Harper Adams University

Resistance and susceptibility in interactions between apple and woolly aphid

A thesis submitted in partial fulfilment of the requirements of Harper Adams University for the degree of Doctor of Philosophy

By

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Declaration

I, Cindayniah Jane Godfrey, hereby declare that this thesis is my own original work unless reported as such in the text. Information from other sources has been fully acknowledged and referenced in the text. None of this work has been submitted for publication or presented for the award of any other degree or diploma at any University.

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Abstract

The woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) is a widespread sap-sucking pest of *Malus* × *domestica* (Borkh.) which has spread from its native range in northeast America worldwide, feeding on all parts of the tree above and belowground year-round. There are four known WAA resistance genes, three of which have been used in rootstock breeding programmes to great commercial success. Reports have emerged of WAA feeding on rootstocks deriving from these three genes, highlighting a need to identify novel resistance sources and to improve genetic resources for existing resistance genes to increase development and release of durably resistant rootstocks. We identified SNP markers significantly linked to the *Er2* gene and a region of the genome in which this gene may lie. We also suggest accessions of the crab apple species *Malus floribunda* as likely novel resistance sources for further investigation. The loss of host-alternation outside of its native range is thought to also indicate exclusive asexual reproduction. We found genetic variation within the UK, and when compared to ten sites abroad, suggestion of sexual reproduction at some sampling locations. We also found varied population structure with some isolated clonal populations. Estimations of individual and population growth metrics suggested reduced performance when feeding on WAA-resistant rootstocks, although traditional metrics were not suitable for a fragile, slow-growing species such as WAA. The findings of this project have been used throughout to inform apple growers of best practice for monitoring and managing WAA.

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Glossary of abbreviations

AFLP	Amplified fragment length polymorphism
AI	Active ingredient
ANOVA	Analysis of Variance
ATPD	V-ATPase subunit D
BAM	Binary Alignment Map
BCF	BIM Collaboration Format
bp	Base pairs
BPH	Brown planthopper
BWA	Burrows-Wheeler Alignment
cM	Centimorgans
CC-type	Coiled coil type
COI	Cytochrome oxidase subunit I
DArT	Diversity Arrays Technology
dsRNA	Double-stranded RNA
EAMU	Extension of Authorisation for Minor Use
EM	East Malling
EMLA	East Malling and Long Ashton virus free rootstock clone
EPG	Electrical Penetration Graph
EST	Expressed sequence tag
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EVA	Ethyl vinyl acetate
F	Fixation index
FB	Fire Blight
FDR	False Discovery Rate
FST	Fixation index
GBS	Genotyping-by-Sequencing
gDNA	Genomic DNA
HAMP	Herbivore-associated molecular pattern
HCl	Hydrochloric acid
hpRNA	Hairpin RNA
HSD	Honestly Significant Difference
HTI	HAMP-triggered immunity
IPM	Integrated Pest Management
ISSR	Inter Simple Sequence Repeat
JA	Jasmonic acid
KASP	Kompetitive allele specific PCR
kb	Kilobases
L:D	Light:Dark
LG	Linkage Group
M.	Malling
M.M.	Malling-Merton
MAS	Marker-assissted selection
Mb	Megabases
MIS	Mildew-immune selection
ML	Maximum Likelihood
MRGR	Mean Relative Growth Rate

NBS-LRR	Nucleotide-binding site leucine-rich repeat
NGS	Next-Generation Sequencing
NIAB	National Institute of Agricultural Biology
NIFTS	National Institute of Fruit Tree and Tea Science
NLR	Nucleotide-binding oligomerisation domain (NOD)-like receptor; aka NBS-LRR
NPQ	Non-phytochemical quenching
OP	Open-pollinated
PAMP	Pathogen-associated molecular pattern
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PNRSV	Prunus necrotic ringspot virus
PPA	*Pinellia pedatisecta* Agglutinin
PSM	Plant Seconday Metabolite
PTI	Pathogen-triggered immunity
PVC	Polyvinyl chloride
Q-Q	Quantile-Quantile
QTL	Quantitative trait locus/loci
R gene	Resistance gene
RAPD	Random Amplified Polymorphic DNA
RH	Relative humidity
rm	Intrinsic rate of natural increase
RNAi	RNA interference
ROS	Reactive oxygen species
RPM	Revolutions-per-minute
SA	Salicylic acid
SAR	Systemic acquired resistance
SCAR	Sequence Characterised Amplified Region
SI	Self-incompatability
siRNAs	Small interfering RNAs
SNP	Single Nucleotide Polymorphism
S-Rnase	Specific ribonuclease
SSR	Simple Sequence Repeat
TASSEL	Trait analysis by ASSociation, Evolution and Linkage software
TMV	Tobacco Mosaic Virus
VCF	Variant Call Format
WAA	Woolly apple aphid
WCR	Wild Crop Relatives

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CHAPTER 1 – Literature Review

1.1. General introduction

Apples are the third most popular fruit in the world, with over 89 million tonnes of apples produced globally each year (FAOSTAT, 2019) and "the most important temperate fruit crop worldwide" (Kellerhals, 2009). They are an important dietary source of flavonoids, phenolic compounds and anti-oxidants, and regular consumption is associated with reduced lung cancer, cardiovascular disease, asthma, and Type II diabetes (Boyer & Liu, 2004). Apples have historically been, and continue to be, an economically important crop in the United Kingdom. In 2021, there were 23000 hectares of orchard fruit (both commercial and non-commercial orchards) in the UK, valued at approximately £287 million, of which £154 million was attributed to dessert apples and £43 million to culinary apples, many of which will be sent for processing or juicing, and £34 million attributed to cider apples and pears (DEFRA, 2022). Apples are widely percieved to be a healthy food; there are many organisations such as the GrEAT British Apples campaign which encourage consuption of, and education around, apples. Apple readily grows in a range of climates, allowing worldwide cropping, but its cold-hardiness means it performs well in temperate environments (Kellerhals, 2009), explaining why apple has historically been such a successful and important crop, especially in temperate areas such as Europe, North America, and New Zealand.

Modern cultivated apple is the result of a series of hybridisations and introgressions of wild crab apple species, namely *Malus baccata* (L. Borkh), *M. orientalis* (Uglitz), and *M. sylvestris* (Mill), with the main apple progenitor *Malus sieversii* (Ledeb. M. Roem.) originating from Siberia, the Caucasus, Western Europe and Central Asia, respectively (Cornille *et al.*, 2012). These domestication events occurred across thousands of years through apple's dispersion along the Silk Road from Asia to Europe, although it is difficult to trace complex domestication events, other than genetically (Duan *et al.*, 2017; Cornille *et al.*, 2019). Hybridization events between the crop and wild relatives are largely responsible for the *M.* × *domestica* genome as we know it today. There are, however, several introgression events which are also responsible for some of the genetic variation seen in apple. Introgression of genes from wild crop relatives (WCRs) to crop plants may have been a major driver in the evolution of perennial crop plants because they are often larger in size and have a longer juvenile phase than annual species (Gaut, Díez & Morrell, 2015; Migicovsky & Myles, 2017). High levels of introgression from *M.* × *domestica* have been observed in *Malus* WCRs, which may threaten the genetic integrity of these WCRs (Cornille *et al.*, 2014). Twenty-seven per cent of wild *M. sylvestris* trees surveyed at the far northwest of its native range in Northern Britain were found to show varying introgression from *M.* × *domestica,* with hybrids occurring

more in areas with intensive land use (Ruhsam *et al.*, 2019). Historical remains of *M.* × *domestica* peak from *ca*. 2100-1500 BCE where cultivated apple was spread around central and Northern Europe, driven by an increase of land management during the Neolithic era (Brozio *et al.*, 2014). Apple has remained an important crop in Europe; in 2017, 473,500 ha of EU land was apple orchards, with an overall increase of 23,900 ha between 2012 and 2017 (Eurostat, 2019).

Orchard management incorporates orchard location, watering, nutrient management, canopy pruning, climate management (where possible, *e.g*. winter fleecing of trees, hail nets and overhead sprinklers to prevent frost damage), and control of pests and pathogens. Apple production in Western Europe decreased between 2014 and 2019, largely because of the unfavourable weather conditions, with a reduction of 2% in apple production in England (Kuden *et al.*, 2023), demonstrating the fine risk margin involved in guaranteeing crop yield. The balance of management concerns is even more critical in organic orchards which do not use conventional synthetic chemical pesticides and therefore rely more heavily on a careful balance of cultural, physical, and biological control to prevent pest and disease outbreaks (Shaw, Nagy & Fountain, 2021). Consumer-driven movement towards food production with reduced environmental impact is likely to increase pressure on the horticultural industry to move towards reduced pesticide usage, making alternate control strategies a major concern. Yield losses, as a result of pest feeding, can drastically affect profit margins, not only through direct fruit yield losses but through other economic expenditure such as pesticide application, especially in the absence of natural enemies (Cross *et al.*, 2015). There is an approximate 20% yield loss with organic cropping systems compared to conventional systems (although this varies depending on crop and region (de Ponti, Rijk & van Ittersum, 2012, which may counteract any financial gain from not applying chemical pesticides. Organic production is considered by some to be at odds with the need to guarantee continued fruit production in the face of a growing global population for whom apples are a major source of nutrition (Vasylieva & James, 2021).

629 Table 1.1: Invertebrate pests of Malus domestica, their taxonomic order and family, and the part or
630 parts of apple trees typically affected by their feeding.

Apple rust mite	*Aculus schlechtendali* Nalepa	Acariformes; Eriophyidae	Leaves
Apple blossom weevil	*Anthonomus pomorum* L.	Coleoptera; Curculionidae	Blossom
Apple bud weevil	*Anthonomus pyri* Kollar	Coleoptera; Curculionidae	Blossom and leaf buds
Vine weevil	*Otiorhynchus sulcatus* Fabricius	Coleoptera; Curculionidae	Rootstock; leaves
Apple fruit weevil	*Tatianaerhynchites aequatus* L.	Coleoptera; Rhynchitidae	Buds; fruit; leaves
Apple leaf midge	*Dasineura mali* Kieffer	Diptera; Cecidomyiidae	Leaves
Green apple aphid	*Aphis pomi* De Geer	Hemiptera; Aphididae	Leaves; shoots
Rosy leaf-curling aphid	*Dysaphis devecta* Walker	Hemiptera; Aphididae	Leaves
Rosy apple aphid	*Dysaphis plantaginea* Passerini	Hemiptera; Aphididae	Leaves
Woolly apple aphid	*Eriosoma lanigerum* Hausmann	Hemiptera; Aphididae	Rootstock; woody tissue
Apple grass aphid	*Rhopalosiphum insertum* Walker	Hemiptera; Aphididae	Leaves
Common green capsid	*Lygocoris pabulinus* L.	Hemiptera; Miridae	Leaves
Brown Marmorated Stink Bug	*Halyomorpha halys* Stål	Hemiptera; Pentatomidae	Fruit
Forest bug	*Pentatoma rufipes* L.	Hemiptera; Pentatomidae	Fruit
Apple sawfly	*Hoplocampa testudinea* Klug	Hymenopotera; Tenthredinidae	Fruit
Brown apple leafminer/spotted tentiform leafminer	*Phyllonorycter blancardella* Fabricius	Lepidoptera; Gracillariidae	Leaves
Apple leafminer	*Lyonetia clerkella* L.	Lepidoptera; Lyonetiidae	Leaves
Summer fruit tortrix moth	*Adozophyes orana* Fischer von Röslerstamm	Lepidoptera; Tortricidae	Fruit; leaves
Tree fruit tortrix moth	*Adozophyes podana* Scopoli	Lepidoptera; Tortricidae	Fruit; leaves
Codling moth	*Cydia pomonella* L.	Lepidoptera; Tortricidae	Fruit
Apple ermine moth	*Yponomeuta malinellus* Zeller	Lepidoptera; Yponomeutidae	Leaves
Fruit tree red spider mite	*Panonychus ulmi* Koch	Trombidiformes; Tetranychidae	Leaves
Two spotted spider mite	*Tetranychus urticae* Koch	Trombidiformes; Tetranychidae	Leaves

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632 ## 1.2. Overview of apple aphids

633 Aphids are soft-bodied, sap-feeding insects of the order Hemiptera, superfamily Aphidoidea.
634 Aphids are small in size (1 – 7 mm long) and often live in colonies containing a large number
635 of individuals feeding in concentrated areas, varying depending on the aphid species and
636 host plant (Barczak *et al.*, 2021). Aphids can spread across the entire plant, depending on
637 the type of tissue fed upon.

Most aphid species show a distinctive life cycle where they alternate between a primary winter host, usually a woody plant, and a secondary summer host which is often herbaceous. Over 4000 species of aphid have been described, which feed exclusively on plants; approximately 250 of these are identified as pests of crop and ornamental plants. Around 40% of species live partly or exclusively on trees, including 66 species which feed on apple (Blackman & Eastop, 2006). Green apple aphid (*Aphis pomi*, de Geer), rosy apple aphid (*Dysaphis plantaginea*, Passerini), and Woolly Apple Aphid (*Eriosoma lanigerum* Hausmann; WAA) are reported as the most common aphid pests of apple in the USA (Blackman & Eastop, 2006). In the United Kingdom, there are several other aphid pests of apple in addition to those listed above, such as the apple grass aphid (*Rhopalosiphum insertum* Walker), but WAA and *D. plantaginea* are the most commonly reported species (Cross *et al.*, 2015; Shaw, Nagy & Fountain, 2021). *Aphis pomi* feeds on apple leaves and does not form colonies, which allows them to colonise a large area of the tree at once. *Dysaphis plantaginea* and *Dysaphis devecta* (Walker) both aggregate on leaves when feeding, inducing leaf curling galls. It is possible for multiple aphid species to feed on apple at one time, creating a serious herbivory pressure on the entire tree. The Spirea aphid (*Aphis spiraecola*, Patch) exhibits similar life history and feeding behaviour to *A. pomi*. *Aphis spiraecola* originates in east Asia, but is becoming increasingly widespread, especially in North America where they have shown lower susceptibility to the neonicotinyl insecticide imidacloprid than *A. pomi* (Lowery *et al.*, 2005). *Aphis spiraecola* has been reported in the UK on both crop species (pear and quince) and non-crop firethorn (*Pyracantha coccinea* M. J. Roemer; Borbély *et al.*, 2020).

Compared to other pests such as *D. plantaginea*, WAA has previously only been considered a serious economic problem in the UK following warm winters which allow earlier spring emergence (AHDB, 2013) but anecdotal evidence suggests that severe WAA infestations are becoming more common. Rising global temperatures, warmer winters, and changing weather conditions may lead to WAA becoming an increasingly common and problematic pest in the UK. The elevated temperature and CO_2 concentration predicted under climate change are expected to be beneficial to aphid numbers and feeding rates, although the actual extent of this will vary depending on the susceptibility of the host species, as well as individual microclimate conditions (Cannon, 1998; Ma & Ma, 2022).

The primary hosts of *Eriosoma* spp. are species of elm (*Ulmus* spp.). *Eriosoma* spp. form galls when they feed (Akimoto, 1981), although the types of gall created vary. There are several notable crop pests within the genus: the woolly pear aphid (*Eriosoma pyricola* Baker & Davidson, also known as the pear root aphid) was first identified in California in 1917 and alternates between cork elm (*Ulmus suberosa* Doud.) and pear (*Pyrus communis* L.).

Eriosoma pyricola produces sexuparae on pear roots (Sethi & Swenson, 1967) and causes serious damage to young nursery pear trees (Westwood & Bjornstad, 1966). *Eriosoma lanuginosum* (Hartig) is a European species which alternates between field elm (*Ulmus minor* Mill.) and pear, although *E. ulmi* alternates between *U. minor* and currant species (Glendenning, 1924). Species related to WAA therefore seem to be primarily pests of elm rather than Rosaceae although alternation between the two plant groups is not unique to WAA.

1.3. Woolly apple aphid

1.3.1. Woolly apple aphid description and biology

Woolly apple aphid belongs to the subfamily Eriosomatinae. These small aphids are *ca*. 1.5 - 2.5 mm long, oval in shape, and dark red to blackish brown (Barbagallo *et al.*, 1997). Woolly apple aphids have a distinctive coating formed of filaments of white wax secreted from glands on the dorsal surface of the abdomen, secreted by all life stages except the first instar (Barbagallo *et al.*, 1997; Smith, 1999). Aphids which produce similar wax coats are spread across different genera and often the structure of wax strands produced varies between species. The wax is thought to primarily protect aphids from getting stuck in their own honeydew by clumping around honeydew droplets, and offer protection from biotic and abiotic stresses, such as waterproofing, isolating a safe microclimate around aphids, and protection from natural enemies (Smith, 1999). Parasitoid wasps locate and identify host aphids through chemical elicitors normally found on the aphid cuticle (Muratori *et al.*, 2006); a thick layer of wax found around WAA may reduce incidence of parasitism not only by physically preventing parasitism but also by disguising or blocking chemical cues which can be used to locate WAA.

Although there are other waxy aphid species which may be encountered within an orchard, including *Eriosoma* spp., WAA wax filaments are longer than the ‘mealy’ wax of other aphids, allowing colonies to be easily recognised (Figure 1.1). Woolly apple aphid wax is made of homologous diketo esters which gives them a similar wax structure to the poplar spiral gall aphid (*Pemphigus spyrothecae*, Passerini), which creates protective galls of plant tissue which surrounding the aphid colony, similar in structure to those created by *E. lanuginosum* (Cameron & Drake, 1976). Woolly apple aphid has completely lost its siphunculi, small tubes on the rear of an aphid’s abdomen which exude pheromones and defensive fluid, instead it has flat siphuncular pores. Waxy aphids and colony-forming aphids often have shorter siphunculi than free-living aphids (Mondor, Roitberg & Stadler, 2002). Typically, longer siphunculi secrete alarm pheromones to warn other aphids of a nearby predator, including by marking the predator with the pheromone. The sugar cane woolly aphid (*Ceratovacuna*

lanigera Zehntner) which also has severely reduced siphunculi produces aphid alarm pheromone when stimulated and will respond to droplets of aphid alarm pheromone when placed onto a natural enemy (Arakaki, 1989), demonstrating that it is possible for an aphid with reduce siphunculi to produce alarm pheromones, although levels may be lower.

1.3.2. Woolly apple aphid life history

Woolly apple aphid is believed to have a centre of origin in Northeast 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. It was first recorded in Australia in 1846 (Nicholls, 1919) and in South Africa in 1895 (Myburgh, Whitehead & Daiber, 1973). Woolly apple aphid can produce up to twenty generations per year, depending on temperature and life cycle (Barbagallo *et al.*, 1997). Optimal WAA growth and reproduction was found to be between 20 - 25 °C across four varieties of apple scion (above-ground portion of the tree, grafted to a rootstock close to soil level) with the lowest generation time (16.1 ± 0.8 days) on the susceptible cultivar Red Fuji seen at 25 °C (Tan *et al.*, 2021).

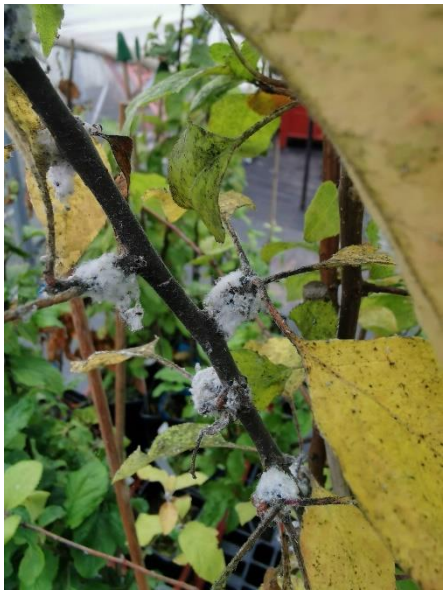

Figure 1.1: Clear and distinct woolly apple aphid colonies on an apple seedling under polytunnel conditions.

In North America, WAA is reported as host-alternating and holocyclic (Figure 1.2) with sexual forms travelling to American elm (*Ulmus americana* L.) and asexual forms reproducing parthenogenetically on apple. In the rest of the world, WAA is reported as feeding only on apple, mostly reproducing parthenogenetically with no successful sexual stages (Eastop, 1966; Blackman & Eastop, 1994). The woolly elm aphid (*Eriosoma americanum*, Riley) also feeds on *U. americana*, and the horticultural pest the elm-currant aphid (*Eriosoma ulmi* L.)

alternates between several *Ulmus* spp. and the roots of currant plants (*Ribes* spp.), and *E. lanuginosum* which feeds on several *Ulmus* spp.

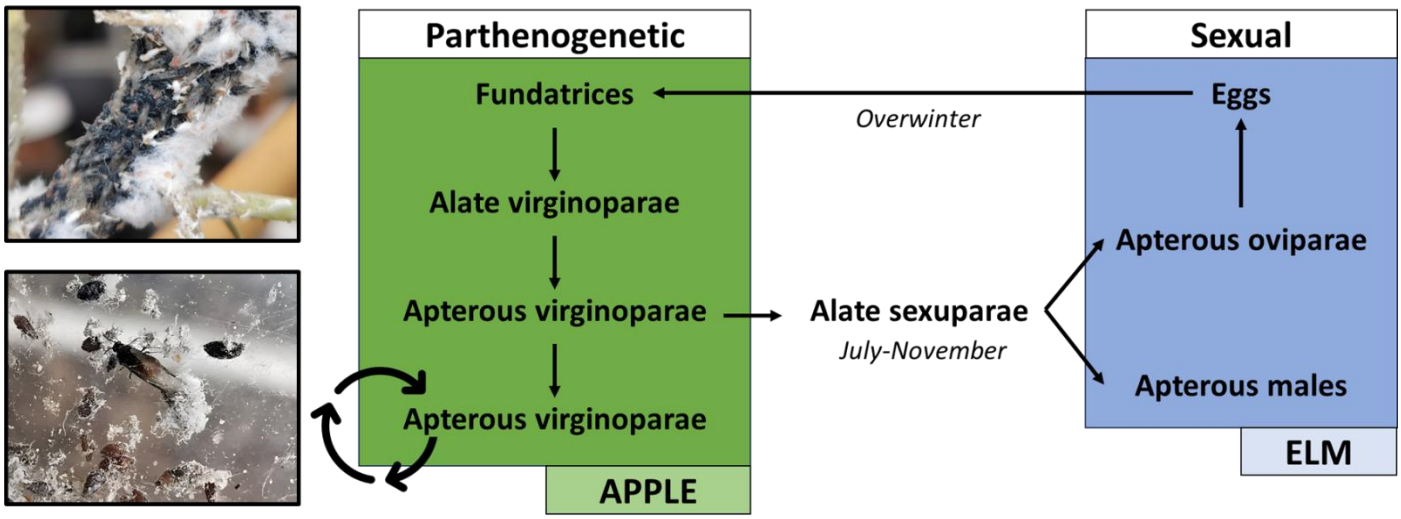

Figure 1.2: Expected lifecycle of the woolly apple aphid in its native range. Lifecycle schematic (right) depicts a heteroecious (host-alternating) lifecycle, as is observed in its host range in North America. Elsewhere, the sexual phase on American Elm has been lost and the aphid exists almost exclusively as apterous virginoparae, feeding on apple (adapted from Sandanayaka, Bus & Connolly (2005). Alate forms have been observed in the UK (photographs, left). These are not necessarily sexuparae, but may be alate virginoparae, produced in response to overcrowding and/or deteriorating host nutritional quality (top left).

Although historically widely reported in the USA, a host-alternating WAA lifecycle is by no means confirmed. American elm is not widely dispersed in the rest of the world, which may in part explain why WAA mostly shows parthenogenetic reproduction (Beers, Cockfield & Gontijo, 2010). There have been reports of males and sexual females (oviparae) being produced but they are not viable, rendering the population functionally asexual (Hely, Pasfield & Gellatley, 1982). It has also been reported that some oviparae survive long enough to produce a single unviable egg, the production of which also kills the mother (Gautam & Verma, 1983; Asante, Danthanarayana & Cairns, 1993). Sexual reproduction has been extensively reported in North America, with some instances of sexuparae reported in other parts of the world (Table 1.1).

Table 1.2.: Life histories of woolly apple aphid as reported in apple growing regions. The observation of sexual forms is not necessarily indicative of a holocyclic lifecycle.

Country	Life cycle	Sexual forms?	References
USA	Holocyclic	Often	(Baker, 1915)
UK	Anholocyclic	Unknown	(Theobald, 1921)
Australia	Anholocyclic	Occasionally	(Nicholls, 1919)
New Zealand	Anholocyclic	Occasionally	(Sandanayaka & Bus, 2005)
India	Anholocyclic	Occasionally	(Gautam & Verma, 1983)
Chile	Anholocyclic	Unknown but expected	(Lavandero *et al.*, 2009a)
South Africa	Anholocyclic	No	(Damavandian, 2000; Heunis, 2001)

Three different WAA biotypes have been reported in key apple growing regions of southeast Australia. It is not known whether these biotypes have arisen through:

1) differential selective pressures in the environment;
2) the arrival of different biotypes in Australia from imported apple material from other countries;
3) another phenomenon such as random genetic drift (Costa, Williams & Powell, 2014).

Biotype, in insect pests of agriculture, refers to sub-populations of a species which share some characteristics, often related to virulence (Claridge & Den Hollander, 1983). It is widely used within WAA literature and is useful to distinguish between populations with different lifecycles and/or host plants, and therefore virulence. The Australian and American biotypes show slight morphological differences; Australian biotypes have spines on their tarsi and tibia and long empodial hairs (bristles on the tarsi or tibia) compared to the American biotype (Eastop, 1966), possibly giving additional protection against natural enemies. In addition to morphological variation between countries, WAA genetic diversity is also connected to geography. Lavandero *et al.* (2009a) found molecular variation among WAA populations from across Chile, partially created by geographic barriers to gene flow within a sexual system, such as rivers and mountains.

Even in instances where sexual forms are produced outside the aphid's native range, they do not show host-alternation (Gautam & Verma, 1983; Asante, Danthanarayana & Cairns, 1993). Woolly apple aphid has historically been more frequently reported as a severe crop pest outside of the USA (Cummins & Aldwinckle, 1983), especially in the southern hemisphere, which is likely a result of the asexual lifecycle. Parthenogenetic reproduction allows large numbers of aphids to rapidly build up on the host plant. In the absence of migration to a secondary host, apple is subject to WAA damage year-round. The aphid's pest status is become increasingly concerning in the northern hemisphere with changing climatic conditions and the withdrawal of control options, making understanding and controlling WAA a global issue.

1.3.3. Lifecycle within the orchard

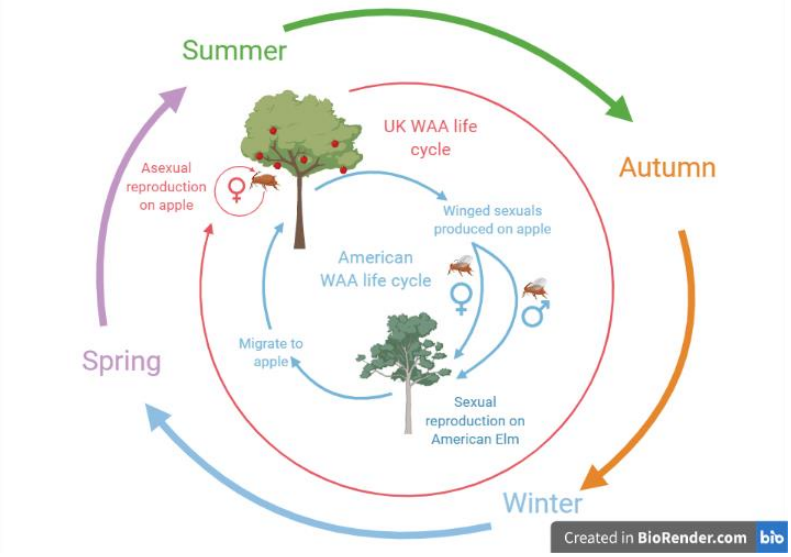

Figure 1.3: Woolly Apple Aphid (*Eriosoma lanigerum;* WAA) seasonal lifecycle. The outermost ring shows the changing seasons. The middle ring shows the expected lifecycle of a non-host-alternating biotype of WAA, such as found in the majority of its global distribution, where it predominantly feeds on apple. The innermost circle represents the lifecycle in the aphid's host range in North America where it exhibits seasonal host-alternation, coupled with sexual reproduction, between apple and American Elm.

Woolly apple aphid overwinters in sheltered positions (*e.g.*, cracks in the bark, below-ground) as first instar nymphs known as "crawlers" which are long-lived and especially tolerant to low

temperatures (Barbagallo *et al.*, 1997). These nymphs are very small (*ca*. 1.2 mm long) and have a flattened oblong shape. Like all WAA life stages, they have short antennae and lack siphunculae, allowing them to travel through soil to feed on the roots (Hetherington, 2009). Crawlers are the key life stage for WAA and are responsible for maintaining the seasonal lifecycle of the species because adult apterous aphids are sessile and do not disperse (Hoyt & Madsen, 1960). As temperatures increase and crawlers leave diapause, they become active and disperse throughout the tree. In spring, WAA above-ground are first seen feeding on pruning injuries and other wounds, and on water shoots and suckers where aphids move up from the roots and establish colonies on the new growth (Barbagallo *et al.*, 1997; Hetherington, 2009). Colonies of WAA form preferentially on the lower trunk and branches, probably to avoid adverse weather conditions, as they have shown limited ability to move at temperatures below 10 °C (Hoyt & Madsen, 1960; Asante, Danthanarayana, & Cairns, 1993).

Crawlers have been observed to disperse through the canopy when triggered by reduced host nutritional quality or high aphid density, for example when WAA numbers reach a peak in late summer or early autumn (Asante, Danthanarayana & Cairns, 1993). The movement of aphids between rootstock and scion continues throughout the spring and summer until lower

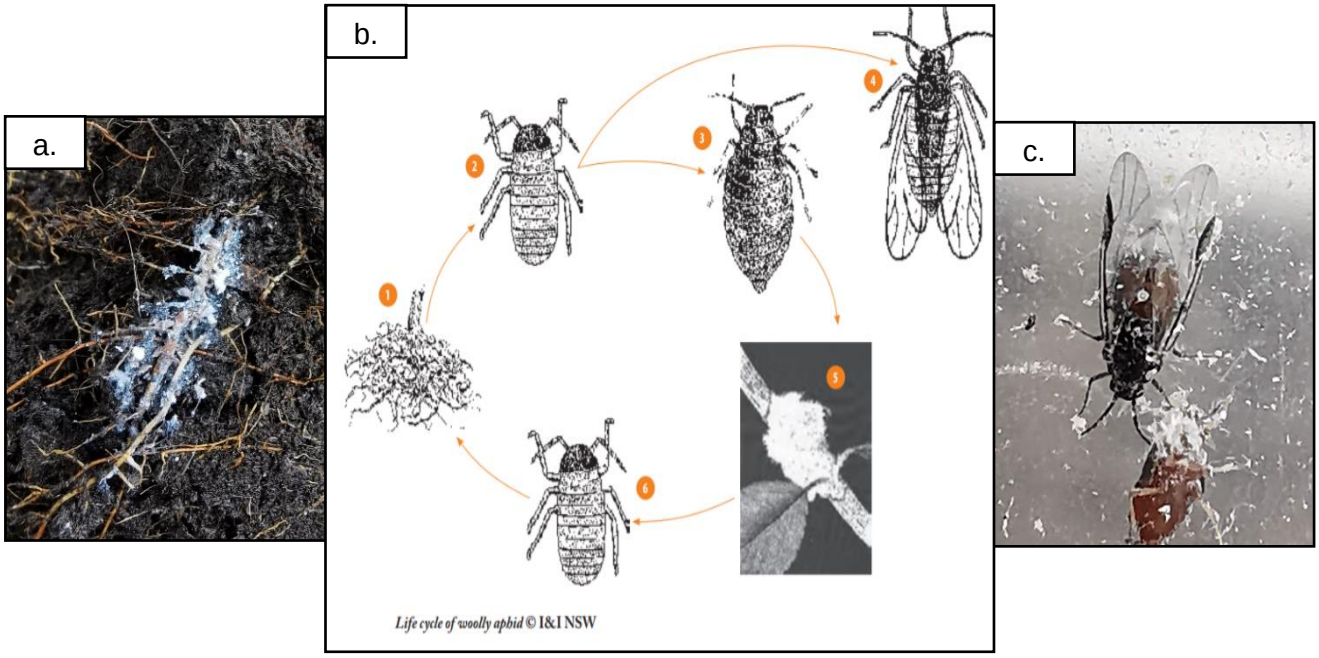

Figure 1.4: **a)** Woolly Apple Aphid (*Eriosoma lanigerum*: WAA) colony feeding on roots of a potted M.9 rootstock under polytunnel conditions (Godfrey, 2020); **b)** Lifecycle of woolly apple aphid in southern Australia with images of each life stage. First instar nymphs ("crawlers") live on roots over winter (1). In spring they disperse to the canopy (2). There are four nymphal instars before adulthood. Asexually reproducing apterous females (3) produce nymphs which cause colonies to build up rapidly (5). In the autumn "crawlers" migrate back down to the roots to overwinter (6). Under crowded conditions and in the autumn alate forms are produced to disperse to other locations (4). It is at this stage that sexual forms may be produced (Hetherington 2009); **c)** Alate and apterous WAA adults taken from a WAA colony on a *Malus* breeding population (Godfrey, 2020).

autumn temperatures trigger crawlers to return to the rootstock for the winter (Hetherington, 2009). The population growth of WAA is closely related to that of its chief natural enemy, the parasitoid wasp *Aphelinus mali* (Haldeman) which also has two seasonal peaks, each shortly after that of WAA (El-Haidari, Georgis & Salam, 1978). In the UK these peaks are typically in mid-spring (May-June) and early autumn (September), depending on temperature in a given year. Declines in WAA numbers after the peaks may therefore also be due to high rates of parasitism which in turn decline as WAA colonies are controlled by *A. mali*. Woolly apple aphids can disperse as alates, presumably triggered by reduced nutritional quality of the plant and/or overcrowding, as is seen in other aphid species (Sutherland & Mittler, 1971). This does occasionally occur in the UK but has not been widely reported.

1.3.4. Woolly apple aphid genetics

The variation in WAA lifecycles shown around the world 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, as seen in the aphid model species the pea aphid (*Acyrthosiphon pisum* Harris; Kanbe & Akimoto, 2009). Eight polymorphic loci have been identified in WAA and microsatellite primers developed and published (Lavandero *et al.*, 2009b). Zhou *et al.* (2015) used these microsatellites 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. The first woolly apple aphid chromosome-level genome assembly was generated in 2020 using 10X Genomics linked reads and in vivo Hi-C data (Biello *et al.*, 2021). The highly complete final assembly was 327 Mb long with 91% of the assembled sequences aligned to 6 chromosomes, ranging in length from 29.68 to 71.23 Mb, which agrees with previous findings that aphids in the *Eriosomatidae* have 2n = 12 chromosomes (Robinson & Chen, 1969; Gautam & Verma, 1982; Gautam & Verma, 1983). This genome is equivalent in size to that estimated for the woolly elm aphid (*E. americanum*) and contains a high number of genes conserved within arthropods. Ninety-seven percent of these conserved genes were found as single copy orthologues, making this the highest number of conserved single-copy Arthropod genes of any published aphid genome. 67% of identified genes have an orthologue in at least one other aphid species. The WAA proteome was compared to the proteomes of nine other aphid species, and to whitefly (*Bemisia tabaci* Gennadius), which placed the tribe Eriosomatini, to which WAA belongs, as an outgroup to the other aphid species investigated. The WAA genome shows high levels of gene duplication across a diverse number of gene functions, giving it 9936 lineage-specific genes which are not similar to genes from other lineages (Biello *et al.*, 2021). Diverse lineage-

specific gene duplication is common within the aphids and may drive evolution of aphid paralogs (Fernández *et al.*, 2020). This genome sequence will be a very useful resource in this project for examining WAA genetic diversity. Understanding the relationship between WAA and other species will help to contextualise variation within the species.

The presence of two bacterial symbionts commonly associated with aphids were found whilst generating this sequence: *Buchnera aphidicola* (Munson) and *Serratia symbiotica* (Moran). Most aphids have an endosymbiotic relationship with intracellular *Buchnera* spp., which are transmitted vertically from mother to daughter and are key in providing amino acids which aphids are unable to source dietarily or to generate themselves (Douglas, 1998). There is also thought to be a role of *Buchnera* symbionts in protection from viral infection. Endosymbiosis with *S. symbiotica* is also associated with increased fatty acid synthesis, resulting in higher nymph weight and shorter development time (Zhou, *et al.*, 2021).

1.3.5. Woolly apple aphid damage

Woolly apple aphid is not known to transmit plant viruses, but rather causes mechanical damage when feeding and can make the host plant vulnerable to secondary pathogen infection (Blackman & Eastop, 2000). When aphids probe plant tissue to locate the phloem, they repeatedly inject saliva into the tissue and siphon a mixture of saliva and cell

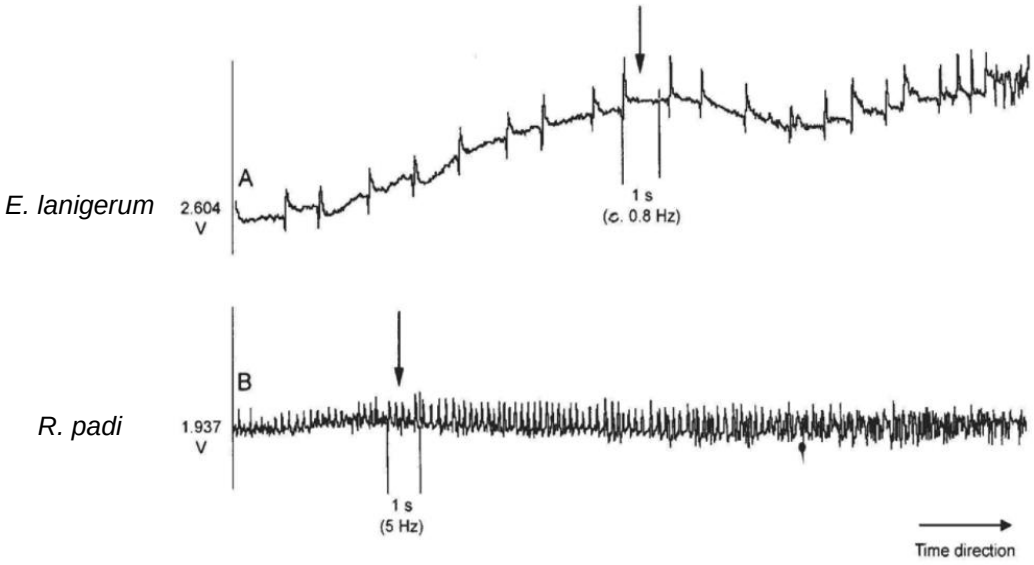

Figure 1.5: EPG outputs from Woolly Apple Aphid (*Eriosoma lanigerum*; WAA; A, top) and the bird cherry-oat aphid (*Rhopalosiphum padi*; B, bottom) during the phloem salivation stage (E1), at which the aphid begins feeding (Sandanayaka & Hale, 2003). The arrows indicate a one second period of time with the waveform patterns and approximate frequency at which probing patterns were recorded in this period indicated between two vertical lines.

components from the plant, allowing them to ‘assess’ what type of cell they have probed and the overall suitability of the host plant (Miles, 1999). Aphids sequentially probe plant cells whilst feeding until they detect phloem sieve tube cells. Most sieve tube elements are pierced and rejected by the aphid as not being phloem tissue, before one is accepted, however these cells do not die (Tjallingii & Esch, 1993). The probing and feeding behaviour of WAA when feeding on ‘Royal Gala’ was measured using Electrical Penetration Graph (EPG) technology (Wageningen, the Netherlands). Woolly apple aphid feeding and probing were broadly similar to those published for other aphid species, but did show two unusual characteristics. Firstly, WAA does not show a high amplitude, which is normally seen at the beginning of aphid pre-probing stage. The aphids were visually observed to carry out the pre-probing stage, but it is unknown what occurs as a part of that phase for WAA. Secondly, the phloem salivation stage, at which the aphid begins to feed on phloem sap, showed a lower frequency of salivation than *Rhopalosiphum padi* (L.) feeding on soft plant tissue, when WAA was feeding on woody tissue (Sandanayaka & Hale, 2003), as shown in Figure 1.5. Low frequency of phloem salivation events indicates that WAA may have reduced feeding rate which may explain the relatively slow growth rate reported for WAA.

