



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

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

3

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8

9 Running head: Apple scab population in mixed orchards

10

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12 race

## 13 **Abstract**

14

15 The ascomycete *Venturia inaequalis* causes annual epidemics of apple scab worldwide. Scab  
16 development is reduced in mixed cultivar orchards compared with monocultures. To use  
17 mixtures in commercial production, we need to understand how the population of scab  
18 changes in a mixed orchard and how likely a super race, with virulence factors overcoming  
19 multiple resistance factors in the mixed orchard, is to emerge and become dominant. We used  
20 short sequence repeat (SSR) markers to investigate the temporal change of scab populations  
21 in two mixed cultivar orchards in the UK to infer the likelihood of emergence of a scab super  
22 race. There were no significant differences between the populations at the two sampling times  
23 (six or seven years apart) in either of the two mixed orchards. In one of the orchards apple  
24 scab populations on different cultivars were significantly different and the differences did not  
25 diminish over time. These results suggest that it is not inevitable that a super race of *V.*  
26 *inaequalis* will become dominant during the lifetime of a commercial apple orchard.

27

## 28 **Introduction**

29

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

36 The pathogen survives the winter primarily as pseudothecia in the leaf litter. Rainfall in  
37 spring, around the time of bud break, causes release of sexually produced ascospores from the

38 leaf litter establishing primary infections on the new season's growth. It is also possible for  
39 the pathogen to overwinter as conidia in dormant buds (Becker *et al.*, 1992; Holb *et al.*,  
40 2004). Primary infection by ascospores or overwintered conidia leads to the production of  
41 conidia in the new lesions that form the basis of re-iterative secondary infection cycles  
42 (Bowen *et al.*, 2011).

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

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

56 Research in identifying and using major R-genes against apple scab has focused on genes  
57 from wild *Malus* species rather than domesticated apple, except for *Rvi1* from Golden  
58 Delicious (Bus *et al.*, 2011). However, seemingly susceptible cultivars may also contain  
59 resistance (Sierotzki *et al.*, 1994; Koch *et al.*, 2000; Barbara *et al.*, 2008) so that scab isolates  
60 from one cultivar may infect another susceptible cultivar weakly or not at all.. One method to  
61 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

87 ineffective as a means of managing scab. A fungal super race may result from a single  
88 mutation or series of mutations, but is more likely to result from recombination of the  
89 necessary virulence factors during sexual reproduction.

90 In this study, Simple Sequence Repeat (SSR) markers were used to genotype scab isolates  
91 from different cultivars in two mixed orchards at two different time points. We aimed to  
92 investigate the extent of differentiation between the scab populations on the different  
93 cultivars within an orchard over time. Should the populations on different cultivars become  
94 more alike over time it would suggest that a super race may have become dominant in the  
95 orchard. Otherwise, as suggested by simulation study (Xu, 2012), we may infer that a super  
96 race has not become dominant.

97

98

## 99 **Materials and Methods**

100

### 101 **Sampling**

102

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

111 Ash Farm and WM132 were sampled in 2005 and 2006, respectively (Xu *et al.*, 2013).  
112 Freeze-dried mycelia from single spore isolates were stored at -20°C and used in the present  
113 study. Both sites were re-sampled in the spring of 2012 when lesions had become visible  
114 from primary infection. At Ash Farm, leaves with freshly sporulating, discrete lesions were  
115 collected from shoots of each of 10 trees per cultivar and placed into paper bags until  
116 isolation. At WM132 a total of 15 shoots were collected from each of 6 trees per cultivar (2  
117 trees per row) and all leaves with discrete sporulating lesions were collected. For all leaves  
118 collected in 2012 a single discrete lesion per leaf was cut out with a 5 mm cork borer, placed  
119 in a 2 ml micro tube, left to air dry at room temperature and then closed and transferred to a -  
120 20°C freezer. Only a single lesion from any one shoot was used to extract DNA.

