Final Report

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Title: Resistance and susceptibility in interactions between apple and woolly aphids

Woolly apple aphid biology, resistance breeding resources

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1. Industry Summary

The woolly apple aphid (WAA) is a widespread sap-sucking apple pest which is becoming increasingly virulent with the reduction in available control options, especially in warmer regions such as South America and Oceana. There are four known WAA resistance genes, including *Er1* which is found in MM106 and M.116, and *Er2* which is used in resistant rootstocks in the Geneva rootstock line. A key aim of this project is to improve genetic resources for rootstock breeding programmes with the long-term goal of increasing the commercial introduction of WAA-resistant rootstocks.

We screened 44 apple accessions for WAA susceptibility with the purpose of identifying WAA resistant trees for use as pollen sources within a commercial crop. Potential novel sources of resistance were selected for further investigation within a rootstock breeding programme. Conventional rootstock breeding often takes at least twenty-five years from initial crossing for a target trait to commercial introduction. A Marker-Assisted Selection (MAS) approach identifies genetic markers closely associated with the target genes and screening seedlings for markers and including/excluding seedlings on this basis, replacing multiple, time-consuming phases of identifying resistant seedlings.

In its native range in Northeast America, WAA alternates between apple and American Elm, but has lost this in the rest of the world and is thought to reproduce exclusively asexually with little to no gene flow. The concern with this is the potential that sexual aphids could spread virulence traits, for example those which allow feeding on resistant rootstocks. There have been published and anecdotal reports of WAA overcoming resistance from both *Er1* and *Er2*, which could be a genetic trait or could be the result of conditions such as temperature and humidity which benefit the aphids. Analysing individual and population growth on different rootstocks found slightly reduced growth on resistant rootstocks, compared to susceptible, although some individuals showed growth on the resistant rootstocks. Woolly apple aphid currently remains on apple year-round, migrating to the rootstock in the autumn and returning to the scion with new spring growth. Sexual reproduction in aphids is coupled with alternating to a second host and could require changes to control measures.

We sampled WAA from 25 sites across England, and 10 sites abroad (spread across Northern Ireland, the Netherlands, Chile, the USA, and New Zealand). We found genetic variation between and within populations which is consistent with some instances of sexual reproduction. This was especially high at sites with varied apple genotypes, such as NIAB East Malling and the National Fruit Collection. This may have created a high-pressure environment, driving sexual reproduction. Some populations were found to be asexual but genetically isolated, which could have occurred through genetic drift over time or from separate introductions/invasions. Some sexual reproduction likely occurs in the UK, although it is not widespread.

2. Introduction

2.1. Impact of the pest

Apples are “the most important temperate fruit crop worldwide” (Kellerhals, 2009) with over 50 million tonnes of apples are produced across the world annually, with over 89 million tonnes of apples produced globally each year (FAOSTAT, 2019). Apple performs especially well in temperate environments such as Europe, North America, and New Zealand (Kellerhals, 2009) and is an economically important crop in the United Kingdom valued at approximately £287 million in 2021, of which £154 million was attributed to dessert apples and £43 million to culinary apples (DEFRA, 2022).

Yield losses, as a result of pest feeding, can drastically effect profit margins, exacerbated by related costs such as pesticide application (Cross *et al.*, 2015). The woolly apple aphid (WAA; *Eriosoma lanigerum*) originates in North East America and was first observed in Britain in 1787 (Theobald, 1921) from where it may have spread to much of the rest of the world. This aphid’s pest status is becoming increasingly concerning in the northern hemisphere with changing climatic conditions and the withdrawal of control options; therefore understanding and controlling WAA is a global issue.

In its native range, WAA exhibits a host-alternating lifecycle where it feeds on apple during the summer before producing sexual forms in the autumn which move to American Elm trees, produce eggs, which then hatch in spring and return to apple. Elsewhere in the world, WAA has lost this stage on American Elm and feeds exclusively on apple, producing up to 20 generations per year (Barbagallo *et al.*, 1997). Rather than switching to an alternative host in autumn, the aphid moves to the rootstock, feeding throughout the winter as adult and nymphal forms. This has led to WAA becoming a more severe crop pest outside North America because of the increased pressure year-round on apple (Cummins & Aldwinckle, 1983).

The international variation in WAA lifecycles poses the question of what impact(s) exclusive asexual reproduction will have on genetic diversity. Sexually reproducing populations of WAA are expected to show higher genetic diversity than populations comprised of parthenogenetically reproducing clones (Kanbe & Akimoto, 2009). Zhou *et al.* (2015) used eight microsatellites primers (Lavandero *et al.*, 2009) to determine the genetic diversity and structure of WAA sampled from twenty-four locations and were able to determine the dispersal routes of WAA through China.

2.2. Woolly apple aphid damage

Aphids sequentially probe plant cells whilst feeding until it detects phloem sieve tube cells, injecting saliva each time (Miles, 1999), inducing a signalling cascade which causes cells around the xylem

and phloem to rapidly divide, forming a gall which spreads to other tissues and breaks down normal plant tissue structure, forming a spongey mass which is easier for aphids to feed on (Staniland, 1924; Barbagallo *et al.*, 1997). The presence of WAA galls on both the scion and rootstock was found to strongly reduce plant growth through reducing water and carbohydrate flow (Weber and Brown, 1988; Brown *et al*., 1995). Galls can create open wounds in plant tissue which are vulnerable to pathogen infection, such as perennial apple canker, outbreaks of which follow severe WAA infestation late in the growing season (Childs, 1929) and are also thought to have a role in spreading fireblight when it shelters under tree collars (Cummins & Aldwinckle, 1983). Woolly apple aphids very rarely infest fruit, except in the case of open-calyx varieties, although fruit can be contaminated with aphids, wax and honeydew. Honeydew can promote the growth of sooty mould which can block photosynthesis if not controlled (Barbagallo *et al.*, 1997; Guerrieri & Digilio, 2008).

2.3. Woolly apple aphid control

It is very difficult to monitor and control aphids when they are in the soil (Hetherington *et al.*, 2009). Pest monitoring is the first step to knowing when to act to control a pest, which is compounded by the complex lifecycle of WAA. It is very rare for root infestations to occur without accompanying aerial infestations. The portion of the year where WAA is feeding below-ground is critical for successful management. Trees which are heavily infested in the autumn are likely to have large numbers of aphids over-wintering on the rootstocks and can be identified for control early in the following season (Hetherington *et al.*, 2009). Suckers and water shoots from the rootstock, and major scaffold limbs are often favoured by first instar nymphs moving back to the canopy in the spring. Rootstocks which are resistant to WAA feeding not only prevent root damage as a result of winter feeding but also remove the reservoir of aphids to re-emerge in the spring.

Contact insecticides, for example FLiPPER and Sentinel are at least partially blocked by the aphid's protective wax coat and colonies may need to be "burnt" using magnesium sulphate or an adjuvant such as a horticultural oil in order to penetrate the wax (Alston *et al.*, 2010; Bird, 2021 pers. comm.; Powell, 2022 pers. comm.). Systemic insecticides, for example spirotetramat and acetamiprid are percieved to be the most effective for WAA control (Bird, 2021 pers. comm.). This is especially beneficial in the case of root-feeding aphids which can be otherwise difficult to treat.

Known predators of WAA include the common earwig, hoverfly and ladybird larvae. The parasitoid wasp *Aphelinus mali* has the same native range as WAA but has since been introduced around the world with widespread success in controlling WAA infestations (Cohen *et al*., 1996; Staniland, 1924). Compared to other natural enemies, *A. mali* is the most effective at controlling WAA, but is its most effective when combined with a generalist predator (Gontijo *et al.*, 2015), as different environmental conditions benefit different natural enemies. It is, therefore, possible to an extent to predict points in

the year at which different natural enemies may be active and to tailor applications of chemical control to fit these time windows.

Rootstocks which are resistant to WAA feeding can effectively control aphids on the rootstock while other control methods, such as the use of *A. mali*, can be used to tackle WAA feeding above ground (Sandanayaka *et al.*, 2005). There is then still the option to use Batavia, or other pesticides, where necessary to control infestation.

2.4. Commercial use of crab apples

Many apple varieties are self-incompatible (Broothaerts *et al*., 2004), meaning that in a single-variety orchard an external pollen source, known as a polliniser, is needed in order to guarantee fruit set. Pollinisers must have a similar flowering time to the crop and must have a compatible pollen type to ensure successful pollination is possible (Sakurai *et al*., 2000) and be spread throughout the main crop to ensure pollen spread, a ratio of 1:5 crab apple pollinisers to crop trees is typical (Free, 1962 Dray & Campbell, 2007). These can, however, create reservoirs for pests and diseases. Resistant pollinisers may help to control pest build up within orchards.

2.5. Mechanisms of woolly apple aphid resistance

Approximately ten cultivars have been reported as showing WAA resistance although they appear to affect aphids differently (Sandanayaka *et al.*, 2005). From these, four resistance genes have been identified: *Er1-4*.

2.5.1. Er1

The American scion cultivar ‘Northern Spy’ shows resistance to WAA and has thickened rings of sclerenchyma around the vascular tissue (Staniland, 1924), shortening the duration of WAA feeding (Abu-Romman & Ateyyat, 2014). Northern Spy was crossed with several rootstocks in the ‘Paradise’ series to create the Malling-Merton (MM) rootstock series (Crane *et al.*, 1937), of which MM106 and M.116 are widely used commercially.

The gene conferring WAA resistance in Northern Spy was identified as a dominant major gene in 1962 and later named *Er1* (Knight *et al*., 1962; King *et al*., 1991) and located to the top of Linkage Group (LG) 8 (Bus *et al*., 2008).

The M.432 family between (‘M.27’ and ‘M.116’) was produced at East Malling Research in 2003 (Evans *et al.*, 2011) and has three genetic maps, which can be used to pinpoint the location of *Er1* in this family (Evans *et al.*, 2011; Antanaviciute *et al*., 2012; Fernández-Fernández *et al*., 2012). MCM007 (‘M.27’ × ‘M.M.106’) is a backcross of ‘M.116’ and useful to understand gene inheritance across families.

2.5.2. Er2

Malus x *robusta* 5 (*M. baccata* x *M. prunifolia* Carr.; Robusta 5) is the source of the second major WAA resistance gene to be identified, *Er2* (King *et al.*, 1991). Robusta 5 is the source of WAA resistance in the Geneva rootstock series developed at Cornell University (Cummins & Aldwinckle, 1983). *Er2* is also believed to reduce WAA feeding (Abu-Romman & Ateyyat, 2014) but is located on a different linkage group (LG 17).

2.6. Resistance-breaking WAA

There have been both published and anecdotal reports of WAA feeding on rootstocks with both *Er1* and *Er2* resistance, mostly in America and the southern hemisphere but more recently in Europe (Self, 1966; Giliomee *et al.*, 1968; Rock & Zeiger, 1974; Jaastad, 2020, pers. comm.). This may be the result of beneficial environmental conditions which favour aphid feeding or which influence the expression of resistance genes within apple (Bus *et al.*, 2008).

2.7. Resistance gene mapping

Microsatellite markers (also known as Simple Sequence Repeats (SSRs)) are short repetitive DNA sequences spread throughout the genome and have been widely used in plant breeding (Mammadov *et al.*, 2012). More recently, single nucleotide polymorphism (SNP) markers have become much more widely used in breeding programmes because they are variations in a single genetic base, and exist across the genome in much higher numbers than microsatellites, allowing the development of saturated genetic maps. Genetic maps with a high density of markers increase the likelihood of identifying markers close to the target gene (Mammadov *et al.*, 2012).

