University of Reading

Population genetics and epidemiological effects on *Venturia inaequalis* from mixed cultivar apple orchards

PhD in Agriculture

School of Agriculture, Policy and Development

Thomas A.J. Passey

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Declaration of original authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Thomas A.J. Passey

Contribution of authors

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Tom Passey and Xiangming Xu were responsible for conception and design of experiment; Tom Passey was responsible for data collection; Tom Passey was responsible for data analysis and interpretation; Tom Passey was responsible for drafting the manuscript; Xiangming Xu and Michael Shaw were responsible for critical revision of the manuscript; all authors approved the final version

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Abstract

The apple industry in the UK produces half a million tonnes of fruit a year; its most economically important disease is apple scab caused by the fungal pathogen *Venturia inaequalis*. Mixing cultivars with differing resistance to the pathogen, in the same orchard, has been shown to reduce the levels of apple scab. In this study we investigated the population genetics and epidemiology of apple scab in mixed cultivar orchards.

Using molecular techniques, populations of *V. inaequalis* differed on the hosts present in an orchard, this difference remained over time. This indicates a "super race" of the pathogen that can infect all of the cultivars present has not emerged and become dominant, therefore making the concept of mixed cultivar orchards more feasible.

The lack of emergence of a super race might be, in part, due to asexual conidia spores forming a proportion of primary inoculum as opposed to being purely from sexually produced ascospores. Conidia accounted for 20-50% of the primary inoculum in the orchard studied. The importance of conidia is likely to be heavily dependent on local conditions.

For a reduction in levels of apple scab in a mixed cultivar orchard it is important that the cultivars present have differing resistance. Comparing scab populations on a number of desert and cider cultivars showed that populations on cider cultivars are most different.

An assembled *V. inaequalis* genome is presented and was used to align isolates from different cultivars within the same orchard. Looking at differences between these isolates has indicated that there is a lack of mating occurring between isolates from the different cultivars present within the same orchard. This indicates that mating is most likely initiated between isolates on the same leaf.

The findings of this thesis could contribute to apple orchard practises, regarding apple scab control, in the short, medium and long term.

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1 Introduction

1.1 Malus – The Host

Malus, a genus of the Rosaceae family, comprises around 55 species of deciduous trees (Phipps et al. 1990). The majority of these are different species of crab apple, but the genus also includes *Malus* x *domestica*, the species that produces the domesticated apple fruit. The origin of the domesticated apple has been a matter of debate for some years. Originally assumed to have evolved through a combination of crab apple species, the main progenitor was reported to have been from *Malus sieversii*, the wild crab apple originating in Central Asia (Harris et al. 2002). It was suggested that *M.* x *domestica* and *M. sieversii* may in fact be the same species and as a result the nomenclature *Malus pumila* should be used (Velasco et al. 2010). However, other research has shown that the domesticated apple has ancestry from other *Malus* spp. and actually has a closer relationship to *Malus sylvestris*, the European crab apple (Coart et al. 2006). It is most likely that apple was domesticated from *M. sieversii* in Central Asia but that *M. sylvestris* has had a significant role as a secondary contributor to the genetic makeup of *M.* x *domestica* (Cornille et al. 2012).
The domesticated apple genome was first published by Velasco et al. (2010) using the cultivar Golden Delicious. They found 57,000 genes on the 17 chromosomes, with around 11,000 specific to apple. A higher quality genome, assembled from a mix of short-read and long-read next generation sequencing (NGS) has recently been published (Daccord et al. 2017). The majority of apple cultivars are diploid. However ca. 14% of the 2162 cultivars at the UK National Fruit Collection have been shown to be polyploid, most of which were triploid, with a few tetraploid (DEFRA 2010).

1.1.1 Cultivation

The apple has been a popular fruit for at least hundreds of years and is likely to have been eaten for thousands (Bultitude 1983). There is evidence of consumption in the First Persian Empire and ancient Greece and popularity spread through Europe and Asia along the Silk Road during the period of the Roman Empire (Morgan & Richards 1993). It was at this time that the cultivated apple was introduced to Britain, although there would have been native

crab apples (*M. sylvestris*) before this. Much of the fruit grown at this time is likely to have been grown from seed. The seed of apple is not true to type with each seed being different from each other and its parents. As a result, the fruit would have been of varying quality. However, in those areas where the popularity of apple was evident there is indication that grafting was used, although it is not known when grafting was first used; it may have been hundreds of years before this known grafting (Mudge et al. 2009). Grafting involves taking material of one plant, normally selected for its flowers/fruits, that doesn't produce true to type seed, and combining it with the roots of another plant. Therefore you can take a shoot from an apple tree (the scion) and graft it to a *Malus* rootstock and get a clone, with true to type fruit, of the donor tree. The rootstock on which the scion is grafted is important too. Rootstocks control the size and vigour of the whole tree. This is important in commercial orchards for uniformity of the trees and therefore making management of the orchard easier. Rootstock breeding, pioneered at the East Malling Research Station (Hatton 1935; Preston 1966; Gregory et al. 2013), has given a range of rootstocks to choose from, ranging from very dwarfing to very vigorous. Rootstock breeding aims to improve tolerance to different soil conditions, confer pest and disease resistance, improve cropping and reduce the cost of pruning and picking.

While there is a range of some tens of rootstocks to choose from to graft apples on to, there are thousands of apple cultivars to choose from as the scion. Cultivar is short for cultivated variety. A new cultivar may result from a chance seedling (chance hybrid of two other cultivars) or from deliberate crossing and selection in a breeding program. Apple cultivars can be split by their use into dessert, cooking and cider, although some cultivars may be multi-use. There are many breeding programs around the world, with around 40 in Europe alone (Sansavini et al. 2005), trying to develop new cultivars to take to either the mass market or with traits specific to conditions in an area, for example, for the state of Washington in the USA (Evans 2013). Desirable traits include taste, appearance, size, high yield, improved storage and resistance to pests and diseases. Traditional breeding involves crossing parents to produce many seedlings and assessing these over time to see which have the best combination of desirable traits. This requires the production and assessment of many seedlings to find one with a good combination of traits from the parents. This process can be sped up by marker assisted breeding where markers for many traits can be looked for

in the DNA of the offspring of crosses very early, thus reducing the number of seedlings grown on for phenotypic assessment (Collard & Mackill 2008).

1.1.2 The apple industry

The world apple industry produces 89 million tonnes of apples a year from 5.3 million ha of land, with China (producing almost half the world's apples), USA and Poland the biggest producers according to FAOSTAT figures (FAOSTAT 2016). The UK produced 481 thousand tonnes of apples in 2016, showing a steady increase in production over the past decade. There are ca. 160 km^2 of apple orchards in England and Wales which amounts to 80% of the total fruit tree orchard area. Cider cultivars account for around half of the land used for growing apples, predominantly in the West Midlands and South West of the country. Bramley is the single cultivar grown on the largest area, with 12.5% of orchard area, 95% of the land used for growing culinary apples. The dessert cultivars Cox (and its clones) and Gala (and clones) are the next most grown with 11.5% and 9% of apple orchard area respectively. The South East of England is the predominant dessert cultivar growing region. The area of land used for growing specific cultivars changes as tastes and demand alter with new cultivars being introduced. The land area growing Cox has dropped by more than a half over the last decade while Gala has doubled in the same period. Braeburn and Jazz both showed big increases in orchard area between 2009 and 2012 (67.5% and 41.4% increases respectively) (DEFRA 2013).

1.2 *Venturia inaequalis* – The Pathogen

Apple scab, also known as black spot in Australasia, is the most important disease of apples globally and as a result has been extensively researched for well over a century. It can infect most tissues of the tree, most severely on young leaves and fruit. Caused by the fungal pathogen *Venturia inaequalis* it was first reported as its anamorph (asexual stage), *Spilocaea pomi,* by Fries in Sweden in 1819 (Fries 1819) and first reported in the United Kingdom in 1855 by Berkeley (as cited in Marsh & Walker 1932). The teleomorph (sexual state) was first described by Cooke in 1866 as *Sphaerella inaequalis* [the spore consisting of two unequal sized cells] (Cooke 1866) while Winter was the first to correctly place the species in the *Venturia* genus (Winter 1875), hence in much of the literature the species is referred to as

Venturia inaequalis (Cke.) Wint. *V. inaequalis* attacks only *Malus* spp. although *formae speciales* of *V. inaequalis* are known on other species of the Rosaceae family such as *Pyracantha* spp. and loquat (*Eriobotrya japonica)* (Le Cam et al. 2002; Gladieux et al. 2010). *V. inaequalis* is closely related to *Venturia pirina*, the causal agent of pear scab, and while the symptoms are very similar the two species are genetically distinct and cannot cross infect, *V. pirina* being specific to *Pyrus* spp.

The pathogen is likely to have originated in Central Asia. Gladieux *et al*. (2008) genotyped commercial apple scab populations from around the world using microsatellite markers and found that genetic variability was greater in populations from Central Asia than in samples from other areas of the world. This indicates that apple scab and the domesticated apple both had the same origin, emerging and spreading in parallel.

1.2.1 Why it is a problem

Annual epidemics of apple scab make it one of the most economically important diseases for apple growers worldwide. It is a problem in most apple growing areas, but especially so in temperate regions with wet and mild climates. In the UK it is one of the three main diseases of apple along with apple powdery mildew (*Podosphaera leucotricha*) and canker (*Neonectria ditissima*).

The largest losses caused by apple scab are from reduction of fruit quality. The requirement for unblemished fruit by customers and, therefore, the supply chain, leads to fruit with scab being rejected. Mycelial growth on the fruit surface leads to an olive-brown coloured lesion which generally forms a brown “scabby” area (Fig. 1 a). Severe scabbing of the fruit can lead to cracking of the fruit skin and a potential route for secondary infection from other pathogens (often post harvest). Infection of young fruit can also lead to a reduction in size and misshapen fruit. Early infection of flowers, petioles and young fruit can lead to fruit drop, while a severe epidemic mid-season can lead to defoliation, potentially reducing wood growth and restricting fruit bud formation for the following year (MacHardy 1996). Infection of shoots by *V. inaequalis* can also provide entry for other pathogens such as *Neonectria ditissima* (syn. *N. galligena*), the apple canker fungus.

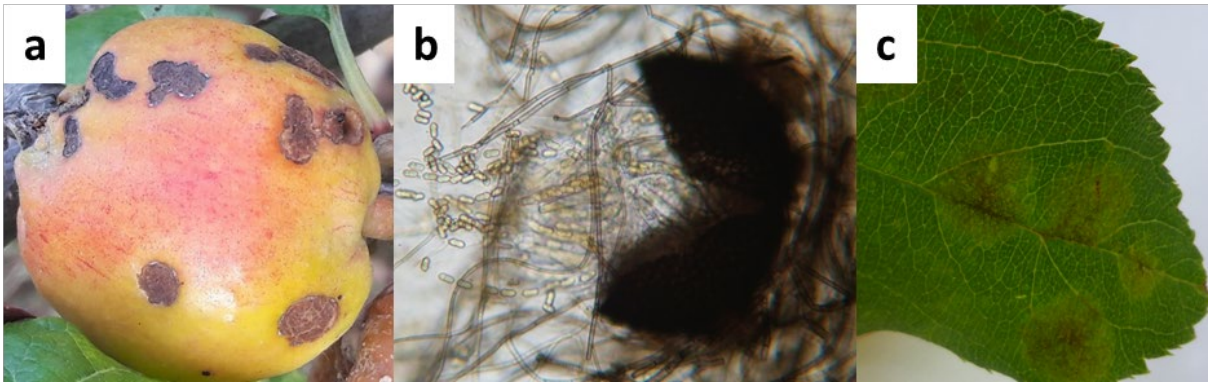

Figure 1. Images of the fungal pathogen *Venturia inaequalis,* the cause of apple scab disease. a) Scab lesions on the surface of apple fruit. b) A Pseudothecia releasing ascospores; image taken from a squash mount of the intersection of mycelia from two isolates of opposite mating type. c) A leaf from a *Malus* tree with multiple olive-green/grey *V. inaequalis* lesions.

1.2.2 Ecology and Epidemiology

1.2.2.1 Primary inoculum of *Venturia inaequalis*

V. inaequalis is an ascomycete fungus. Sexual reproduction between two isolates leads to the formation of pseudothecial fruiting bodies which in turn produce many asci; each ascus contains eight ascospores (Fig. 1 b). Keitt & Palmiter (1937) were the first to show that clones of a single genetic isolate could not self-mate, and showed that cross mating isolates fell into two distinct groups; crossing isolates between groups formed mature pseudothecia (referred to as perithecia by Keitt) that bore ascospores, but crossing isolates within a group did not. This showed that *V. inaequalis* is heterothallic.

Ascospores are discharged from overwintering leaf litter after sufficient rainfall at bud burst and then wind dispersed, landing on the new leaves as primary inoculum. Much of the research on early season infection has assumed that ascospores are the main (or only) source of primary inoculum for apple scab. As a result, modelling of early season spore release has focused on the release and dispersal of ascospores from leaf litter (Gadoury & MacHardy 1986; Beresford & Manktelow 1994; Berrie & Xu 2003). However, there is also evidence that asexual conidia, formed by lesions throughout the growing season (see life cycle section below), overwinter in the buds and are then dispersed by water splash at bud burst, thereby contributing to the primary inoculum (Holb et al. 2004; Holb et al. 2005; Becker et al. 1992; Stensvand et al. 1997).

1.2.2.2 Life cycle

Infection by both potential primary spore types, ascospores and conidia, is very similar, forming olive-green/grey lesions on leaves (Fig. 1 c). The spores land on susceptible tissue, germinate and penetrate the cuticle. Hyphae grow between the cuticle and epidermal cell wall resulting in the development of stromata. Conidiophores and, thus, conidia form and rupture the cuticle. These spores, clonal copies of the primary spore that initiated the lesion, are released and dispersed by water splash leading to further lesions on susceptible tissue (Fig. 2). It is this process that leads to rounds of secondary infection on leaf and fruit throughout the growing season (MacHardy 1996).

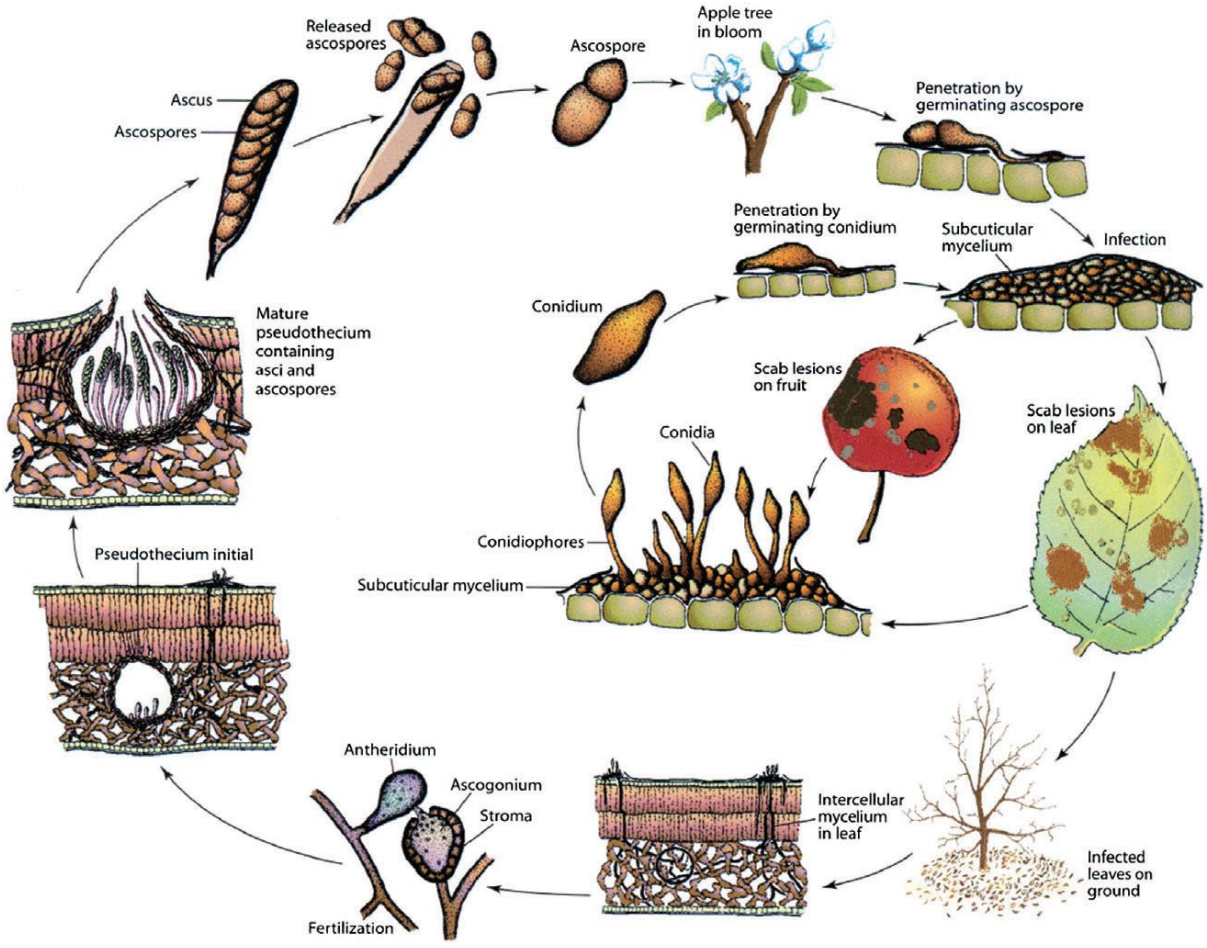

Figure 2. Life cycle as presented in Plant Pathology 5th Edition (Agrios 2005). Note that the assumption here is that the primary inoculum is from sexually produced ascospores only, with asexual conidia only involved in secondary cycles of infection from lesions on leaf and fruit.

1.2.3 *Venturia inaequalis* genome

V. inaequalis is a haploid organism with conventional cytology suggesting it to have seven chromosomes (Day et al. 1956). A linkage map of *V. inaequalis* by Xu et al. (2009) produced 11 linkage groups spanning 1106 cM in length, while Broggini et al. (2011) mapped 14 avirulence genes on a linkage map of 15 linkage groups spanning 972 cM. Construction of a Bacterial Artificial Chromosome (BAC) library estimated the genome size to be 102 Mb (Broggini et al. 2007), however this was calculated by multiplying the number of clones of BAC library (7680) by the average insert size (80kb) and dividing by estimated number of haploid genome equivalents (6) and therefore is open to statistical variation. Whole genome sequencing has estimated the genome size to be between 40 Mb and 72 Mb (Shiller et al. 2015; Deng et al. 2017); these isolates were sequenced on short read next generation sequencing platforms, thus making it difficult to assemble genomes from the many short fragments, in fact none of the isolates sequenced were assembled into less than 1000 scaffolds. It is estimated that 36 Mb of the genome is non-repeated DNA (Bowen et al. 2011), making assembly more complicated. The different strategies and software for sequencing and assembly are the likely reason for the difference in genome size of these isolates (Deng et al. 2017). The de novo assembly and annotation of the *V. inaequalis* transcriptome has been published (Thakur et al. 2013); however, although useful for RNA-seq data and comparison of protein expression, it does not cover sufficient amounts of the whole genome for carrying out population studies. Only one *V. inaequalis* genome is publicly available, the Vi 1 isolate from Deng et al. (2017).

1.3 Control of apple scab

As one of the most important diseases of apple it is unsurprising that measures to control scab are imperative in a commercial orchard. Management of scab on susceptible cultivars is vital and the most effective control is through a programme of chemical sprays; however, many alternative methods have been researched and implemented as alternatives or complementary to fungicide use.

1.3.1 Fungicide control and forecast models

The need for chemical control of apple scab started in the late 19th Century after a number of years of favourable weather for scab in Europe in the 1880s, with extensive damage reported in Australia, Canada, England and the USA in the 1890s (MacHardy 1996). There

was also a growing requirement from the consumer for unblemished fruit (Large 1946). The first successful chemical control for scab was the use of Bordeaux mixture, a mix of Copper (II) Sulphate [$CuSO_4$], lime [$Ca(OH)_2$] and water, that had first been discovered to be an effective treatment against downy mildew (*Plasmopara viticola*) in grape (as cited in Millardet 1933).

Modern fungicide control of scab is based around decades of research since the first experiments using Bordeaux mixture on apple orchards. Introduction of organic fungicides in the mid-20th century (Morton & Staub 2008) led to a larger range of available products for growers and understanding of their different modes of action was important for maximising their effectiveness at different stages of the epidemic.

It was Mills in the 1920s who began research on the importance of timing of sprays against scab (MacHardy 1996). Since then a number of management programmes have been developed around the world for example, Schwabe (1980), MacHardy & Jeger (1983) and Beresford & Manktelow (1994). Scab infection models have been used as the basis for computer programmes, such as ADEM (Berrie & Xu 2003) and RIMpro (http://www.rimpro.eu/) which relate phenology and weather to indicate to growers when it will be most effective to apply sprays as well as the type of spray (protectant, curative or eradicant).

At the start of the 2017 growing season 74 Ministerially Approved Pesticide Products (MAPP) were licensed for use on apple in the UK, 65 of which have an active ingredient that can have an effect (if only incidental) on scab levels (https://secure.pesticides.gov.uk/pestreg/prodsearch.asp). It is not unusual for 12-18 spray applications to be applied to an orchard every year and a typical spray programme can be seen in Table 1. Fungicides account for around two thirds of the of total pesticide-treated area of orchard crops in the United Kingdom (Garthwaite et al. 2016).

There is tight legislation on the use of pesticides. Pesticide labels have specific maximum rates of use, as well as harvest intervals, to minimise any potential health threat. In the UK there are a number of regulations that growers must adhere to backed up by law, including use, storage and record keeping. All EU member states are required to monitor food for pesticide residue and carry out surveys every year. However there is a drive within crop protection, due to a number of potential environmental and economic costs, to reduce the use of pesticides (Pimentel et al. 1992). The use of pesticides is unpopular with consumers

and retailers with press reporting of pesticide use often focusing on the negatives, strong opposition to their use from environmental groups and research led environmental (Aktar et al. 2009) and potential health factors (Nicolopoulou-Stamati et al. 2016) associated with their use. As a result there is ever increasing legislation around the use of pesticides and a decreasing number of products available. The Andersons Centre (2011) report suggested 87 out of 250 active ingredients approved in the UK at the time were under threat due to new legislation, with 12 fungicides highly likely to be banned. The number of sprays required for acceptable levels of scab (Table 1) is indicative of the economic costs of chemical control in an average year and therefore a reduction in pesticide use would be appreciated if other measures were available giving the same or similar control.

Resistance of scab to dodine (Szkolnik & Gilpatrick 1969; Köller et al. 1999) and demethylation inhibitors (DMI) (Stanis & Jones 1985) such as myclobutanil (Braun & McRae 1992; Gao et al. 2009) has been shown, however they are still recommended for use (Table 1) and will provide acceptable control of scab for commercial production in many orchards. The Fungicide Resistance Action Committee (FRAC) recommends mixing the use of fungicides with differing modes of action for pathogen control (Fungicide Resistance Action Committee 2010). The mix might be a pre-mixed product, tank mixed or alternating the use of two or more products. The type of mix will differ between crop and pathogen as well as the time in the season and life cycle of the pathogen. Mixes are recommended for three reasons:

1) They can improve disease control; by combining the characteristics of the components in the mixture there may be better control, for example mixing a fungicide with a curative mode of action with one with protectant activity would be good for controlling a pathogen, like scab, where there are secondary rounds of infection through the growing season.

2) Minimise crop losses due to fungicide resistance; if there is a breakdown in the effectiveness of one of the products in the mix due to resistance in the pathogen population the other product(s) will provide back up for continued control, thus keeping control of the pathogen levels and minimising crop losses. The potential drawback of this in combined application mixes is that the resistance to that product will be slower to be recognised, as well as not knowing which product in the mix has failed. If mixing products by alternating and resistance to one of the products develops then control will not be as good (although gives the option to substitute an alternative product).

3) Better resistance management; an over reliance on chemical control with one mode of action is likely to lead to more rapid resistance in the pathogen to the pesticides with that mode of action, thus leading to faster dominance of the resistant race. Spray programmes with multiple modes of action are less likely to have resistance occur as if resistant isolates to a specific product emerge they may be eradicated by the other product(s) in the mix and therefore not allow the development and domination of a resistant race in the crop.

1.3.2 Spray control alternatives to commercially used fungicides

Alternatives are required to the common commercially used fungicides, due to the increased legislation mentioned previously and also for scab control in organic orchards. Of the fungicides licensed for apple in the UK only sulphur based products are available for use in organic orchards during the growing season (http://apples.ahdb.org.uk/apple-scab.asp), although they tend to be less effective and can be more phytotoxic than fungicides available to non-organic growers (Ellis et al. 1998; Holb et al. 2003).

The use of alternative controls to fungicides up to the end of the 20th Century is well reviewed by Carisse & Dewdney (2002). Biological control is a method of pest, weed and disease management using natural enemies (Barratt et al. 2018). In plant pathology this applies to the use of a microbial antagonist, known as a biocontrol agent (BCA), to suppress disease (Pal & McSpadden Gardener 2006). The antagonism caused by a BCA can be direct through hyperparasitism (e.g. mycoviruses), mixed-path, for example the release of antibiotics, lytic enzymes, waste products or chemical interference, or indirect, by competition or induction of host resistance (Pal & McSpadden Gardener 2006). The use of BCAs for use against apple scab has been widely investigated in the past couple of decades. For example, an isolate of *Cladosporium cladosporioides* was selected as the most effective fungal coloniser, from a range of those found, at reducing conidial production (Köhl et al. 2009) and then shown to reduce the severity of apple scab on leaf and fruit in orchard conditions (Köhl et al. 2015).

The study of plant extracts *in vitro* to slow down germination of conidia led to 1% populin, an extract from black poplar (*Populus nigra*), being shown to reduce scab on leaves and fruits (Bálint et al. 2014), when applied to run-off. Plant retardant Prohexadione-Ca could reduce scab incidence (Bazzi et al. 2003) most likely by upregulating pathogenesis related proteins and inducing an increase in host resistance (Bini et al. 2008).