In the absence of virus transmission, saliva is widely accepted to be the causal agent of aphid damage as salivary elicitors induce drastic changes in plant physiology (Miles, 1999). Gall induction is triggered by elicitors in aphid saliva as it is repeatedly injected into plant tissue when the aphid is probing to find the phloem, although it is also dependent on the susceptibility of the target plant tissue to galling which varies greatly depending on the type of tissue attacked (Maresquelle & Meyer, 1965; Madden & Stone, 1985; Jiang & Miles, 1993). Salivary injection is likely to induce a signalling cascade which ultimately leads to gall formation. *Hormaphis cornu* (Shimer), the witch-hazel cone gall aphid (Hemiptera: Hormaphidinae) injects proteins associated with anthocyanin pigment synthesis when feeding on witch hazel (*Hamamelis virginiana* L.). Anthocyanin pigments are deposited in the leaf galls, giving them a red colour but the injection of anthocyanin synthase proteins also causes differential expression of plant genes and the formation of distinctive cone-shaped galls which *H. cornu* colonies live within (Korgaonkar *et al.*, 2021). These genes are members of the *bicycle* gene family which are strongly expressed in the salivary glands of gall-forming aphids, suggesting that they are likely involved in gall development (Korgaonkar *et al.*, 2021).

Shortly after feeding initiation, elicitor molecules in WAA saliva induce transcriptional changes in apple, related to both plant defence signalling and gall induction pathways (Wemmer, 2019). Defence signalling mediated by reactive oxygen species (ROS), including downregulation of photosynthesis and non-photochemical quenching (NPQ) is observed,

along with upregulation of inter-cellular transduction, including of ROS generators and R genes. The upregulation of proteins involved in cell wall loosening, and the induction of xylem differentiation following feeding causes rapid proliferation of cambium cells surrounding the phloem and the spread of gall tissue (Staniland, 1924; Wemmer, 2019). Normal tissue structure within the gall breaks down, creating masses of undifferentiated tissue known as neoplasm (Staniland, 1924; Barbagallo *et al.*, 1997).

Many gall-forming aphids are sheltered within the galls they create. This is not the case for WAA, but galling also offers nutritional benefit to aphids, especially those which feed in dense colonies. There are three broad mechanisms by which aphids are able to secure sufficient nutrition for high numbers of individuals feeding in a small area, as is associated with the high population growth rates seen in parthenogenetically reproducing aphids: host alternation; group feeding; and bacterial symbionts (Koyama, Yao & Akimoto, 2004):

1. Host alternation;
    - Alternating between host plants offers an alternate food source for aphids to migrate to in the instance of a decline in nutritional quality in the primary host, for example during changing seasons, most commonly in the autumn (Kundu & Dixon, 1995). This is the “normal” life cycle for WAA in its native range.
2. Group feeding;
    - Group feeding increases herbivory pressure on the plant at specific sites, especially in the instance of colony feeding. The sugar beet root aphid *Pemphigus betae* (Doane) induces galls on narrowleaf cottonwood (*Populus angustifolia* James) which the aphid colony lives within. These galls act as photosynthate sinks within *P. angustifolia*, sequestering carbon from neighbouring leaves and carbon reserves in the stem, and providing high photosynthate levels in the gall to feed the aphid colony (Larson & Whitham, 1991).
3. Bacterial symbionts.
    - Endosymbiotic *Buchnera* spp. can convert non-essential amino acids into essential amino acids which the aphid cannot synthesise and are not available in phloem sap (Douglas, 1993). Aphids reared without *Buchnera* symbionts grow slowly and perform poorly, reflecting the nutritional benefits of the endosymbiosis (Douglas, 1992).

Tissue disruption occurs soon after aphid infestation, with root galls visible four weeks after inoculation with WAA and the bark of infested stems can be seen to be cracked after eight weeks (Weber & Brown, 1988). Adult WAA prefers to feed on young woody tissue rather than leaves or fruit tissue, as was discovered by EPG analysis of WAA feeding on different tissues, although in an orchard environment WAA colonies containing mixed aged aphids

were also found to preferentially feed on young tissue (Zhou, H., *et al.*, 2021). The overall dry biomass of apple roots and shoots begins to increase four weeks after infection and continues to increase over time, indicating that galls are building up within the vascular tissue, even when not visible, with root gall abundance proportional to the WAA infestation (Weber & Brown, 1988). Although the presence of WAA galls was found to strongly reduce plant growth, it is not clear whether this is due to carbohydrate withdrawal, tissue disruption, or an interaction between factors (Weber & Brown, 1988). Disrupted vascular tissue and enlarged sclerenchyma reduce water flow through the root, reducing growth especially in young trees (Brown, Glenn & Wisniewski, 1991). Root-feeding withdraws carbohydrates from photosynthate transport from leaves to roots (Weber & Brown, 1988).

Edaphic WAA were found to significantly reduce linear growth of branches in mature trees, causing significant yield loss per tree in one growing season by reducing fruit set (Brown *et al.*, 1995). In young, non-fruit bearing trees, edaphic WAA have been observed to reduce trunk diameter growth, which is correlated with fruit yield production (Waring, 1920; Westwood & Roberts, 1970; Brown & Schmitt, 1990). Edaphic WAA can have long-term effects on growth if left unchecked; after three years of WAA feeding both trunk diameter and scion biomass were found to be significantly reduced (Brown & Schmitt, 1990) which would have reduced fruit yield. Galls in axils disrupt the production of fruit and vegetative buds which may seriously disfigure young trees and nursery stock (Barbagallo *et al.*, 1997). Tissue disruption combined with reduction in growth means that WAA infestation is a very serious threat to fruit yield. Ultimately, the amount of damage caused depends on the seriousness of the attack and the susceptibility of the cultivar (Barbagallo *et al.*, 1997).

Figure 1.6*:* Example of severe galling on a young tree (7 years old) under polytunnel conditions at the National Fruit Collection. Galls can clearly been seen on the shoots and main stem, and the tree lacks leaves compared to its neighbours which were not as severely infested.

Secondary damage

The injuries caused by aphid feeding can make the plant vulnerable to secondary damage. As galls grow, enlarged cambium cells exert pressure on the inside of the gall which collapses. This may make aphid feeding easier as the structure of the gall is soft and pulpy (Staniland, 1924). Galls can then crack, especially when thawing after periods of low temperatures (Childs, 1929) creating open wounds which, combined with reduced plant fitness from aphid infestation (Sandanayaka, Bus & Connolly, 2005), makes infested trees vulnerable to secondary pathogen infection. Woolly apple aphid galls are prone to splitting after freezing and thawing at temperatures below 0 °C, predisposing trees to secondary pathogen infections in colder environments (Hetherington, 2009). Outbreaks of perennial apple canker (*Neonectria ditissima* Tul. & C. Tul.) have been found to follow severe WAA infestation late in the growing season (Childs, 1929) and can infect gall tissue, exacerbating the negative effects of aphid feeding (Barbagallo *et al.*, 1997). Woolly apple aphids often shelter under tree collars which provides a protected environment for both WAA and the bacterium *Erwinia amylovora* (Winslow), the causative agent of fire blight, a serious disease in orchards (Cummins & Aldwinckle, 1983). Aphids are then able to mechanically spread the bacterium when leaving the tree collar.

Large aerial WAA colonies above ground produce a layer of honeydew which falls from colonies feeding on woody tissue onto leaf surfaces where it remains, promoting the growth of sooty mould which blocks stomata and prevents light from reaching photosystems (Barbagallo *et al.*, 1997; Guerrieri & Digilio, 2008). Although WAA are not recorded to feed on fruits, apples are often contaminated by WAA wax, honeydew, and sooty mould. It is possible for WAA to infest the apple calyx, especially in open-calyx varieties and under high aphid numbers, although this is rare.

1.3.6. Woolly apple aphid control

Because of its unusual lifecycle, the key to controlling WAA is thought to lie in controlling the root feeding life stage because it should allow for prevention of spring emergence, although it is very difficult to monitor and control aphids when they are in the soil (Hetherington, 2009). Spring emergence is a potential monitoring point to determine the expected severity of WAA infestation, and to apply control mechanisms. Pest monitoring is the first step to know when to act to control a pest, which is compounded by the lifecycle of WAA which is not well understood and varies between regions. Fifteen pest management decision-makers in Washington State, USA, were interviewed about their opinions of WAA control and only six of the fifteen gave a specific action threshold for WAA management, and these threshold levels

varied between individuals (Orpet *et al.*, 2019a). Although this survey was carried out in the USA, the pest's native range is in the north east of the country, and this is where sexual reproduction has been reported, whereas Washington State is in the north west. Although sexual WAA do host-alternate in the autumn, they also overwinter on apple rootstocks and decision-makers will have been used to developing threshold levels for this lifecycle.

Root galling is a little more difficult to distinguish from galling caused by nematodes, crown gall, or a number of other pests. However, it is very rare for root infestations to occur without accompanying aerial infestations. Where root galls are found, the presence of aerial colonies is adequate confirmation that woolly aphid is responsible in Australian orchards (Hetherington, 2009). Woolly apple aphids stop producing wax in the autumn and winter, and the majority of aphids above ground die, allowing galls to be clearly seen in the winter (Hetherington, 2009) and the extent of the previous season's damage to be assessed.

Chemical control

Traditional synthetic pesticide use has historically been disproportionately high in orchards compared to other sectors (Vysini *et al.*, 2011). This may be because orchard crops are perennial, often remaining in the ground for decades, and therefore need protection to ensure crop longevity. In any instance of a severe infestation either in or outside of the growing season, control mechanisms will need to be used to prevent loss of crop trees. There is therefore a year-round window in which synthetic pesticides can be applied compared to an annual crop, such as strawberries, where there is less need to protect crop plants.

Chlorpyrifos, an organophosphate acetylcholinesterase inhibitor, was used historically to control WAA but was withdrawn in the UK in 2016 (Bayer, 2019). Insecticides which require physical contact with the pest, for example FLiPPER and Sentinel (Unsaturated fatty acid and bifenthrin active ingredients, respectively), tend to be less effective than systemic insecticides because of the aphid's protective wax coat. The active ingredient (AI) of FLiPPER, Unsaturated Carboxylic Acids, disrupts cell membranes which prevents feeding and respiration. Sentinel is a bifenthrin pyrethroid insecticide which inhibits sodium ion channels, disrupting insect neuron function. Contact insecticides are only effective on the scion and colonies may need to be "burnt" using magnesium sulphate pre-application or an adjuvant which can be mixed and applied with the insecticide, such as a horticultural oil in order to remove the wax for the insecticide to reach the insect cuticle and to be effective, as has been reported by UK growers (Alston, Reding & Murray, 2010; Bird, 2021 *pers. comm*.; Powell, 2022 *pers. comm*.).

Soil drenches of imidacloprid (neonicotinoid; inhibits nicotinic acetylcholine receptors causing nerve disruption) and clothianidin (neonicotinoid) are used in other countries such as Australia to control below-ground populations and can be an effective method for controlling WAA on the entire tree, rendering aerial applications for WAA control unnecessary (Hetherington, 2009). Avoiding aerial sprays for WAA may reduce the risk of damage to non-target insects, although soil drenches may negatively impact ground dwelling insects.

Insecticides with the active ingredient spirotetramat are percieved to be the most effective under UK regulation for WAA control, with acetamiprid insecticides the second most effective (Bird, 2021 *pers. comm*.). Spirotetramat is a keto-enol insecticide, known to be especially effective against juvenile sap-feeding pests and has been shown to be effective against WAA in field trials in Germany (Nauen *et al.*, 2007). It inhibits acetyl CoA carboxylase which then disrupts lipid biosynthesis, affecting insect metabolism, metamorphosis, and especially juvenile development. Acetamirid is a chloropryidinyl neonicotinoid which inhibits nicotinic acetylcholine receptors, disrupting the insect nervous system. Both spirotetramat and acetamiprid are systemic and are absorbed into the plant and translocated through all tissues, so that aphids feeding at any location will ingest the insecticide. This is especially beneficial in the case of root-feeding aphids which can be otherwise difficult to treat. Spirotetramat has been reported to bioaccumulate in high concentrations in the roots of lettuce (*Lactuca sativa* L.) following application of the insecticide directly to bare roots, which may also be the case for apple rootstocks, pre-planting, although they may need to be exposed directly to spirotetramat (Liu *et al.*, 2023). Spirotetramat is currently authorized for use on aphids of outdoor tree fruit and both indoor and outdoor strawberries, with a recent EAMU (Extension of Authorisation for Minor Use) for WAA control in the UK (Bayer, 2019). Two applications of spirotetramat insecticides are permitted in the UK per year. These must be applied to apple before the onset of fruit maturity, which in the UK usually occurs between August and October, allowing it to be applied during the peak in WAA often seen at spring emergence.

Spirotetramat presents a viable chemical control option because it is effective against WAA feeding on all parts of the plant, including the rootstock, as reported from Washington State (Beers & Cockfield, 2007) but, as with all chemical pesticides, its use is limited because of the constant risk of withdrawal. Whilst resistant rootstocks can control edaphic WAA, other control methods, such as the use of *A. mali*, can be used to tackle WAA feeding above ground in New Zealand (Sandanayaka, Bus & Connolly, 2005), which is similar in climate to the UK, allowing us to expect the same control. There is then still the option to use spirotetramat, or other synthetic insecticides, where necessary, to control infestation. The

best solution, therefore, for controlling WAA is as part of an Integrated Pest Management (IPM) strategy.

Biological control

The common earwig (*Forficula auricularia* L.) is known to predate WAA in mature orchards. When earwigs are excluded from trees in an experimental orchard in the Netherlands, WAA colonies were found to infest 20 - 25% more new growth than trees where earwigs were present (Mueller, Blommers & Mols, 1988). Whilst predation can be an effective control mechanism, there is however some negative perception associated with earwigs in the orchard. When pest-management decision makers in Washington State were interviewed about their opinions of WAA control, one third of interviewees thought *F. auricularia* were pests of apple, and fourteen of the fifteen decision makers reported having seen earwigs on damaged apple (Orpet *et al.*, 2019a). Whilst earwigs have been recorded to cause some damage to fruits (Carroll, Walker & Hoyt, 1985; Huth *et al.*, 2011), this is minimal in apple and must be evaluated for potential WAA biological control. Orchard management is crucial to encourage the presence of earwigs in the orchard. Sticky banding is sometimes applied to tree trunks in order to exclude WAA from the canopy, but in the Netherlands and the USA this practice has been observed to lead to an increase in WAA colonies as the banding also excludes earwigs, and likely other non-flying natural enemies (Mueller, Blommers & Mols, 1988; Orpet *et al.*, 2019b). Woodland habitats near to or connected to IPM-managed orchards in Germany were found to decrease *F. auricularia* abundance in the orchard, likely because the use of traditional pesticides in the crop drives natural enemies into surrounding areas (Happe *et al.*, 2018). This was not observed in organic orchards with nearby woodland habitats, confirming that pest management choices are likely influencing natural enemy presence in the orchard.

Adult female parasitoid wasps lay eggs into the body of its host insect and the larva consumes the host aphid as it develops. The larva spins its cocoon within the aphid's cuticle which dries and hardens into a 'mummy' where the wasp pupates until it emerges as an adult and the lifecycle begins again (Boivin, Hance & Brodeur, 2012). This is especially effective in enclosed environments such as polytunnels where the elevated temperatures are beneficial for *A. mali* development in temperate countries, such as Australia (Asante & Danthanarayana, 1992). The first emergence of adult *A. mali* of the season has been accurately modelled using flight data collected in the field in Belgium for ten years and compared against historical data (Bangels *et al.*, 2021). Accurately predicting when natural enemies will be active in the orchard is key for an IPM strategy because it allows management of pesticide sprays to avoid application when beneficial species (chiefly pollinators and natural enemies) are present in the orchard.

Aphelinus mali has been widely introduced from its host range in North America to control WAA around the world (Cohen *et al.*, 1996). For example, *A. mali* was introduced into Australia from New Zealand in 1923 and over the next fifteen years became widely established in apple-growing regions causing a large decline in reports of WAA infestations, especially in warmer regions (Staniland, 1923). Woolly apple aphid is not as effectively controlled by *A. mali* in cooler environments, such as New Zealand (Sandanayaka, Bus, Connolly, 2005) or Northwest Europe, as the developmental temperature threshold for *A. mali* is higher than that of WAA. It therefore takes *A. mali* more day degrees to reach its developmental peak, allowing WAA numbers to build before *A. mali* emergence, a pattern observed in both the USA and Europe (Asante & Danthanarayana, 1992; Mols & Boers, 2001). The temperature acclimatisation of different *A. mali* strains affects their ability to control WAA outbreaks. Modelling of two *A. mali* strains found that Canadian *A. mali* were better at controlling WAA outbreaks in the Netherlands than a known Dutch strain of *A. mali.* This was likely due to them being adapted to colder winter temperatures and therefore their spring development was not impacted by the warmer winters seen in Europe (Mols & Boers, 2001). By July in Northeast America, parasitism by *A. mali* was observed on over half of all WAA colonies surveyed (Brown & Schmitt, 1994). Wild populations of *A. mali* often form around orchards, but controlled releases of adult *A. mali* have been found to reduce WAA infestation to 19% from 93% in untreated orchards in northeast China (Lung, Wang & Tang, 1960).

Other common aphid natural enemies have been reported as successfully controlling WAA. Effective control has been achieved by larvae of two hoverfly species: *Heringia calcarata* (Loew) and *Eupeodes americanus* (Weidemann) in the USA (Bergh, 2008). Twenty per cent of WAA colonies surveyed in West Virginia, USA, in the month of June were found to have syrphid larvae present (Brown & Schmitt, 1994). Although a reduction in WAA infestation is desirable, in Australia it is thought to be unlikely that these predators alone can effectively control WAA (Nicholas, Spooner-Hart & Vickers, 2005) and they are better utilised in combination with other natural enemies. There are differing reports of the most effective natural enemy for WAA control. Nicholas, Spooner-Hart & Vickers (2005) reported *F. auricularia* as the principal component in an IPM strategy for WAA in Australia and that *A. mali* and flying predators (e.g. adult ladybirds and hoverflies) could only effectively control WAA in combination with earwigs. Gontijo, Beers & Snyder (2015) in contrast found that *A. mali* shows the most significant slowing of WAA population growth in Washington State compared to other natural enemies, but agreed that a consistent WAA decline was only seen in conjunction with a generalist predator (Gontijo, Beers & Snyder, 2015). Natural enemies contribute to the control of WAA differentially throughout the year as environmental

conditions and relative predator-prey abundances interact to benefit different natural enemies at different times. Woolly apple aphid control by *F. auricularia* is most effective in the early spring in Spain (Lordan *et al.*, 2015), whereas *A. mali* has a higher developmental temperature threshold than WAA, making it a beneficial natural enemy under summer conditions, after WAA has reached high population growth in the spring in Australia (Asante & Danthanarayana, 1992). When used in combination *F. auricularia* and *A. mali* can keep WAA below threshold levels in Australian orchards (Nicholas, Spooner-Hart & Vickers, 2005; Quarrell, Corkrey & Allen, 2017), highlighting the need for multiple natural enemies for effective biological control. Understanding of variation in both WAA and natural enemies throughout the year can also allow for the prediction of when beneficial insects are likely to be present in the orchards, giving time windows to remove chemical control (Bangels *et al.*, 2021). Removing the use of broad-spectrum insecticide for control of codling moth (*Cydia pomonella* L.) has been observed to reduce WAA numbers in Australian orchards, as a result of the increased abundance of natural enemies associated with a reduction in pesticide spraying (Nicholas, Spooner-Hart & Vickers, 2005). Maintaining a balance of synthetic pesticides and biological control agents is crucial for an effective IPM strategy. Application of oil products (paraffin and/or neem oil) to apple trees was found to reduce WAA infestation which, when combined with a controlled release of earwigs, resulted in a reduction in WAA infestation coverage by almost 100% at one site in Germany (Toups *et al.*, 2008).

Alternative forms of biological control have been tested against WAA with some success, although widespread use of these other methods is yet to be seen. Biological control of root-feeding aphids can be achieved using the entomopathogenic nematode worm *Steinernema carpocapsae* (Weiser). Exposing WAA to *S. carpocapsae* in water under laboratory conditions lead to significant WAA mortality (Brown *et al.*, 1992). When *S. carpocapsae* was applied to trees in West Virginia using a broadcast spray treatment, a significant reduction in WAA root colonies was seen although no reduction was seen when the nematodes were applied as a topdressing treatment (Brown *et al.*, 1992).

Woolly apple aphid control using RNA interference (RNAi) has been achieved under laboratory conditions. RNAi functions by introducing RNA to a target organism, which disrupts the normal functioning of the corresponding gene and subsequently silencing it. When carried out for a critical gene this can lead to organism death (Cagliari *et al.*, 2019). Using RNAi to target the V-ATPase subunit D (ATPD), an essential proton pump for proton transport across cellular membranes (Collaco *et al.*, 2013), would be beneficial due to its vital role in the function of most organelles (Mohan *et al.*, 2021). Topical application of double stranded RNA (dsRNA) for ATPD resulted in 40.5% silencing for the ATPD gene in WAA

after twenty-four hours under laboratory conditions, although when combined with nanocarrier transport molecules this increased to 98.5% gene silencing (Guo *et al.*, 2022). The WAA treated with dsRNA and nanocarriers showed 55.75% mortality five days after treatment, demonstrating that RNAi has potential for WAA control through topical dsRNA application. Field applications of RNAi for pest control have been explored in many insect species and are broadly successful, although further work into field stability and safety of RNAi treatment for non-target organisms is needed for successful widespread use of RNAi for WAA control (Cagliari *et al.*, 2019).

Cultural control

Commercial use of crab apples

Many apple varieties are self-incompatible (Broothaerts, Nerum & Keulemans, 2004), meaning that in a single-variety orchard an external pollen source is needed in order to guarantee pollination and fruit set; these are commonly referred to as pollinisers. It is common practice in apple production to introduce crab apple (Malus) species which are compatible and flower at the same time as the main commercial apple variety. They are also easily distinguishable from the main crop, which other varieties of *M.* × *domestica* may not be (Kendall & Smith, 1975) and often have many blossoms which act as a vital pollen source (Church, Goodall & Williams 1974).

Pollinisers must have a similar flowering time and a compatible pollen type to the crop to ensure successful pollination is possible (Sakurai, Brown & Weeden, 2000). It is crucial that pollinisers be close enough to the main crop to ensure pollen spreads to all crop trees through pollinator activity (Free, 1962), therefore pollinisers must be planted regularly throughout the crop. These can, however, create reservoirs for pests and diseases if the pollinisers are susceptible. Resistant pollinisers may also help to control pest build up within orchards. When selecting a polliniser for commercial use, a number of considerations must be made: pollen and cropping season compatibility with the cropping variety; attractiveness to pollinators; ease of cultural management and crab apple placement within the orchard. A ratio of polliniser to cropping trees of 1:5 crab apple pollinisers to crop trees is typical (Dray & Campbell, 2007). Any efforts made to use cropping scion and rootstock varieties carrying pest and/or disease resistance should also be considered for pollinisers to prevent them from becoming reservoirs of a pest or disease within the crop.

Pest dispersal and transmission

Woolly apple aphids are mostly distributed by infected nursery stock, providing several points for phytosanitary checks to be carried out to reduce their spread (Barbagallo *et al.*, 1997; Hetherington, 2009). Woolly apple aphids can produce asexual alates, however they are not

especially mobile; apterous adults are mostly sessile and do not readily disperse around the orchard. Alate dispersal can be aided by wind flow, although this can be impeded by geographical features such as mountain ranges and rivers in Chile, where this study was carried out (Lavandero *et al.*, 2009a). The Rothamsted Insect Survey has operated a system of twelve-metre-tall suction traps in the United Kingdom since 1964, which continuously suck air from their immediate areas, indiscriminatingly trapping any small insects nearby, especially aphids. The survey has been monitoring for WAA since 2018, but has not yet recorded any individuals (Greenslade, 2021, *pers*. *comm*.). This may be the result of WAA alate production being a much less common occurrence than for other aphid species, or because of the poor flight shown by these alates, although the survey has recorded other *Eriosoma* spp. including *E. patchiae* and *E. ulmi* (Greenslade, 2021, *pers*. *comm*.).

Orchard management

Tree and orchard management is key at all points throughout the year to control population build up and to prevent aphid movement around individual trees and the orchard. Woolly apple aphid is a largely sessile species, except for its first instar “crawlers” and therefore is unlikely to travel long distances. Aphid spread through an orchard can be reduced by low-density planting where canopies do not overlap, or selective pruning of overlapping branches which removes a physical pathway for the aphid, although this does have implications for reduced yield (Hetherington, 2009). The slow-moving nature of WAA does allow targeted insecticide application only to affected trees, rather than blanket spraying, which reduces the cost and negative side effects associated with pesticide applications. Summer pruning can be utilised both to remove large WAA colonies and to thin the canopy to allow better penetration by aerial insecticide sprays. Suckers and water shoots from the base of the tree and major scaffold limbs are often favoured by first instar WAA nymphs as a readily available source of phloem sap when moving back to the canopy from the rootstock in the spring, making these sites ideal targets for hygiene pruning and/or targeted pesticide application. Pruning injuries can be a vulnerable site for aphid feeding, but painting large wounds with a wound sealer can discourage aphid colonies from establishing, which has been trialled in Australia (Hetherington, 2009).

Although reported mostly on apple, WAA has several other hosts which may act as pest reservoirs if found near apple orchards. *Pyracantha coccinea* is a rosaceous tree which has been found as hedgerows flanking apple orchards in Chile and can act as a host for WAA. It is, however, thought to be unlikely in this case to act as a pest reservoir as WAA reared on *P. coccinea* showed less rejection of *M.* × *domestica* than for *P. coccinea* whereas WAA reared on *M.* × *domestica* maintained a preference for apple (Lavandero *et al.*, 2011). This

suggests that WAA are unlikely to be moving from the main apple crop into *P. coccinea* hedges, whereas there is a slight chance that they may move from the hedge into the crop.

The presence of wildflower strips in orchards has been widely investigated with regards to benefitting pollinator diversity, but there is also an observed benefit to natural enemies in the orchard. Planting of sweet alyssum flowers (*Lobularia maritima* L. Desv.) near to orchards lead to significant and rapid reduction in WAA numbers on apple trees (Gontijo, Beers & Snyder, 2013). This was found to be a direct result of sweet alyssum flowers attracting generalist natural enemies, which subsequently moved from the sweet alyssum plantings into the orchard in West Virginia, providing some WAA control (Gontijo, Beers & Snyder, 2013). The beneficial effects of wildflower plantings appear to be variable, as in some cases perennial wildflower sowing in UK orchard alleyways has been observed to have no effect on WAA numbers in crop trees or on incidence of WAA parasitism by *A. mali* (McKerchar *et al.*, 2020). In a separate study, WAA incidence was found to be significantly higher in plots where annual and perennial flowering plants had been sown in the alleyways than when alleyways were either bare ground or mowed grass (Markó *et al.*, 2013). This may be because floral alleyways create cool and moist conditions which are beneficial to WAA, compared to mown grass or bare ground.

The use of cover cropping in California orchards, however, was observed to lead to reduced pest levels, including aphids, leafhoppers and codling moth and greater incidence and diversity of soil-dwelling natural enemies (Altieri & Schmidt, 1986). This appeared to function through attracting pest species into the cover crops where they were then predated by natural enemies, rather than encouraging the movement of natural enemies into the crop.

Woolly apple aphid resistant rootstocks

The practice of grafting two portions of an apple tree, the scion and rootstock, to create a tree with multiple agronomically advantageous traits has been in practice since ancient Greece (Cummins & Aldwinckle, 1983). Scions are chosen for fruit quality, weather tolerance, and pest resistance; beneficial rootstock traits include influence on scion productivity and adaptations to soil conditions (Cummins & Aldwinckle, 1983). Dwarfing is a key trait, especially for dessert apple growers to control overall tree size, making harvesting fruit by hand much easier. Rootstock breeding has always aimed to incorporate multiple desirable traits for commercial use, for example WAA resistance, fruit quality, and dwarfing (Agapito-Tenfen *et al.*, 2015). The rootstock M.M.106, for example, confers WAA resistance and is semi-vigorous, but shows a much lower yield than other dwarfing rootstocks, making the choice to use M.M.106 in an orchard dependent on the individual needs of the grower (Kosina, 2010).

The first recorded rootstock breeding programme began at the East Malling Research Station in 1917, which joined the John Innes Institute at Merton after the first World War to form a national rootstock breeding programme. The programme became commercially funded in the 1990s and has existed as the East Malling Rootstock Breeding Club from 2008 until 2021 (Cummins & Aldwinckle, 1983; Evans *et al*, 2011; Fernández Fernández, 2024, *pers*. *comm*.). Multiple other rootstock breeding programmes have arisen around the world, each with different priorities for breeding. Increased dwarfing and productivity are often key targets, although increased cold hardiness was the main objective of the Budagovsky rootstock series, established at Michurinsk College in the USSR, now Russia (Crassweller & Schupp, 2021). Cold tolerance was of interest to the Polish rootstock breeding programme which was developed between 1998 - 2006, although yield, dwarfing and fire blight resistance were also investigated (Czynczyk & Bielicki, 2012; Zurawicz *et al.*, 2011). Canadian rootstock breeding programmes have also incorporated cold tolerance. The rootstock Ottawa 3 was introduced in 1974 and is cold hardy with resistance to the oomycete pathogen *Phytophthora cactorum* (Lebert & Cohn) and some apple viruses, but is susceptible to WAA (Spangelo *et al.*, 1974)**.** Ottawa 3 has been used as a parent several times in the Geneva Rootstock Breeding Programme which is run by Cornell University from the New York State Agricultural Experiment Station in Geneva, New York State. This programme began in 1968 and merged with the United States Department of Agriculture in 1998. Key goals of this programme include selecting for resistance to fireblight, crown, and root rots (caused by *P. cactorum*), and apple replant disease, although they also select for other disease and pest resistance (Crassweller & Schupp, 2021). The Geneva Rootstock Breeding Programme has generated WAA-resistant rootstocks including G.41 and G.202 (‘M.27’ × ‘Robusta 5’), G.222 (‘Robusta 5’ × ‘M.27’), and G.214, G.890 and G.969 (‘Robusta 5’ × Ottawa 3). Chinese rootstock breeding programmes have been mostly focused on abiotic stressors (Wang *et al.*, 2019). The JM rootstock series started at the Apple Research Centre NIFTS in Morioka, Japan in 1972 with a range of objectives, including WAA resistance which three of the rootstock series carry, conferred by *Malus prunifolia* (Borkh.) ‘Seishi’ (Soejima *et al.*, 2010). A new wave of rootstock breeding projects began at New Zealand Plant and Food Research in 1993 with specific goals of moving away from dependence on ‘M.9’ rootstocks and to improve pest and disease resistance (Wang *et al.*, 2019). To the author’s knowledge, there are currently two active rootstock breeding programmes which cite WAA resistance as a research objective: the Geneva Rootstock Breeding Programme, and the rootstock breeding programme at New Zealand Plant and Food Research.

There is some debate as to whether rootstocks and scions can confer resistance to each other. Small interfering RNAs (siRNAs) are generated in plants as an RNA interference (RNAi)-based defence response to viral infection. A sweet cherry (*Prunus avium* L.) rootstock transformed with hairpin RNA (hpRNA) to carry resistance to the Prunus necrotic ringspot virus (PNRSV) has been recorded to confer PNRSV resistance to the scion up to 1.2 m above the graft union (Zhao & Song, 2014). This demonstrates that it is possible for resistance genes carried in the rootstock to mediate resistance in a grafted scion. This work used transgenic rootstocks, which had been transformed with PNSRV-hpRNA, mediated by the bacterium *Agrobacterium tumefaciens* (Smith & Turner) and it may be that conventional breeding does not generate sufficient siRNAs to confer resistance. Woolly apple aphid resistance is thought to predominantly be conferred through structural variation factors, and is therefore not likely to be transmissible (NIAB, 2022), compared to molecular variation in plant defence, such as that required for pathogen resistance. This, combined with the reduction in available control methods for the canopy highlights the need for scion breeding to also incorporate WAA resistance.

1.4. Pest and disease resistance in apple

1.4.1. Plant-insect interactions

"Knowledge of the biochemical and molecular background of insect-plant interactions is a prerequisite for optimising breeding for resistance" (Qubbaj, Reineke & Zebitz, 2005).

Historically, discussion of plant resistance to herbivores has focused on three broad mechanisms of resistance, as suggested by Painter (1951):

1. Non-preference; deters aphids settling on a plant (later named antixenosis by Kogan & Ortman (1978);
2. Antibiosis; inhibits insect growth, development, reproduction etc.;
3. Tolerance: a trait which does not prevent herbivory, but allows the plant to withstand injury without affecting yield.

More recently, however, issues with using a simplistic approach to plant-insect interactions have been debated, as this forces resistance into artificial categories whereas, in real life, there are some mechanisms which do not fit into these categories and some which overlap between them (Stout, 2013). Figure 1.8 shows a comparison of these two resistance classification principles.

Plant-aphid interactions are based on a molecular exchange between host and herbivore which leads to variation in aphid virulence depending on the specific combination of host and herbivore genotypes (Kanvil, Powell & Turnbull, 2014). A resistance phenotype in plants suggests an incompatibility between herbivore and host, which has reduced aphid growth and reproduction to a point where herbivory is no longer a threat to the plant. When heritable, this suggests a genetic component such as the presence of one or more resistance genes, or of a polygenic trait (Dogimont *et al.*, 2010).

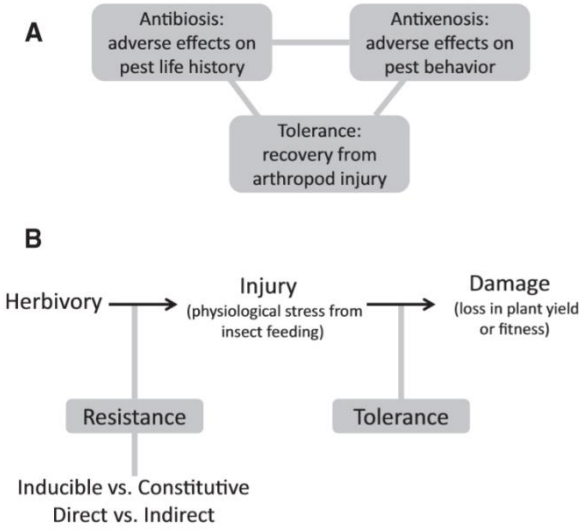

Figure 1.7: Schematic showing the comparison between traditional (A) and more up-to-date categorisations (B) of host-plant resistances (Stout, 2013).

1.4.2. Marker technologies

Microsatellite markers (also known as simple sequence repeats (SSRs)) are short repetitive DNA sequences spread throughout the genome. Simple sequence repeats are highly reproducible and polymorphic and have been widely used in plant breeding as they are an improvement on earlier RAPDs and AFLPs (Mammadov *et al.*, 2012). Because of their prevalence in the genome and high polymorphism between individuals, SSRs are excellent candidates for genetic mapping.

More recently, single nucleotide polymorphism (SNP) markers have become much more widely used. Single nucelotide polymorphisms are substitutions of a single nucleotide at a specific position for another nucleotide and are much more widespread throughout the genome than microsatellites, allowing the development of saturated genetic maps. Saturated maps offer greater marker coverage of the genome, increasing the likelihood and speed of identifying markers closely associated with the gene(s) of interest (Mammadov *et al.*, 2012). Maps with high coverage can not only identify markers for single genes but also give more precise locations of QTLs (Ganal *et al.*, 2012), which are often responsible for pest and pathogen resistance.

Diversity Arrays Technology (DArT) allows whole-genome identification of SNPs for species with complex genomes and little prior knowledge of their genetic structure, making them especially useful for crop species with complex genomes, including apple (Wenzl *et al.*, 2004; Velasco *et al.*, 2010). Schouten *et al.* (2012) created the first DArT linkage maps for apple, generating high quality DArT markers. A segregating cross of *Malus fusca* (Schneid) and *M.* × *domestica* was mapped for fire blight resistance and DArT markers were used to identify a linkage group which was significantly linked to a QTL for fire blight resistance (Emeriewen *et al.*, 2014). Using the 'Golden Delicious' genome sequence (Velasco *et al.*, 2010) the genomic locations of the DArT markers were found and SSRs developed at those sites to determine the region of the QTL associated with the fire blight resistance. This study demonstrates how different marker technologies can be integrated to identify and locate resistance QTLs. The use of the more recently published apple genome (Daccord et al., 2017a) with SNPs would be useful in future studies of this type to give more precise mapping of the traits of interest.

Genotyping-by-Sequencing (GBS) is a Next-Generation Sequencing (NGS) approach which uses a combination of specific restriction endonucleases and barcode adapters to generate genomic libraries which are sequenced with NGS technologies to generate large numbers of SNPs which can be used for high coverage 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). Novaes *et al.* (2008) used high-throughput SNP identification on a Eucalyptus species with no prior sequence information to generate many SNPs which were later used as part of a full sequence of the genome. The potential to use SNPs on genomes with little to no genetic information available highlights its potential usage in identification of novel genotypes, for example for resistance mapping. The woolly poplar aphid (*Phloeomyzus passerinii* Signoret) is a major pest of cultivated species in the genus *Populus* (poplars). Simple sequence repeats and GBS-based mapping of a cross between resistant *Populus deltoides* (Bartr. ex Marsh) and susceptible *Populus nigra* L. found three QTLs associated with genetic variance following attack by *Ph. passerinii* (Carletti *et al.*, 2016). Within these QTL regions, three genes associated with disease resistance were identified, and may be candidates for *Ph. passerinii* resistance.

The published maps to date for all four WAA resistance genes use microsatellite markers (Table 1.2). For these genes, the use of SNPs would allow fine mapping to give more accurate gene positioning, as seen in the fine mapping of the *Rag1* gene for soybean aphid (*Aphis glycines* Matsumura) resistance in soybean (*Glycine max* L.). The *Rag1* resistance gene was first identified in the cultivar 'Dowling' and mapped using SSRs to a 12 cM interval

on soybean chromosome 7 (Hill, Li & Hartman, 2006; Li *et al.*, 2007). Using SNPs it was possible to generate a fine map of the region containing *Rag1*, narrowed down to approximately 0.12 cM (115 kb), a *ca*. 10-fold narrower interval than identified by SSRs (Kim *et al.*, 2010). Fine mapping of WAA resistance genes using SNPs is hoped to produce similar results.

The self-incompatibility (SI) locus encodes a pistil-specific ribonuclease (S-RNase) which rejects pollen tubes growing from pollen with SI locus incompatible with that the pistil, preventing successful pollination (Brancher *et al.*, 2020). There are 120 possible combinations of the 16 most common SI alleles in apple, making it relatively rare to find incompatible pairs of cultivars which are not related (Hegedűs, 2006). Traditionally, SI could be determined by measuring pollen tube growth and staining for *S-RNase* (Bošković & Tobutt, 1999) but molecular markers can now be used to identify SI loci and therefore remove incompatible pairings (Brancher *et al.*, 2020).