Genotyping-By-Sequencing (GBS) is a Next-Generation Sequencing (NGS) approach to generate large numbers of SNPs for linkage mapping (Poland & Rife, 2012). This approach has been successful in key crop plants such as *Zea mays* L. (Beissinger *et al.*, 2013), as well as being useful for SNP identification in previously uncharacterised species (Poland & Rife, 2012). Fine mapping of WAA resistance genes using SNPs will narrow down the areas in which the genes lie.

Traditional breeding programmes are time- and labour-intensive, especially rootstock traits which must also be evaluated through their effects on the scion, as well as their own benefits. Resistance breeding can require multiple generations of back-crossing to guarantee a commercial product with the desired trait(s) (Bianco *et al.*, 2014).

Marker-Assisted Selection (MAS) uses the presence or absence of a marker or markers linked to a target gene to determine whether or not the gene is present, reducing the time taken to ensure a desired trait is present (Collard *et al.*, 2005). SNPs have become widely used in MAS programmes because of how widespread they are in the genome (Mammadov *et al.*, 2012), allowing

inclusion/exclusion of seedlings from a breeding programme without lengthy field assessments (Hamblin *et al.*, 2011).

Resistance mediated by a single gene is not considered to be an effective long-term solution as they present only a single mode of resistance and therefore can be easily overcome by pests. Resistance gene pyramiding combines multiple resistance genes from different sources, to confer resistance which is both more difficult to break and can protect against all strains of the pest (Van der Plank, 1963; Servin *et al.*, 2004; Bus *et al.*, 2008).

Genetic markers identified through this project will be good candidates for future gene pyramiding to prevent feeding of multiple biotypes of WAA, potentially including some strains with a resistance-breaking phenotype.

2.8. Research outline

This project has two main research objectives: to better understand WAA biology, and to refine genetic positions of WAA resistance genes to improve rootstock breeding. These objectives were broken down into five experimental chapters, each with their own aims.

1. Screening *Malus* accessions for WAA resistance
    a. Aim: identifying WAA resistant or tolerant accessions for use as pollen sources within a commercial crop.
    b. Aim: selecting potential novel sources of resistance for inclusion in resistance breeding programmes.
2. Mapping the *Er1* gene
    a. Aim: refining the genetic position of the *Er1* gene to aid with marker-assisted selection of this gene for rootstock breeding.
3. Mapping the *Er2* gene
    a. Aim: refining the genetic position of *Er2*, as above but with the addition of generating the first SNP map for this gene.
4. WAA genetic diversity
    a. Aim: to investigate the genetic diversity of WAA within the UK and compared to other key apple-growing countries; using this information to infer how likely it is that WAA is sexually reproducing in the UK.
5. WAA performance on different rootstocks
    a. Aim: to assess the effects of feeding on resistant and susceptible rootstocks on WAA growth and reproduction.

3. Materials and methods

3.1. Phenotyping of woolly apple aphid susceptibility through controlled inoculation with aphid material

The WAAs used for screening were collected around from NIAB East Malling from a wide range of apple genotypes. Aphids were collected with great care taken not to damage them, and used the same day to inoculate apple material.

Apple material was inoculated with WAA and colony growth assessed to provide each seedling with a susceptibility score. Two inoculation sites were selected for each tree, spaced at least 10 cm apart, except in the case of very small seedlings where this was not possible. Care was taken to ensure that inoculation sites were above the graft union (where applicable) and that there were no WAA colonies currently feeding at or around these sites. A refuge for the WAA was created at each inoculation site with 1 cm width PVC tape to secure a petiole to the main stem creating a covered space on all sides except above (see Figure 3). A pea-sized amount of mixed life stage WAA was placed into this refuge using a dry, fine paintbrush. The refuge keeps the aphids in position, allowing colonies to feed and build, and offering some protection from abiotic stressors and natural enemies. Woolly apple aphid colonisation was assessed using the criteria given in Table 1 before inoculation and two weeks after inoculation. Those which were classified as resistant or intermediate were re-inoculated to ensure WAA was given an opportunity to develop and scored after an additional two weeks of incubation.

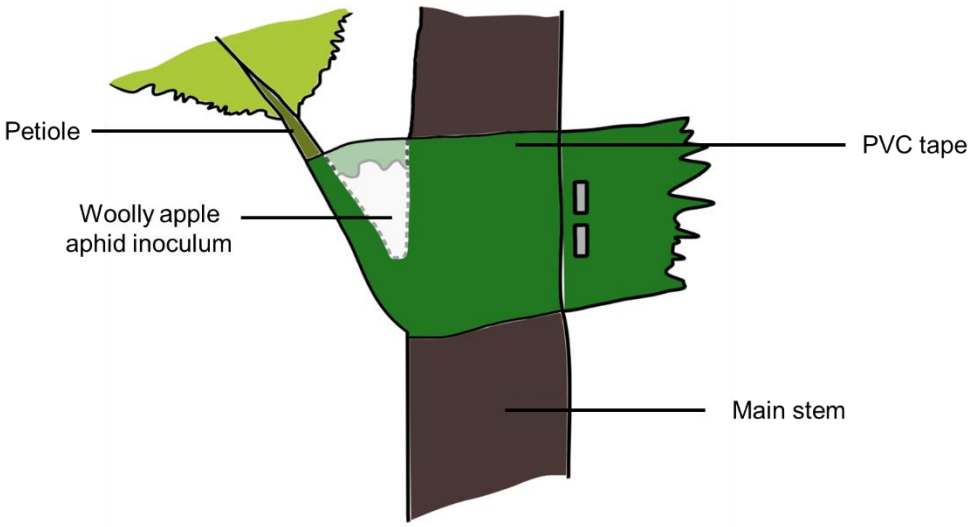

Figure 1- Woolly apple aphid refuge for inoculation with woolly apple aphid. Two refuges were created per tree, spaced well apart. PVC tape was used to create a refuge between the petiole and main stem. A pea-sized amount of aphids was placed into each refuge the inoculum from biotic and abiotic stressors.

Table 1- Scoring criteria for WAA colonisation of apple material. Individuals were assessed per the description given here, given a susceptibility score and assigned a class. This was carried out prior to inoculation, and two weeks after controlled inoculation with aphid material.

Score	Description	Classification
0	No colonies	Resistant
1	Single colony less than 1cm in diameter	
	Colony located near an inoculation site	
	Colony does not persist beyond the end of the growing season	
2	Two to three colonies 1 cm or more in diameter	Intermediate
	Colonies located around inoculation sites	
	Colonies do not persist beyond the end of the growing season	
3	Four or more small colonies less than 1 cm in diameter or two to three colonies greater than 1 cm in diameter	
	Colonies beginning to spread away from inoculation sites	
	Colonies persist into autumn	
4	Four or more large colonies greater than 1 cm in diameter	Susceptible
	Colonies may have begun to join up	
	Colonies well spread over the plant	
	Colonies persist into autumn	
5	Five or more large colonies greater than 1 cm in diameter	
	Many smaller colonies	
	Colonies have often begun to join	
	There are few parts of the plant without aphids	
	Colonies persist into autumn	

3.2. Husbandry

Inoculated trees were kept in glasshouse and/or polytunnel conditions and watered daily. Limited plant protection products were applied, in order to prevent damage to developing WAA colonies. No synthetic insecticides were used, but ladybird larvae were used to predate infestations of green apple aphid. Across the duration of the project some applications of synthetic fungicide were required to control powdery mildew.

3.3. Screening Malus accessions for woolly apple aphid resistance

3.3.1. Plant material

A range of apple accessions were selected to be screened for WAA resistance, based on their potential for use as WAA-resistant crab apple pollinisers. Known susceptible accessions were included, along with sources of resistance genes, to act as positive and negative controls,

respectively. A total of 59 genotypes were selected, of which 41 remained healthy enough for analysis, detailed in Table 2. Graft wood was collected in late February from Frank P. Matthews nurseries and at NIAB East Malling, and grafted onto M.9 rootstocks.

3.3.2. Statistical analysis

The highest scores for each individual across all scoring events were analysed with a Chi-square test using R Studio with R version 4.2.1. (RStudio Team, 2022).

Table 2- Apple accessions screened for woolly apple aphid susceptibilitity. The number of initial and successful screening repeats is given, as some grafts were unsuccessful. Details of parentage and resistance to WAA are given, where known, along with where the graftwood was sourced from. The second portion of the table, below, includes details of accessions selected for screening but which had no successful grafts. EMLA denotes a virus free rootstock clone developed at East Malling and Long Ashton Research Stations. EM germplasm accession denotes material collected from a gene bank at NIAB East Malling Research Station. Crab apple species endemic to, and accessions bred in, North America are indicated in the parentage column.

Accessions successfully phenotyped						
Variety	***n* grafted**	***n* phenotyped**	**Type**	**Parentage**	**Source**	**Resistance status**
Alnarp 2	3	2	Rootstock	Selected from mixed dwarf trees	NIAB East Malling	Susceptible
Geneva 11	3	2	Rootstock	M.26 × 'Robusta 5' **Bred in North America**	NIAB East Malling	Moderately resistant; R gene known
Geneva 202	3	2	Rootstock	M.27 × 'Robusta 5' **Bred in North America**	NIAB East Malling	Resistant; R gene known
Malus × *atrosanguinea* 'Gorgeous'	3	3	Commercial crab apple		F. P. Matthews	Unknown
Hashabi MH10.1	3	3	Rootstock		NIAB East Malling	Unknown
'Indian Magic'	3	3	Commercial crab apple	**Bred in North America**	F. P. Matthews	Unknown
'Louisa'	3	1	Commercial crab apple	**Bred in North America**	F. P. Matthews	Unknown
M. baccata	1	1	EM germplasm accession	Wild type	NIAB East Malling	Unknown; parent of *M. robusta*
M. baccata flexilis	2	2	EM germplasm accession		NIAB East Malling	Unknown; parent of *M. robusta*

M. 'Baskatong'	2	2	Commercial crab apple	*M.* 'Simcoe' × *M.* 'Meach' **Bred in North America**	F. P. Matthews	Unknown
M. coronaria 'Elk River'	3	3	Commercial crab apple	Wild type **Native to North America**	F. P. Matthews	Unknown
M. florentina	3	2	EM germplasm accession	Wild type	NIAB East Malling	Unknown
M. floribunda	6	6	EM germplasm accession	Wild type, likely *M. toringo* × *M. baccata* hybrid	NIAB East Malling	Resistant; R gene unknown
M. floribunda J	2	1	EM germplasm accession	Wild type, likely *M. toringo* × *M. baccata* hybrid	NIAB East Malling	Resistant; R gene unknown
M. fusca M	3	3	EM germplasm accession	**Native to North America**	NIAB East Malling	Low to zero colonisation reported (Cummins *et al*., 1981)
M. halliana	3	2	EM germplasm accession		NIAB East Malling	Low colonisation (Cummins *et al*., 1981)
M. hupehensis (EMLA)	3	3	EM germplasm accession	Wild type	NIAB East Malling	Resistant; R gene unknown
M. kansuensis	3	1	Commercial crab apple	Wild type	F. P. Matthews	Susceptible
M. koreana	1	1	EM germplasm accession		NIAB East Malling	Resistant; R gene unknown
M. niedzwetzkyana	3	3	Commercial crab apple	Wild type	F. P. Matthews	Unknown
M. platycarpa (EMLA)	3	3	EM germplasm accession	*M. coronaria* × *M. domestica* **Native to North America**	NIAB East Malling	Susceptible