Table 1. Typical fungicide spray programme for Cox in a UK orchard. In the first instance of product name it is followed by its chemical group name in bold and target site of action in italics

Timing	Target diseases	Fungicide product	Comments
Pre-bud burst	Overwintering scab on tree/canker	Copper fungicide (**Inorganic**; *Multi-site contact activity*)	e.g., Cuprokylt®
Bud burst	Scab/canker	Dodine (**guanidine**; *proposed: cell membrane disruption*) or Dithianon (**quinone** - *Multi-site contact activity***)**	If canker is not a problem Pyrimethanil (**anilino-pyrimidine**; *proposed: methionine biosynthesis*) is an alternative
Mouse ear	Scab/canker	Dodine or Dithianon	
Green cluster	Scab/mildew	Myclobutanil (**DeMethylation Inhibitors**; *C14- demethylase in sterol biosynthesis*) + Captan (**phthalimide**; *Multi-site contact activity***)**	Other DMI, e.g. Penconazole, + Captan is an alternative
Pink bud	Scab/mildew	Myclobutanil + Captan	
Blossom	Scab/mildew	Myclobutanil + Captan	Where mildew incidence is low then Kresoxim-methyl, e.g. Stroby®, (**Quinone outside Inhibitor fungicides**; *cytochrome bc1 at Qo site*) is an alternative
Petal fall↓ repeat 10 day intervals to late May/mid June	Scab/mildew	Myclobutanil + Captan	
Fruitlet (mid June)	Mildew	Bupirimate (**hydroxy- (2-amino-) pyrimidines**; *adenosindeaminase*) OR Boscalid (**Succinate-dehydrogenase inhibitors**; *succinate-dehydrogenase*) + pyraclostrobin (**Quinone outside Inhibitor**; *cytochrome bc1 at Qo site*) if Bupirimate not registered on apple	Include scab protectant fungicide only if scab present
June↓ continued at 10 day intervals to August	Mildew	Bupirimate or Penconazole or Sulphur (**Inorganic**; *Multi-site contact activity*), if bupirimate not registered on apple	Include scab fungicide only if scab risk. NB Do not use triazole DMIs on Cox after mid June if risk of Diffuse Browning Disorder
August	Mildew/ storage rots	Boscalid + Pyraclostrobin	
Post-harvest	Scab	Urea	To encourage leaf rotting and elimination of overwintering scab

Table adapted from http://apples.ahdb.org.uk/apple-scab-additional-information.asp#link11
Chemical information from FRAC Code List 2018 http://www.phi-base.org/images/fracCodeList.pdf

Although these alternative methods show promise in lab and field trials they are rarely adopted in practice. Their success in commercial practice is often not as good as it is in a controlled research trial. This can be due to a number of factors including; use of other products that might compete against, or render ineffective, the treatment (e.g. using chemical control that will inhibit a BCA); differing environmental factors between the area the product was developed and where it is used; and the application process used in development cannot be repeated in practise.

The efficacy and reliability of these alternative products is often less than that of chemical control. Fluctuations of the environmental conditions, within and between seasons, means that conditions are often far from optimal for successful disease control by these products. BCAs are living organisms and therefore their biology can be as difficult to predict and model as the pathogen that needs controlled. Like chemical products there is the chance the pathogen will develop resistance to these products. Although, BCAs are thought to be more durable than chemical control, the limited, available information suggests this assumption is not always justified (Bardin et al. 2015).

These products are often more expensive than fungicides. BCAs for use in agriculture require strict pathogenicity testing, for both human safety and plant pathogenicity (hypersensitivity), before product development (Velivelli et al. 2014). Registration procedures can be complicated (Velivelli et al. 2014) and expensive (Barratt et al. 2018), although debatable if any more or less expensive than registering a new fungicide.

Perhaps these alternative products are too often compared to chemical control for a pathogen and instead need to be viewed only as an important tool in an integrated pest management (IPM) approach, alongside other practices such as sanitation.

1.3.3 Orchard sanitation

Keitt, in 1936, discussed how after 50 years of the “splendid achievements of protectant spraying” there remained serious limitations. Although some of the limitations discussed might have been overcome in the last 80 years (Morton & Staub 2008), at least in part, it is important to supplement fungicide control with other methods to reduce primary inoculum. The level of apple scab inoculum will increase during the epidemic phase of the life cycle with each new lesion producing conidia for secondary rounds of infection, although the actual rate of infection will vary with environmental conditions and the proportion of ageing

leaves with ontogenic resistance (MacHardy et al. 2001). It is imperative therefore to reduce the level of primary inoculum as much as possible for better control of scab through the rest of the season.

Ascospores are released from the overwintered leaf litter in spring and therefore removal of leaves is a crucial factor in reducing primary inoculum levels. Rudimentary practices such as removal of leaves from the orchard or raking and burning leaves are not likely to be practical for commercial growers (although may be of help to gardeners). Making leaf material smaller and softer allows better maceration by earthworms. Sutton et al. (2000), in orchards in New Hampshire and Maine in the USA, showed shredding all leaf litter with a flail mower in autumn reduced the risk of scab by up to 90%; while shredding as little as 65% of the litter could reduce the risk by 50%. Sweeping up leaves with a lawn sweeper and ploughing them in to the row led to a 80% reduction in fruit scab incidence compared to not removing leaves, although this figure dropped to 50% in the following year when the orchard had a higher scab incidence and severity (Gomez et al. 2007); i.e. a faster exponential rate of scab development in a season reduces the effect of reducing primary inoculum. Carisse & Dewdney (2002) suggest that many growers do not carry out this physical leaf removal due to a combination of lack of equipment (shredders etc.), the lack of acceptable scab control and a belief that these practices do not lead to a reduction in the use of fungicides for sufficient scab control.

A practice that is commonly used in commercial orchards is the use of a 5% urea spray, with application recommended between harvest and leaf fall. Burchill *et al*. (1965) observed a "noticeable effect on the decomposition of leaves" when treated with 5% urea and a 97% reduction in ascospores liberated per cm^2 of leaf material compared to leaves treated with just water. The use of urea has been shown to reduce ascospore number and/or scab incidence to varying degrees in a number of other studies both as an individual treatment or as part of a multifaceted approach (Sutton et al. 2000; Vincent et al. 2004: Holb et al. 2006;). Urea application can be difficult with cultivars, such as Bramley's Seedling, which can retain their leaves in to early winter, in the maritime climate of Northern Ireland (Mac an tSaoir et al. 2010). Bramley, as often with triploid cultivars, have thicker leaves than many cultivars and therefore urea has less of an effect at breaking down the leaves. Urea appears to decrease scab levels through a number of routes. Ross (1961) showed that excess nitrogen inhibits formation of pseudothecia (perithecia in the paper); while urea can also soften

leaves making them more palatable to earthworms, therefore increasing breakdown and changing the microbial population for faster maceration and inhibition of *V. inaequalis* (Carisse & Dewdney 2002).

Appropriate pruning increases air circulation in the tree canopy and therefore reduces leaf wetness (Carisse & Dewdney 2002), an important aspect in incidence and severity of scab (MacHardy 1996). Tree architecture (Simon et al. 2006) and both winter (Holb 2005) and summer (Cooley & Autio 2011) pruning can have effects on leaf wetness which reduce scab levels, as well as allowing better spray deposition for more effective chemical control. Winter pruning to decrease levels of overwintered conidia in bud scale is also an important sanitation method in orchards where overwintering of this type is likely (Holb 2005; Holb et al. 2005). Pruning is carried out predominantly for improved fruit quality with a positive impact on disease control an additional benefit, although it can also lead to an increase in some diseases such as fire blight (caused by the bacteria *Erwinia amylovora* – an important disease in North America) and apple canker (*Neonectria ditissima)* (Cooley & Autio 2011).

1.3.4 Host resistance

Flor was the first person to fully report on the concept of Gene-for-Gene (GfG) relationships in the interaction of pathogens with their host. He had initially developed the idea working on the interaction between flax (*Linum usitatissimum*) and the flax rust pathogen *Melampsora lini* (Flor 1956). *V. inaequalis* was shown to have avirulence genes that matched resistance genes in *Malus* (Boone 1971) , indicating that apple and scab have a GfG relationship.

Since then 18 GfG relationships have been identified in the *Malus* – *V. inaequalis* pathosystem. A new nomenclature was recently set out for the first 17 GfG pairings (Bus et al. 2011) to which *Rvi18* has now been added (Soriano et al. 2014). The nomenclature used in this thesis follows that set out by Bus et al. in 2011, where necessary followed by historical names in brackets. A number of these R genes have now been cloned and characterised, including *Rvi1* (Cova et al. 2015), *Rvi6* (Belfanti et al. 2004; Malnoy et al. 2008) and *Rvi15* (Schouten et al. 2014). Other workers have investigated mapping regions and development of markers of R genes for their potential use in scab resistant breeding, for example Padmarasu et al. (2014) fine mapped the *Rvi12* resistance locus. Research in identifying and using major R genes against apple scab in commercial breeding has focused on genes from

wild *Malus* species rather than domesticated apple. The only exception is *Rvi1* (formerly *Vg*) from Golden Delicious, but this gene can be overcome by 87% of the scab population in Europe and is described as "an exception to the premise that narrow spectrum R genes should be excluded from the nomenclature" (Bus et al. 2011). The inclusion of this gene is due to the extensive characterisation of both the gene and Golden Delicious (the differential host) and it is therefore deemed an important model to understand the difference between ephemeral and durable R genes (Bus et al. 2011). The only major R gene that has been incorporated into commercial apple cultivars is the *Rvi6* (formerly *Vf*) gene. Rvi6 was first used in the PRI breeding programme in the 1940s after recognising that material crossed in the 1910s-1920s showed resistance to scab. These original crosses were between the cultivar Rome Beauty and *M. floribunda* 821 and further crosses of the offspring (Reviewed by Gessler & Pertot 2011). Although the *Rvi6* resistance was durable for around 50 years (Crosby et al. 1992), breakdown was first seen in Europe in the last decade of the 20th Century (Parisi et al. 1993; Roberts & Crute 1994). Breakdown in Europe has now been reported in many countries, but predominantly in organic orchards; no confirmed breakdown has yet been reported in North American commercial orchards (Gessler & Pertot 2011). An international monitoring scheme known as VINQUEST (http://www.vinquest.ch/index.html) has been implemented to assess any global strains of *V. inaequalis* able to overcome the R genes of 15 differential hosts and assess the temporal breakdown after emergence. The aim of the project is for a better understanding of durability of these R genes and their sustainability if implemented into apple breeding programmes (Patocchi et al. 2009).

Although only a few cultivars with the *Rvi6* gene are grown commercially (e.g. Topaz is a relatively popular cultivar in Germany) as named varieties, the vast majority of apple cultivars grown commercially do not have the *Rvi6* resistance gene, hence are susceptible to scab. However there is a range of susceptibility to apple scab across domesticated apple cultivars with seemingly susceptible cultivars containing potential resistance factors (Sierotzki et al. 1994; Koch et al. 2000; Barbara et al. 2008; Papp et al. 2016). Partial resistance to scab in cultivars, not conditioned by a (known) R gene, might come from multiple loci (including "defeated" R genes) that have a quantitative effect. A number of quantitative trait loci (QTLs) have been identified in a cross between the cv. Discovery, assigned polygenic resistance, and the susceptible cv. Fiesta (Liebhard et al. 2003), as well as

in a cross between Discovery and apple hybrid "TN10-8" (Calenge et al. 2004). Bastiaanse et al. (2016) suggested that this partial resistance from QTLs is more durable than that of the qualitative resistance of an R gene and although Caffier et al. (2014) suggested resistance from QTL could be subject to erosion, the same group showed that this erosion could be slow (Caffier et al. 2016).

The breakdown of R-gene resistance, as seen with *Rvi6* breakdown in a number of orchards around the world, shows that the use of a single R gene to confer resistance of a host to a pathogen is likely to lead towards selection of pathogenicity and so a lack of durability of the resistance. Pyramiding resistance genes to *V. inaequalis,* i.e. incorporating a number of resistance genes within a single cultivar, has been suggested as a way of breeding durable scab resistance with cultivars already containing *Rvi6* being used as a first source for resistance (Gessler & Pertot 2011). However, starting with a gene that has already been overcome in many areas may well be a mistake and looking at alternative resistance would be prudent. Perhaps the *Rvi6* gene should be used until full breakdown while in the meantime pyramiding of numerous resistance genes can be developed. It is important that the resistance genes used in pyramiding have good molecular markers, to allow marker assisted breeding (Patocchi et al. 2009). Laloi et al. (2017) showed pyramiding of three QTLs for resistance increased efficiency of resistance compared to any of the independent QTL. It is important to note that the three QTLs in the Laloi et al. study affected the development of the pathogen at different stages and it is likely that any pyramiding will benefit from resistance targeted at different stages of pathogen development. Should one of the resistance factors (i.e. QTL in this case) fail, due to the pathogen gaining the necessary virulence to overcome that resistance at that stage of pathogen development, or there are favourable conditions for the pathogen to overcome resistance in that instance (e.g. if the plant is under stress), one of the other resistance factors is likely to still be able to halt the development of the lesion. This is in much the same way as the use of pesticides with different modes of action is advised for use throughout the season.

Understanding how the QTL work is therefore important and so too is the breadth of the scab inoculum spectrum they have resistance to. Lê Van et al. (2013) used the same progeny from the cross between Discovery and "TN10-8" used by Calenge et al. (2004) to identify QTL. They monitored *V. inaequalis* isolates on diseased apple leaves of the progeny using quantitative pyrosequencing technology and QTL mapping to investigate selection pressures

on the *V. inaequalis* population exerted by these QTL. The seven QTL identified can be divided into groups of those specific to only one or two isolates (four QTL), two QTL with moderate specificity (QTL identified on four or five isolates out of the six) and one with broad spectrum specificity. From their findings they suggest differing strategies for the use of broad-spectrum and narrow-spectrum resistance factors. Pyramiding broad spectrum resistance factors is a possible strategy for the minimisation of resistance erosion; the erosion of a single broad-spectrum resistance factor in a genotype could allow a rapid epidemic of the resistant race, whereas pyramiding these resistance factors would require an isolate to emerge with all the necessary virulence factors to overcome the differing resistance factors. However, they suggest that mixing apple genotypes with narrow-spectrum resistance factors is a better strategy for this type of resistance factor.

1.4 Cultivar mixtures

1.4.1 What are mixtures?

Agriculture has become reliant on the use of monoculture growing systems over the last 200 years (Wolfe 1985). While this is driven by a range of factors the major drawback of this homogeneity is the potentially rapid increase in pest and disease. Having a single genotype in the growing system means that there is high selection pressure exerted, with the breakdown of any host resistance allowing a rapid increase in a race with increased fitness. Mixing species is not practicable so one way to increase genetic diversity is to mix cultivars with differing characteristics, such as differing resistance to a pathogen; although crucially this cannot be a random mixture as disease reductions will not be obtained with all mixtures (Mundt 2002).

Reduction of disease levels has been shown in a range of crops, predominantly in foliar diseases of cereals, for example rust diseases (caused by *Puccinia spp.*) of wheat (Cox et al. 2004; Finckh & Mundt 1992) and powdery mildew (*Blumeria graminis f. sp. hordei*) of Barley (Finckh et al. 1999; Tratwal & Bocianowski 2018). Reductions due to cultivar mixtures have also been seen in late blight of potato (*Phytophthora infestans*) (Pilet et al. 2006) and blast of rice (*Magnaporthe grisea*) (Zhu et al. 2005), as well as reductions of rusts in perennials such as coffee rust (caused by *Hemileia vastatrix*) (Reviewed by Mundt 2002) and willow coppice rust (caused by *Melampsora epitea)* (McCracken & Dawson 1998).

The spread of the pathogen population is reduced by three mechanisms (Wolfe 1985). The first is the dilution factor; increasing the distance between susceptible plants and reducing the amount of susceptible material limits the maximum extent to which the pathogen can increase. The second factor is the barrier effect; by placing resistant plants in place of susceptible they act as a physical barrier against spore dispersal. The third factor is resistance induced by non-pathogenic spores; avirulent spores landing on the host will trigger host defences which may limit the effectiveness of infection by a virulent spore in the same area. Other potential mechanisms include architectural differences in cultivars, causing altered environment in the canopy, and compensation between cultivars with different micro-niches in a varied area (Mundt 2002). Each of these mechanisms on their own in a single generation of the pathogen is unlikely to have a significant effect. However, the multiplicative effect of the the mechanisms over a number of generations leads to the reduction of the disease (Wolfe 1985).

Garrett & Mundt (1999) suggest there are five characteristics of a host-pathogen system that indicate the potential success of a mixture on disease reduction:

1) The Genotype Unit Area (GUA): this might be a single plant, a row or block of genotypically identical material. The larger the GUA the higher the chance of autoinfection and therefore the smaller the likely disease reduction (Mundt 2002).

2) The steepness of the dispersal gradient: how spores are dispersed will affect the chance of autoinfection, for example, wind dispersed spores are less likely to land on the genotype they originated from than splash dispersed spores.

3) Lesion size: mixtures are likely to be less effective on pathogens with expanding lesions as a higher number of smaller lesions are required for similar disease progress in pathogens with non-expanding lesions.

4) Pathogen generation time: the shorter the pathogen generation time, the larger the host-diversity effects.

5) Host specialisation: the greater the specialisation of populations on the differing hosts the more likely the mix will reduce disease levels. The relative importance of these five characteristics is hard to calculate due to the huge variety in a single host-pathogen system let alone when comparing between different host-pathogen systems (Garrett & Mundt 1999).

1.4.2 Mixed cultivar apple orchards

Apple trees do not immediately appear to be strong candidates for mixing to reduce pathogen attack. Plants larger than small grain cereals will have a much larger GUA; success in mixing cultivars for disease control in these crops has varied (Mundt 2002). Orchard design will also have a large effect on GUA with within-row mixtures (i.e. alternating trees of different cultivars) having a smaller GUA than row-by-row mixtures (i.e. alternating rows of differing cultivars, but the row is monoculture). The wind dispersal of ascospores is a favourable characteristic in support of mixture benefit, but less favourable is splash dispersal of conidia. However, because cultivar resistance to apple scab varies because of both gene-for-gene and differential quantitative resistance, the potential for scab control by mixing has been investigated.

The potential for mixed cultivar orchards to reduce scab development in apple was first assessed by simulation (Blaise & Gessler, 1994) and then tested in a field trial (Bousset et al. 1997). The field trial mixed susceptible and R-gene carrying resistant cultivars and provided evidence that the number of scab lesions per shoot was lower on Golden Delicious and Elstar in mixtures than when these cultivars were in monoculture. Didelot et al. (2007) calculated the area under the disease progress curve (AUDPC) on leaves in monoculture, within-row and row-by-row mixed plots in two years. The AUDPC of scab leaf incidence, compared with the mean of monoculture plots, was reduced in within row mixture and row-by-row mixture by similar amounts (ca. 8% and 22% in the more severe year and in the less severe year respectively). The AUDPC of scab leaf severity, compared with the mean of monoculture plots, was reduced more in the within row mixture (19% and 35% in the more severe year and in the less severe year respectively) than in the row-by-row mixture (16% and 15% in the more severe year and in the less severe year respectively). The reduction of scab is greater where the rate of disease progress is already low. Parisi et al. (2013), in 2008, found 9% of scabbed fruits at harvest in a mixed orchard compared with a mean of 15% in pure stands, a 40% reduction; the following year also showed a significant reduction in incidence on scabbed fruit in the mixed orchard (76% compared with 82% in the pure stands). The scabbed fruit incidence in 2009 was reduced further (70% scabbed fruit) when combined with sanitation methods (dead leaves removed with lawn sweeper and buried within the row). However there was no significant reduction in the severity of scab on fruits, or the

incidence and severity of scab on shoots, between monoculture and mixtures, with significant reduction only seen when mixtures were combined with sanitation practices. Although often significant, the reductions seen in these mixed cultivar orchard trials, especially in high incidence years, are not sufficient for commercial practice. However, they could be of benefit if implemented as part of an IPM regime alongside other strategies for reducing scab incidence, such as sanitation and BCAs, backed up by the use of chemical control in high risk periods, thus reducing the use of chemicals while maintaining adequate disease control. It is possible that in cider production, where the stringency of fruit quality is not as strict as with dessert apples, the plausibility of mixed cultivars is higher.

Parisi et al. (2013) used a within-row mix, after Didelot et al. (2007) showed a within-row mixture is the most effective mixture type, with a smaller GUA than row-by-row mixes; both studies used mixtures of an *Rvi6* cultivar and susceptible cultivar. But, in commercial orchards, with current management methods, an intimate mix of this kind is not economically feasible. The management costs of a mixed cultivar orchard are likely to be higher than that of monoculture due to differences between the timing of bud-break, flowering and fruit development between cultivars (Table 2), leading to complications in pest and disease control, crop husbandry (e.g. irrigation, fertilisation) and harvesting. Dessert cultivars are sold as named varieties and therefore fruit needs to be separated into individual cultivars on harvesting. As a result of increased management costs, the benefit of reduced scab in a mixture must both offset the increased management cost and be long lasting relative to the life of commercial apple orchards (ca. 20 years).

Where *Rvi6* has been overcome in an orchard also containing non-*Rvi6* cultivars, the scab population has been seen to split, showing strong and stable differentiation between the two populations in the same orchards over several years (Gladieux et al., 2011). A major concern in the viability of orchards with mixed susceptible cultivars is that a 'super race' of scab, which has virulence factors which overcome most or all of the resistance genes in the host cultivars present in the mixture, might emerge and become dominant in the orchard, rendering the mixture ineffective as a means of managing scab. A fungal super race is most likely to result from recombination of the necessary virulence factors during sexual reproduction although it is possible a super race might occur through mutations. Xu's (2012) simulation study suggested that a super race is not likely to occur within a life time (ca. 20 years) of a perennial tree plantation of cultivar mixtures where population crashes occur

(mimicking overwintering). The bottleneck caused by overwintering led to a single fungal genotype dominating a population, with cost of virulence determining whether this genotype can infect both cultivars. However, due to genetic drift, the dominant genotype is unlikely to accumulate all the necessary virulence alleles to infect both cultivars. It has been shown, through artificial crossing in the laboratory, that it is possible for ascospore progenies to be produced that combine virulence factors to overcome varying resistance (i.e. a "super race") (Barbara et al. 2008).

Table 2. Typical flowering dates and picking times for a selection of apple cultivars grown at the National Fruit Collection, Brogdale, Kent, UK

Cultivar	10% flowering	Full (80%) flowering	90% petal fall	Picking time
Beauty of Bath	02-May	08-May	16-May	Early August
Bramley's Seedling	06-May	12-May	18-May	Early October
Cox's Orange Pippin	09-May	13-May	20-May	Late September
Golden Delicious	07-May	11-May	19-May	Late October
Red Falstaff	25-Apr	28-Apr	07-May	Early October
Royal Gala	02-May	05-May	11-May	Early October
Worcester Pearmain	02-May	04-May	13-May	Early September

Data taken from NFC fruit (undated) unpublished characterisation by staff at NFC, Brogdale http://www.nationalfruitcollection.org.uk/

1.5 Aims of Project

It has been shown that mixed cultivar orchards can reduce the levels of apple scab compared to monoculture; however a number of questions require investigation before mixed cultivars can be implemented into commercial practice. Will a super race of the pathogen emerge and dominate an orchard rendering the benefits of the mix obsolete? If not, why? On current understanding of reproduction of the pathogen it should be feasible for a super race to emerge. If mixed orchards remain viable then which cultivars have the largest difference in scab populations? The larger the difference, the greater the potential range of resistance composition in that candidate cultivar mix.

This thesis is presented as a collection of published papers and a submitted manuscript covering the following topics:

1. **"Differentiation in populations of the apple scab fungus *Venturia inaequalis* on cultivars in a mixed orchard remain over time" (Passey et al. 2016a)**
   This work explores if differences between populations of *V. inaequalis* on different cultivars in the same orchard change over time. They did not and the results suggest that a super race of the pathogen is unlikely to emerge and dominate in the lifetime of an orchard.

2. **"The relative importance of conidia and ascospores as primary inoculum of *Venturia inaequalis* in a Southeast England orchard" (Passey et al. 2017)**
   A super race might not emerge and dominate an orchard if the effective recombination rate is lower and clonal mixing less than if ascospores dominate primary inoculum. On the other hand, once a super race has established in the orchard, increasing asexual spores as primary inoculum may accelerate the subsequent expansion of the super race. The importance of conidia relative to ascospores in primary inoculum was tested by indirect means due to the impracticalities of trapping primary conidia spores. Overwintered, asexual conidia formed a high proportion of pathogen primary inoculum in spring.

3. **"Population difference of the apple scab fungus *Venturia inaequalis* on cultivars within a mixed cultivar orchard" (Passey et al. 2016b)**
   If the emergence of a super race of *V. inaequalis* is not likely then mixed cultivar orchards could be a sustainable option as part of a multi-faceted approach to the control of apple scab. However, it is imperative that the cultivars present have differing resistance to scab for the mixture to have the desired effect. In this work we compared the populations of *V. inaequalis* on different popular cultivars within an orchard as an indirect assessment of cultivar differences in scab resistance. Populations from many of the cultivars did differ from those populations on other cultivars indicating that they would make a good mix for reduction of scab. This was especially the case for populations from cider cultivars.

4. **"Annotated draft genome sequence of the apple scab pathogen *Venturia inaequalis*" (Passey et al. 2018)**

   At the start of this piece of work only a de novo assembly of the *V. inaequalis* transcriptome was available. In order to compare sequences from a number of isolates from different cultivars, we needed a good whole genome sequence assembly. Although during the process of this work a WGS was made available, the *V. inaequalis* genome we present is more complete.

5. **"Genomic sequencing indicates non-random mating of *Venturia inaequalis* in a mixed cultivar orchard" (To be submitted 2019)**

   The assembled genome (paper 4) was used as a reference to align isolates from the mixed cultivar orchard used for the population temporal change work in paper 1. Genotype specific allele loci (conditioned on all SNPs) are clustered within the genome indicating non-random mating among isolates in an orchard. It is theorised that this non-random mating is most likely due to a much higher probability of successful mating between isolates on the same leaf than between isolates from different leaves.

The final chapter of the thesis discusses how the findings in this body of work are relevant to the current knowledge and future apple orchard practices regarding apple scab control in the short, medium and long term.

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Differentiation in populations of the apple scab fungus *Venturia inaequalis* on cultivars in a mixed orchard remain over time

T. A. J. Passey[ab]*, M. W. Shaw[b] and X.-M. Xu[a]

[a]*East Malling Research, New Road, East Malling, ME19 6BJ; and* [b]*School of Agriculture, Policy and Development, University of Reading, Reading, RG6 6AR, UK*

The ascomycete *Venturia inaequalis* causes annual epidemics of apple scab worldwide. Scab development is reduced in mixed cultivar orchards compared with monocultures. In order to use mixtures in commercial production, how the population of scab changes in a mixed orchard needs to be understood, together with how likely a super race, with virulence factors overcoming multiple resistance factors in the mixed orchard, is to emerge and become dominant. This study used simple sequence repeat (SSR) markers to investigate the temporal change of scab populations in two mixed cultivar orchards in the UK to infer the likelihood of emergence of a scab super race. There were no significant differences between the populations at the two sampling times (6 or 7 years apart) in either of the two mixed orchards. In one of the orchards, apple scab populations on different cultivars were significantly different and the differences did not diminish over time. These results suggest that it is not inevitable that a super race of *V. inaequalis* will become dominant during the lifetime of a commercial apple orchard.

Keywords: apple black spot, microsatellites, population genetics, SSR, super race, *Venturia inaequalis*

Introduction

Apple scab, caused by *Venturia inaequalis*, is one of the most important diseases on apple. Emerging and spreading from central Asia (Gladieux *et al.*, 2008), the centre of origin for the domesticated apple *Malus* × *domestica* (Harris *et al.*, 2002), it is found across apple growing regions worldwide. Annual epidemics can lead to large losses of marketable fruit and severe attack may lead to young fruit dropping and to defoliation, which can cause a decline in yield in subsequent seasons (MacHardy, 1996).

The pathogen survives the winter primarily as pseudothecia in the leaf litter. Rainfall in spring, around the time of bud break, causes release of sexually produced ascospores from the leaf litter, establishing primary infections on the new season's growth. It is also possible for the pathogen to overwinter as conidia in dormant buds (Becker *et al.*, 1992; Holb *et al.*, 2004). Primary infection by ascospores or overwintered conidia leads to the production of conidia in the new lesions that form the basis of reiterative secondary infection cycles (Bowen *et al.*, 2011).