Self-incompatibility was thought to be an issue of concern in WAA resistance breeding because it was reported by Knight *et al.* (1962), that *Er1* was likely linked to the SI locus but this has, however, been more recently established as not the case, as *Er1* has been mapped to LG 08 and the SI locus to LG 17 (Tobutt, Boškovic & Roche, 2000*; Bus et al.*, 2008). *Er2* is also located on LG 17, but the genetic distance between them is > 50 cM and therefore the regions segregate independently, suggesting that the strong segregation distortion shown by *Er2* is not related to its proximity to the SI locus (Bus *et al.*, 2008). Significant marker distortion was found at the bottom of LG 17 around the SI locus in M432 (Evans *et al.*, 2011) but this was to be expected in a semi-incompatible backcross.

1.4.3. Pathogen resistance in apple

Historically, disease resistance in plants has been more thoroughly studied than herbivore or insect resistance and therefore the two disciplines often have very similar aims. Aphid feeding induces both pathogen and herbivore defence responses (Korban & Tartarini, 2009), meaning that the processes of identifying and mapping disease resistance genes can therefore offer a template of how aphid resistance gene mapping can progress.

Apple scab (*Venturia inequalis,* Winter) is a major fungal disease of temperate apple production. Scab resistance genes were identified by Hough (1944) and later named *Rvi6* (*Venturia floribunda* after *M. floribunda*) (Williams, 1966). The prevalence of scab and the severity of the disease means that scab resistance has been well studied and the process of resistance gene identification, marker development and mapping has been completed, making it a good template for resistance gene identification and location in the genome. Scab

resistance occurs in several *Malus* spp. (e.g. *M. prunifolia, M. baccata*) and was found to be conferred by the same allelic genes as *Rvi6* resistance in *M. floribunda* (Williams, 1966). Isozyme analysis identified molecular markers associated with *Rvi6* resistance, located approximately 8 cM from the *Rvi6* gene (Manganaris *et al.*, 1994). Other marker types have subsequently been identified for *Rvi6,* which have narrowed down the region of the genome in which *Rvi6* is located (SCAR markers developed by Tartarini *et al.*, 1999). Map-based cloning of two markers located by Tartarini *et al.* (1999) gave a fine-scale physical map of the area around *Rvi6* between these flanking markers (Patocchi, Gianfranceschi & Gessler, 1999). These and other maps identified multiple genes in the *Rvi6* region (Xu & Korban, 2002). SNP markers (single nucleotide polymorphisms) have been developed for three of these genes and have been validated for future use in Marker Assisted Selection (Chagné *et al.*, . One of these genes (*HcrRvi62*) has been successfully used to transform the susceptible variety 'Gala' to give scab resistance (Vanblaere, 2011). Some breakdown of *Rvi6* resistance has been reported, which is suspected to be the result of widespread use of scab-resistant cultivars (Lespinasse, 1989; Schouten & Schenk, 1997). A future aim of breeding programmes working on scab resistance is to pyramid *Rvi6*-associated genes from different resistant cultivars to give durable resistance (Gessler & Pertot, 2012).

Fire blight, caused by the bacterium *E. amylovora*, can rapidly cause widespread cankering resulting in huge crop destruction and major economic losses (Vanneste, 2000). *Malus* × *robusta* (Rehder) shows Fire Blight (FB) resistance which Gardner, Cummins & Aldwinckle (1980) suggested was conferred by a single major gene. Fire blight resistance has since been found to be controlled by Quantitative Trait Loci (QTLs) located across several Linkage Groups (LGs), making it a much more complex trait than previously thought (Korban *et al.*, 1988; Calenge *et al.*, 2005). Simple sequence repeat (SSR) markers across all apple LGs were used to identify alleles from *M. robusta* which were associated with FB resistance and to locate a major FB resistance gene (Peil *et al.*, 2007). One of these markers, CH03e03, was found to be diagnostic for FB resistance, hence any trees carrying this marker are highly likely to carry the FB resistance trait. In more recent years SNP markers have been developed for *M. robusta*-derived FB resistance which have also been approved for marker assisted selection (Chagné *et al.*, 2019). Analysis of the *Malus* × *arnoldiana* accession MAL0004 which segregates for FB resistance was located to the 0.57 cM region with SSRs and aligned to the 'Golden Delicious' genome, giving a similar region to where FB resistant loci were identified in both *M. floribunda* 821 and 'Evereste' (Emeriewen *et al.*, 2014). Fine-mapping of 2,133 'Robusta 5' progeny identified flanking SSR and SNP markers for a region associated with FB resistance and identified an NBS–LRR (nucleotide-binding site leucine rich repeat) protein within this region (Fahrentrapp *et al.*, 2013).

Linkage group is the term used to refer to groups of genes which are inherited together, in linkage. Positions of genes in a linkage map, usually given in cM, are determined by their linkage to other genes. These often correspond with chromosomes, although the specific physical positions of genes on a chromosome cannot be determined without genetic sequencing.

1.4.4. Host pathogen/aphid resistance

Aphid feeding is unusual in that it is perceived by the plant as a mid-point between herbivore and pathogen damage (Kaloshian & Walling, 2005) and induces both the Jasmonic Acid (JA) and Salicylic Acid (SA) pathways respectively, causing large-scale transcriptomic changes in plants (Schoonhoven *et al.*, 2005). The specific pathways affected will vary depending on the specific combination of host and aphid. Jasmonic acid- and SA-regulated genes associated with defence against disease development in sorghum (*Sorghum bicolor* (L.) Moench) are expressed following feeding by the wheat aphid (*Schizaphis graminum* Rondani; Zhu-Salzman *et al.*, 2004). *Acyrthosiphon pisum* also induces JA and SA pathways when feeding on barrel clover (*Medicago truncatula* Gaertn.; Stewart *et al.*, 2016).

Plant systemic acquired resistance (SAR) is analogous to mammalian acquired immunity insofar as following inoculation with pathogen material, a systemic immune response is seen which prevents disease emerging both after this and any subsequent inoculations (Cruickshank & Mandryk, 1960). The resistance conferred by SAR is systemic, long-lasting and non-specific, offering protection across the whole plant for up to months at a time and across pathogens which are unrelated to that which caused the original infection (Lucas, 1999). In Burley tobacco (*Nicotiana rustca* L.), this induced immunity has been shown to spread across a scion/rootstock graft union (Tuzun & Kuć, 1985). SAR has been reported in apple leaves against *Erwinia amylovora* under field conditions within 10 days after injection with a preventative fungicide, acibenzolar-S-methyl, which significantly induced the induction of protein genes related to SAR response (Aćimović *et al.*, 2015).

The largest group of genes associated with aphid immunity and resistance in plants are the nucleotide-binding site leucine-rich repeat (NBS-LRR) genes. Activation of these genes is triggered by effectors in aphid saliva and initiates effector-triggered immunity (ETI) to aphids (Boissot, 2023). These genes are typically associated with pathogen-triggered immunity (PTI) but are also triggered by piercing aphid damage. The zigzag model, proposed by Jones and Dangl (2006), outlined a two-part model of the innate immune response observed in plants upon perception of pathogen effector molecules. Pattern-triggered immunity (PTI) is the recognition of pathogen-associated molecular patterns (PAMPs), molecular triggers which are conserved across classes of microbes, both pathogenic and not. Herbivores, both

chewing and piercing, produce herbivore-associated molecular patterns (HAMPs) in saliva (Hougenhout & Bos, 2011). Aphids which are unable to secrete effectors which suppress HAMP-triggered immunity (HTI) will be affected by the HTI response in plants and cease feeding. This can lead to an adaptive “arms race” between plants, creating the second part of the zigzag model. In response to PTI, pathogens may produce effectors which interfere with and overcome PTI, leading to effector-triggered susceptibility (ETS) of the host plant. Lastly, selective pressure from pathogen infection and ETS may lead to emergence of plant NBS-LRR alleles which recognise and suppress the effectors involved in ETS, leading to effector-triggered immunity (ETI) (Jones & Dangl, 2006). This is repeated as new pathogen effectors emerge and require plant immune responses to those new effectors. Aphids also invoke HTI followed by ETI, similarly to the zigzag model developed for pathogen infection (Hougenhout & Bos, 2011). It is also possible for HTI to be suppressed by the aphid but for these effectors to the be recognised by *R* genes, such as NBS-LRR genes, which will trigger plant resistance to the aphid.

The brown planthopper (*Nilaparvata lugens* Stål; BPH) is a sap-sucking Hemipteran pest of rice (*Oryza sativa* L.), causing both mechanical damage and through transmission of the rice grassy stunt virus which reduces rice growth and yield (Hibino, 1986). At least 24 resistance genes have been identified from domesticated *Oryza sativa* L. and wild *Oryza* species (Cheng, Zhu & He, 2013), named *Bph* (brown plant hopper) and their numerical identifier. Du *et al.* (2009) identified and characterised the resistance gene *Bph14* from rice which confers resistance to BPH through activation of SA-pathways which increased deposition of the plant cell wall component, callose into phloem cells, preventing BPH feeding. *Bph14* is expressed in vascular bundles and encodes a CC-type NLR gene, the LRR domain of which is thought to recognise BPH feeding and activate the SA pathway. Salicylic acid pathways are normally associated with pathogen defence, demonstrating how the BPH are perceived, at least partly, by plants as pathogens, as observed in aphids. Brown planthopper salivary molecules are recognised by a lectin receptor kinase (LecRK), encoded by *Bph15*, which function as pattern recognition receptors (PRRs) which are activated following HAMP recognition (Hones & Dangl, 2006), fitting the zigzag model. Virulence against the major resistance gene *Bph1* is thought to be conferred by a single recessive gene which overcomes or circumvents the resistance conferred by *Bph1* (Kobayashi *et al.*, 2014). The emergence of gene-for-gene relationships between rice and an insect pest demonstrates the potential for this system to exist for herbivory, although historically more reported in host/pathogen relationships. *Bph14* and *Bph15* have been successfully pyramided using Marker-Assisted Selection introgression methods to improve BPH-resistance while maintaining rice yield, under field conditions (Hu *et*

al., 2012). This provides a framework for how pyramiding resistance genes can be utilised to overcome a resistance-breaking virulence trait.

The *Vat* (virus aphid transmission) gene is a coiled coil (CC)-type NLF gene. The *Vat* gene, identified from melon (*Cucumis melo* L.) inhibits non-persistant virus transmission by the melon-cotton aphid (*Aphis gossypii* Glover), but not by *M. persicae* (Chen *et al*., 1997). The nematode-resistance CC-type NLR gene *Mi*, derived from tomato (*Solanum lycopersicum* L.) was found to also confer resistance to the potato aphid (*Macrosiphum euphorbiae* Thomas); the first demonstration that plants can confer resistance across multiple phyla (Rossi *et al.*, 1998). *Mi*-mediated resistance is highly specific to individual aphid isolates and varies with aphid species and biotype. When transformed with *Mi*, previously susceptible tomato lines showed resistance to the same *M. euphorbiae* biotypes previously used when developing resistant lines (Rossi *et al.*, 1998). It is not, however, effective against *M. persicae* or *M. euphorbiae* biotypes sourced from America but was effective against French and Dutch biotypes (Goggin, Williamson & Ullman, 2001).

A massive immune response is observed after feeding of the peach-potato aphid (*Myzus persicae* Sulzer) on Rubira, a *M. persicae*-resistant rootstock of peach (*Prunus persica* L. Batch; Le Boulch *et al.*, 2022). Genes for cell surface receptors involved in pattern-triggered immunity, and cytoplasmic NBS-LRRs for effector-triggered immunity were upregulated within 48 hours of *M. persicae* feeding. Localised cell death and the oxidative stress response are observed around feeding punctures, including further NBS-LRRs, and production of hydrogen peroxide. These factors lead to anti-xenosis of *M. persicae* which, combined with associated reduced nutritional quality of the apices, prevents aphids from establishing on Rubira, and therefore feeding for long enough to transmit plant viruses, for example the Peach Latent Mosaic Viroid (Jo *et al.*, 2015).

Pinellia pedatisecta Agglutinin (*PPA*) is a lectin molecule isolated from *Pinellia pedatisecta* (Schott) which, when transformed into sugarcane (*Saccharum officinarum* L.), is effective at preventing feeding of the sugarcane woolly aphid (*Ceratovacuna lanigera* Zehntner) (Zhao *et al.,* 2022). *PPA* transgenic sugarcane shows increased stomata number, but decreased individual stomata size which affects the transpiration rate of the plant and thereby the passive feeding of the aphids. *PPA* is also associated with increased antioxidant and tannin production in sugarcane leaf material, and reduced sugar production, which further deters aphid feeding.

Resistance to apple aphids

Phloem-related resistance in apple has been shown in response to multiple aphid species including *D. devecta* and *D. plantaginea*, and to *A. pomi* (Stöckli, 2008). A segregating F_1

population of crosses of ‘Fiesta’ and ‘Discovery’ were surveyed and scored for infestation with *D. devecta*. Parental linkage maps were used with scores for *D. devecta* infestation to carry out QTL analysis for aphid resistance and amplified fragment length polymorphism (AFLP) markers, which were linked to a QTL for *D. devecta* resistance were identified (Liebhard *et al.*, 2003a). These markers selectively amplify fragmented genomic DNA (gDNA) using a Polymerase Chain Reaction (PCR) approach and offer very sensitive detection of genetic polymorphisms when visualised using gel or capillary based electrophoresis.

Dysaphis plantaginea resistance is phloem-related and likely determined by a resistance factor in the sieve element sap, preventing effective feeding (Marchetti *et al.*, 2009). When infested with *D. plantaginea*, *Malus* × *domestica* ‘Florida’ (*Malus floribunda* 821 × Rome Beauty), an aphid resistant cultivar, showed up- and down-regulation of several defensive genes related to wound-signalling pathways and defensive cytotoxicity, showing differential expression between infested and non-infested leaves and resistant and susceptible cultivars (Schaller & Oecking, 1999; Qubbaj, Reineke & Zebitz, 2005). Aphid resistance in plants usually come from a relatively small number of resistance alleles (Dogimont *et al.*, 2010), limiting available material for resistance breeding.

1.4.5. Proposed mechanisms of woolly apple aphid resistance

An early study of WAA resistant cultivars found that the mechanism of resistance was phloem related (Staniland, 1924). Unlike *D. plantaginea* resistance, it is related to the sclerenchyma rather than the sieve tube element. Resistant genotypes show thickened masses of sclerenchyma without gaps, a pattern which is also seen in younger tissue which has smaller, more dispersed masses of sclerenchyma and is more susceptible to WAA than more mature tissue (Staniland, 1924). Large masses of sclerenchyma physically prevent feeding because the aphid’s stylets cannot penetrate or circumvent the mass to reach the phloem; gaps in the sclerenchyma increase the likelihood of successful attack. Sclerenchyma thickness also confers resistance in wound tissue, a frequent site of WAA colony formation. The resistant cultivar ‘Transparente de Croncel’ was found to have eight layers of sclerenchyma within wound tissue whereas susceptible Cox’s Orange Pippin had two rows with irregular masses of sclerenchyma with large gaps (Staniland, 1924).

Sclerenchyma is a specialised plant tissue with a lignified secondary cell wall consisting of cellulose and hemicellulose fibres which provide tissue stiffness and strength, but are also flexible, allowing movement, for example, under windy conditions (Jarvis, 2012). Sclerenchyma in *Malus* × *domestica* are formed of an open ring of fibres and a thick-walled, open ring of sclerids. Crab apple species seem to have more intact sclerenchyma than

cultivated apple; *Malus sylvestris, M. sieversii, M. orientalis*, and *M. hupehensis* (Rehder) have an open ring fibre arrangement and thick-walled closed rings of sclerids, whereas *M. baccata* has a closed ring of fibres and a thin-walled closed ring of sclerids (Horbens *et al.*, 2014). It is possible that commercial breeding has led to thinner sclerenchyma, which may make *M.* × *domestica* vulnerable to aphid feeding. Crab apple species with intact sclerenchyma may be an important genetic resource for breeding resistance to phloem-feeding pests.

Electrical Penetration Graph (EPG) analysis found that feeding duration and relative proportions of different types of feeding varied between four scion cultivars with varying reported WAA resistance (Hao *et al.*, 2020). The pre-phloem stage observed before probing is often shortened when aphids are unable to find a suitable feeding location because of a physical resistance or repellent. Resistance factors associated with sieve tube elements lengthen the duration of the pre-feeding stage. Both the pre-phloem and pre-feeding stages were observed to be shorter in the highly susceptible variety Red Fuji than in the more tolerant 'Ralls Genet', suggesting that 'Ralls Genet' carries a resistance factor likely associated with preventing phloem access (Hao *et al.*, 2020). Preference to feed on young tissue was confirmed by EPG analysis of individual adult WAA feeding on shoots, trunks and leaves of potted 'Starkrimson' seedlings (Zhou *et al.*, 2021) which found significantly longer phloem ingestion when feeding on shoots than on trunk or leaf material

Whist approximately ten cultivars have been reported as showing variable levels of WAA resistance or tolerance, which affect aphid growth and behaviour in different manners, four resistance sources have been postulated as single major genes and their inheritance described. Parameters used to measure WAA resistance include rate of growth and/or reproduction, gall formation, colony size, and wool production (Sandanayaka *et al.,* 2003).

1.5. Resistance breeding

Single gene WAA resistance has been found in both cultivated and wild apple genotypes (Korban & Tartatini, 2009) and to date four distinct resistance genes have been identified, named *Er1* to *Er4*.

1.5.1. *Er1*

The American scion cultivar 'Northern Spy' shows resistance to WAA, but has poor root anchorage and sub-standard production for modern commercial requirements, however it has been valued in resistance breeding since the early 20th century (Cummins & Aldwinckle, 1983). 'Northern Spy' has thickened sclerenchyma rings compared to susceptible cultivars (Staniland, 1924) and WAA feeding on 'Northern Spy' showed a short period of phloem ingestion, suggesting that *Er1*-mediated resistance is phloem-related (Sandanayaka *et al.*,

2003). In order to combine WAA resistance with other beneficial rootstock traits, 'Northern Spy' was crossed with several rootstocks in the 'Paradise' series to create the Malling (M.) and Malling Merton (M.M.) rootstock series (Crane *et al.*, 1937). Rootstocks from the M.M. series have had widespread commercial success, including 'M.116' and 'M.M.106'.

The gene conferring WAA resistance in 'Northern Spy' was identified as a single dominant major gene in 1962 and later named *Er1* with the discovery of more genes which confer WAA resistance (Knight *et al.*, 1962; King *et al.*, 1991). Bus *et al.* (2008) refined the position of *Er1* with six families deriving from 'Northern Spy' F1 and F2 populations assessed in a single year. Families were inoculated with WAA under orchard or glasshouse conditions and scored on a six-point scale after 3 - 4 months, from 0 - 5 where 0 represents complete immunity to WAA and 5 represents complete susceptibility. Strong bimodal segregation of *Er1* was found, but some seedlings scored as resistant had WAA colonies and/or galls present suggesting that *Er1* may not be a single major gene. The authors also generated SCAR (Sequence Characterised Amplified Region) and RAPD (Random Amplified Polymorphic DNA) markers and found that *Er1* maps to the top of linkage group (LG) 08 in 'Northern Spy'.

'M.M.106' ('Northern Spy' × 'M.1') and 'M.116' (M.M.106 × 'M.27') have been widely used commercially, making them key rootstocks for further research. The M432 family is the progeny of a backcross between 'M.27' and 'M.116' produced at East Malling Research in 2003 (Evans *et al*, 2011). These parents were chosen to generate progeny which would segregate for a range of traits of interest to rootstock breeding including dwarfing, root architecture, anchorage, water use efficiency, and pest and disease resistance. The first two linkage maps developed for the M432 population comprised of 116 and 324 SSR loci and covered 1,191 cM and 1,229 cM, respectively (Evans *et al.*, 2011; Fernández-Fernández *et al.*, 2012). Antanaviciute *et al.* (2012) significantly improved marker density on the map by adding a further 3069 SNP markers, of which 107 were located on LG 08. The family MCM007 ('M.27' × 'M.M.106') is a reciprocal cross of 'M.116' and can be used to generate further markers in the region of *Er1*, and validated in M432, narrowing the interval around the gene which can be identified with flanking markers

The *Er1* gene has also been identified in the *Malus prunifolia* var. ringo rootstock (also known as 'Maruba Kaido') with the SNP marker NZsn_O05, which is linked to *Er1* in 'Northern Spy'-derived accessions, found amplified in 'Maruba Kaido' and resistant progeny (Agapito-Tenfen *et al.*, 2015). This may offer further study into differential inheritance and expression of *Er1* across different families.

1.5.2. *Er2*

An accession of the hybrid species *Malus* × *robusta*, *Malus* × *robusta* '5' (*M. baccata* × *M. prunifolia* Carr.; 'Robusta 5') is the source of the second major WAA resistance gene to be identified, *Er2*, which was first described by (King *et al.*, 1991) and attributed to accession 3762 (apple × *M.* × *robusta*). The source of the gene has subsequently been corrected to 'Robusta 5' (Bus *et al.*, 2008). Inheritance and segregation of *Er2* were described using six F1 families with 'Robusta 5' as the male parent. Seedlings were inoculated with WAA under glasshouse, nursery, or stoolbed conditions in a single year (full details in Bus *et al.*, 2008). Susceptibility phenotypes were assigned from a six-point scale after 3 - 4 months.

Er2 has been used as the source of WAA resistance in the Geneva rootstock series developed at Cornell University (Cummins & Aldwinckle, 1983). Similarly to *Er1*, *Er2* also showed slightly weaker segregation distortion than expected Mendelian ratios, with some individuals displaying minor WAA colonisation (Bus *et al.*, 2008). *Er2*-mediated resistance has also been hypothesised to be phloem-related; aphids feeding on 'Robusta 5' showed a short period of phloem ingestion (Sandanayaka *et al.*, 2003). *Er2*, however, maps to a different location on the genome, the top of LG 17 (Bus *et al.*, 2008), and has been observed to show different inheritance patterns compared to *Er1* (Mackenzie & Cummins, 1982).

1.5.3. *Er3*

The third major WAA resistance gene, *Er3*, was identified from an open-pollinated *Malus sieboldii* (Rehder) accession 'Aotea 1' (Bus *et al.*, 2002). The Aotea rootstock series was created as part of a breeding programme at New Zealand Plant & Food Research, prioritising resistance to WAA and *P. cactorum* root rot (Taylor, 1981). Inheritance of *Er3* was studied across 15 families deriving from 'Aotea 1', across three generations (F1-F3). Seedlings were screened in nursery, orchard, or stoolbed in a single year (see Bus *et al.* (2008) for details) and scored for WAA susceptibility with a six-point scale from 0 - 5. Individuals carrying *Er3* show much stronger bimodal segregation than *Er1* and *Er2*, with most individuals either completely susceptible or resistant, rather than showing Medelian susceptibility (Bus *et al.*, 2008). *Er3* maps to LG 08 of 'Aotea 1' in a similar position to *Er1* in 'Northern Spy' (Bus *et al.*, 2008). *Er1* and *Er3* are likely to be alleles with different functionality or closely-linked loci, rather than alleles conferring the same function, as *Er1* resistance has been broken by a biotype of WAA which was not able to break *Er3* (Sandanayaka *et al.*, 2003; Bus *et al.*, 2008).

1.5.4. *Er4*

The fourth and final major WAA resistance gene identified to date, *Er4*, was identified from the progeny of an open-pollinated (OP) family derived from an open pollinated selection of

'Delicious', which was developed to select for mildew immunity (Dayton, 1977). Seedlings of a cross between 'Fuji' and the accession MIS OP 93.051 G02-054 were inoculated with shoot sections infested with WAA across one growing season and scored after three months using a 0 - 5 scale of susceptibility (Bus *et al.*, 2008, 2010). *Er4* was identified, named and mapped to LG 07 from this family by Bus *et al.* (2010), near to previously mapped genes *Sd-1* and *Sd-2,* which confer resistance to *D. devecta* (Cevik & King, 2002), although *Er4* is not linked to these genes. *Er4* segregates independently from *Er1-3,* and likely confers a different, non phloem-related mode of resistance (Bus *et al.*, 2010). Although *Er4* would be a strong candidate for future work for WAA resistance, it is derived from an unknown pollen parent and the existing germplasm carrying this gene is not available in the UK at present.

Table 1.32: Details of closest flanking markers published to date for each of the four identified woolly apple aphid resistance genes, on their respective linkage groups.

Gene	Marker name	Marker type	Distance between marker and target gene (cM)	
Er1	CH01c06	RAPD	2.9	(Bus *et al.*, 2008)
	NZsc_O05	SCAR	7.9	
Er2	NZms_EB145764	SSR	5.5	(Bus *et al.*, 2008)
	GD153	SSR	17.6	
Er3	NZra_A01	RAPD	4.0	(Bus *et al.*, 2008)
	NZsc_O05	SCAR	4.1	
Er4	NZscA4F3R3	SCAR	1.8	(Bus *et al.*, 2010)
	CH04e05	SSR	9.0	

1.5.5. Single-gene resistance and gene pyramiding

Single gene WAA resistance has been found and mapped in both cultivated and wild apple genotypes (Korban & Tartatini, 2009) but generally resistance mediated by a single gene is not considered to be an effective long-term solution as they can be more easily overcome by pests and pathogens than multiple modes of action. Resistance gene pyramiding combines genes from different parents, both of which carry a target gene, to confer durable horizontal resistance (Servin *et al.*, 2004; Bus *et al.*, 2008).

Horizontal resistance protects against attack from all races or biotypes of a pest or pathogen, affecting the rate at which the host is infected, and often shows polygenic inheritance (van der Plank, 1963). This is in contrast to vertical resistance which only protects against attacks from a single pathogen race (van der Plank, 1963), although these definitions have been controversial. Nelson (1978) argued that horizontal resistance should be considered as resistance which reduces infection rate and vertical resistance reduces the amount of effective initial inoculum by removing a race from this inoculum. A desirable outcome for this project is moving towards finding durable horizontal resistance which should prevent the feeding of multiple WAA biotypes.

Whilst it is possible for single genes to show durable resistance, for example, several durable genes for disease resistance are identified in many wheat cultivars (Johnson, 2000), it is more common for single resistance genes to be broken by pathogens (Bus *et al.*, 2009). Conventional apple breeding has successfully pyramided double and triple resistances against apple scab, powdery mildew, and fire blight (Fischer, 1994). Genes *Er1* and *Er3* are good candidates for gene pyramiding because they lie in similar positions on the same linkage group. Bus *et al.* (2009) successfully pyramided these 2 genes, with 35 of the 38 progeny of a cross between 'Northern Spy' and S26R01T053 (*Er3*) found to be homozygous for the SNP marker NZsn O05, located on LG 08 and known to be associated with both *Er1* and *Er3*. There was slightly higher susceptibility to WAA than had been expected, although the authors acknowledge that this may be due to ineffective phenotyping, or the presence of sub lethal genes linked to *Er3*.

1.5.6. Resistance-breaking WAA

'Northern Spy'-derived rootstocks have been used globally with much success to prevent WAA feeding, but there have been some isolated reports of WAA observed to be feeding on these rootstocks. These reports have mostly been from the southern hemisphere, namely Australia (Self, 1966), South Africa (Giliomee *et al.*, 1968), and South America although they have also been found in North America (Rock & Zeiger, 1974) and are moving further north, with some recent observation in Norway (Jaastad, 2020, *pers*. *comm*.) and increasing anecdotal reports in the United Kingdom. This phenomenon is often reported on 'M.116' rootstocks in regions where they are not widely used and therefore represent potential pockets of resistance breaking aphids, but this is still an issue of concern for areas which widely use rootstocks with *Er1* resistance.

'Robusta 5'-mediated resistance had been thought to be immune to WAA, making the Geneva rootstock line often considered the more reliable source of WAA resistance, especially in areas with *Er1*-breaking aphids (Young *et al.*, 1982; Cummins & Aldwinckle,

1983). Reports of WAA feeding on *Er2* rootstocks began more recently and are less widespread but are an area of concern (Cummins & Aldwinckle, 1983). Since the development of the Aotea rootstock series in the 1980s, WAA have already been observed feeding on 'Aotea 1', demonstrating that *Er3* has been broken (Sandanayaka *et al.*, 2003). Woolly apple aphid feeding was measured using EPG analysis over an eight-hour period and showed a much longer feeding period on *Er3* than *Er1* or *Er2*, suggesting that *Er3* may confer a different mechanism of resistance (Sandanayaka *et al.*, 2003). Woolly apple aphid biotypes identified from New York and North Carolina (USA) showed different colonisation patterns when feeding on *Er1* rootstocks (Young *et al.*, 1982), suggesting multiple mechanisms of resistance breaking.

Woolly apple aphid with resistance breaking genotypes may be able to feed on supposedly resistant apple because varying environmental conditions can influence the expression of resistance genes (Bus *et al.*, 2008). There is some evidence for gene-for-gene co-evolution between plants and insects (Edger *et al.*, 2015). In theory this could lead to a co-evolutionary 'arms race' between host and herbivore where each trophic group co-evolves adaptations against the other. Given that there are reports of many different biotypes of WAA, it is more likely that WAA is a plastic species and will continue to overcome single gene resistances it encounters. This may, however, be slowed by the lack of sexual reproduction seen in the majority of WAA. The potential of an 'arms race' between plants and aphids was well summarised in a review by Züst and Agrawal (2016) who stated that aphids will always be able to overcome plant defence mechanisms largely through manipulating host plant defences, but that the plant will always be able to recognise aphid feeding and develop defence responses, for example initiating phloem sealing and/or the release of defensive Plant Secondary Metabolites (PSMs).

1.6. Apple genetics, genomics and breeding

1.6.1. Genome sequences of *Malus* × *domestica*

An early reference map for *M.* × *domestica* used a cross between 'Fiesta' and 'Discovery' to generate linkage maps of these two parents of length 1140 cM and 1450 cM respectively (Liebhard *et al.*, 2003b). These maps were comprised of 475 AFLPs, 235 RAPDs, 129 SSRs and 1 SCAR marker. The key advantage of this map was that it provided a framework of SSRs which would be transferrable to other cultivars, which could then be saturated with AFLPs to provide deeper resolution. Linkage mapping of the progeny of a cross between 'M.9' and **'**Robusta 5' increased marker coverage of these parental accessions with maps spanning 1175.7 cM and 1086.7 cM, respectively and comprised of 224 SSRs, 42 RAPDs, 18 SCARs and 14 SNPs (Celton *et al.*, 2009). Forty-seven new polymorphic SSRs were

identified from an EST database (expressed sequence tag) and used in the construction of these maps. Two of the SSRs identified on ‘Robusta 5’ through this mapping were later discovered to be the closest flanking markers for *Er2* on LG 17 (Bus *et al.*, 2008).

In 2010 a high-quality draft genome sequence of ‘Golden Delicious’ was generated using whole-genome shotgun sequencing, covering 603.9 cM (*ca*. 71.2 % of the genome) and comprised entirely of SNPs (Velasco *et al.*, 2010). This work was expanded in 2017 to produce a high-quality *de novo* assembly of length 625.2 Mb (Daccord et al., 2017a). Having such a comprehensive genome available is essential to limit issues within apple breeding. Not only is conventional breeding a long-term process, apple has a highly heterozygous genome and the intensive crosses involved in domestication can lead to inbreeding depression, causing gamete-incompatibility between some pairs of accessions, subsequently preventing crosses between those cultivars (Lawson, Hemmat & Weeden, 1995; Tobutt, Boškovic & Roche, 2000*;* Clark *et al.*, 2014).

1.6.2. Marker assisted selection

Once a desirable characteristic has been identified it can take 20 to 25 years from the initial breeding cross to commercial introduction and is an expensive process in terms of time, space, and money (Bianco *et al.*, 2014; Clark *et al.*, 2014). The selection of rootstock genotypes frequently takes longer than that of scion varieties as rootstock traits must also be evaluated through their effect on the scion, as well as their own characteristics. Breeding pest and disease resistance can take even longer because after resistance gene(s) have been identified, several generations of back-crossing may be required in order to guarantee a commercial product, depending on the complexity of the trait (Bianco *et al.*, 2014).

Marker-assisted selection (MAS) involves using the presence or absence of a marker or markers linked to the gene(s) encoding a desired phenotype to determine whether or not it is present. It is more efficient and cost-effective than traditional breeding methods as it reduces the time taken to ensure a desired trait is present in the crop (Collard *et al.*, 2005). Traditional breeding requires multiple stages of phenotyping and genotyping, each of which can only take place once a season for apple breeding. Marker-assisted selection will increase the precision of selection in breeding which will speed up breeding programmes as the inclusion/exclusion of an individual for a specific trait can be rapidly decided (Antanaviciute *et al.*, 2012).

SNPs have become widely used in MAS programmes because of their advantages previously discussed (Mammadov *et al.*, 2012). Genotyping arrays have been developed using SNPs for several important crop plants including apple (*e.g*. Antanaviciute *et al.*, 2012; Chagné *et al.*, 2012). Maps with high SNP density for their target genotype mean that

predictions can be made for those markers as to the phenotype of the individual, allowing inclusion/exclusion from a breeding programme without the actual process of phenotyping (Hamblin, Buckler & Jannink, 2011).

1.7. Conclusions and research outline

Woolly apple aphid on domesticated and wild apple is likely to increase with changing climatic conditions and further removal and limitation of control measures. This project has two main research objectives: to refine genetic positions of WAA resistance genes to improve rootstock breeding and to better understand WAA biology.

Identification of novel sources of resistance will always be key in a breeding programme but is especially vital once the first instances of resistance gene breakdown has been observed, as is the case with WAA resistance genes. Suggestion of a novel resistance source(s) will directly benefit future breeding programmes. The improvement of molecular breeding techniques such as marker-assisted selection for previously-characterised WAA resistance genes which have not yet had close flanking markers identified. Achieving MAS is a goal of resistance breeding, the largest obstruction to which is the identification and development of robust genetic markers for the trait(s) of interest. The existing linkage maps of the WAA resistance genes *Er1* and *Er2* provide a framework from which to identify SNP markers for both genes which have not yet been identified for *Er2* and are limited for *Er1*.

Key gaps in the current knowledge of WAA include misconceptions and a lack of understanding of the pest's life history and how it varies outside of its native range. Woolly apple aphid is a dynamic species with differing lifecycles but much published literature is not up to date and/or feature data not collected in Europe. Improving the available data on the expected lifecycle of the aphid in an orchard context by determining how likely it is that sexual reproduction will chiefly inform apple growers and agronomists of how to approach the pest in the UK, although will also be applicable to other climates. Whilst the use of WAA-resistant rootstocks, especially M.116 and MM106, is widespread in the UK, it is unclear the extent to which these rootstocks can control aphid feeding, especially under changing environmental conditions and increasingly widespread resistance-breaking aphids. Determining the extent of WAA colony control by these rootstocks will provide an expected level of control offered by resistant rootstocks. This can then influence application of synthetic plant protection products in an informed manner as a treatment rather than prevention, perhaps without WAA present.

This research aims to contribute to the understanding of WAA biology and to improve genetic resources for rootstock resistance breeding in the following experimental chapters:

Chapter 2: Materials and methodology shared across multiple chapters

Chapter 3: Phenotyping of *Malus* accessions for WAA resistance

Chapter 4: Mapping or *Er1* and *Er2* resistance genes

Chapter 5: WAA genetic diversity

Chapter 6: WAA performance on different rootstocks

CHAPTER 2 – Shared Methodologies

2.1. Phenotyping

For two rootstock breeding families, clonally propagated replicates of 139 seedlings for MCM007 and 111 seedlings for M639 were inoculated with Woolly Apple Aphids (WAA) and colony growth assessed to score each individual replicate (seedling) for susceptibility. Rootstock material was visually scored before inoculation (Table 1). All plants were inoculated with WAA, regardless of their initial score. Two inoculation sites were selected for each tree, spaced well apart, *i.e*. one inoculation site at the base of the scion and the other site on the stem. 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 near that site. A refuge for WAA was created at each inoculation site using the Duratool™ J99 Tapetool (Duratool, Taiwan) with 1 cm width PVC tape (Duratool, Taiwan) to secure a petiole to the main stem, such that the space between is covered on all sides except above (see Figure 2.1). A pea-sized amount of mixed life stage WAA was placed into this refuge using a dry, fine paintbrush. The refuge was provided to keep the aphids in position, allowing colonies to feed and build, as well as offering some protection from abiotic stressors and natural enemies.

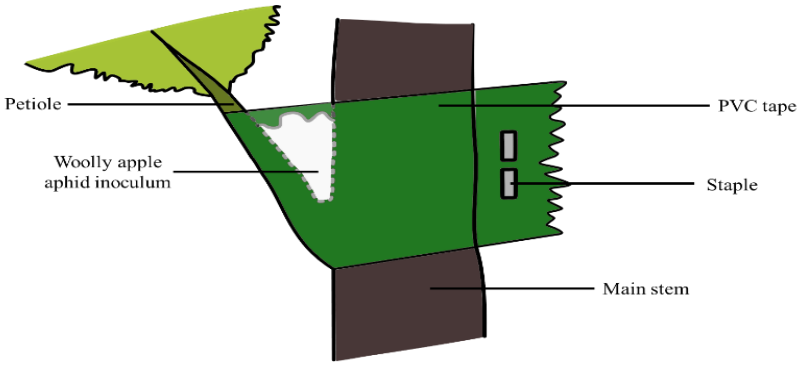

Figure 2.1: Woolly apple aphid refuge for inoculation. Two refuges were created per tree, spaced well apart. PVC tape was used to create a refuge between the petiole and main stem. A pea-sized amount of aphids was placed into each refuge to protect aphids from biotic and abiotic stressors.

After a two-week period under glasshouse or polytunnel conditions (see individual experimental chapters for details), colony number and size were assessed and interpreted as a single susceptibility score (Table 2.1). Those trees, which were classified as resistant or intermediate, were re-inoculated to ensure WAA was given an opportunity to colonise and avoid false resistance scores. Re-inoculation was carried out with new refuges created at sites without WAA present, if necessary. After an additional two weeks, plants were scored as before (Table 2.1). The scoring criteria are adapted from those published in Bus *et al.* (2008) but modified to exclude gall number and size and to be carried out over a shorter

period of time (two weeks rather than three to four months). This allows phenotyping of a greater number of seedlings in a single season. Counting gall number and size is not necessarily indicative of damage caused as galling is induced soon after feeding initiates and may not be visible to an assessor.

Table 2.1: Scoring criteria for woolly apple aphid (*Eriosoma lanigerum*; 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 1 cm 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 beyond end of season	
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 beyond end of season	
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 beyond end of season	

2.2. 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 family, parents, and grandparents, and dried in a 1.5 ml Eppendorf tube filled with *ca*. 1 ml grade 40 silica gel (6-14 mesh; Sigma-Aldrich, USA) for at least 24 hours to ensure all moisture was removed.

2.3. DNA extraction

Dried leaf discs were transferred to 1.2 ml polypropylene collection microtubes (Qiagen, Hilden, Germany) and a single 3 mm stainless steel ball-bearing (Qiagen) was dispensed into each microtube using a Qiagen 96-well TissueLyser 3 mm Bead Dispenser (Qiagen). Samples were disrupted in a Geno/Grind*Er2*010 (SPEX SamplePrep, Stanmore, UK) tissue homogeniser at 1500 RPM for one minute, samples were inverted, and homogenisation was repeated. Total genomic DNA (gDNA) was extracted following the protocol described by Edge-Garza *et al.* (2014) but with 5 M sodium chloride used instead of 6 M ammonium acetate. DNA was subsequently re-suspended in 150 µl of 1 mM Tris HCl.