M. praecox	3	3	EM germplasm accession	Wild type	NIAB East Malling	Unknown
M. pumilla 7728	3	3	Germplasm accession		F. P. Matthews	Susceptible
M. robusta (EMLA)	3	1	EM germplasm accession	*M. baccata* × *M. prunifolia*	NIAB East Malling	Resistant; R gene known
M. rubra 'Evelyn'	3	3	Commercial crab apple	*M. ioensis* × *M. purpurea* **Native to North America**	NIAB East Malling	
M. transitoria	3	3	Germplasm accession	Wild type	F. P. Matthews	Unknown
M. tschonoskii	3	1	EM germplasm accession	Wild type	NIAB East Malling	Resistant; R gene unknown
M.9	6	6	Rootstock		NIAB East Malling	Susceptible
Mac 24	3	3	Rootstock		NIAB East Malling	Unknown
Mac 4	3	2	Rootstock		NIAB East Malling	Unknown
Mac 9	3	2	Rootstock		NIAB East Malling	Unknown
Malling Crab 'C'	3	3	EM germplasm accession		NIAB East Malling	Intermediate (Cummins *et al*., 1981)
Malus × *magdeburgensis*	3	2	Commercial crab apple	*M. domestica* × *M. spectabilis*	F. P. Matthews	Resistant or tolerant
Malus × *robusta* 5a	3	2	EM germplasm accession		NIAB East Malling	Resistant; R gene known
Malus × *robusta* f. *erecta* (EMLA)	1	1	EM germplasm accession		NIAB East Malling	Resistant; R gene known

Malus × *robusta* 'Red Sentinel'	1	1	Commercial crab apple		F. P. Matthews	Resistant; R gene known
Malus × *zumi* 'calocarpa'	3	2	EM germplasm accession		NIAB East Malling	Unknown
Mokum	2	1	Commercial crab apple	'Profusion' × 'Liset'	F. P. Matthews	Unknown
Northern Spy	6	6	Scion variety	Unknown **Bred in North America**	NIAB East Malling	Resistant; R gene known
Novole	3	3	Rootstock		NIAB East Malling	Unknown
Polish 22	3	1	Rootstock	M.9 × 'Common Antonovka'	NIAB East Malling	Susceptible
Scarlet Sentinel	3	3	Commercial crab apple		F. P. Matthews	Unknown
White Angel	3	3	Commercial crab apple	**Bred in North America**	F. P. Matthews	Unknown
White Star	3	3	Commercial crab apple		F. P. Matthews	Unknown

Accessions selected but not phenotyped

Variety	*n* grafted	Parentage	Type
Hashabi MH14.5	3		Rootstock
M778	3		Rootstock
M789	3		Rootstock
M793	3		Rootstock
'Admiration'	3	OP seedling of *M. halliana* 'Koehne' **Bred in North America**	Commercial crab apple
M. baccata 'Gracilis'	3		Commercial crab apple

M. brevipes	3		Commercial crab apple
M. denticulata	3		Crab apple
M. floribunda (EMLA)	3	Wild type, likely *M. toringo* × *M. baccata* hybrid	EM germplasm accession
M. baccata mandschurica	3	Wild type	Crab apple
'Pink Pearl'	3	*M. niedzwetskyana* ancestry **Bred in North America**	Scion variety
M. spectabilis 'Riversii'	3	Wild type	Crab apple
Malus × *robusta* 'Persicifolia'	3		EM germplasm accession
M. toringoides 'Mandarin'	3	Clone of *M. bhutanica*	Commercial crab apple
Malus × *zumi*	3	*M. mandschurica* × *M. sieboldii*	

3.4. Woolly apple aphid resistance gene mapping

3.4.1. Preparation of plant material

MCM007 family for* Er1 *mapping

Second year seedling graftwood was collected in January and February 2020 with three repeats collected for each seedling and grafted onto M.9 rootstocks. The grafts were kept under polytunnel conditions for three months to ensure successful grafting.

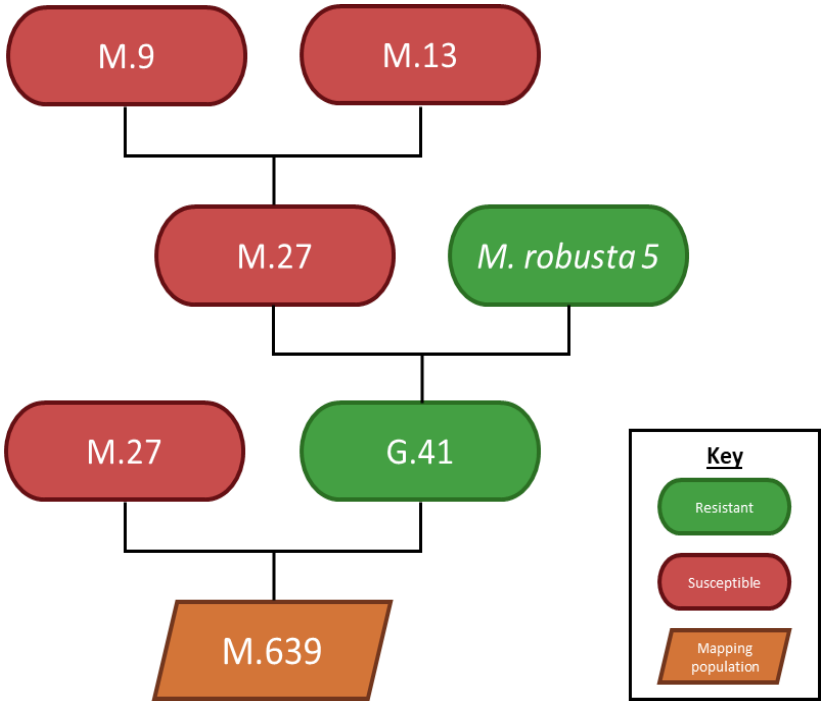

Figure 2 - Breeding pedigree for the experimental *Er2* mapping population, M.639.

M.639 family for* Er2 *mapping

The M.639 family was generated by a controlled full emasculation cross of M.27 × G.41 in the spring of 2020. Mature seeds were extracted from fruit manually, sown, stratified in coldstore conditions and moved into polytunnel conditions in the spring of 2021. Graftwood was collected from these seedlings in February 2022 with four repeats of each seedling taken, where possible, and grafted onto M.9 rootstocks. Trees were transferred to polytunnel conditions in April 2022 for three months for the scion to establish.

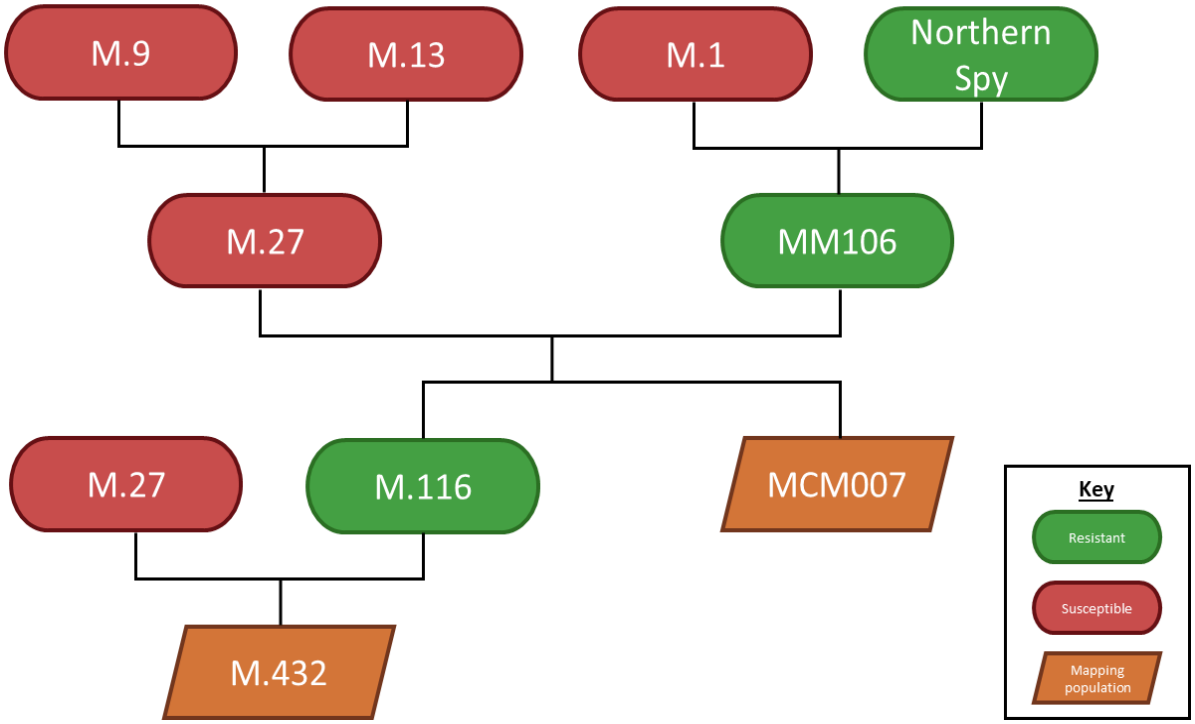

Figure 3- Pedigree of *Er1* lines and experimental mapping populations.

3.4.2. Phenotyping for woolly apple aphid susceptibility

Phenotyping was carried out as in section 3.1.

Details of phenotyping for individual families

M.639 seedlings were phenotyped between August and October 2021. Grafted trees were phenotyped between July and October 2022.

First year MCM007 grafted trees were phenotyped between July and October of 2020, under glasshouse conditions. Second year grafted trees were kept under polytunnel conditions and scored for WAA infestation once per month between June and November of 2021, but not inoculated with WAA at any point in that year. This was to ensure that resistance-breaking WAA which had been observed on site at NIAB East Malling were not widespread before continuing phenotyping. These trees were properly inoculated and scored between May and June of 2022.

3.4.3. DNA extraction and preparation for sequencing

Collection of leaf material

Two leaf discs ca. 1 cm in diameter were taken from the youngest available healthy leaves of each seedling of the breeding families, parents and grandparents, and dried with silica gel.

DNA extraction

Dried leaf discs were homogenised to a fine dust and total genomic DNA (gDNA) extracted following the protocol described by Edge-Garza *et al.* (2014).

Mapping population identification

Extracted gDNA samples were screened to identify and remove out-crosses by Polymerase Chain Reaction (PCR) amplification using eight multiplexed microsatellite markers (Table 2). PCR success was determined using gel electrophoresis, and fragment size of PCR products were analysed by ABI Analyzer.

Table 3- Details of microsatellite primers used in multiplex to identify progeny of a successful cross in *Malus* spp.

