

Project title: Novel ways of managing tree crop fungal diseases: Using precision diagnostic technologies to tailor disease management strategies

Project number: CTP_FCR_2018_1

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Report: Annual report, Oct 2021

Previous report: Annual report, Oct 2020

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Location of project: NIAB EMR

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Date project commenced: 01 October 2018

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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

High coverage genome assemblies have been generated for powdery mildew of apple, strawberry and raspberry. These are the first genomes available for strawberry and raspberry mildew and the first generated from a European apple powdery mildew sample. A population of apple scab segregating for fungicide resistance has been sequenced.

Background

To better inform the disease management strategies of growers there is an immediate need for improved diagnostic testing for disease. Specifically, a test is needed that provides a quick diagnosis at a low cost to growers, ideally simultaneously, for a panel of common diseases. This test also needs to be precise enough to identify key pathogen characteristics such as resistance to certain fungicides and virulence. One approach is a diagnosis by sequencing method which identifies genetic markers associated with resistance and virulence traits. Such a diagnostic could also be used to better track the effects of different planting decisions and agronomic practices, informing disease management over the longer term.

Among those diseases affecting UK horticulture are the fungal pathogens *Venturia inaequalis*, *Podosphaera leucotricha* and *Podosphaera aphanis*. These are responsible for apple scab, powdery mildew of apple and powdery mildew of strawberry, raspberry and blackberry respectively. Successful control of these pathogens is paramount for growers. Production losses can be as high as 70% in orchards affected by apple scab (Biggs 1990; MacHardy 1996) and powdery mildew has been rated as the most important aerial disease for UK strawberry growers (Calleja, 2011). Diagnosis by sequencing has been proposed for these diseases.

Unfortunately, the requisite genomic resources for diagnosis by sequencing of these pathogens lags behind those available for their respective host plants or for other pathogenic fungi. Indeed, there were no genome assemblies available for any *Podosphaera* species prior to 2020. The genetic basis for resistance to commonly used fungicides is not well understood in either scab or powdery mildew. Similarly, no virulence genes have been characterised that facilitate the suppression of a host's natural immune response. There is significant interest in developing new varieties combining the fruit quality of commercial cultivars with the superior disease resistance of wild species. However, in the past the resistance of cultivars has quickly been broken in field conditions, to prevent this from happening in the future, a better understanding of these pathogens is needed (Caffier et al. 2014, Caffier and Laurens 2005).

The objective of this project is to generate some of the first genomic resources for the study of apple, strawberry and raspberry powdery mildew. We aim to improve the understanding of virulence in apple scab and mildew by identifying putative virulence and avirulence genes that determine isolate host specificity as well as mutations leading to fungicide resistance. This will inform future management strategies and breeding efforts.

Summary

In the first three years of this PhD, techniques for collection, culture and DNA extraction from apple scab and powdery mildew have been refined. These methods have facilitated DNA and RNA sequencing of the apple, strawberry and raspberry powdery mildew genomes. Comparison of these species genomes and transcriptomes will enable identification of key virulence and fungicide resistance factors. Additionally, 50 apple scab isolates displaying different levels of fungicide susceptibility have been sequenced enabling identification of fungicide resistance markers.

- Apple Scab was sampled from indicator trees known to carry resistance genes
- Apple Scab was sampled from problem orchards and confirmed to be fungicide resistant
- Fungicide resistant and susceptible strains of apple scab were crossed to generate a mapping population for the fungicide resistance trait
- A fungicide resistant population was sequenced
- Samples of powdery mildew were collected from susceptible apple, strawberry and raspberry plants
- DNA extraction and sequencing was performed on apple, strawberry and raspberry powdery mildew samples
- Genome assemblies were generated for apple, strawberry and raspberry powdery mildew
- RNA extraction and sequencing was performed on strawberry powdery mildew samples
- A mapping population of apple trees were scored for susceptibility to apple powdery mildew in 2020 and 2021

Financial Benefits

Apple growers are currently heavily dependent upon frequent fungicide applications to control disease in orchards. Without these products, outbreaks inflict serious crop losses of up to 70%. There is increasing pressure to reduce the use of such crop protection products. This

project lays the groundwork for diagnosis by sequencing of the two pathogens behind: apple powdery mildew and scab. This kind of rapid and precise diagnosis would allow the effects of management strategies to be tracked and modified, supporting the maximisation of yield whilst minimising the application of fungicides.

Action Points

There are no grower actions points at this stage of the project.

SCIENCE SECTION

Introduction

Apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) are key pathogens of apple cultivation. Currently, growers use a range of integrated pest management strategies to control this disease but remain heavily dependent upon fungicide use, applying pesticides throughout the season to limit damage to their crops (Roßberg 2003). Apple is among the most important fruit crops globally, ranked third in terms of annual production at 84.6 million tonnes (FAOSTAT 2014) and it is thought that apple producers in the eastern United States spend as much as 18.6 million USD per year on scab control alone (Cox 2015).

Powdery mildew caused by *Podosphaera aphanis* (formerly *Sphaerotheca macularis*) has been rated as the most important aerial disease for UK strawberry growers, it also affects raspberry and blackberry (Calleja, 2011). Epidemics can lead to severe yield loss and unmarketable fruit. As with apple scab disease control is primarily achieved through foliar fungicide applications.

