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

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

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

[a]*East Malling Research, 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

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Abstract

The ascomycete *Venturia inaequalis* causes annual epidemics of apple scab worldwide. Scab development is reduced in mixed cultivar orchards compared with monocultures. To use mixtures in commercial production, we need to understand how the population of scab changes in a mixed orchard and how likely a super race, with virulence factors overcoming multiple resistance factors in the mixed orchard, is to emerge and become dominant. We used short 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 (six or seven 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.

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* x *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 re-iterative 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). However, 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,

62 known as gene pyramiding (Gessler *et al.*, 2006). However this process is slow and it may
63 take a long time before new scab resistant cultivars can be released commercially.
64 An alternative use for the difference in resistance factors between cultivars, including those
65 regarded as susceptible, is to plant these cultivars in the same orchards. Mixing cultivars of a
66 crop with varying resistance has been shown, predominantly in arable crops, to reduce
67 disease development compared to monoculture (Wolfe, 1985; Mundt, 2002). The potential
68 for mixed cultivar orchards to reduce scab development has been demonstrated by simulation
69 (Blaise & Gessler, 1994) and supported by field trials. Didelot *et al*. (2007) calculated the
70 area under the disease progress curve (AUDPC) on leaves in monoculture and row-by-row
71 mixed plots in two years. The AUDPC of scab leaf incidence, compared with the mean of
72 monoculture plots, was reduced in the mixture by 8.9% in the more severe year and 22.5% in
73 the less severe year. Parisi *et al*. (2013) found 9% of scabbed fruits at harvest in a mixed
74 orchard compared with a mean of 15% in pure stands in 2008, with the following year also
75 showing a reduced incidence in the mixed orchard (76% compared with 82% in the pure
76 stands). This study used a within-row mix as did Didelot *et al*. (2007). This is the most
77 effective mixture type, but in commercial orchards with current management methods an
78 intimate mix of this kind of mix is not economically feasible.
79 The management costs of a mixed cultivar orchard are likely to be higher than that of
80 monoculture due to differences between the timing of bud-breaking, flowering and fruit
81 development between cultivars, leading to complications in pest and disease control and
82 harvesting. As a result the benefit of reduced scab must both offset the increased management
83 cost and be long lasting relative to the life of commercial apple orchards (ca. 20 years). A
84 major concern in the use of mixed cultivars is that a ‘super race’ of scab, which has combined
85 virulence factors to overcome the differing resistance genes in the host cultivars, may emerge
86 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. We aimed 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 may have become dominant in the orchard. Otherwise, as suggested by simulation study (Xu, 2012), we may infer 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* 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. This orchard has never been sprayed or recently pruned and is ca. 45-50 years old. WM132 has a block of three rows of cv. Cox next to a block of three rows of cv. Royal Gala on M9 rootstocks. This orchard was not sprayed with fungicides, but pruned annually, and is ca.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 re-sampled 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 6 trees per cultivar (2 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 micro tube, 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 likely 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 micro tube 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 micro tube. 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 (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, followed by 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, followed by a final extension at 60°C for 30 min. One µl of PCR products (diluted 1/10 for 2005/6 samples, undiluted for 2012 samples) were 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, we also conducted statistical analyses 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, we assessed the significance of the interaction between years and cultivars by a permutation test as for the main year or cultivar effect. For a given dataset

186 (observed or permuted) the sum of squares (SS) due to the interactions was calculated as the
187 differences between among-cultivar-within-year SS and among-cultivar SS. The removal of
188 very rare alleles would have given a large amount of missing data for some loci and therefore
189 all data were used for AMOVA.
190 Arlequin version 3.5 (Excoffier & Lischer, 2010) was used to compute pairwise F_{ST}
191 between populations on each cultivar at each time point based on 110 permutations. Under
192 the null hypothesis of random mating among isolates from all cultivars, all pairwise F_{ST}
193 values would be expected to be similar. An Unweighted Pair Group Method with Arithmetic
194 Mean (UPGMA) tree was produced to present F_{ST} data using the software Mega (Tamura *et*
195 *al.*, 2013).
196 Structure 2.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003) uses a Bayesian approach to
197 determine the number of clusters (K) present in a set of individuals. Under the hypothesis of
198 random mating, there should be one population (i.e. K = 1); if there is sufficient population
199 differentiation, K is expected to be greater than one. To estimate the number of clusters in the
200 Ash Farm or WM132 orchards, an admixture model with correlated allele frequencies was
201 run 10 times, with a burn-in period of 10 000 followed by 50 000 Markov chain Monte Carlo
202 iterations for K = 1 to 10. Inference of the 'true' number of populations (K) was based on the
203 second order rate of change of the likelihood ΔK (Evanno *et al.*, 2005) and the test re-run
204 with 20 runs of 500 000 iterations after an initial burn-in of 50 000 iterations on a reduced
205 range of K values.
206 Multi-locus Linkage Disequilibrium (LD) was calculated for fungal populations from
207 individual orchards and from individual cultivars in a given year. When in linkage
208 equilibrium, the genotype frequency is equal to that of the product of the allele frequencies
209 (Liu & Muse, 2005). Powermarker software 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