Current control can include a number of non-pesticide methods, such as accelerating decomposition of leaf litter by urea spray (Carisse & Dewdney, 2002). The predominant control method is frequent fungicide application aided by forecasting systems (Berrie & Xu, 2003). However, due to fungal resistance to pesticides, costs incurred by their heavy use, consumer pressure on reducing fungicide use and ever-decreasing number of available fungicides due to regulations, alternative measures are being sought for scab management.

An effective scab management strategy is to breed cultivars with durable resistance to the pathogen. The only major *R* gene that has been incorporated into commercial apple cultivars is the *Rvi6* (*Vf*) gene from *M. floribunda*, but this gene has been overcome in several regions (Parisi *et al.*, 1993; Roberts & Crute, 1994), raising the question about the longevity of *Rvi6*. Where *Rvi6* has been overcome in an orchard also containing non-*Rvi6* cultivars, the scab population has been seen to split (Gladieux *et al.*, 2011).

Research in identifying and using major *R* genes against apple scab has focused on genes from wild *Malus* species rather than domesticated apple, except for *Rvi1* from Golden Delicious (Bus *et al.*, 2011). However, seemingly susceptible cultivars may also contain resistance (Sierotzki *et al.*, 1994; Koch *et al.*, 2000; Barbara *et al.*, 2008), so that scab isolates from one cultivar may infect another susceptible cultivar weakly or not at all. One method to potentially achieve durable resistance is to combine resistance genes into a single genotype, known as gene pyramiding (Gessler *et al.*, 2006). However, this process is slow and it may take a long time before new scab resistant cultivars can be released commercially.

An alternative use for the difference in resistance factors between cultivars, including those regarded as

*E-mail: tom.passey@emr.ac.uk

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susceptible, is to plant these cultivars in the same orchards. Mixing cultivars of a crop with varying resistance has been shown, predominantly in arable crops, to reduce disease development compared to monoculture (Wolfe, 1985; Mundt, 2002). The potential for mixed cultivar orchards to reduce scab development has been demonstrated by simulation (Blaise & Gessler, 1994) and supported by field trials. Didelot *et al.* (2007) calculated the area under the disease progress curve (AUDPC) on leaves in monoculture and row-by-row mixed plots in 2 years. The AUDPC of scab leaf incidence, compared with the mean of monoculture plots, was reduced in the mixture by 8.9% in the more severe year and 22.5% in the less severe year. Parisi *et al.* (2013) found 9% of scabbed fruits at harvest in a mixed orchard compared with a mean of 15% in pure stands in 2008, with the following year also showing a reduced incidence in the mixed orchard (76% compared with 82% in the pure stands). This study used a within-row mix, as did Didelot *et al.* (2007). This is the most effective mixture type, but in commercial orchards with current management methods, an intimate mix of this kind is not economically feasible.

The management costs of a mixed cultivar orchard are likely to be higher than that of monoculture due to differences between the timing of bud-breaking, flowering and fruit development between cultivars, leading to complications in pest and disease control and harvesting. As a result, the benefit of reduced scab must both offset the increased management cost and be long lasting relative to the life of commercial apple orchards (*c.* 20 years). A major concern in the use of mixed cultivars is that a 'super race' of scab, which has combined virulence factors to overcome the differing resistance genes in the host cultivars, may emerge and become dominant in the orchard within a short period of time, rendering the mixture ineffective as a means of managing scab. A fungal super race may result from a single mutation or series of mutations, but is more likely to result from recombination of the necessary virulence factors during sexual reproduction.

In this study, simple sequence repeat (SSR) markers were used to genotype scab isolates from different cultivars in two mixed orchards at two different time points. The aim was to investigate the extent of differentiation between the scab populations on the different cultivars within an orchard over time. Should the populations on different cultivars become more alike over time, it would suggest that a super race might have become dominant in the orchard. Otherwise, as suggested by simulation study (Xu, 2012), it may be inferred that a super race has not become dominant.

Materials and methods

Sampling

Two mixed orchards in the UK, namely Ash Farm, Worcestershire and WM132 at East Malling Research, Kent, were sampled. The Ash Farm orchard has a mix of *Malus* × *domestica* 'Bramley's Seedling' (Bramley), 'Cox's Orange Pippin' (Cox) and 'Worcester Pearmain' (Worcester) on non-dwarfing rootstocks; each cultivar has two rows with no cultivar being in consecutive rows. This orchard has never been sprayed or recently pruned and is *c.* 45–50 years old. WM132 has a block of three rows of Cox next to a block of three rows of cultivar Royal Gala on M9 rootstocks. This orchard was not sprayed with fungicides, but pruned annually, and is *c.* 15 years old.

Ash Farm and WM132 were sampled in 2005 and 2006, respectively (Xu *et al.*, 2013). Freeze-dried mycelia from single-spore isolates were stored at −20°C and used in the present study. Both sites were resampled in the spring of 2012 when lesions had become visible from primary infection. At Ash Farm, leaves with freshly sporulating, discrete lesions were collected from shoots of each of 10 trees per cultivar and placed into paper bags until isolation. At WM132, a total of 15 shoots were collected from each of six trees per cultivar (two trees per row) and all leaves with discrete sporulating lesions were collected. For all leaves collected in 2012, a single discrete lesion per leaf was cut out with a 5 mm cork borer, placed in a 2 mL microtube, left to air dry at room temperature and then closed and transferred to a −20°C freezer. Only a single lesion from any one shoot was used to extract DNA.

DNA extraction and screening

DNA was extracted from approximately 50–100 mg of freeze-dried mycelia for the 2005/6 samples, while the samples collected in 2012 had DNA extracted directly from the lesion on the leaf disc (Table 1). The samples from 2005/6 had previously been used in other experiments where single-spore isolates were required for inoculation tests. For the 2012 samples, it was quicker and cheaper to extract DNA directly from the lesion on a leaf disc than to produce single-spore isolates *in vitro*, which was done previously (Xu *et al.*, 2008). As discrete lesions were selected early in the season it is probable that the lesion will have resulted from infection by a single spore. Should the lesion have had multiple origins it would be detectable as described below.

The freeze-dried mycelia or infected leaf disc were placed in a 2 mL microtube with two 4 mm ball bearings and disrupted in an MM2 oscillating mill (Retsch). DNA was then extracted using a DNeasy Plant Mini kit (QIAGEN) following the manufacturer's instructions with all optional steps. DNA was eluted with 100 μL elution buffer into a 1·5 mL microtube. DNA was quantified and quality-checked using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and stored at −20°C.

The targets of a number of published SSR primer pairs (Tenzer *et al.*, 1999; Guérin *et al.*, 2004; Xu *et al.*, 2009) were tested for polymorphism against a range of scab isolates. Following this primary screening, eight SSR markers were selected to genotype the populations (Table 2). SSRs, labelled on the forward primer with either 6-FAM or HEX fluorescent dyes

Table 1 Number of scab lesions sampled from each apple cultivar in each of two mixed cultivar orchards in 2 years

	Ash Farm			W132	
Year	Bramley	Cox	Worcester	Cox	Gala
2005/6	36	27	31	20	23
2012	35	35	35	36	36

Table 2 Sequences for SSR primer pairs used to genotype apple scab isolates

SSR	Fluorescent label-forward primer (5′–3′)	Reverse primer (5′–3′)	Allele size range (bp)
EMVi029[a]	HEX-ACGAGTCCCAGGTCTCACAG	TGTTGACGGTCACGGTGTAT	164–248
Vica9/X[b]	FAM-TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGAAG	219–243
Vica10/154[b]	HEX-CCTCCTTCCTATTACTCTCG	CTGAAGCGAACCTATGTCC	104–168
Vicacg8/42[b]	FAM-TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	198–234
Vict1/130[b]	FAM-GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	148–156
Vitc1/82[b]	HEX-ACTGTCTCTAGGCGAAAG	ACTTGGAAGCTCGCTAAG	223–241
Vitc2/16[b]	FAM-ACATTGACGAAGACGAGC	TACAATTGAGGCGTGTCC	153–169
Vitg9/129[b]	FAM-CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	277–291

[a]Xu *et al.* (2009).
[b]Guérin *et al.* (2004).

(Integrated DNA Technologies), were split into two multiplexes of four primer pairs. PCR was performed using 6·25 μL Type-it microsatellite PCR master mix, 3·5 μL water (both QIAGEN), 1·25 μL 2 μM SSR primer mix, and 2 μL DNA. Due to the high concentration of the DNA extracted from mycelium (2005/6 samples) the DNA was diluted 1/10 before PCR, whereas the DNA extracted from leaf discs was added undiluted as the concentration was lower, and a proportion of the DNA extracted will have been from the apple leaf. Touchdown PCR was performed on a DYAD thermal cycler (MJ Research) using the following cycle: an initial 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 90 s (decreasing 0·5°C per cycle until 50°C) and 72°C for 60 s; then a final extension at 60°C for 30 min. One microlitre of each PCR product (diluted 1/10 for 2005/6 samples, undiluted for 2012 samples) was run on an ABI 3130xl sequencer with GeneScan 500 LIZ size standard (Life Technologies). Alleles were then scored using GENESCAN and GENOTYPER software conforming to the stepwise mutation model, i.e. to ensure allele sizes fit into a stepwise model (however, it should be noted that in practice nearly all alleles varied by integral multiples of the repeat length). PCR was repeated on any samples with no product for an SSR marker, alongside a positive control (s), so as to score a null allele, rather than a failed PCR, for that primer pair.

Statistical analysis

Allele frequencies for an orchard were calculated using POWERMARKER software (Liu & Muse, 2005). Rare alleles, ≤0·01 of the population of an orchard (in the present study an allele that only appears once in the orchard), were recorded as missing values. These were removed from the data set as they contribute very little information towards assessing genetic diversity and population structure (Hale *et al.*, 2012). Null alleles were included as a single allele at a locus. Null alleles occur when a mutation in the flanking region of the sequence repeat stops the annealing of the primer and therefore no amplification during PCR, or when the SSR region is deleted, resulting in a very short fragment not scored. In the present study, null alleles were treated as a single allele for that marker, but they may in fact include different sequences. Therefore, statistical analyses were also conducted with all null alleles excluded. If there were two alleles at a locus it was assumed that the lesion had resulted from infection by more than one spore. If a sample only had one locus with two alleles, one was randomly selected for inclusion in statistical analysis. If a sample had multiple loci with more than one allele then the sample was discarded.

To assess if the scab populations in the two orchards had changed between the two temporal sampling points, population differentiation was assessed by a two-hierarchical level AMOVA (analysis of molecular variance; Excoffier *et al.*, 1992) using the POPPR package in R (Kamvar *et al.*, 2014). AMOVA was carried out separately for each orchard, first with the 'years/cultivars' hierarchical structure, i.e. cultivars are nested within each year (2005 vs 2012 for Ash Farm, 2006 vs 2012 for WM132) and then the 'cultivars/years' structure. Significance of population differentiation was assessed with a permutation test (a total of 999 permutations). In addition, the significance of the interaction between years and cultivars was assessed by a permutation test as for the main year or cultivar effect. For a given data set (observed or permuted) the sum of squares (SS) due to the interactions was calculated as the differences between among-cultivar-within-year SS and among-cultivar SS. The removal of very rare alleles would have given a large amount of missing data for some loci and therefore all data were used for AMOVA.

ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010) was used to compute pairwise F_{ST} between populations on each cultivar at each time point based on 110 permutations. Under the null hypothesis of random mating among isolates from all cultivars, all pairwise F_{ST} values would be expected to be similar. An unweighted pair group method with arithmetic mean (UPGMA) tree was produced to present F_{ST} data using the software MEGA (Tamura *et al.*, 2013).

STRUCTURE v. 2.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003) uses a Bayesian approach to determine the number of clusters (K) present in a set of individuals. Under the hypothesis of random mating, there should be one population (i.e. $K = 1$); if there is sufficient population differentiation, K is expected to be greater than one. To estimate the number of clusters in the Ash Farm or WM132 orchards, an admixture model with correlated allele frequencies was run 10 times, with a burn-in period of 10 000 followed by 50 000 Markov chain Monte Carlo iterations for $K = 1$ to 10. Inference of the 'true' number of populations (K) was based on the second order rate of change of the likelihood ΔK (Evanno *et al.*, 2005) and the test rerun with 20 runs of 500 000 iterations after an initial burn-in of 50 000 iterations on a reduced range of K values.

Multilocus linkage disequilibrium (LD) was calculated for fungal populations from individual orchards and from individual cultivars in a given year. When in linkage equilibrium, the genotype frequency is equal to that of the product of the allele frequencies (Liu & Muse, 2005). POWERMARKER was used for testing LD with a permutation test (1000 permutations) to infer whether random mating took place among specific groups of isolates.

Results

General

All statistical analyses were run with or without null alleles, with very little difference in the results. Therefore, only results with null alleles included are presented. Of the 199 samples from Ash Farm, 196 gave useable data. Three Cox samples from 2012 were excluded as they had multiple loci with more than one allele. In addition there were seven samples where it was not possible to determine the size of the SSR band at one of the loci; these were scored as missing values. One hundred out of 115 samples from the WM132 orchard were included in statistical analysis. Seven of the 2012 samples and two of the 2006 samples were discarded as they had multiple loci with more than one allele. A further six samples failed to amplify during PCR. There were two samples for which one SSR locus was not reliably scored; these were marked as missing values. Summary allele data for both orchards are given in Table 3.

Two of the SSR markers used in this study, vitg9/129 and EMVi029, mapped at the same locus in the linkage map of Xu *et al.* (2009). The alleles were in strong LD at Ash Farm ($P < 0{\cdot}0001$) but not at WM132 ($P = 0{\cdot}7$). Both markers were used in the subsequent analysis.

Differences between populations

Two-hierarchal level AMOVA of the Ash Farm data showed no significant differences between the scab populations sampled in 2005 and 2012 ($P = 0{\cdot}5$), but the populations from the three cultivars were clearly different ($P \leq 0{\cdot}001$). There was no evidence for cultivar differences changing between 2005 and 2012, i.e. no significant interactions between years and cultivars ($P = 0{\cdot}2$; Table 4). F_{ST} pairwise comparison of populations on different cultivars showed Bramley in 2005 was distinct from those of both Cox and Worcester ($P < 0{\cdot}001$), and the differences between the populations on Cox and Worcester were also close to statistical significance ($P = 0{\cdot}04$). In 2012 the populations on the three cultivars remained different ($P < 0{\cdot}01$); while the populations of Bramley and Cox were more alike than in 2005, the scab population on Worcester was more different from those on the other two cultivars (Fig. 1). The inferred number of populations for Ash Farm, using the Evanno *et al.* (2005) method, is $K = 2$. If K is increased above two, these clusters are subdivided but remain as homogeneous groupings without creating clearly distinct clusters, supporting the inference of $K = 2$. The scab samples from Bramley were distinctly different to those of Worcester, whereas samples from Cox appeared to be an admixture between the two (Fig. 2).

In WM132, AMOVA showed no evidence for significant differences between the samples from 2006 and 2012 ($P = 0{\cdot}4$) or for differences between cultivars ($P = 0{\cdot}1$). There was weak evidence for interaction between years and cultivars ($P = 0{\cdot}06$; Table 4). F_{ST} pairwise comparison suggested population differentiation between Cox and Gala ($P = 0{\cdot}03$) in 2006 but not in 2012 ($P = 0{\cdot}5$; Fig. 1). It was not possible to obtain a consistent peak in the (very low) ΔK values to determine the number of clusters present in the WM132 orchard.

The scab population as a whole on Bramley in Ash Farm was more like the populations in WM132, some 200 km away, than it was like the scab population on Worcester in the same orchard (Fig. 3).

Assessment of random mating

The Ash Farm orchard population was in LD ($P < 0{\cdot}001$) in both years. There was evidence for LD in the scab population on Bramley in 2005 ($P < 0{\cdot}001$) but not in 2012 ($P = 0{\cdot}2$). LD in the population on Worcester was significant in both years ($P < 0{\cdot}05$) and appeared to have increased between 2005 and 2012. LD in the population on Cox at Ash Farm was clear ($P < 0{\cdot}002$) and did not change much with time (Table 5).

The WM132 population was in linkage equilibrium in both years, indicating random mating in the orchard; there was no evidence for LD in the populations from individual cultivars at either time point (Table 5).

Discussion

The present results suggest that the scab populations in two mixed orchards have not changed in ways that indicate wider host adaptation by the pathogen over a period of 6–7 years. The differences between scab populations on different cultivars within one of the mixed orchards showed no sign of decreasing. This suggests that scab in a mixed orchard may remain adapted to individual cultivars and a super race of scab becoming dominant in an orchard, with row alternation rather than the commercially impractical within-row mixing, is not inevitable, substantiating the simulation study by Xu (2012).

The Ash Farm orchard is the same as that used to collect samples for *in vivo* inoculation virulence testing (Barbara *et al.*, 2008); unfortunately these samples were not available for use in this study. The 2005 samples used in this study are the same as those used for molecular population studies using AFLP and SSR markers (Xu *et al.*, 2013). The results presented here from the 2012 resampling support the earlier findings with molecular markers that showed scab populations on different cultivars, namely Bramley, Cox and Worcester, were significantly different (Xu *et al.*, 2013). Furthermore, isolates from one cultivar could not necessarily infect leaves of the other cultivars in virulence tests, confirming distinctness (Barbara *et al.*, 2008). Both previous studies showed that the scab populations on Bramley and Worcester are the most different, and present findings concur. Barbara *et al.* (2008) suggest that there are at least one, two and three resistance factors in Bramley, Cox and Worcester, respectively. Therefore it could be conjectured that Bramley and Worcester do not share any of these resistance factors while Cox could share a differing resistance

Table 3 Summary for eight SSR markers used to genotype apple scab populations from different cultivars in two mixed orchards

		EMVi029		Vica9/X		Vica10/154		Vicacg8/42		Vict1/130		Vitc1/82		Vitc2/16		Vitg9/129	
SSR	N^a	Range[b]	n_a[c]	Range	n_a	Range	n_a	Range	n_a	Range	n_a	Range	n_a	Range	n_a	Range	n_a
Ash Farm																	
Bramley 2005	36	176–178–210	11	229–231–235	4	104–122 136–160	13	200–206–218	5	148–150–152	3	223-231-235	6	153–153–169	3	277–277 279–285	4
Bramley 2012	35	172–178–228	10	229–231–243	5	104–134–164	14	200–206–234	5	148–150–156	4	229–231–239	5	153–153–167	2	277–279–291	4
Cox 2005	27	178–192–196	4	229–229–231	2	104–108–148	7	206–206–212	2	150	1	229–231–231	3	153	1	277–279–291	4
Cox 2012	32	176–178–200	7	229–231–235	3	106–122–166	12	200–206–218	4	148–150–154	3	223–231–241	7	153–153–167	2	277–277–281	3
Worcester 2005	31	178–192–196	4	229–231–235	3	120–120–148	6	200–206–212	3	150	1	229–231–233	4	153	1	277–277–279	2
Worcester 2012	35	178–192–200	4	229–229–231	2	120–120–136	5	206–206–212	2	150	1	231–231–233	3	153	1	277–277–279	2
All	196	172–192–228	16(4)	229–231–243	5(1)	104–120–166	21(6)	200–206–234	6(0)	148–150–156	5(2)	223–231–241	9(3)	153–153–169	3(0)	277–277–291	5(0)
WM132																	
Cox 2006	20	172–178–202	7	229–231–235	4	104–118–156	11	200–206–218	4	148–150–152	3	231–231–239	5	153–153–167	2	277–277–285	5
Cox 2012	31	176–178–202	7	229–231–243	5	106–118–150	16	200–206–218	5	148–150–154	3	229–231–237	5	153	1	277–277–285	4
Gala 2006	21	164–176 178–248	10	219–231–231	3	104–134–168	13	198–206–212	3	148–150–154	4	229–233–239	4	153–153–169	5	277–277–283	4
Gala 2012	28	172–178–216	7	219–231–243	4	104–118–156	14	200–206–222	4	148–150–154	4	229–231–241	5	153–153–167	3	277–277–281	3
All	100	164–178–248	13(3)	219–231–243	6(0)	104–118–168	26(9)	198–206–222	7(2)	148–150–154	4(0)	229–231–241	8(3)	153–153–169	5(1)	277–277–285	5(0)

[a]Number of scab isolates genotyped.
[b]Range (in bp): smallest allele size – mode allele size (two numbers indicates two alleles present in equal numbers) – largest allele size.
[c]Number of distinct alleles; number in parentheses = the rare alleles removed from orchard data set.

Table 4 Two-level hierarchical analysis of molecular variance (AMOVA) of apple scab populations in different years and on different cultivars in two mixed cultivar orchards

Orchard	Term	d.f.	Sum of squares	*P*-value[a]
Ash Farm	Years (2005 vs 2012)	1	3·7	0·47
	Between cultivars	2	25·5	≤0·001
	Year × Cultivar	2	9·0	0·21
WM132	Years (2006 vs 2012)	1	2·3	0·40
	Between cultivars	1	2·8	0·14
	Year × Cultivar	1	3·3	0·06

[a]Based on 999 permutations.

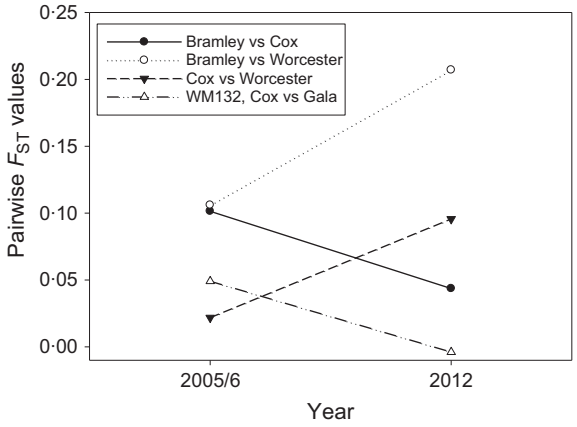

Figure 1 Pairwise differences (F_{ST}) between apple scab populations on different cultivars within the same orchard (Bramley, Cox, Worcester in Ash Farm; Cox and Gala in WM132) in 2005(Ash Farm)/2006 (WM132) and 2012.

factor with each of these two cultivars. Although there has been much research into resistance mediated by known *R* genes, only a few studies (Liebhard *et al.*, 2003; Calenge *et al.*, 2004) have investigated quantitative resistance in cultivars not carrying a known major *R* gene, so there is limited knowledge of hidden resistance factors present in susceptible cultivars. There was no significant difference in the scab population of 2005 and that of 2012 at Ash Farm. Although the gap in sampling (7 years) is short, relative to the life of commercial orchards, it should be noted that this orchard is about 40–45 years old and has not been subjected to any control measures. Although the scab populations on Bramley and Cox appeared to become more alike between the two sampling points, they were still significantly different. The differences in scab populations between Worcester and the other two cultivars appear to have increased, suggesting that the scab population, especially on Worcester, is probably becoming increasingly adapted to specific cultivars. Although it is also possible that other evolutionary forces, such as migration, are having an effect, adaptation is the simplest explanation. It may therefore be inferred from these results that a super race has not prevailed in the life of the Ash Farm orchard, which is around twice that of a commercial orchard.

The scab populations in WM132 did not differ between the two sampling years. The STRUCTURE analysis failed to give a consistent result on the number of clusters, suggesting that there is just one population present ($K = 1$). Although the populations on the two cultivars were different in 2006, the multilocus LD test indicates that the scab population in the orchard was already in linkage equilibrium. This is most probably explained by the fact that Gala is susceptible to almost all known scab isolates regardless of the host it was isolated from (Bus *et al.*, 2011), i.e. 'universally' susceptible, despite carrying two QTL for resistance (Soufflet-Freslon *et al.*, 2008). Thus isolates infecting Cox can infect Gala as well and therefore recombination among these isolates can take place. It is also possible that the initial scab founders of the orchard were randomly drawn from a randomly mating population and the orchard population has not yet adapted to the cultivars present. The difference between the cultivars in 2006 was not strongly significant and can be explained by the possibility that a considerable number of isolates sampled from Gala in 2006 may not be able to infect Cox.

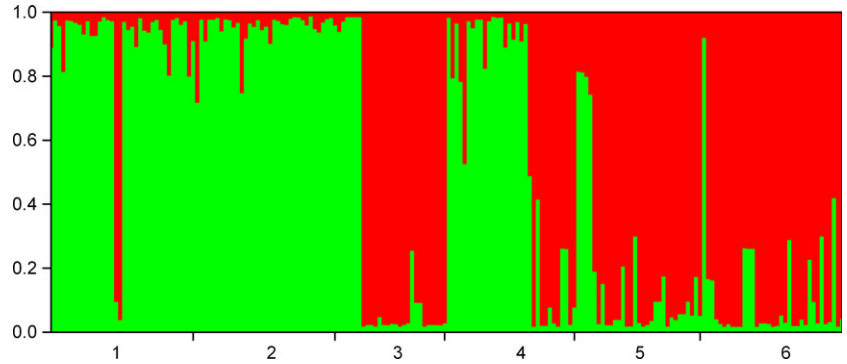

Figure 2 STRUCTURE bar plot assuming two populations ($K = 2$) in Ash Farm orchard sampled in 2005 and 2012, in which individual isolates were plotted as a vertical bar representing the probability of being from one or the other population (shown in different colours). Population (*x*-axis): 1 = Bramley 2005, 2 = Bramley 2012, 3 = Cox 2005, 4 = Cox 2012, 5 = Worcester 2005, 6 = Worcester 2012.

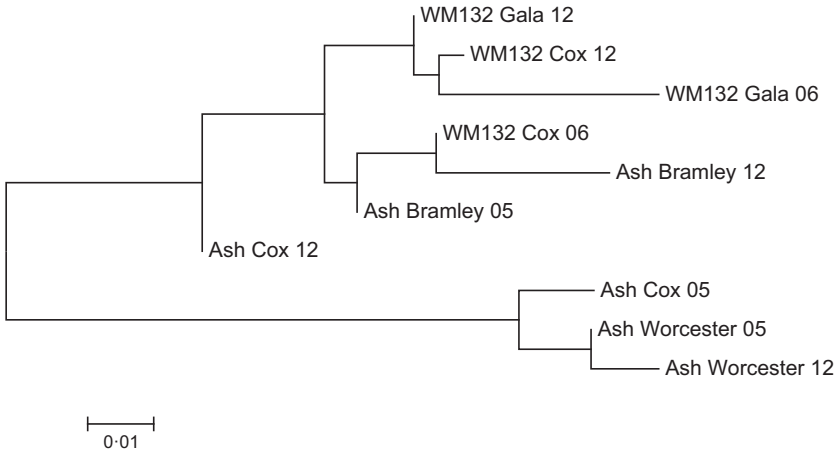

Figure 3 An UPGMA tree grouping scab populations based on similarity at eight SSR markers scored on samples from cultivars in two apple orchards *c.* 200 km apart (Ash Farm sampled in 2005 and 2012; WM132 sampled in 2006 and 2012).