Diagnosis by sequencing of these pathogens requires knowledge of the genetics underlying key traits such as host range and fungicide susceptibility.

Control through frequent fungicide application may be unsustainable, tighter legislative restrictions on fungicide spraying is anticipated. Additionally, reduced sensitivity to fungicides has been reported in field populations of *P. aphanis*, *P. leucotricha* and *V. inaequalis* (Brent and Hollomon 1995; Lesemann et al. 2006; Palmer and Holmes, 2021). It is thought that fungicide resistance can arise via several mechanisms; through alterations in the fungicide binding site, increased production of a targeted protein or an improved ability of fungi to remove toxins (Koenraad et al. 1992; Hamamoto et al. 2000; Deising et al. 2008). Resistance against some classes of fungicide have been associated with mutations in specific target regions, for example non-synonymous mutations in the CYP51A1 gene region have been associated with resistance to demethylase inhibitor fungicides (Villani et al. 2016). However, this does not fully explain resistance in apple scab. Detection of fungicide resistance often still relies upon microbiological methods for many fungicide classes where the genetic basis for resistance remains unclear.

Powdery mildew species are biotrophic fungi and are known to be highly host specific. This host specificity is thought to arise from each pathogen's ability to suppress or avoid recognition by a given host's immune system. Fungi secrete toxins and proteins to aid infection. These are termed effectors. However, some of these effectors may be recognised

by the host plant if it carries certain resistance genes. This results in effector triggered immunity. Fungi may gain or lose effectors to avoid recognition by the plant, allowing them to overcome resistance. New patterns of virulence may occur due to the disruption of genes via the insertion of a transposon or because of mutation. The unique effector complement of different isolates is an important feature which could be used to predict host range. Whilst they are considered the same species strawberry and raspberry powdery mildew isolates have been shown to be genetically distinct and it has been demonstrated that *P. aphanis* isolates from strawberry are unable to infect raspberry and vice versa (Harvey and Xu, 2010; Martin et al. 2017). The ability of an isolate to overcome known resistance genes in a host is used to define different races in a pathogen population. A race structure is well established in the apple scab pathosystem (Bowen et al. 2011). Investigations into the population structure of powdery mildews have been hampered by a lack of genomic resources.

This project aims to identify key genetic markers in *V. inaequalis*, *P. leucotricha* and *P. aphanis*, conferring traits such as host specificity and fungicide resistance. This lays the groundwork for diagnosis by sequencing. We have collected samples of *P. leucotricha* and *P. aphanis* as well as isolating *V. inaequalis* strains that display different virulence and fungicide resistance characteristics. Sequencing and analysis of these samples is now underway.

Project aims:

- To identify key virulence factors in resistance breaking strains of apple scab (*V. inaequalis*)
- To identify genetic markers for fungicide resistance in *V. inaequalis*
- To generate the first draft genome and transcriptome for a European isolate of apple powdery mildew (*P. leucotricha*)
- To generate the first draft genome and transcriptome for strawberry powdery mildew (*P. aphanis*)
- To generate the first draft genome for raspberry powdery mildew (*P. aphanis*)

Materials and methods

Powdery mildew Sampling:

Heavily infected leaves were removed and transported to the lab in paper envelopes taking care to retain as much mildew material as possible in transit. Leaves were then placed into a two-litre glass beaker containing one litre of water and stirred vigorously to wash off conidia. Conidial suspensions were then sieved into 50 ml falcon tubes which were centrifuged at

5000 g for 5 mins. Following this the supernatant was removed via stripette leaving isolated conidia. Washing of leaves was repeated into the same falcon tubes until all conidia had been dislodged and subsequent washes gave no increase in conidial yield. Any remaining water was removed from the samples by pipette following a final centrifugation at 5000 g for 5 mins. Samples were then freeze dried overnight and the fully dried conidial powder transferred to 1.5 ml Eppendorf tubes stored at -80 °C.

V. inaequalis Sampling and Culture:

Scab was collected from the field by using cork borers to excise single scab lesions from infected leaves, the resulting leaf discs were kept at -20 °C for long term storage. To bulk up individual isolates from these stocks the fungus was cultured from single spores grown on PDA plates containing 60 ppm rifamycin in 24-hour darkness at 17 °C; individual leaf discs collected from a given cultivar were defrosted and placed into 2 ml Eppendorf tubes with 300 µl of sterile water, discs were then vortexed for 1 min to wash off conidia. An optical microscope was used to confirm the presence of spores suspended in the water. 50 µl of spore suspension was then spread onto a plate. Another 300 µl of sterile water was then added to the remaining suspension and 50 µl of the more dilute spores spread onto a second plate. Germination was observed under a dissecting microscope and a scalpel was used to excise individual germinated spores onto fresh individual plates, these were sealed with parafilm and left to develop for 6 weeks. For collection of mycelia the cultures were transferred to cellophane disc plates. These were prepared by pipetting 1 ml of sterile water onto standard PDA + rifamycin plates before spreading a cellophane disc flat across the agar surface. Plugs of mycelium were taken from uncontaminated plates mixed with 1 ml of sterile water and spread over the top of the cellophane discs. These plates were then sealed with parafilm and the cultures grown until the disc was covered by mycelium, at this point sub culturing and mycelial harvest took place. Mycelium was harvested by scraping from the surface of a given cellophane disc and freeze drying in 1.5 ml Eppendorf tubes overnight. These stocks were then stored at -20 °C until use for either DNA extraction or for recovering growing cultures of contaminated isolates.