Name	**Primer sequence (5'-3')**	**Size range (bp)**	**T_a (°C)**
CH04c07	F: GGCCTTCCATGTCTCAGAAG R: CCTCATGCCCTCCACTAACA	94-149	60
CH01h10	F: TGCAAAGATAGGTAGATATATGCCA R: AGGAGGGATTGTTTGTGCAC	84-137	60
CH01h01	F: GAAAGACTTGCAGTGGGAGC R: GGAGTGGGTTTGAGAAGGTT	86-143	58
Hi02c07	F: AGAGCTACGGGGATCCAAAT R: GTTTAAGCATCCCGATTGAAAGG	106-152	60
Ch04e05	F: AGGCTAACAGAAATGTGGTTTG R: ATGGCTCCTATTGCCATCAT	152-246	60
CH02d08	F: TCCAAAATGGCGTACCTCTC R: GCAGACACTCACTCACTATCTCTC	154-258	60
CH02c11	F: TGAAGGCAATCACTCTGTGC R: TTCCGAGAATCCTCTTCGAC	198-259	60
Ch02C09	F: TTATGTACCAACTTTGCTAACCTC R: AGAAGCAGCAGAGGAGGATG	224-264	60

Samples were removed from the data set if they differed from the parents at more than one locus, as this indicates the seedling is likely an outcross with an unknown pollen doner. The quality and quantity of the extracted gDNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and samples were rejected if the concentration and quality of DNA was below set thresholds. The ninety-two samples with the highest quality and concentration of DNA, and which had clearly resistant or susceptible phenotyping scores were selected to proceed.

Preparation for Genotyping-by-Sequencing

Extracted gDNA was diluted to a 10 ng/µl concentration and prepared into libraries according to Elshire *et al*. (2011) with ApeKI restriction enzyme. Library quality and concentration was assessed on a TapeStation 4200 (Agilent, USA) and a Qubit 2.0 Fluorometer (Invitrogen) before shipping samples to Novogene for genotyping-by-sequencing (GBS).

3.4.4. Data analysis and linkage mapping

This work is still underway but will involve cleaning and filtering the data, before identifying variations in SNPs and where on the genome they lie. This will allow us to identify variations between resistant and susceptible accessions and therefore markers close to our target genes.

3.5. Woolly apple aphid population genetics

3.5.1. Sample collection

Mixed age aphids were collected from a single, distinct colony by brushing aphids from plant material with a soft paintbrush into Eppendorf tubes filled with silica gel to dry for at least 72 hours. Excess wax was removed from samples before collection by gentle brushing.

3.5.2. gDNA extraction and product amplification

DNA extraction

Total genomic DNA was extracted from mixed age samples collected above by homogenising dried aphids to a fine powder and extracting gDNA using the Qiagen DNeasy Blood and Tissue kit using the supplementary protocol for purification of total DNA from insects.

Genotyping with microsatellite markers

Extracted DNA was amplified by PCR using the Qiagen Type-it Microsatellite PCR Kit with markers from Lavandero *et al.* (2009b) in two multiplexes (Table 5). PCR products were prepared for fragment size analysis using ABI PRISM® sequence analysis and the resulting peaks were classified using GeneScan® and Genotyper® Analysis Software (Applied Biosystems Inc).

Table 4- Microsatellite markers used in two multiplexes to analyse genetic diversity of woolly apple aphid samples. Markers originally published in (Lavandero *et al*., 2009).

Multiplex	**Name**	**Primer sequences (5'-3')**	**Size range (bp)**	**T_a (°C)**
A	*Erio3*	F: GCCAAACAGTCTTATCTTTCC	147-163	60
		R: GAATTCGCTGGCTCTCTCTCT		
	Erio33	F: TCAATGGCAACCGAAGTGTA	159-183	60
		R: GCAACAGTGGCGTCATCC		
	Erio72	F: GCTGTAGCGGGCGTAATAAT	148-170	60
		R: AACCTTAACCGCCCCTCTAA		
	Erio75	F: ACGGAGATGAAGGCGTTATG	134-166	60
		R: TCTCTCCGTCTTTCCGTCTC		
B	*Erio20*	F: CGACCTTGAGCCTTTGAAAC	161-179	59
		R: CTGGCTCACTTCCTGGTAGC		
	Erio25	F: TTGTCACGAACATAAACGTA	100-106	50
		R: GTACATATTACAACAACAAC		
	Erio29	F: TACTCATCGCGAAAACGAGA	171-189	60
		R: AGTCTCGTCCGATGTTGTTG		
	Erio78	F: AAGTTTAATGGCGTGGGCTA	143-175	60
		R: GGGATGGTAAACGAGTGTGTG		

Table 5- Subsection of WAA samples chosen for genotyping using a GBS approach. Sample codes and approximate latitude and longitude of sampling locations are given in Table A. Samples with codes EMR M.9, EMR MM106, and EMR M.116 were collected from the same WAA culture at NIAB East Malling and were feeding on those respective potted rootstocks. Table B gives the 96-well plate of samples sent for GBS, with repeated samples indicated with and a or b.

A.

Code	Sampling location and approximate co-ordinates	Code	Sampling location and approximate co-ordinates
GNY	Geneva, New York, USA (42.903, -77.029)	WOT	Walton-on-Thames, Surrey, England (51.386, -0.431)
SNY	Sodus, New York, USA (43.210, -77.016)	WFG	Woodford Green, London, England (51.600, 0.055)
TLC	Talca, Maule Region, Chile (-35.418, -71.664)	WSB	Whitstable, Kent, England (51.357, 1.019)
SOC	San Fernando, O'Higgins Region, Chile (-34.585,-70.565)	PRS	Pagehurst Road, Staplehurst, Kent, England (51.161, 0.519)
MMC	Molina, Maule Region, Chile (-35.174,-71.189)	HPW	Honoton Farm, Paddock Wood, Kent, England (51.146, 0.413)
GMC	Guaico, Romeral, Maule Region, Chile (-35.003,-71.034)	CHF	Clockhouse Farm, Penshurst, Kent, England (51.227, 0.498)
HVN	Plant & Food Research, Havelock North, New Zealand (-39.654, 176.876)	NFC	Ambient polytunnel, National Fruit Collection, Brogdale, Kent, England (51.296, 0.883)
FAN	Floriade Expo, Almere, Netherlands (52.355, 5.227)	NFC +2	Ambient + 2°C polytunnel, National Fruit Collection, Brogdale, Kent, England (51.296, 0.882)
NIL	Loughall, County Armagh, Northern Ireland (54.410,-6.603)	NFC +4	Ambient + 4°C polytunnel, National Fruit Collection, Brogdale, Kent, England (51.296, 0.882)
LSF	Lincolnshire, England (52.941, -0.255)	MST	Loose, Maidstone, Kent, England (51.250, 0.531)
WMC	Alan Hudson Ltd, Wisbech St Mary, Cambridgeshire, England (52.641, 0.117)	WMK	West Malling, Kent, England (51.296, 0.403)
LBH	Ledbury, Herefordshire, England (52.037, -2.457)	EMS	Railway Station, East Malling, Kent, England (51.285, 0.442)
EVW	Evesham, Worcestershire, England (52.134, -1.934)	OGB	Apple gene bank, NIAB, East Malling, Kent, England (51.288, 0.442)
AIH	Aston Ingham, Herefordshire, England (51.923, -2.462)	WSM	Wiseman orchard, NIAB, East Malling, Kent, England (51.287, 0.466)
LIH	Linton, Herefordshire, England (51.926, -2.467)	GHJ	NIAB glasshouse, East Malling, Kent, England (51.285, 0.450)
PSH	Peterstow, Herefordshire, England (51.919, -2.656)	EMR M.9	WAA culture on M.9 rootstock, NIAB, East Malling, Kent (51.286, 0.453)
MSM	Minehead, Somerset, England (51.202, -3.480)	EMR MM106	WAA culture on MM106 rootstock, NIAB, East Malling, Kent (51.286, 0.453)
LFS	Lydford-on-Fosse, Somerset, England (51.084, -2.633)	EMR M.116	WAA culture on M.116 rootstock, NIAB, East Malling, Kent (51.286, 0.453)
TSS	Thatchers Cider, Sandford, Somerset, England (51.320, -2.845)		

B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	GNY-1a	TLC-1	GMC-1	NIL-4	LBH-2	PSH-2	WOT-3b	HPW-1	NFC+2-66a	MST-6	WSM-3	MM106-9b
B	GNY-1b	TLC-2	GMC-2	NIL-10	EVW-1	MSM-1	WOT-4	CHF-1	NFC+2-66b	WMK-2	WSM-4	MM106-10
C	GNY-2	TLC-3	HVN-1	LSF-1	EVW-2	MSM-5	WFG-2	NFC-76	NFC+2-75	WMK-5a	GHJ-7	MM106-11a
D	GNY-6	TLC-5	HVN-7	LSF-2	AIH-1	LFS-2	WFG-3	NFC-77	NFC+4-15	WMK-5b	GHJ-8	MM106-11b
E	SNY-1a	SOC-2	HVN-8	WMC-2a	AIH-2	LFS-6	WSB-1	NFC-81	NFC+4-26	EMS-2	M.9-2a	M.116-12a
F	SNY-1b	SOC-3	FAN-1a	WMC-2b	LIH-1	TSS-1	WSB-3	NFC-84	NFC+4-28	EMS-3	M.9-2b	M.116-12b
G	SNY-2	MMC-1	FAN-1b	WMC-8	LIH-2	TSS-2	PRS-5	NFC+2-43	NFC+4-33	OGB-2	MM106-8	M.116-13a
H	SNY-3	MMC-2	NIL-2	LBH-1	PSH-1	WOT-3a	PRS-9	NFC+2-61	MST-1	OGB-7	MM106-9a	M.116-13b

Genotyping-by-sequencing

A sub-section of the above samples was selected for more detailed analysis using a genotyping-by-sequencing (GBS) approach to generate large numbers of single nucleotide polymorphism (SNP) markers saturated across the genome. This approach will allow smaller variations between samples to be detected across the genome, especially compared to the microsatellite approach above which can only identify variation at eight loci across the genome. 37 samples were selected from the larger dataset for GBS analysis (Table 5). Repeats were included for samples of special interest.

Extracted gDNA was diluted to a 10 ng/µl concentration and prepared into a single library according to Elshire *et al.* (2011) with ApeKI restriction enzyme. Library quality and concentration was assessed on a TapeStation 4200 (Agilent, USA) and a Qubit 2.0 Fluorometer (Invitrogen) before shipping samples to Novogene for genotyping-by-sequencing (GBS).

3.5.3. Data analysis

Population assignment

Population structure was inferred using the software STRUCTURE version 2.3.4 (Pritchard *et al*., 2000; Falush *et al*., 2003, 2007; Hubisz *et al*., 2009). An assumed number of genetic populations, K, was selected based on the number of sample collection sites. For each value of K, six independent runs of the STRUCTURE algorithm were carried out with a burn-in period of 20,000 and 50,000 Markov Chain Monte Carlo (MCMC) repetitions and assuming population admixture. The data generated were further analysed by STRUCTURE HARVESTER to generate mean likelihood values for each value of K value that was tested using the Evanno *et al.* (2005) method.

Generation of summary population statistics

The following population statistics were generated using GenAlEx (Peakall & Smouse, 2006, 2012): the observed number of alleles (Na); the effective number of alleles (Ne); the observed heterozygosity (Ho); the effective heterozygosity (He); unbiased expected heterozygosity (uHe); and the fixation index (F). GenAlEx was also used to calculate pairwise FST and private allele summaries.

3.6. Effects of host rootstock on woolly apple aphid growth

3.6.1. Plant material

Three rootstock accessions were used: M.9, M.116 (MM106 × M.27), and MM106 (Northern Spy × M.1). Both M.116 and MM106 are known to carry the Northern Spy-derived WAA resistance gene *Er1* and M.9 is known to be susceptible to WAA feeding.

Bare-rooted rootstocks were potted into 0.5 L pots and kept in controlled long day conditions (16:8 L:D, c. 20°C).

3.6.2. Aphid material

The aphids used were all taken from a clonal culture kept at NIAB East Malling and reared on potted M.9 rootstocks. The aphids used were derived from a starting population which were the same age (to a 24-hour period), to reduce the potential effects of aphid age on growth.