We ran all statistical analysis 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 ($P < 0.0001$) but not for WM132 ($P = 0.7$). 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 ($P = 0.5$), but the populations from the three cultivars were clearly different ($P \leq 0.001$). There was no evidence for cultivar differences changing between 2005 and 2012, i.e. no significant interactions between years and cultivars ($P = 0.2$) (Table 4). F_{ST} pairwise comparison of populations on different cultivars showed Bramley in 2005 was distinct from those of both Cox and Worcester ($P < 0.001$), and the differences between the populations on Cox and Worcester were also close to statistical significance ($P = 0.04$). In 2012 the populations on the three cultivars remained different ($P < 0.01$); 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 (Figure 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 are distinctly different to those of Worcester, whereas samples from Cox appears to be an admixture between the two (Figure 2).

In WM132 AMOVA showed no evidence for significant differences between the samples from 2006 and 2012 ($P = 0.4$) or for differences between cultivars ($P = 0.1$). There was weak evidence for interaction between years and cultivars ($P = 0.06$) (Table 4). F_{ST} pairwise comparison suggested population differentiation between Cox and Gala ($P = 0.03$) in 2006 but not in 2012 ($P = 0.5$) (Figure 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 (Figure 3).

Assessment of random mating

The Ash Farm orchard population was in LD ($P < 0.001$) in both years. There was evidence for LD in the scab population on Bramley in 2005 ($P < 0.001$) but not in 2012 ($P = 0.2$). LD in the population on Worcester was significant in both years ($P < 0.05$) and appeared to have increased between 2005 and 2012. LD in the population on Cox at Ash Farm was clear ($P < 0.002$) 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, even with row alternation rather than the commercially impractical within-row mixing, is not inevitable, substantiating Xu's (2012) simulation study.

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

285 molecular population studies using AFLP and SSR markers (Xu *et al.*, 2013). The results
286 presented here from the 2012 resampling support the earlier findings with molecular markers
287 that showed scab populations on different cultivars, namely Bramley, Cox and Worcester,
288 were significantly different (Xu *et al.*, 2013). Further, isolates from one cultivar could not
289 necessarily infect leaves of the other cultivars in virulence tests, confirming distinctness
290 (Barbara *et al.*, 2008). Both previous studies showed that the scab populations on Bramley
291 and Worcester are the most different and present findings concur. Barbara *et al.* (2008)
292 suggest that there are at least one, two and three resistance factors in Bramley, Cox and
293 Worcester respectively. Therefore it could be conjectured that Bramley and Worcester do not
294 share any of these resistance factors while Cox could share a differing resistance factor with
295 each of these two cultivars. Although there has been much research into resistance mediated
296 by known R genes, only a few studies (Liebhard *et al.*, 2003; Calenge *et al.*, 2004) have
297 investigated quantitative resistance in cultivars not carrying a known major R gene, so there
298 is limited knowledge of hidden resistance factors present in susceptible cultivars. There was
299 no significant difference in the scab population of 2005 and that of 2012 at Ash Farm.
300 Although the gap in sampling (seven years) is short, relative to the life of commercial
301 orchards, it should be noted that this orchard is about 40-45 years old and has not been
302 subjected to any control measures. Although the scab populations on Bramley and Cox
303 appear to become more alike between the two sampling points, they were still significantly
304 different. The differences in scab populations between Worcester and the other two cultivars
305 appear to have increased, suggesting that the scab population, especially on Worcester, is
306 probably becoming increasingly adapted to specific cultivars. Although it is also possible that
307 other evolutionary forces, such as migration, are having an effect, adaptation is the simplest
308 explanation. We may therefore infer from these results that a super race has not prevailed in
309 the life of the Ash Farm orchard, which is around twice that of a commercial orchard.