Table 5 Multilocus linkage disequilibrium test run on two orchard populations of apple scab, and scab population on individual cultivars in two different years. Null hypothesis is the population is in linkage equilibrium

Orchard	Cultivar(s)	Year	*P*-value[a]	Loci
Ash Farm	Whole orchard	2005	<0·01	8
		2012	<0·01	8
	Bramley	2005	<0·01	8
		2012	0·21	8
	Cox	2005	<0·01	6[b]
		2012	<0·01	8
	Worcester	2005	0·04	6[b]
		2012	<0·01	6[b]
WM132	Whole orchard	2006	1·00	8
		2012	0·17	8
	Cox	2006	1·00	7[c]
		2012	1·00	7[c]
	Gala	2006	1·00	8
		2012	0·11	8

[a]Tests run on 1000 permutations.
[b]Full 8 loci could not be run due to lack of polymorphism in Vict1/130 and Vitc2/16.
[c]Full 8 loci could not be run due to lack of polymorphism in Vitc2/16.

It may take a long time for a super race to form in an orchard, depending on the nature of mutations required and sexual reproduction. If several mutational steps are required, formation of the genotype will be expected to be vanishingly slow (Hedrick, 2011). In this case, appearance of a super race requires recombination between strains possessing different sets of virulence factors. Only in the leaf litter does the annual sexual reproduction occur. It is not known whether mating only occurs between lesions on the same leaf or whether mating can occur between lesions on different leaves but physically in contact. If the former is true, then a super race could only develop either by multiple mutations or by an opportunistic infection by non-adapted isolates in conditions where the effectiveness of resistance was reduced; both routes to recombination are likely to be rare. If the mating between strains on distinct leaves in the litter is possible, the chance that two infected leaves from different cultivars have sufficient physical contact to mate would still be less than mating between lesions on the same leaf. Whether or not the two exceptional processes occur, mating in the scab fungus favours recombination among strains infecting the same host.

Another important factor determining how likely a super race is to emerge and spread in an orchard is the proportion of primary inoculum resulting from conidia overwintered in buds and as wood scab. Conidia do not survive on leaves and fruits that have fallen to the orchard floor in the autumn (MacHardy, 1996). However, they have been shown to survive the winter, predominantly on the inside tissues of buds and wood pustules (Becker *et al.*, 1992; Holb *et al.*, 2004). The survival of conidia and their impact in the following season as part of primary infection is dependent on factors such as weather, orchard management and the previous year's incidence (Holb *et al.*, 2005). The ratio of sexual to asexual spores as a source of primary inoculum also depends on the amount of leaf litter in the orchard. The higher the proportion of primary infection from asexual conidial spores, the higher will be the proportion of primary inoculum that is genetically identical to spores from the previous year. As a super race is most likely to develop from sexual reproduction in the leaf litter, if the relative importance of the primary inoculum from ascospores is less than currently expected, a super race is expected to be less likely to occur. However, this also means that should a super race develop, significant primary inoculum from overwintered conidia would accelerate the race towards dominance in the orchard. The relative importance of overwintered conidia and ascospores as the dominant source of primary inoculum is likely to be region specific. In areas with warm winters, primary inoculum from conidia is most important (Boehm *et al.*, 2003). The advantage of implementing mixtures is less if conidia are the predominant source of primary inoculum, as conidia are distributed by water splash and therefore are mainly likely to infect the same

row and therefore the same cultivar (assuming row-by-row mixing).

Should a fungal genotype be present with necessary virulence to infect multiple cultivars in the orchard it still does not mean it will inevitably become dominant. An increase in virulence may come with a cost in fitness, as demonstrated in other pathogens (Bahri *et al.*, 2009; Montarry *et al.*, 2010). If the cost is sufficient, a super race may never dominate, or it may increase only slowly; if the emergence were longer than 20–30 years, it would still be commercially feasible to reduce scab by using mixed orchards.

This study demonstrated that differentiation between *V. inaequalis* populations on different cultivars did not decrease over time in mixed orchards, indicating that a super race, if present, has not become common. This agrees with inoculation studies of isolates from the mixed orchard and other monoculture orchards (Barbara *et al.*, 2008). Therefore, it may be concluded that mixed apple orchards could be a feasible component of an integrated management scheme. Although the reductions of 10–30% in scab are modest, it is likely that mixed cultivar orchards are beneficial in managing other pests and diseases too (Parisi *et al.*, 2013). Implementation is particularly suited for cider and juicing apples, because cosmetic damage is unimportant and disease management need not be as stringent as for dessert apples.

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The relative importance of conidia and ascospores as primary inoculum of *Venturia inaequalis* in a southeast England orchard

T. A. J. Passey[ab]*, J. D. Robinson[a], M. W. Shaw[b] and X.-M. Xu[a]

[a]*NIAB EMR, New Road, East Malling, ME19 6BJ; and* [b]*School of Agriculture, Policy and Development, University of Reading, Reading, RG6 6AR, UK*

Apple scab, caused by *Venturia inaequalis*, can lead to large losses of marketable fruit if left uncontrolled. The disease appears in orchards during spring as lesions on leaves. These primary lesions are caused by spores released at bud burst from overwintering sources; these spores can be sexually produced ascospores from the leaf litter or asexual conidia from mycelium in wood scab or within buds. The relative importance of conidia and ascospores as primary inoculum were investigated in an orchard in southeast England, UK. Potted trees not previously exposed to apple scab were placed next to (*c.* 1 m) orchard trees to trap air-dispersed ascospores. Number and position of scab lesions were assessed on the leaves of shoots from both the potted trees (infection by airborne ascospores) and neighbouring orchard trees (infection by both ascospores and splash-dispersed, overwintered conidia). The distribution and population similarity of scab lesions were compared in the two tree types by molecular analysis and through modelling of scab incidence and count data. Molecular analysis was inconclusive. Statistical modelling of results suggested that conidia may have contributed approximately 20–50% of the primary inoculum in early spring within this orchard: incidence was estimated to be reduced by 20% on potted trees, and lesion number by 50%. These results indicate that, although conidia are still a minority contributor to primary inoculum, their contribution in this orchard is sufficient to require current management to be reviewed. This might also be true of other orchards with a similar climate.

Keywords: apple scab, asexual overwintering, *Fusicladium dendriticum*, *Spilocaea pomi*

Introduction

Annual epidemics of apple scab, caused by the ascomycete *Venturia inaequalis* (anamorph *Spilocaea pomi*, *Fusicladium dendriticum*), lead to large losses of marketable fruit worldwide if uncontrolled. In the *V. inaequalis* life cycle, overwintered spores are released in the spring to infect newly emerged leaves. Lesions from these infections produce conidia that are dispersed by water splash, leading to secondary infections, which in turn continue the secondary inoculum cycle throughout the growing season (MacHardy, 1996). There are two possible sources of overwintered inoculum, one sexual and the other asexual. Sexually produced ascospores, released during spring rainfall from leaf litter and wind dispersed (most likely within the orchard of origin (MacHardy, 1994)), have traditionally been believed to be the most important primary inoculum of *V. inaequalis*. As a result, the majority of research into apple scab control has focused on reducing leaf litter in orchards (Sutton *et al.*, 2000; Vincent *et al.*, 2004; Gomez *et al.*, 2007) and inoculum forecasting, based on ascospore development and release, to aid the application of chemical control (Gadoury & MacHardy, 1986; Beresford & Manktelow, 1994; Berrie & Xu, 2003).

Venturia inaequalis can also overwinter asexually, potentially as stromata on twigs (Cook, 1974; Hill, 1975), or more probably as viable inoculum (most probably conidia) between bud scales (Becker *et al.*, 1992). As with conidia from lesions in the main epidemic phase, it is probable that conidia from overwintered sources will infect within an area close to the source of overwintered scab. This may be by conidia being washed on to leaves nearby, or by their germination to form a lesion on or around the bud, producing conidia that are then released and dispersed by water splash. In contrast, airborne ascospores will be turbulently dispersed or advected over longer distances.

Studies (Holb *et al.*, 2004, 2005; Gao *et al.*, 2009) suggest that conidial sources may be a significant part of the primary inoculum. This is important because reduction of overwintering inoculum and early season control measures differ for the two sources of inoculum. Furthermore, the evolution of virulence to resistant cultivars or resistance to fungicides in pathogen populations may depend on the relative ratios between sexual and asexual reproduction. If conidia contribute to primary inoculum it means that a proportion of the lesions present in an orchard are not recombinant products of meiosis and

*E-mail: tom.passey@emr.ac.uk

therefore a certain proportion of the primary inoculum have identical genotypes to the previous year. As a result, the population as a whole will evolve at a different rate than the population of an orchard where ascospores are the sole primary inoculum. The extent to which the rate differs will depend on the genetic architecture of the trait under study. In addition, if a race of *V. inaequalis* with superior fitness (e.g. virulence towards a resistant cultivar or resistance to a fungicide) caused by several weakly linked polymorphic loci develops in an orchard, it is likely to become dominant in the orchard faster, as more of the primary inoculum in successive seasons will be of the favoured genotype.

The aim of this study was to investigate the relative importance of conidia and ascospores as sources of primary inoculum in an orchard in southeast England. It is possible to trap ascospores from the air, but it is difficult to trap overwintered conidia reliably in splash water in the early season. Instead, an indirect method was developed, placing potted trees in an orchard with a history of scab. Scab on potted trees not previously exposed to scab should result from ascospores only, because they are airborne and travel longer distances than conidia; however, scab on orchard trees may result from both ascospores and overwintered conidia. Therefore, scab incidence and clustering on the two types of recipient tree were compared in order to infer the relative importance of the two sources of primary infection. In addition, the genetic structures of the *V. inaequalis* populations from potted and orchard trees were compared using simple sequence repeat (SSR) markers to determine clonality of the populations and the linkage disequilibrium (LD).

Materials and methods

Sampling and lesion assessments

Orchard WM132 at East Malling Research (Kent, UK) has three consecutive rows of *Malus* × *domestica* 'Cox's Orange Pippin' (Cox) next to three consecutive rows of *Malus* × *domestica* 'Royal Gala' (Gala) on M9 rootstocks (rows 4 m apart); each row (running north to south) has 12 trees planted 1.75 m apart. This orchard is not sprayed with fungicides, but is pruned, and is *c.* 15 years old. At bud burst in 2012, 2013 and 2014, six potted trees of each of Cox and Gala on M9 rootstocks (*c.* 10–12 years old) in 10 L pots were placed within the rows of orchard trees of the same cultivar at two positions randomly chosen in each row (these positions remained the same for all 3 years); subsequent observations were carried out between paired samples, a potted tree with a partner orchard tree. Potted trees had been kept in a polytunnel, except for the experimental exposure period, to prevent surface wetness and so prevent *V. inaequalis* infection (hence removing the possibility of overwintering conidia from previous years). The distance between the potted tree and the nearest orchard tree was *c.* 1 m; potted trees were secured to the west of the post of an orchard tree but the trees were arranged and pruned so that no branches of an orchard tree touched or were directly above a branch of the corresponding potted tree. Trees of both types were around 180–200 cm tall, with the lowest shoots about 80 cm above ground level. Potted trees were watered (*c.* 500 mL) three times a week, directly onto the compost in the pot. The potted trees were returned to a polytunnel after sufficient infection events (3–5 weeks, depending on the weather), but before the first generation of conidia (i.e. visible lesions resulting from infection by primary inoculum) was produced, to ensure that infection on the potted trees all resulted from primary sources. The number of potential infection periods were 12, 3 and 3 for 2012, 2013 and 2014, respectively. Two weeks later, up to 15 shoots (flower trusses) were randomly sampled from each potted tree and the nearest orchard tree (all available shoots were sampled when less than 15 were available). The number of scab lesions was counted on both sides of every leaf and the position (counting from the base of the shoot noting the absolute position of every leaf to the newest unfurled leaf) of infected leaves on the shoot noted. On the few occasions when the scab was so severe that discrete lesions could not be defined, an estimate of the percentage of leaf covered in scab was made and this was converted to an estimated number of lesions (assuming a single lesion corresponds to 1% scabbed area, based on empirical experiences). From each infected leaf, the most clearly separated scab lesion was selected and cut out with a 5 mm cork-borer, placed in a 2 mL microtube, left to air dry at room temperature and then transferred to a −20 °C freezer until DNA extraction.

DNA extraction and screening

DNA was extracted from six lesions (where possible) per tree, no more than one lesion from any one shoot. As lesions were relatively sparse, few lesions will have resulted from infection by more than one spore; the rate at which this occurred was estimable from the genotype data. Therefore, DNA was extracted directly from the lesion on the leaf disc. Two 4 mm ball bearings were added to the leaf disc in the microtube and disrupted in an MM2 oscillating mill (Retsch). DNA was then extracted using a DNeasy Plant Mini kit (QIAGEN) following the manufacturer's instructions with all optional steps. DNA was quantified and quality-checked using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and stored at −20 °C.

The SSR primers used (Table 1), PCR and thermal cycle conditions, as well as the procedure for genotyping were all carried out as set out in Passey *et al.* (2016). PCR was repeated on any samples with no product for an SSR marker, alongside a positive control(s), so as to score a null allele, rather than a failed PCR, for that primer pair.

Statistical analysis

Molecular data

Allele frequencies were estimated using PowerMarker software (Liu & Muse, 2005). Analysis was run with and without rare alleles (frequency ≤0.01; i.e. an allele appearing only once in the orchard in any given year) as very rare alleles have little effect on genetic diversity (Hale *et al.*, 2012). If two alleles were present at a locus it was assumed that the lesion had resulted from infection by more than one spore. If a sample had only one locus with two alleles, one was randomly selected. If a sample had multiple loci with more than one allele, the sample was discarded.

Differentiation between populations on the potted trees and the orchard trees was assessed by amova (analysis of molecular variance) in Arlequin v. 3.5 (Excoffier & Lischer, 2010). An amova hypothesis test, based on 1023 permutations, was carried out for 'among tree type populations (orchard versus potted)';

Table 1 Sequences for simple sequence repeat (SSR) marker primer pairs used to genotype apple scab isolates

SSR	Fluorescent label-forward primer (5′–3′)	Reverse primer (5′–3′)	Allele range (bp)
EMVi029[a]	HEX-ACGAGTCCCAGGTCTCACAG	TGTTGACGGTCACGGTGTAT	170–252
Vica9/X[b]	FAM-TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGAAG	219–247
Vica10/154[b]	HEX-CCTCCTTCCTATTACTCTCG	CTGAAGCGAACCTATGTCC	100–168
Vicacg8/42[b]	FAM-TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	200–240
Vict1/130[b]	FAM-GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	148–164
Vitc1/82[b]	HEX-ACTGTCTCTAGGCGAAAG	ACTTGGAAGCTCGCTAAG	227–243
Vitc2/16[b]	FAM-ACATTGACGAAGACGAGC	TACAATTGAGGCGTGTCC	153–169
Vitg9/129[b]	FAM-CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	277–291

[a]Xu *et al.* (2009).
[b]Guérin *et al.* (2004).

whole populations were used for analysis, as the number of isolates per tree was too small to compare individual tree pairs.

Multilocus linkage disequilibrium (LD) was estimated for scab populations on each tree type for each cultivar to determine whether associations between alleles were compatible with sexual reproduction. LD was calculated by a permutation test (1000 permutations) with PowerMarker software. The null hypothesis of the test was that scab from a particular group was in linkage equilibrium, i.e. that the genotype frequency was equal to the product of the allele frequencies (Liu & Muse, 2005).

Infection data

Cultivar or year were not compared explicitly in the analysis because the purpose of the present investigation was to study the overall difference in scab development between the potted and orchard trees to infer the importance of conidia as primary inoculum. Year, cultivar and location in the orchard were included in the analysis to compare scab development between the potted and orchard trees, but they were represented by a single factor of tree pairs: six locations (pairs of trees) within each of the Cox and Gala sections within each year (giving 36 levels for the factor 'tree pairs'). Therefore the effects of the three factors (years, position in the orchard and cultivars) and their interactions were already accounted for by the 'tree pairs' factor.

Lesion distribution

Lesions were expected to be more aggregated within an individual leaf on orchard trees than potted trees because of additional conidia in the orchard trees. However, it was not possible to have a direct statistical test to compare the lesion distribution between the two types of trees. Aggregation was assessed by fitting the distribution of lesion counts on leaves to a Poisson or negative binomial distribution (separately for potted and orchard trees). Generalized linear modelling (GLM) was used to make the fits. In the GLM analysis, tree pairs were treated as a blocking factor. Errors were assumed to follow either a Poisson or a negative binomial distribution. The best fitting distribution was used in subsequent work.

Incidence and lesion density

The density of lesions was expected to be higher on leaves of orchard trees than on potted trees because of additional overwintered conidia in the orchard trees. Mean lesion counts per leaf were tested using a hurdle model to determine whether they were significantly greater for the orchard than for the potted trees. A limitation of standard count models is that the zeros and the non-zeros (positives) are assumed to come from the same data-generating process; often this type of model cannot account for an excess of zero counts in the data. To overcome this shortcoming, two types of models have been proposed: hurdle models and zero-inflated models (Cameron & Trivedi, 1998, 2005). For hurdle models, a Bernoulli probability governs the binary outcome of whether a count variate has a zero or positive realization, similar to the common logistic modelling in GLM. If the realization is positive (i.e. the hurdle is crossed), positive count data are assumed to be governed by a truncated-at-zero count data model (e.g. Poisson or negative binomial model). On the other hand, zero-inflated models assume that the response variable is a mixture of a Bernoulli distribution and a discrete data-generating process (e.g. Poisson) distribution. Therefore, zero counts can result from a discrete data generating process as well as a Bernoulli process for the zero-inflated models, but only from a Bernoulli process for hurdle models.

The hurdle models were chosen because they enable easy interpretation of differences between potted and orchard trees in the incidence of scabbed leaves and in average lesion counts per scabbed leaf. The incidence of scabbed leaves was modelled as a binomial process and lesion density per scabbed leaf as a negative binomial process. When fitting hurdle models, the origin of leaves (potted or orchard trees) was used as a factor in both parts of the hurdle model: incidence (logistic model) and density (truncated positive counts model). GLM was carried out using the MASS package (Venables & Ripley, 2002) and hurdle models using the PSCL package (Zeileis *et al.*, 2008) in R v. 3.2.

Aggregation of scabbed leaves

The variance in the number of infected leaves on a shoot would be expected to be greater in orchard trees due to additional conidial infection localized on particular shoots. For each tree, there were 12–15 shoots. The variances between trees could not be directly compared for two reasons. First, shoots had an unequal number of leaves. Secondly, the variance of the distribution depended on the mean by the nature of binomial distribution. Therefore, a permutation test, conditioned on the total number of scabbed leaves in a tree, was used to compare the number of infected leaves in each shoot with that expected under the assumption of a random distribution of infected leaves. For each tree, the following analysis was first conducted: (i) the total number of scabbed leaves was found; (ii) for trees with more than one infected leaf, the same number of infected leaves was randomly assigned to the shoots (taking into account the number of leaves on each shoot), (iii) the variance among shoots on each tree in the number of scabbed leaves on a shoot

Table 2 Number of leaf discs with scab lesions screened for simple sequence repeat (SSR) markers to compare populations from potted and orchard apple trees (cultivars Cox and Gala)

	2012		2013		2014	
Type	Cox	Gala	Cox	Gala	Cox	Gala
Potted	36	36	25	35	35	35
Orchard	31	29	34	31	36	33

was calculated, (iv) steps one to three were repeated 999 times, (v) the variance of the observed data was calculated (1000 variance values for each tree: 999 variances for simulated datasets and one for the observed), (vi) the rank of the observed variance in the 1000 values was calculated (if there were ties in ranking, the average rank was used; rank was calculated in descending order, i.e. the largest value had a rank of 1), and (vii) the ratio of the observed variance to the mean of the 999 permutated values was calculated. Thus, for each tree, the analysis resulted in two values: the rank (frequency with which the observed variance would be seen if the pattern were random), and the relative size of the observed variance compared to the mean of a random pattern. Then, ANOVA was applied to assess whether the rank (ln-transformed) or the ratio of variances differed significantly between potted and orchard trees. For the same reasons as outlined above, only tree pairs and the type of tree were included as factors in ANOVA of permutated data. Permutation and ANOVA were implemented in R v. 3.2.

Results

Molecular data

In total, 396 sampled leaf discs were screened over the 3 years (2012–14, Table 2): 202 and 194 samples from potted and orchard trees, respectively. Some populations had fewer than 36 samples analysed due to: a lack of scab (two potted Cox trees in 2013), samples failing to amplify, or removal of samples from analysis because they had multiple alleles at more than one locus. A change of capillary in the ABI 3130xl, after the 2012 samples were analysed, led to a + 2 bp shift in markers Vica9/X, Vitc1/82 and Vitg9/129. This was ascertained by running a subset of the 2012 samples and cross-checking against their original allele sizes; an appropriate correction was made to the data. Tests were run with and without rare alleles (frequency ≤0.01) of the orchard population in a given year; however, there was no difference in results. Null alleles occur when a mutation in the flanking region of the sequence repeat stops the annealing of the primer and therefore stops amplification during PCR. Statistical tests were run twice, including the null as an extra allele for that marker or excluding the isolate. There were no differences that affected inferences.

AMOVA showed no evidence of difference between the orchard trees and the potted trees in any of the 3 years ($P > 0.3$).

In 2012 and 2014, all of the multilocus LD tests showed that the populations were in linkage equilibrium, indicating random mating (Table 3). In 2013, the *V. inaequalis* populations on Gala potted trees were in linkage equilibrium but the scab populations on the Cox potted and orchard trees and the Gala orchard trees were in linkage disequilibrium (Table 3).

Table 3 Significance results of test for linkage disequilibrium of *Venturia inaequalis* populations of potted and orchard trees in different cultivars in an orchard in southeast England

Cultivar	Population	2012	2013	2014
Cox	Orchard	1.00	0.01**	1.00
	Potted	1.00	<0.001***	1.00
Gala	Orchard	1.00	0.01**	1.00
	Potted	1.00	1.00	1.00

Multilocus linkage disequilibrium was calculated by a permutation test (1000 permutations) using PowerMarker. Null hypothesis of random mating rejected at $**P = 0.01$, or $***P < 0.001$.

Infection data

Lesion distribution

A Poisson distribution fitted the count data from potted trees reasonably well (average residual deviance 1.58) but not for the data from orchard trees (average residual deviance 3.24). The lack of fit of a Poisson distribution can be seen in Figure 1, particularly for Gala. Both sets of lesion data were well described by a negative binomial distribution: average residual deviances were 0.327 and 0.343 for the potted and orchard trees, respectively.

Incidence and lesion density

More scab was observed on orchard trees than on potted trees in 2012; however, in 2014, slightly more scab was seen on potted trees than on orchard trees (Table 4). In 2013, there were only slight differences in the overall scab incidence and density between potted and orchard trees (Table 4). Average number of lesions on the scabbed leaves was 4.61 (± 0.224).

Generalized linear modelling analysis, using hurdle models, showed that the incidence of leaves with scab was significantly ($P < 0.001$) greater on the orchard trees than on the potted trees. For the binomial part of the hurdle model, the parameter estimate for potted trees was 0.206 (± 0.063) less than that of orchard trees; that is, the odds ratio of being scabbed for potted trees was *c.* 80% of corresponding orchard trees. The negative binomial part of the fitted hurdle model indicated that average lesion counts on infected leaves were greater ($P < 0.001$) on the orchard trees than on the potted trees. Potted trees had an intercept 0.701 (± 0.140) less than that of orchard trees; that is, the average lesion number on potted trees was about 50% of that on the corresponding orchard trees.

Aggregation of scabbed leaves

The variance in the number of infected leaves on a shoot (expressed as ratio of the observed to the mean of the permuted values) and the rank in a list of random

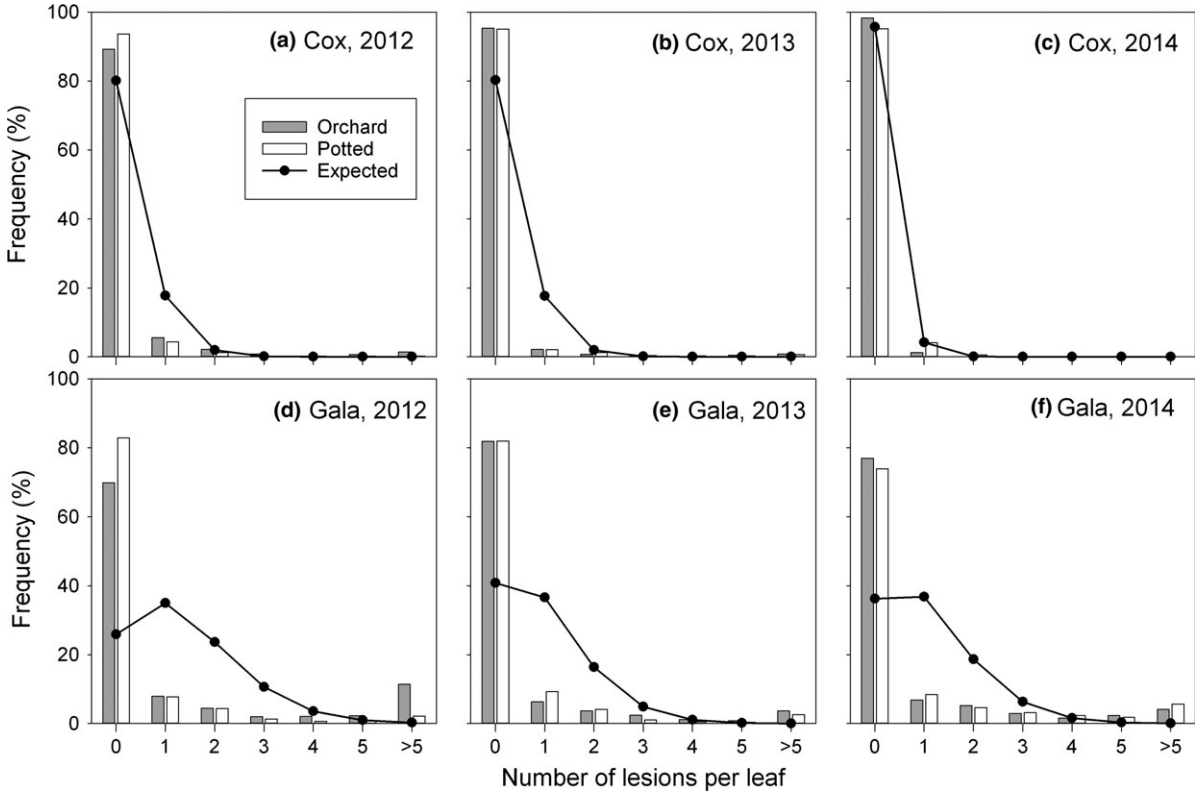

Figure 1 Distribution, in 3 years, of apple scab lesions on individual leaves collected from potted trees (non-shaded bar) and corresponding orchard trees (shaded bar) of cultivars Cox and Gala. Both types of tree were exposed to the same conditions at the same location. In addition the expected frequency, assuming a Poisson (random) distribution for number of lesions on individual leaves, is shown (line). Observed data has a higher frequency than expected for leaves with no lesions and more than four lesions per leaf indicating aggregation of lesions within a single leaf.