121

#### 122 **DNA extraction and screening**

123

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

133 The freeze dried mycelia or infected leaf disc were placed in a 2 ml micro tube with two 4  
134 mm ball bearings and disrupted in an MM2 oscillating mill (Retsch). DNA was then  
135 extracted using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions



136 with all optional steps. DNA was eluted with 100  $\mu$ l elution buffer into a 1.5 ml micro tube.  
137 DNA was quantified and quality-checked using a Nanodrop 1000 spectrophotometer  
138 (Thermo Scientific) and stored at -20°C.

139 The targets of a number of published SSR primer pairs (Tenzer *et al.*, 1999; Guérin *et al.*,  
140 2004; Xu *et al.*, 2009) were tested for polymorphism against a range of scab isolates.  
141 Following this primary screening eight SSR markers were selected to genotype the  
142 populations (Table 2). SSRs, labelled on the forward primer with either 6-FAM or HEX  
143 fluorescent dyes (Integrated DNA Technologies), were split into two multiplexes of four  
144 primer pairs. PCR was performed using 6.25  $\mu$ l Type-it microsatellite PCR master mix, 3.5  $\mu$ l  
145 water (both Qiagen), 1.25  $\mu$ l 2  $\mu$ M SSR primer mix, and 2  $\mu$ l DNA. Due to the high  
146 concentration of the DNA extracted from mycelium (2005/6 samples) the DNA was diluted  
147 1/10 before PCR, whereas the DNA extracted from leaf discs was added undiluted as the  
148 concentration was lower and a proportion of the DNA extracted will have been from the  
149 apple leaf. Touchdown PCR was performed on a DYAD thermal cycler (MJ Research) using  
150 the following cycle: an initial 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C  
151 for 90 s (decreasing 0.5°C per cycle until 50°C) and 72°C for 60 s, followed by a final  
152 extension at 60°C for 30 min. One  $\mu$ l of PCR products (diluted 1/10 for 2005/6 samples,  
153 undiluted for 2012 samples) were run on an ABI 3130xl sequencer with GeneScan 500 LIZ  
154 size standard (Life Technologies). Alleles were then scored using GENESCAN and  
155 GENOTYPER software conforming to the stepwise mutation model, i.e. to ensure allele sizes  
156 fit into a stepwise model (however, it should be noted that in practice nearly all alleles varied  
157 by integral multiples of the repeat length). PCR was repeated on any samples with no product  
158 for an SSR marker, alongside a positive control(s), so as to score a null allele, rather than a  
159 failed PCR, for that primer pair.

160

161 **Statistical analysis**

162

163 Allele frequencies for an orchard were calculated using Powermarker software (Liu & Muse,  
164 2005). Rare alleles,  $\leq 0.01$  of the population of an orchard (in the present study an allele that  
165 only appears once in the orchard), were recorded as missing values. These were removed  
166 from the data set as they contribute very little information towards assessing genetic diversity  
167 and population structure (Hale *et al.*, 2012). Null alleles were included as a single allele at a  
168 locus. Null alleles occur when a mutation in the flanking region of the sequence repeat stops  
169 the annealing of the primer and therefore no amplification during PCR, or when the SSR  
170 region is deleted, resulting in a very short fragment not scored. In the present study, null  
171 alleles were treated as a single allele for that marker, but they may in fact include different  
172 sequences. Therefore, we also conducted statistical analyses with all null alleles excluded. If  
173 there were two alleles at a locus it was assumed that the lesion had resulted from infection by  
174 more than one spore. If a sample only had one locus with two alleles, one was randomly  
175 selected for inclusion in statistical analysis. If a sample had multiple loci with more than one  
176 allele then the sample was discarded.

177 To assess if the scab populations in the two orchards had changed between the two temporal  
178 sampling points, population differentiation was assessed by a two-hierarchical level AMOVA  
179 (Analysis of Molecular Variance) (Excoffier *et al.*, 1992) using the Poppr package in R  
180 (Kamvar *et al.*, 2014). AMOVA was carried out separately for each orchard first with the  
181 ‘years/cultivars’ hierarchical structure, i.e. cultivars are nested within each year (2005 vs.  
182 2012 for Ash Farm, 2006 vs. 2012 for WM132) and then the ‘cultivars/years’ structure.  
183 Significance of population differentiation was assessed with a permutation test (a total of 999  
184 permutations). In addition, we assessed the significance of the interaction between years and  
185 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  $\Delta K$  (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

210 (1000 permutations) to infer whether random mating took place among specific groups of  
211 isolates.