2.4. Verification of mapping populations

A total of 285 seedlings from two different segregating families (MCM007 and M639) raised from controlled crossing in the rootstock breeding programme were screened with eight highly polymorphic microsatellites (Table 2.2; Fernández Fernández, 2013) to confirm paternity and identify and remove potential out-crosses. Extracted gDNA concentration and quality were assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and normalised with ultrapure water to a 5 ng/µl concentration. Amplification by Polymerase Chain Reaction (PCR) was completed using the Qiagen Type-it Microsatellite PCR Kit (Qiagen) under standard 50 – 55 °C annealing stage PCR cycling conditions in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems). PCR success was determined by gel electrophoresis on a 1.5% agarose gel at 150 V for 50 minutes with Fisherbrand™ horizontal gel electrophoresis systems with a PowerPro 300 power supply (Fisher Scientific). PCR products were diluted using ultrapure distilled water and denatured at 90 °C for three minutes using a Thermal Cycler with GeneScan™ 500 LIZ® Size Standard and Hi-Di Formamide (ThermoFisher Scientific). Fragment size analysis was carried out by ABI 3730 DNA Analyzer (Applied Biosystems) at the John Innes Centre and the resulting peaks were classified at NIAB using GeneMapper™ v. 4.0 software (Applied Biosystems).

Table 2.2: The sequences, size ranges and annealing temperatures in Celsius of the eight microsatellite primers used in multiplex to identify variation in *Malus* seedlings from their parental genotypes.

Name	Primer sequence (5' - 3')	Size range (bp)	Annealing temperature (°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

2.5. Preparation for Genotyping-by-Sequencing

Samples were removed from further analysis in this dataset if their allele combination was incompatible with that of parental genotypes at more than one locus, or if data were missing or indeterminate at more than two loci. For each breeding family, 92 seedlings were chosen for mapping, prioritising samples with better quality DNA without altering phenotype segregation ratios from expected 1:1 Mendelian segregation ratios. Priority was also given to those seedlings which had shown clear phenotypic resistance or susceptibility.

Extracted gDNA was normalised to give 100 ng of DNA in a 10 µl volume and library preparation was carried out according to Elshire *et al.* (2011) with ApeKI restriction enzyme (New England Biosciences). Each sample was ligated to a common adaptor and a unique barcode adaptor with T4 ligase (New England Biosciences) before purification using QIAquick PCR Purification Kit (Qiagen). Fragment size of pooled library products was checked before and after purification with a 1.5% agarose gel to confirm successful ApeKI digestion of gDNA into short fragments. Fragment size and concentration of the purified

library was assessed on a TapeStation 4200 (Agilent, USA) and concentration checked on a Qubit 2.0 Fluorometer (Invitrogen). Pooled libraries were sent to NovoGene for whole genome sequencing using Illumina NovaSeq6000 paired-end sequencing.

2.6. SNP alignment and filtering

Paired-end reads were demultiplexed from the unassigned index bins from sequencing runs shared with indexed libraries using a bespoke Python script. The script selects reads where the i7 index contained 6 consecutive Gs and the read 2s begin with the partial ApeKI cutsite C followed by A or T and then G. Reads were then binned according to their inline barcode followed by the partial ApeKI cutsite G followed by A or T and then C. Demultipexed paired-end reads were then trimmed using Trim Galore version 0.6.5. (Krueger, 2019) to remove adapter sequences and quality filtered (-q 25), retaining only reads with a length greater or equal to 25 bp (-l 25) before aligning with BWA mem (Li, 2013) against reference genomes. For *Malus* linkage mapping, the *Malus* x *domestica* genome assembly (Daccord et al., 2017b) was used. Woolly apple aphid samples were aligned against the WAA genome assembly (Biello *et al.*, 2021). Following BWA alignment, SAMtools was used to fix and fill in mate information before merging BAMs of the same sample and filtering to retain only read 2s from correctly read pairs while removing non-primary and supplementary alignments before indexing (Li *et al.*, 2009). Variant calling was performed using BCFtools mpileup v. 1.17 (Li, 2011), mpileup was employed with a mapping quality threshold of 20 (-q 20) and allelic and total depth information was computed (-a AD,DP) before calling using BCFtools call in consensus-caller mode (-c) to identify variants.

CHAPTER 3 - Identification of *Eriosoma lanigerum* resistant *Malus* spp. to inform breeding programmes and orchard design.

3.1. Abstract

Woolly apple aphid (WAA; *Eriosoma lanigerum*, Hausman.) is a major economic pest of domesticated apple (*Malus* × *domestica* Borkh.) around the world. Four WAA resistance genes have been identified and mapped, and resistant rootstock lines developed; there are reports of WAA biotypes able to feed on rootstock carrying one of three of these four genes. To our knowledge, there are no reports of WAA biotypes feeding on genotypes carrying multiple resistance genes (pyramided resistances). Here 45 *Malus* spp. (crab apples) and *M.* × *domestica* breeding accessions were screened for WAA resistance at NIAB East Malling in Kent, UK. Based on a "worst case scenario", six accessions were susceptible to WAA and 23 resistant, tolerant, or immune, with a further 15 classed as intermediate and warranting further scoring. The identification of potentially resistant *Malus* spp. will aid the potential description of novel resistance genes to inform breeding programmes, and inform selection of polliniser crab apples within commercial orchards, which could be a source of WAA infestation. Both accessions of *Malus floribunda* tested were categorised as immune, making the species an excellent candidate for future work provided the results of this study can be replicated across multiple seasons.

3.2. Introduction

Cultivated apple (*Malus* × *domestica* Borkh.) is the result of repeated hybridisation and introgression events between *Malus baccata* (L. Borkh), *M. orientalis* (Uglitz), *M. sylvestris* (Mill) and *M. sieversii* (Ledeb. M. Roem.) across thousands of years (Cornille *et al.*, 2012). Apple is an important temperate crop, both for Class I dessert fruit, and juicing and processing, with over 89 million tonnes of apples produced globally each year, across 97 countries (Cornille *et al.*, 2012; FAOSTAT, 2019).

In 2021, 23,000 hectares of commercial and non-commercial orchard fruit were harvested in the UK (DEFRA, 2022), requiring careful management to assure good crop yield and quality. Many commercial apple varieties are self-incompatible, meaning they cannot pollinate themselves (Broothaerts, Nerum & Keulemans, 2004). The mechanism is controlled genetically by a multiallelic self-incompatibility (SI) locus; if the allele carried by a pollen grain matches one of the two alleles present in the stigma of the pollen recipient flower, the growth of pollen tubes is aborted preventing pollination (Brancher *et al.*, 2020). In a single-variety orchard, or a mixed-variety crop with incompatible pollen types, an external pollen source with compatible SI alleles and a similar flowering time is needed in order to guarantee

pollination and fruit set, in the absence of compatible pollen brought into the crop by flying pollinators (Sakurai, Brown & Weeden, 2000). It is common practice in top fruit (tree fruit) production to use ornamental crab apple species of a compatible pollen type as pollen sources, they are known as pollinisers. Wild *Malus* spp. have several advantages over the use of *M.* × *domestica* accessions: they are easily distinguishable from the main crop (Kendall & Smith, 1975) and often have a high blossom number, improving pollen supply for a large number of cropping trees with less area/crop sacrifice (Church, Goodall & Williams, 1974). Crab apple polliniser trees are planted regularly throughout the crop to ensure pollen availability during blossom and to encourage pollinator activity across the orchard (Free, 1962) but can, however, create reservoirs for pests and disease. Planting resistant pollinisers in orchards may help to control pest build-up within orchards.

The woolly apple aphid (WAA; *Eriosoma lanigerum* Hausmann; Hemiptera: Aphididae) is a widespread global pest of *M.* × *domestica*, feeding on woody tissue of both the scion and rootstock, especially during the winter as the first-instar nymphs over-winter on roots and sheltered areas on the tree bark, emerging in the spring. Elicitor molecules in WAA saliva induce gall formation through rapid proliferation of cells around the vascular tissue, which begins rapidly after feeding (Staniland, 1924; Wemmer, 2019). Tissue damage and disruption of water and photosynthate transport as a result of galling can severely reduce plant growth and fruit yield (Weber & Brown, 1988; Brown, Glenn & Wisniewski, 1991; Brown *et al.* 1995). Tissue collapse within the gall creates wounds for secondary pathogen infection, for example from European apple canker (*Neonectria ditissima*: Childs, 1929; Sandanayaka, Bus & Connolly, 2005). Woolly apple aphid feeds predominantly on woody tissue and root tissue, whereas other apple aphid species tend to inhabit soft tissue and extension growth. *Dysaphis devecta* (Walker) and *Dysaphis plantaginea* (Passerini) induces leaf curling galls, and *Aphis pomi* (de Geer) feed primarily on young woody tissue and buds, creating the potential for a single tree to be overwhelmed by multiple species.

Commercial control options for WAA include systemic spirotetramat insecticides (e.g. the commercial products Batavia or Movento) which can control aphids feeding on any part of the tree. Natural enemies can control WAA in apple orchards, including the parasitoid wasp *Aphelinus mali* (Haldemann), and common predators such as the European earwig (*Forficula auricularia* L.), and larvae of Coccinellidae and Syrphidae species (Cohen *et al.*, 1996; Sandanayaka, Bus & Connolly, 2005; Bergh, 2008). These can be encouraged into the orchard via various practices, for instance, through the use of wildflower strips, refuges, and natural enemy attractants *e.g*. methyl salicilate (Fountain, 2022). Aphids feeding below-

ground overwinter creating a reservoir of WAA which emerge in the spring to feed on new scion growth before natural enemies are active.

Monitoring rootstock-feeding WAA presents a significant challenge in their control. Although systemic spirotetramat insecticides are expected to reduce rootstock-feeding WAA, resistant rootstocks can consistently prevent feeding, offering long-term control. Avoiding the establishment of WAA below-ground, combined with targeted control on the scion, would achieve an integrated pest management (IPM) strategy for WAA on apple.

Woolly apple aphid resistance is therefore a priority for commercial rootstock breeding programmes. Tolerance or resistance to WAA has been anecdotally reported in approximately ten crab apple species and domestic cultivars but not yet characterised genetically, giving a potential source of novel resistance genes. Four WAA resistance genes have been identified, and their genetic location and segregation patterns characterised to date: *Er1-4*, of which two were identified from crab apple species;

1. *Er1* (Knight *et al.*, 1962) (*Eriosoma* resistance) characterised in the scion variety 'Northern Spy' and its derivatives was the first resistance incorporated in rootstock breeding.
2. *Er2* was identified from the crab apple *Malus* × *robusta* 5 (*M. baccata* × *M. prunifolia* Carr.; Robusta 5a) (King *et al.*, 1991) and has been used as the source of WAA resistance in the Geneva rootstock series developed at Cornell University in the 1950s (Cummins & Aldwinckle, 1983).
3. *Er3* was isolated from an open-pollinated accession of *Malus sieboldii* (Rehder) and is used in the commercial rootstock 'Aotea 1' (Bus *et al.*, 2002).
4. *Er4* was identified from an open-pollinated mildew-immune selection of 'Delicious', and mapped to LG 07 (Dayton, 1977; Bus *et al.*, 2010).

Woolly apple aphid has been observed feeding on resistant rootstocks carrying either *Er1, Er2 or Er3* (Giliomee, Strydom & van Zyl, 1968; Rock & Zeiger, 1974; Sandanayaka & Hale, 2003; Cummins & Aldwinckle, 1983). Pyramiding of multiple resistance genes is expected to increase durability of resistance to multiple genotypes of WAA. *Er1* and *Er3* have been successfully pyramided (Bus *et al.*, 2009), but the discovery of novel resistance genes suitable for pyramiding will increase opportunities for resistance breeding programmes. The identification of novel resistance genes would increase opportunities for gene pyramiding and contribute to long-term rootstock breeding programmes.

Stout (2013) adapted the traditional categories of non-preference, antibiosis and tolerance, suggested by Painter (1951) to two categories: resistance, where herbivory is prevented, and

tolerance, where herbivory injury occurs but plant material can withstand the damage without loss of yield. The distinction between resistant and tolerant plants is difficult to estimate without long term studies of plant health, but it is important to highlight variable susceptibility in resistance breeding as divergence from expected Mendelian segregation ratios can be indicative of the presence of a resistance QTL, or a resistance gene cluster, rather than single major genes. In order to differentiate between high and low levels of colonisation, the terms “resistant” and “tolerant” can be used, assuming that plant material which is tolerant to aphid feeding will show some colonisation, but otherwise perform well. ‘Robusta 5’ has been reported as immune to WAA feeding, *i.e*. showing zero colonisation by WAA, even when inoculated with a biotype of WAA able to colonise rootstocks with *Er1*-derived resistance (Young *et al.*, 1982). Within categorisation of resistance as outlined by Stout (2013), “immune” would refer to plant material which was highly resistant.

The aim of this study was to identify WAA-resistant candidates as the first stage in a resistance breeding programme to identify potential sources of novel resistance genes. This was achieved through screening 59 crab apple species and *M.* × *domestica* accessions, including four crab apple species endemic to, and nine accessions bred in, North America, as representative host plants available in the WAAs native range.

3.3. Materials and Methods

3.3.1. Plant material

Plant material was selected based on the following criteria: previously reported as having WAA tolerance or resistance; having a flowering time compatible with commonly used crop accessions; reported as having disease resistance, which is often also observed in WAA-resistant accessions (*e.g*. apple scab resistance, Miñarro & Dapena, 2009). Accessions known to be susceptible were included, along with the sources of identified resistance genes, as positive and negative pseudo-controls respectively. In addition to the 41 accessions phenotyped for their WAA susceptibility (Table 3.1), 18 accessions were selected for analysis but either did not survive the grafting process or had their new season growth destroyed by severe *A. pomi* infestation.

Graft wood was collected in late February 2020. Fifteen accessions were provided by Frank P. Matthews nurseries (Tenbury Wells, Worcestershire) and the remaining 26 were collected on site at NIAB East Malling (Table 3.1). Graft wood cuttings were *ca*. 1 cm in diameter and *ca*. 10 cm long, depending on available material, grafted onto M.9 rootstocks (Frank P. Matthews nurseries, Tenbury Wells, Worcestershire) and potted into 3 L square pots with

potting compost. The grafts were kept under polytunnel conditions for three months to ensure successful grafting.

Table 3.1: Apple accessions screened for Woolly Apple Aphid (WAA) susceptibility. The number of initial and successful screening repeats is given, as some grafts were unsuccessful. The number of grafts made and the number of grafts which survived in a healthy enough condition to be inoculated with WAA are given. Details of parentage and resistance to WAA are given, where known, along with where the graft wood 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. All varieties were grafted onto M.9 rootstocks and treated as scions, regardless of their normal usage, for WAA susceptibility phenotyping.

Variety	***n* repeats of each variety grafted**	***n* grafts successfully phenotyped**	**Normal usage of variety**	**Parentage**	**Source**	**Reported resistance status**
'Admiration'	3	Not phenotyped	Commercial crab apple	OP seedling of *M. halliana* 'Koehne' **Bred in North America**	F. P. Matthews	Unknown
Alnarp 2	3	2	Rootstock	Selected from Doucin dwarf trees released in Sweden	NIAB East Malling	Susceptible (Cummins, Forsline & Mackenzie, 1981)
G.11	3	2	Rootstock	M.26 × 'Robusta 5' **Bred in North America**	NIAB East Malling	Susceptible (Lyga,2018)
G.202	3	2	Rootstock	M.27 × 'Robusta 5' **Bred in North America**	NIAB East Malling	Resistant; *Er2* (Lyga,2018)
Malus × *atrosanguinea* 'Gorgeous'	3	3	Commercial crab apple	Unknown	F. P. Matthews	Unknown
Hashabi MH10.1	3	3	Rootstock	Unknown	NIAB East Malling	Unknown

Hashabi MH14.5	3	Not phenotyped	Rootstock	Unknown	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
M. baccata flexilis	2	2	EM germplasm accession	Unknown	NIAB East Malling	Unknown
M. baccata mandschurica	3	0	Crab apple	Wild type	F. P. Matthews	Unknown
M. baccata 'Gracilis'	3	Not phenotyped	Commercial crab apple	Unknown	NIAB East Malling	Unknown
M. 'Baskatong'	2	2	Commercial crab apple	*M.* 'Simcoe' × *M.* 'Meach' **Bred in North America**	F. P. Matthews	Unknown
M. brevipes	3	Not phenotyped	Commercial crab apple	Unknown	NIAB East Malling	Unknown
M. coronaria 'Elk River'	3	3	Commercial crab apple	Wild type **Native to North America**	F. P. Matthews	Unknown

M. denticulata	3	Not phenotyped	Crab apple	Unknown	NIAB East Malling	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 (Miñarro & Dapena, 2009)
M. floribunda (EMLA)	3	Not phenotyped	EM germplasm accession	Wild type, likely *M. toringo* × *M. baccata* hybrid	NIAB East Malling	Unknown
M. floribunda 'J'	2	1	EM germplasm accession	Wild type, likely *M. toringo* × *M. baccata* hybrid	NIAB East Malling	Resistant; R gene unknown (Miñarro & Dapena, 2009)
M. fusca M	3	3	EM germplasm accession	**Native to North America**	NIAB East Malling	Low/zero colonisation (Cummins, Forsline & Mackenzie, 1981)
M. halliana	3	2	EM germplasm accession	Unknown	NIAB East Malling	Low colonisation (Cummins, Forsline & Mackenzie, 1981))
M. hupehensis (EMLA)	3	3	EM germplasm accession	Wild type	NIAB East Malling	Resistant; R gene unknown (Cummins, Forsline & Mackenzie, 1981)

M. kansuensis	3	1	Commercial crab apple	Wild type	F. P. Matthews	Susceptible (Cummins, Forsline & Mackenzie, 1981)
M. koreana	1	1	EM germplasm accession	Unknown	NIAB East Malling	Resistant; R gene unknown (Fernández Fernández, 2020, pers. comm.)
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 (Cummins, Forsline & Mackenzie, 1981)
M. praecox	3	3	EM germplasm accession	Wild type	NIAB East Malling	Unknown
M. pumilla 7728	3	3	Germplasm accession	Unknown	F. P. Matthews	Susceptible (Cummins, Forsline & Mackenzie, 1981)
M. robusta (EMLA)	3	1	EM germplasm accession	*M. baccata* × *M. prunifolia*	NIAB East Malling	Resistant; R gene known (King *et al.*, 1991; Bus *et al.*, 2008)
M. rubra 'Evelyn'	3	3	Commercial crab apple	*M. ioensis* × *M. purpurea* **Native to North America**	NIAB East Malling	Unknown

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 (Cummins, Forsline & Mackenzie, 1981)
M.9	6	6	Rootstock	Unknown	NIAB East Malling	Susceptible
M778	3	Not phenotyped	Rootstock	Unknown	NIAB East Malling	Unknown
M789	3	Not phenotyped	Rootstock	Unknown	NIAB East Malling	Unknown
M793	3	Not phenotyped	Rootstock	Unknown	NIAB East Malling	Unknown
Mac 24	3	3	Rootstock	Unknown	NIAB East Malling	Unknown
Mac 4	3	2	Rootstock	Unknown	NIAB East Malling	Unknown
Mac 9	3	2	Rootstock	Unknown	NIAB East Malling	Unknown

Malling Crab 'C'	3	3	EM germplasm accession	Unknown	NIAB East Malling	Intermediate (Cummins, Forsline & Mackenzie, 1981)
Malus × *magdeburgensis*	3	2	Commercial crab apple	*M. domestica* × *M. spectabilis*	F. P. Matthews	Resistant or tolerant (Cummins, Forsline & Mackenzie, 1981)
Malus × *robusta* 5a	3	2	EM germplasm accession	Unknown	NIAB East Malling	Resistant; *Er2* source (Bus *et al.*, 2008)
Malus × *robusta* f. *erecta* (EMLA)	1	1	EM germplasm accession	Unknown	NIAB East Malling	Unknown
Malus × *robusta* 'Persicifolia'	3	Not phenotyped	EM germplasm accession	Unknown	NIAB East Malling	Unknown
Malus × *robusta* 'Red Sentinel'	1	1	Commercial crab apple	Unknown	F. P. Matthews	Unknown
M. spectabilis 'Riversii'	3	Not phenotyped	Crab apple	Wild type	F. P. Matthews	Unknown
M. toringoides 'Mandarin'	3	Not phenotyped	Commercial crab apple	Clone of *M. bhutanica*	F. P. Matthews	Unknown
Malus × *zumi*	3	Not phenotyped	*M. mandschurica* × *M. sieboldii*	Unknown	NIAB East Malling	Unknown

Malus × *zumi* 'Calocarpa'	3	2	EM germplasm accession	Unknown	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; *Er1* source (Knight *et al.*, 1962; King *et al.*, 1991)
Novole	3	3	Rootstock	Unknown	NIAB East Malling	Unknown
‘Pink Pearl’	3	Not phenotyped	Scion variety	*M. niedzwetskyana* ancestry **Bred in North America**	F. P. Matthews	Unknown
Polish 22	3	1	Rootstock	M.9 × ‘Common Antonovka’	NIAB East Malling	Susceptible (eApples, 2019)
Scarlet Sentinel	3	3	Commercial crab apple	Unknown	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	Unknown	F. P. Matthews	Unknown

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3.4. Results

The highest susceptibility score by individual across all scoring events is presented as a boxplot of highest score by accession in Figure 3.1. The difference in high-score between accessions was found to be significant ($p = 0.003$) with a Chi-squared analysis and the difference between categories (crab apple, breeding material sourced from germplasm, rootstock, scion) was found to be close to significant ($p = 0.058$).

Paired t-tests of accession category with Bonferroni correction found a significant difference in high-score between rootstock and crab apple accessions ($p = 0.040$). No significant differences were found between any of the other categories.

The positive control, M.9, showed a median high-score of 4 (*n* = 6), categorising it as "susceptible" within this analysis, as expected. Of the known sources of WAA resistance genes included, 'Northern Spy' had a median highest score of 1 (*n* = 6). The median 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).

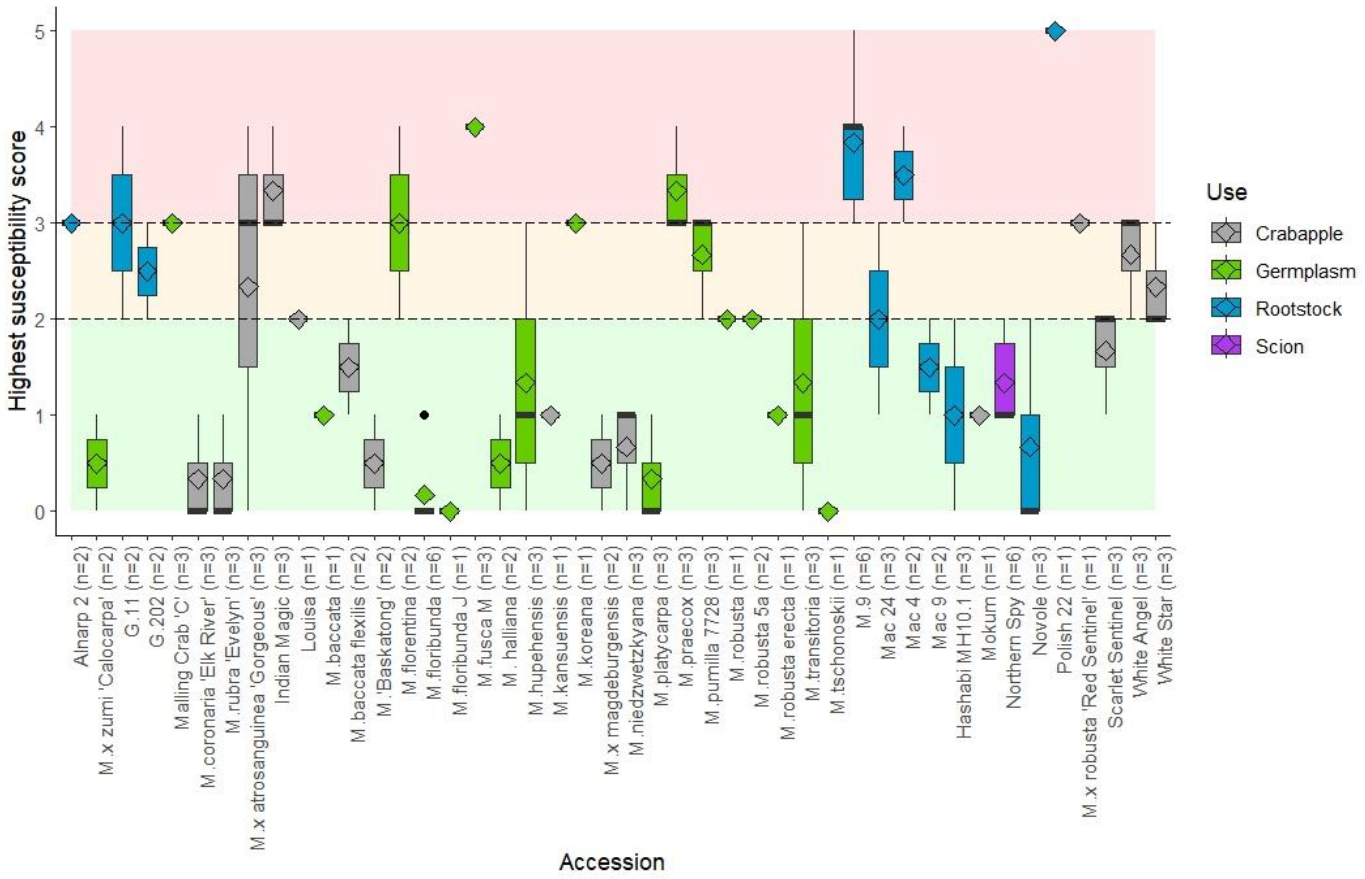

Figure 3.1- Boxplot generated from the highest susceptibility score recorded across all scoring events pre- and post- inoculation(s) with woolly apple aphid material, for each graft of each accession.
Material of each genotype was collected and grafted onto M.9 rootstocks. All grafts were treated as scions, regardless of their normal usage. The numbers of grafts successfully phenotyped is indicated in brackets by each accession name. The thick central line indicates the median value recorded and the mean 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 normal usage of each genotype are indicated in the legend. "Scion" and "rootstock" represent their normal usage in commercial orchards. "Crabapples" may be found in the wild or as ornamental trees. "Germplasm" indicated crabapple species and domesticated accessions which are commonly found as germplasm material for breeding.

Of the 45 accessions successfully screened, 28 were classified as "immune", "resistant", "or tolerant", based on the mean highest susceptibility score recorded, four as "susceptible", and the remaining 12 as an intermediate classification. The widely used WAA-susceptible rootstock accession, M.9, was categorised as "susceptible", as expected. None of the accessions known to be resistant showed WAA immunity. 'Northern Spy' showed resistance, although the mean highest score was 1.3, towards the upper end of the "resistant" range.

Malus robusta varieties also showed highest susceptibility scores between 1 and 2, which would categorise it as “tolerant” by classifications used here.

2187 Table 3.2: Median susceptibility scores of *Malus* accessions for woolly apple aphid (*Eriosoma*
2188 *lanigerum*, WAA) resistance and suggested categories for those resistances.

Immune (susceptibility = 0)	**Resistant (susceptibility ≤ 1)**	**Tolerant (susceptibility ≤ 2)**	**Intermediate (susceptibility >2, ≤ 3)**	**Susceptible (susceptibility > 3)**
M. coronaria 'Elk River'	Hashabi MH10.1	'Louisa'	Alnarp 2	*M. fusca* M
M. floribunda	*M. baccata*	*M. baccata flexilis*	G.11	M.9
M. floribunda 'J'	*M. baskatong*	*M. robusta* (EMLA)	G.202	Mac 4
M. platycarpa	*M. halliana*	*Malus* × *robusta* 5a	Malling Crab 'C'	Polish 22
M. rubra 'Evelyn'	*M. hupehensis* (EMLA)	Mac 24	*Malus* × *atrosanguinea* 'Gorgeous'	
M. tschonoskii	*M. kansuensis*	Mac 9	Indian Magic	
'Novole'	*M.* × *magdeburgensis*	*Malus* × *robusta* 'Red Sentinel'	*M. florentina*	
	M. niedzwetzkyana	'White Star'	*M. koreana*	
	M. × *robusta* f. *erecta* (EMLA)		*M. praecox*	
	M. transitoria		*M. pumilla* 7728	
	M. × *zumi* 'calocarpa'		*Malus* × *robusta* 'Red Sentinel'	
	Mokum		'White Angel'	
	'Northern Spy'			

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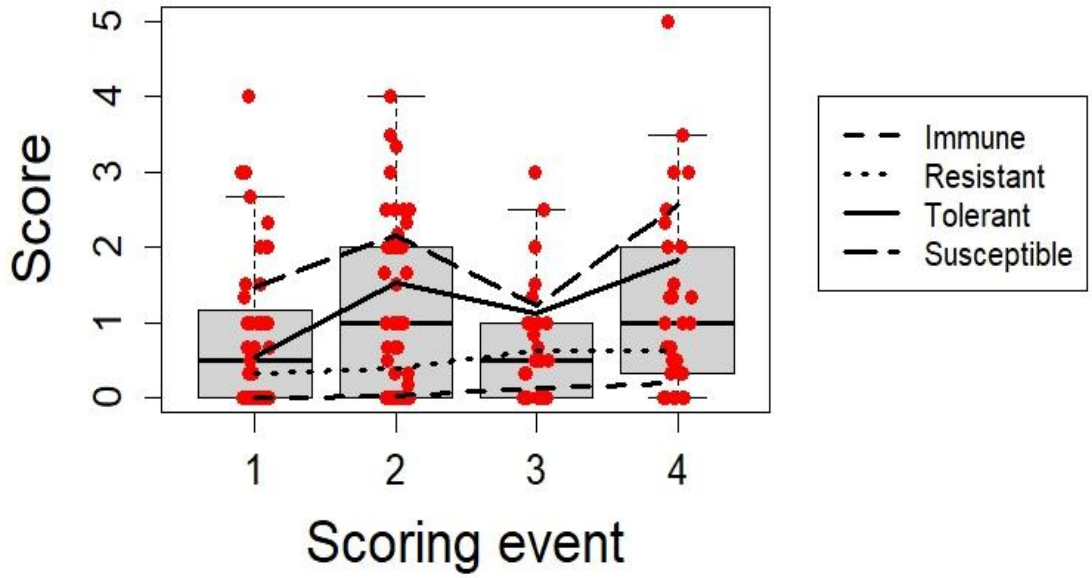

Figure 3.2 - Change in mean susceptibility scores across all accessions over the four scoring events, pre- and post-inoculation. Points represent the mean score for each accession at those scoring events, with the distribution at each time point, and associated statistics, are represented as box plots. Mean susceptibility score for each category of susceptibility, as indicated in Table 3.2, are also indicated.

These results have been shared directly with apple growers through in-person presentations and published as reports which are accessible to the public and more specifically, AHDB horticulture levy payers. Further information has also been requested verbally by growers who were interested in the potential to replace their pollinisers, which they had observed were highly susceptible to WAA, with resistant varieties to remove a reservoir of infection material. These data were also shared directly with F. P. Matthews Tree Nursery to allow them to advise their customers (both commercial and retail) on the WAA susceptibility of their nursery stock.

3.5. Discussion

'Robusta 5' has previously been reported to be immune to multiple biotypes of WAA when feeding on apple material in outdoor stoolbeds (Young *et al.*, 1982), although *M. robusta* varieties were only classified as "tolerant" in this here. Growing under glasshouse conditions affects both plant and aphid material differently than an open air climate. It is likely that high pressure from elevated temperature and the presence of other herbivore pests (*A. pomi* and two-spotted spider mite, *Tetranychus urticae* Koch) reduced the fitness of 'Robusta 5' that it can be infested by WAA.

Colonies on ‘Northern Spy’ were persistent and all grafts showed higher WAA susceptibility at the end of the experiment, although the final score was always within the resistant or low intermediate category (data not shown). Colony persistence varied on the three accessions of *M. robusta*. Colonies on *M. robusta* persisted at an intermediate susceptibility throughout, although on ‘Robusta 5a’, colonies reduced in severity across the time points of the experiment (data not shown). It is possible that environmental conditions were favourable for aphid growth, allowing them to thrive on otherwise resistant material (Bus *et al.*, 2008). This would, however, be in contradiction to the lower-than-expected scores across the full range of accessions used. The persistence of colonies on ‘Northern Spy’ but not *M. robusta* accessions is supportive of the presence of a resistance-breaking strain of WAA which is able to colonise rootstocks with *Er1*-derived resistance, to an extent, but cannot overcome *Er2*. A clade of WAA which presented similarly were reported in New York State and North Carolina, and ‘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).

High levels of WAA parasitism by *A. mali* were observed in the glasshouse compartment, likely due to the developmental temperature threshold for *A. mali* being higher than that of WAA (Asante & Danthanarayana, 1992), allowing it to out-perform WAA in warmer environments. The high temperatures and enclosed glasshouse conditions may have also led to lower WAA colonisation, and therefore lower susceptibility scores than in an open-air orchard context.

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. The highest score was not always recorded at the last scoring event (Figure 3.2), and not every seedling had four scoring events; those which scored as susceptible after the first inoculation did not receive a second inoculation. The mean susceptibility scores, as shown in Figure 3.2, may therefore be lower for the third and fourth scoring events because more resistant accessions with low susceptibility scores were progressed to this stage.

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. It is difficult to determine

whether there is a resistance-breaking clade of WAA present but further testing is needed into the effects of feeding on resistant rootstocks on growth and reproduction both for individuals and at a population level. There may also be a genetic component to resistance breaking traits, discussed further in Chapter 5.

Woolly apple aphid resistance conferred by both *Er1* and *Er2* is thought to be phloem-related; *Er1* resistant varieties show thickened bundles of sclerenchyma around vascular tissue, mechanically preventing aphid feeding (Staniland, 1924), and both *Er1* and *Er2* reduce the duration of WAA feeding (Sandanayaka *et al.*, 2003). 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 (as observed by Staniland, 1924).

Both rootstock accessions of *M. floribunda* screened (*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, Bus & Connolly, 2005). *Malus floribunda* 821 has been 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-incompatibility locus of *M. floribunda* 821 is known (Verdoodt *et al.*, 1998) which, if compatible with the crop variety, would make it an ideal candidate for a resistant polliniser. The significantly lower susceptibility score in crab apples compared to rootstock accessions indicates that future resistance breeding may wish to focus on crab apple species as potential wild sources of novel resistance genes. Of the accessions which scored as immune to WAA, three are native to North America, namely *M. coronaria* 'Elk River', *M. platycarpa* (EMLA), and *M. rubra* 'Evelyn'. It is possible that accessions which are endemic in the WAA host range may be more likely to carry resistance to WAA and therefore would be good candidates for further investigation to widen the resistance gene pool. It may, however, be the case that WAA not in North America have not been exposed to, and therefore not able to evolve the ability to overcome these resistances.

Although susceptibility criteria are defined (Table 2.1) there is still a degree of subjectivity; the number of aphids used to inoculate with is technically challenging 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. 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.

Broady speaking, it is most likely for a seedling to be resistant or susceptible, in alignment with Mendelian segregation patterns seen with a single major resistance gene. A resistance QTL may exhibit differing segregation across multiple populations (Cui *et al.*, 2015), which could lead to differential resistance expression and therefore some individuals showing greater colonisation than others. Intermediate scores in this study 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. It may also be suggestive of resistance QTLs present in these accessions. Future phenotyping repeated across multiple seasons outdoors in a commercial orchard situation would help to reduce the influence that environmental conditions had on the outcomes of this experiment.

This study has identified as WAA resistant *Malus* species that can be used to investigate their potential as novel sources of WAA resistance as part of a longer-term breeding programme; ultimately using genetic markers to determine the genomic location of the novel resistance gene of interest. There is special interest in the crab apples *M. floribunda* and *M. floribunda* 'J'. because they are different accessions of the same parentage, and both scored as completely resistant to WAA. In order to determine whether or not these are truly a novel source of resistance, *M. floribunda* can be screened with existing markers for the four published WAA resistance genes to see if this resistance is in fact attributable to a known gene. Accessions native to, or bred in, North America may offer a wider gene pool with WAA resistance.

This work can also be used to directly inform growers of resistant accessions which may be suitable for use as polliniser trees within a crop. Selection of the most suitable polliniser will vary depending on compatibility and peak bloom, but the results summarised here may offer a resource while breeding programmes develop resistant crop varieties or rootstocks. This is especially important in future orchards where the chemistry to control WAA is increasingly being withdrawn to minimise impacts on the environment. Through discussions with chiefly apple growers and agronomists, the findings of this work have been disseminated and positive feedback received from growers that these data will be considered when selecting pollinisers in future.

CHAPTER 4 – Genetic mapping of the woolly apple aphid (Hemiptera: Aphididae) resistance genes *Er1* and *Er2*, using SNP markers.

4.1. Abstract

Identification of genetic markers closely associated with genes of interest is a key tool in resistance breeding, allowing for marker-assisted selection of traits which would otherwise require generations of screening using traditional techniques which are time- and money-expensive. Here we generated 3613 SNPs for chromosome 17 of the rootstock breeding family M629, using a GBS approach. A region was identified containing SNPs significantly associated with resistance to the woolly apple aphid (*Eriosoma lanigerum*), which approximately aligned with the location identified for this gene with linkage mapping.

4.2. Introduction

4.2.1. Mechanism of resistance

Tolerance or resistance to the woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) has been reported in at least ten accessions of cultivated apple (*Malus* × *domestica* Borkh.) and crab apple (*Malus sylvestris* L.), species (Sandanayaka *et al.*, 2005), however, resistance mechanisms and effects on aphid colonisation, feeding, growth and reproduction are not known (Sandanayaka *et al.*, 2003).

The scion variety ‘Northern Spy’ has been identified as a source of mechanical resistance, showing thickened, intact rings of sclerenchyma around vascular tissue, which are less vulnerable to aphid penetration (Staniland, 1924; Horbens *et al.*, 2014). ‘Northern Spy’ is resistant to WAA feeding, although there have been reports of ‘Northern Spy’-derived resistant rootstocks being fed on by WAA, indicating resistance can be overcome (Self, 1966; Giliomee *et al.*, 1968; Rock & Zeiger, 1974). Electrical Penetration Graph (EPG) analysis found that fewer WAA on ‘Northern Spy’ were able to reach sustained phloem feeding than on susceptible Royal Gala (Sandanayaka *et al.*, 2003). These aphids fed for a shorter duration overall, especially in this sustained phloem ingestion phase, further suggesting that ‘Northern Spy’ resistance is a phloem-related factor.

Malus x *robusta* 5a (*M. baccata* x *M. prunifolia*, Carr.; ‘Robusta 5a’) is a crab apple hybrid widely used in apple breeding programmes for both scion and rootstock varieties. It carries well characterised resistance genes to fungal pathogens including fireblight (*Erwinia amylovora* Burrill), European apple canker (*Neonectria ditissima*), and powdery mildew (Markussen *et al.*, 1995; Peil *et al.*, 2007; Bus *et al.*, 2017).

‘Robusta 5a’ has been reported as showing complete immunity to WAA feeding (Mackenzie & Cummins, 1982), although there are anecdotal reports that this resistance may have been broken in commercial conditions (Cummins & Aldwinckle, 1983). Minor colonisation and/or galling has been seen in rootstock breeding lines derived from ‘Robusta 5’, suggesting it does not provide full immunity to WAA feeding (Bus *et al.*, 2008). Apart from minor colonisation by some strains of WAA, ‘Robusta 5a’ is regarded as resistant (Young *et al.*, 1982). Aphids feeding on ‘Robusta 5a’ did not show sustained phloem ingestion and fed for a shorter duration than on susceptible Royal Gala, when assessed with EPG analysis (Sandanayaka *et al.*, 2003). This suggests that resistance within ‘Robusta 5a’ is likely phloem related, as is theorised for *Er1*-mediated resistance (Staniland, 1924). Phloem-related resistances have been observed in apple for multiple pest aphids on *Malus spp.*, for example, resistance to *Dysaphis plantaginea* (Passerini; the rosy apple aphid) is thought to be conferred by a resistance factor present in the sieve element (Marchetti *et al.*, 2009).