Table 4 Incidence of leaves with scab and average number of lesions per leaf across all leaves on orchard and potted trees of cultivars Cox and Gala in an orchard in southeast England

	2012		2013		2014	
Type	Cox	Gala	Cox	Gala	Cox	Gala
Number of leaves assessed						
Potted	1201	1105	738	687	1051	602
Orchard	917	850	830	951	797	686
Incidence of leaves with scab						
Potted	0.063	0.171	0.049	0.180	0.049	0.261
Orchard	0.108	0.301	0.047	0.181	0.017	0.230
Average number of lesions per leaf						
Potted	0.118	0.536	0.172	0.646	0.059	1.228
Orchard	0.358	2.414	0.263	1.077	0.025	0.828

permutations of the observations both differed greatly between potted and orchard trees (Fig. 2). For both variance ratio and log-transform rank variables, residual plots did not suggest any apparent violations of ANOVA assumptions. For potted trees, the ratio of the observed variance in the number of infected leaves on a shoot within each tree to the mean of the permuted values was 0.98, close to the expected value of 1.0. For the orchard trees, this ratio was much greater, at 1.63 ($F_{1,35} = 27.2$, $P < 0.001$), than for the potted trees. The rank of the observed variance in a permuted dataset (Fig. 2b) was much greater in orchard trees (208) than in potted trees (533) ($F_{1,35} = 25.1$, $P < 0.001$); the average rank of variance of the permuted datasets was necessarily 500 (note: the lower the rank value the greater the variance).

Discussion

Previous molecular comparisons of isolates from different cultivars within the same orchard indicated that conidia might overwinter in bud and/or wood scab and act, in addition to ascospores, as a source of primary inoculum (Xu *et al.*, 2013). Several other studies have also suggested overwintered conidia are a source of primary

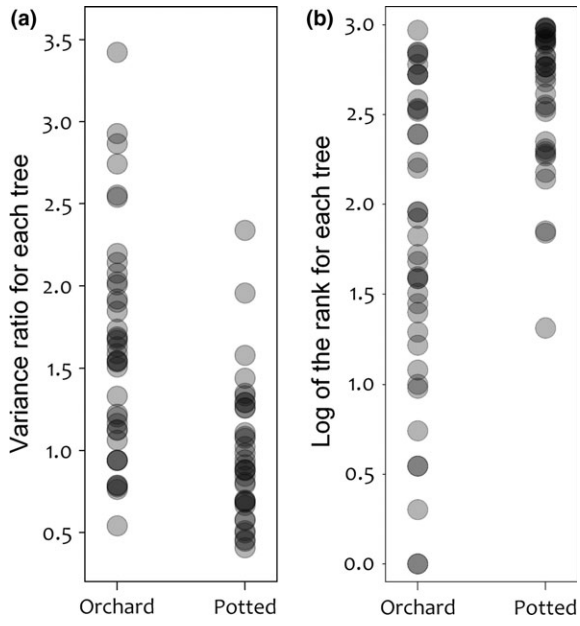

Figure 2 (a) Plot of the ratio between the observed variance in the number of scabbed apple leaves in each shoot within each tree, with the average variance of 999 permutations, assuming random distribution of infected leaves. (b) The log of the rank of the observed variance among the 1000 variance values (999 permutated and one observed; in descending order, i.e. the largest has the rank of 1). Depth of grey indicates overlaying of observations. The rank of observed variance was significantly different ($P < 0.001$) between orchard and potted trees in this southeast England orchard.

inoculum (Becker *et al.*, 1992; Holb *et al.*, 2004, 2005; Gao *et al.*, 2009). In this study, it was shown that scab lesions on orchard trees were more aggregated on their leaves and shoots than on adjacent potted trees not previously exposed to scab (i.e. not exposed to overwintered conidia). Both scab incidence and count data suggest that conidial primary inoculum may have contributed approximately 20–50% of the primary infection in early spring: incidence was estimated to be reduced by 20% on potted trees, and lesion number by 50%, averaged over the 3 years of the study. This interpretation is assuming that infection efficiencies by conidia and ascospores on orchard and potted trees are the same and that both potted and orchard trees are equally susceptible to infection. Infection efficiency in the spring temperatures that the orchard experienced is considered to be similar for ascospores and conidia (reviewed by MacHardy, 1996). However, the greater susceptibility expected of 'softer' tissue in potted plants, as well as the possible lack of resistance priming and induced resistance from phylloplane organisms in these plants, would both result in more scab on the potted trees. Hence, this would lead to an underestimation of the importance of conidia as primary inoculum if equal-susceptibility is assumed (i.e. there would be more lesions on potted trees than on the orchard trees for a given dose of inoculum). The initial infection process should have been completed when the potted trees were returned to the polytunnel; subsequent temperature should not have affected the number of lesions, because sufficient time was allowed for all infections to become visible, predicted on the basis of the relationship of incubation time to temperature (MacHardy, 1996).

The scab populations on potted and orchard trees were in linkage equilibrium in both 2012 and 2014. This fits the hypotheses of either predominantly ascospore primary inoculum or no deviation from linkage equilibrium within the conidial primary inoculum (presumably due to no detectable selective changes in the population the previous year). In 2013, the population of *V. inaequalis* on potted trees of Gala was in linkage equilibrium, but the orchard trees were in linkage disequilibrium. This would be expected if conidia were an important part of the primary inoculum, as the scab on the potted trees would be from sexually produced ascospores and therefore from independent sampling, whereas the scab on the orchard trees would be from both (freely recombinant) ascospores and clonal conidia. However, in the same year, the populations of *V. inaequalis* on both potted and orchard trees of Cox were in linkage disequilibrium, the potted trees more significantly than the orchard trees. This suggests that unexplained factors influenced the estimates of linkage disequilibrium, so no secure inferences can be drawn.

Although wood scab in heavily infected orchards is commonly observed, it is believed that very few of these wood scab lesions produce viable conidia in spring, indicating that asexually overwintering scab is most likely to result from overwintering in buds (Becker *et al.*, 1992). Although the present study was conducted in an unsprayed orchard (WM132), scab was not very severe and there was no evidence of wood scab present. Furthermore, commercial pruning was applied to the orchard, so heavily infected shoots were likely to have been removed. Thus, conidia that overwintered in the buds are probably the main source of overwintered conidial inoculum in the spring.

It can be concluded that ascospores are the main source of primary inoculum (*c.* 80% in this specific orchard) in the spring for temperate growing regions such as southeast England. Therefore, the current management practice of eliminating leaf debris in late autumn (MacHardy, 1996) needs to be retained. However, conidia as primary inoculum cannot be ignored. The relative importance of conidia and ascospores as primary inoculum is likely to vary between orchards and years. In this study, the differences between years, cultivar or position within an orchard have not been compared, as the aim was to assess the overall importance of conidia as primary inoculum. There are many other factors that could affect the relative proportion of conidia as primary inoculum, including pruning, leaf degradation, in-season control efficacy, cultivar and epidemic severity. Most of the studies suggesting the importance of conidia as part of primary inoculum have been in areas with wet and mild winters such as the UK (present study; Cook, 1974; Hill, 1975), the Netherlands (Holb *et al.*, 2004, 2005)

and west Norway (Stensvand *et al.*, 1996). Conditions in these regions are likely to be more conducive to faster decomposition of leaf material, reduction of ascospore levels, and survival of conidia or mycelia in buds, than regions with colder winters. Warmer growing regions, where the necessary winter chill for development of pseudothecia does not occur, only have clonal lineages of the apple scab pathogen (Boehm *et al.*, 2003).

Reducing the amount of inoculum in early season is paramount to good scab control. Currently, the main focus of forecast programmes, designed to aid effective application of chemical control in spring, is ascospore release. However, even with perfect elimination of leaf debris, scab control in the early season is still essential as, based on the present study, overwintered conidia are likely to be a source of primary inoculum. Consideration of release of overwintered conidia should be incorporated into spray guidance programmes. Furthermore, it may also be useful to spray when buds are forming. A similar strategy is currently being evaluated for reducing overwintering of powdery mildew (*Podosphaera leucotricha*) in apple buds at East Malling, while dormant season sprays for powdery mildew have shown some success (Frick & Burchill, 1972; Hislop & Clifford, 1976).

In summary, it has been shown that conidia play an important role as part of the primary inoculum of apple scab in the orchard studied; however, ascospores are still the predominant source. Due to the many factors that can affect the amount of overwintering conidia in orchards, the overall contribution of conidia as primary inoculum is expected to vary considerably with orchards and seasons. Sanitation practices are imperative, for example good winter pruning and removal of leaf litter are both important. Early season sprays are necessary for successful control of scab whether the primary inoculum is from ascospores or overwintered conidia; however, traditional spray programmes may have to be revisited in light of these findings.

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POPULATION DIFFERENCE OF THE APPLE SCAB FUNGUS *VENTURIA INAEQUALIS* ON CULTIVARS WITHIN A MIXED CULTIVAR ORCHARD

T.A.J. PASSEY[1,2], M.W. SHAW[2] and X.-M. XU[1]
[1]NIAB EMR, East Malling, Kent, ME19 6BJ, United Kingdom
[2]University of Reading, School of Agriculture, Policy and Development, Reading, RG6 6AR, United Kingdom
Corresponding author E-mail: Tom.Passey@emr.ac.uk

SUMMARY

Apple cultivars differ in their resistance to the fungal pathogen *Venturia inaequalis*, the causal agent of apple scab. Mixed cultivar orchards, where the cultivars present have differing resistance to *V. inaequalis,* have been shown to reduce the levels of scab compared to monoculture. To maximise the mixture effect of reducing scab development, cultivars need to be selected with maximum differences in their scab resistance. One indirect, yet efficient, method of selecting such cultivars is to quantify population differences of scab from different cultivars, which are expected to largely reflect the differences in the resistance to the pathogen.

We sampled early season scab lesions from different cultivars in two mixed cider cultivar orchards in the Southwest of England and from various desert and cider cultivars in an orchard in the Southeast of England. Using Simple Sequence Repeat (SSR) markers we compared the scab populations sampled from the different cultivars.

Scab populations from different cultivars differed significantly, depending on specific pairs of cultivars; however, larger differences appear to be among fungal populations from different sites. The results demonstrate that certain cultivars likely share much of their genetic resistance factors to *V. inaequalis*. For dessert apple the scab populations on Cox, Gala, Bramley and Fiesta were not different and therefore it is not advisable to plant these cultivars in the same orchard with a view to reduce scab development. On the other hand, a reduction in scab would be more likely if one of the above cultivars is planted together with Golden Delicious, Red Falstaff or Spartan.

There was a very large difference in the scab population from cv. Three Counties and populations from all other cider cultivars. This was particularly surprising given the shared parentage (Dabinett x James Grieve) between Three Counties and all but one of the other cider cultivars sampled, suggesting considerable differences in the resistance to *V. inaequalis* between the two parents. It also indicates that selection of cultivars for inclusion in mixed orchards cannot be reliably made based on pedigree information alone.

Key words: apple scab, cultivar mixtures, population comparison, resistance

INTRODUCTION

Apple scab is one of the most important diseases economically to the apple industry worldwide. Caused by the ascomycete *Venturia inaequalis*, annual epidemics lead to large losses of marketable fruit due to the unsightly lesions not accepted by retailers and consumers. Left uncontrolled the disease can lead to poor tree health and reduced yields. Current control is predominantly through a programme of fungicide sprays aided by forecasting models (Beresford and Manktelow, 1994; Berrie and Xu, 2003). Emergence of resistance to fungicides from the pathogen, the demand from consumers and policy makers to reduce levels of pesticide use and the cost of chemical control of scab make the need for alternatives a necessity.

The natural resistance of a host is often used to control disease development. The only R-gene that has been bred into new commercial cultivars of apple (*Malus* x *domestica)* is the Rvi6 (Vf) gene from *M. Floribunda.* However this resistance has been overcome (Parisi *et al.*, 1993;

Roberts and Crute, 1994) and is only an option for newly bred cultivars not for popular current cultivars. However it is known that scab isolates from one cultivar cannot necessarily infect another cultivar (Sierotzki *et al.*, 1994; Koch *et al.*, 2000; Barbara *et al.*, 2008). This indicates that susceptible apple cultivars may have differing resistance factors to certain strains of scab. Incorporating cultivars in an orchard with these differences in resistance factors can reduce the incidence of scab. Mixing cultivars to reduce levels of scab has been shown to work in an orchard with a susceptible cultivar and a cultivar carrying the Rvi6 R-gene reducing leaf scab incidence in the range of 7.3 to 22.5% (depending on year and orchard design) compared to monoculture, improved further when a spray programme was applied (14.8 to 75.1% reduction) (Didelot *et al*. 2007). Similarly, this can lead to reduced incidence of fruit scab, further improved when coupled with sanitation (Parisi *et al*. 2013).

The design of a mixed orchard is important to achieve disease reduction compared to monoculture. Mixing within rows is more effective than alternate rows of cultivars, which in turn is more effective than blocks (Blaise and Gessler, 1994; Didelot *et al.*, 2007). This is due to the Genotype Unit Area (GUA) where the larger the area of host with the same genotype the smaller the disease reduction (Mundt, 2002). Therefore the closer the trees of the same cultivar are together the larger the GUA and the higher chance of autoinfection. Within row mixtures are not likely to be introduced into a commercial setting as increased management costs would be too high compared to other types of mixture, let alone monoculture.

One necessary requirement for using mixture to manage diseases is that the cultivars to be used in a mixture must differ in their resistance factors to the target pathogen(s). In this study we compared scab populations on different cultivars within an orchard and used that knowledge to infer which cultivars have differential resistance to scab (hence can be used together in a mixture orchard). Fungal population differences were determined at the molecular level (using Simple Sequence Repeat (SSR) markers). Leaf scab lesions were sampled from several popular desert cultivars and cider cultivars.

MATERIALS AND METHODS

Sampling

Orchard WM132 at NIAB EMR (Kent, UK) has blocks of three rows of *Malus* x *domestica* cv. Cox and three rows of *Malus* x *domestica* cv. Fiesta separated by a block of three rows of *Malus* x *domestica* cv. Royal Gala (Gala); each row has 12 trees. This orchard (ca. 15 years old) has had no fungicide programmes applied since planting, but has received minimal orchard husbandry (pruning, mowing). Scabbed leaves were sampled from the trees in late spring 2012. In 2013 potted trees of each of the three cultivars were placed in the orchard within their own cultivar. At the same time, potted trees of *Malus* x *domestica* cv. Bramley, *Malus* x *domestica* cv. Golden Delicious, *Malus* x *domestica* cv. Red Falstaff, *Malus* x *domestica* cv. Rosette and *Malus* x *domestica* cv. Spartan were placed in this orchard, two trees within each of Cox and Gala orchard trees and one within Fiesta. These potted trees were put in the same orchard again in 2014, this time one tree in each of Cox and Gala, and three within Fiesta. In addition, two potted trees of each of three cider cultivars, *Malus* x *domestica* cv. Angela, *Malus* x *domestica* cv. Dabinett and *Malus* x *domestica* cv. Somerset Redstreak were placed within each of the three cultivars in 2014 (six trees in total). All potted trees used were placed into the orchard at bud burst (approximately 1 m from the orchard tree). They were moved back to a polytunnel after 3-4 weeks and lesions sampled from these trees after a further three weeks.

Two commercially managed cider orchards in Southwest England were also sampled. First, an experimental orchard planted in 2008 in Staunton-on-Wye, Herefordshire was sampled at the end of spring 2012. This orchard was designed as a complete randomised block. The orchard contained the cultivars Angela, Dabinett, *Malus* x *domestica* cv. Katja (known as Katy in the UK and in this study), *Malus* x *domestica* cv. Lizzy and *Malus* x *domestica* cv. Tina on six different rootstock/interstock combinations. We sampled scab lesions from two plots (two different rootstocks) of each cultivar within one block, where all trees sampled were all in an area approx. 12 m x 70 m. Second, St Monica's Orchard, planted in 2009 in Sandford, Somerset, was sampled at the end of spring 2014. This is a mixed orchard of 24 new cider cultivars (colloquially known as "The Girls"), one row (approx. 120 m) of each between rows of Katy. Seven of the 24 cultivars had been identified in a previous scab survey as susceptible to scab, however only *Malus* x *domestica* cv. Gilly, *Malus* x *domestica* cv. Three Counties, Tina and *Malus* x *domestica* cv. Vicky had enough scab to sample in 2014. Infected leaves from Katy were also sampled.

For sampling of all sites, leaves with freshly sporulating, discrete scab lesions, were selected and placed into paper bags, no more than one leaf per shoot. For all leaves collected, a single discrete lesion per leaf was cut out with a 5 mm cork borer, placed in a 2 ml micro tube, left to air dry at room temperature, 2x 4 mm ball bearings added to the tube and then transferred to a -20 °C freezer.

DNA extraction and screening

DNA was extracted directly from the lesion on the leaf disc. The leaf disc was disrupted in a MM2 oscillating mill (Retsch, Haan, Germany) and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions with all optional steps. DNA was eluted with 100 µl elution buffer into a 1.5ml micro tube. DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and stored at -20 °C.

The SSR primers used (Table 1), PCR and thermal cycle conditions, as well as the procedure for genotyping followed published protocols (Passey *et al.* 2016).

Table 1 Simple Sequence Repeat primer pairs (5' to 3') used for screening apple scab isolates and the range of the alleles in present study

SSR	Fluorescent label-Forward primer	Reverse Primer	Allele range
EMVi029[a]	HEX-ACGAGTCCCAGGTCTCACAG	TGTTGACGGTCACGGTGTAT	164-248
Vica9/X[b]	FAM-TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGAAG	219-247
Vica10/154[b]	HEX-CCTCCTTCCTATTACTCTCG	CTGAAGCGAACCTATGTCC	102-190
Vicacg8/42[b]	FAM-TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	194-242
Vict1/130[b]	FAM-GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	142-164
Vitc1/82[b]	HEX-ACTGTCTCTAGGCGAAAG	ACTTGGAAGCTCGCTAAG	225-243
Vitc2/16[b]	FAM-ACATTGACGAAGACGAGC	TACAATTGAGGCGTGTCC	153-169
Vitg9/129[b]	FAM-CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	277-293

[A] XU *ET AL*. (2009)
[B] GUERIN *ET AL*. (2004)

Determination of parentage

The parentage and family pedigree for each cultivar was assessed based on the National Fruit Collection database (DEFRA, 2010). It was not possible to assess Rosette as it was not in the

database. The database also did not include new cider cultivars Angela, Gilly, Lizzy, Three Counties, Tina and Vicky, which derived from a cross between Dabinett or Michelin with James Grieve or Worcester Pearmain (Liz Copas – personal communication). To confirm their parentage, we fingerprinted these new cultivars using the same SSR markers and methods as used for fingerprinting the National Fruit Collection (DEFRA, 2010) and comparing the allele sizes to those of the four possible parents from the collection.

Statistical analysis

Allele frequencies for an orchard were calculated using Powermarker software (Liu and Muse, 2005). Rare alleles ≤ 0.01 of the population of an orchard were recorded as missing values. If there were two alleles at a locus it was assumed that the lesion had resulted from infection by two different strains - only one was randomly selected for inclusion in analysis. If a sample had multiple loci with more than one allele then the sample was discarded. Null alleles occur when a mutation in the flanking region of the sequence repeat stops the annealing of the primer and therefore no amplification during PCR. In this study we included null alleles as a single allele for that marker although it is possible that the sequences in these samples differed.

To assess if scab populations on different cultivars within an orchard differ from each other, pairwise F_{ST} values were calculated and significance testing based on 1023 permutations using Arlequin version 3.5 (Excoffier and Lischer, 2010). Data from all sites, along with 2012 sample data from Ash Farm, Worcestershire, UK (a mixed orchard of Bramley, Cox and Worcester) (Passey *et al*. 2016), were then combined to calculate F_{ST} values, which were subjected to cluster analysis based on the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm using the software Mega (Tamura *et al.*, 2013).

RESULTS

Determination of Parentage

There is generally little known shared parentage among the cultivars sampled (potted and orchard trees) in the WM132 orchard (results not shown). Fiesta and Gala both have Cox in their genealogy; however most of the other cultivars are older, resulting from chance/raised seedlings. Of the cider cultivars in the Southwest of England Dabinett is from a chance seedling while Katy is from a Swedish cross between James Grieve and Worcester. Fingerprinting of "The Girls" showed that all six susceptible cultivars shared James Grieve as a common parent; Angela and Lizzy were from a cross between James Grieve and Michelin and the other four (Gilly, Three Counties, Tina and Vicky) from a cross of James Grieve with Dabinett.

General results

The numbers of isolates sampled varied greatly between cultivar and between sites due to differing scab severity (Tables 2 and 3). The difference between the number of isolates used in statistical analysis and the number of isolates with DNA extracted is due to the fact that a few samples failed to amplify or that a few samples had multiple alleles at more than one locus. Only one lesion was found on Dabinett in the Staunton-on-Wye orchard and therefore omitted from statistical analysis. There was no scab on the potted trees of Rosette in WM132 in 2013, or on the Dabinett trees placed in the same orchard in 2014.

Table 2 Number of apple scab isolates successfully screened with SSR markers from trees within WM132 orchard, Kent

Apple type	Cultivar sampled	2012[a]	2013[b]				2014[b]			
			Cox	Fiesta	Gala	Total	Cox	Fiesta	Gala	Total
Dessert	Cox	31(36)	25(27)	---	---	25(27)	---	---	---	---
	Fiesta	36(36)	---	35(35)	---	35(35)	---	---	---	---
	Gala	28(36)	---	---	35(36)	35(36)	---	---	---	---
	Golden Delicious	---	10(12)	9	14	35(37)	10	16	10	36
	Red Falstaff	---	14	9	14	37	8(9)	14(15)	6	28(30)
	Rosette	---	0	0	0	0	1	9(10)	2	12(13)
	Spartan	---	13	5	17	35	10	11(13)	10	31(33)
Culinary	Bramley	---	14	8	14	36	4(5)	6(10)	9(10)	19(25)
Cider	Angela	---	---	---	---	---	3	5	7	15
	Dabinett	---	---	---	---	---	0	0	0	0
	Somerset Redstreak	---	---	---	---	---	8	8	7	23

Number in the brackets is the number of isolates from which DNA was extracted; otherwise, all isolates with DNA extracted were used in statistical analysis. [a]2012 samples collected from orchard trees; [b]2013 and 2014 sampling from potted trees placed within the blocks of Cox, Fiesta or Gala orchard trees

Table 3 Number of apple scab isolates genotyped with SSR markers from commercial cider orchards in Southwest England

Staunton-on-Wye		Sandford	
Angela	10	Gilly	8
Dabinett	0(1)	Katy	6
Katy	29(36)	Three Counties	10
Lizzy	7(14)	Tina	26
Tina	20(35)	Vicky	26

Number in the brackets is the number of isolates from which DNA was extracted; otherwise, all isolates with DNA extracted were used in statistical analysis

Population pairwise comparisons

Pairwise F_{ST} values between populations from Fiesta and Gala in WM132 showed that the two populations differed significantly ($p = 0.03$) in 2012; however, this was not the case for the lesions from potted trees in 2013 ($p = 0.8$). The population from Cox was not different to that from either Gala or Fiesta in both years ($p > 0.05$).

Pairwise comparisons of the populations from Golden Delicious, Red Falstaff and Spartan showed they were significantly different in both 2013 and 2014 ($p < 0.05$; Table 4). The scab population from Golden Delicious did not differ from populations from the other cultivars. The scab population from Red Falstaff was different to that of Somerset Redstreak in 2014 ($p = 0.04$) and Bramley in 2013 ($p < 0.001$) and 2014 ($p = 0.01$). Although the scab population from Bramley in 2013 differed from that from either Golden Delicious or Spartan, there were no significant differences between these populations in 2014; nor between the Bramley population and any other cultivar's population (other than the aforementioned Red Falstaff). The only other cultivar that Spartan's scab population was different to was Angela's ($p < 0.001$). The population from Angela was not significantly different to that of any other cultivar, nor was that of the other cider cultivar Somerset Redstreak (other than that previously stated with Red Falstaff). The Rosette scab population showed no significant difference to any of the other cultivars.

Pairwise F_{ST} values between populations in the cider orchards are shown in Table 5. The only common cultivar comparison in the two commercial orchards in Southwest England was between the populations of Katy and Tina, which showed no significant difference in either orchard ($p > 0.1$). In the Staunton-on-Wye orchard, the population on Angela was significantly different to that of Lizzy ($p = 0.04$) and Tina ($p = 0.01$) and close to significance with the population from Katy ($p = 0.06$). There were no other significant pairwise differences. The scab population on Three Counties differed ($p < 0.001$) from the other four cultivars sampled in the Sandford orchard. The population on Gilly was close to being significantly different to that of Vicky ($p = 0.07$) and differed from that of Tina ($p = 0.01$).

A UPGMA tree (Figure 1) illustrates the differences between all populations in this study, including those 2012 samples from another mixed orchard as reported in Passey *et al*. (2016). The result indicates a general geographical difference between scab populations in the sampled orchards and a greater difference between populations on cider cultivars than on desert cultivars.

Table 4 Estimated pairwise F_{ST} values from AMOVA between scab isolates sampled from potted trees of different cultivars placed within the WM132 mixed cultivar orchard: upper diagonal - 2013 samples; lower diagonal - 2014 samples

	Angela	Bramley	Golden Delicious	Red Falstaff	Rosette	Somerset Redstreak	Spartan
Angela		---	---	---	---	---	---
Bramley	0.00		0.08***	0.07***	---	---	0.03*
Golden Delicious	0.01	0.00		0.07***	---	---	0.04**
Red Falstaff	0.01	0.04*	0.03**		---	---	0.03*
Rosette	0.00	0.00	0.00	0.02		---	---
Somerset Redstreak	0.01	0.00	0.01	0.03*	0.00		---
Spartan	0.07***	0.01	0.03**	0.05***	0.00	0.02	

* Significant at 0.05; ** Significant at 0.01; *** Significant at 0.001

Table 5 Estimated pairwise F_{ST} values from AMOVA of scab isolates sampled from different cultivars in mixed cider cultivar orchards in Southwest England: upper diagonal Staunton-on-Wye - 2012 samples; lower diagonal Sandford - 2014 samples

	Angela	Gilly	Katy	Lizzy	Three Counties	Tina	Vicky
Angela		---	0.05	0.13*	---	0.11*	---
Gilly	---		---	---	---	---	---
Katy	---	0.00		0.03	---	0.02	---
Lizzy	---	---			---	0.04	---
Three Counties	---	0.47***	0.48***	---		---	---
Tina	---	0.09*	0.03	---	0.39***		---
Vicky	---	0.04	0.00	---	0.36***	0.00	

* Significant at 0.05; ** Significant at 0.01; *** Significant at 0.001

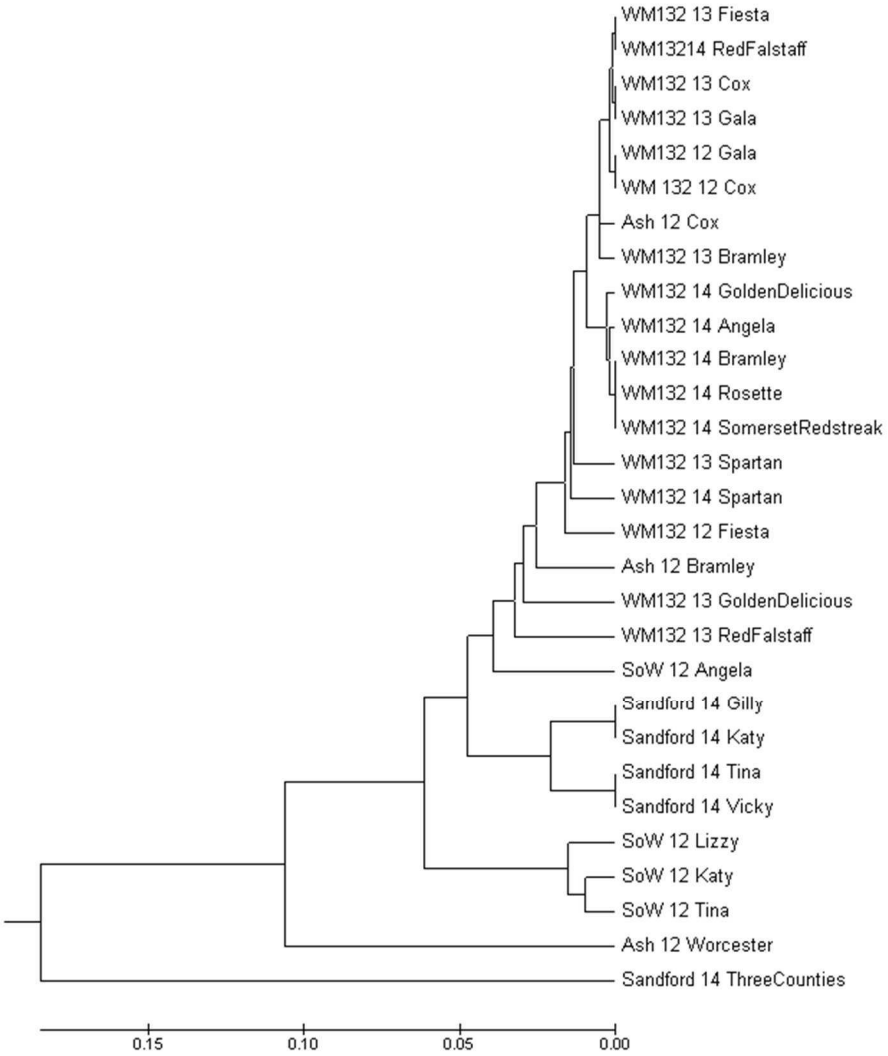

Figure 1 A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree generated from pairwise F_{ST} values of scab isolates sampled from different cultivars in three mixed orchards in three years; Population nomenclature: Orchard (SoW = Staunton-on-Wye), Sampling year, Cultivar

DISCUSSION

In this study we have shown that there are significant differences between some scab populations from different cultivars in the same orchard. However, it should be noted that population differences between orchards were much greater than between cultivars in the same orchard.