212

213

## 214 **Results**

215

### 216 **General**

217

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

228 Two of the SSR markers used in this study, vitg9/129 and EMVi029, mapped at the same  
229 locus in the linkage map of Xu et al. (2009). The alleles were in strong LD at Ash Farm ( $P <$   
230  $0.0001$ ) but not for WM132 ( $P = 0.7$ ). Both markers were used in the subsequent analysis.

231

232

### 233 **Differences between populations**

234

235 Two-hierarchical level AMOVA of the Ash Farm data showed no significant differences  
236 between the scab populations sampled in 2005 and 2012 ( $P = 0.5$ ), but the populations from  
237 the three cultivars were clearly different ( $P \leq 0.001$ ). There was no evidence for cultivar  
238 differences changing between 2005 and 2012, i.e. no significant interactions between years  
239 and cultivars ( $P = 0.2$ ) (Table 4).  $F_{ST}$  pairwise comparison of populations on different  
240 cultivars showed Bramley in 2005 was distinct from those of both Cox and Worcester ( $P <$   
241  $0.001$ ), and the differences between the populations on Cox and Worcester were also close to  
242 statistical significance ( $P = 0.04$ ). In 2012 the populations on the three cultivars remained  
243 different ( $P < 0.01$ ); while the populations of Bramley and Cox were more alike than in 2005,  
244 the scab population on Worcester was more different from those on the other two cultivars  
245 (Figure 1). The inferred number of populations for Ash Farm, using the Evanno *et al.* (2005)  
246 method, is  $K = 2$ . If  $K$  is increased above two these clusters are subdivided but remain as  
247 homogeneous groupings without creating clearly distinct clusters, supporting the inference of  
248  $K=2$ . The scab samples from Bramley are distinctly different to those of Worcester, whereas  
249 samples from Cox appears to be an admixture between the two (Figure 2).

250 In WM132 AMOVA showed no evidence for significant differences between the samples  
251 from 2006 and 2012 ( $P = 0.4$ ) or for differences between cultivars ( $P = 0.1$ ). There was weak  
252 evidence for interaction between years and cultivars ( $P = 0.06$ ) (Table 4).  $F_{ST}$  pairwise  
253 comparison suggested population differentiation between Cox and Gala ( $P = 0.03$ ) in 2006  
254 but not in 2012 ( $P = 0.5$ ) (Figure 1). It was not possible to obtain a consistent peak in the  
255 (very low)  $\Delta K$  values to determine the number of clusters present in the WM132 orchard.

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

259

260

## 261 **Assessment of random mating**

262

263 The Ash Farm orchard population was in LD ( $P < 0.001$ ) in both years. There was evidence  
264 for LD in the scab population on Bramley in 2005 ( $P < 0.001$ ) but not in 2012 ( $P = 0.2$ ). LD  
265 in the population on Worcester was significant in both years ( $P < 0.05$ ) and appeared to have  
266 increased between 2005 and 2012. LD in the population on Cox at Ash Farm was clear ( $P <$   
267  $0.002$ ) and did not change much with time (Table 5).

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

271

272

## 273 **Discussion**

274

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

282 The Ash Farm orchard is the same as that used to collect samples for *in vivo* inoculation  
283 virulence testing (Barbara *et al.*, 2008), unfortunately these samples were not available for  
284 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



335 lesions on the same leaf. Whether or not the two exceptional processes occur, mating in the  
336 scab fungus favours recombination among strains infecting the same host.