V. inaequalis Crossing:

Four fungicide resistant *V. inaequalis* isolates were crossed with seven susceptible isolates. An apple leaf decoction media was prepared; senescent apple leaves were boiled in distilled water for 15 mins, 25 g/L, the resulting decoction was filtered through muslin and used to prepare malt extract media (5 g/L malt extract, 17 g/L agar). Crossing of two strains was achieved by subculturing mycelial plugs of each parent onto the same leaf decoction plate amended with 60 ppm rifamycin. Plugs were placed approximately 15 mm apart from each

other and left for one month at room temperature to grow together. Once colonies from the two isolates had grown sufficiently to meet each other plates were moved to chilled 4 °C conditions for 6 months to facilitate sexual reproduction.

After 6 months the meeting point of two colonies was excised using a scalpel blade and placed on a microscope slide, sexual pseudothecial structures were separated from as much media as possible. Following this a few drops of sterile deionised water were added, and samples were crushed, the release of ascospores was confirmed under via optical microscope. Spores were washed into Eppendorf tubes using sterile water. This spore suspension was then pipetted onto rifomycin amended PDA plates and allowed to dry before plates were sealed. 24-48 hours later plates were checked for germinating spores which were then isolated and removed to individual plates for culture as described previously.

Short read DNA Extraction:

DNA extraction was performed using a nucleospin plant II extraction kit following the manufacturer's instructions for fungal samples, with the modification that homogenization and lysis was performed using a geno/grinder with 2 ball bearings inserted into 2 ml Eppendorf tubes at 1500 rpm in 20 s bursts for a total of 2 mins chilling with liquid nitrogen between bursts. RNase A and proteinase K were used as described in step 2 of the manufacturer's instructions.

High molecular weight DNA Extraction:

High molecular weight DNA extractions were based upon the CTAB extraction protocol of Schwessinger (2016) which was progressively modified to obtain high quality DNA from mildew. Premade buffers were combined to form lysis buffer (Buffer A; 0.35 M sorbitol, 0.1 M TrisHCl, 5 mM EDTA pH 8 Buffer B; 0.2 M Tris-HCl, 50 mM EDTA pH 8, 2 M NaCl, 2% CTAB Buffer C; 5% Sarkosyl N-lauroylsarcosine sodium salt Buffer D; PVP40 10% Buffer E PVP10 10%) in the ratios 5:5:2:1:1, 10 µL (10kU) RNase A was added to this. Fungal samples were ground in a geno/grinder with 2 ball bearings inserted into 2 ml Eppendorf tubes at 1500 rpm in 20 s bursts for a total of 2 mins chilling with liquid nitrogen between bursts. They were then incubated at room temperature for 30 mins in 1.5 ml lysis buffer whilst being inverted, ball bearings were left in the tubes to increase mixing, 20 µL of proteinase K was then added and incubation continued for another 30 mins. Following this, samples were cooled on ice for 5 mins before 250 µl of 5 M potassium acetate was added and cooling continued for an additional 5 mins. Samples were then centrifuged for 12 mins at 5000 g and the supernatant removed to safe lock tubes. Washing was then carried out by the 1:1 addition of Phenol:Chloroform:Isoamylalcohol 100 mM Tris-EDTA pH 8 (P:C:I), samples were mixed by inversion for 2 hours and centrifuged for 10 mins at 4000 g before transfer of the supernatant

to a fresh tube. This wash was repeated three times per sample, followed by a fourth wash using Chloroform:Isoamylalcohol (C:I) in place of P:C:I. DNA precipitation was performed with 200 µl of Sodium Acetate (3 M pH 5.2) and 800 µl Isopropanol, added to approximately 1 ml of supernatant and mixed by inversion at room temperature for 10 mins. DNA was pelleted by centrifugation at 8000 g for 30 mins. The supernatant was discarded and the pellet washed three times by resuspension in 1.5 ml of 70% ethanol, centrifugation at 13000 g for 5 mins and discarding of the supernatant. Following the final wash step remaining ethanol was allowed to evaporate for 30 mins before the DNA pellet was dissolved in 100 µL of 10 mM Tris pH 8.5 at room temperature overnight. DNA was then re-precipitated using 20 µl Sodium Acetate (3 M pH 5.2) and 1.5 ml 100% ethanol overnight at - 20 °C. Following this the previous washing steps were repeated; x3 P:C:I, x1 C:I, isopropanol precipitation, x3 70% ethanol and DNA was redissolved in 100 µL of 10 mM Tris pH 8.5 at room temperature overnight.

The quality of extracted DNA was assessed initially using a Nanodrop 1000 spectrophotometer (Thermo Scientific), if these results were in the target range then quality was further assessed using a Qubit 2.0 fluorometer (Invitrogen) and using these results 100 ng of DNA was run on a 1.5% agarose gel with GelRed to assess contamination with RNA and finally these results were compared to a qubit RNA reading.