3.6.3. Intrinsic rate of increase

Individual adult aphids were transferred to an M.9 rootstock leaf cutting placed in wet oasis and isolated inside two clear plastic pots for 24 hours to produce nymphs, after which the adult and all but one nymph removed. This single nymph was reared to adulthood and the number of days from birth until it reached reproductive maturity and produced its first nymph, *d*, was recorded. The number of nymphs produced in the *d* number of days following this was recorded and used to calculate the Intrinsic Rate of Natural Increase (r_m) using the formula from Wyatt & White (1977):

$r_m = 0.738((\ln M_d)/d)$

Where *d* = pre-reproductive time (days) from birth to first nymph produced, M_d = number of progeny produced in the time period after first reproduction, of length *d*. The constant 0.738 is the approximate proportion of a female's total offspring, produced in the first few days of reproduction, in this case *d* = 7 (Castle *et al.*, 1998). This was successfully repeated for five individuals.

3.6.4. Mean relative growth rate

Weight gain of an individual aphid over a fixed time can suggest the impact of feeding conditions on aphid growth.

1. As above for intrinsic rate of natural increase, a single adult aphid isolated on a rootstock leaf cutting of M.9, M.116, or MM106 in an enclosure to compare the effects of feeding on these different rootstocks on mean relative growth rate (MRGR).
2. After 24 hours the adult and all but one nymph were removed.
3. Nymphs were weighed as a group to give a mean starting weight.
4. The isolated nymph was left to feed on the cutting for a set period of three or seven days.
5. At the end of this period the aphid was removed from the cutting, its wax removed with a damp paintbrush, and the aphid weighed to give the end weight.
6. This was repeated for 20 individuals on M.9 rootstock cuttings, 16 on M.116, and 11 on MM106.

Mean relative growth rate (MRGR) was calculated using the following formula (Radford, 1967):

MRGR ($\mu g/\mu g$/day) = $(logW_2 - logW_1)/t_2 - t_1$

Where W_1 = first weight, W_2 = final weight (without wax), $t_2 - t_1$ = the time in days between weighing events.

MRGR = ln(W1) – ln(W0)/d

Where W1 = adult weight; W0 = birth weight; d = developmental time from nymph to reproductive maturity (Castle & Berger, 1993).

3.6.5. Aphid mortality

The percentage of WAA nymphs which were still alive after n days was recorded for both r_m and MRGR for a total of thirty-four individuals.

3.6.6. Wax weight

Individual WAA nymphs were removed from the plant gently using a damp paintbrush after n days of feeding and weighed using the Cahn 29 microbalance. Care was taken to weigh all wax produced by the nymph in that time. After weighing all wax was carefully removed using a damp paintbrush and the aphid weighed again. The approximate weight of the wax was estimated by subtracting the weight of the aphid with wax removed from the weight of the aphid and wax combined.

3.6.7. Statistical analysis

Statistical analysis was carried out using R version 4.2.2 (R Core Team, 2022). A two-way ANOVA (analysis of variance) was used to analyse the effects of rootstock and days after inoculation on percentage nymph survival. A one-way ANOVA were carried out for both the effects of rootstock on MRGR and wax weight.

4. Results

4.1. Susceptibility screening of Malus accessions

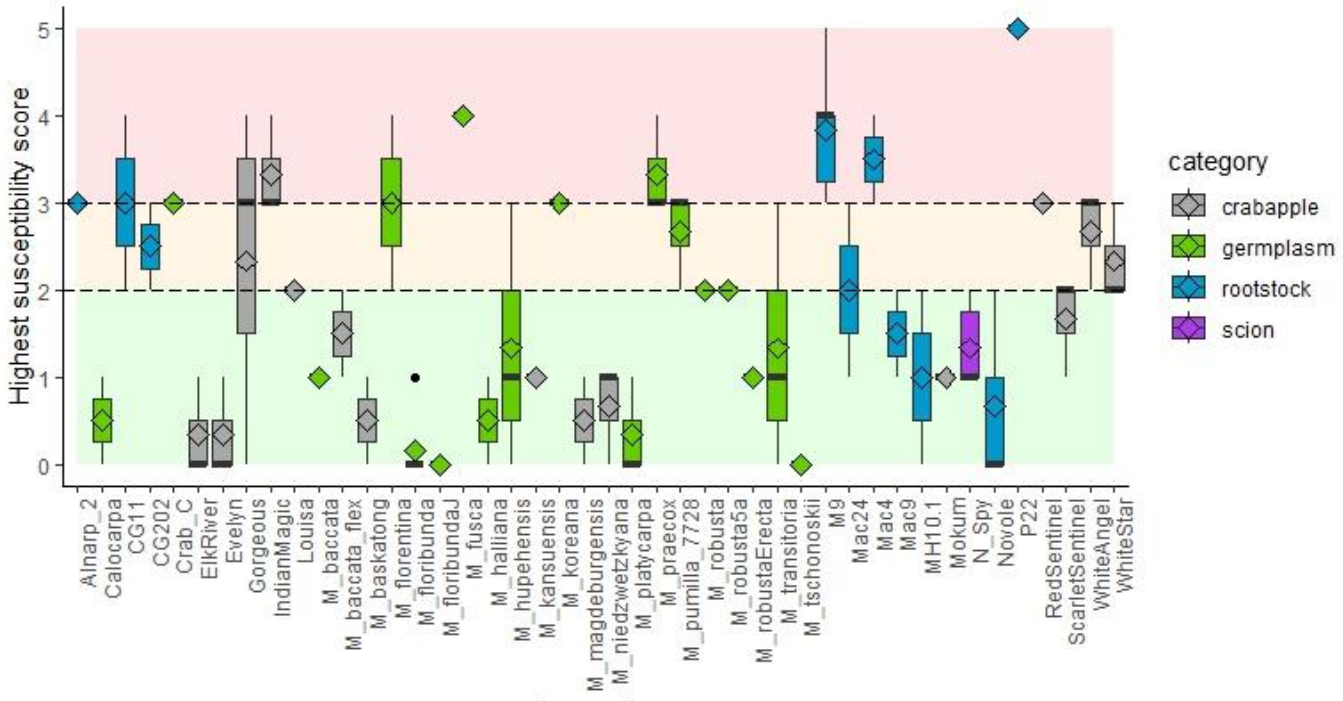

Figure 4- Boxplot of highest recorded susceptibility score across all scoring events pre- and post inoculation(s). The mean highest score is indicated by diamonds. Arbitrary category of susceptibility is indicated by red, orange, and green areas, representing susceptible, intermediate, and resistant categorisation, respectively.

The highest susceptibility score by individual across all scoring events presents a worst-case scenario of the susceptibility of each apple accession, and is presented as a boxplot of highest score by accession in Figure 1. The difference in the highscore between accessions was significant ($p < 0.01$) and the difference between categories was close tosignificance ($p = 0.058$).

The negative control, M.9, showed a mean highscore of 3.83 ($n = 6$), categorising it as “susceptible” within this analysis..

Of the known sources of WAA resistance genes included, ‘Northern Spy’ had a mean highest score of 1.3 ($n = 6$). The mean highest scores of *M. robusta* varieties were: *M. robusta*: 2 ($n = 1$), *M. robusta* 5a: 2 ($n = 2$), and *M. robusta erecta*: 1 ($n = 1$).

Table 6- Accessions which had a low median susceptibility score and suggested categories for those resistances.

Immune (susceptibility = 0)	**Resistant (susceptibility ≤ 1)**	**Tolerant (susceptibility ≤ 2)**
M. coronaria 'Elk River'	Hashabi MH10.1	'Louisa'
M. floribunda	*M. baccata*	*M. baccata flexilis*
M. floribunda J	*M. baskatong*	*M. robusta* (EMLA)
M. platycarpa	*M. halliana*	*Malus* × *robusta* 5a
M. rubra 'Evelyn'	*M. hupehensis* (EMLA)	Mac 24
M. tschonoskii	*M. kansuensis*	Mac 9
'Novole'	*M.* × *magdeburgensis*	*Malus* × *robusta* 'Red Sentinel'
	M. niedzwetzkyana	'White Star'
	M. × *robusta* f. *erecta* (EMLA)	
	M. transitoria	
	M. × *zumi* 'calocarpa'	
	Mokum	
	'Northern Spy'	

4.2. Resistance gene mapping

The phenotyping portions of this work has been completed and samples processed using Genotyping-by-Sequencing to generate SNP markers. We are still in the process of identifying variations between markers to create linkage maps for the MCM007 and M.639 families, and identifying flanking markers for *Er1* and *Er2*.

4.3. Woolly apple aphid population genetics

4.3.1. STRUCTURE analysis

STRUCTURE HARVESTER found the most likely number of populations (K) from those tested, to be two (Figure 5). The smaller peaks in ΔK at K = 8, K = 24, and K = 26 suggests the presence of sub-structuring within the populations, with the most likely number of subpopulations being 26.

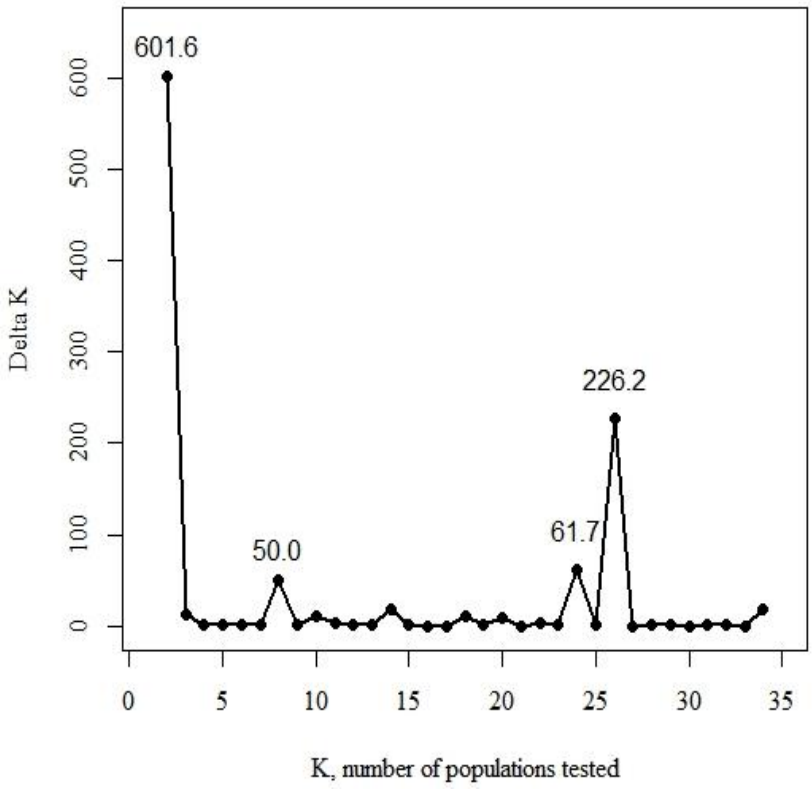

Figure 5- Likelihood (delta K), of the number of genetic populations (K) being present within the woolly apple aphid samples analysed with SSR markers.

The output of STRUCTURE analysis for K=2 (probability that there are 2 populations present) shows that most sampling locations contained a mixture of the two putative populations, although some sampling sites are comprised of a single population. The outputs for K = 8, 14 and 26 show much more complex population assignment, with many individual samples assigned to multiple populations. Most sampling locations showed some samples which were assigned to a single population. Across all four values of K, the following sampling locations showed a single population assignment for all samples from that site: HVN, FAN, NIL, LFS, TSS, WFG, WSB, EMS (Figure 6).