In the UK, WAA feeds exclusively on apple, moving from the canopy to the rootstock in autumn to over-winter on the rootstock, before returning to the scion in spring to feed on new growth (Hetherington *et al.*, 2009). Monitoring and control of rootstock-feeding aphids is challenging, especially in commercial orchards. Aphid-resistant rootstocks allow WAA control below-ground and remove a reservoir of WAA material when the pest re-emerges in the spring, making rootstocks a key component in an integrated pest management (IPM) programme for WAA control. ‘Northern Spy’ is the source of WAA resistance in the widely-used Malling-Merton (MM) rootstock series (Crane *et al.*, 1937); the most common of which include MM106 (‘Northern Spy’ x M.1), and MM111 (‘Northern Spy’ x M.2); as well as in M.116 (MM106 x M.27) ‘Robusta 5’ is the source of WAA resistance in the Geneva rootstock series developed at Cornell University in the 1950s, and is now commercially available in rootstocks such as ‘G.41’ and ‘G.202’ (reciprocal crosses of ‘M.27’ × ‘Robusta 5a’; Cummins & Aldwinckle, 1983).

4.2.2. Resistance gene mapping

‘Northern Spy’-derived resistance was found to be mediated by the dominant major gene *Er1* (Knight *et al.*, 1962; King *et al.*, 1991) which was assigned to the top of Linkage Group (LG) 08 in ‘Northern Spy’ with flanking Sequence Characterised Amplified Region (SCAR) markers at 2.9 cM and 7.9 cM away from *Er1* (Bus *et al.*, 2008). The M432 breeding population (M.27 × M.116) has been mapped with both Simple Sequence Repeat (SSR; microsatellite) and Single Nucelotide Polymorphism (SNP) markers (Evans et al, 2011; Antanaviciute *et al.*, 2012; Fernández-Fernández *et al.*, 2012), giving dense marker

coverage of LG 08. *Er1* is thought to be a single major gene because of the strong bimodal segregation seen in its F1 progeny when crossed with a susceptible parent (Bus *et al.*, 2008).

Resistance in 'Robusta 5a' is conferred by the major gene, *Er2* (King *et al.*, 1991) which Bus *et al.* (2008) determined originated from 'Robusta 5' and mapped to linkage group (LG) 17 using 178 seedlings of 'M.9' × 'Robusta 5a'. *Er2* was designated as a single major gene because of its strong WAA resistance phenotype and because progeny of a cross between 'Robusta 5a' and susceptible parent historically have shown strong bimodal segregation for a WAA resistant phenotype (King *et al.*, 1991). Subsequent *Er2* breeding populations have, however, not showed the expected bimodal pattern, likely a result of scoring criteria and the decision point at which "susceptible" and "resistant" classifications are differentiated (Bus *et al.*, 2008). This does not explain all variation away from the expected ratios as re-classification of seedlings increased segregation distortion for some families. It is therefore likely that *Er2* is not a single major gene but rather a complex of resistance genes or a QTL.

The closest transferrable markers identified for *Er2* so far are flanking Simple Sequence Repeat (SSR) markers located at 5.5 cM and 17.6 cM away from *Er2* (Bus *et al.*, 2008). The current closest flanking markers are indicated in Figure 4.1. Three nucleotide binding site-leucine rich repeat (NBS-LRR) genes have been identified within this genomic region (Calenge *et al.*, 2005), which are associated with pathogen resistance and the innate immune response. This may constitute a resistance gene cluster which has been seen for resistance to apple scab (*Venturia inaequalis* Cooke) where five resistance loci were located in the same region as nine candidate NBS-LRR genes (Bastiaanse *et al.*, 2016).

The breeding family M639 is an F1 backcross between 'M.27' and 'G.41' ('M.27 × 'Robusta 5a') (Figure 4.2) and incorporates WAA resistance with other desirable characteristics, such as extreme dwarfing from 'M.27'. An updated map of LG 17 with high-density coverage of SNP markers will allow identification of SNPs more closely associated with *Er2* than existing SSR markers. SNPs in close linkage with *Er2* are an essential requirement of marker-assisted selection (MAS) which will reduce the time and resource input required in conventional breeding, increasing the frequency at which resistant rootstocks can be released commercially. Traditional rootstock breeding would be expected to take at least 20 years from initial cross to commercial introduction, given that apple can only produce a single generation per year. Markers closely associated with *Er2* will also facilitate its pyramiding with other WAA resistance genes, located on different LGs (Bus *et al.*, 2008 2010), to create rootstocks with long-lasting resistance to multiple WAA clades. Genotyping-by-Sequencing (GBS) generates large numbers of SNPs in array, which can be used for high coverage linkage mapping, both for organisms with no existing genome

sequence, and to increase the detail in existing resistance loci (Novaes *et al.*, 2008; Poland & Rife, 2012). The high quality assembly of 'Golden Delicious' created a genome of 625.2 Mb, aligned into 17 chromosomes (Daccord et al., 2017a), which can be used to align linkage maps to an assembled genome to give genomic positions of markers associated with the target gene. This approach has been successfully used with SNPs generated by GBS to align with linkage maps to correlate genetic and physical marker positions (*e.g.* Felcher *et al.*, 2012).

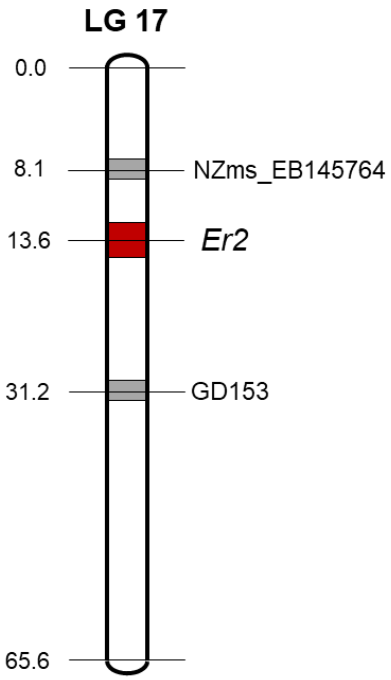

Figure 4.1 - The current closest flanking markers identified to the *Er2* gene to date (Bus *et al.*, 2008). Both are Simple Sequence Repeat (SSR) markers.

This chapter aims to identify SNP markers closely associated with *Er1* and *Er2* using a GBS approach to generate a high-density of SNPs across the target linkage groups. Identification of the SNPs most strongly associated with WAA resistance through linkage and association mapping.

4.3. Materials and Methods

4.3.1. Plant material

Selection of breeding population for mapping of Er1 and clonal propagation

In this work, one line each of two mapping populations were used to study the inheritance of *Er1* and to generate markers closely associated with the gene which are conserved and heritable across generations: MCM007 and M432. MCM007 (M.27 × M.M.106) is a reciprocal cross of M.116 (Figure 4.2) and is therefore useful to investigate the effect(s) of which parent carries *Er1* on its inheritance and variation in resistance expression (Mukanga *et al.*, 2010).

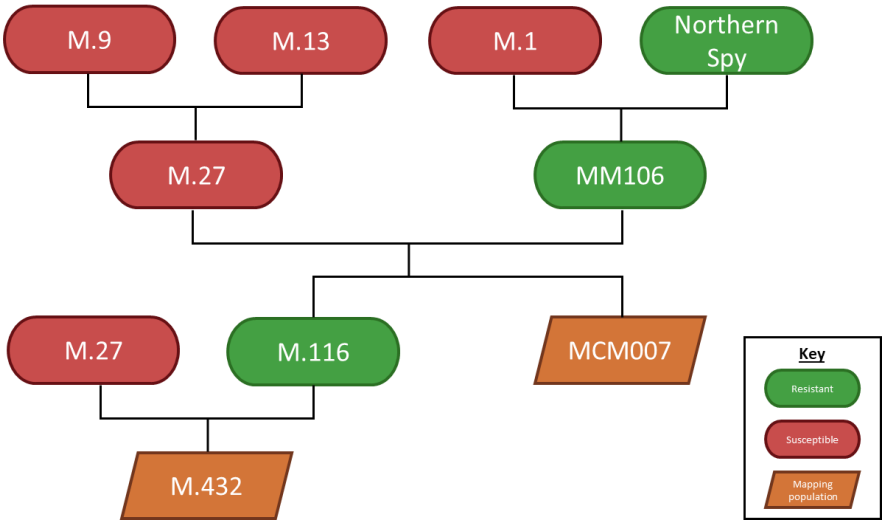

Figure 4.2 – Pedigree chart for the *Malus* rootstock breeding populations MCM007 and M432, both raised to study the inheritance of the *Er1* gene, derived from 'Northern Spy' and conferring resistance to the woolly apple aphid (*Eriosoma lanigerum*).

Second year seedling graftwood was collected in January and February 2020. Three repeats of each seedling were taken for grafting, where possible. Graftwood cuttings were *ca*. 1 cm in diameter and *ca*. 10 cm long, depending on available material. Cuttings were then grafted onto M.9 rootstocks (Frank P. Matthews nurseries, Tenbury Wells, Worcestershire), and potted into 3 L square pots with potting compost. Grafted trees were transferred to a polytunnel in April 2020 for three months to allow the scion to establish.

First year grafted trees of both families were scored between July and October of 2020, under glasshouse conditions in a 3 x 5 m compartment, equipped with ventilation fans and manually watered daily. No conventional or biological plant protection products were applied, to prevent damage to developing WAA colonies. Severe outbreaks of *A. pomi* were controlled using manual application of soapy water to colonies, with care taken to avoid WAA.

Second year MCM007 and M432 grafted trees were transferred in September 2020 to a single span polytunnel with side vent netting, a door at one end and ventilation louvres at the opposite end. This tunnel had additional fan ventilation only during the summer of 2021. Temperature (°C) and relative humidity (% RH) were recorded every hour from 1st of July to 23rd of November in 2021 and from 27th of April to 25th of November in 2022 with two Elitech RC-51H data loggers placed inside white Delta traps to protect from UV damage and located at opposite ends of the polytunnel.

Raising a seedling population to map Er2 and clonal propagation

Controlled crossing

The M639 apple family was generated by a controlled cross of M.27 × G.41 in the spring of 2020 (see Figure 4.3 for pedigree details) as part of the rootstock breeding programme at NIAB East Malling. Pollen was prepared in advance by removing anthers from paternal G.41 blossoms using sterilized tweezers which were then placed into a 35 mm diameter Petri dish and desiccated in room temperature conditions for 2 - 3 days with the Petri dish lid partially covering the dish. The Petri dish was sealed with PVC electrical tape and stored in a glass dome desiccator under coldstore conditions (*ca*. 4 °C) for up to three years.

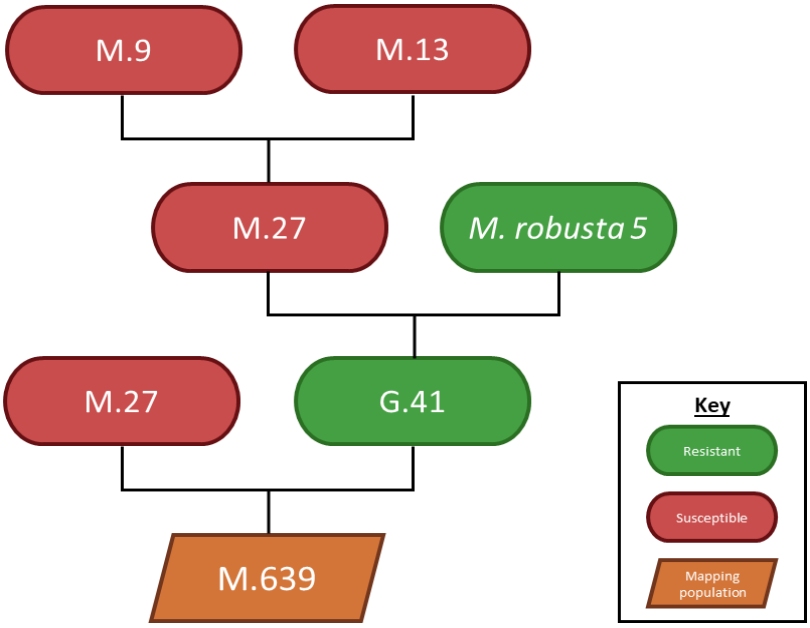

Figure 4.3 - Breeding pedigree for the *Malus* rootstock breeding family M639, raised to study the inheritance of the gene *Er2* for resistance to the woolly apple aphid (*Eriosoma lanigerum*).

Full emasculation pollination was carried out in the spring once most M.27 blossom clusters had at least one blossom at balloon stage or more. Branches were prepared by removing all leaves. One or two blossoms at advanced balloon stage were selected per cluster and the petals, anthers and filaments were removed. All remaining blossoms further or less

developed than balloon stage were removed. This was repeated for all clusters on the branch or bush, depending on tree size. After blossom preparation, a small quantity of pollen was transferred from the Petri dish (described above) to the stigmata of prepared blossoms using a sterilised index finger. The number of crosses made was recorded and trees fleeced to prevent frost damage. Fleecing was removed once the risk of frost was deemed suitably low and to prevent damage due overheating. Trees were checked weekly to remove any blossoms which had bloomed since crossing. Fruit set was recorded in August 2020 and manually harvested in October 2020 (details in experimental chapters) and stored in coldstore conditions until seed extraction.

Seed extraction and sowing

Mature seeds were extracted from fruit manually, rinsed to remove fruit flesh and submerged in distilled water in unlidded Petri dishes at room temperature to leach out germination inhibiting compounds. This water was discarded, seeds washed with fresh distilled water, and submerged again every two to three days, until the water remained clear. The seeds were rinsed and sterilised in a 10% bleach solution for ten minutes before thorough rinsing with distilled water. The cleaned seeds were placed in labelled containers lined with laboratory blue roll and left to dry uncovered in ambient room conditions. Once dry, they were placed into paper envelopes and kept at *ca*. 4 °C in coldstore conditions until sowing. Seeds were sown in February 2021 in a moist 50:50 peat to perlite mix and stratified in coldstore at 2 °C for 12 – 16 weeks before being moved into polytunnel conditions when day temperature was over 18 °C, and night temperature over 15 °C, in the spring of 2021. Seedlings were phenotyped for WAA resistance across the summer of 2021 per methods given in Chapter 2, Section 2.1.

Table 4.1: Numbers of flowers crossed, successful fruit set, and final number of seedlings generated for the M639 rootstock breeding population (M.27 × G.41).

No. flowers crossed	113
No. fruit set	157
No. viable seeds	694
Seedlings germinated	212
No. seedlings with available wood collected for grafting	175
No. seedling genotypes successfully grafted	169

Grafting

Wood was collected for grafting in February 2022. Four repeats of each seedling were taken for grafting, where possible. For each graft, second year seedling graftwood was collected, *ca*. 1 cm in diameter and *ca*. 10 cm long, although this varied depending on available material. Graftwood was grafted onto M.9 rootstocks (Frank P. Matthews nurseries, Tenbury Wells, Worcestershire) and potted into 3 L square pots with potting compost. Trees were transferred to polytunnel conditions in April 2022 for three months for the scion to establish.

Polytunnel conditions

Phenotyping was carried out in a single span polytunnel, as described above in Section 3.3.1.3., across 2021 and 2022. Grafted trees were arranged in a randomised block design as four trees per box, fourteen boxes per palette, arranged with seven boxes on each side of the palette (Figure 4.4). A gap was left between each set of four trees to allow for appropriate air flow to reduce build-up of pathogens and other pests. Trees were manually watered daily. No fertigation or insecticides were applied, but fungicides were applied for control of powdery mildew and scab six and five times, respectively during 2021 and 13 and three times, respectively during 2022.

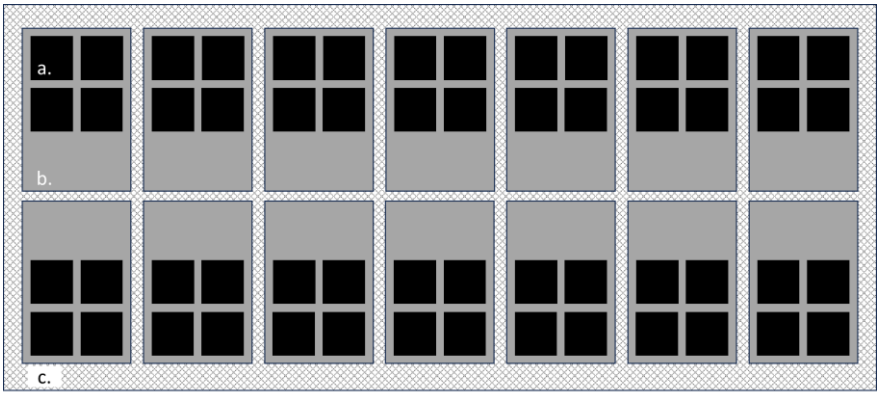

Figure 4.4- Experimental layout for phenotyping grafted trees of the rootstock breeding family, M639. Four potted trees (a.) per crate (b.) were randomised across four palettes (c.), arranged in two pairs such that each block contained two grafted trees of each seedling. The two pairs of palettes were located at opposite ends of a polytunnel, and therefore subject to slightly different microclimates, but otherwise treated the same.

Grafted trees were separated into two blocks such that each block contained half of the grafted trees from any one seedling. Repeats were divided across the two blocks such that half of the repeats for each seedling were assigned to each block. These blocks were located at opposite ends of the polytunnel, one block near the door and one by the ventilation louvres. Trees were randomised within each block.

4.3.2. Phenotyping events following controlled inoculation with aphid material

Families segregating for Er1

For the MCM007 population, grafted trees were inoculated with WAA material and scored, as described in Chapter 2, Section 2.1 in between July and October of 2020 and May and June of 2022. Second year grafted trees were 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.

Family segregating for Er2

A total of 111 M639 first year seedlings were phenotyped between August and October 2021 following Chapter 2 Section 2.1. 544 grafted trees were phenotyped between July and October 2022. Inoculation was carried out as described in Chapter 2 Section 2.1, with an additional scoring stage two weeks after the second score, to check WAA colonisation was progressing at an acceptable level. A fifth scoring event was carried out two weeks after the fourth score, and therefore four weeks after the second inoculation event.

Genotyping

Leaf discs were taken from 145 seedlings of MCM007 and 140 seedlings of M639, gDNA extracted and outcrosses removed following Chapter 2 Sections 2.2-2.4. A subsection of 92 samples were prepared for genotyping-by-sequencing GBS (Chapter 2 Section 2.5).

4.3.3. Data Analysis

Susceptibility scores were plotted using the ggplot2 package (v. 3.5.) with R Studio, v. 4.2.1. (R Core Team, 2022).

Mapping and marker association

The results of SNP generation were de-multiplexed, trimmed, and filtered following protocol given in Chapter 2, Section 2.6, by Dr Lawrence Percival-Alwyn.

Variant files were filtered to give only those aligned to LGs 8 and 17 for MCM007 (*Er1*) and M639 (*Er2*), respectively. This was carried out by Dr Greg Deakin. Trimmed and filtered reads in BCF format were converted to VCF format using BCFtools (Danecek *et al.*, 2021) and further transformed to a Hapmap Diploid format using Tassel (Trait analysis by ASSociation, Evolution and Linkage) software, v. 5.2.93 (Bradbury *et al.*, 2007). Libraries for generation of SNPs for the MCM007 family had very low read depth and quality and

therefore were not suitable for further analysis. Methodology henceforth refers only to the M639 population.

The resulting diploid SNP data were converted manually to a format compatible with JoinMap software and linkage mapping carried out with JoinMap v. 4.1 (Van Ooijen, 2006). These SNP data were filtered to remove loci with missing values for at least 10 seedlings, and any seedlings which were missing SNP data at over 100 loci. Adjacent loci with identical genotype data for all markers were removed from the data set. Maximum likelihood association mapping carried out for a LOD of > 4.

SNP calls were tested against the highest WAA susceptibility score for each seedling with a Kruskal-Wallace analysis in R Studio v. 4.2.1. (R Core Team, 2022) using packages data.table v. 1.15.2 (Barrett *et al.*, 2024) and dplyr v. 1.1.4. (Wickham *et al.*, 2023). *p*-values were subsequently adjusted using a False Discovery Rate (FDR) correction, and Manhattan and Quantile-Quantile (Q-Q) plots generated using the package qqman (Turner, 2018).

4.4. Results

4.4.1. Phenotyping following controlled inoculation with aphid material

Frequency of MCM007 phenotypic scores

Frequency of all phenotype scores recorded revealed a single peak across all three scoring years, at 1 for 2020 and 0 for 2021 and 2022 (Figure 4.5). The number of inoculations received by each seedling varied between one and two per season, except in 2021 when no trees were inoculated.

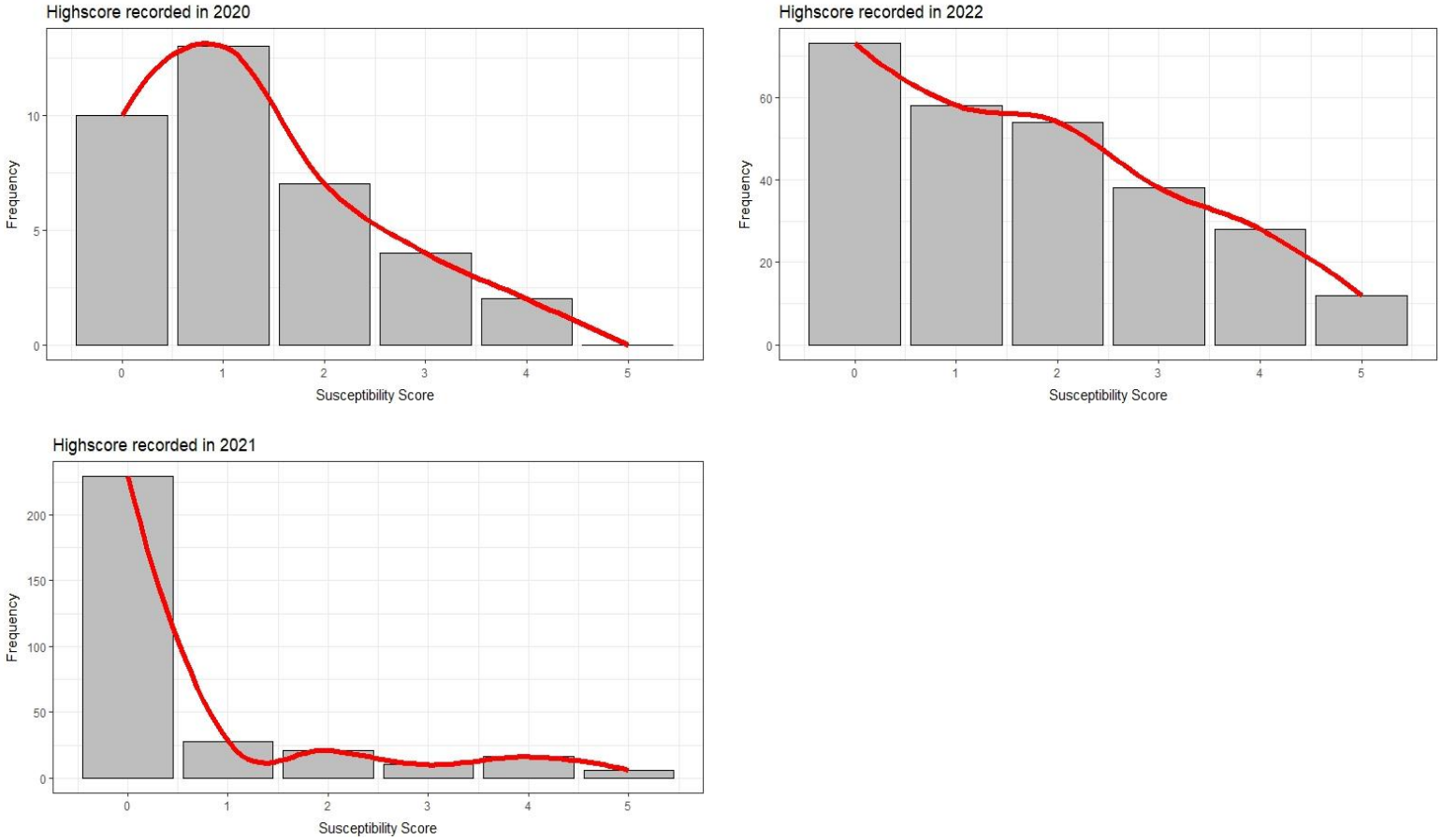

Figure 4.5 - Frequency graphs of the highest score recorded for susceptibility to the woolly apple aphid (*Eriosoma lanigerum*; WAA) in seedlings of the MCM007 rootstock breeding population (M.27 × MM106). Seedlings were scored across three summer seasons: 2020, 2021, 2022, indicated here, treated as scions and grafted onto M.9 rootstocks. Susceptibility scores were recorded from 0 to 5, where 0 indicates immunity to WAA and 5 indicates complete susceptibility. The highest score recorded across all scoring events is presented here, regardless of how many inoculations with WAA (one or two per season, except for 2021 where trees were not inoculated) each plant received.

Frequency of M639 phenotypic scores

Frequency of all phenotype scores recorded revealed a single peak for both years, at 0 and 1 for 2021 and 2022, respectively (Figure 4.6).

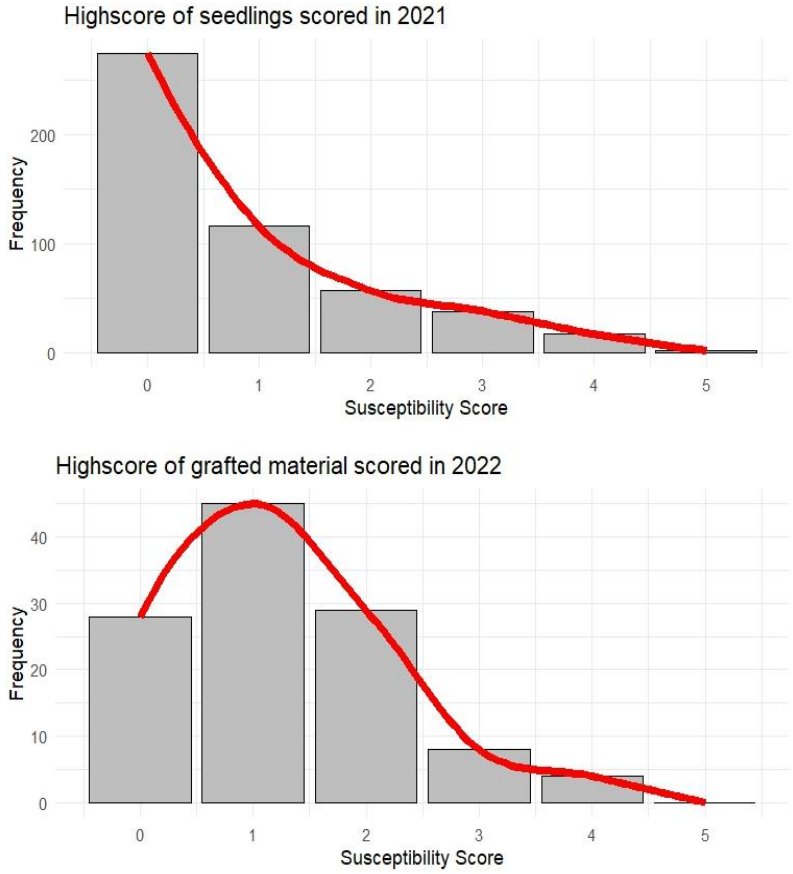

Figure 4.6: The frequency recorded of susceptibility scores recorded for seedlings, and grafted material of the rootstock breeding population M639 ('M.27' × 'G.41'). Score for susceptibility to the woolly apple aphid (*Eriosoma lanigerum*; WAA) from 0 to 5, where 0 indicates immunity to WAA and 5 indicates complete susceptibility.

4.4.2. SNP identification

Libraries for generation of SNPs for the MCM007 family had very low read depth and quality and therefore were not suitable for further analysis.

For the M639 family, after filtering, a total of 9927 SNPs were identified on LG 17, aligned across 34.731 Mb. After removing instances where both parents were homozygous, a total of 3613 SNP loci were identified, spanning from 0.036 to 34.731 Mb. SNPs were removed if both parents were homozygous because no segregation would been seen for the trait in the progeny.

4.4.3. JoinMap outputs

After filtering, a total of 1524 SNP loci were identified, of which 610 SNP loci were close to the top of LG 17 were subjected to Maximum Likelihood (ML) linkage mapping with JoinMap, generating a linkage map of LG 17 spanning a total of 75658.8 cM. Assuming *Er2* is a single major gene, as has previously been reported, it was located to 53703.5 cM with the closest flanking SNPs at 53645.7 and 53703.6 cM being at 0.480 and 9.017 Mb, respectively.

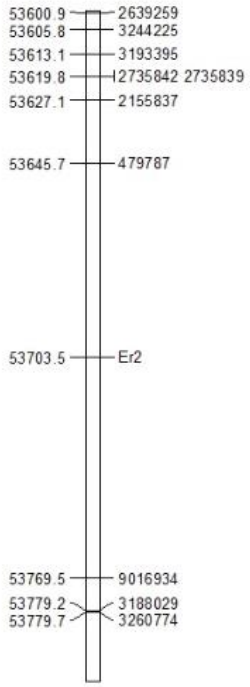

Figure 4.7 - Closest flanking markers in cM to *Er2*, a gene which confers resistance to the woolly apple aphid (*Eriosoma lanigerum*, WAA). This linkage map was generated from 610 SNPs identified across 92 seedlings of the *Er2* rootstock breeding family M639 (M.27 × G.41). The name of each SNP is its position in bp in LG 17.

4.4.4. Manhattan plot

An additional SNP locus was removed from the dataset prior to Kruskal-Wallis analysis as all seedlings showed the same genotype.

Of the 3613 SNPs identified, Kruskal-Wallis analysis found 333 SNPs with a *p*-value < 0.05, 72 < 0.01, and six < 0.001.

When plotted as a Q-Q plot, data showed a slight negative skew (Figure 4.8b), indicative of non-normal distribution. The smallest values are in-line with normal distribution but the largest values are larger than expected for a normal distribution, suggesting that the data are

skewing slightly towards the higher end of the scale. This is supportive of a QTL or resistance gene cluster because significant SNPs are not associated with a single point on the chromosome, as would be expected with a sngle gene.

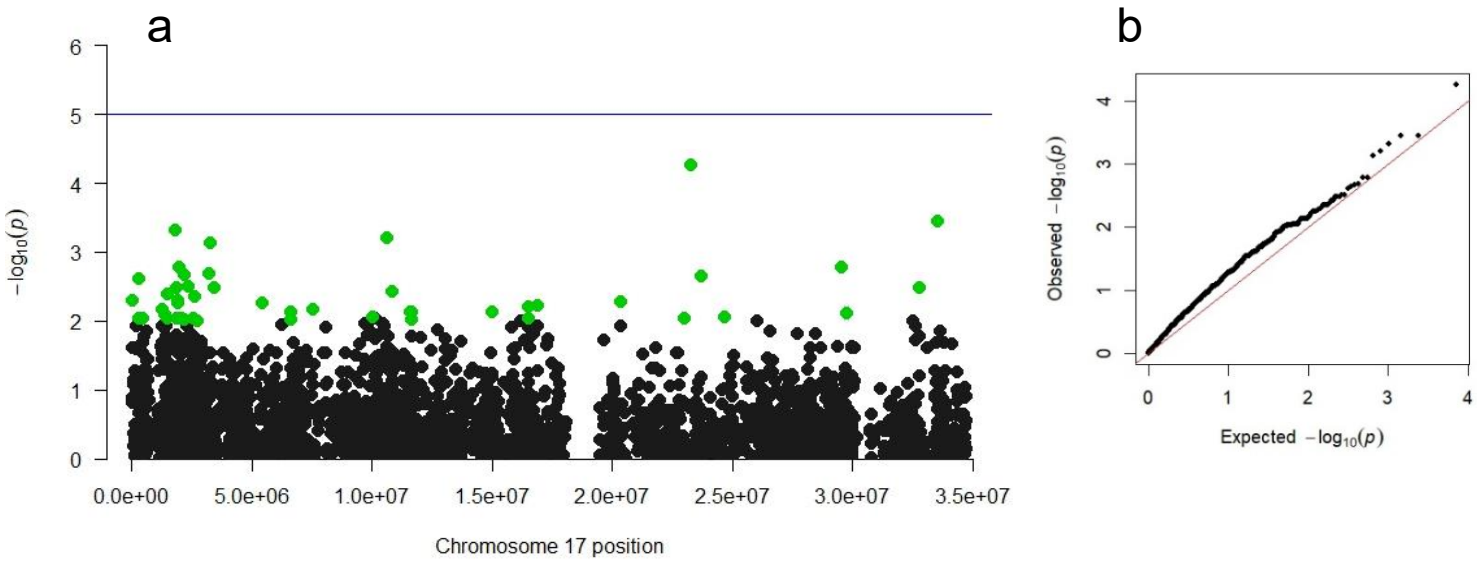

Figure 4.8- a). Manhattan plot ($-\log_{10}$[P] genome-wide association plot) of SNPs generated for 80 seedlings of the apple rootstock breeding population, M639 (M.27 × G.41), the parents of this population, and the source of the woolly apple aphid resistance gene *Er2*, *Malus* x *robusta* 5a. Position in chromosome 17 is given in base pairs. *p* values were generated through Kruskal-Wallis analysis of SNP variation against seedling classification of resistant or susceptible to the woolly apple aphid (*Eriosoma lanigerum*; WAA), and adjusted using False Discovery Rate (FDR). SNPs with a significance greater than 0.01 are highlighted in green.

b). Quantity-Quantity (Q-Q) plot of observed vs. expected *p* values output by the Kruskal-Wallis analysis. Normal distribution is indicated by the red line where observed values = expected values. Deviance of data points away from this line indicates deviation of the data from a normal distribution.

Table 4.2: The position (Mb) of the 10 most significant SNPs associated with WAA resistance to woolly apple aphid (*Eriosoma lanigerum*; WAA), in the M639 rootstock breeding population. *P* values calculated via Kruskal-Wallis analysis of SNP loci against seedling susceptibility to WAA across three conditions; two-factor classification as resistant or susceptible; the highest susceptibility score recorded for each seedling (0 - 5) across all WAA inoculations; the mean susceptibility score (0 - 5) across scoring events and repeats. SNP loci which were significantly associated with *Er2* resistance across both analysis conditions are indicated by ^.

	Two factor scoring (resistant or susceptible)		Highest susceptibility score (0-5)		Mean susceptibility score	
	Position (Mb)	*p* value	Position (Mb)	*p* value	Position (Mb)	*p* value
1	23.273	0.000056	0.586 ^	0.00000105	4.316	0.000784
2	33.529	0.000362	0.586	0.00000105	21.1089	0.000784
3	33.529	0.000362	0.586 ^	0.00000105	6.274 ^	0.000784
4	1.792	0.000481	2.930 ^	0.00000105	6.274	0.000784
5	10.646	0.000626	2.930 ^	0.00000105	6.274	0.000784
6	3.261	0.000739	6.274	0.00000105	8.828	0.000784
7	29.553	0.001658	6.910	0.00000105	0.586 ^	0.000784
8	1.981	0.00167	27.945	0.00000105	0.586 ^	0.000784
9	3.236	0.002052	31.814	0.00000105	2.930 ^	0.000784
10	2.206	0.002142	6.274 ^	0.000706614	2.930 ^	0.000784

The region between 589478 and 6274028 base pairs were aligned against the 'Golden Delicious' genome to detect relevant genes which may be linked to *Er2* resistance. 58 genes were described in this region, including 11 uncharacterised genes, 10 of which were protein-coding and one ncRNA gene. Two genes encoding DNA repair proteins, a zinc finger CCCH

protein involved in abiotic stress response, and TMV resistance protein N, which confers resistance to the tobacco mosaic virus were present in this region.

4.5. Discussion

For a single major gene exhibiting Mendelian segregation, we would expect to observe two peaks of susceptibility scores, indicating equal distribution of resistant and susceptible phenotypes. The skew towards resistance seen in Figures 4.5. and 4.6. may be the result of conditions unfavourable for aphid survival, including high temperatures under polytunnel cover, which are both a stressor for WAA and beneficial for its parasitoid wasp *Aphelinus mali* (Haldeman), which has a higher developmental temperature threshold than WAA (El-Haidari *et al.*, 1978). The time of year of phenotyping can therefore influence the scores seen; scoring events carried out in spring or autumn are more likely to yield high susceptibility scores than those carried out mid-season when parasitoid wasp numbers are highest.

The repeat inoculation event for seedlings which score as “resistant” after the first scoring (Chapter 2, Section 2.1), increases the frequency of low susceptibility scores within the dataset, *i.e.* a high score of four or five would be recorded a maximum of twice, whereas a resistant seedling could be scored as zero up to five times. Distortion away from expected ratios is not unusual; a skew towards susceptibility was reported in the progeny of a cross of M.9 × ‘Robusta 5a’ cross (Bus *et al.*, 2008). This was, however, most likely a factor of the scoring classification used, which classified only seedlings which were completely immune to WAA feeding as “resistant” with the presence of any aphids or visible galling as “susceptible”. We have used instead a classification more similar to that of Knight *et al.* (1962): a six-point scale of variable susceptibility, allowing for variable susceptibility. In the raw susceptibility score data there is an under-representation of high susceptibility scores because after receiving a score of four or five, trees would not be given a second inoculation of WAA material, leading to only two “susceptible” scores recorded. Plants deemed “resistant” or “intermediate” after their initial inoculation were inoculated a second time, allowing for low scores to be recorded up to four times (first pre-score, first post-inoculation score, second pre-score, second post-inoculation score). This creates over-representation of lower susceptibility (higher resistance) scores within the data set. This has been combatted in this data set by using the highest score recorded across all scoring events as the score taken for each plant. This should reduce skew towards low scores created by over-representation, as discussed, but also reduce instances where a aphid colonisation was present but had been knocked down by *e.g.* unfavourable temperature conditions. Parental material was only included in the 2021 season for MCM007 and in the 2022 season for

M639, as controls. The highest score recorded for M.27, the susceptible parent for both families, was lower than expected, at a three. This supports the over-representation of low susceptibility/high resistance scores recorded for the breeding populations which deviated from expected segregation ratios. This re-enforces the theory that the glasshouse and polytunnel conditions the breeding populations were kept in were unfavourable for WAA growth. Resistant parents showed no WAA colonisation, as would have been expected, but also potentially a factor of the unfavourable growth conditions, rather than a strong resistant phenotype.

The ten markers most significantly linked with WAA susceptibility varied between scoring techniques used, although those identified when using highest and mean susceptibility scores were more similar than a resistant/susceptible scoring system. Across the 10 most significant SNPs identified in each of the three analysis conditions for Kruskal-Wallis test, all identical SNPs were located between 0.586 and 5.274 Mb, suggesting that *Er2* lies within this region. Although there were no shared SNPs identified when the phenotype was analysed as resistant/susceptible, five of the ten most significant markers identified for that analysis were located within that region. Both instances of susceptibility scoring using a six-point scale identified eight of the ten most significant markers in this region. The differences in both individual SNPs and regions significantly associated with the trait of interest suggests that careful consideration is required when selecting parameters for association analysis. A simple categorisation of resistant or susceptible may be expected to show a more clear, dichotomous assignment of SNPs associated with each trait, rather than multiple scorings. JoinMap 4.1 requires trait data to be coded as either the resistant or susceptible parent, in this way. In this study, Kruskal-Wallis analysis better identified a region of the chromosome with SNPs significantly linked to the WAA resistance trait when using multiple scoring values, which may contribute to the alignment of SNPs with *Er2* out of chromosome position order by JoinMap.