A number of populations from different cultivars were not different, suggesting that these cultivars probably share similar genetic factors of resistance (and therefore susceptibility) to

V. inaequalis and would therefore not make desirable candidates for use in a mixture for scab management. Thus, in desert apple it would not be advisable to plant Cox, Fiesta or Gala in the same orchard if the aim is to reduce scab development. On the other hand, Golden Delicious, Red Falstaff or Spartan are good candidates for such a purpose.

For the cider cultivars there were no significant differences in the scab populations from Katy, Tina and Vicky, but there was a large difference in the population from Three Counties with the populations of all other cider cultivars. This is surprising given that Gilly, Tina, Vicky and Three Counties all come from the same cross (Dabinett x James Grieve). In fact, of the 24 "The Girls" cultivars in the Sandford orchard we found a sufficient amount of scab for sampling on only four. All "The Girls" cultivars are from four possible crosses indicating that there is likely to be considerable differences in genetic control of the response to scab within the parents, leading to segregation of this character in the progeny. There were no scab lesions on potted trees of Dabinett in the WM132 orchard and only one lesion in the Staunton-on-Wye orchard; however, four of the six "The Girls" cultivars sampled had Dabinett as a parent. Worcester is likely to have at least three resistance factors (Barbara *et al.*, 2008) and the population on Worcester is quite different to the other populations presented. These results indicate that selection of cultivars for inclusion in mixture cannot be reliably made based solely on pedigree information.

The greater differences between scab populations on cider cultivars compared to the differences between desert cultivars suggest that there is less variability in resistance factors among the desert cultivars in the present study. Breeding for improved dessert cultivars has been much more extensive than cider apples, which may have reduced genetic variability among commercial desert cultivars for scab resistance. It would be advantageous to expand the genetic base from which desert cultivars are bred to increase the pool of resistance factors to *V. inaequalis* and potentially other pathogens.

The use of SSR markers is a good way to initially assess how similar populations of scab are from different cultivars and therefore discount those susceptible cultivars which show no differences as being incompatible for a successful mixed orchard for disease management. Of course, at this stage those cultivars that showed limited scab development remain viable candidates for mixture. It is possible to select potential mixture components based only on the SSR results when scab populations from those cultivars are different. Nevertheless, some controlled cross inoculation with isolates from these candidates may be advisable. Of course, any susceptible cultivar can be mixed with resistant cultivars.

The selection of cultivars for mixture is not decided on scab resistance alone, other traits have to be considered in order to maximise profitability. For example, Red Falstaff flowers almost two weeks before Golden Delicious and Spartan, which may impose extra cost in orchard management, e.g. pest and disease control. There is also some evidence that mixed cultivar apple orchards might reduce levels of powdery mildew and rosy apple aphid (Parisi *et al.*, 2013) and therefore comparing and selecting cultivars with differing resistance to other pests and diseases will further increase the benefit of mixture.

The cider industry remains the most obvious area of apple production for implementation of mixed cultivar orchards. Not only is fruit quality not as stringent as for desert apples, much mass produced cider includes a blend of apple cultivars and therefore requires different cultivars to be grown, as opposed to desert growers who are often more restricted in which cultivars they grow for the mass market. As we have shown in this study cider growers also seem to have the advantage of a more diverse genetic background in cultivars for reduction in scab.

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GENOME SEQUENCES

Annotated Draft Genome Sequence of the Apple Scab Pathogen *Venturia inaequalis*

Thomas A. J. Passey,[a,b] Andrew D. Armitage,[a] Xiangming Xu[a]

[a]NIAB EMR, East Malling, Kent, United Kingdom

[b]School of Agriculture, Policy and Development, University of Reading, Reading, United Kingdom

ABSTRACT Apple scab is one of the most economically important diseases of apples worldwide. The disease is caused by the haploid ascomycete *Venturia inaequalis*. We present here an annotated *V. inaequalis* whole-genome sequence of 72 Mb, assembled into 238 contigs, with 13,761 predicted genes.

Venturia inaequalis (phylum *Ascomycota*, class *Dothideomycetes*) is the causal agent of apple scab, one of the most important diseases of apples worldwide, and, as a result, has been extensively researched for well over a century (1). If not managed, annual epidemics can result in large numbers of unmarketable fruit. Previously published annotated genome sequences for *V. inaequalis* have between 1,012 and 1,680 scaffolds (2, 3).

A single-spore isolate of *V. inaequalis* (05/172) was obtained in 2005 from a lesion on a leaf of *Malus x domestica* cv. Worcester Pearmain from Ash Farm in Worcestershire, United Kingdom (4). DNA was extracted and sequenced by two methods: (i) DNA was extracted from mycelium using a Qiagen Genomic-tip 100/G kit; the tissue method of sample preparation was used according to the manufacturer's protocol with options 3B and 4B (adapted to 200 μl proteinase K). Isolation of DNA followed the manufacturer's protocol with options 5B and 6B. DNA was sent to the Earlham Institute (Norwich, UK), for sequencing using the Pacific Biosciences (PacBio) platform. (ii) DNA of the isolate was extracted for Passey et al. (5). Paired-end genomic libraries were prepared using a NEXTflex Rapid DNA-Seq version 14.02 library prep kit (Bioo Scientific) following the manufacturer's protocol but modified by using Illumina adapters rather than NEXTflex barcodes. Libraries were validated using a fragment analyzer (Advanced Analytical Technologies), which confirmed a high proportion of library DNA fragments between 600 and 900 bp long. Libraries were sequenced using 2 $\times$ 300-bp reads on an Illumina MiSeq platform. Illumina adapters and low-quality base pairs were trimmed from 1,281,750 MiSeq reads with fastq-mcf version 1.04.636 (6).

PacBio sequencing reads (944,907 reads) were corrected, trimmed, and assembled with Canu version 1.2 (7), and the assembly was corrected with MiSeq reads using Pilon version 1.17 (8). Hybrid assembly with both PacBio and MiSeq reads was performed with SPAdes version 3.9.0 (9) and then merged with the Canu assembly using quickmerge version 0.2 (10); the merged assembly was corrected with the MiSeq reads using Pilon. The genome was assembled into 72.3 Mb in 238 contigs (Table 1). Repetitive and low-complexity regions of the merged assembly were identified by repeat masking with RepeatMasker version 4.0.6 (http://www.repeatmasker.org) and TransposonPSI (release 08222010; http://transposonpsi.sourceforge.net), masking 34.2 Mb (47.3%) of the genome, of which 98.7% was due to transposable elements. Quality of the genome assembly was assessed by looking for benchmarking universal single-copy orthologs (BUSCO) with BUSCO version 3 (11) against the *Ascomycota* odb9 data set, identifying 1,286 (out of 1,315) as present in the assembly. Gene prediction was performed with

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Address correspondence to Thomas A. J. Passey, tom.passey@emr.ac.uk.

TABLE 1 *V. inaequalis* isolate 05/172 genome assembly statistics[a]

Statistic	Value for isolate 05/172
No. of contigs	238
Total length (bp)	72,310,420
Largest contig (bp)	3,847,617
GC content (%)	42.75
N_{50} (bp)	953,805
N_{75} (bp)	531,805
L_{50}	23
L_{75}	49

[a]Assembly produced by merged Canu and SPAdes assemblies using PacBio- and MiSeq-generated sequencing reads.

the use of RNA sequencing (RNA-seq) data from Thakur et al. (12); RNA-seq data were aligned to the genome by STAR version 2.6 (13). A predicted 13,761 genes are present in the assembled genome; 11,597 genes were predicted by Braker1 (14), supplemented by 2,164 genes predicted by CodingQuarry (15) (in pathogen mode) in the intergenic regions of Braker1 gene models. Functional annotation of the genome was performed using Interproscan version 5.18-57.0 (16) and the July 2016 release of the Swiss-Prot database (17).

Data availability. The Sequence Read Archive accession numbers are SRR5183052 for the Illumina MiSeq reads and SRR5183051 for the PacBio reads. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number QFBF00000000 (BioProject number PRJNA354841). The version described in this paper is the first version, QFBF01000000.

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Title: Genomic sequencing indicates non-random mating of *Venturia inaequalis* in a mixed cultivar orchard

Authors: Thomas A. J. Passey[ab], Andrew D. Armitage[a], Maria K. Sobczyk[a], Michael W. Shaw[b], Xiangming Xu[a]

[a]*NIAB EMR, New Road, East Malling, Kent, ME19 6BJ, UK;* [b]*School of Agriculture, Policy and Development, University of Reading, Reading, RG6 6AR, UK*

E-mail: tom.passey@emr.ac.uk (Corresponding author)

xiangming.xu@emr.ac.uk

Abstract

Apple scab is one of the most economically important diseases of apples worldwide. The disease is caused by the haploid ascomycete *Venturia inaequalis*. Growing apples in cultivar mixtures may reduce disease severity. To determine how the pathogen population structure is affected by host mixtures we studied 24 *V. inaequalis* isolates sampled from three different apple cultivars (Bramley, Cox and Worcester) growing in a mixed orchard approximately 50 years old. The isolates were aligned against a reference genome and Single Nucleotide Polymorphisms (SNPs) were called between the isolates. The populations isolated from Bramley and Worcester were distinct, while Cox isolates were an admixture. This supports previous tests of the ability of isolates to cross-infect hosts, and molecular comparisons using Simple Sequence Repeats (SSRs). Genotype specific allele (GSA) loci were not distributed randomly across contigs in proportion to contig length, but were clustered. Furthermore, within individual contigs those GSA loci were much closer to each other than expected from random placement, indicating lack of crossing-over events (i.e. recombination) within the GSA blocks. This lack of crossing-over events between Bramley and Worcester isolates is probably due to physical separation effects: sexual mating is more likely to take place and succeed between isolates from lesions on the same leaf than contact between independently infected leaves in leaf litter on the orchard floor. This is especially the case if sexual reproduction is initiated before leaf-fall.

Highlights

- **1** Host genotype specific alleles in populations of *Venturia inaequalis* are aggregated on the genome

- **2** Lack of mating between *V. inaequalis* populations from different host cultivars in same orchard
- **3** Likely that sexual mating is mainly between isolates on the same leaf

Keywords

Apple scab; Apple Black spot; ascospore production; super race; host mixture;

1. Introduction

The ascomycete *Venturia inaequalis* is the causal agent of apple scab, one of the most important diseases of apples worldwide and as a result has been extensively researched for well over a century (MacHardy, 1996). The primary inoculum of the pathogen is predominantly from sexually produced ascospores released from overwintered leaf litter, although, probably depending on climate, some may be from overwintering asexual conidia (Holb et al., 2004, 2005; Passey et al., 2017). If not adequately managed, rounds of secondary infections from conidia can result in large numbers of unmarketable fruit due to unsightly lesions and regular high incidence can lead to premature leaf fall, reduced cumulative growth and very low yields (MacHardy, 1996). Sufficient control to achieve high quality scab-free fruit requires optimum use of numerous fungicide spray rounds through orchard monitoring and disease forecasting systems.

An alternative, or supplementary, method of disease control is the use of mixing together cultivars of a crop with differing resistance factors (Mundt, 2002; Wolfe, 1985). The potential for cultivar mixture to reduce scab development in apple orchards was first assessed by simulation (Blaise & Gessler, 1994) and then tested with a field trial (Bousset et al. 1997).

The field trial mixed susceptible and R-gene carrying resistant cultivars and provided evidence that the number of scab lesions per shoot was lower on cvs. Golden Delicious and Elstar in mixtures than when these cultivars were in monoculture. The potential of mixtures for scab management was further investigated with a mixture of the susceptible cultivar Smoothee and the *Rvi6* R-gene carrying cultivar Baujade (Didelot et al., 2007). Scab leaf incidence was reduced on cv. Smoothee in mixture by 7% - 21% compared with the mean of monoculture plots, while scab leaf severity was reduced by 15 – 35%, depending on mixture type and annual epidemic severity. Parisi et al. (2013) investigated scab levels in a mixed orchard of cv. Melrouge, a low susceptibility cultivar, and Pitchounette, a resistant cultivar again carrying the *Rvi6* gene. In 2008 they found 9% of scabbed fruits at harvest in a mixed orchard compared with a mean of 15% in pure stands. In the following year conditions led to much greater incidence; this was slightly reduced in the mixed orchard, 76% compared with 82% in the pure stands.

These studies involved mixing a susceptible cultivar with an R-gene carrying cultivar; however, susceptible cultivars are also known to have differential resistance to apple scab (Koch et al., 2000; Sierotzki et al., 1994). Barbara et al. (2008) showed that isolates of scab sampled from different susceptible cultivars growing within the same orchard could not necessarily infect all other cultivars present. Laboratory crossing between such isolates led to ascospore progenies containing individuals that could infect the whole range of cultivars present in the orchard. Using simple sequence repeat (SSR) markers to look for changes in the *V. inaequalis* populations on the different cultivars indicated that the population difference did not reduce over about a decade, indicating lack of recombination between isolates from different cultivars (Passey et al., 2016). This suggests that sexual reproduction in *V. inaequalis* may be conditioned on isolates being on the same leaf (being physically close

and in contact for a long time) rather than occurring at random among isolates present in a population.

We have obtained further genomic data to confirm this inference of non-random mating. Specifically, we sequenced *V. inaequalis* isolates from different apple cultivars within a single mixed orchard to identify single nucleotide polymorphisms (SNPs) present for subsequent investigation of population differentiation. Of these SNPs, we were specifically interested in any SNP which had the same allele among isolates from a single host cultivar, but differed from an allele that shared among all isolates from another cultivar; the alleles at these SNP loci are referred to as genotype (cultivar) specific alleles (GSA). We studied whether these GSA loci were randomly distributed within genomic regions. Non-random distribution may indicate non-random mating among isolates from different cultivars.

2. Materials and methods

2.1. Isolates and DNA extraction

Ash Farm, Worcestershire, UK has a 6-row mixed orchard of *Malus* x *domestica* cv. Bramley's Seedling (Bramley), cv. Cox's Orange Pippin (Cox) and cv. Worcester Pearmain (Worcester) on non-dwarfing rootstocks; each cultivar has two rows with no cultivar being in consecutive rows - Worcester, Cox, Bramley, Cox, Bramley, Worcester. This orchard is ca. 45-50 years old. It has never been sprayed and has not recently been pruned. Scab lesions were sampled from this orchard in 2005 and single spore isolates obtained (Xu *et al.*, 2013). In previous work DNA was extracted from freeze-dried mycelia of single spore isolates for comparison of scab populations on the different cultivars using SSR markers (Passey et al., 2016). The eight isolates from each of the three cultivars with the highest DNA concentrations [quantified

and quality-checked using a Nanodrop 1000 spectrophotometer (Thermo Scientific)] were selected for Next Generation Sequencing (NGS) on the Illumina MiSeq platform (Supplementary table A).

2.2. Library preparation and sequencing

All isolates were sequenced with an Illumina MiSeq. Paired-end genomic libraries were prepared using NEXTflex Rapid DNA-Seq library prep kit Version 14.02 (Bioo Scientific) following the manufacturer's protocol modified by using Illumina adapters rather than the NEXTflex Barcodes. Libraries were validated using a Fragment Analyzer (Advanced Analytical Technologies) confirming a high proportion of library DNA fragments between 600 and 900 bp long. Library concentrations were quantified using a Qubit 2.0 (Invitrogen/Thermo Fisher), standardised to 9nM before pooled and then diluted to 4nM (libraries of 5 isolates). Denatured, pooled libraries at 20pM were sequenced using 300 bp reads on an Illumina MiSeq.

2.3 Alignment of MiSeq reads to reference genome and SNP calling

MiSeq reads for all of the isolates were trimmed to remove adaptors and poor quality data from the sequences using fastq-mcf v1.04.636 (Aronesty, 2013). Alignment of the trimmed reads of the isolates to the reference genome of isolate 05/172 (Passey et al., 2018) was performed with Bowtie2 (Langmead and Salzberg, 2012). After removing multimapping and discordant reads from the isolates with SAMtools v.1.3.1, SNPs were called with GATK v.3.6 (Van der Auwera et al., 2013) and then filtered to retain only high-quality SNPs, using VCFtools (Danecek et al., 2011) with no missing data for genetic analyses.

2.4 Determining genetic structure

Previous work comparing isolates from this orchard using AFLP and SSR screening clearly showed a difference between isolates from different cultivars, particularly between Bramley and Worcester (Xu et al., 2013). To confirm this differentiation was true of isolates genome-wide, identity-by-state (IBS) was calculated based on the percentage similarity of shared alleles between samples to produce a SNP matrix, visualised using R as a heatmap and dendogram. A Neighbour joining (NJ) tree based on 100 bootstrap replicates was produced using the ape package in R and visualised using Figtree v.1.4.3, to show unrooted phylogeny of the isolates.

For isolates originating from the three different cultivars we ran pairwise searches (i.e. isolates from Bramley and Cox; Bramley and Worcester; Cox and Worcester) for those SNPs where isolates from a single host cultivar shared the same allele at a locus, but the allele differed from those in isolates from other populations (i.e. GSAs). Using a custom Python script we set the GSA threshold to be the major allele in a given population with observed frequency of at least 0.95 [given the small sample sizes, this meant that this allele was present in all isolates in a sample from a given host cultivar]. The number and positions of all SNP loci, GSA loci, GSA loci in genes and nonsynonymous GSA loci in genes were recorded for each contig.

2.5. Aggregation of GSA loci

The number of GSA loci between Bramley and Cox, and between Cox and Worcester, was small. Thus subsequent aggregation analysis of GSA loci was only applied to GSA loci between Bramley and Worcester.

2.5.1. Number of GSAs within a contig

We wanted to assess whether the GSA loci in the Bramley and Worcester isolates were randomly distributed among the contigs, given the number of total SNP loci in each contig. GSA loci are more likely to be aggregated – that is, more likely to be in the same contig than expected under the assumption of independence among GSA loci - if the frequency of sexual mating between isolates from different cultivars is less than expected under the assumption of random mating assuming equal viability of resulting ascospores.

We ran a permutation test to test for aggregation of GSA loci. Specifically, we tested whether the observed variance in the number of GSA loci between contigs was greater than expected under the assumption of random positioning of GSA loci, conditioned on the total number of SNP loci in the Bramley and Worcester isolates in each contig. We excluded contigs with fewer than 100 SNP loci within the Bramley and Worcester isolates from the permutation test. Such contigs are likely to be either highly conserved regions of DNA and therefore unrepresentative, or poorly sequenced (leading to SNPs being removed during filtering).

Each permutation consisted of the following steps: (i) the observed number of SNP loci of all types in the first contig were randomly sampled from the entire set of SNP loci in the Bramley and Worcester isolates (initial source of SNP loci), without replacement; (ii) the number of GSA loci in this random sample of SNP loci was counted; (iii) the sampled SNP loci were removed from the initial source of SNP loci (i.e. sampling without replacement) to form the new source for subsequent sampling; (iv) the above three steps were repeated on the next contig until random samples for all contigs had been constructed; (v) finally, variance in the number of GSA loci on each contig was calculated. A total 999 permutations were conducted to generate a frequency distribution of variance in the number of GSA loci

expected under the assumption of random distribution of GSA loci among contigs. The observed variance in the number of GSA loci among the contigs was then compared with this distribution.

2.5.2. Distribution of GSA loci within a contig

We would expect that GSA loci would be closer to each other within a contig if mating between isolates from different cultivars is infrequent, within the life time of a commercial orchard (15-20 years), relative to mating between isolates within the same cultivars. This is because there would not have been enough crossing-over events in the region to disrupt a block of GSAs. Specifically, we used a permutation test to assess whether individual GSA loci were randomly distributed within a contig conditioned on the number of SNP loci observed on each contig. Non-GSA SNP loci were coded '0' while GSA loci were coded '1' as for a run test (Sprent and Smeeton, 2007). As before, we excluded contigs with less than 100 SNPs from the permutation analysis. For each permutation, the observed number of GSA loci on each contig was randomly distributed among the positions of all the SNP loci on the contig. Next, the number of consecutive 1s (i.e., GSAs) was calculated for two consecutive 1s up to eleven consecutive 1s. Only non-overlapping consecutive 1s were counted; thus, for instance, '1111' had two counts of '11' and one count each of '111' and '1111', rather than three counts of '11', two counts of '111' and one count of '1111'. This process of redistributing GSAs and counting consecutive GSAs was repeated 1000 times for each contig. The maximum number of two consecutive 1s up to eleven consecutive 1s from the 1000 permutations was calculated and compared to the observed value for each contig.

3. Results

3.1. Determining genetic structure

Isolates 05/036, 05/057 (both isolated from Bramley) and 05/118 (isolated from Cox) had insufficient sequencing coverage and had to be removed from the analyses. Isolates from Bramley grouped separately in neighbour-joining cluster diagrams from isolates from Worcester while isolates from Cox appear to be a mixture, thus supporting previous findings with SSRs (Figures 1 and 2).

No GSA loci were observed between populations from Cox and Worcester, while 160 GSA loci (0.03% of all SNP loci) were found between Cox and Bramley populations, and 7168 (1.15% of all SNP loci) between Bramley and Worcester (Table 1). Of the GSA loci between populations from Bramley and Worcester, 3821 were in the regions of predicted genes, of which 1019 were nonsynonymous; these proportions are similar to the proportion of total SNPs that are in genes (50%) and those that are non-synonymous (14%). The breakdown of total SNP and GSA loci for individual contigs is given in Supplementary table B.

3.2 Aggregation of GSAs

3.2.1. Number of GSAs within a contig between Bramley and Worcester

After removal of contigs with less than 100 SNP loci, 99.96% of SNP loci remained across 92 contigs, covering 90.4% of the total genome length. The GSA loci follow an extremely aggregated distribution (Permutation test $P < 0.001$; Fig. 3).

3.2.2. Distribution of GSAs within a contig

In all contigs, with more than 100 SNP loci, the observed number of consecutive GSA loci was much greater than the maximum of the corresponding values in the 1000 permutations

(Supplementary table C). For example, for contig 47 (Supplementary information D) in 1000 permutations, two consecutive GSAs occurred a maximum 17 times (122 observed), three consecutive GSAs a maximum three times (77 observed) and just once four consecutive GSA loci (53 observed); but the observed contig has a single run of 52 consecutive GSA loci.

Across all contigs, the most consecutive GSA loci observed in 1000 permutations was six, on contig 65. The longest consecutive run of GSAs observed was on contig 8, with 121 consecutive GSA loci. Contig 8 is 1.5 Mb long (2.0% of the genome) and has a total of 13278 SNPs within the combined Bramley and Worcester populations (2.1% of all SNPs across the genome), of which 339 are GSA loci (4.7% of all GSAs across the genome). However, 70% of contig 8 GSA loci are between positions 770024 and 781706, comprising only 0.8% of the total contig length.

4. Discussion

Aligning isolates of *V. inaequalis* taken from three different cultivars within a single orchard and comparing SNPs between them showed that isolates from Bramley and Worcester differed significantly. This supports previous findings based on artificial inoculation studies (Barbara et al., 2008) and molecular comparisons using SSRs (Xu et al., 2013). Pairwise comparisons show that the number of GSAs is much higher between Bramley and Worcester than between Cox and Bramley or Cox and Worcester. Furthermore, these GSA loci between Worcester and Bramley, when conditioned on all SNPs (i.e. so to exclude situations due to highly conserved regions of DNA), are spatially aggregated along the genome, i.e. forming blocks of GSAs. The sample size in this study is small and therefore is likely to overestimate the number of GSA loci; however, our inference is not about the number of GSAs but the

aggregation of GSA loci conditioned on the number of observed GSA and non-GSA loci. Previously, we showed that fungal population differences in the same mixed orchard decreased over time between Cox and Bramley, but, increased in the same period between Bramley and Worcester (Passey et al., 2016). Previous *in vitro* crossing results showed that there are no barriers to sexual recombination between isolates from Worcester and Bramley and that recombination of virulence factors against both cultivars did take place (Barbara et al., 2008). Together with the present results on the spatial aggregation of GSA loci within a contig, we may conclude that sexual mating between isolates from Worcester and Bramley is far less frequent than between isolates from Bramley and Cox. The mixed orchard where scab isolates were sampled has not received any sanitation or leaf degradation management. Thus, there have been plenty of fallen leaves from all cultivars on which sexual processes of *V. inaequalis* are believed to take place (MacHardy, 1996).