4.3.2. F statistic

Pairwise FST values calculated with GenAlEx ranged from 0.000 to 0.310 with a mean of 0.227 (Table 7). Of 745 FST outputs, 134 were below 0.1 and five were greater than 0.5.

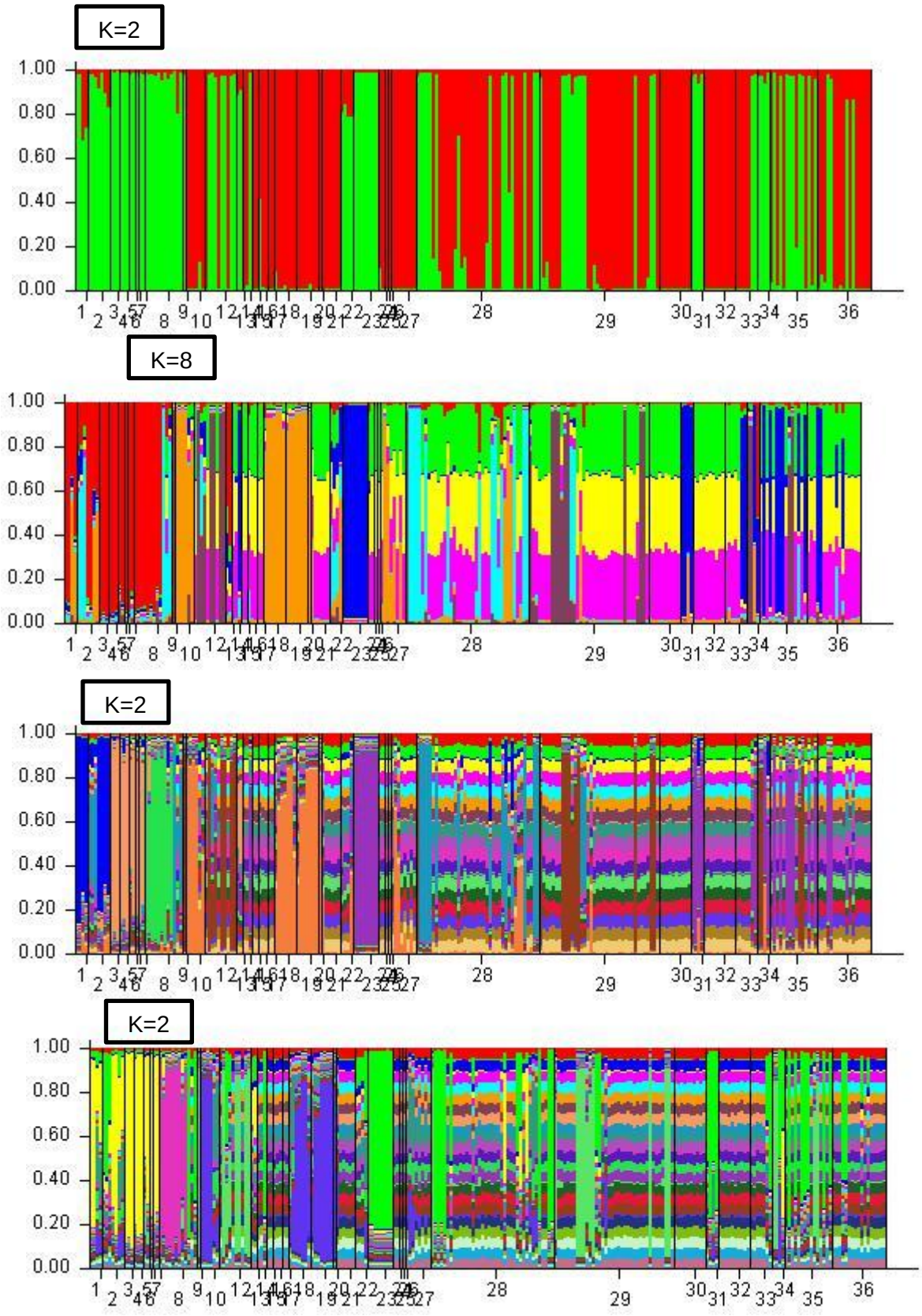

Figure 6 - Population assignment graphs generated by STRUCTURE for putative populations (K) of 2, 8, 24 and 26. Each vertical line represents a single sample, with sampling location indicated on the x axis. The y axis gives the probability of a sample being assigned to a population, with each population tested indicated in a different colour.

Table 7-Matrix of pairwise FST values for all sampling locations. Values with a low FST below 0.1 are indicated by *. Values with a high FST value above 0.5 are indicated by **.

	GNY	SNY	TLC	SOC	RMC	MMC	GMC	HVN	FAN	NIL	WMC	LBH	EVW	AIH	LIH	PSH	MSM	LFS	TSS	WFG	WOT	PRS	WSB	HPW	CHF	NFC	NFC + 2	NFC + 4	MST	WMK	EMS	OGB	WSM	GHJ	EMR
GNY	-																																		
SNY	0.07*	-																																	
TLC	0.26	0.18	-																																
SOC	0.22	0.15	0.20	-																															
RMC	0.42	0.34	0.42	0.25	-																														
MMC	0.27	0.19	0.19	0.14	0.34	-																													
GMC	0.22	0.18	0.25	0.05*	0.29	0.20	-																												
HVN	0.27	0.21	0.31	0.18	0.36	0.21	0.19	-																											
FAN	0.39	0.32	0.45	0.36	0.46	0.33	0.36	0.27	-																										
NIL	0.26	0.18	0.36	0.32	0.46	0.33	0.34	0.33	0.38	-																									
WMC	0.24	0.20	0.32	0.24	0.37	0.24	0.23	0.18	0.07*	0.25	-																								
LBH	0.27	0.21	0.28	0.21	0.34	0.24	0.21	0.24	0.35	0.28	0.23	-																							
EVW	0.22	0.16	0.31	0.23	0.40	0.25	0.21	0.19	0.22	0.21	0.07*	0.17	-																						
AIH	0.26	0.20	0.37	0.27	0.46	0.30	0.24	0.22	0.30	0.24	0.11	0.23	0.06*	-																					
LIH	0.26	0.20	0.37	0.26	0.46	0.30	0.23	0.21	0.29	0.24	0.11	0.22	0.06*	0.01*	-																				
PSH	0.26	0.20	0.37	0.27	0.46	0.30	0.24	0.22	0.30	0.24	0.11	0.23	0.06*	0.00*	0.01*	-																			
MSM	0.28	0.21	0.38	0.34	0.48	0.35	0.37	0.34	0.41	0.14	0.29	0.29	0.25	0.27	0.27	0.27	-																		
LFS	0.33	0.25	0.44	0.41	0.56**	0.41	0.44	0.40	0.48	0.20	0.35	0.34	0.31	0.33	0.34	0.33	0.16	-																	
TSS	0.37	0.27	0.47	0.47	0.61**	0.45	0.50	0.46	0.53**	0.25	0.40	0.37	0.34	0.37	0.38	0.37	0.26	0.30	-																
WFG	0.23	0.18	0.34	0.25	0.43	0.28	0.22	0.20	0.27	0.21	0.11	0.20	0.06*	0.01*	0.02*	0.01*	0.24	0.30	0.35	-															
WOT	0.26	0.22	0.40	0.40	0.60**	0.42	0.40	0.39	0.39	0.25	0.22	0.39	0.20	0.26	0.27	0.26	0.31	0.41	0.39	0.24	-														
PRS	0.20	0.14	0.27	0.22	0.00	0.24	0.25	0.21	0.30	0.28	0.18	0.17	0.12	0.23	0.22	0.23	0.32	0.36	0.38	0.21	0.32	-													
WSB	0.23	0.18	0.34	0.24	0.43	0.27	0.21	0.20	0.28	0.22	0.12	0.20	0.07*	0.02*	0.03*	0.02*	0.24	0.30	0.34	0.01*	0.25	0.19	-												
HPW	0.21	0.17	0.34	0.24	0.42	0.27	0.21	0.20	0.30	0.22	0.14	0.19	0.09*	0.05*	0.06*	0.05*	0.24	0.29	0.34	0.03*	0.27	0.21	0.03*	-											
CHF	0.26	0.20	0.37	0.27	0.46	0.30	0.24	0.22	0.30	0.24	0.11	0.23	0.06*	0.00*	0.01*	0.00*	0.27	0.33	0.37	0.01*	0.26	0.23	0.02*	0.05*	-										
NFC	0.30	0.23	0.43	0.35	0.52**	0.37	0.34	0.32	0.37	0.21	0.21	0.29	0.15	0.15	0.16	0.15	0.25	0.32	0.35	0.14	0.26	0.29	0.15	0.17	0.15	-									
NFC + 2	0.14	0.09*	0.25	0.18	0.35	0.22	0.15	0.18	0.21	0.18	0.07*	0.17	0.04*	0.06*	0.06*	0.06*	0.21	0.27	0.30	0.05*	0.15	0.14	0.06*	0.06*	0.06*	0.14	-								
NFC + 4	0.19	0.14	0.28	0.19	0.35	0.21	0.17	0.15	0.17	0.20	0.06*	0.17	0.06*	0.05*	0.05*	0.05*	0.22	0.28	0.33	0.04*	0.21	0.16	0.04*	0.03*	0.05*	0.15	0.04*	-							
MST	0.26	0.20	0.37	0.27	0.46	0.30	0.24	0.22	0.30	0.24	0.11	0.23	0.06*	0.00*	0.01*	0.00*	0.27	0.33	0.37	0.01*	0.26	0.23	0.02*	0.05*	0.00*	0.15	0.06*	0.05*	-						
WMK	0.25	0.18	0.34	0.29	0.43	0.32	0.30	0.26	0.32	0.27	0.19	0.21	0.12	0.23	0.23	0.23	0.31	0.36	0.39	0.21	0.29	0.10	0.22	0.24	0.23	0.27	0.15	0.18	0.23	-					
EMS	0.26	0.20	0.37	0.27	0.45	0.29	0.24	0.22	0.29	0.24	0.11	0.22	0.06*	0.00*	0.01*	0.00*	0.27	0.33	0.37	0.01*	0.27	0.22	0.02*	0.05*	0.00*	0.15	0.06*	0.05*	0.00*	0.23	-				
OGB	0.27	0.23	0.38	0.29	0.49	0.32	0.28	0.25	0.32	0.31	0.18	0.25	0.15	0.14	0.13	0.14	0.34	0.39	0.42	0.12	0.32	0.22	0.12	0.12	0.14	0.25	0.12	0.11	0.14	0.27	0.14	-			
WSM	0.29	0.21	0.34	0.28	0.40	0.27	0.29	0.20	0.11	0.28	0.08*	0.24	0.13	0.20	0.21	0.20	0.32	0.37	0.40	0.19	0.29	0.16	0.20	0.22	0.20	0.28	0.14	0.13	0.20	0.14	0.20	0.24	-		
GHJ	0.22	0.15	0.28	0.21	0.37	0.23	0.21	0.19	0.20	0.22	0.07*	0.17	0.02*	0.10	0.11	0.10	0.26	0.32	0.35	0.10	0.22	0.09*	0.11	0.13	0.10	0.18	0.06*	0.08*	0.10	0.08*	0.10	0.18	0.10	-	
EMR	0.22	0.16	0.33	0.23	0.41	0.26	0.21	0.19	0.23	0.20	0.09*	0.18	0.02*	0.03*	0.04*	0.03*	0.23	0.29	0.33	0.03*	0.20	0.15	0.03*	0.05*	0.03*	0.12	0.04*	0.04*	0.03*	0.15	0.03*	0.13	0.14	0.05*	-

4.3.3. Summary of population genetic diversity statistics

The mean observed number of alleles (N_a) across all marker loci ranges from 1.75 to 7.75. The effective number of alleles (N_e) ranges from 1.33 to 2.76. The observed heterozygosity (H_o) ranges from 0.31 to 1.00. The effective heterozygosity (H_e) ranges from 0.20 to 0.62. Unbiased expected heterozygosity (uH_e) ranges from 0.27 to 0.88. The fixation index (F) ranges from 0.01 to -1.00. The value of H_e is lower than the value of H_o for every sampling location.