The map position generated for *Er2* by JoinMap was exceptionally large, because of the large number of SNPs identified. The existing map of *Er2* places it at 13.6 cM on a map spanning 65.6 cM, generated with seven SSR markers. Whilst more dense coverage is expected with SNP generation (Poland & Rife, 2012), this quantity of SNPs is difficult to display with JoinMap. Whilst JoinMap has been used successfully for linkage mapping in apple (Evans *et al.*, 2011), these have mostly used SSR markers. The closest flanking markers to *Er2* identified by JoinMap 4.1 are out of alignment with their genomic positions, as are many of the other markers in the linkage map (Figure 4.7). If the SNPs were in their

correct order we would expect to see SNP names, giving their genomic position, increasing numerically along the map but in this instance we observed variation in

JoinMap 4.1 struggles to compute large marker datasets, presenting a challenge when analysing SNP datasets (Liu *et al.*, 2014), even when using strict filtering conditions. Other mapping technologies which are better suited to managing large datasets should be trialled with this dataset to generate a linkage map. HighMap has been used successfully with SNPs generated by NGS and was able to generate a linkage map with three times as many markers as JoinMap 4.1 could construct (Liu *et al.*, 2014). The R package OneMap uses the mapping functions of JoinMap, implemented into R for linkage mapping, although the original version was not able to analyse F1 populations from two outbred parents (Margarido, Souza & Garcia, 2007). The package BatchMap optimises OneMap to use high-throughput sequencing outputs to rapidly generate high density linkage maps for F1 populations (Schiffthaler *et al.*, 2017).

No SNPs had previously been developed for *Er2*. SNPs identified in the M432 (Antanaviciute *et al.*, 2012) may no be longer reproducible as they were generated using an older 8K SNP array. SNP generation through a GBS approach generates specific, highly reproducible markers (He *et al.*, 2014), which have greater longevity for use in MAS. SNPs found by Kruskal-Wallis analysis were significantly associated with WAA susceptibility spread across the chromosome (Figure 4.6), with the majority found at the top of LG 17, in agreement with the position found by Bus *et al.* (2008). The genomic positions of the 10 most significant SNPs identified for all Kruskal-Wallis analysis conditions varied, suggesting that there may not be a single major resistance gene. The region in which the highest number of significant SNPs identified two DNA repair genes, one gene linked to abiotic stress tolerance, and one gene for Tobacco Mosaic Virus resistance. Aphid feeding induces both herbivore and pathogen responses in plant tissue (Moran & Thompson, 2001) and whilst a single virus defence gene is not strong evidence of a resistance cluster, there is potential to investigate this region further.

It is not clear what caused the MCM007 family to generate lower quality SNPs than M639. Library preparation was carried out identically across both families, although MCM007 gDNA was extracted by two individuals in two separate years. This may have led to poor quality DNA for library prep, but we would in that case expect to see some successful SNP reads. This work was carried out alongside another student who also found low read depth in some libraries. It seems therefore possible that some regents and/or enzymes were unsuitable for library preparation. With more time and money remaining in the project, this population could have been run through a SNP array to detect any varying SNPs which were not able to be identified through the poor library read depths.

Robust arrays for flanking SNPs would allow MAS of *Er2* as a single gene, and for pyramiding multiple resistance genes to give durable horizontal resistance. Horizontal resistance confers resistance, or tolerance, to all strains of a pest or pathogen, in contrast to vertical resistance which is only conferred to a single strain. Horizontal resistance may allow infestation but prevents disease colonisation and spread (Dyck & Kerber, 1985). While it is desirable that WAA be prevented entirely from feeding on apple rootstocks, there may be a trade-off for future resistant rootstocks whereby durable tolerance is achieved, but that some WAA will be able to feed, but not establish. Following verification of flanking markers using a KASP array system, these markers would be tested on progeny of an *Er2* cross across a range of WAA susceptibility phenotypes to determine how accurately these markers can predict WAA resistance.

Marker verification in a related breeding population, *e.g*. M432 would be used to verify markers for MCM007. The M432 family is a backcross between M.27 and M.116 (MM106 x M.27; Figure 4.2), generated to segregate for a range of traits of interest to rootstock breeding including dwarfing, root architecture, and anchorage (Evans *et al*, 2011).

CHAPTER 5 - The identification of genetic variation and inference of population structure of the woolly apple aphid (Hemiptera: Aphididae) within the United Kingdom, and compared to international sampling locations using microsatellite markers.

5.1. Abstract

The woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) is a widespread pest of apple (*Malus* × *domestica* Borkh.). Woolly apple aphid originates in North America where it has a heteroecious lifecycle, alternating between asexual reproduction on apple and sexual reproduction on American Elm (*Ulmus americana* L.) but has lost this lifecycle elsewhere. The species feeds exclusively on apple and is thought to be predominantly, if not entirely, asexual. This work aims to determine whether UK populations of WAA show genetic variation within the country and when compared to samples from other apple-growing countries, and to infer to what extent this variation is the result of sexual reproduction. One hundred and eighty-seven WAA samples were collected from thirty-five locations (mean population size *n*= 5). Assuming asexual reproduction of WAA and a single colonisation event, a single genetic population of WAA was expected. Analysis with the software STRUCTURE tested between one and thirty-five putative populations and found the most likely number of populations to be two, with the presence of likely sub-structuring. This alone is not evidence of functional sexual reproduction but suggests the potential for previously unknown geneflow between WAA populations within the UK, to an extent unlikely to be caused solely by genetic drift or multiple invasions of the pest. This is a concern for pest control because of the potential for spreading genes which confer the ability to feed on resistant rootstocks, as has been reported in several apple-growing regions.

5.2. Introduction

The woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) (Hemiptera: Aphididae) is a sap-feeding pest of domesticated apple (*Malus* × *domestica* Borkh.), first reported in Britain in 1787 (Theobald, 1921). In its native North America WAA exhibits a heteroecious lifecycle, alternating between sexual reproduction on American Elm (*Ulmus americana* L.) and asexual reproduction on *M. domestica* (Baker, 1915) but elsewhere appears to have lost its sexual phase, feeding exclusively on apple. Woolly apple aphid causes damage through the injection of elicitors in saliva whilst feeding which causes cambium cells to rapidly divide, creating a gall which can spread to other vascular tissues (Staniland, 1924; Miles, 1999) blocking photosynthate transport and leading to reduced growth and yield, especially in

younger trees (Weber & Brown, 1988; Brown *et al.*, 1991). The loss of the sexual stage may have increased pressure on commercial apple production through rapid build-up of asexually produced nymphs, leading to increased galling. Gall formation can promote group feeding in aphids by creating a photosynthate sink from neighbouring tissues, sustaining a large number of aphids feeding at that site (Larson & Whitham, 1991).

Constant asexual reproduction is expected to have reduced the genetic variation within the species. Microsatellite analysis of both sexual and asexual populations of the bird cherry-oat aphid (*R. padi*) found high allelic polymorphism and heterozygote deficiency in sexual populations, compared to asexual populations which showed much less polymorphism but large amounts of heterozygosity (Delmotte *et al.*, 2002). Little geographic differentiation was found between sexual populations, suggesting that *R. padi* can disperse over large areas which, combined with sexual reproduction, has led to widespread varied genotypes of *R. padi*. Obligate sexual populations of the pea aphid (*Acyrthosiphon pisum* Harris) show lower allelic diversity per locus but higher overall genotypic diversity than asexual populations (Kanbe & Akimoto, 2009).

Genetic diversity of WAA measured by polymorphic loci identified by Inter Simple Sequence Repeat (ISSR) markers found four distinct genetic clusters in central Chile, corresponding to landscape features in that region, such as rivers and areas of high ground (Lavandero *et al.*, 2009a). Spatial separation of individuals by geographic barriers creates isolated habitats which prevent gene flow and drives the development of distinct populations (Coyne & Orr, 2004). This variation is not necessarily indicative of sexual reproduction but suggests that it may be present because no linkage disequilibrium with codominant markers was observed (Lavandero *et al.*, 2009a). As WAA is often distributed on infected rootstocks, wind patterns and geographic barriers cannot entirely explain the genetic variation seen as there is always the potential for populations to be moved against geographic barriers through human intervention.

Greater variation can be detected through higher marker coverage, for example through generation of single nucleotide polymorphism (SNP) markers. The generation of SNPs by Genotyping-by-Sequencing (GBS) has been successfully used to investigate genetic variation of the Argentine stem weevil (*Listronotus bonariensis* Kuschel) in New Zealand (Harrop *et al.*, 2020). Variation was found between the north and south islands of New Zealand, consistent with both multiple invasions of *L. bonarensis* and a single invasion followed by genetic diversification. This approach identified higher variation within and between populations of *L. bonariensis* than previously found with RAPD and COI markers.

A GBS approach found that *Sitobion miscanthi* (Takahashi; the Indian grain aphid) in China mostly show cyclical parthenogenesis, with six distinct genetic sub-populations observed, correlated strongly with sampling location (Morales-Hojas *et al.*, 2020). *Sitobion avenae* (Fabricius; the English grain aphid) in England mostly reproduce asexually, forming a single genetic cluster, which could be consistent with the insecticide resistant *S. avenae* clone identified previously (Malloch *et al.*, 2016).

Several WAA-resistant rootstocks are commercially available for WAA control but there have been reports, both published and anecdotal, of WAA feeding on these rootstocks. Most of these have been from the southern hemisphere where conditions may be more favourable for aphid growth, and more recently in Europe (Giliomee *et al.*, 1968; Rock & Zeiger, 1974; Jaastad, 2020, *pers*. *comm*.). Woolly apple aphid, feeding on M.116 and MM106 rootstocks have been observed at NIAB East Malling in Kent, UK but this has not been formally reported. If such a resistance-breaking biotype of WAA does exist within the UK it could pose a serious threat to UK apple production, especially if the species is also able to sexually reproduce and spread a potential virulence gene(s). Alleles for insecticide resistance increase in frequency in populations of *Myzus persicae* (Sulzer) following sexual reproduction (Guillemaud *et al.*, 2003).

The presence of sexual reproduction and the associated increase in genotypic diversity and gene flow raises the potential spread of virulence genotypes. The spread of a pyrethroid-resistant clone of *S. avenae* in the UK has been limited so far by the fact that this clone is anholocyclic and thus lacks the sexual recombination required to spread this trait (Malloch *et al.*, 2016). As with the WAA, the emergence of sexual reproduction within this clone could rapidly spread this trait. At least three biotypes of WAA have been discovered in Australia, each with measurably different performance (Costa *et al.*, 2014). The variation between biotypes is suggested to be linked to different modes of host plant resistance to aphid feeding which may drive selection of virulence phenotypes. The pea aphid has genetically determined differences in performance depending on its host plant which selects for aphids which remain on their host rather than those which move between hosts and are not able to improve their performance through experience (Via, 1991). This promotes genetic diversity between populations which may be geographically close but separated by host plant.

We expect that there is not functional sexual reproduction in the UK based on previous observations in other countries outside of the USA. Because of this we hypothesise that there is little to no genetic variation between samples collected within the UK. This research sets out to test two questions:

i. To what extent is there genetic variation within UK WAA? Is it alternatively possible that there are several distinct populations?

ii. In the instance of genetic variation, how likely is it that this variation is caused by sexual reproduction? The use of a GBS approach will allow identification of smaller differences between populations and highlight potential regions of high variation.

5.3. Materials and Methods

5.3.1. Sample collection

Each sample collected consisted of multiple, mixed-age aphids of no defined size from a single, distinct colony, assumed to be the result of a single asexual mother aphid. Aphids were brushed from plant material with a soft paintbrush into collection tubes.

Samples from GNY, SNY, TLC, and MSM (see Table 5.1 for details of sampling location) were collected and stored in > 96% ethanol. Before gDNA extraction samples were removed from ethanol, dried briefly on Fisherbrand Grade 601 filter paper (Fisher Scientific) to remove excess ethanol, and transferred to 1.5 ml Eppendorf tubes filled with *ca*. 1 ml grade 40, 6-14 mesh silica gel (Sigma-Aldrich) to fully desiccate. All other samples were collected directly into Eppendorfs with silica gel to dry.

5.3.2. gDNA extraction and product amplification

Dried aphids were removed from the silica gel and transferred to clean 2 ml Eppendorf tubes. Two 5 mm steel ball-bearings (Simply Bearings) were added to each tube and tissue homogenization was carried out using a Geno/Grinder 2010 tissue homogenizer (SPEX SamplePrep, USA) at 1500 RPM for 90 seconds. This was repeated, as necessary, until samples reached a homogenous fine powder. Total genomic DNA (gDNA) was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden) using the supplementary protocol for purification of total DNA from insects and eluted into a final volume of 200 µl. Total gDNA quantity and quality was estimated using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). gDNA extraction products were normalised with ultrapure water to a 5 ng/µl concentration and amplified by Polymerase Chain Reaction (PCR) using the Qiagen Type-it Microsatellite PCR Kit (Qiagen, Hilden) under standard 50-55 °C PCR cycling conditions in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems). Microsatellite markers from Lavandero *et al.* (2009b) were used in two multiplexes (Table 5.2).

5.3.3. Microsatellite analysis

PCR products were diluted with ultrapure distilled water and denatured at 90 °C for three minutes using a Thermal Cycler with GeneScan™ 500 LIZ® Size Standard and Hi-Di Formamide (ThermoFisher Scientific) for fragment amplification analysis using ABI PRISM® DNA Sequencing Analysis which was carried out both at NIAB East Malling and the John Innes Centre. For samples sent away, PCR success was determined by gel electrophoresis on a 1.5% agarose gel at 150 V for 50 minutes with Fisherbrand™ horizontal gel electrophoresis systems with a PowerPro 300 power supply (Fisher Scientific). Fragment size analysis was carried out by ABI 3730 DNA Analyzer (Applied Biosystems) at the John Innes Centre for 72 samples from 16 locations, and by ABI 3130 DNA Analyzer (Applied Biosystems) at NIAB East Malling for 200 samples from 20 locations. Details of analysis method for each sampling location are given in Table 5.1. The resulting peaks were classified at NIAB using GeneMapper™ 4.0 software (Applied Biosystems).

2922 Table 5.3- Details of woolly apple aphid (*Eriosoma lanigerum*, WAA) samples analysed with microsatellites to determine population genetic structure. Numeric and letter codes are given for each location and used throughout the text and other figures. Identifying location, region, country and approximate latitude and longitude co-ordinates are given, with sampling date. The number of samples remaining after quality control checks is given and number of samples analysed by STRUCTURE software, given that any loci with more than two alleles must be inputted as multiple samples.

Numeric code	Sample code	Sampling location and approximate co-ordinates	Sampling date	No. samples	No. STRUCTURE inputs
1	GNY	Geneva, New York, USA (42.903, -77.029)	07.07.2020	4	4
2	SNY	Sodus, New York, USA (43.210, -77.016)	07.07.2020	4	7
3	TLC	Talca, Maule Region, Chile (-35.418, -71.664)	22.01.2020	3	3
6	MMC	Molina, Maule Region, Chile (-35.174, -71.189)	14.03.2022	3	3
7	GMC	Guaico, Romeral, Maule Region, Chile (-35.003, -71.034)	28.03.2022	1	2
5	RMC	Romeral, Maule Region, Chile (-35.034, -71.085)	28.03.2022	1	1
4	SOC	San Fernando, O'Higgins Region, Chile (-34.58535, -70.56521)	28.03.2022	2	2
8	HVN	Crosses & St George's Rd Orchard, Plant & Food Research, Havelock North, New Zealand (-39.654, 176.876)	15.03.2021	8	12
9	FAN	Floriade Expo, Almere, Netherlands (52.355, 5.227)	26.08.2022	1	1
10	NIL	Loughall, County Armagh, Northern Ireland (54.410, -6.603)	17.11.2021	4	6
11	WMC	Alan Hudson Ltd, Wisbech St Mary, Cambridgeshire, England (52.641, 0.117)	07.06.2022	9	10
12	EVW	Evesham, Worcestershire, England (52.134, -1.934)	08.06.2022	2	2
13	LBH	Ledbury, Herefordshire, England (52.037, -2.457)	07.06.2022	2	3
14	AIH	Aston Ingham, Herefordshire, England (51.923, -2.462)	09.06.2022	2	2
15	LIH	Linton, Herefordshire, England (51.926, -2.467)	09.06.2022	2	3
16	PSH	Peterstow, Herefordshire, England (51.919, -2.656)	09.06.2022	2	2
17	MSM	Minehead, Somerset, England (51.202, -3.480)	08.2020	5	7
18	LFS	Lydford-on-Fosse, Somerset, England (51.084, -2.633)	29.09.2021	5	7
19	TSS	Thatchers Cider, Sandford, Somerset, England (51.320, -2.845)	06.10.2021	1	1
20	WFG	Woodford Green, London, England (51.600, 0.055)	25.06.2022	5	6
21	WOT	Walton-on-Thames, Surrey, England (51.386, -0.431)	13.07.2020	4	4
22	PRS	Pagehurst Road, Staplehurst, Kent, England (51.160, 0.519)	17.06.2022	8	8
23	WSB	Whitstable, Kent, England (51.357, 1.019)	11.06.2021	2	2
24	HPW	Honoton Farm, Paddock Wood, Kent, England (51.146, 0.413)	12.07.2020	1	1
25	CHF	Clockhouse Farm, Penshurst, Kent, England (51.227, 0.498)	08.06.2020	1	1
26	NFC	Ambient polytunnel, National Fruit Collection, Brogdale, Kent, England (51.296, 0.883)	10.06.2021	5	8
27	NFC +2	Polytunnel at ambient + 2°C, National Fruit Collection, Brogdale, Kent, England (51.296, 0.882)	10.06.2021	26	39
28	NFC +4	Polytunnel at ambient + 4°C, National Fruit Collection, Brogdale, Kent, England (51.296, 0.882)	10.06.2021	34	38
29	MST	Loose, Maidstone, Kent, England (51.250, 0.531)	07.06.2022	10	10
30	WMK	West Malling, Kent, England (51.296, 0.403)	14.05.2020	3	4
31	EMS	Railway Station, East Malling, Kent, England (51.285, 0.442)	07.06.2022	10	10
32	OGB	Apple gene bank, NIAB, East Malling, Kent, England (51.288, 0.442)	06.06.2020	6	7
33	WSM	Wiseman orchard, NIAB, East Malling, Kent, England (51.287, 0.466)	01.06.2021	2	4
34	GHJ	NIAB glasshouse, East Malling, Kent, England (51.285, 0.450)	18.02.2020	8	15
35	EMR	WAA culture on resistant and susceptible rootstocks, NIAB, East Malling, Kent (51.286, 0.453)	27.03.2021	14	17

Table 5.2: Microsatellite markers used for population genetic analysis of woolly apple aphid (*Eriosoma lanigerum*, WAA). Markers from (Lavandero *et al.*, 2009b) were used in two multiplexes, A and B, based on complimentary size ranges.

Multiplex	Locus	Repeat	GenBank Accession no.	Primer sequences (5'-3')	Size range (bp)	T_a (°C)
A	*Erio3*	$(TC)_9(CTAT)_6$	EU410510	F: GCCAAACAGTCTTATCTTTCC	147-163	60
				R: GAATTCGCTGGCTCTCTCTCT		
	Erio33	$(CAA)_{12}$	EU410514	F: TCAATGGCAACCGAAGTGTA	159-183	60
				R: GCAACAGTGGCGTCATCC		
	Erio72	$(CT)_{13}$	EU410515	F: GCTGTAGCGGGCGTAATAAT	148-170	60
				R: AACCTTAACCGCCCCTCTAA		
	Erio75	$(TC)_{12}(CT)_7$	EU410516	F: ACGGAGATGAAGGCGTTATG	134-166	60
				R: TCTCTCCGTCTTTCCGTCTC		
B	*Erio20*	$(CAA)_{10}$	EU410511	F: CGACCTTGAGCCTTTGAAAC	161-179	59
				R: CTGGCTCACTTCCTGGTAGC		
	Erio25	$(CAA)_{10}$	EU410512	F: TTGTCACGAACATAAACGTA	100-106	50
				R: GTACATATTACAACAACAAC		
	Erio29	$(GTT)_8$	EU410513	F: TACTCATCGCGAAAACGAGA	171-189	60
				R: AGTCTCGTCCGATGTTGTTG		
	Erio78	$(AG)_{12}$	EU410517	F: AAGTTTAATGGCGTGGGCTA	143-175	60
				R: GGGATGGTAAACGAGTGTGTG		

5.3.4. Analysis of microsatellite diversity

From an initial data set of 272, samples were rejected if they had missing data for at least half of the eight loci. Alleles were classified to a single base-pair position, rounded up or down within a four base-pair range. In the 47 instances where a single WAA sample had more than two alleles present, this was split into multiple samples to allow analysis as

software used were only suitable for diploid samples. Sample details, including those with duplicates for data analysis, are given in Table 5.1.

Population assignment

Population structure was inferred using the software STRUCTURE version 2.3 (Pritchard, Stepehns & Donnelly, 2000; *Hubisz et al.*, 2009). An assumed number of genetic populations, K, was selected based on the number of sampling locations, in this case from one to 35. 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 (Zhou *et al.*, 2015).

The data generated by STRUCTURE were further analysed by STRUCTURE HARVESTER to generate mean likelihood values for each K value tested (Earl & vonHoldt, 2012) using the Evanno, Regnaut & Goudet, (2005) method. Here values for the change in log probability of the data for each putative value of K, ΔK, are calculated. ΔK represents an ad hoc statistic based on the rate of change of the likelihood function with respect to the value of K being tested (Evanno, Regnaut & Goudet, 2005).

STRUCTURE software was developed for sexual populations and therefore may not be able to capture population differences in asexual populations. An artificial clonal dataset was created using identical marker information for all individuals. The same number of individuals, population data, and parameters were used for STRUCTURE analysis.

Principal Component Analysis

Principal Component Analysis (PCA) was conducted and visualised using R v. 4.1.2 (R Core Team, 2021) with the following packages: ade4 (v. 1.7-19; Dray & Dufour, 2007), adegenet (v.1.3-1; Jombart, 2008), factoextra (v.1.0.7; Kassambara & Mundt, 2020).

Generation of population statistics using GenAlEx

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

The above methods were applied to a sub-section of samples collected from Southeast England and published in Acta Horticulturae (Godfrey *et al.*, 2023), attached here as appendix 3.

5.3.5. Genotyping-by-Sequencing

Sample selection

A sub-section of the above samples was selected for SNP generation using a Genotyping-By-Sequencing (GBS) approach (Table 5.3a). Priority was given to sampling a range of locations, with samples ordered from the WAA native range in North America to NIAB East Malling. Samples were rejected if their estimated DNA concentration was less than 10 ng/µl, or if their 260/280 or 260/230 ratios were below 1.8 and 1.0 respectively.

Once these criteria had been filled the remaining samples were further streamlined to a limit of two samples per sampling location in most cases with preference given to samples with a higher DNA concentration to ensure the required amount of DNA for GBS could be reached. Where possible, samples with a DNA concentration above 100 ng/µl were chosen. After this, samples with higher 260/280 and 260/230 ratios were chosen. Repeats were included for some sites of interest to prevent loss of data through unsuccessful runs and to check for variation across repeats (Table 5.3b).

Library preparation

Library preparation was carried out following the method given in Chapter 2, Section 2.5 and whole genome sequencing carried out by Illumina NovaSeq6000 paired-end sequencing at NovoGene. Demultiplexing, trimming and alignment to the WAA genome assembly (Biello *et al.*, 2021) follow the protocol given in chapter 2, section 2.6.

SNP genotyping

SNP genotyping and population analysis were not able to be completed in the timeframe of the project.

Table 5.3: Subsection of woolly apple aphid (*Eriosoma lanigerum*; 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

5.4. Results

5.4.1. STRUCTURE analysis

STRUCTURE HARVESTER found the most likely number of populations (K) from those tested, to be two (Figure 5.1). 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. The most likely number of populations identified in the artificial clonal dataset created as a control population was eight and the second most likely as 15 (Figure 5.1.b).The outputs of the STRUCTURE analysis show assignment of microsatellite data for K values from 2 to 10, and 26 as this was the second most likely number of

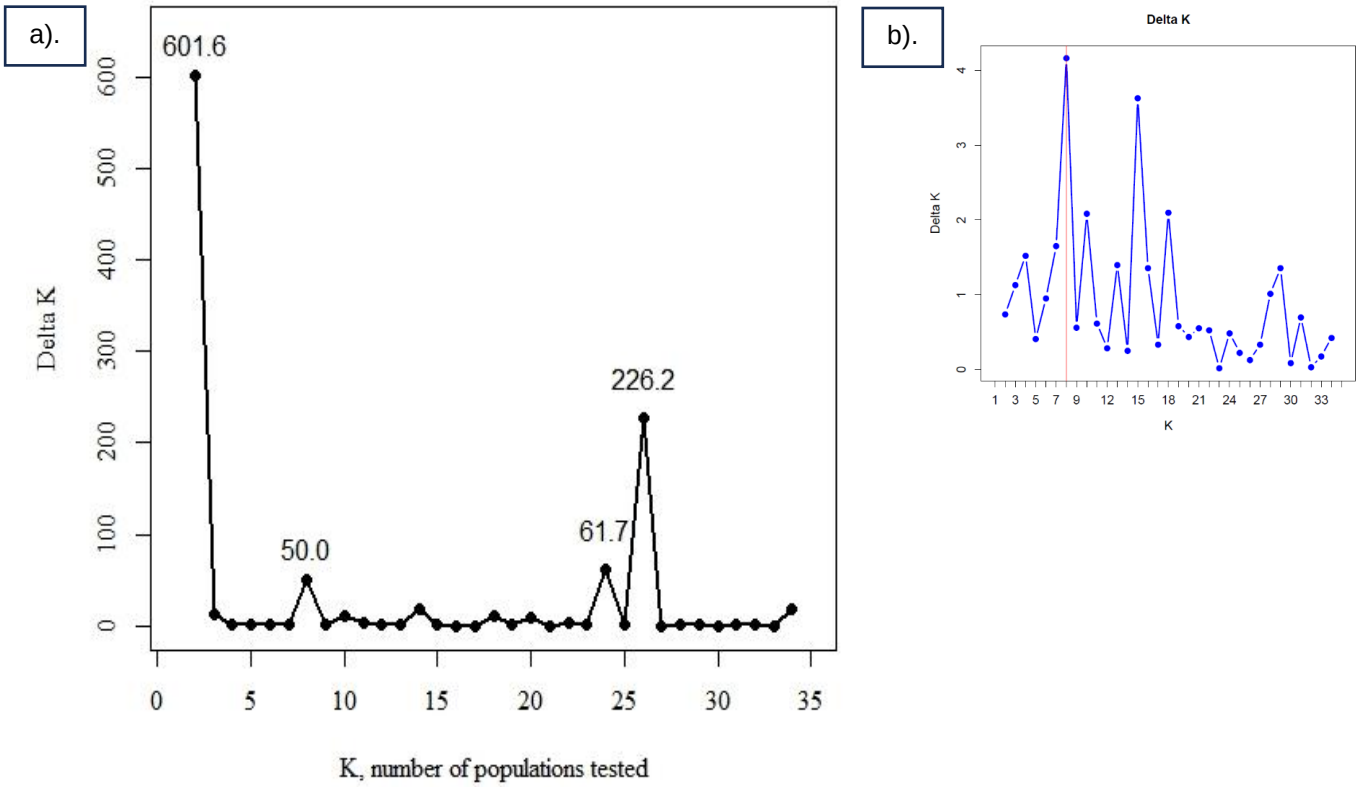

Figure 5.11: a). Likelihood (Delta K), of woolly apple aphid (*Eriosoma lanigerum*, WAA) samples being comprised of K populations, when tested on K = 1 to K =34, based on the number of locations from which samples were collected.
b). Delta K outputs for an artificial clonal dataset developed from the same populations as in a). but with the same marker values inputted for all individuals. The most likely number of populations present across the samples is 8, as indicated by the red vertical line. Figure 5.1.b. Generated by StructureSelector (Li & Liu, 2018).

populations as determined by STRUCTURE HARVESTER (Figure 5.2). The output from K=2 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.

5.4.2. F statistic

Pairwise FST values calculated with GenAlEx ranged from 0.000 to 0.310 with a mean of 0.227 (Table 5.4). Of 745 FST outputs, 134 were below 0.1 and five were greater than 0.5.

5.4.3. Summary of population genetic diversity statistics

The mean observed number of alleles (N_a) across all marker loci ranged 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 HE is lower than the value for HO for every sampling location.

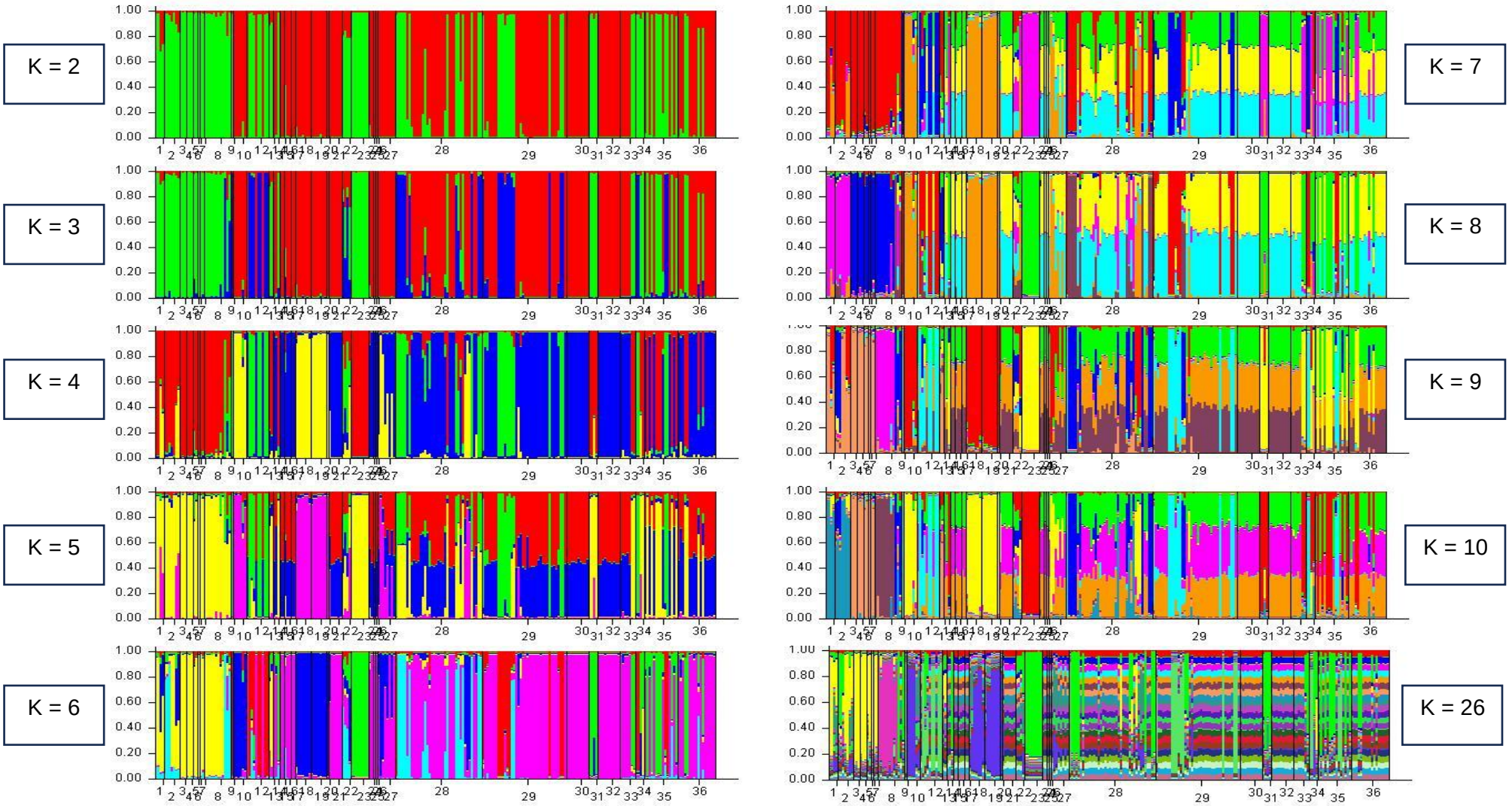

Figure 5.2 - Population assignment graphs generated by STRUCTURE for putative populations (K) of 2-10 and 26 of the woolly apple aphid (*Eriosoma lanigerum*, WAA). Each vertical line represents a single sample, with sampling location indicated on the x axis. The y axis gives the probability of a sample being assigned to a population, with each population tested indicated in a different colour.

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Table 5.4- 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*	-

Table 5.5 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

5.4.4. 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 5.6: 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

5.4.5. Principal Component Analysis

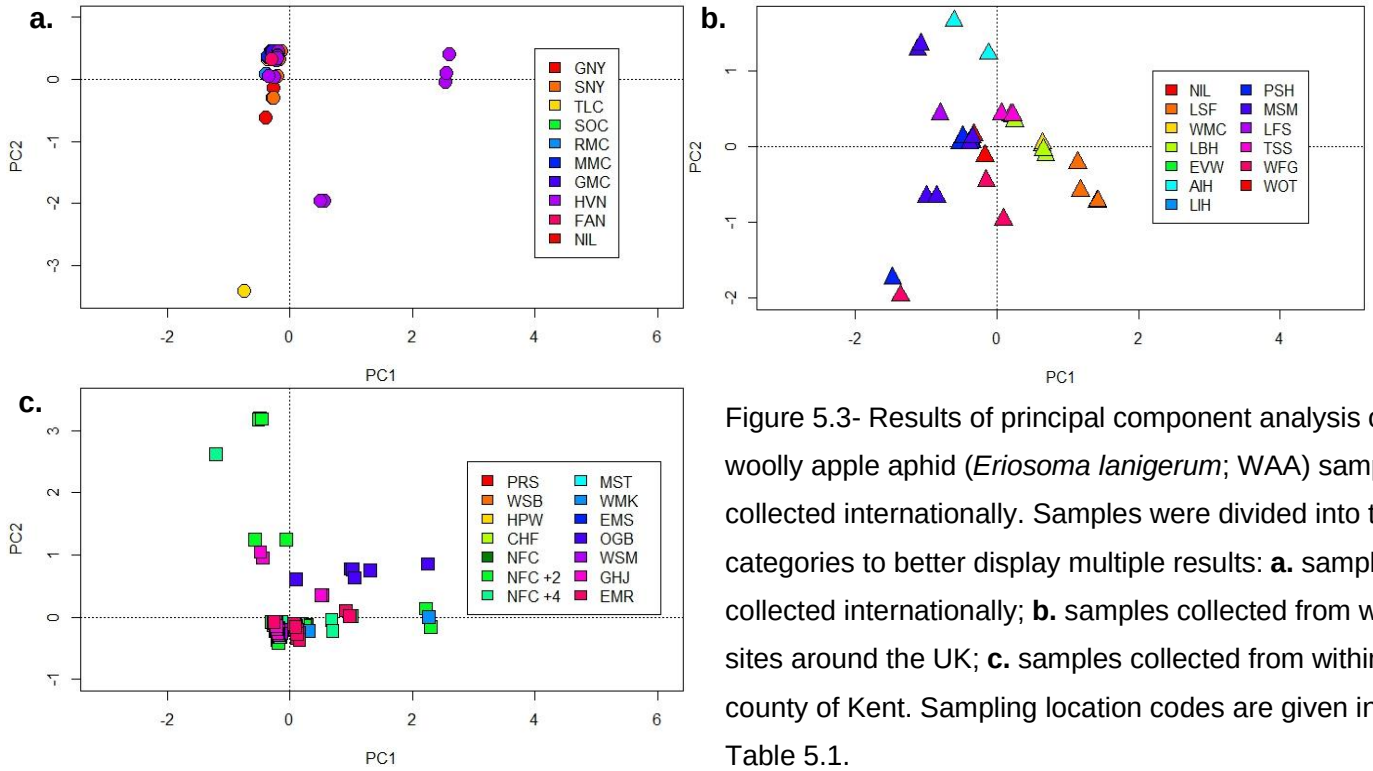

Figure 5.3- Results of principal component analysis of woolly apple aphid (*Eriosoma lanigerum*; WAA) samples collected internationally. Samples were divided into three categories to better display multiple results: **a.** samples collected internationally; **b.** samples collected from wider sites around the UK; **c.** samples collected from within the county of Kent. Sampling location codes are given in Table 5.1.

5.4.6. SNP markers generated

16383 SNP loci were identified for WAA across 71.22 Mb, in 7120 scaffolds with a mean read depth of 379 (min. = 20, max. = 999).

5.5 Discussion

The first objective of this work was to determine the extent of genetic variation in WAA within the UK and compared to samples collected from other countries. Outputs of population structure analyses with STRUCTURE and STRUCTURE HARVESTER software suggest that WAA collected across England and from other apple growing regions form two broad genetic clusters with smaller sub-populations at higher putative population numbers. Estimation of true value of K by calculation of ΔK is almost always accurate, except in instances where there is small marker and/or population size, or partial sampling (Evanno, Regnaut & Goudet, 2005). The exception to this is when testing uneven sample sizes for which STRUCTURE predicts fewer sub-populations than may be present, often merging small but distinct sub-populations and diving large but uniform sub-populations (Puechmaille, 2016). In this instance only eight microsatellite marker loci were used, spread across only three of the six WAA chromosomes, of which five are located on chromosome 3. Analysis

with more informative markers, for example generated with a GBS approach, may give deeper insights into population structure and the putative sub-populations suggested here.

The STRUCTURE outputs suggest that different population types are present in these samples, both exclusively asexual and with multiple genotypes present. This was seen even at K = 2 and single genotype blocks align with single genotypes observed in higher values of K. 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. Individual samples which were assigned to a single population may indicate the presence of clonal individuals within samples which are otherwise mixed. Individuals assigned to a single population existed both in single-genotype sampling locations, and within otherwise mixed sampling locations. Sampling locations with a single population assignment throughout tested values of K all had ten or fewer samples, with the exception of the 12 samples collected in New Zealand (sampling location 8). The sites at which the highest variation was observed, the National Fruit Collection, and East Malling, also had the highest numbers of samples collected, 85 and 53, respectively. Large sample sizes are more likely to capture variation present in the data set.

The most likely number of populations was found to be two, based on Delta K values calculated by STRUCTURE HARVESTER. The second most likely number of populations was 26, which may indicate the presence of sub-populations of that number. The original authors of the STRUCTURE programme said “We may not always be able to know the TRUE value of K, but we should aim for the smallest value of K that captures the major structure in the data” (Pritchard, Stephens & Donnelly, 2000). It is therefore more likely that the presence of sub-populations is eight, as this is the smallest value of K other than K = 2.

STRUCTURE was built to analyse sexual datasets and may struggle to accurately predict population structure of asexual populations. An artificial clonal population was included to act as a control dataset to test the accuracy of STRUCTURE when analysing populations which are likely to be asexual. The expected result would be that STRUCTURE harvester would identify that there was only a single population present within the dataset. The most likely of populations was, however, estimated to be eight, with a lot of distortion seen at other values of K tested. The only value of K identified as not at all likely was 23 (Figure 5.1.b.). This was in disagreement with the Delta K calculated with a “real” dataset (e.g. Figure 5.2.a. or Figure 1 in Appendix 3) where Delta K values are typically zero except for values of K where there is any value of Delta K has been recorded. The incorrect assignment of most likely K, and the distortion of Delta K values suggests that STRUCTURE may not be suitable for analysing asexual populations and that there may be over-estimation of population number.