RNA Extraction

RNA Extraction was performed using 3% CTAB extraction buffer as described in Yu et al. 2012 with the following modifications; chloroform:isoamyl alcohol (24:1) washing was omitted, precipitation was performed at -20°C for four hours. Resulting RNA concentration and RNA integrity number (RIN) of samples was assessed using the Agilent RNA ScreenTape System with a 2,200 TapeStation (Agilent Technologies, Germany) according to the manufacturers protocols. DNA concentration was assessed via Qubit dsDNA HS assay kit with a Qubit 3.0 fluorometer (Life Technologies, Waltham, MA USA).

ITS sequencing:

The ITS region of isolates was amplified using serial dilutions of DNA samples. PCR was performed using 5 µL Taq 5X master Mix (NEB), 16 µL water, 1 µL ITS-1 primer (TCCGTAGGTGAACCTGCGG), 1 µL ITS-4 primer (TCCTCCGCTTATTGATATGC) and 2 µL DNA dilutions. PCR was performed on a Veriti thermal cycler (Applied biosystems) using the following cycle: an initial 95 °C for 3 min; 35 cycles of 95 °C for 20 s, 60 °C for 15 s and 68 °C for 2 mins; then a final extension at 68 °C for 2 mins. PCR products were visualised on a 1.5% agarose gel with GelRed (0.5 µl/L) before being purified using a monarch PCR & DNA clean-up kit (5 µg) following the manufacturer's instructions. Purified PCR products were submitted for sequencing by Eurofins genomics LIGHTrun tube service. The resulting

sequences were aligned to a reference ITS region (Locus EU035437 for scab and JQ999954 for mildew) downloaded from NCBI and analysed using the program Geneious V10.0.2.

DNA/RNASeq Sequencing

DNA and RNA samples were submitted to the commercial sequencing company Novogene for total RNA depletion, purification, fragmentation, cDNA library construction, enrichment and sequencing. Sequencing was performed on an illumina HiSeq platform using PE150 chemistry.

Bioinformatic pipelines

Our mildew genome assembly pipeline was as follows; raw sequencing reads were subjected to a quality control check using FastQC v0.11.9 (Andrews, 2010). Sequences were trimmed and adapters removed using Trimmomatic v0.39 (Bolger et al, 2014). Reads were then aligned to the respective host genome; apple (Daccord et al, 2017), strawberry (Edger et al., 2019) or raspberry (Wight et al., 2019). Alignments were also made to the apple powdery mildew genome (JAATOF000000000, Gañán et al. 2020) using bowtie2 v2.4.2 for coverage estimation. Coverage was assessed using samtools v1.1 (Li et al, 2009) 'coverage' function and the kmer analysis toolkit v2.4.2 (Mapleson et al, 2017) function 'kat plot spectra-cn'. Those reads not aligning to the host genome were carried forward for de novo genome assembly using the program SPAdes v3.14.1 (Bankevich et al, 2012). Kraken2 v2.1.1 (Wood et al. 2019) was used to taxonomically classify contigs, a custom database was constructed including the standard databases for; archaea, bacteria, fungi, plants, protozoa, viral and vertebrate mammals with the addition of the host genomes and the 30 powdery mildew genomes publicly available in the NCBI database, those contigs classified to taxa other than fungi were removed from the assembly. Quality of the resulting genome was assessed by looking for benchmarking universal single-copy orthologs (BUSCO) with BUSCO v 4.0.6 (Simão et al. 2015). A *de novo* prediction of repetitive elements was performed using RepeatModeler v2.0.2 (Flynn et al. 2020) and TransposonPSI (TransposonPSI: An Application of PSI-Blast to Mine (Retro-)Transposon ORF Homologies, 2021), and the resulting library masked using bedtools v2.30.0 (Quinlan and Hall 2010). Sequence reads from RNA-seq were subjected to a quality control check using FastQC v0.11.9. Sequences were trimmed and adapters removed using Trimmomatic v0.39. Reads were then aligned to the draft genome assembly using STAR v2.7.3 (Dobin et al. 2013). The BRAKER v1.9 (Hoff et al. 2019) pipeline was then used to make gene predictions using these alignments, these predictions were supplemented by predictions made using Codingquarry v2.0 (Testa et al. 2015) in pathogen mode. Functional annotation of proteins was performed via Interproscan 5 v44-79.0 (Jones et al. 2014).

Our apple scab SNP calling pipeline was as follows; raw sequencing reads were subjected to a quality control check using FastQC v0.11.9 (Andrews, 2010). Sequences were trimmed and adapters removed using Trimmomatic v0.39 (Bolger et al, 2014). Coverage was assessed using the kmer analysis toolkit v2.4.2 (Mapleson et al, 2017) function 'kat plot spectra-cn' and bowtie2 v2.4.2 aligning to a scab reference genome (Passey et al. 2018). SNPs were called using GATK following the developers' best practices for pre-processing and germline short variant discovery (DePristo et al., 2011).