Table 8- Mean population genetic diversity statistics across the eight marker loci for each sampling location. Observed number of alleles (N_a); effective number of alleles (N_e); observed heterozygosity (H_o); effective heterozygosity (H_e); unbiased expected heterozygosity (uH_e); fixation index (F).

Sampling location	**N_a**	**N_e**	**H_o**	**H_e**	**uH_e**	**F**
GNY	2.25	2.16	0.77	0.49	0.56	-0.63
SNY	3.25	2.74	0.79	0.62	0.68	-0.28
TLC	1.88	1.78	0.67	0.41	0.51	-0.62
SOC	2.25	1.89	0.63	0.42	0.51	-0.47
RMC	1.38	1.33	0.31	0.20	0.27	-0.60
MMC	1.88	1.88	0.88	0.44	0.88	-1.00
GMC	2.00	1.87	0.69	0.39	0.52	-0.76
HVN	3.63	2.24	0.52	0.44	0.46	0.01
FAN	1.63	1.63	0.63	0.31	0.63	-1.00
NIL	2.25	2.01	0.73	0.49	0.53	-0.55
WMC	2.88	2.19	0.71	0.50	0.52	-0.41
LBH	2.00	1.90	0.88	0.47	0.63	-0.83
EVW	2.50	2.40	0.69	0.51	0.64	-0.39
AIH	1.75	1.75	0.75	0.38	0.50	-1.00
LIH	1.88	1.88	0.71	0.40	0.53	-0.83
PSH	1.75	1.75	0.75	0.38	0.50	-1.00
MSM	2.13	1.84	0.64	0.45	0.49	-0.40
LFS	1.75	1.54	0.55	0.35	0.38	-0.54
TSS	1.38	1.38	0.63	0.31	0.63	-1.00
WFG	2.00	1.81	0.78	0.42	0.46	-0.80
WTN	1.88	1.72	0.44	0.32	0.39	-0.30
PRS	2.00	2.00	1.00	0.50	0.53	-1.00
WSB	1.88	1.83	0.81	0.42	0.56	-0.90
HPW	1.88	1.88	0.88	0.44	0.88	-1.00

CHF	1.75	1.75	0.75	0.38	0.75	-1.00
BR0	1.75	1.63	0.59	0.37	0.40	-0.57
BR2	4.00	2.76	0.75	0.60	0.61	-0.23
BR4	3.63	2.38	0.78	0.55	0.56	-0.40
MST	1.75	1.75	0.75	0.38	0.39	-1.00
UWM	2.13	2.11	0.64	0.44	0.52	-0.52
EMS	1.75	1.75	0.74	0.37	0.39	-0.97
OGB	2.38	1.93	0.62	0.42	0.46	-0.47
WSM	2.88	2.59	0.59	0.47	0.54	-0.26
GHJ	3.13	2.59	0.70	0.55	0.57	-0.31
EMR	2.88	2.28	0.71	0.49	0.53	-0.27

4.3.4. Private allele summaries

Eleven private alleles were found at four of the 35 sampling locations and across four SSR loci. The frequency of private alleles found ranged from 0.059 to 0.857.

Table 9- positions in base pairs (bp) of private alleles identified with their respective loci and frequency of each private allele.

Sampling location	**Locus**	**Allele (bp)**	**Frequency**
MMC	Erio75	165	0.500
HVN	Erio33	164	0.857
BR2	Erio33	158	0.059
EMR M.9	Erio25	112	0.250
EMR M.9	Erio78	179	0.077

4.4. Effects of host rootstock on population growth

4.4.1. Aphid mortality

Nymph survival significantly decreased over time for all rootstocks ($p < 0.05$) but there was no significant effect of rootstock on percentage survival ($p = 0.0971$).

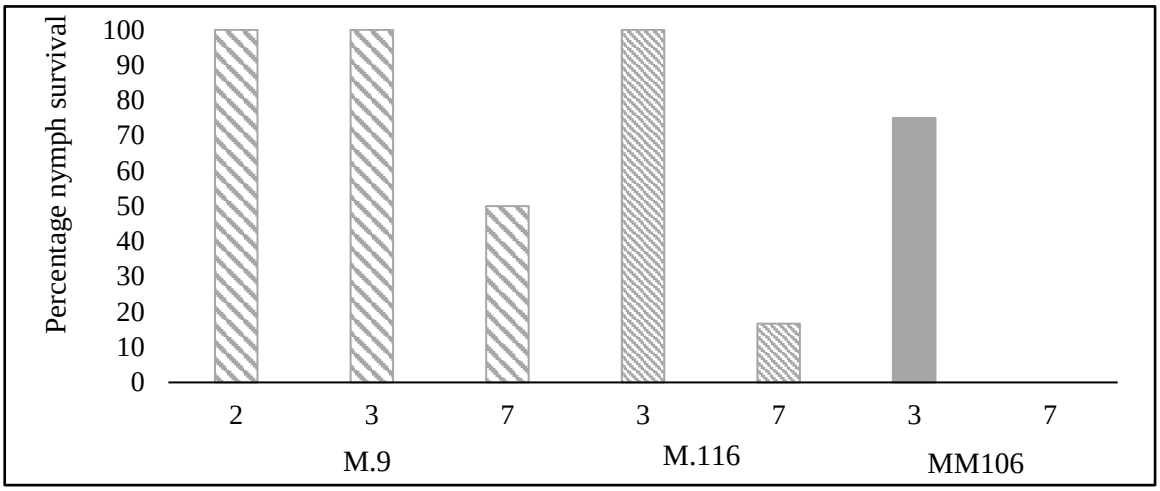

Figure 7- The percentage of nymphs surviving on rootstock cuttings after n days, of which M.9 is woolly apple aphid susceptible and M.116 and MM106 are WAA resistance.

4.4.2. Intrinsic rate of increase

Mean r_m of WAA feeding on M.9 rootstocks for seven days was 0.2966608 (s.d. = 0.07174681).

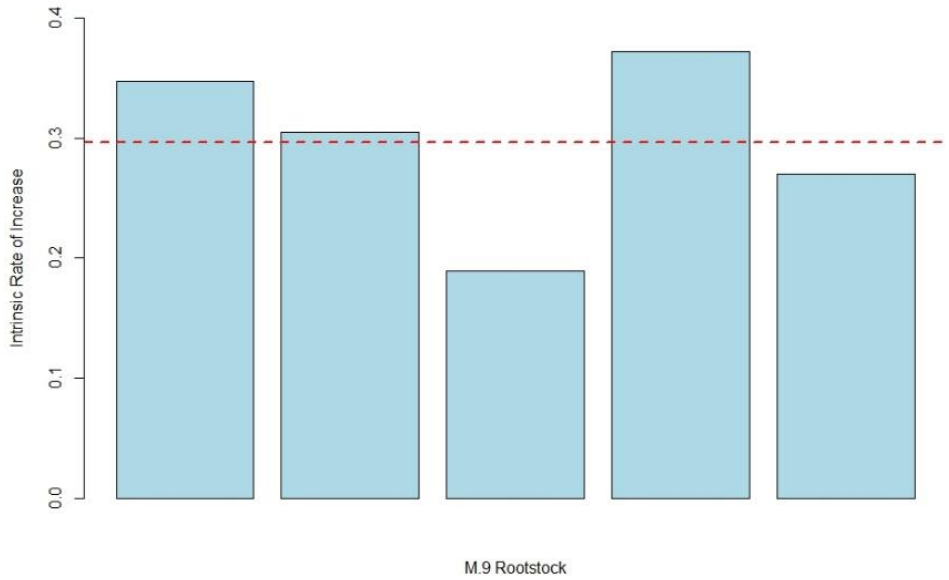

Figure 8- Intrinsic rate of increase of woolly apple aphids feeding on M.9 rootstocks. The mean value is indicated by the red dashed line.

4.4.3. Mean relative growth rate

Woolly apple aphid feeding on M.9 rootstocks showed the highest MRGR which was only slightly higher than that of WAA feeding on MM106. Both were higher than that for M.116, although this result was not significant (p = 0.463).

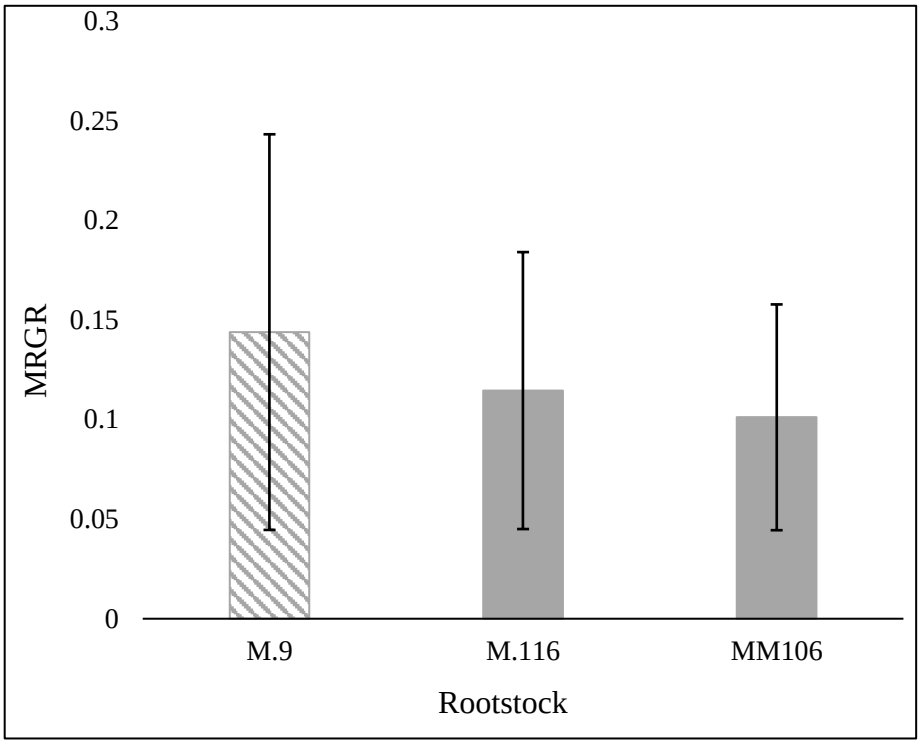

Figure 9- Mean Relative Growth Rate of woolly apple aphid feeding on rootstocks. M.9 is susceptible to WAA feeding whereas M.116 and MM106 are resistant.

4.4.4. **Wax weight**

No significant difference was found in wax weight between rootstocks ($p = 0.462$). The wax produced by WAA nymphs feeding on M.9 and M.116 rootstocks was similar but less wax was produced by those feeding on MM106.