There are several possible causes for the apparent lack of mating between isolates from Bramley and Worcester. The first is the fitness cost associated with the combined virulence required to infect both cultivars. However, fitness cost is unlikely to be the main explanation. Seven out of 53 viable ascospores from three crosses between isolates in the same orchard from Cox, Bramley and Worcester can infect both Bramley and Worcester with no obvious difference in lesion development (Barbara et al., 2008). Furthermore, the aggregation of GSAs was found in all contigs with a sufficient number of SNPs (ca. 90% of the total genome); it would be difficult to imagine that such strong selection (fitness cost) across ca. 90% of the whole genome would be necessary to ensure infection of both commonly regarded, susceptible cultivars. Another possible explanation is complex epistasis among virulence factors overcoming Bramley and Worcester, reducing the proportion of progeny ascospores that could infect both cultivars. However, simple models without epistasis can satisfactorily

explain segregation of virulence factors among three crosses between isolates in the same orchard from Cox, Bramley and Worcester (Barbara et al., 2008). A more plausible explanation, without resort to genetics, for the lack of sexual mating between isolates from Bramley and Worcester, is that sexual mating is more likely to succeed between isolates (of opposite mating type) on the same leaf than between isolates from different leaves. Indeed, if the sex is initiated before leaf-fall, only isolates with similar genetic backgrounds for virulence have the opportunity to mate. If the sexual mating is initiated after leaf-fall (i.e. on fallen leaves), the chance of successful mating is still likely to be much higher between isolates on the same leaf than from different leaves since sufficient contact time is needed to initiate the mating process. Further research is necessary to investigate if mating initials occur before and/or after leaf fall and the minimum contact time between isolates from different leaves to initiate the mating process. This knowledge has significant implications not only on understanding pathogen evolution but also on developing practical disease management, e.g. timing of end of season control measures for reducing sexual reproduction and therefore primary inoculum the following season.

The number of GSA loci separating the isolates from Cox and isolates of the other two cultivars were too low to look at aggregation. This is probably due to the isolates of Cox being an admixture, with some grouping more closely with isolates from Bramley and some more closely with isolates from Worcester; which is supported by both artificial inoculation studies (Barbara et al., 2008) and population comparisons based on SSR markers (Passey et al., 2016; Xu et al., 2013). As all three cultivars are commonly regarded as susceptible to scab, we may speculate that partial resistance (hence polygenic) may operate on these cultivars. Cox may have some common factors contributing to scab resistance with Bramley and Worcester; whereas Bramley share very few, or no, resistance factors with Worcester. If

isolates with virulence to Bramley and others with virulence to Worcester can infect the same Cox leaf then this could allow mating to take place and the possible emergence of an isolate that infect all three cultivars. Indeed, artificial crossing has shown that the recombination of virulence against all three cultivars can take place (Barbara et al., 2008). However, the chance of this event (recombining virulence against Bramley and Worcester, or all three cultivars) occurring in the orchard is very small relative to other scenarios. Firstly, two isolates of opposite mating types must infect the same Cox leaf – one with virulence against both Cox and Worcester, and one with virulence against both Cox and Bramley. Secondly, artificial inoculation indicated that at least three virulence factors may need to overcome Worcester (Barbara et al., 2008). Thus, even if the necessary cross did take place, the probability of having three virulence factors against Worcester, combined with that against Bramley, is far lower than combining the three virulence factors against Worcester in crosses between two Worcester isolates. Thus, given the sample size we took, we may have missed scab lesions containing signatures of recombination between isolates from Worcester and Bramley that occur less frequently than other scenarios. It is an important consideration of implementing a mixed cultivar orchard that the cultivars have differing resistance factors and although, we have not shown a breakdown in the difference between Bramley and Worcester in this orchard, it would be prudent not to have a potential intermediate cultivar.

Conidia are likely to have a role in primary inoculum in some regions (Holb et al., 2004, 2005; Passey et al., 2017) and if this site does not favour ascospore production and only has asexual clonal races of the pathogen it would explain the lack of reproduction and no super race, even with an intermediate cultivar present. However, this is highly unlikely. Although no pseudothecia have ever been recovered in areas of Israel which lack the necessary lower

winter temperatures, with inoculum only consisting of conidia (Boehm et al., 2003), there is no indication that this has happened in orchards of temperate regions and all the evidence is that ascospores are the main, or only, source of inoculum in these areas. Indeed, the decreased population differentiation between scab isolates from Cox and Bramley over time (Passey et al., 2016), indicate that ascospores are an important primary inoculum in this orchard.

If mating among scab isolates within an orchard is not random, as often assumed, due to the simple physical separation of isolates with opposite mating types, one consequence would be that we would overestimate the rate in which virulence factors against different resistance genes can recombine to form super races. Another consequence would be persistent differentiation of scab populations from different cultivars in the same orchard over time. This would explain why populations on different cultivars within the same orchard remain different after ca. 50 years and indeed the differences between scab populations from Worcester and Bramley may have increased in the period from 2005 to 2012 (Passey et al., 2016). The implementation of orchards with mixed cultivars of differing resistance, shown to decrease levels of apple scab compared to monoculture (Didelot et al., 2007; Parisi et al., 2013), are therefore a more attractive option if the risk of super races emerging is much less than predicted on the assumption of random mating. This is particularly the case since current commercial apple orchards are replaced after only 15-20 years.

The findings presented here suggest that there is a lack of random mating between isolates from specific cultivars within a mixed orchard, which may be explained by the reduced chance of mating between isolates on different leaves than on the same leaf. One consequence of this physical separation effect on mating is that sexual mating is more likely to take place between isolates of *V. inaequalis* with similar virulence factors against scab.

This suggests that the risk of super-races in mixed orchards may be low enough for mixtures that reduce apple scab to remain viable for the lifetime of commercial orchards (15-20 years). A similar conclusion would follow for other pathosystems requiring hyphal mating on living tissue.

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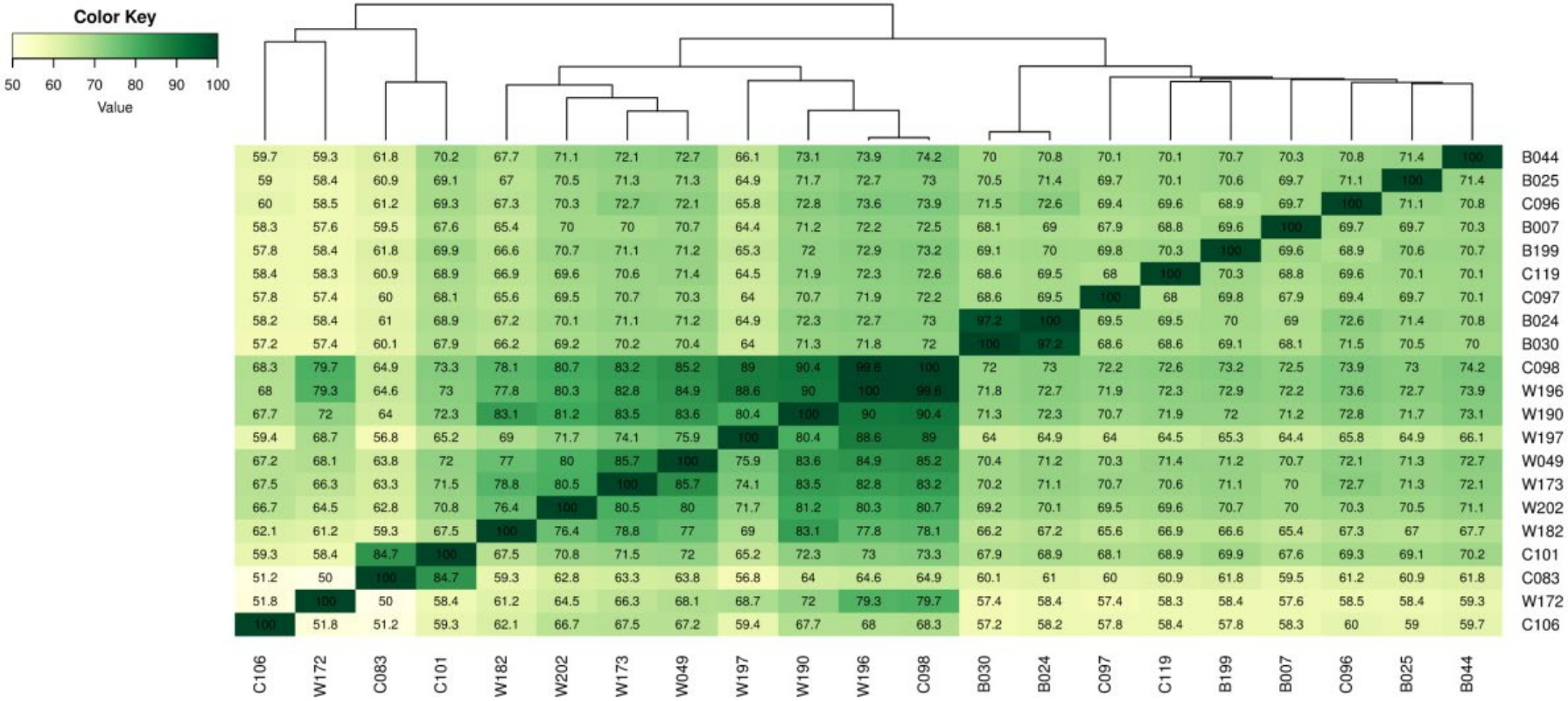

Figure 1. Heatmap and dendogram to represent clustering of 21 *V. inaequalis* isolates from three different apple cultivars, Bramley (B), Cox (C) and Worcester (W), present in the same orchard. Data from identity-by-state (IBS) calculated on the percentage similarity of shared alleles between samples to produce a SNP matrix (the darker the shading the more alike the isolates)

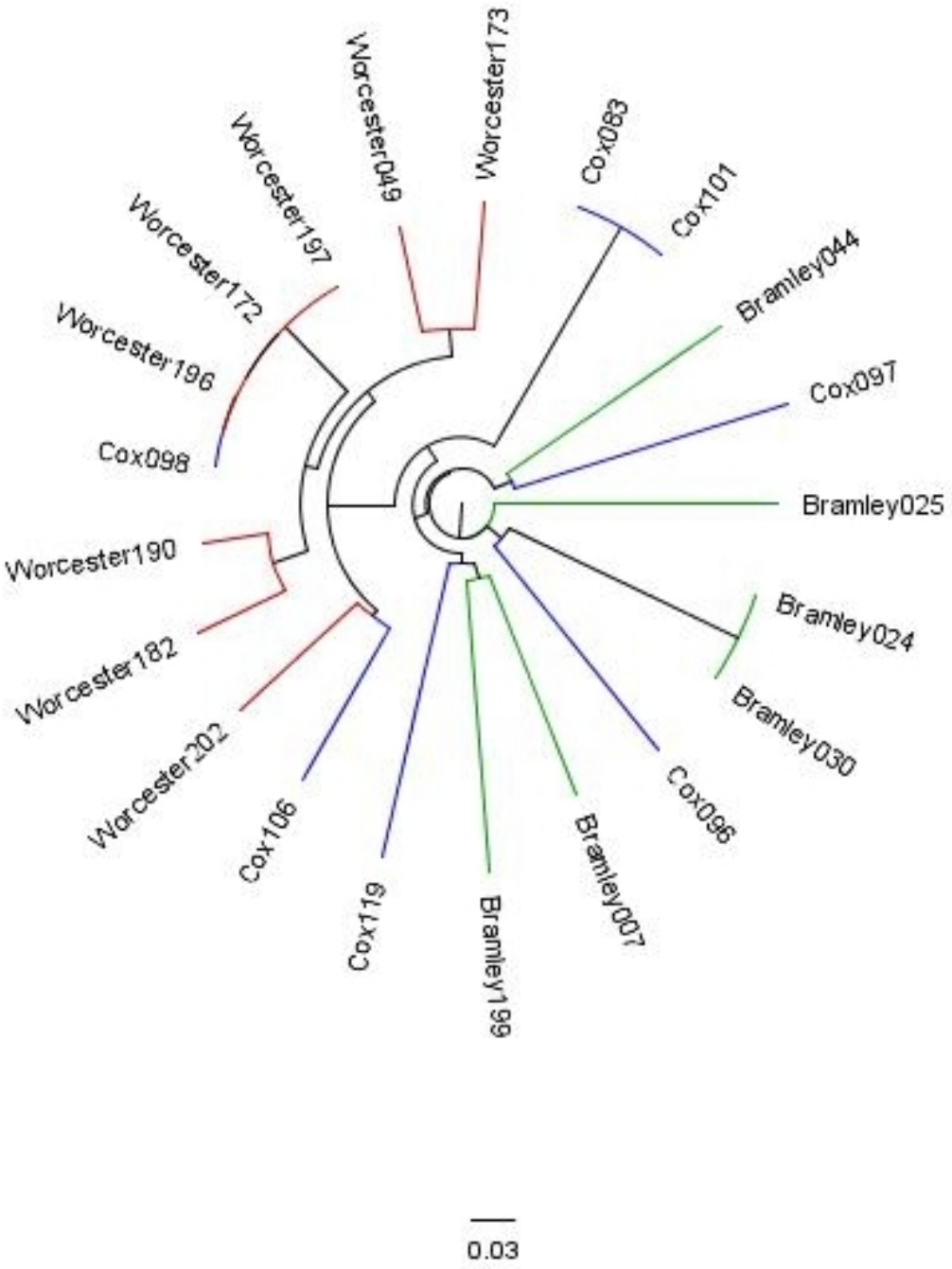

Figure 2. A Neighbour joining (NJ) tree, in polar format, showing clustering of *Venturia inaequalis* isolates from three different apple cultivars, Bramley, Cox and Worcester, present in the same orchard

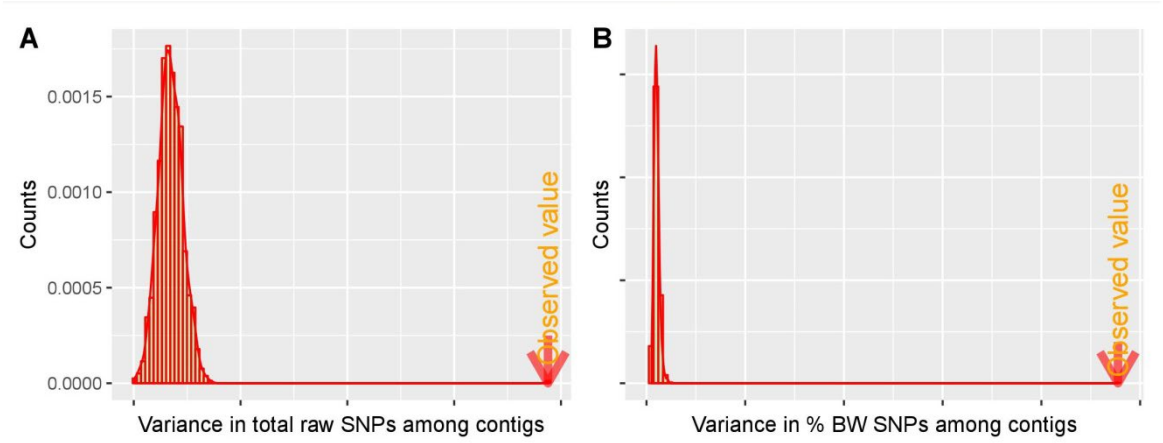

Figure 3. Variance plots comparing the distribution of all genotype specific allele (GSA) loci with that expected under the assumption of random distribution of GSA loci across the contigs. Variance values from 999 permutations test conditioned on the total number of SNPs for *V. inaequalis* isolates sampled from Bramley and Worcester trees within the same orchard

Table 1. Pairwise comparison of total SNP loci (lower diagonal) and loci with genome specific alleles (upper diagonal) between isolates from different cultivars within the same orchard

	Bramley	Cox	Worcester
Bramley		160	7168
Cox	584854		0
Worcester	625550	605764	

Appendix A. Supplementary data

Supplementary table A. *Venturia inaequalis* isolates from Ash Farm, UK, a mixed cultivar orchard of Bramley, Cox and Worcester. Genomes of all isolates were sequenced on the Illumina MiSeq platform

Isolate ID	Host cultivar	MiSeq run	Amount of data from MiSeq run (bp)
05/007	Bramley	2	13136208
05/024	Bramley	4	12453319
05/025	Bramley	3	14340023
05/030	Bramley	4	11430665
05/036	Bramley	5	2029822
05/044	Bramley	2	19637369
05/049	Worcester[b]	1	13700931
05/057	Bramley	1	13336096
05/083	Cox	2	12003095
05/096	Cox	3	13063352
05/097	Cox	5	12123271
05/098	Cox	1	17982080
05/101	Cox	4	13983601
05/106	Cox	4	9259589
05/118	Cox	3	11758642
05/119	Cox	1	14438772
05/172[a]	Worcester	2	12672586
05/173	Worcester	5	19115153
05/182	Worcester	2	15752464
05/190	Worcester	5	13003150
05/196	Worcester	3	11826771
05/197	Worcester	4	13898993
05/199	Bramley[b]	1	21335557
05/202	Worcester	3	14365407

[a]The genome of isolate 05/172 was also sequenced by the PacBio platform and used to assemble the reference genome used in this publication. [b]Isolates were mislabelled during library prep; this was picked up and checked during data analysis and as such the isolate codes for this publication have been switched; i.e. the isolate in 05/199 in this publication is actually the isolate 05/049 from Bramley and vice versa

Supplementary table B. The whole genome sequence (WGS) of 14 *V. inaequalis* isolates (6 from Bramley, 8 from Worcester), sequenced on the Illumina Miseq platform, were aligned to the 05/172 reference genome (one of the 8 Worcester isolates). The total number of SNPs between the 14 isolates were called and then the number of genotype specific polymorphic alleles (GSAs) (i.e. isolates from Bramley shared the same allele at a locus but this differed to that shared among the Worcester isolates) in the whole genome, those just in genes and those nonsynonymous

Contig	Length (bp)	Total SNPs in B/W populations	Total GSA loci in genome	GSA loci in genes	Genotype specific nonsynonymous polymorphic loci in genes
1	3847617	39750	88	24	6
2	2883036	35428	367	162	53
3	2469270	25959	294	156	39
4	1643167	18383	88	31	10
5	1572910	16462	483	204	55
6	1553562	13901	48	21	6
7	1545189	15053	290	158	27
8	1540187	13278	339	307	79
9	1520579	11419	249	120	36
10	1471990	11801	18	4	0
11	1469107	18420	209	105	29
12	1466925	16430	79	32	12
13	1444683	16911	254	90	29
14	1434827	7843	47	11	7
15	1433712	13375	42	24	6
16	1432488	15795	149	73	19
17	1345551	15547	176	116	27
18	1224983	11025	22	11	5
19	1201024	13765	133	52	13
20	1189902	12346	529	259	55
21	989026	8262	12	5	3
22	960501	13438	94	48	18
23	953805	6543	61	40	3
24	887866	7589	2	1	1
25	878632	7455	87	38	5
26	877845	11043	11	0	0
27	830644	7224	45	30	4
28	802546	8038	59	48	7
29	775226	5108	49	32	3
30	757030	5133	1	0	0
31	757014	4206	42	13	6
32	742314	3749	62	24	4
33	713553	9208	46	5	4
34	702798	6598	10	4	3

35	667479	8316	14	11	3
36	664565	4321	0	0	0
37	642819	6378	170	94	17
38	633838	4775	3	0	0
39	631560	8285	399	158	36
40	616300	5535	8	0	0
41	615051	4108	0	0	0
42	612523	2330	0	0	0
43	606529	8417	25	13	8
44	599691	7232	81	45	25
45	572125	7163	237	171	62
46	570550	7497	257	151	28
47	548817	8366	270	193	33
48	544245	5585	231	141	34
49	531805	7819	42	36	9
50	510422	1584	0	0	0
51	486582	2049	2	1	1
52	483498	4553	1	0	0
53	467987	1618	0	0	0
54	448816	6344	175	98	48
55	403330	2362	1	0	0
56	400139	3177	18	11	5
57	386935	1347	1	0	0
58	359892	4218	107	61	20
59	347592	2998	1	0	0
60	334733	2927	0	0	0
61	334267	2826	1	0	0
62	316014	1835	0	0	0
63	307156	4892	4	0	0
64	259082	2355	1	0	0
65	255655	2879	235	152	37
66	249385	2827	2	1	1
67	246485	1439	77	37	13
68	243293	2172	112	93	17
70	239636	796	0	0	0
71	239618	1123	2	1	1
72	238937	2896	53	16	1
73	236519	2721	0	0	0
74	227977	1006	2	2	2
75	223988	1210	0	0	0
76	222240	1059	29	15	11
77	205344	1583	2	0	0
79	194393	1952	0	0	0
80	193792	10	0	0	0
81	190172	79	0	0	0
82	189157	1573	4	4	0

83	188550	1504	21	6	4
85	173860	1172	0	0	0
86	165300	764	0	0	0
88	156979	1207	1	0	0
89	148886	320	0	0	0
90	145307	1200	1	0	0
91	144156	783	6	6	5
92	144025	1500	69	44	12
93	142133	584	0	0	0
94	140254	568	0	0	0
96	133811	28	0	0	0
97	123707	1353	1	1	1
99	110078	497	0	0	0
101	98753	4	0	0	0
102	89478	278	1	1	1
120	60568	634	16	10	10
144	44087	12	0	0	0
145	43653	1	0	0	0
166	34764	21	0	0	0
183	29119	86	0	0	0
190	28319	12	0	0	0
Remaining contigs[a]	6144191	0	0	0	0
Total	72310420	625550	7168	3821	1019

[a]Contigs containing no SNPs, equating to 8.5% of the genome

Supplementary table C. Number in brackets is the maximum number of consecutive genotype specific allelic (GSA) loci in a contig from 1000 permutations of the allocation of GSA to positions occupied by a single nucleotide polymorphism; a lack of number on top row for each contig indicates there was no occurrence in the permutation for that number of consecutive GSA loci. The main entry (second row for each contig) is the observed number of consecutive GSA on each contig. Only contigs with any GSA loci presented

		Number of consecutive Genotype Specific Alleles									
Contig number	No. of GSAs	2	3	4	5	6	7	8	9	10	11
1	88	(3)	(1)								
		29	15	9	7	5	4	2	2	2	2
2	367	(11)	(1)	(1)							
		108	54	29	15	9	8	5	5	3	2
3	294	(12)	(1)								
		111	60	40	29	18	16	12	10	8	7
4	88	(4)	(1)								
		33	15	12	5	3	3	3	1	1	1
5	483	(26)	(4)	(1)							
		164	87	56	38	27	21	18	15	14	12
6	48	(3)	(1)								
		12	6	4	4	1	1	1	1	1	1
7	290	(15)	(2)	(1)							
		98	52	32	26	20	13	10	8	7	6
8	339	(19)	(2)	(1)	(1)						
		137	80	57	44	34	27	24	19	16	13
9	249	(13)	(2)	(1)							
		87	44	28	17	14	10	9	6	4	4
10	18	(1)									
		5	2	1	0	0	0	0	0	0	0
11	209	(8)	(2)								
		72	37	22	12	9	5	4	3	3	3
12	79	(4)									
		25	14	6	4	3	3	1	1	1	1
13	254	(11)	(2)								
		90	49	31	20	16	13	11	9	7	6
14	47	(3)									
		9	6	2	1	0	0	0	0	0	0
15	42	(2)									
		17	9	5	3	2	2	1	1	1	1
16	149	(5)	(2)								
		60	38	27	20	18	14	12	11	8	7
17	176	(7)	(2)								
		60	33	23	13	12	8	8	7	5	4

18	22	(2)									
		6	3	1	1	1	1	0	0	0	0
19	133	(5)	(1)								
		44	19	14	8	6	4	4	3	2	1
20	529	(37)	(6)	(1)	(1)						
		189	90	55	35	26	18	13	10	9	7
21	12	(1)									
		2	1	0	0	0	0	0	0	0	0
22	94	(5)	(1)								
		25	11	8	4	2	2	2	1	1	1
23	61	(3)	(1)								
		20	11	6	3	3	2	2	2	1	1
24	2										
		0	0	0	0	0	0	0	0	0	0
25	87	(5)	(1)	(1)	(1)						
		32	18	12	9	6	5	4	4	3	2
26	11	(1)									
		3	1	1	0	0	0	0	0	0	0
27	45	(4)									
		17	8	6	4	1	1	1	0	0	0
28	59	(4)	(1)								
		23	12	8	6	4	2	2	2	2	2
29	49	(3)	(1)								
		17	8	7	4	2	2	2	1	1	1
31	42	(4)	(1)								
		10	3	1	1	1	1	0	0	0	0
32	62	(5)	(1)								
		25	12	7	6	5	2	0	0	0	0
33	46	(3)	(1)								
		19	12	9	6	4	3	3	2	2	2
34	10	(1)									
		2	0	0	0	0	0	0	0	0	0
35	14	(1)									
		2	0	0	0	0	0	0	0	0	0
37	170	(13)	(3)	(1)							
		54	25	14	6	5	3	3	1	0	0
38	3										
		1	0	0	0	0	0	0	0	0	0
39	399	(30)	(4)	(2)	(1)						
		148	88	54	35	27	21	17	15	13	9
40	8	(1)									
		3	2	1	1	1	0	0	0	0	0
43	25	(2)									
		8	3	1	0	0	0	0	0	0	0
44	81	(5)	(1)								
		24	11	4	3	2	2	1	1	1	0

45	237	(15)	(3)	(1)	(1)						
		99	59	43	32	24	20	18	13	12	10
46	257	(18)	(3)	(1)							
		103	58	41	26	21	18	14	10	9	9
47	270	(17)	(3)	(1)							
		122	77	53	39	33	24	21	19	16	11
48	231	(20)	(5)	(1)	(1)						
		87	47	30	23	18	12	7	5	5	4
49	42	(2)	(1)								
		8	3	1	0	0	0	0	0	0	0
51	2	(1)									
		1	0	0	0	0	0	0	0	0	0
54	175	(14)	(3)	(1)							
		70	45	28	21	16	14	12	11	8	7
55	1										
		0	0	0	0	0	0	0	0	0	0
56	18	(2)	(1)								
		2	0	0	0	0	0	0	0	0	0
58	107	(9)	(2)	(1)							
		47	30	21	16	13	10	9	6	6	6
63	4	(1)									
		1	1	0	0	0	0	0	0	0	0
65	235	(31)	(7)	(3)	(1)	(1)					
		65	24	15	10	4	3	3	1	1	1
66	2										
		0	0	0	0	0	0	0	0	0	0
67	77	(10)	(2)	(1)							
		17	4	2	2	1	1	0	0	0	0
68	112	(13)	(3)	(1)	(1)						
		51	30	21	16	12	9	8	6	5	4
71	2										
		0	0	0	0	0	0	0	0	0	0
72	53	(6)	(1)	(1)							
		15	6	4	2	2	0	0	0	0	0
74	2	(1)									
		0	0	0	0	0	0	0	0	0	0
76	29	(4)	(1)	(1)							
		13	9	6	4	4	2	2	2	1	1
77	2	(1)									
		1	0	0	0	0	0	0	0	0	0
82	4	(1)									
		0	0	0	0	0	0	0	0	0	0
83	21	(3)	(1)								
		3	0	0	0	0	0	0	0	0	0
91	6	(1)									
		2	1	1	1	0	0	0	0	0	0

92	69	(9)	(2)	(1)							
		24	13	7	4	4	3	3	3	1	1
120	16	(3)	(1)								
		4	2	1	1	0	0	0	0	0	0

Supplementary information D. Input data of genotype specific allele (GSA) (1) and non-GSA SNPs (0) of contig 47 for GSA redistribution analysis of GSAs within a contig . Each number represent a SNP within the 14 isolates (6 from Bramley, 8 from Worcester) sequenced and aligned to the assembled genome of *Venturia inaequalis* isolate 05/172.