Results

Apple scab:

Scab isolates have been collected from two orchards; one problem orchard containing scab isolates displaying resistance to fungicides and one orchard which has never been sprayed and contains isolates presumed to be fungicide susceptible. Isolates taken from the problem orchard were first assessed for their ability to germinate in the presence of different chemicals. Successfully germinated spores displaying resistance to 6 chemicals (myclobutanil, tebuconazole, pyraclostrobin, cyprodinil, difenoconazole and pyrimethanil) were cultured. These represent strobilurin, demethylase inhibitor and anilinopyrimidine class fungicides. Resistant and susceptible isolates were then crossed to produce ascospores segregating for the resistance trait. Five mapping populations were generated, four as part of this project and one by a previous student. Work performed prior to the start of this project generated a population of 81 ascospores that was tested to determine the ED₅₀ of tebuconazole and myclobutanil for each isolate, however, genotyping was limited to PCR amplification of select target sites. Investigation of this pre-existing population was continued in this project; DNA was extracted from each isolate and submitted for skim sequencing to around 15 times coverage. Raw sequencing data was processed through the SNP calling pipeline detailed above, some 600,000 SNPs have been identified between these isolates and the reference apple scab genome. These SNPs will be used with existing ED₅₀ data to map QTL for the fungicide resistance trait.

Additionally, race 7 scab isolates have been sampled from naturally occurring 2019 and 2021 outbreaks of the disease on apple trees carrying the major resistance gene Rvi7. Cultures of these isolates have been established and their DNA extracted for future sequencing.

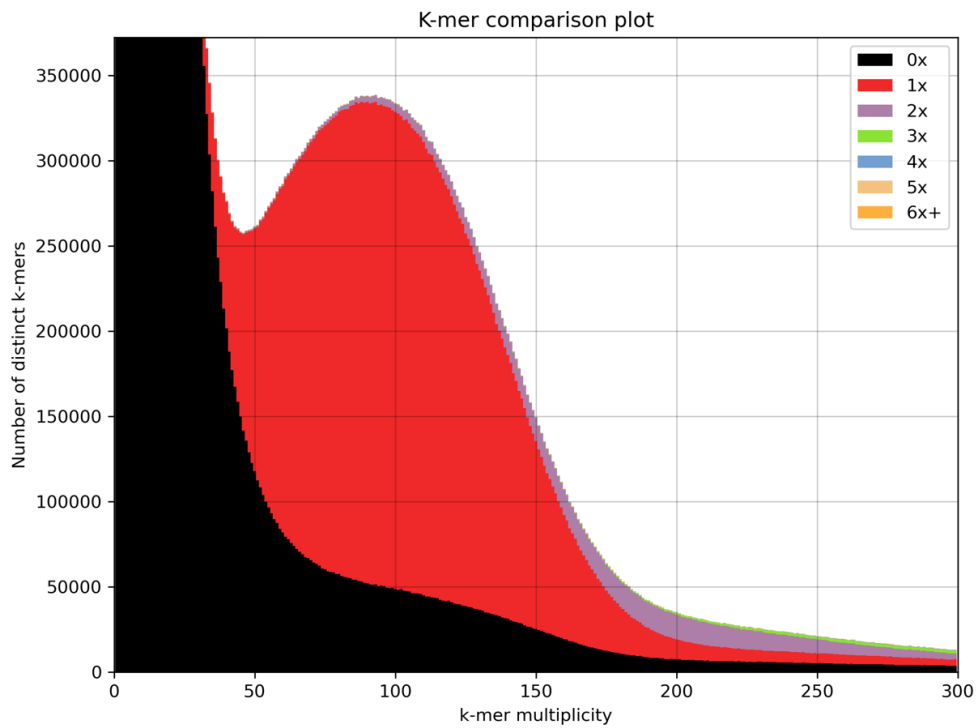
Powdery mildew

Mildew samples have been collected from field outbreaks of the pathogen *P. leucotricha* affecting apple trees and the pathogen *Podosphaera aphanis* infecting both strawberry and raspberry. DNA has been extracted and short read Illumina sequencing completed for each of these three pathosystems.

Apple powdery mildew

DNA sequencing of apple powdery mildew yielded 121,174,496 raw reads. Following quality control, we found that 13.67% of trimmed reads aligned to the apple genome. By contrast, 64.15% of reads aligned to the available *P. leucotricha* genome. Alignment to the available *P. leucotricha* genome provided an estimated a median sequencing coverage of x90. Some 22% of reads do not align to either the apple host or existing *P. leucotricha* genome assembly. The quality of reads was assessed using the program KAT (kmer analysis toolkit), much of our read dataset represents sequences that appear as single copies in the available apple powdery mildew genome and a far lower proportion represent sequences that are found in the available apple genome (Figure 1). This shows that we have successfully extracted a relatively pure sample of apple mildew.

A)



B)