Principal component analysis outputs showed the greatest variation is seen within samples from the UK (excluding Kent) where there was wide variation between all sampling locations without clear grouping based on geographic region. The international samples which group separately are those from Talca, Chile, and some of those from New Zealand. This suggests that there is genetic variation within these countries. Chilean samples were collected from multiple sites across the country, all except Talca clustered together, suggesting that aphids at this divergent site may be the result of a separate invasion of WAA into that region. The large variation within the New Zealand samples, which were all collected at the same location, suggests that there is sexual reproduction occurring. This is in agreement with Sandanayaka and Bus (2005) who reported capturing sexual WAA in New Zealand, although these individuals had not been viable, it demonstrates that the initiation of sexuparae is likely occurring. There were no out-groups identified in the PCA analyses in the samples from Europe or the wider UK, although there was some grouping observed based on geographic proximity of sampling locations. Figure 5.3.b. shows approximate geographic segregation between the east and west of England, most clearly seen in samples from Minehead (MSM) and Lincolnshire (LSF), the south west and east of the country, respectively. The PCA data suggest multiple population clusters, both those mentioned above and some samples from the National Fruit Collection, in Kent. This is not consistent with the two populations suggested by the Delta K analysis but more likely one large genetic population consisting of most samples, separate from samples from Talca, and some from New Zealand and the National Fruit Collection. These out-groups may represent the potential sub-structuring detected by Delta K analysis, or may be a product of STRUCTURE struggling to accurately analyse (partially) asexual datasets. Zhou *et al.* (2015) were successful in analysing WAA population genetics in China using the same SSR markers as here, and also using STRUTURE analysis. This may have been more successful than here because the projects used different numbers of samples and sampling locations.

Low FST values (>0.1) are indicative of wild type sexual reproduction or recent divergence of populations (Latch *et al.*, 2006). 129 of the 745 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. The majority of FST values were > 0.1 indicating that these populations differ genetically. Five of the 745 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.

Private alleles are those found only in one (sub-)population (Neel, 1973) and can be indicative of heritable alleles. One private allele was found from the National Fruit Collection which is consistent with the high diversity found in those samples (Figure 5.2) and the large sample sizes which has been found to increase the number of private alleles identified, suggesting higher gene flow (Slatkin, 1985). Collecting more, larger data sets of WAA genomic material may reveal further genetic variation between populations than previously thought.

The only site with multiple private alleles identified was at NIAB East Malling, within the same sampling location, and existed at two different loci. The private alleles were detected in WAA feeding on susceptible M.9 rootstocks, rather than the resistant MM106 and M.116 which were within the same enclosed culture. 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. The pairwise FST values for this population were below 0.1 for 14 of the 34 populations, all of which were in England and eight of which were within Kent. This is consistent with expected patterns of reduced genetic variation with increased geographic proximity. Internationally, samples from Molina in Chile and New Zealand each had a private allele present. The private allele identified in New Zealand samples had a high frequency of 0.857 and was present in eight of the 12 samples. This is consistent with the isolation of New Zealand samples observed in PCA and the single genotype which emerges for this sampling location in STRUCTURE outputs. This is consistent with expectations that geographically distinct samples will also be genetically isolated.

The second objective of this work was to determine, as far as possible, the extent to which genetic variation is likely to be the result of sexual reproduction. Organisms which reproduce both sexually and asexually can exist in populations which are wholly sexual or asexual, or partially sexual populations (Delmotte *et al.*, 2002). It is possible that high heterozygosity and low allelic polymorphisms in sexual populations of *R. padi* is a result of either long-term asexuality leading to high genetic diversity (Bengtsson, 2003), or of asexual lineage which have since hybridised (Delmotte *et al.*, 2002). The samples from this study found high observed heterozygosity in all cases (Table 5.4) which is consistent with highly heterozygous asexual populations of the bird cherry-oat aphid (Delmotte *et al.*, 2002).

This study did find genetic variation in WAA samples collected from within the UK, compared to other apple-growing regions. It is likely that there is some instance of sexual reproduction occurring within these samples, and that there have been multiple invasions of WAA, creating complex population structures. Higher genetic variation is likely to be identified by SNPs identified by GBS compared to traditional molecular markers (Harrop *et al.*, 2020).

These SNPs are available and further genetic analysis should be straightforward for future work.

CHAPTER 6 - Individual and population growth of WAA on apple rootstocks with *Er1* resistance, estimated by mean relative growth rate and intrinsic rate of increase

6.1 Abstract

Woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) is an economically important pest of *Malus* spp., which feeds on all parts of the plant, including on the rootstock. This pest has been widely controlled through the use of aphid-resistant rootstocks. There are increasing numbers of reports of WAA biotypes, which can overcome resistant rootstocks. These biotypes were first reported in North America and apple growing regions in the southern hemisphere but are emerging in Europe. Individual and population growth were estimated to understand the extent to which aphid growth is affected by feeding on resistant host material. The effect on individual aphids of feeding on susceptible M.9, or resistant M.116 and MM106 rootstock material was estimated through mean relative growth rate and wax production over time. Population growth on M.9 rootstock material was estimated under different experimental conditions by intrinsic rate of increase. Low replication numbers were generated, owing to WAA being a slow-growing species, which habitually lives as a colony. Suitable methodology for analysis of similar species has not yet been developed, which has contributed to low replication. No significant differences were found in either MRGR or wax production when feeding on susceptible or resistant rootstocks. There was, however, a significantly higher intrinsic rate of increase found when reducing the study duration to account for WAA life history, compared to duration. Future measures of WAA growth and reproduction, and that of similar species, will need adaptation to accurately estimate population growth, especially when feeding on resistant host material.

6.2 Introduction

Woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) feeds on both the scion and rootstock of *Malus* × *domestica* (Borkh.) and often forms colonies at vulnerable points on the tree such as new growth and pruning injuries. Below-ground feeding, vascular tissue disruption and rootstock galling reduce water flow and availability, strongly reducing vegetative growth, especially in young trees (Weber & Brown, 1988; Brown *et al.*, 1991). Aphid colonies often exert very high herbivory pressure at feeding sites and can establish large colonies on gall sites by creating a photosynthate sink from nearby tissue, which increases nutrient availability (Larson & Whitham, 1991). A key element of the rapid population growth, which allows aphids to become such serious agricultural and horticultural pests, is their high reproductive rate. Parthenogenesis removes the need for sexual

reproduction and telescoping of generations (*i.e*. a viviparous asexual female aphid grows parthenogenetic daughters which in turn carry parthenogenetic grand-daughters), allowing development of the next generation to begin before birth (Leather, Awmack & Garratt, 2017). Woolly apple aphid has an unusual lifecycle compared to many other aphid species, having lost host-alternation outside of its native range, and feeding exclusively on all parts of the tree (Eastop, 1966; Blackman & Eastop, 1994). In its native range of northeast America, the species alternates between apple in the summer and American Elm (*Ulmus americana* L.) in winter. In regions where it feeds exclusively on apple, WAA shows seasonal variation within the orchard; early instar nymphs migrate below-ground to the rootstock to feed over winter and subsequently return to the canopy in spring (Barbagallo *et al.*, 1997; Hetherington, 2009).

Control options for WAA are limited; contact insecticides are not effective against WAA due to their protective wax coat (Alston, Reding, & Murray, 2010; Bird, 2021 *pers. comm*.; Powell, 2022 *pers. comm*.). The systemic insecticide spirotetramat allows control of rootstock-feeding aphids without contact (Beers & Cockfield, 2007), but is expensive and requires either prophylactic application or visible aerial colonies to have formed. Selecting an appropriate rootstock is thus key for ensuring good tree anchorage and crop yield and reducing the need for conventional pest and disease control. The WAA-resistance gene *Er1* is from the scion accession 'Northern Spy', which shows thicker rings of sclerenchyma than susceptible accessions (Staniland, 1924), and is the source of WAA-resistance in the Malling-Merton rootstock lines (King *et al.*, 1991). WAA has a significantly shorter phloem feeding phase and poorer development and survival on 'Northern Spy' compared to susceptible 'Royal Gala' (Sandanayaka *et al.*, 2003). There have, however, been reports of WAA feeding on these rootstocks, mostly from the southern hemisphere and the aphid's host range in northeast America; although there is recent anecdotal evidence that these resistance-breaking aphids are present in Europe (Jaastad, 2020, *pers*. *comm*.). Differential aphid performance on resistant rootstocks may appear under varying environmental conditions, for instance seasonally variable weather and temperature conditions, influencing resistance gene expression (Bus *et al.*, 2008).

As outlined above, the high rate of aphid reproduction, and telescoping of generations means that aphid populations may show exponential growth under ideal conditions (Birch, 1948). Estimation of population growth and reproduction when feeding on different *Malus* varieties or accessions can inform the extent to which host plants affect colony growth. The intrinsic rate of natural increase (r_m) of the green apple aphid (*Aphis pomi* de Geer) when isolated on *Malus pumila* L. leaves under laboratory conditions was reported as 0.396 ± 0.015 (Madahi & Sahragard, 2012). Values of r_m have been estimated for WAA when

feeding on different scion varieties; when feeding on the highly susceptible 'Red Fuji', WAA had an r_m of 0.30 ± 0.01, under optimal temperature conditions (Tan *et al.*, 2021). This value was found to be lower under orchard conditions; WAA feeding on apple twigs across all four seasons in the Yunnan province, China (Kuang, Shan & Tang, 1990). The maximum r_m recorded under these conditions was 0.265, in the autumn, and the minimum was –0.0591 in winter; negative values of r_m are possible when host nutritional quality is especially poor, indicating a population decline (Kuang, Shan & Tang, 1990). Aphids feeding on resistant rootstocks in laboratory conditions and in polytunnel conditions under high temperatures were observed to produce little to no wax, despite overall morphology appearing normal (Figure 6.1). It is thought, therefore, that reduced WAA wax production may be a sub-lethal effect of living under stressful conditions.

Figure 6.1- A large Woolly Apple Aphid (*Eriosoma lanigerum*; WAA) colony feeding on a susceptible individual of a rootstock breeding population. A colony of this size would normally be covered in wax, rather than the very small quantity which appears to be located only at the cornicle of each aphid, perhaps clumping around honeydew. This photograph was taken in June 2021 when mean temperatures in the polytunnel for the previous ten days was approximately 19.3 °C with a maximum temperature recorded five days before this photo of 32.7 °C (Godfrey, 2021).

Understanding the effects of feeding on resistant rootstocks on WAA growth and reproduction will inform how we expect WAA populations to behave when developing resistant rootstocks with the *Er1* gene. This chapter aims to understand both individual and population growth and the extent to which feeding on rootstocks known to carry the *Er1*

WAA-resistance gene inhibits aphid growth. This was carried out through estimating both individual and population growth when feeding on resistant or susceptible rootstock material through intrinsic rate of natural increase, mean relative growth rate, and weight of wax produced.

6.3 Materials and Methods

6.3.1. Plant material

Bare-rooted rootstocks were purchased from Frank P. Matthews nurseries (Tenbury Wells, Worcestershire) and cold stored (*ca*. 5 °C and in darkness) until use. Rootstocks were subsequently potted in 0.5 L pots with potting compost, as required for this work *(i.e.* after no fixed time in cold store conditions). To induce rootstocks to leave dormancy and produce leaves, they were moved into long-day conditions, either in an outdoor polytunnel during summer months, or into controlled long-day conditions (16:8 Light:Dark (L:D), *ca*. 20 °C) during winter months when the external conditions were below these values.

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

6.3.2. Aphid material

The aphids were taken from clonal culture kept at NIAB East Malling on potted M.9 rootstocks under controlled growth chamber conditions (*ca*. 17 °C, 75% Relative Humidity (RH), 16:8 L:D).

A fixed age population was used to reduce the effects of parental age on offspring growth. A population of the same age should assume a fixed stable age distribution when reproducing in an unlimited environment (Birch, 1948). Apterous adult females were placed using a damp paintbrush onto a potted M.9 rootstock and left under controlled conditions (*ca*. 20 °C, 16:8 L:D) for 24-hours, after which adults were removed, and nymphs left on the plant to develop.

6.3.3. Intrinsic rate of increase measured on potted susceptible rootstocks

Individual age-synchronised adults were removed from culture and isolated on potted experimental rootstocks, using a damp paintbrush to move aphids carefully, and placed on the woody portion of the rootstock. The potted rootstocks were placed within two 300 mm × 450 mm breadbags (Lakeland), one from above and one below, such that all parts of the plant and the pot were covered, and the bags were secured around the base of the trunk

with a twist tie. Rootstocks were transferred to controlled conditions (*ca*. 20 °C, 16:8 L:D) for 24-hours, after which the adult and all but one nymph were removed and the remaining nymph reared on the rootstock. The number of days from birth to reproductive maturity, *d*, was recorded. The number of nymphs produced in *d* days after reaching reproductive maturity was recorded and used to calculate the Intrinsic Rate of Natural Increase (r_m) using the formula from Wyatt and 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 period after first reproduction, of length d. The constant 0.738 is the approximate proportion of a female's total fecundity which is produced in the first few days of reproduction. Thirteen, nine, and seven replicates were set up for M.9, M.116, and MM106, respectively.

6.3.4. Intrinsic rate of increase measured on susceptible rootstock leaf cuttings

To reduce the chance of "losing" nymphs on potted sticks, the decision was made to use leaf cuttings with a petiole *ca*. 2 mm in diameter. Individual cuttings were placed in wet floral foam and isolated inside two clear plastic pots (*ca*. 30 mm diameter in the centre, 20 x 20 mm base, 55 mm in height each (110 mm total height; Figure 6.2), creating an enclosure in which the nymph could move freely on the rootstock cutting whilst confined in an area which was easy to monitor.

Individual age-synchronised adults were transferred to one of these enclosures with a damp paintbrush and placed on the petiole to feed. Enclosures were closed, numbered, and organised into a randomised design within the growth room.
These adults were allowed to produce nymphs under these conditions for 24-hours and then the adult and all but one nymph were removed. Calculation of r_m was carried out as in section 6.2.3.4, for 16 replicates, all feeding on M.9 material.

Because of the low number of successful repeats achieved (Table 6.1), these methods were not suitable for WAA, as aphids were not able to survive for a full period of *d* days after beginning to reproduce. The value of *d* was, therefore, changed to a fixed value of seven days, as used in Castle, Mowry & Berger (1998), for eight replicates, all feeding on M.9 material.

Figure 6.2 - Experimental enclosure set up to isolate individual Woolly Apple Aphid (*Eriosoma lanigerum*; WAA) nymphs for life history studies. Each enclosure consisted of two plastic pots, inverted, and secured together with electrical tape (clear sticky tape in this image). Containers were *ca*. 30 mm diameter in the centre, 20 x 20 mm base, 55 mm in height each (110 mm total height). A piece of floral foam the diameter of the containers was cut and soaked in water. A small reservoir of water was filled underneath the floral foam such that the end of the petiole cutting can reach the water. The floral foam functions to prevent aphids from drowning in the water and maintains humidity for the leaves. Enclosures were arranged in a complete randomised design within a growth room.

3322

6.3.5. Mean relative growth rate

Individual adult apterous aphids of a synchronised age were transferred to a rootstock leaf cutting of M.9, M.116, or MM106 to compare the effects of feeding on these different rootstocks on WAA mean relative growth rate. Enclosures for individual nymphs were prepared and arranged in a randomised block, as before, with individual adults isolated under these conditions and allowed to produce nymphs for 24 hours. One of these nymphs was left on the cutting undisturbed and the other nymphs removed, pooled and weighed as a group using a Cahn 29 microbalance (Thermo Electron) to give a mean starting weight. The single isolated nymph was left to feed on the petiole for a set period of two, three or seven days. At the end of this period, the aphid including all of its wax, was carefully removed using a damp paint brush and weighed using the Cahn 29 microbalance to give the end weight. The wax was then carefully removed from the aphid using a damp paintbrush and the aphid re-weighed. The weight of the wax was estimated by subtracting the weight of the nymph with the wax removed from the weight of the aphid with wax present. The number of replicates established for each rootstock and duration of Mean Relative Growth Rate (MRGR) period is given in Table 6.2. MRGR was calculated using the following formula (Castle & Berger, 1993, developed from (Radford, 1967):

$$MRGR = \ln(W_1) - \ln(W_0)/d$$

Where W_0 = initial weight; W_1 = weight at end of developmental time; d = the time in days between weighing events.

6.3.6. Statistical analysis

Statistical analysis was carried out using R v. 4.2.2 (R Core Team, 2022). The effects of experimental conditions on r_m, and the effects of host rootstock on MRGR and wax production, were analysed by one-way ANOVA (Analysis of Variance). Tukey Honestly Significant Difference (HSD) tests were carried out after ANOVA to determine pairwise differences.

6.4 Results

6.4.1. Intrinsic rate of increase

Table 6.1: Number of repeats, and their success in terms of the number of woolly apple aphid (*Eriosoma lanigerum*; WAA) nymphs produced, and surviving until reproductive maturity, when feeding on whole potted rootstocks. M.9 rootstocks are known to be WAA susceptible, M.116 and MM106 both carry the resistance gene, *Er1.*

Treatment	**Rootstock**	**No. individual adults isolated onto rootstock material**	**No. isolated adults which were able to produce nymphs**	**No. nymphs surviving to reproductive maturity. Each nymph was the daughter of a different mother**	**No. instances for which a value of r_m could be calculated**
Whole plant	M.9	13	6	2	1
	M.116	9	4	0	0
	MM106	7	4	0	0
Leaf cutting in enclosure	M.9	16	7	3	3
Leaf cutting in enclosure for 7 days	M.9	8	8	7	5

Separate experiments using different conditions to estimate r_m all yielded a very low number of repeats (Table 6.1). Comparison of r_m across these treatments in order to determine which would be most suitable for r_m estimation for WAA found a significant difference (p = 0.044*);* whole plant feeding, petiole cutting feeding, and seven-day post-reproductive period*.* Pairwise comparison with Tukey only found a significant difference in r_m between the two treatments feeding on leaf cuttings (F-statistic = 5.021, p = 0.044*)*. The highest median r_m (0.305) was found when the reproductive period (d days) was limited to seven days. The second highest r_m (0.173) was found when nymphs were allowed a whole potted rootstock to feed on, and the lowest value was found when nymphs were isolated on leaf cuttings for a longer d period (Figure 6.3).

The highest number of successful repeats where the nymph survived long enough to produce a value for r_m was observed when using a fixed post-reproductive period of seven days. Of the eight aphids isolated on M.9 leaf cuttings, seven reached reproductive maturity and five survived the seven-day period, generating values for r_m.

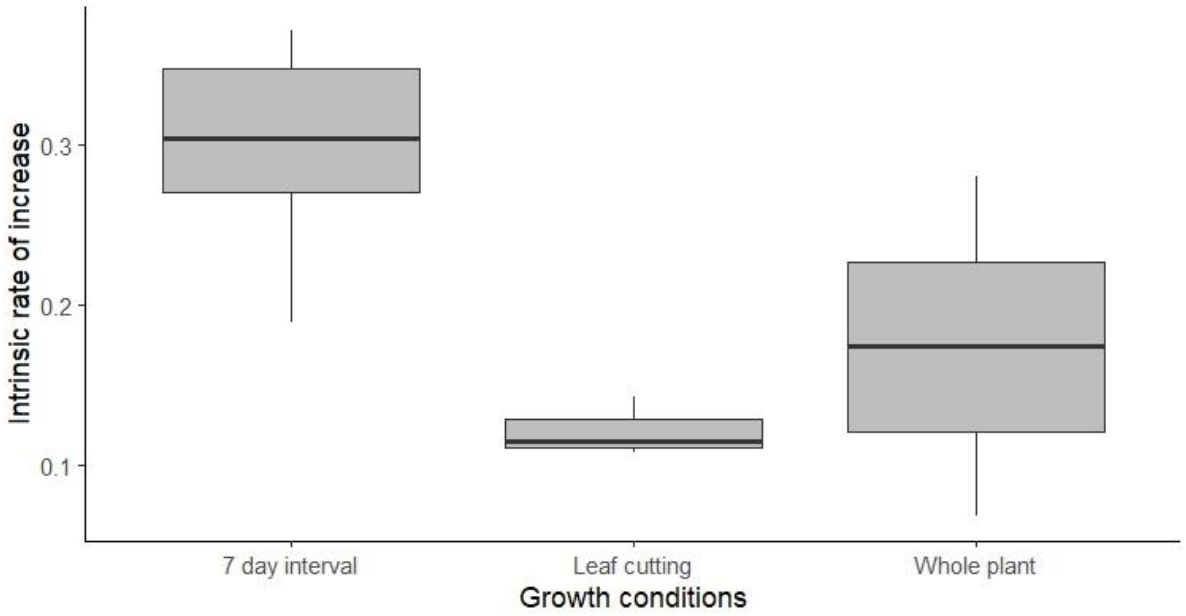

Figure 6.3 - Intrinsic rate of increase values for Woolly Apple Aphid *(Eriosoma lanigerum; WAA)* nymphs when feeding on plant material from the susceptible apple rootstock, M.9. The experimental conditions tested were carried out using a single WAA nymph isolated on (from right to left): whole potted M.9 rootstocks; M.9 petiole cuttings over a period of *d* days, where *d* = the pre-reproductive period (in days) of each aphid; M.9 petiole cuttings over a period of 7 days. The maximum and minimum values recorded are shown, along with distribution around the median.

6.4.2. Mean relative growth rate

There was no significant difference in MRGR between WAA on resistant and susceptible host rootstocks (*F*-statistic = 0.426, *p* = 0.659). Tukey HSD comparison found no significant differences between pairs of rootstocks. Woolly apple aphid feeding on M.9 rootstocks did, however, show the highest median MRGR of 0.15.

Rootstock	No. adults inoculated onto rootstock material	No. nymphs established at beginning of MRGR calculation	No. nymphs for which values of MRGR and wax weight were calculated	No. repeats carried out for each duration of MRGR (days)		
				2	3	7
M.9	20	16	13	3	7	3
M.116	16	11	6	0	5	1
MM106	11	8	3	0	3	0

3387

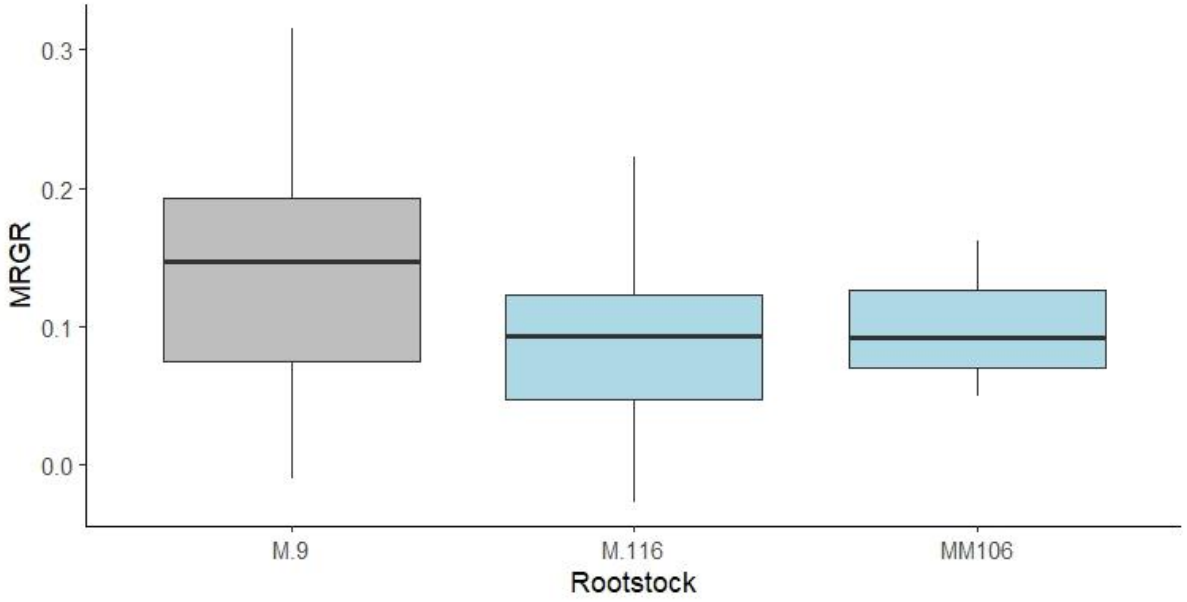

Figure 6.4 - Mean Relative Growth Rate values for isolated individual Woolly Apple Aphid *(Eriosoma lanigerum; WAA)* adults feeding on the susceptible rootstock, M.9 (indicated in grey) and the resistant rootstocks M.116 and MM106 (indicated in light blue). The maximum and minimum values recorded are shown, along with distribution around the median.

3388

3389 **6.4.3. Wax weight**

3390 No significant difference was found in wax weight between rootstocks (*F*-statistic = 0.587, *p*
3391 = 0.566). No pairwise significant differences were found between rootstocks with Tukey HSD
3392 correction. The median weight of wax produced per day by WAA nymphs feeding on M.9
3393 and M.116 rootstocks were similar (0.001 mg and 0.0008 mg, respectively), but less wax
3394 was produced by those feeding on MM106.

3395

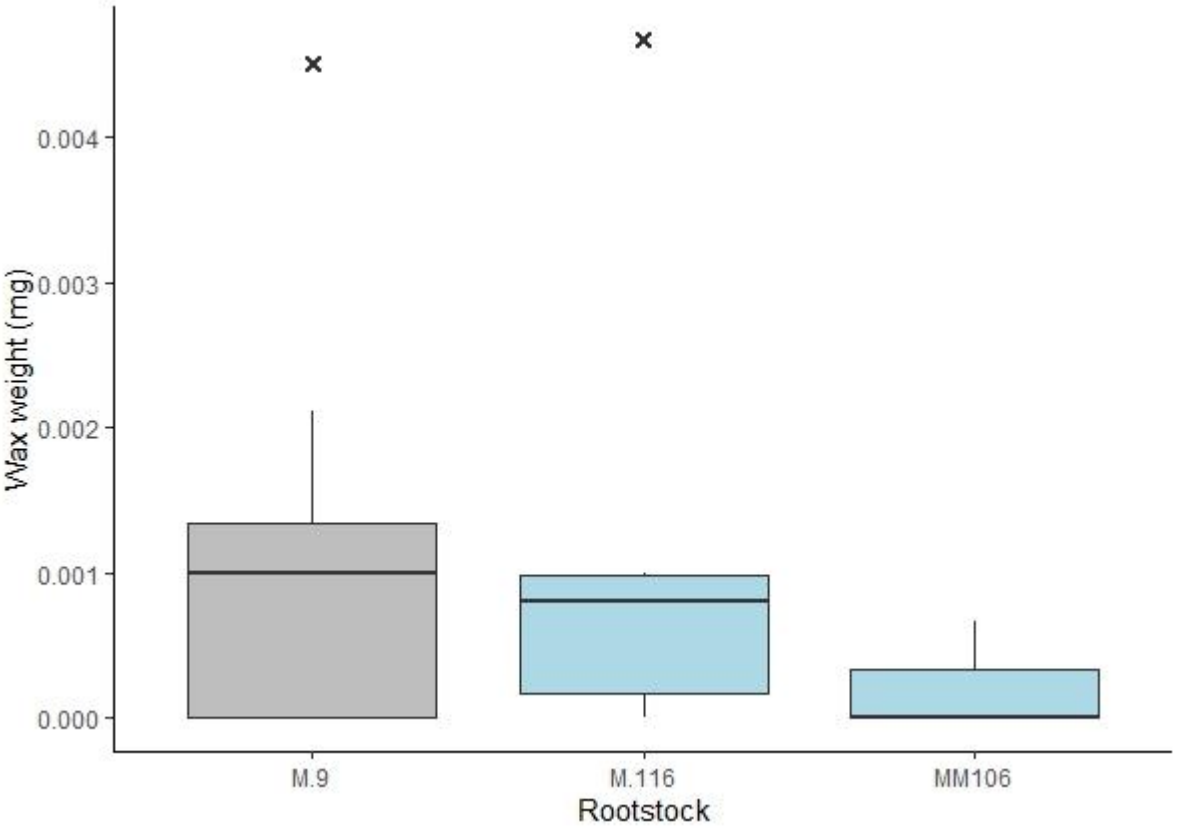

Figure 6.5 - Weight of wax in mg produced per day by a single Woolly Apple Aphid *(Eriosoma lanigerum;* WAA) nymph while feeding on leaf and petiole cuttings of the susceptible M.9 rootstock, in grey, and the resistant M.116 and MM106 rootstocks, in blue. The maximum and minimum values recorded are shown, along with distribution around the median. Outliers are indicated by ×.

6.5 Discussion

6.4.4. Intrinsic rate of increase

Intrinsic rate of increase was measured only on M.9 as testing showed this technique would be difficult to sustain for aphids feeding on resistant rootstocks. It is difficult to infer r_m values for WAA feeding on M.116 and MM106 from the other metrics measured here due to the relationship between MRGR and r_m varying depending on the aphid species, the plant cultivar, and the plant growth stage (Guldemond, den Brink & den Belder, 1998). It is, however, reasonable to assume that feeding on these rootstocks would lead to lower r_m, given the lower values for these rootstocks in all other values measured. Of the 29 replicates set up across all three rootstock varieties, only one value of r_m was calculated, largely because it was difficult to reliably locate a single aphid on a potted rootstock, especially first instar nymphs which are very small and can easily hide in cracks in the bark, *etc*. There is also the potential for WAA to move below the soil level, which would be difficult to prevent because of the small body size of first instar WAA nymphs.

The constant 0.738 used in the calculation of r_m represents the percentage of an aphid's nymphs which will be produced in the first few days of reproductive maturity. The exact figure of this would be expected to vary between species depending on lifecycle i.e. aphids with a longer and slower reproductive lifecycle may expect a lower value as they may produce nymphs over a longer period of time and at a lower rate. Wyatt and White (1977) calculated the r_m standard from an average population slope across four aphid species: *Myzus persicae*, *Brachycaidus helichrysi* (Kaltenbach), *Macrosiphoniella sanborni* (Gilette), and *Aphis gossypii* which had population slope values of 0.736, 0.745, 0.738, and 0.765 respectively. *Brachycaidus helichrysi*, the leaf-curling plum aphid, is a member of the Macrosiphini which are more distantly related to the Eriosomatini than the Aphidini but have a similar lifecycle to WAA with regards to both feeding on woody plants and induce galls when feeding. The use of multiple species with different life histories to estimate this value, including another tree-feeding species, increases the applicability of this constant for use with WAA to a greater extent than calculation using only the value for *i.e. M. persicae*. Given the exceptionally slow growth and low establishment of WAA in the r_m study performed here,

Figure 6.6- An enclosure made to isolate Woolly Apple Aphid (*Eriosoma lanigerum*; WAA) nymphs on apple material in outdoor settings or on larger plants. This enclosure is a modification of a standard clip cage design, developed to fit around woody material. It is constructed of two sheets of 10 mm thick EVA foam (80 mm × 80 mm) joined with contact cement on the right-hand side. A 25 mm × 25 mm window was cut in one piece and covered with fine mesh, *ca*. 50 - 100 µm and secured with contact cement, to allow viewing of the aphid colony and air flow, but to prevent nymphs from escaping. The cage can be placed around a WAA colony feeding on woody material and secured so that it sits tight to the stem with for instance bulldog clips and cable ties, as shown in the figure, depending on the diameter of the woody material.

a specific population sloe for WAA may also be difficult to calculate, necessitating the use of such a constant.

More realistic values for r_m are estimated when measured on whole plants (Guldemond, den Brink & den Belder, 1998), which was difficult to achieve on potted rootstocks. The use of petiole cuttings isolates the nymphs in a controlled area, allowing them to be monitored more closely. In the future, isolating WAA in a specific area of the plant, combined with a more favourable duration of *d* would likely improve the number of successful repeats generated. For a predominantly leaf-feeding aphid, a clip cage could be used for this purpose, although it is difficult for species feeding on woody tissue. Some success has been observed when using a custom “cage” constructed from mesh and foam to isolate WAA colonies when inoculating apple trees outdoors (Figure 6.6). This, or a similar technique, could be used to prevent WAA individuals from moving or being predated on potted rootstocks, as was an issue in these experiments. Creating wounds and inoculating aphids directly onto these wounds may discourage aphids from moving around the plant and becoming lost, although this may reduce plant fitness. Given the context of this work is to inform rootstock breeding programmes, trees should be kept as healthy as possible and creating an open wound in the plant will increase vulnerability to pathogens.

Isolating individual aphids so that they live and feed alone does give more accurate values of r_m (Guldemond, den Brink & den Belder, 1998) and is therefore recommended for life history studies. In the case of colony-forming aphids which are known or suspected to perform poorly when isolated (*e.g.* Hayamizu, 1984), a standardised approach using multiple aphids could be very useful. Typically, 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 a fragile and slow-growing species, which showed significantly higher r_m on leaf cuttings when the duration of the post-reproductive period was reduced to seven days.

The telescoping of generations means that while aphids are developing they are exposed to the nutritional conditions of their mother, and grandmother. Those whose mother developed on a susceptible rootstock, as here with stock WAA reared on susceptible M.9, have been “primed” to feed on M.9. Hu *et al.* (2016) investigated the effects of maternal and offspring diet on life history traits of *Rhopalosihum padi* when feeding on *R. padi* resistant and susceptible wheat lines. Aphids feeding on the resistant wheat variety Xiaoyan22 produced the highest number of alate (winged) offspring, likely as an attempt to migrate away from the poor source of nutrition. These alates themselves showed high r_m, to establish a colony on a

new host plant. There was, however, no significant effect of maternal diet on alate fecundity and r_m but there was a significant of offspring diet on its own fecundity and r_m. The only significant instance of an interaction between maternal and offspring diets occurred when mothers and nymphs were both raised on Xioyan22, the r_m was 13% lower than when mothers were raised on Xioyan22 but nymphs were transferred to the susceptible variety Batis. This suggests that we had not "primed" aphids to perform better when they and their mother were both raised on M.9 and we are therefore seeing the effect of feeding on a resistant rootstock on aphid performance.

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, Awmack & Garratt, 2017). Seven (Castle Mowry & Berger, 1998) and five (Dahlin & Ninkovic, 2013) day periods have been used to accurately measure r_m. The mean r_m value for WAA nymphs when feeding on M.9 cuttings for a fixed seven day period was 0.30, which similar to that found for WAA feeding on twigs of the susceptible variety 'Red Fuji', which was 0.30 ± 0.01 (Tan *et al.*, 2021). Tan *et al.* (2021) also used potted apple material when estimating r_m, although using two year old seedlings *ca*. 1 m in height and in a 20 cm diameter × 25 cm high pot. Aphids were inoculated onto wounds created on the stem of each seedling, and the authors did not report losing any nymphs. Providing nymphs with a feeding site may prevent them from moving around the plant to find a suitable feeding location.

6.4.5. Mean relative growth rate

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 aphid feeding (Staniland, 1924). The lack of a significant difference between the three rootstocks initially suggests that feeding on rootstocks carrying the *Er1* gene has no effect on individual WAA growth. However, the time period over which MRGR was measured was very short (2, 3, or 7 days), which, especially for a slow-growing species, meant only a small amount of weight gain was possible. Although this is accounted for within the MRGR formula, a shorter time frame is less desirable for several reasons. Firstly, differences in aphid weight between treatments will be smaller and therefore more likely to be obscured by inaccuracies in measurements, or by random variation. Secondly, reduced phloem sap availability affects aphids chronically and therefore differences in weight gain will continue to diverge over time and may not diverge in a linear fashion.

Measurements of individual aphid growth may be more suitable for WAA and similar species than methods which measure fecundity. Methods such as MRGR could be supported in future with other measures, for instance allometry, which investigates the relationship between the size of an individual body part and the organism as a whole (Stern & Emlen, 1999). Individual aphid body size is strongly positively correlated with fecundity, allowing prediction of population growth from individual aphid growth using Mean Relative Growth Rate (MRGR) of a single aphid over a predefined time period (Leather & Dixon, 1984; Leather, Awmack & Garratt, 2017).

The nature of WAA as colony-forming, sedentary aphids means that individuals may perform poorly when isolated. Individual nymphs of the mealy cabbage aphid (*Brevicoryne brassicae* L.), which also typically form colonies protected by wax, perform much worse when alone than when feeding as part of an aggregation (Hayamizu, 1984). This is thought to occur as an adaptive trait, because colony feeding improves the food quality of the host plant (Way & Cammell, 1970) and can sequester photosynthates from neighbouring tissues, providing high carbohydrate levels for the colony (Larson & Whitham, 1991). It is likely that isolating individual WAA nymphs to carry out these growth studies reduces their ability to effectively feed. An age-synchronised population of ten WAA nymphs were isolated on potted rootstocks, across three wounds cut in the stem, to calculate r_m (Tan *et al.*, 2021). Information is not provided as to whether these nymphs formed colonies, but does demonstrate that it is possible to estimate r_m from multiple individuals, although values cannot be calculated for individual aphids. Aphid wax functions to protect colonies from natural enemies and abiotic stressors, and is especially important to prevent aphids from drowning in their own honeydew, by forming the honeydew into hydrophobic droplets (Smith, 1999). When calculating MRGR, nymphs had all wax removed prior to feeding, which may have negatively impacted their weight gain.

6.4.6. Wax weight

No significant difference was found in wax weight and any non-significant difference observed was very small. This is most likely an issue with the methods used as the wax is incredibly light, and the nymphs were only producing wax for a short period of time (2, 3, or 7 days). Most microbalances are likely to struggle with the sensitivity needed to precisely weigh the wax, and it is difficult to guarantee all wax has been removed for weighing. It had been expected that we would see a reduction in wax produced by aphids feeding on resistant rootstocks, as a sub-lethal effect of reduced phloem availability. The black bean aphid (*Aphis fabae* Scopoli) increases wax production under stressful conditions such as

overcrowding and low temperatures (Pope, 1983). The wax produced by *A. fabae* is, however, powdery and forms a less dense layer than WAA wax, and may therefore require less energy to produce and be less severely affected by reduced sap availability.

Woolly apple aphid resistance can be estimated using a number of different parameters, including gall formation, colony size, wax production, and aphid growth and reproduction (Sandanayaka *et al.*, 2003). It is important to consider multiple factors when determining whether a rootstock is resistant to WAA feeding. Commercial breeding programmes often use the number and size of both WAA colonies and galls to determine resistance (Bus *et al.*, 2008) but an understanding of the effect(s) of resistance genes on pest population growth will help to determine to what extent resistant rootstocks are preventing or hindering aphid growth, which can inform pest control choices.

6.4.7. Future perspectives

The results of this chapter show that whilst it was not possible to collect many replicates, WAA do appear to perform poorly on rootstocks carrying *Er1* resistance, evidenced by the difficulties collecting data on these rootstocks, which was even greater than for aphids feeding on susceptible rootstock material. Traditional population growth metrics are likely unsuitable for slow-growing, colony-forming aphids, such as WAA, but adaptations can be made to increase the likelihood of generating usable results. Use of multiple aphids may help to reduce individual nymph death when isolated, but will require values for r_m in particular to be calculated for a group and then averaged. For future studies, greater aphid longevity could be achieved through reducing the duration of life history studies, combined with isolating aphids on a whole plant with a clip cage equivalent. Adaptation of these methods for multiple individuals would require standardisation both to justify the benefits of not isolating aphids and to ensure the estimation of population growth is accurate.

7. CHAPTER 7 – General discussion

7.1. Resistance gene identification and mapping

The objectives of this project spanned almost the entire process of identifying WAA resistant apple genotypes, scoring for a resistance phenotype, and categorising potential marker loci for marker-assisted selection.