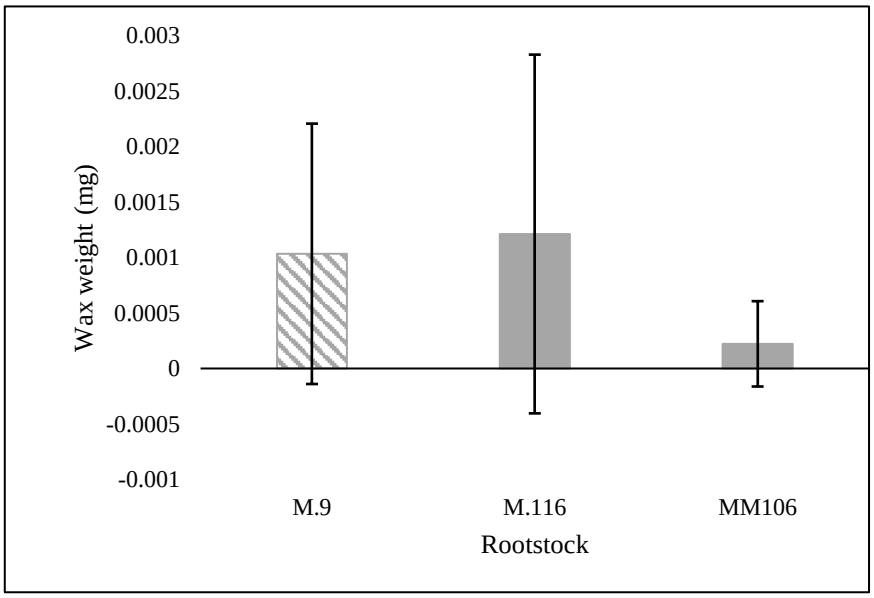

Figure 10- Mean weight of wax produced over time by individual woolly apple aphid nyphs while feeding on apple rootstocks, of which M.9 is susceptible and M.116 and MM106 are resistant.

5. Discussion

5.1. Susceptibility screening of Malus accessions

Of the 44 accessions screened, 23 were classified as “resistant”, based on the mean highest susceptibility score recorded, six as “susceptible”, and the remaining 15 as an intermediate classification.

Colonies on ‘Northern Spy’ persisted and increased slightly in susceptibility across the experiment. Colonies on *M. robusta* also persisted throughout, although on ‘Robusta 5a’, aphid colonisation decreased over time. This supports previous reports of a WAA strain which is able to overcome *Er1* resistance but not *Er2*. ‘Robusta 5’ is reported as being immune to WAA feeding, whereas ‘Northern Spy’ and its derivatives are more commonly reported as tolerant or resistant (Young *et al.*, 1982; Cummins & Aldwinckle, 1983; Bus *et al.*, 2008).

The scoring criteria used are similar to those used by Bus *et al.* (2008) when mapping *Er1-3*, who used colony and gall number and size over a period of months to assess susceptibility. Assessing

galling over time can give a good indication of the actual damage caused to the plant but in the short time frame used here, we could not have accurately assessed gall formation. The absence of visible galling is not necessarily indicative of no damage caused; internal galling is induced quickly and stunts growth through reduced water and photosynthate flow, and piercing wounds offer entry sites. The highest value across all scoring events was chosen to present a “worst case scenario” of susceptibility. Within commercial breeding programmes it is important to only invest resources in breeding populations which show clear resistance. By selecting the highest susceptibility score recorded, it allows the true extent of colonisation to be seen whereas using an average score or a final score would include instances where aphids had died because of external factors.

Host plant resistance does not necessarily prevent pest feeding but can merely reduce growth and reproduction, leading to eventual population decline. It may be possible for aphids to feed on resistant host plants for a short period of time.

Increased WAA resistance in crab apple species may be the result of increased sclerenchyma thickening in wild species, and that the process of domestication may have reduced sclerenchyma bundle thickness, allowing WAA colonisation.

Both accessions of *M. floribunda* (*M. floribunda* and *M. floribunda* J) showed strong resistance to WAA, in agreement with previous findings of reduced WAA settlement on *M. floribunda*, compared to commercial varieties, including ‘Royal Gala’ (Sandanayaka *et al*., 2005). *Malus floribunda* 821 is used in the rootstock breeding programme at NIAB East Malling (Fernández Fernández, 2020, pers. comm.) and accessions of *M. floribunda* could in future be used to identify and map a potential novel resistance gene. The self-incompatability locus of *M. floribunda* 821 is known (Verdoot *et al.*, 1998) which, if compatible with the crop variety, would make it an ideal candidate for a resistant polliniser.

This work shows the full range of susceptibility completed to the scoring criteria given in Table 1. Although these criteria and the inoculation protocol are defined, there is still a degree of subjectivity: the number of aphids used to inoculate with is hard to standardise, given that WAA are incredibly fragile, the temperature conditions across the study period varied considerably, as did the time period between first inoculation and final scoring.

Intermediate scores are likely to be the result of varying environmental conditions which benefitted aphid feeding on tolerant accessions or, more likely given the high temperatures and presence of natural enemies, colonies were unable to establish on otherwise susceptible accessions.

Standardisation of the time for full completion of the work would help to eliminate some variation but this was not possible because of a shortage of WAA for inoculation. A third inoculation event at the end of the season may help to clarify some intermediate genotypes.

5.2. Woolly apple aphid population genetics

5.2.1. STRUCTURE outputs

These population structure analyses suggest that WAA collected across England and from other apple growing regions form two broad genetic clusters with smaller sub-populations, as indicated by STRUCTURE outputs (Figure 2). Several clusters emerge when considering population sub-structuring at higher values of K.

Individual samples which were assigned to a single population may indicate the presence of clonal lines within the samples. Individuals assigned to a single population existed both in single-genotype sampling locations, and within otherwise mixed sampling locations. Sampling locations assigned to a single population were consistent across all values of K presented here, suggesting that samples collected from these sites (HVN, FAN, NIL, LFS, TSS, WFG, WSB, EMS) are exclusively asexual. Of the five other countries with samples analysed here, HVN, FAN and NIL consistently showed a single assigned population. Samples from Chile and the USA showed mixed populations at all values of K presented here and grouped together at all except K = 24 where they group separately.

Using ΔK to estimate the most likely number of populations is almost always accurate, except when there is small marker and/or population size, or partial sampling (Evanno *et al*., 2005). In this instance only eight microsatellite markers were used, spread across only three of the six WAA chromosomes. This, combined with the fact that many sampling locations had a small number of samples may mean that the estimated likely value of K = 2 is not accurate.

5.2.2. F statistic

Low F statistic values (> 0.1) are indicative of wild type sexual reproduction or recent divergence of populations (Latch *et al*., 2006). One hundred and twenty nine of the seven hundred and fourty five pairwise population tests from these samples were below this threshold suggesting that the samples collected at these locations are very similar and may either be part of the same genetic population or have only recently diverged.

Of 745, five had a pairwise FST > 0.5, suggesting that these populations were completely isolated from each other. Four of these five were between sampling location 5, in Chile, and locations in England, and the remaining one was between sites in the Netherlands and England.

5.2.3. Private alleles summaries

Private alleles are those found only in one population (of those analysed) (Neel, 1973) and can be indicative of an isolated population with heritable genes. One private allele was found from the National Fruit Collection, which is consistent with the high diversity found in those samples.

The only site with multiple private alleles identified was at NIAB East Malling, within the same sampling location, and found at two different loci. The private alleles were detected in WAA feeding on susceptible M.9 rootstocks, in the same enclosed culture as WAA on the resistant MM106 and M.116. Resistance-breaking aphids are still able to feed on susceptible rootstocks, so it is possible that these private alleles represent variation in a resistance-breaking phenotype.

Samples from Molina in Chile and New Zealand each had a private allele present, demonstrating some international variation. The private allele identified in New Zealand samples had high frequency and were present in 67% of the samples, suggesting that these samples from New Zealand are the most isolated.

5.3. Effects of host rootstock on population growth

Estimation of intrinsic rate of increase and MRGR is more realistic when measured on whole plants (Guldemond *et al*., 1998) which is however difficult to achieve with a large plant such as apple, hence the use of petiole cuttings in an enclosed area. Woolly apple aphids are, however, naturally a colony-forming aphid and may perform poorly when isolated from other aphids, as is seen in similar species (Hayamizu, 1984), explaining the overall low survival rates, even when feeding on M.9.
Traditionally, when calculating intrinsic rate of increase the number of days from a nymph emerging until it reaches reproductive maturity would be recorded (*d*) and the number of nymphs produced in the subsequent *d* days counted (Wyatt & White, 1977). Woolly apple aphid is, however, very fragile and slow-growing which, combined with the reduced growth expected from isolation, meant that most adults did not survive *d* days of reproduction. Intrinsic rate of increase can be accurately measured using a shorter, fixed period after reaching reproductive maturity, for example five or seven days, as used here (Castle *et al*., 1998; Dahlin & Ninkovic, 2013). Although this does not capture the entire reproductive period of an individual adult, nymph production is highest immediately after maturity has been reached, making this a good estimate of maximum population growth (Leather *et al.*, 2017).

Reduction of MRGR when feeding on resistant rootstocks was expected as the resistance factor conferred by *Er1* in both MM106 and M.116 is known to be phloem-related and thought to prevent aphids aphid feeding (Staniland, 1924).

We would expect to have seen reduced wax production from aphids feeding on resistant rootstocks, as under limited nutritional availability we would expect to see a reduction in non-essential processes. The lack of a significant difference in wax weight is likely to be because of difficulties in accurately measuring the weight of the wax. The single, isolated aphids only had a short period of time to produce wax, which is very light, making it difficult for the microbalance used to accurately determine the weight. We do see a reduction in wax produced when feeding on MM106 compared to M.9, but not M.116, despite the two being closely related. Across all metrics of growth and reproduction included here, aphids feeding on MM106 always performed slightly worse than those on M.116. This may be because MM106 is closer to the source of resistance, 'Northern Spy' (see Figure 4, Section 3.4.1.).

5.4. Recommendations for future study

5.4.1. Resistance breeding

Identification of flanking markers more closely associated with the target genes than those currently available will inform future breeding attempts. The use of SNPs here will give much greater coverage of the genome and may therefore identify other sites of variation indicative of a resistance complex. The identification of immune and resistant accessions will also be useful in resistance breeding programmes. The consistent low susceptibility score of *M. floribunda* accessions makes it a good candidate for future investigation.

5.4.2. WAA genetics

SNP-based analysis of the WAA samples will give greater definition of differences between samples. We have only been able to capture a limited amount of variation with the SSR markers currently available but variation across the whole genome may identify regions with a lot of differences between aphid populations. It would be of special interest to identify SNPs in regions associated with stylet structures or feeding to determine if resistance-breaking WAA have developed any genetic adaptations.

5.4.3. Rootstock feeding

The methods trialled here were not effective at determining the effects of feeding on resistant rootstocks on WAA growth and reproduction, largely because these techniques were created using species which are more robust and perform better when isolated. Despite these limitations it may be possible to use some other, more gentle techniques may be successful. Electrical Penetration Graph analysis has been used successfully in the past to determine the feeding patterns of WAA and could be useful here to discover whether resistant apple accessions prevent WAA feeding from being initiated or if they slow or interrupt feeding.

Continuing to monitor for WAA feeding on resistant rootstocks will help us to infer what effects they have on population growth i.e. if WAA are observed on MM106 in the field but are not persistent then we could predict that perhaps their growth is merely slowed, not prevented. Aphid monitoring may also help to record the spread of resistance-breaking WAA and any potential sexual forms.

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