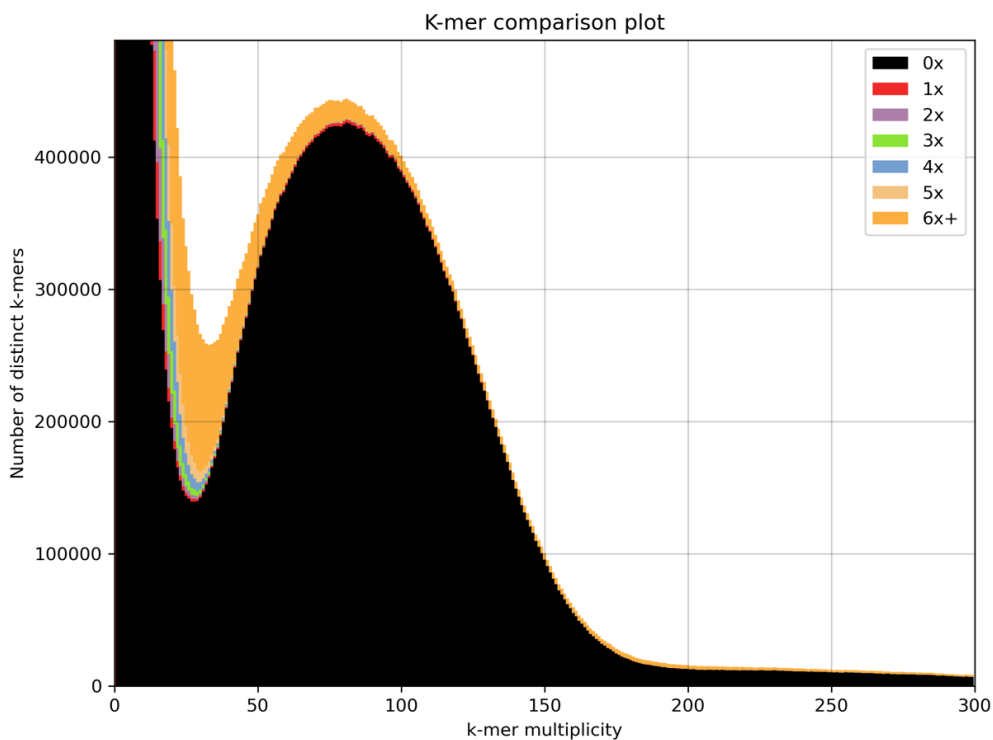


Figure 1. Read kmer comparison plots: Number of distinct kmers against their coverage in the trimmed apple mildew sequencing read dataset; A) Coloured for the number of times a given kmer appears in the available apple powdery mildew genome B) Coloured for the number of times a given kmer appears in the available apple genome.

Genome assembly was performed using the program 'SPAdes', resulting contigs were then filtered for contaminants using 'Kraken2'. Contamination was found to be minimal, classified nonfungal contigs were removed. The resulting assembly consisted of 7,163 contigs with an N50 of 19,981 and a genome size of 49.4 Mb. BUSCO analysis was performed using databases of conserved genes for the kingdom: fungi, division: ascomycota and order: erysiphales to which *P. leucotricha* belongs found that, respectively, 98.8%, 97.6% and 92.2% of conserved genes were represented in this genome assembly. Repeat regions were identified and masked, 47.1 % of the genome was found to be made up of repeats.

Strawberry powdery mildew

DNA sequencing of strawberry powdery mildew yielded 698,565,358 raw reads. Following quality control 14.04% aligned to the strawberry genome, there is no pre-existing *P. aphanis* genome available and so no alignments to the pathogen genome could be made. A KAT plot of reads versus the host genome indicate an average sequencing coverage of around 400 (Figure 2).