We identified *M. floribunda* and *M. floribunda* J. as ideal candidates to identify novel sources of resistance, which would be good avenue for continuation of this project. The Rootstock Breeding Club at East Malling has unfortunately closed since this project began; hence accessions of this species have currently not been investigated further. *Malus floribunda* 821 was used in the rootstock breeding programme at East Malling, but no resistance gene had been identified (Fernández Fernández, 2020, *pers*. *comm*.). The self-incompatibility locus of *M. floribunda* 821 is known (Verdoodt *et al.*, 1998) which, if compatible with commercial crop varieties, would make it an ideal candidate for a resistant polliniser variety for industry use. This work did fulfil the aim to identify novel sources of resistance from *Malus* WCRs which is already useful for a resistance breeding programme however unfortunately we have not been able to carry this work on to identify a putative resistance gene, because the EMR RBC has been closed.

7.2. Limitations of inoculation and scoring

A longer duration between inoculation and scoring would allow WAA to establish colonies and may reduce some variation in scores caused by climate, *i.e*. a two-week incubation at high temperatures, will likely reduce aphid growth more drastically than the same incubation period in conditions beneficial to aphids. A longer period between inoculation and scoring, for example, 3 - 4 months (Bus *et al.*, 2008) would not only subject repeats to similar climatic conditions, but would also reduce the difference in time between the first inoculation and the final scoring. Incorporating assessment of galling alongside WAA colony size and number would help to determine the long-term impacts of aphid feeding over this extended incubation period, as used when mapping *Er1*, *Er2*, and *Er3* (Bus *et al.*, 2008).

The parasitoid wasp *A. mali* can be very effective in its control of WAA and shows population peaks after those of WAA, tending to peak under high temperatures in mid-summer. Depending on when inoculation occurs, the effects of *A. mali* on WAA establishment will therefore vary. Yellow sticky traps attached to tree trunks have been shown to be attractive to *A. mali* (Beers, 2012), and could be used to monitor their population when inoculating.

This could inform both the best inoculation windows to avoid predation pressure from *A. mali,* and the application of selective insecticides for *A. mali* (as in Bus *et al.*, 2008).

7.3. Limitations in mapping process and technologies

Using the highest score recorded across all scoring events to represent a “worst case scenario” of susceptibility for each seedling may have skewed phenotypes towards the susceptible classification.. This stringent scoring is, however, useful to ensure that no susceptible seedlings are mis-characterised as resistant with only a single colony present. Linkage mapping with JoinMap, as was attempted first, requires phenotypic data to be input as a two-factor scale of resistant/susceptible. Identification of significant SNPs with Kruskal-Wallis analysis found identical significant SNPs within a similar region when the highest overall score and mean highest score were used as phenotypic data, from a six-point scale of susceptibility. No identical SNPs were found when the phenotype was coded as two-factor resistant or susceptible.

Within this region containing the most significantly linked SNPs to *Er2* resistance there is an NBS-LRR gene previously identified in apple. These genes, as discussed in section 1.4.3., and reported by Hougenhout and Bos (2011), recognise effector molecules produced by aphids when feeding and initiate effective immune responses, and resistance, in the plant. *Mi* and *Vat* NBS-LRR genes have been reported as conferring clone-specific resistance to potato and melon aphids, respectively. A resistance-breaking strain of WAA identified in the USA, known as the North Carolina biotype, was able to colonise *Er1* but not *Er2* rootstocks (Young *et al.*, 1982), in contrast to a New York WAA biotype which could not colonise either rootstock line. *Er1* may therefore mediate clone-specific resistance, suggestive of HTI. Although mostly anecdotally reported, the break down of *Er2* resistance may also be specific to a limited number of WAA clones, in agreement with a HAMP-triggered resistance mechanism, which may involve the NBS-LRR gene identified in the region *Er2* was estimated to lie in. This system is not as widely reported for aphid pests as for pathogens, but suggests that there may be gene-for-gene interaction between WAA and *Malus* spp. This will require further investigation to determine the presence of any other genes associated with HTI, for example PPRs to recognise WAA feeding patterns.

These are the first SNPs identified for an *Er2* population. Integration of known microsatellite markers for this gene with these SNPs could offer further information on linkage and inheritance of *Er2*. SNPs identified by GBS have good longevity for use in MAS. The region identified as the most likely to contain *Er2* was similar to the location in cM identified by linkage mapping (Bus *et al.*, 2008), both being located towards the top of LG 17. This work is

highly relevant to WAA-resistance breeding worldwide and, although we were not able to define and validate flanking SNP markers for either *Er1* and *Er2*, we have increased the availability of SNP markers for *Er2*, a gene for which no SNP markers have previously been characterised.

7.4. Woolly apple aphid population genetics

The findings of this study, that WAA populations in the UK show genetic variation within geographic regions but also compared to other samples collected from other apple-growing countries. Without finding male alate WAA, or eggs, it is very difficult to definitively say that sexual reproduction is occurring. The results of this work are hence treated with caution, but the high levels of variation detected suggest more population mixing than would be expected in asexual populations. The presence of sampling locations comprised of a single genotype suggests that these samples comprised of isolated asexual clones. It is reasonable then to extrapolate that locations with multiple genotypes present are the result of sexual reproduction. The population genetic analysis methods used here were not designed for use with populations which are, or may be, asexual, and therefore must be treated with caution. Further analysis using software designed for population genetic analysis of aphids, or of other organisms which predominantly reproduce asexually, may clarify findings.

We had assumed, when beginning the resistance phenotyping portion of this project, that WAA in the UK and on site at NIAB East Malling in particular, were reproducing asexually and that we would only have one population of WAA present. After phenotyping had begun we discovered firstly that WAA at East Malling showed higher genetic diversity than expected, likely inconsistent with exclusively asexual reproduction, and secondly that there were WAA on site which could feed on material with *Er1*-mediated resistance. Phenotyping was suspended for the 2021 season to determine how widespread the resistance-breaking biotype were, as it became a concern that not only were they present but that they may be able to sexually reproduce and spread the trait. It was determined that the resistance-breaking WAA were an isolated outbreak located away from the breeding families and phenotyping continued. It is unlikely, therefore, that the skew towards resistant phenotypes which we observed was a result of resistance-breaking WAA. For future WAA screening at East Malling it is important that WAA inoculation material is monitored to check for resistance-breaking aphid biotypes and for alate aphids in the autumn which may be sexual forms.

7.5. Woolly apple aphid growth and reproduction

Although values for intrinsic rate of increase of WAA have been successfully estimated while feeding on potted apple material (Tan *et al.*, 2021), the methodology used here was chosen before this paper was published and is more similar to traditional population growth methods. It became clear that traditional population growth methods are unsuitable for slow-growing aphids, such as WAA. The traditional methods were chosen because high numbers of replicates are theoretically possible when using small potted rootstocks. In practice, however, we found that the negative effects of isolated feeding in this manner on WAA outweighed any benefit of being able to establish high numbers of replicates. In future, inclusion of further measures of insect growth, for example allometry, may strengthen findings found here, without requiring population growth. Previous EPG of WAA found reduced frequency of phloem salivation and therefore lower feeding rate, compared to *R. padi*, outlined in Section 1.3.5. and Figure 1.5 (Sandanayaka & Hale, 2003). Although attempted several times in this project, we were not able to complete EPG analysis of WAA feeding. This would be a good area for further analysis, as hoped, to determine the extent, if any, of reduction in feeding rate observed when the host plant is reported to be WAA-resistant.

We were successful in establishing a WAA culture feeding on resistant M.116 and MM106, both carrying the *Er1* resistance gene. It was, however, not possible to carry out growth and reproduction life studies because the aphids were less robust than those reared on M.9 and did not survive transfer onto new rootstock material. These cultures were persistent over many months but grew much more slowly than those on M.9 and would not have exceeded a susceptibility score of two following the scoring criteria we have used. Given that cultures were maintained under laboratory conditions with stable temperature and RH, and not exposed to natural enemies, it is likely that any resistance-breaking aphids in polytunnel conditions would not have been detectable and resistant trees mis-scored as susceptible.

7.6. Applications for industry

Throughout this project there has been interest from growers in increasing their understanding of WAA in order to better protect their crops. Disseminating information around the possibility of WAA sexual reproduction, and the potential for this to spread virulence traits such as resistance-breaking is crucial for improving cultural control of this pest. Whilst the results of the microsatellite analysis of population genetics could not conclusively state whether WAA sexual reproduction is widespread in Britain, the presence of genetic variation goes against the common assumption that WAA is asexual and therefore non-varying. When discussing this project with growers throughout, WAA has always been

reported as a severe orchard pest, which changes with climate conditions, and so increasing awareness of potential sexually reproducing aphids is likely to increase monitoring and control.

As discussed in section 3.4., the *Malus* accession screening data were shared with growers to inform practices when selecting polliniser crabapples to remove sources of WAA in the orchard. This information was also requested by F. P. Matthews tree nursery to allow them to advise their customers of WAA resistant varieties. The results of the WAA population genetics has been an excellent source of communication with growers with some specific farm managers following up after providing aphid samples to ask after the results from their farm.

7.7. Recommendations for future study

7.7.1. Resistance gene mapping

The poor performance of WAA when isolated on potted rootstocks or leaf cuttings, alongside the skew towards resistance seen when phenotyping rootstocks for WAA resistance, suggest that WAA studies would benefit from being carried out over longer periods of time. Carrying out susceptibility scoring across multiple seasons will account for the slow-growing nature of WAA and unfavourable climatic conditions , allowing WAA populations to establish on susceptible material. When scoring WAA infestation across conditions *e.g*. seedlings in glasshouse, field, or stool bed conditions, Bus *et al.* (2008) assessed WAA 3 - 4 months after inoculation, giving a greater opportunity for colonies to establish than the two-week period used here. The duration of a PhD project is relatively short compared to the expected time usually required to release a rootstock commercially with a trait of interest. Two inoculation and scoring events per year, re-inoculating seedlings which initially scored as resistant, would take 6-8 months which would be pushing the extremes of the apple and WAA seasons in southeast England. This could be accounted for by replicating over multiple years but, as mentioned, this would not be suitable for a short term project.

Validation of SNP markers identified as significantly linked to WAA resistance in the *Er2* mapping family M639 across a range of susceptibilities will be key for determining the predictability of these markers. 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.

7.7.2. Woolly apple aphid genetics

SNPs were generated using a GBS approach for a subsection of the samples analysed with microsatellites here, although these data have not yet been processed with population genetic methods. These SNPs are expected to show greater definition between samples, as only eight microsatellite loci were used in this study. These samples included WAA collected whilst feeding on rootstocks carrying the *Er1* resistance gene. Aligning SNPs generated from these samples with the WAA reference genome would be of special interest, to identify any SNPs in regions associated with aphid feeding morphology, and thus determine if resistance-breaking WAA biotypes have any specific genetic adaptations.

7.7.3. Woolly apple aphid individual and population growth

There is a need to create and standardise adapted insect growth methodology which is more suitable for slow-growing, fragile species such as the WAA. This will be useful not only for future studies of WAA but also for other, similar species. Implementing methods similar to those used by Tan *et al.* (2021), where seedlings in larger pots were inoculated with multiple aphids, should remove the loss in longevity seen in isolated aphids. Creating wounds in the seedling stem to encourage WAA feeding may reduce rootstock fitness. Using another technique to keep WAA in place for observations, such as the modified clip cages suggested in Chapter 6, would therefore be preferable in this instance. Continuing to monitor for WAA feeding on resistant rootstocks will help us to infer what effects *Er1* rootstock resistance has 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.

7.8. FINAL REMARKS

This project has begun to unravel some of the complexities of both the woolly apple aphid and resistant apple rootstock material. This will guide future research into genetic markers which can be diagnostic for WAA resistance in rootstock breeding programmes which the author hopes will re-establish in the UK. Further understanding of the lifecycle of WAA will inform research into its control, both for scientific researchers and apple growers, especially in the face of the spread of resistance-breaking biotypes.

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Appendices

Appendix 1:

Average temperature and relative humidity recorded in the polytunnel across the two growing seasons over which all phenotyping was carried out for the M639 breeding population, and the second and third seasons of MCM007 phenotying. Means were taken across two data loggers, spaced apart within the polytunnel, and the maximum and minimum values presented here. The daily temperature and RH values across both data loggers is also given.

	Year	Average min.	Average max.	Daily average
Temperature	2021	-0.2	37.3	15.3
	2022	0.7	45.6	17.0
Relative Humidity	2021	31.7	100.0	84.2
	2022	17.4	99.8	76.3

Appendix 2: Fungicides applied to breeding populations

Date	Trade Name	Active	Purpose
24/06/2021	Pyrus	pyrimethanil	Scab
	Flint	trifloxystrobin	Mildew
06/07/2021	Stroby	kresoxim-methyl	Mildew
	Captan 80 WG	captan	Scab
22/07/2021	Systhane 20	myclobutanil	Mildew
	Mulan	Dithianon	Scab
04/08/2021	Captan 80 WG	captan	Scab
	Topas	Peconazole	Mildew
11/08/2021	Fontellis	penthiopyrad	Mildew
23/08/2021	Luna Privalige	fluopyram	Mildew
	Difference	difenoconazole	Scab
14/04/2022	Nimrod	bupirimate	Mildew
19/05/2022	Sythane 20	myclobutanil	Mildew
	Pyrus	pyrimethanil	Mildew
08/06/2022	Flint	trifloxystrobin	Mildew
	Difference	difenoconazole	Scab
22/06/2022	Fontellis	penthiopyrd	Mildew
	Mandrake	mancozeb	Mildew
29/06/2022	Secardis	fluxapyroxad	Mildew
	Captan 80 WG	Captan	Scab
06/07/2022	Nimrod	bupirimate	Mildew
	Systhane 20	myclobutanil	Mildew
21/07/2022	Flint	trifloxystrobin	Mildew
	Difference	difenoconazole	Scab
03/08/2022	Potassium Bicarb	Potassium Bicarb	Mildew
09/08/2022	Nimrod	bupirimate	Mildew
27/09/2022	Nimrod	bupirimate	Mildew

Appendix 3:

The identification of genetic variation within populations of the woolly apple aphid (Hemiptera: Aphididae) in the United Kingdom using microsatellite markers.

ABSTRACT

The woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) is a widespread pest of apple (*Malus* × *domestica* Borkh.). Woolly apple aphid originates in North America where it has a heteroecious lifecycle, alternating between asexual reproduction on apple and sexual reproduction on American Elm (*Ulmus americana* L.). Elsewhere WAA appears to have lost the sexual stage, living entirely on apple where reproduction is thought to be predominantly, if not entirely, asexual. Both male and female sexuales have been captured in three countries outside of the USA, although in all cases offspring were not viable under laboratory conditions. This work aims to determine the extent of any genetic diversity within UK WAA populations and to examine the possibility that any variation found may be the result of sexual reproduction. Two hundred and two WAA samples were analysed using eight microsatellite markers. Samples were collected from twelve locations in South East England (mean population size *n*= 17). Assuming asexual reproduction of WAA and a single colonisation event, a single genetic population of WAA was expected. Analysis with the software STRUCTURE tested between one and twelve putative populations and found the most likely number of populations to be two, with the presence of likely sub-structuring. This alone is not evidence of functional sexual reproduction but suggests the potential for previously unknown geneflow between WAA populations in orchards in South East England. This is a concern for pest control because of the potential for spreading genes which confer the ability to feed on resistant rootstocks, as has been reported in several apple-growing regions.

Keywords: woolly apple aphid; *Eriosoma lanigerum*; population genetics; microsatellite; apple; aphid pest; asexual reproduction; sexual reproduction

INTRODUCTION

The woolly apple aphid (*Eriosoma lanigerum* Hausmann; WAA) (Hemiptera: Aphididae) is a sap-feeding pest of domesticated apple (*Malus* × *domestica* Borkh.). In its native North America WAA exhibits a heteroecious lifecycle, alternating between sexual reproduction on American Elm (*Ulmus americana* L.) and asexual reproduction on *M. domestica* (Baker, 1915). Elsewhere WAA appears to have lost its sexual phase and feeds exclusively on apple. Woolly apple aphid causes damage through the injection of elicitors in saliva whilst feeding which causes cambium cells to rapidly divide, creating a gall which can spread to other vascular tissues (Staniland, 1924; Miles, 1999) blocking photosynthate transport and leading to reduced plant growth, especially in younger trees (Weber & Brown, 1988; Brown *et al.*, 1991).

It is hypothesised that the loss of its sexual phase has increased pressure on commercial apple production through rapid build-up of asexually produced nymphs, leading to increased galling. This constant asexual reproduction is also expected to have reduced the genetic variation within the species. Microsatellite analysis of both sexual and asexual populations of the bird cherry-oat aphid (*Rhopalosiphum padi* L.) found high allelic polymorphism and a lack of heterozygotes in sexual populations, compared to asexual populations which showed much less polymorphism but large amounts of heterozygosity (Delmotte *et al.*, 2002). It was also found that

little geographic differentiation existed between sexual populations, suggesting that *R. padi* is able to disperse over large areas and reproduce sexually, leading to varied genotypes. A similar pattern was found in the pea aphid (*Acyrthosiphon pisum* Harris) where higher allelic diversity per locus but lower genotypic diversity was found in an asexual population, compared to an obligate sexual population (Kanbe & Akimoto, 2009).

Genetic diversity of WAA measured by polymorphic loci identified by Inter Simple Sequence Repeat (ISSR) markers found four distinct genetic clusters in central Chile (Lavandero *et al.*, 2009a). The patterns of genetic variation observed correlated with landscape features in that region, such as rivers and higher ground. Spatial separation of individuals by geographic barriers creates isolated habitats which prevent gene flow and drives the development of distinct populations (Coyne & Orr, 2004). This variation is not necessarily indicative of sexual reproduction but suggests that it may be present because no linkage disequilibrium with codominant markers was observed (Lavandero *et al.*, 2009a). As WAA is often distributed on infected rootstocks, wind patterns and geographic barriers cannot entirely explain the genetic variation seen as there is always the potential for populations to be moved against geographic barriers through human intervention.

The most obvious indication of sexual reproduction is the presence of sexual forms. Both male and oviparous WAA have been reported outside of North America. In Australia, both sexual forms have been reported although they had degenerate mouthparts and were not thought able to feed (Asante, 1994). The offspring of these sexuales produced eggs which were not viable under laboratory conditions, although they were not tested under field conditions. Similar observations have also been made in India (Gautam & Verma, 1983) and New Zealand (Sandanayaka & Bus, 2005).

The presence of sexual reproduction and the associated increase in genotypic diversity and gene flow raises the potential spread of virulence genotypes. At least three biotypes of WAA have been discovered in Australia, each with measurably different performance (Costa *et al.*, 2014). The variation between biotypes is suggested to be linked to different modes of host plant resistance to aphid feeding which may drive selection of virulence phenotypes. The pea aphid has genetically determined differences in performance depending on its host plant which selects for aphids which remain on their host rather than those which move between hosts and are not able to improve their performance through experience (Via, 1991). This promotes genetic diversity between populations which may be geographically close but separated by host plant.

Several WAA-resistant rootstocks are commercially available for WAA control but there have been reports, both published and anecdotal, of WAA feeding on these rootstocks. Most of these have been from the southern hemisphere where conditions may be more favourable for aphid growth, and more recently in Europe (Giliomee *et al.*, 1968; Rock & Zeiger, 1974; Jaastad, 2020, pers. comm.). Woolly apple aphid, feeding on M.116 and MM106 rootstocks have been observed at NIAB East Malling in Kent, UK but this has not been formally reported. If such a resistance-breaking biotype of WAA does exist within the UK it could pose a serious threat to UK apple production, especially if the species is also able to sexually reproduce and spread a potential virulence gene(s). The WAA resistance gene *Er1* originates from the scion cultivar *Malus* × *domestica* 'Northern Spy' (Knight *et al.*, 1962) which shows thickened rings of sclerenchyma around the phloem which block sap feeding (Staniland, 1924). Rootstocks carrying *Er1* are expected to also prevent aphid feeding in this manner. It is unknown how WAA can overcome this resistance or whether there may be any sub-lethal effects of the resistance on WAA populations.

We hypothesise that there is not functional sexual reproduction in the UK based on previous observations in other countries outside of the USA. Because of this we expect to see little to no genetic variation between samples collected within the UK. This analysis sets out to test two questions: To what extent is there genetic variation within UK WAA? Or is it possible that there are several distinct populations; In the instance of genetic variation, how likely is it that this variation is caused by sexual reproduction?

MATERIALS AND METHODS

Sample collection

Table 5-Sample identifiers and collection details.

Multiplex	Locus	Repeat	GenBank Accession no.	Primer sequences (5'-3')	Size range (bp)	T_a (°C)
A	*Erio3*	$(TC)_9(CTAT)_6$	EU410510	F: GCCAAACAGTCTTATCTTTCC R: GAATTCGCTGGCTCTCTCTCT	147-163	60
	Erio33	$(CAA)_{12}$	EU410514	F: TCAATGGCAACCGAAGTGTA R: GCAACAGTGGCGTCATCC	159-183	60
	Erio72	$(CT)_{13}$	EU410515	F: GCTGTAGCGGGCGTAATAAT R: AACCTTAACCGCCCCTCTAA	148-170	60
	Erio75	$(TC)_{12}(CT)_7$	EU410516	F: ACGGAGATGAAGGCGTTATG R: TCTCTCCGTCTTTCCGTCTC	134-166	60
B	*Erio20*	$(CAA)_{10}$	EU410511	F: CGACCTTGAGCCTTTGAAAC R: CTGGCTCACTTCCTGGTAGC	161-179	59
	Erio25	$(CAA)_{10}$	EU410512	F: TTGTCACGAACATAAACGTA R: GTACATATTACAACAACAAC	100-106	50
	Erio29	$(GTT)_8$	EU410513	F: TACTCATCGCGAAAACGAGA R: AGTCTCGTCCGATGTTGTTG	171-189	60
	Erio78	$(AG)_{12}$	EU410517	F: AAGTTTAATGGCGTGGGCTA R: GGGATGGTAAACGAGTGTGTG	143-175	60

Table 2-Details of microsatellite markers used (Lavandero *et al.*, 2009b).

Num. code	Letter code	Sampling location and approximate co-ordinates	Date of sampling	No. samples
1	GHJ	NIAB East Malling glasshouse (51.284623, 0.449558)	18.02.2020	14
2	OGB	NIAB East Malling apple gene bank (51.287592, 0.441731)	06.06.2020	8
3	WMK	West Malling, Kent (51.295752, 0.402833)	14.05.2020	5
4	NFC	National Fruit Collection polytunnel (51.296155, 0.882794)	10.06.2021	14
5	NFC +2	National Fruit Collection polytunnel +2°C above ambient (51.296231, 0.881980)	08.07.2020	64
6	NFC +4	National Fruit Collection polytunnel +4°C above ambient (51.296224, 0.882149)	08.06.2020	57
7	CHF	Clockhouse Farm (51.227388, 0.498149)	08.06.2020	1
8	HPW	Honoton Farm, Paddock Wood (51.146229, 0.412999)	12.07.2020	1
9	EMR	WAA culture at NIAB East Malling (51.285892, 0.453019)	27.03.2021	19
10	WSB	Whitstable (51.357181, 1.018644)	11.06.2021	4
11	WSM	Wiseman orchard NIAB East Malling (51.286482, 0.465539)	01.06.2021	9
12	WOT	Walton-on-Thames (51.386154, -0.431309)	13.07.2020	5

Aphids were collected by brushing aphids from plant material with a soft paintbrush into 1.5ml Eppendorf tubes filled with c. 1ml grade 40, 6-14 mesh silica gel (Sigma-Aldrich) for drying.

Excess wax was removed from samples before collection by gentle brushing with a soft paintbrush. Individual, mixed age samples of no defined size were taken from a single, distinct colony, assumed to be the result of a single asexual mother aphid.

gDNA extraction and product amplification

DNA was extracted from multiple individuals from each sample. Two metal ball bearings were added per dried aphid sample and tissue homogenization was carried out using a Geno/Grinder tissue homogenizer at 1500 RPM for 90 seconds. gDNA was extracted using the Qiagen DNeasy Blood and Tissue kit using the supplementary protocol for purification of total DNA from insects. gDNA extraction products were diluted using distilled water to a 5 ng/µl concentration and were then amplified by Polymerase Chain Reaction (PCR) using the Qiagen Type-it Microsatellite PCR Kit under standard 50-55°C PCR cycling conditions. Microsatellite markers from Lavandero *et al.* (2009b) were used in two multiplexes (see Table 2). PCR products were diluted 110% using ultrapure distilled water to prepare samples for fragment amplification analysis using ABI PRISM® DNA Sequencing Analysis Software. Diluted PCR products were denatured at 90°C for three minutes using a PCR machine with GeneScan™ 500 LIZ® Size Standard and Hi-Di Formamide (ThermoFisher Scientific). Fragment size analysis was carried out by ABI PRISM® sequence analysis and the resulting peaks were classified using GeneScan® and Genotyper® Analysis Software (Applied Biosystems Inc).

Data analysis

Population structure was inferred using the software STRUCTURE version 2.3 (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009). An assumed number of genetic populations, K, was selected based on the number of collection sites. In this case K was tested from one to twelve. 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 (Zhou *et al.*, 2015). The data generated by STRUCTURE were further analysed by STRUCTURE HARVESTER to generate mean likelihood values for each K value tested (Earl & vonHoldt, 2012) using the Evanno *et al.* (2005) method. Here values for the change in log probability of the data for each putative value of K, ΔK, are calculated. ΔK represents an ad hoc statistic based on the rate of change of the likelihood function with respect to the value of K being tested (Evanno *et al.*, 2005).

1. Principal Component Analysis

Principal Component Analysis (PCA) was conducted and visualised using R version 4.1.2 (R Core Team, 2021) with the following packages: ade4 (v1.7-19; Dray & Dufour, 2007), adegenet (v.1.3-1; Jombart, 2008), factoextra (v.1.0.7; Kassambara & Mundt, 2020).

2. Population analysis using GenAlEx

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

RESULTS

STRUCTURE analysis

STRUCTURE HARVESTER found the most likely number of populations (K) from those tested, to be two (Figure 1). The smaller peak in ΔK at K=9 suggests the presence of sub-structuring within the populations.

The outputs of the STRUCTURE analysis (Figure 2) shows assignment of microsatellite data to both two and nine populations, as suggested to be the most likely value of K and a potential number of sub-populations (Figure 1). The output from K=2 shows that most sampling locations contained a mixture of the two putative populations with the exceptions being sampling locations

three, eleven and twelve, and locations seven and eight which are assigned almost entirely as populations single populations, represented in green and red respectively.

The output from K=9 has complex population assignment. Whilst many individuals are assigned to multiple populations, clustering appears for some populations. Sampling locations four, five, six and nine show some blocks of solid colour suggesting the presence of distinct genetic clusters within the sampled populations. Woolly apple aphid collected from sampling location twelve (Walton-on-Thames, Surrey, UK) have a distinct population when K=9.

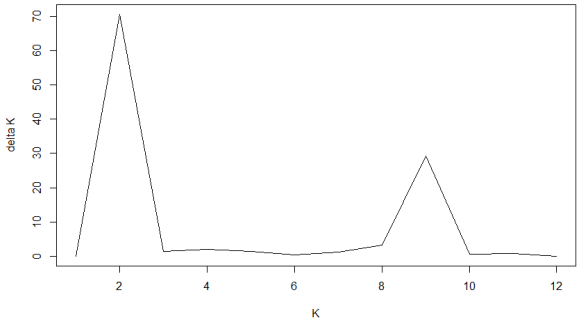

Figure 113- ΔK values for tested numbers of populations from 2-16 as generated by STRUCTURE HARVESTER

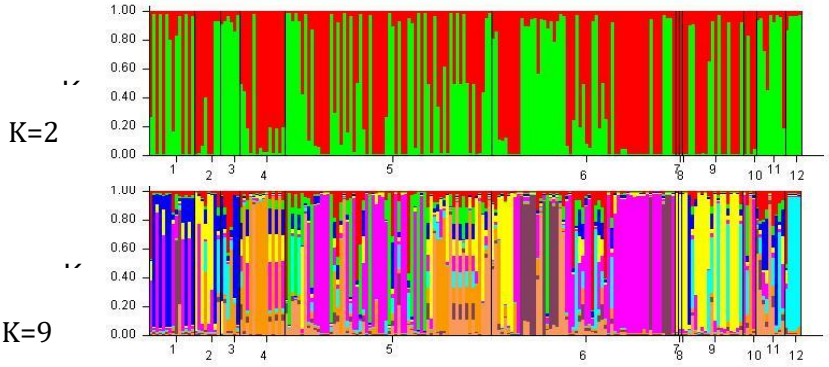

Figure 2- Outputs from STRUCTURE software testing assignment of individual samples to a number of populations (K). Each vertical line represents a single sample.

F statistic

Pairwise FST values calculated with GenAlEx ranged from 0.024 to 0.403 with an average of 0.205.

Table 3-Matrix of pairwise FST values for all sampling locations. Values with a low FST below 0.1 are indicated by *

	GHJ	OGB	WMK	NFC	NFC +2	NFC +4	CHF	HPW	EMR	WSB	WSM	WOT
GHJ	0.000											
OGB	0.213	0.000										
WMK	0.224	0.334	0.000									
NFC	0.227	0.273	0.338	0.000								
NFC +2	0.055*	0.154	0.215	0.159	0.000							
NFC +4	0.069*	0.140	0.250	0.170	0.024*	0.000						
CHF	0.222	0.199	0.276	0.352	0.177	0.152	0.000					
HPW	0.187	0.143	0.403	0.262	0.125	0.097	0.200	0.000				
EMR	0.208	0.235	0.256	0.302	0.172	0.167	0.144	0.246	0.000			
WSB	0.096*	0.168	0.280	0.148	0.041*	0.032*	0.201	0.125	0.198	0.000		
WSM	0.119	0.255	0.224	0.223	0.098*	0.094*	0.267	0.260	0.234	0.130	0.000	
WOT	0.232	0.281	0.370	0.275	0.161	0.195	0.378	0.270	0.352	0.212	0.257	0.000

Summary of population genetic diversity statistics

The observed number of alleles (N_a) ranges from 1.75 to 7.75. The effective number of alleles (N_e) ranges from 1.75 to 4.25. The observed heterozygosity (H_o) ranges from 0.51 to 0.96. The effective heterozygosity (H_e) ranges from 0.38 to 0.74. Unbiased expected heterozygosity (uH_e) ranges from 0.50 to 0.80. The fixation index (F) ranges from -0.15 to -0.92. The value of HE is lower than the value for HO for every sampling location.

Private allele summaries

Twenty one private alleles were found at seven of the twelve locations sampled and across all marker loci tested. The frequency of private alleles found ranged from 0.011 to 0.438.

Table 4- Mean population genetic diversity statistics across the eight marker loci for each sampling location. Observed number of alleles (Na); effective number of alleles (Ne); observed heterozygosity (Ho); effective heterozygosity (He); unbiased expected heterozygosity (uHe); fixation index (F).

Sampling location	N_a	N_e	H_o	H_e	uH_e	F
1	3.88	3.00	0.79	0.65	0.69	-0.24
2	3.14	2.41	0.72	0.52	0.57	-0.30
3	2.75	2.24	0.75	0.45	0.56	-0.43
4	2.86	2.31	0.93	0.51	0.55	-0.49
5	7.75	4.25	0.90	0.74	0.75	-0.23
6	7.25	3.19	0.96	0.67	0.68	-0.45
7	1.75	1.75	0.88	0.38	0.75	-0.75
8	1.75	1.75	0.75	0.38	0.75	-0.75
9	4.00	2.52	0.93	0.55	0.57	-0.92
10	2.75	2.62	0.88	0.56	0.71	-0.55
11	4.00	3.43	0.85	0.66	0.80	-0.49
12	2.25	1.97	0.51	0.42	0.50	-0.15

Table 5- positions in base pairs (bp) of private alleles identified with their respective loci and frequency of each private allele

Sampling location	Marker locus	Allele (bp)	Frequency
1	Erio25	92	0.438
1	Erio25	112	0.063
1	Erio78	158	0.063
3	Erio3	162	0.200
3	Erio3	163	0.200
3	Erio75	131	0.167
5	Erio20	168	0.012
5	Erio20	183	0.023
5	Erio20	185	0.047
5	Erio29	181	0.016
5	Erio29	182	0.016
5	Erio33	158	0.023
5	Erio75	150	0.079
5	Erio75	156	0.145
6	Erio25	109	0.011
6	Erio75	137	0.014
9	Erio29	176	0.077
9	Erio78	177	0.083
11	Erio78	169	0.286
12	Erio72	161	0.400
12	Erio75	152	0.100

Principal Component Analysis

Principal Component Analysis (PCA) found that the markers used formed two groups each comprised of both alleles of four marker loci (Figure 3) found that half of the markers used aligned positively, for both alleles. Individual samples are widely dispersed with several clusters of samples.

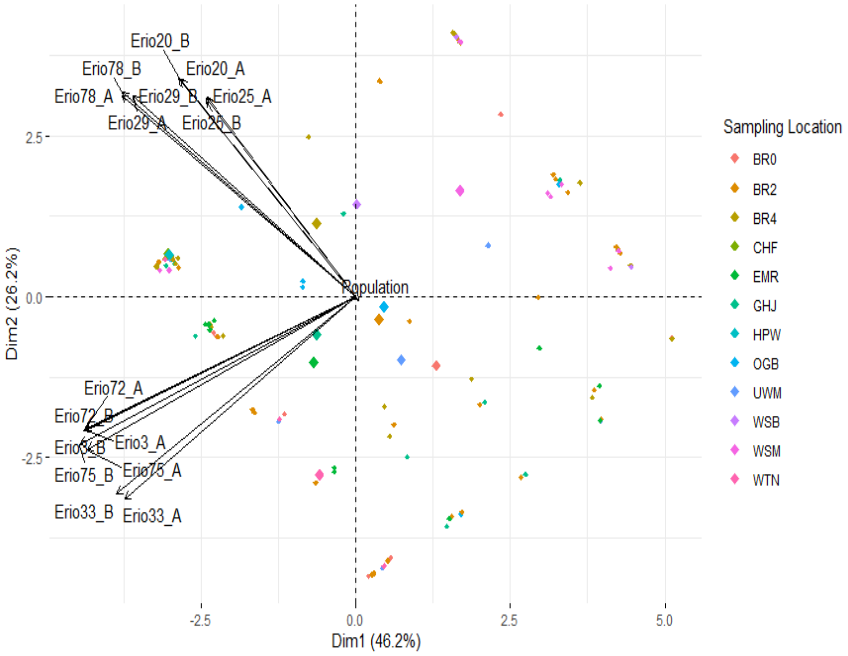

Figure 3- Correlation circle plotting of a principal component analysis of genetic data. The point size varies according to the quality of representation of each variable. A larger point indicates good representation of that variable on the principal component. Variation caused to each marker locus caused by the first to principal components is represented by arrows.

DISCUSSION

These population structure analyses suggest that WAA in South East England show two broad genetic clusters with smaller sub-populations, as indicated by STRUCTURE output at K=9 (Figure 1) and also visualised with PCA (Figure 3). Several clusters emerge when considering population sub-structuring at K=9. Samples collected at the National Fruit Collection showed the largest number of assigned populations at K=9, with multiple clusters assigned solely to one population. This may indicate the presence of clonal lines within the samples, along with samples assigned to multiple populations which may stem from multiple genotypes collected within one mixed-aphid sample. Population clustering for K=2 also has some areas of mixed assignment and some clusters of individuals assigned solely to one population, often which align with single blocks for K=9. The only location not in Kent is sampling location twelve (Walton-on-Thames), approximately seventy miles away from the other sites. These samples are assigned to a single population for both K=2 and K=9 suggesting that the population sampled was entirely clonal. For K=9 these samples are assigned to a population which only appears partially in other samples, suggesting that it has a unique population structure to those collected in Kent. This samples show unique population assignment from K=6 (data not shown).

Estimations of true value of K by calculation of ΔK is almost always accurate, except in instances where there is small marker and/or population size, or partial sampling (Evanno *et al.*, 2005). The exception to this is when testing uneven sample sizes for which STRUCTURE predicts fewer sub-populations than may be present, often merging small but distinct sub-populations and diving large but uniform sub-populations (Puechmaille, 2016). In this instance only eight microsatellite marker loci were used and the number of samples analysed varied (Table 1). This

may mean that the estimated likely value of K=2 is not accurate in this instance and the real number of populations is likely to be higher. Increasing the number of samples or the marker coverage in the future may help to refine the ΔK analysis.

STRUCTURE operates using Bayesian clustering methods of analysing genetic population structure which are advantageous over other data analysis methods such as AMOVA (analysis of molecular variance) as they do not require samples to be pre-assigned into populations, instead assigning groupings based on genetic differences (Lavandero *et al.*, 2009a). Although a potential number of populations must be given for the analysis, these do not affect the population number selected but merely test the likelihood that each putative number of populations is true. Unlike STRUCTURE, GenAlEx requires populations to be pre-defined before analysis and can only compare samples to these defined populations.

Low FST values (>0.1) are indicative of wild type sexual reproduction or recent divergence of populations (Latch *et al.*, 2006). Some 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. The majority of FST values were >0.1 indicating that these populations differ genetically. Although all were <0.5 suggesting that there are no populations completely isolated from each other it does demonstrate genetic diversity within WAA populations collected in SE England and the potential for partial sexual reproduction.

Private alleles are those found only in one (sub-)population (Neel, 1973) and can be indicative of heritable alleles. Ten private alleles were found in samples collected from the National Fruit Collection which is consistent with the high diversity found in those samples (Figure 2) and the large sample sizes which has been found to increase the number of private alleles identified, suggesting higher gene flow (Slatkin, 1985). Collecting more, larger data sets of WAA genomic material may reveal further genetic variation between populations than previously thought. Six private alleles were identified at NIAB East Malling, suggesting a degree of genetic isolation at these two sites. Two private alleles were identified for the samples from Walton-on-Thames. These samples appear to be clonal based on population assignment by STRUCTURE software (Figure 4) but the presence of private alleles and the pairwise FST values for this location suggest that this population is genetically isolated from others included here.

Organisms which reproduce both sexually and asexually can exist in populations which are wholly sexual or asexual, or partially sexual populations (Delmotte *et al.*, 2002). It is possible that high heterozygosity and low allelic polymorphisms in sexual populations of *R. padi* is a result of either long-term asexuality leading to high genetic diversity (Bengtsson, 2003), or of asexual lineage which have since hybridised (Delmotte *et al.*, 2002). The samples from this study found high observed heterozygosity in all cases (Table 4) which is consistent with highly heterozygous asexual populations of the bird cherry-oat aphid (Delmotte *et al.*, 2002). In cases where there is higher observed than expected heterozygosity it may be the result of 'isolation breaking' which is observed when previously isolated populations have begun to mix (Hamilton, 2021). It is clear that WAA in South East England have a dynamic genetic structure which may be the result of multiple invasions of WAA from the USA at different time points.

CONCLUSIONS

It is likely that WAA in South East England are producing partially sexually which has resulted in two distinct genetic clusters with potential sub-populations. There are potential explanations: spontaneous mutation or genetic drift in obligate asexual populations; multiple points of invasion of WAA from the USA where it does sexually reproduce; and lastly that there is WAA sexual reproduction in the UK. Assuming sexual reproduction had been occurring in the USA continuously in the meantime, each invasion to the UK which then became functionally asexual would have a different genotype than the previous invasion. This may explain genetic variation between locations but is more tenuous when considering that some sampling locations of this study were close together and still showed allelic polymorphisms, where it would be unlikely that multiple invasions had occurred.

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