in the present study.

Only a few specific microbial groups were significantly correlated with canker size or percent canopy cover. Interestingly, all significant correlations with bacterial groups indicated that higher relative abundance of these groups is associated with less canker

development, mostly with better canopy coverage and, in a few instances, negatively with canker size. In contrast, there is no such consistency in the correlation of fungal groups with canker development: both positive and negative correlation with canker development were observed. Moreover, the inconsistency exists even for the same fungal group across different canker variables or parametric or rank correlations, or at the tree or genotypic levels. Reasons

TABLE 6

Significant Pearson and Spearman (in parentheses) correlation coefficients of canker size, canopy cover and percent shoots with canker 20 months postinoculation with most abundant bacterial operational taxonomic units (OTUs) based on individual trees or F₁ genotypic means

OTU ID	Taxonomy	Quantitative PCR normalized data	Median of ratio normalized data	
		Genotypic	Individual	Genotypic
		Canker size	Canopy	Canopy
OTU58	<i>Abditibacteriota</i>	–	–	(0.43)
OTU93	<i>Actinobacteria</i>	–	–	0.47 (0.41)
OTU165	<i>Actinomycetospora</i>	–	0.25	–
OTU67	<i>Allobranchiibius</i>	–	–	(0.43)
OTU107	<i>Armatimonadetes_gp5</i>	–	–	0.45 (0.43)
OTU108	<i>Bacteria</i>	–	–	(0.47)
OTU1181	<i>Bacteria</i>	–	0.22	–
OTU170	<i>Dyadobacter</i>	–	–	0.50 (0.42)
OTU154	Methylobacteriaceae	–	–	(0.50)
OTU70	<i>Methylobacterium</i>	–	–	(0.42)
OTU106	Propionibacteriales	–0.49 (–0.53)	–	–
OTU85	<i>Pseudokineococcus</i>	–0.50 (–0.54)	0.22	–
OTU94	<i>Pseudomonas</i>	–	0.23	(0.41)
OTU72	<i>Sphingomonas</i>	–	–	0.46 (0.51)
OTU87	<i>Spirosoma</i>	–	0.23	–
OTU545	<i>Tepidimonas</i>	–	0.23	–

Fig. 6. Principal component 7 (PC7) scores of the median of ratio normalized bacterial operational taxonomic unit data plotted against canopy coverage 20 months postinoculation.

for these observed differences are not obvious. As discussed above, recruitment of endophytes may be considerably influenced by the local aerosol microbiome, not necessarily affected by tree genotypes. Thus, individual trees may differ greatly in relative abundance for some endophytes not because of genetics but because of variabilities in the aerosol microbiome. If these microbes have direct or indirect effects on *N. ditissima*, we may expect inconsistent correlations at the individual and genotypic levels. This may be further complicated by possible differences in the stability or persistence of individual endophytes in the leaf scar tissues at a given endophyte background. For example, fungal endophyte composition might be more easily influenced by differences in aerosol microbiome or external conditions than bacteria. Communities of endophytic fungi assembling in several plant species depend significantly on proximity to the inoculum source as well as the identity of the plant species (Ricks and Koide 2019). It is not known what fraction of the endophytic bacterial microbiome is dispersed via the atmosphere or originated from the atmosphere (Frank et al. 2017). Further research is needed to understand the relative importance of sources for plant endophytes and the effects of biotic and abiotic factors on the stability of the endophyte microbiome over time before we can amend the phytobiome with a reasonable level of predictability to improve crop performance.

Most of those bacterial groups with significant correlations with canker development cannot be classified into genus. *Dyadobacter* isolates are one of the major cohorts of bacteria in the plant phyllosphere (Delmotte et al. 2009; Reisberg et al. 2012) but what role *Dyadobacter* spp. play in these communities is currently unknown. The relative abundance of a *Pseudomonas* OTU (OTU94) and *Sphingomonas* OTU (OTU72) is positively correlated with canopy coverage in the present study. A *Pseudomonas* strain isolated from apple endophytes was shown to be antagonistic toward *N. ditissima* in in vitro tests (Liu et al. 2020). A seed-endophytic strain (*Sphingomonas melonis* ZJ26) confers rice with disease resistance against a bacterial pathogen and is vertically transmitted among plant generations via their seeds (Matsumoto et al. 2021). A couple of *Sphingomonas* groups were more abundant in canker-susceptible cultivars than in resistant cultivars (Olivieri et al. 2021a); however, that particular study did not consider the actual canker severity. *Methylobacterium* normally resides in soil and water but has also been identified as a contaminant of DNA extraction kit reagents, which may lead to its erroneous appearance in microbiota or metagenomic datasets (Salter et al. 2014). Given that these bacterial groups are mostly correlated with canopy cover but not with canker size, we may speculate that they may not be effective as direct competitors of *N. ditissima* but, rather, improve plant tolerance against consequences on plant development due to canker.

Only 3 of the 16 fungal groups with significant correlations with canker development can be classified into species: *Vishniacozyma carnescens* (syn = *Cryptococcus carnescens*), *Pseudoophiobolus rosae*, and *Sporobolomyces roseus*. Of all 16 groups, only *V. carnescens* is associated with reduced canker damage (but only at the individual tree level); unfortunately, there is no published information on its ecology in relation to plants. An *Entyloma* sp. (OTU35) is positively correlated with canker tolerance but *Entyloma* is a genus of plant-pathogenic smut fungi. Thus far, these fungal groups have not been associated with a direct antagonistic effect against *N. ditissima* (Liu et al. 2020) or associated with cultivars with differential susceptibility to *N. ditissima* (Olivieri et al. 2021a). A couple of groups appear to facilitate canker development, including *P. rosae* and Exobasidiomycetes (OTU59). Exobasidiomycetes are a class of fungi sometimes associated with galls of

plant tissues. Further metagenome sequencing and isolation combined with challenging assays against *N. ditissima* are needed to make further progress in this area.

In summary, some components of the apple endophyte microbiome as well as individual microbial groups around leaf scar tissues are partially controlled genetically by apple genotypes. Several microbial groups had significant correlation with canker development. Bacterial groups appear to be positively associated with canker tolerance. On the other hand, a few fungal groups may facilitate canker development whereas a few others may compete with the canker pathogen. The present results may be used to inform targeted approaches to further research in biocontrol of *N. ditissima* with specific microbes and breeding for resistance.

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