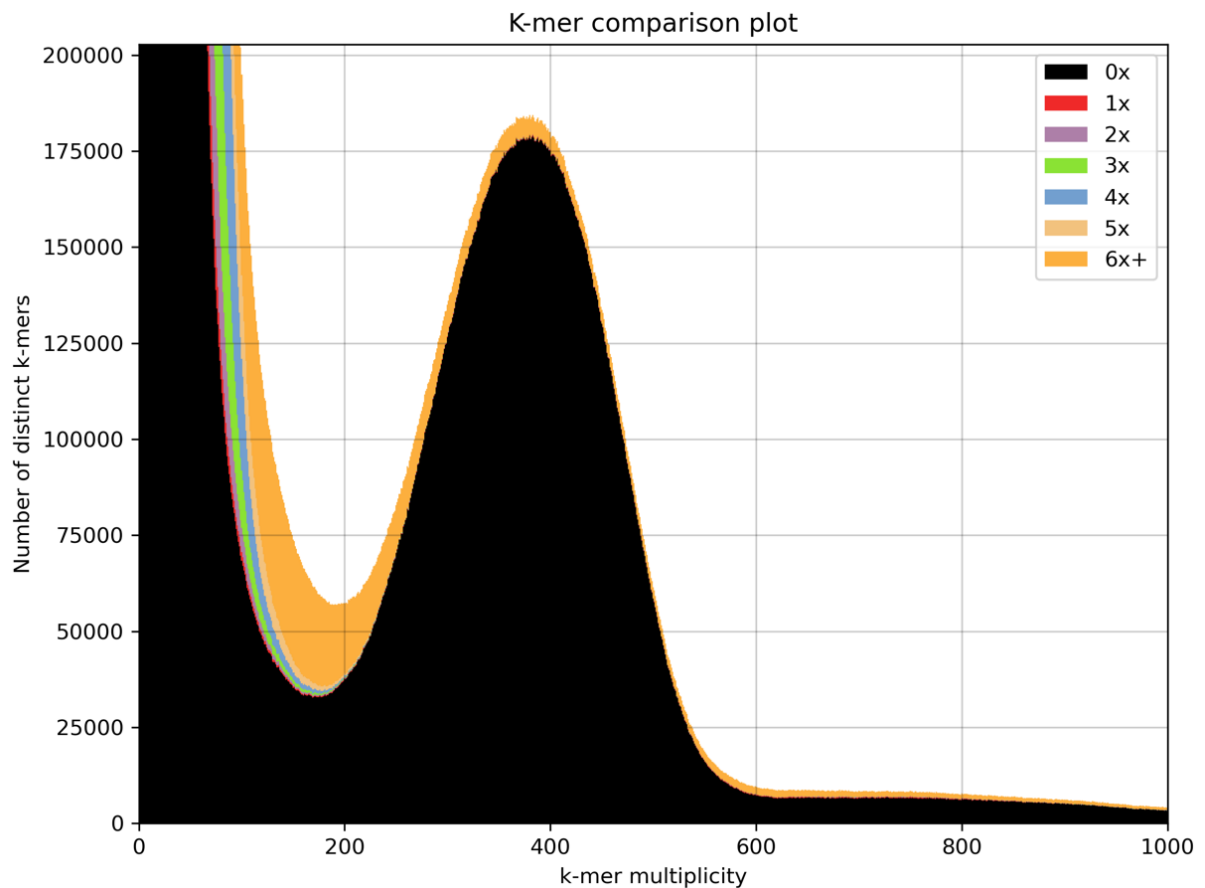


Figure 2. Read kmer comparison plot: Number of distinct kmers against their coverage in the trimmed strawberry mildew sequencing read dataset. Coloured for the number of times a given kmer appears in the host *Fragaria ananassa* genome. A peak of reads around 400 times coverage implies this is the average coverage in the dataset.

Genome assembly was performed using the program 'SPAdes' and contigs filtered using 'Kraken2', a small number of contaminant contigs were removed. The resulting strawberry powdery mildew genome consisted of 12,702 contigs with an N50 of 11,343 and a genome size of 56.2 Mb. BUSCO analysis found 98.8 % representation for a fungal database, 97.5 % representation for ascomycota and 91.9% representation for a leotiomycetes database. Repeat regions were identified and masked, 53.32 % of the genome was found to consist of repetitive elements. To facilitate gene prediction RNAseq was performed. The resulting 88,436,408 read pairs were aligned to the genome assembly and used to make gene predictions. A total of 17,565 putative protein-coding genes were identified.

Raspberry powdery mildew

DNA sequencing yielded 129,833,014 raw read from raspberry mildew. Following quality control 16.1% of trimmed reads aligned to the red raspberry genome, no alignments could be made to the pathogen genome as there is no pre-existing *P. aphanis* genome available. We estimate a sequencing coverage of 40 times from the results of a KAT plot of read against the raspberry genome (Figure 3).

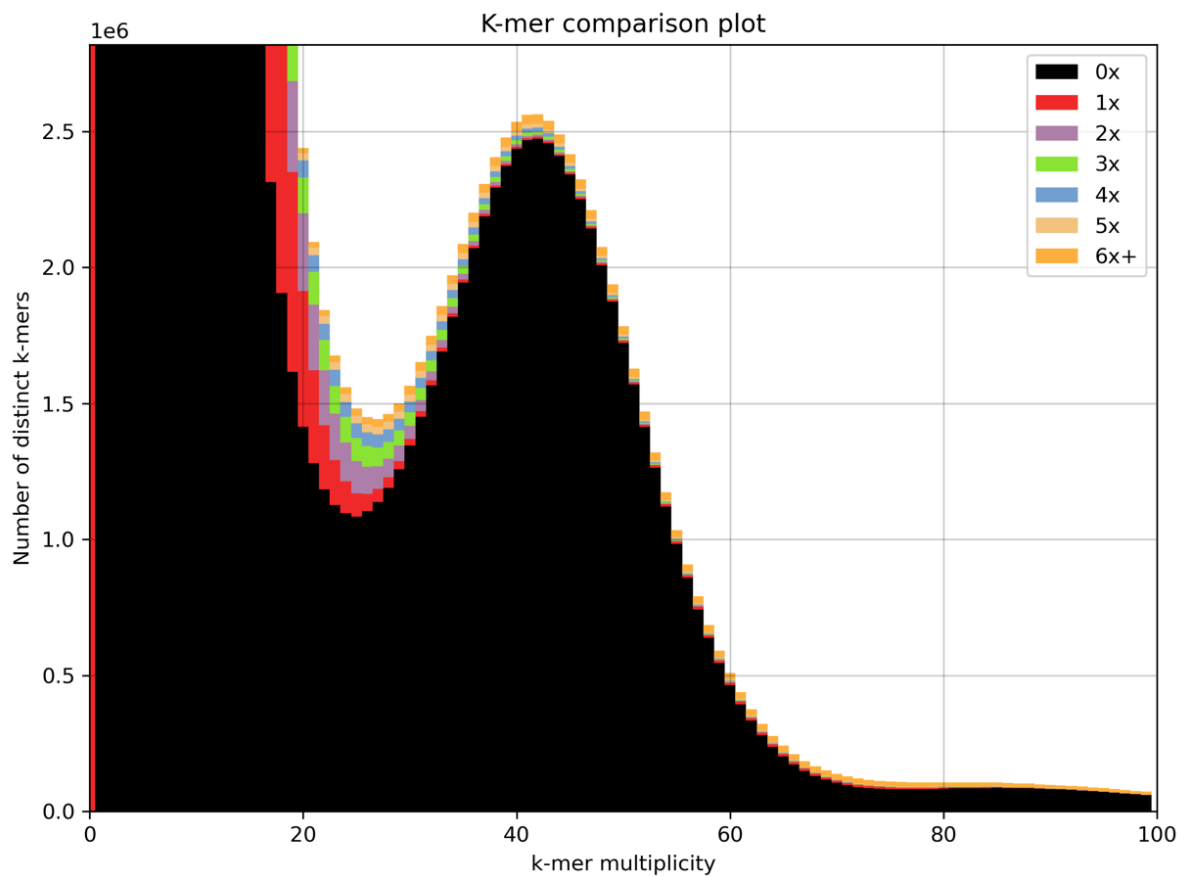


Figure 3. Read kmer comparison plot: Number of distinct kmers against their coverage in the trimmed raspberry mildew sequencing read dataset. Coloured for the number of times a given kmer appears in the host *Rubus idaeus* genome. A peak of reads around 40 times coverage implies this is the average coverage in the dataset.