310 The scab populations in WM132 did not differ between the two sampling years. The
311 Structure analysis failed to give a consistent result on the number of clusters, suggesting that
312 there is just one population present (K = 1). Although the populations on the two cultivars
313 were different in 2006, the multi-locus LD test indicates that the scab population in the
314 orchard was already in linkage equilibrium. This is most likely explained by the fact that Gala
315 is susceptible to almost all known scab isolates regardless of the host it was isolated from
316 (Bus *et al.*, 2011), i.e. 'universally' susceptible, despite carrying two QTL for resistance
317 (Soufflet-Freslon *et al.*, 2008). Thus isolates infecting Cox can infect Gala as well and
318 therefore recombination among these isolates can take place. It is also possible that the initial
319 scab founders of the orchard were randomly drawn from a randomly mating population and
320 the orchard population has not yet adapted to the cultivars present. The difference between
321 the cultivars in 2006 was not strongly significant and can be explained by the possibility that
322 a considerable number of isolates sampled from Gala in 2006 may not be able to infect Cox.
323 It may take a long time for a super race to form in an orchard depending on the nature of
324 mutations required and sexual reproduction. If several mutational steps are required,
325 formation of the genotype will be expected to be vanishingly slow (Hedrick, 2011). In this
326 case, appearance of a super race requires recombination between strains possessing different
327 sets of virulence factors. Only in the leaf litter does the annual sexual reproduction occur. It is
328 not known whether mating only occurs between lesions on the same leaf or whether mating
329 can occur between lesions on different leaves but physically in contact. If the former is true,
330 then a super race could only develop either, by multiple mutations or by an opportunistic
331 infection by non-adapted isolates in conditions where the effectiveness of resistance was
332 reduced; both routes to recombination are likely to be rare. If the mating between strains on
333 distinct leaves in the litter is possible, the chance that two infected leaves from different
334 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 was 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, we may conclude 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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Table 1 Number of scab lesions sampled from each cultivar in each of two mixed cultivar orchards in two years.

	Ash Farm			**W132**	
	Bramley	Cox	Worcester	Cox	Gala
2005/2006	36	27	31	20	23
2012	35	35	35	36	36

Table 2 Sequences (5'-3') for SSR primer pairs used to genotype apple scab isolates

SSR	Fluorescent label-Forward primer	Reverse Primer	Allele range[c]
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
[c]In this study

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

SSR	EMVi029			Vica9/X		Vica10/154		Vicacg8/42		Vict1/130		Vitc1/82		Vitc2/16		Vitg9/129	
	N_i[a]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]	Range[b]	N_a[c]
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: 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 brackets - the rare alleles removed from orchard dataset

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

Orchard	Terms	Degrees of freedom	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 x Cultivar	2	9.0	0.21
WM132	Years (2006 Vs 2012)	1	2.3	0.40
	Between cultivars	1	2.8	0.14
	Year x Cultivar	1	3.3	0.06