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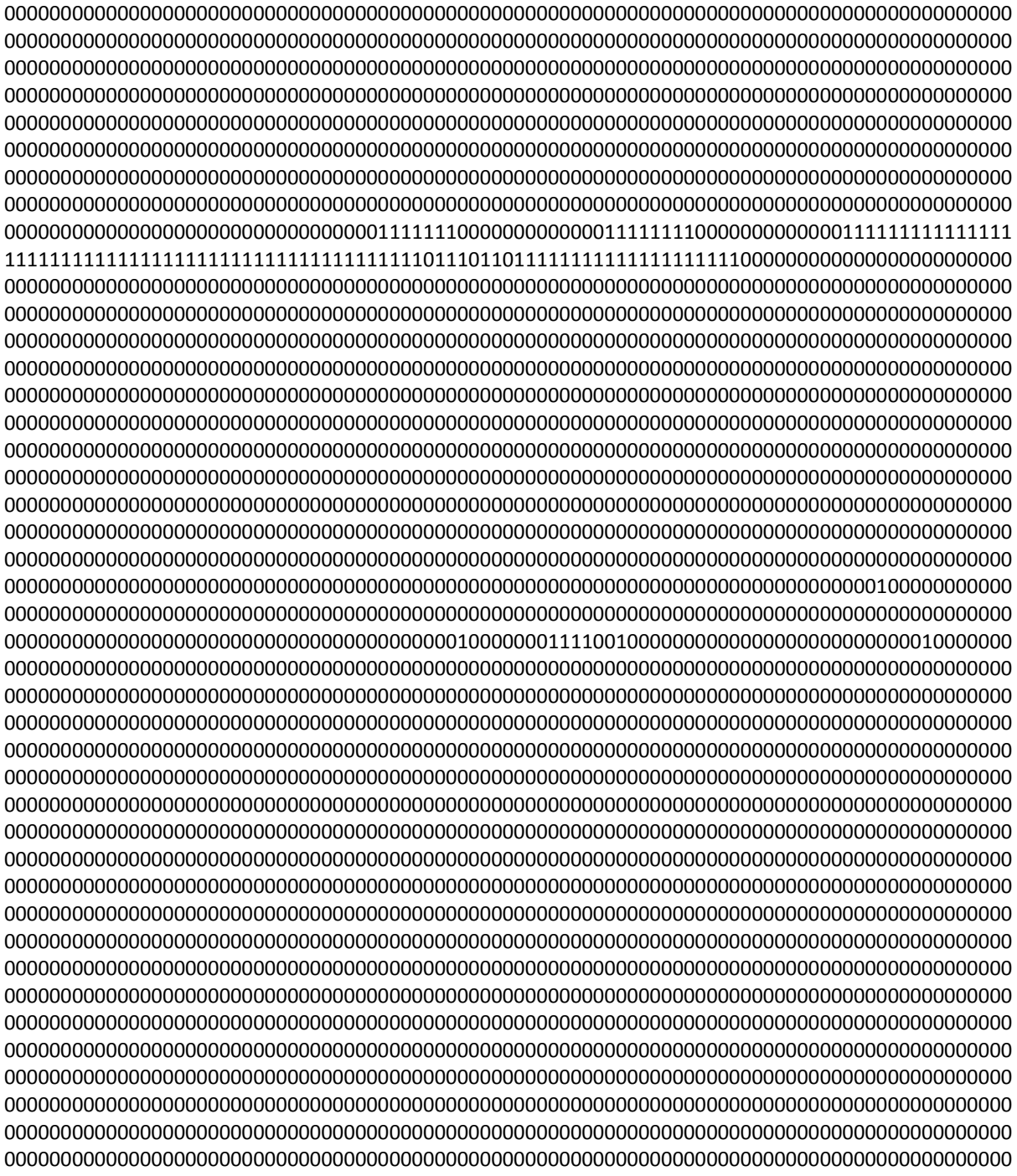

7 General discussion

7.1 General overview of findings

Although it has been shown that mixed cultivar orchards reduce the levels of scab compared to monoculture (Didelot *et al.*, 2007; Parisi *et al.*, 2013), the long term dynamics of the differing populations in the orchard have only been investigated by modelling (Xu, 2012). The results presented in this thesis show that the populations remain different and suggest a super race is unlikely to occur in the length of a commercial orchard. For a super race to occur either, many mutational events are required, which is unlikely, especially in the life of a commercial orchard, or, recombination is required between the strains differing in the virulence/pathogenicity in a population. In the discussion of this piece of work possible reasons were presented as to why sufficient sexual reproduction was not occurring for a super race to become dominant. Two of these reasons were investigated further.

Firstly the importance of overwintered conidia in primary inoculum of the pathogen was considered. The higher the proportion of primary inoculum from asexual conidial spores, the higher will be the proportion of primary inoculum that is genetically identical to lesions from the previous year. If the relative importance of the primary inoculum from sexually produced ascospores in the leaf litter is less than currently expected, a super race is expected to be less likely to occur. Although around 20 – 50 % of primary lesions were shown to have probably resulted from overwintered conidia in the orchard of study, the resulting 50 – 80 % of the remaining primary lesions resulted from ascospores. Therefore there should be sufficient recombination for a super race to occur if random mating of lesions had taken place as commonly assumed. Should a super race emerge in an orchard, with 20 – 50 % of primary infection coming from clonal conidia, it would likely increase the rate to dominance compared to primary infection from ascospores alone. As discussed in the paper on this work the finding is important in terms of orchard management and potential impact on population genetics; however, it is debatable whether it has much effect on the emergence of a super race.

The likely explanation as to why a super race of the pathogen is unlikely to occur and become dominant in an orchard is because there is insufficient sexual mating between

isolates from different cultivars. If isolates from different cultivars are unlikely to mate then the ascospores that form primary inoculum the following spring are likely to be from crosses of isolates with similar virulence; although the case of an intermediate cultivar is a potential issue as considered in the genotype specific allele (GSA) chapter discussion. It is likely that sex is initiated between isolates on the same leaf; however, an issue for future research is when mating is initiated relative to leaf fall. The results presented in this thesis would be more easily explained if sex is initiated between lesions on the same leaf before leaf-fall. If mating initials occur after leaf-fall it is possible that leaves from the same tree might be more likely to fall and mix on the orchard floor than to mix with leaves from all over the orchard, in the orchard of study this is less likely as the trees are tall and well-spaced allowing plenty of airflow in the orchard, thus likely to blow leaves around and mix them. Modelling leaf mixing would help answer this question and should be possible for this single orchard. However, to model turbulence would be difficult with the variation possible in the many different orchard designs and climates, meaning the model would need quite specific data from different orchards. However, if sex is initiated after leaf-fall it is still more likely that mating will occur between lesions on the same leaf than between mycelial growth of isolates on different leaves, which would require sufficient contact time for pseudothecia production. This is unlikely, especially in climates, such as northwest Europe (e.g. UK and Netherlands), where there is little snow cover stopping movement of leaves, but plenty of wind to move leaves around in the orchard.

For mixed cultivar orchards to remain viable without the emergence of a super race the selection of cultivars is imperative. Cultivar selection for mixed orchards cannot be guaranteed with molecular methods alone. For example, in 2013 the *V. inaequalis* population comparison with SSRs between Bramley and Red Falstaff showed they were significantly different, however, when trees of these cultivars were put into the same orchard the following year the pathogen populations from these two cultivars were not significantly different. So, although differences were found between the scab populations of some of the cultivars when in the same orchard, especially between populations on some of the cider cultivars, it would be remiss to say that these cultivars would make a good mix for a reduction of scab based purely on these results. However, it indicates that cultivars which have differing scab populations are likely to have differing resistance factors. Those

combinations of cultivars with differences can then be tested with cross inoculations as carried out by Barbara *et al.* (2008) between Bramley, Cox and Worcester.

Mixed cultivar orchards have been shown to work where one of the cultivars carries R gene resistance (Bousset *et al.*, 1997; Didelot *et al.*, 2007; Parisi *et al.*, 2013), with relative reductions greatest when the background disease pressure is lower. However, most of our work has been with susceptible cultivars, often in orchards with high disease pressure. Although in theory, an orchard with two susceptible cultivars with differing resistance, and therefore isolates from each that cannot cross infect, should give a reduction in the same way as an orchard with an R gene and a susceptible cultivar, this has not been tested. No scab incidence or severity assessment was done when sampling Ash Farm but there were plenty of isolates to sample from. It is, however, likely that this type of orchard with polygenic resistance will gill give more durable resistance than that which includes a single R-gene (Lê Van *et al.*, 2013).

It is possible to have single cultivars that do not have an R gene but do have polygenic resistance to *V. inaequalis* , and therefore show high levels of resistance to scab such as Discovery (Liebhard *et al.*, 2003; Calenge *et al.*, 2004). In St Monica's orchard, Sandford, we found that populations on some of the cultivars were different, but in more cultivars there was no scab, so population differences for those cultivars could not be determined. This was an assessment of primary inoculum in early summer and if we had returned later in the season we might have found lesions on those other cultivars. However, it shows that although the cultivars had very similar parentage there are potentially multiple combinations of QTL in play in the orchard. No scab was found on either Jenny or Hannah, and only a limited amount on Katy in the same orchard (although higher incidence was found in the Staunton-on-Wye orchard). All three of these cultivars share Worcester as a parent. It has been suggested that Worcester might have three resistance factors towards scab virulence (Barbara *et al.*, 2008). Worcester and another cultivar known for good scab resistance, Beauty of Bath, are the parents of Discovery.

Crop mixing can be a useful IPM tool for a decrease of insect pests (Tooker & Frank, 2012) as well as disease suppression. For example Parisi *et al.* (2013) showed evidence of a positive effect of mixture on Rosy apple aphid (*Dysaphis plantaginea*). The same investigation also suggested a benefit of mixture against powdery mildew (*Podosphaera leucotricha*), although significant reduction was only seen in one cultivar when mixed with sanitation practices.

However, the mixture was picked for apple scab and not powdery mildew, and therefore mixtures of other cultivars might give better control of powdery mildew; of course, a mixture where the cultivars have differing resistance for both scab and powdery mildew would be ideal but this makes selection even more difficult than for a single pathogen. Powdery mildew of apple occurs from asexual conidia of *P. leucotricha* with no observed sexual stage in the UK, therefore variability is only through mutations and the chance of a super race of mildew is not likely to be higher within a mixed cultivar orchard.

Presented in this thesis is the most complete *V. inaequalis* genome currently available. The genome size of 72 Mb is comparable, although in some cases larger than, genomes assembled by short read sequencing only, ranging from 40 to 72 Mb (Deng et al., 2017; Shiller et al., 2015). However, our assembly has a quarter of the number of contigs of these whole genome sequences (WGS). Repeat masking of the 05/172 isolate shows 38 Mb of non-repeated DNA, similar to that estimated previously (Bowen et al., 2011), so almost half the genome is repeated DNA. The majority of this repetitive material (98.7%) is made up of transposable elements. This is likely to explain why it is more difficult to obtain a good assembly for the *V. inaequalis* genome than similar ascomycetes. For example the apple canker fungal pathogen - *Neonectria ditissima* - has been sequenced and assembled by a comparable method to that reported in this study to give a genome of 45 Mb assembled over 48 contigs (Gómez-Cortecero – Personal communication). However, only 12% of the *N. ditissima* genome is repeat-masked (Gómez-Cortecero et al., 2015). Increased sequencing would give more depth but 238 contigs is still a long way from the 11 linkage groups (Xu et al., 2009) or seven chromosomes (Day et al., 1956) reported previously for *V. inaequalis*.

Having identified nonsynonymous GSA loci it should now be possible to see which genes they are present in and whether they code for proteins involved in the host-pathogen interaction. A number of different protein types have been implicated in pathogenicity. Fungal effectors suppress plant defence and facilitate pathogenicity (Lo Presti et al., 2015; Stukenbrock and McDonald, 2009); putative effector prediction is based on searching for small, cysteine rich proteins found in the secretome. Identification of these effectors could be used to discover host resistance factors which in turn could be a useful tool in disease resistance breeding (Vleeshouwers and Oliver, 2014). Gene clusters coding secondary metabolites, such as polyketide synthases (PKS) and non-ribosomal protein synthases (NRPS), play an important role in fungal-plant interactions (Pusztahelyi et al., 2015), while

carbohydrate active enzymes (so called CAZymes) are involved in plant cell wall degradation (Kubicek et al., 2014). If our nonsynonymous GSAs are enriched in these pathogenicity related genes it would provide evidence of how natural selection was involved in adaptive divergence to different apple cultivars.

7.2 The potential of mixed cultivar apple orchards

Apple production in the United Kingdom has significantly increased in the last decade, due predominantly to the increase of yield per unit area (FAOSTAT, 2016). It is imperative that crop yields continue to increase around the world, as highlighted by the Global food and farming futures Foresight project (Beddington, 2011). With uncertainty around what a post-Brexit Britain will look like, as well as a demand for less "food miles", it is important that the UK continues to be at the forefront of this push. The UK currently imports around two thirds of the apples purchased (DEFRA, 2017), with around 70% of those imports from the EU (International Trade Centre, 2018).

One of the cornerstones of increased yield is the control of pest and disease; however, this is likely to become more difficult with increased regulation of the use of pesticides and therefore alternatives need to be investigated. The work reported in this thesis has been looking at the population genetics and epidemiology of *V. inaequalis* in the context of mixed cultivar apple orchards. It is important to think about how these findings could fit in to apple orchards of the future, specifically related to apple scab control, but with many themes applicable to other pest and disease control.

7.2.1 Short term – Cider

Apple orchards supplying the cider industry, as opposed to dessert cultivars grown for eating, offer the most viable use of mixed cultivar orchards at the current time. The greatest differences seen between *V. inaequalis* populations were those from cider cultivars; therefore it is more likely that a mix of these cultivars would see a useful reduction in apple scab levels than a mix of dessert cultivars. Breeding for cider cultivars is not as intensive as that for dessert cultivars, which might explain the greater variability available in scab resistance. This could also mean that there are QTL available from cider cultivars for use in dessert cultivar breeding.

The majority of cider consumed is mixed variety and although each brand will have specific recipes, it would be easier for mixed cultivar orchards to be incorporated on their land, or by their growers, than it would for dessert cultivar growers who are likely to grow just a handful of cultivars specified by their market.

Scab control in cider orchards is important, as an early epidemic of infection of flowers, petioles or young fruit could lead to fruit drop and reduced yield, while a severe mid-season epidemic could lead to defoliation of trees and lead to poor tree health in subsequent seasons. High scab incidence could also lead to an increase in spoilt fruit through secondary infection of pathogens entering through cracked fruit from the scab lesions. However, unlike dessert orchards, where all the fruit must be blemish free, some scab lesions on the fruit from a cider cultivar would not be a problem and therefore cider orchards are less stringent in terms of fruit quality. As long as scab levels in the orchard are managed to an acceptable level, total control is not necessary and therefore the use of orchards with mixed susceptible cultivars with differing resistance is a viable option.

Traditionally the life of a cider orchard is longer than that of a dessert orchard as the market preferences do not change as fast. Breeding for dessert cultivars sees new cultivars introduced and replacing previously popular cultivars relatively frequently, although nowhere near as fast as annual crops. In comparison there is very little cider cultivar breeding. The trees in cider orchards are often grown on more vigorous rootstocks and therefore have much larger trees. This decreases the potential impact of a mixture as the Genotype Unit Area is larger and therefore self-infection is more likely (Wolfe, 1985). Cider orchards would perhaps be better served using semi-dwarfing or dwarfing rootstocks if they were to implement cultivar mixing for scab reduction, although if smaller trees led to more trees planted closer together the GUA would increase thus negating the advantage from using smaller trees.

Many of the issues with the implementation of any mixed orchard are still likely to be barriers towards growers of dessert, or cider, apples adopting these orchards. After selecting cultivars with differing resistance to scab for the benefit of reduced incidence, it is still necessary to consider how compatible the cultivars will be in a number of other phenotypic traits. As mentioned previously, scab levels would still need to be managed in a mixed orchard and although this might mean fewer fungicide treatments it is likely growers would still need to spray, especially if the cultivars being grown are very susceptible. This

will be made more difficult if development stages, for example in flowering and fruit ripening, are different, as sprays are often targeted at specific development stages and a need for precise timing would be even more likely if the aim was to use less sprays. Although most popular cider brands are mixed variety it would still be important to know what cultivar the fruit is coming from to fulfil certain recipes. This might rule out within-row mixtures but, as cider orchards are likely to be mechanically picked, row-by-row mixtures could be implemented with greater ease compared to dessert orchards that are picked by hand.

7.2.2 Medium term – Orchard management of the future

The increase in yield from the same land area in the UK over the last decade has been largely due to orchard intensification. Tree planting space has generally reduced and post and wire systems form more of a wall of trees trained to allow in sunlight, airflow and easier picking. This type of orchard is different to those used in the mixed cultivar studies presented here, where trees were generally spaced further apart. How these orchards might increase or decrease the ability of a row-by-row cultivar mixture to reduce levels of apple scab is debatable; in theory by the trees being a continuous block you have increased the Genotype Unit Area (GUA) thus the chance of conidial autoinfection is increased. However, these systems might also lend themselves to better pruning and tree architecture. Pruning of trees can be an important part of scab control, both in the summer (Cooley & Autio, 2011) and winter (Holb, 2005). Changing tree architecture has been shown to reduce levels of scab, although only in two of the three years investigated (Simon *et al.*, 2006). The effects of adapting pruning and architecture to reduce scab levels include decreasing humidity and duration of leaf wetness, both of which are important for the development of scab lesions, and increasing the deposition of fungicides on to the tree, so making them more effective. Summer pruning will remove some of the lesions from the tree, reducing rounds of secondary infection, while strong winter pruning has been shown to reduce scab levels the following year, presumably by removal of conidia overwintering in buds (Holb, 2005, 2008). It is believed that only conidia that overwinter in buds are viable in the spring and having shown in this thesis the potential importance that overwintering conidia can have as primary inoculum, it follows that pruning out these buds should lead to a decrease in scab

incidence. However it is impossible to know how many, and which, buds contain conidia. They cannot all be pruned out and therefore a future technology that could tell if there are viable conidia present in a bud would then allow its removal. This is unlikely to be in the form of hyperspectral imaging, a technology currently being widely investigated for its potential applications in plant health (Lowe *et al.*, 2017; Thomas *et al.*, 2018), including scanning scab inoculations on leaves (Nouri *et al.*, 2018), as it is not likely to pick up dormant spores in buds. Perhaps a new technology that could be fitted to a drone might be able to fly over an orchard and pick out particular 'hotspots' of conidia in bud, although imaging from such a distance might again be infeasible.

The likely use of drones in the more immediate future is to image an orchard during the growing season to aid disease pressure assessment, giving both earlier detection and more precise data to indicate concentrated outbreaks, thus allowing faster and more precise fungicide applications. Systems for imaging scab using drones, or unmanned aerial vehicles (UAV), are already being investigated (Wallhead, 2016).

As well as drones flying overhead, the orchards of the future might be more like an Amazon warehouse with robots moving up and down the rows of trees, replacing the need, and increasing lack, of human labour. Robots that can be programmed to know specific orchard designs would make mixed orchards more feasible, removing the human error in classifying fruit harvested from a mixture. There are a number of complex issues to be overcome, including recognition of ripeness and ability to pick the fruit without causing any damage. Minor damage is less of a problem in the cider industry where fruit is pressed soon after harvest and therefore some damage is not as important. A robot with imaging capability could pinpoint lesions to allow precision fungicide spraying, thus vastly reducing the volume of pesticide required, although this would only be viable if and when the price of the robots became low enough.

With increased technology the potential for mixed orchards in dessert apple production increases. However, the lack of sufficient differing resistance in popular susceptible cultivars would still remain the main obstacle to mixed cultivar orchards. For example, Gala is fast becoming the most widely grown cultivar in the UK, yet it is susceptible to almost all known scab isolates regardless of the host they were isolated from (Bus et al., 2011), despite it carrying two QTL for resistance (Soufflet-Freslon et al., 2008). It is therefore imperative that scab resistance be an important trait in apple breeding. Although scab resistance for a new

cultivar will be assessed it will not take precedence over shape, flavour and yield while the disease is successfully controlled by chemical treatment. However with the use of marker assisted breeding it would be possible to know earlier in the breeding process if known resistance factors for scab are present. Better understanding of genes involved in scab resistance would increase this potential further and allow removal early in the breeding process of any seedlings which do not have sufficient scab resistance factors. This understanding of the differing resistance between new cultivars could then be used in the selection of cultivars to mix.

7.2.3 Long term – Mixed monocultivar orchards

This section has been titled as long term, but this is largely due to politics rather than the time needed to develop technologies and therefore the time frame for implementation of the following ideas is completely unknown. In the European Union (EU), and any countries who want to trade with the EU, the regulations on the use of genetically modified organisms (GMOs) are extremely stringent and after a ruling in July 2018, now also include gene editing technologies such as the use of CRISPR-Cas9. After leaving the EU it is possible that the UK government might allow greater research into genetic modification (GM), especially in less contentious areas of the science such as cisgenesis. A cisgenic plant is defined by Schouten *et al.*, (2006) as "a crop plant that has been genetically modified with one or more genes (containing introns and flanking regions such as native promoter and terminator regions in a sense orientation) isolated from a crossable donor plant. In contrast, transgenic plants contain genes from noncrossable organisms (e.g. a selection marker gene originating from a microorganism), synthetic genes or artificial combinations of a coding gene with regulatory sequences, such as a promoter, from another gene." They argue that cisgenesis is akin to traditional breeding and thus should have less strict regulations. However, they also state "As the process of genetic modification itself may lead to mutations and rearrangements, cisgenic plants should be screened for unwanted changes in a similar way as plants derived from mutagenesis are screened and selected". However any attempt to implement such technologies into growing for the consumer is likely to face stiff resistance from organisations such as Greenpeace (and many others), as well as the EU if the UK wants to export crops to the continent. The opinion of the British public was heavily against GM

for much of the 1990s and the 2000s but polls in 2016 (Populus, 2016) and of Millenials in March 2018 (Populus, 2018) suggest opinion is swinging in favour of the "safe use" of GM crops. The science community needs to gain greater evidence that cisgenesis is as safe as traditional breeding and then disseminate that knowledge to the general public.

The main restrictions to using mixed cultivar orchards are the increased management problems of having cultivars with differing development stages and having apple varieties in the same orchard that need to be kept separate for market. Although some of these problems could be overcome with new ideas and technologies mentioned previously, the costs involved in the use of some of these are likely to be cost prohibitive, at least while the technology is new. Mixed cultivar orchards also need to have differing resistance factors and this is difficult to do with an industry that is slow to move away from cultivars that are traditionally popular. This is especially a problem with a cultivar as popular as Gala which is universally susceptible to races of scab and therefore of little use in a mixed orchard. A possibility would be a "mixed monocultivar" orchard where the cultivar type is the same through the orchard, but the trees have differing resistance factors (R genes or QTL) to scab within their genomes. This would allow the positive effects of a mixed cultivar orchard but with the ease of a monoculture orchard. Pyramiding of resistance genes to *V. inaequalis,* i.e. incorporating a number of resistance genes within a single cultivar, has been suggested as a way of breeding durable scab resistance (Gessler & Pertot, 2011). Laloi et al. (2017) showed pyramiding of three QTLs for resistance increased efficiency of resistance compared to any of the independent QTL. Although pyramiding is likely to make resistance more durable, should a virulent race develop on these trees a breakdown in the whole orchard is likely. However, having shown that populations in a mixed orchard do not become more alike, if resistance factors could be pyramided within trees and within an orchard (i.e. numerous resistance factors split among different trees), should a breakdown occur in one of the pyramided genotypes, it is highly unlikely that a super race which can overcome all the differing resistance factors would occur in the orchard.

There are two factors that need to be considered for the implementation of such an orchard. Firstly is the insertion of genes for resistance into the genome of a susceptible cultivar. The *Rvi6* (formerly *HcrVf2*) gene has already been inserted into Gala lines by cisgenesis (Vanblaere *et al.*, 2011). One of the cisgenic Gala lines was shown not to have full scab control compared to the traditionally bred cv. Florina carrying *Rvi6*, most likely due to

expression being 500 fold lower in the cisgenic line due to an issue inserting the promoter. However, a significant level of resistance was observed compared to wild type Gala (Vanblaere *et al.*, 2014). The same cisgenic line was then compared to wild type Gala for a number of other phenotypic traits. A few were significantly different but this was explained as a difference in the quality of budwood used for grafting (Jänsch *et al.*, 2014). Future insertions for good quality resistance will need to have similar gene expression to that in the traditionally bred cultivars they are isolated from and would need stringent testing to show that there are no significant downstream effects that cause significant changes to traits from the wild type, especially when concerning apple allergens.

The second factor for implementation of a mixed monocultivar orchard is the necessity for sufficient resistance factors to be identified. As well as the 18 gene-for-gene R-genes identified in *Malus* species against *V. inaequalis* (Bus *et al.*, 2011; Soriano *et al.*, 2014), a number of QTL have also been identified (Liebhard *et al.*, 2003; Calenge *et al.*, 2004; Soufflet-Freslon *et al.*, 2008). Understanding how these resistance factors, as well as those identified in the future, work against the pathogen is imperative in selection for implementation in pyramiding and/or mixed orchards. This is so that the resistance factors chosen are working in different ways to target the pathogen for better efficiency and also for the minimal chance of resistance erosion.

Beyond these strategies, and as a way to revive genes that have been overcome, Gessler & Pertot (2011) suggest that the identification and sequencing of avirulence genes of *V. inaequalis*, and their mutated alleles whose product is no longer recognised, will help to understand the binding patterns and changes that lead to non-recognition. They suggest that artificial resistance alleles could then be developed by making small changes in the Leucine Rich Repeat (LRR) recognition zones and selecting artificial mutations of the gene so the pathogen can be recognised again. With more whole genome sequencing of *V. inaequalis* isolates, as we have in this thesis, perhaps this idea is closer to feasibility.

7.3 Main Conclusions

- The populations of *Venturia inaequalis* from different cultivars within the same orchard can differ from each other and the difference does not reduce over time. This implies that a super race of the pathogen is unlikely to emerge and dominate in the lifetime of an orchard.
- One possible reason that a super race might only slowly emerge and dominate an orchard is that asexual conidia relative to sexually produced ascospores can contribute to the pathogen primary inoculum in spring. In the orchard of study an estimated 20-50% of primary infections are likely to have been from conidia.
- It is imperative that the cultivars present in a mixed orchard have differing resistance to scab for the mixture to have the desired reduction in scab levels. In this study differences were found between populations from different cultivars in the same orchard. The largest differences were seen in populations from cider cultivars in the same orchard, indicating the greatest difference in host resistance is likely to be in cider cultivars.
- A 72 Mb *V. inaequalis* genome assembled into 238 contigs and annotated is presented.
- Sexual reproduction between isolates from different leaves is much less than we commonly assumed (random mating). The present results can be explained by a much higher mating success rate among strains on the same leaves than between leaves. This is particularly the case if the sex is initiated before leaf-fall, which needs further confirmation.
- Mixed cultivar orchards are currently more likely to be implemented in the cider industry than they are in growing apples for eating. With increased technologies it might be possible for mixed cultivars to become a part of an integrated strategy for control of scab, as well as other pests and diseases, in all commercial settings.

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