Genome assembly was performed using the program ‘SPAdes’. For raspberry ‘Kraken2’ classified 12.26% of assembled contigs as *Sporobolomyces roseus*, filtering of this assembly was therefore more stringent and only those contigs positively identified as fungi outside the basidiomycota clade were included in our draft assembly. The resulting raspberry powdery mildew assembly consisted of 11,513 contigs with an N50 of 8,377 and a genome size of 52.4 Mb. The completeness of the assembly was assessed via BUSCO analysis, 99.2 % of fungal BUSCOs were found, 97.5% of Ascomycotal BUSCOs and 92.2% of leotiomycete BUSCOs (Table 1).

Table 1. Genome assemblies: Basic quality measurements for our genome assemblies and the published *P. leucotricha* genome assembly for comparison, including species, coverage, contig number, N50, genome size and BUSCO score versus an Ascomycota dataset.

Mildew Species:	Coverage:	Contig no.:	N50	Size (Mb):	BUSCO %
<i>P. leucotricha</i> (published)	80	8,921	8,371	43.8	96.1
<i>P. leucotricha</i>	90	7,163	19,981	49.4	97.6
<i>P. aphanis</i> (strawberry)	400	12,702	11,343	56.2	91.9
<i>P. aphanis</i> (raspberry)	40	11,513	8,377	52.4	92.2

Discussion

Apple Scab:

The aim of our work with fungicide resistant scab is to map QTL for fungicide resistance. It is hypothesized, based on previous, work that two separate genetic locations are responsible for resistance to DMI fungicides in apple scab. Our approach has been to generate mapping populations from resistant and susceptible parent isolates, in which progeny isolates segregate for fungicide resistance. By combining phenotypic data of the ED₅₀ for each isolate with genotypic data we can determine which genomic regions are inherited from the fungicide resistant parent of the population are responsible for the fungicide resistance trait. We now have both phenotypic data and genotypic data available, having completed 10X coverage sequencing of the mapping population. 600,000 SNPs have been identified in these isolates when called against a reference scab genome, these SNPs can be used as genetic markers to track where recombination has taken place between the two parent isolates. In the future we will identify which of these SNPs are unique to each parent and perform QTL mapping for the population.

Powdery mildew

In our previous report sampling and DNA extraction methods had been optimized for powdery mildew fungi, and genome assembly had been completed for *P. leucotricha*. Alignment of our

apple powdery mildew sequencing reads against the available apple and apple powdery mildew genomes demonstrates that our sampling method has successfully isolating mildew material. Many more reads align to the apple powdery mildew genome than to the apple genome. KAT analysis further supports this as most reads aligning to the apple genome align multiple times, indicating that they are repetitive sequences, whereas those reads aligning to the apple powdery mildew genome are unique, aligning only once. The success of our sampling method is further supported by taxonomic classification of our assembled contigs where 'kraken2' identified >90% of contigs as belonging to *Podosphaera* species.

We have now completed genome assembly for apple, strawberry and raspberry powdery mildew, and these assemblies have been successfully submitted to the NCBI database. High BUSCO scores for each of these assemblies indicate that our genomes are relatively complete and of good quality. The quality of our assemblies is further supported by comparison to published *Podosphaera* genomes which have similar contiguity and N50 metrics.

Gene prediction has so far been completed for strawberry powdery mildew, in the immediate future this will be performed for our other assemblies. Following gene prediction, genome comparisons will be made between assemblies in order to determine unique features identifying each species, this will be aided by the recent release of genome assemblies for *Podosphaera xanthii* and *Podosphaera cerasii* as well as the published *P. leucotricha* genome (Ganan et al. 2020; Kim et al. 2021; Polonio et al. 2021). With these genomes and our own it will be possible to draw comparisons between the *Podosphaera* clade and the more widely studied cereal grain powdery mildews as well as other fungi.

Whilst it is possible to generate genome assemblies using only Illumina sequence data, powdery mildew genomes are known to contain many repetitive sequences that are hard to resolve using only short read sequencing technologies (Frantzeskakis et al. 2018). To produce more contiguous genome assemblies, we hope to use the nanopore sequencing platforms. Further DNA extractions for each pathosystem will be performed for Oxford Nanopore long-read sequencing.

Conclusions

- Different races of apple scab have been sampled and cultured
- Fungicide resistant and susceptible scab isolates have been sequenced and SNPs called

- A first genome assembly has been constructed for apple, strawberry and raspberry powdery mildew

Knowledge and Technology Transfer

The student has presented this project internally and at CTP (July) and AHDB (January) meetings, unfortunately COVID 19 has led to the cancellation of other conferences.

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