[a]based on 999 permutations

Table 5 Multi-locus 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

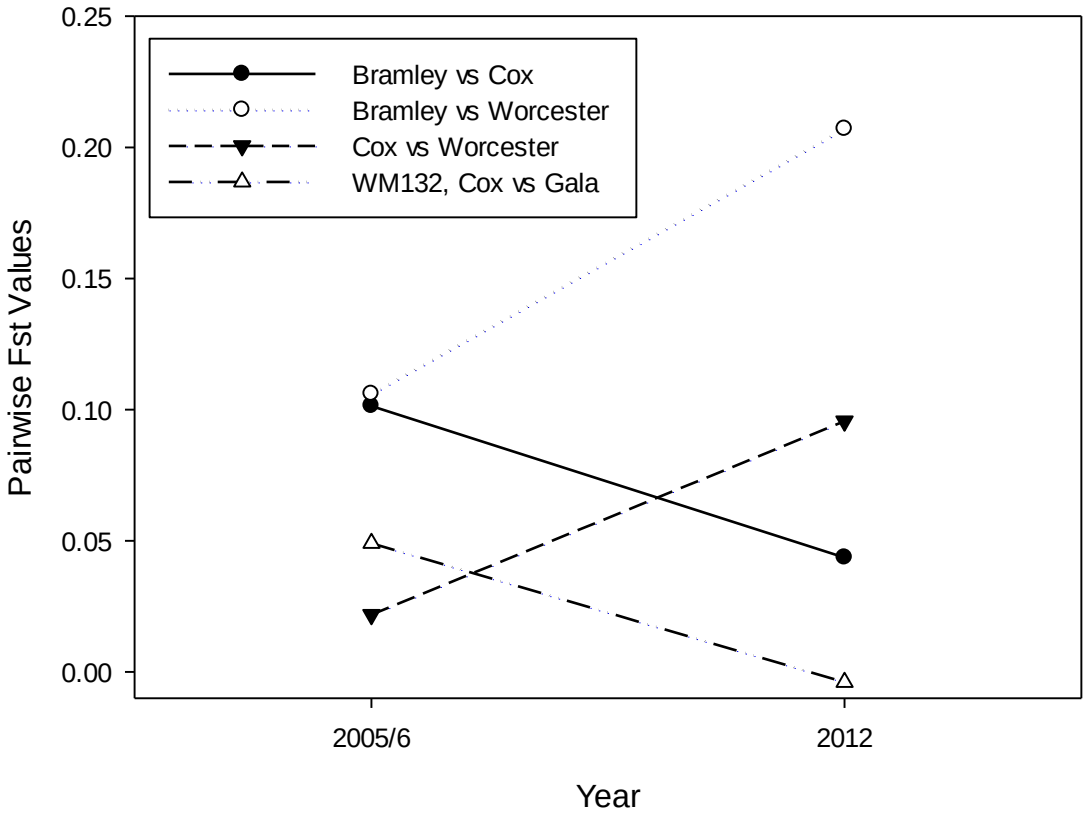

Figure 1 Pairwise differences (F_{ST}) between apple scab populations on different cultivars within the same orchard (Bramley, Cox, Worcester in Ash Farm; Cox and Gala in WM132) in 2005(Ash Farm)/2006(WM132) and 2012

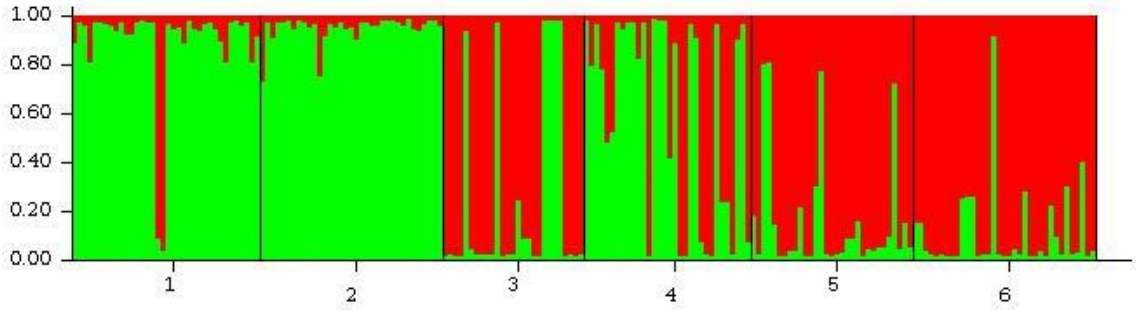

Figure 2 Structure bar plot assuming two populations (K = 2) in Ash Farm orchard sampled in 2005 and 2012, in which individual isolates were plotted as a vertical bar representing the probability of being from one (green) or the other population (red)**.** Population (x-axis): 1 = Bramley 2005, 2 = Bramley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

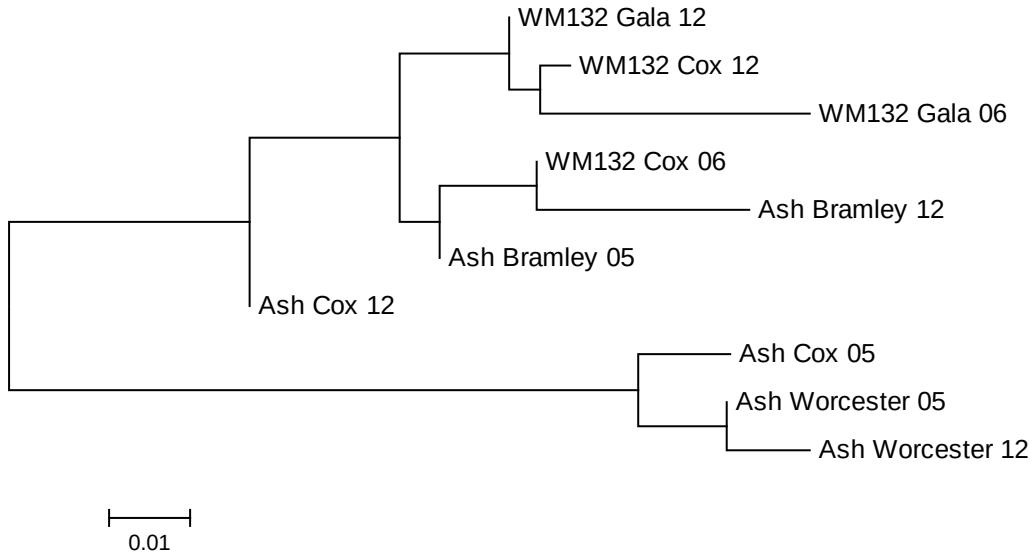

Figure 3 An UPGMA tree grouping scab populations based on similarity at eight SSR markers scored on samples from cultivars in two apple orchards ca. 200 km apart (Ash Farm sampled in 2005 and 2012; WM132 sampled in 2006 and 2012)