337 Another important factor determining how likely a super race is to emerge and spread in an  
338 orchard is the proportion of primary inoculum resulting from conidia overwintered in buds  
339 and as wood scab. Conidia do not survive on leaves and fruits that have fallen to the orchard  
340 floor in the autumn (MacHardy, 1996). However they have been shown to survive the winter,  
341 predominantly on the inside tissues of buds and wood pustules (Becker *et al.*, 1992; Holb *et*  
342 *al.*, 2004). The survival of conidia and their impact in the following season as part of primary  
343 infection is dependent on factors such as weather, orchard management and the previous  
344 year's incidence (Holb *et al.*, 2005). The ratio of sexual to asexual spores as a source of  
345 primary inoculum also depends on the amount of leaf litter in the orchard. The higher the  
346 proportion of primary infection from asexual conidial spores, the higher will be the  
347 proportion of primary inoculum that is genetically identical to spores from the previous year.  
348 As a super race is most likely to develop from sexual reproduction in the leaf litter, if the  
349 relative importance of the primary inoculum from ascospores is less than currently expected,  
350 a super race is expected to be less likely to occur. However this also means that should a  
351 super race develop, significant primary inoculum from overwintered conidia would accelerate  
352 the race towards dominance in the orchard. The relative importance of overwintered conidia  
353 and ascospores as the dominant source of primary inoculum is likely to be region specific. In  
354 areas with warm winters, primary inoculum from conidia is most important (Boehm *et al.*,  
355 2003). The advantage of implementing mixtures is less if conidia are the predominant source  
356 of primary inoculum, as conidia are distributed by water splash and therefore are mainly  
357 likely to infect the same row and therefore the same cultivar (assuming row-by row mixing).

358 Should a fungal genotype be present with necessary virulence to infect multiple cultivars in  
359 the orchard it still does not mean it will inevitably become dominant. An increase in virulence

360 may come with a cost in fitness, as demonstrated in other pathogens (Bahri *et al.*, 2009;  
361 Montarry *et al.*, 2010). If the cost is sufficient, a super race may never dominate, or it may  
362 increase only slowly; if the emergence was longer than 20-30 years, it would still be  
363 commercially feasible to reduce scab by using mixed orchards.

364 This study demonstrated that differentiation between *V. inaequalis* populations on different  
365 cultivars did not decrease over time in mixed orchards, indicating that a super race, if present,  
366 has not become common. This agrees with inoculation studies of isolates from the mixed  
367 orchard and other monoculture orchards (Barbara *et al.*, 2008). Therefore, we may conclude  
368 that mixed apple orchards could be a feasible component of an integrated management  
369 scheme. Although the reductions of 10-30% in scab are modest, it is likely that mixed  
370 cultivar orchards are beneficial in managing other pests and diseases too (Parisi *et al.*, 2013).  
371 Implementation is particularly suited for cider and juicing apples because cosmetic damage is  
372 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.

	<b>Ash Farm</b>			<b>W132</b>	
	Bramley	Cox	Worcester	Cox	Gala
<b>2005/2006</b>	36	27	31	20	23
<b>2012</b>	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 <sup>c</sup>
EMVi029 <sup>a</sup>	HEX-ACGAGTCCCAGGTCTCACAG	TGTTGACGGTCACGGTGTAT	164-248
Vica9/X <sup>b</sup>	FAM-TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGGAAG	219-243
Vica10/154 <sup>b</sup>	HEX-CCTCCTTCCTATTACTCTCG	CTGAAGCGAACCTATGTCC	104-168
Vicacg8/42 <sup>b</sup>	FAM-TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	198-234
Vict1/130 <sup>b</sup>	FAM-GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	148-156
Vitc1/82 <sup>b</sup>	HEX-ACTGTCTCTAGGCGAAAG	ACTTGGAAGCTCGCTAAG	223-241
Vitc2/16 <sup>b</sup>	FAM-ACATTGACGAAGACGAGC	TACAATTGAGGCGTGTCC	153-169
Vitg9/129 <sup>b</sup>	FAM-CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	277-291

<sup>a</sup>Xu *et al.*, 2009<sup>b</sup>Guérin *et al.*, 2004<sup>c</sup>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 <sub>i</sub> <sup>a</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>	Range <sup>b</sup>	N <sub>a</sub> <sup>c</sup>
<b>Ash Farm</b>																	
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)
<b>WM132</b>																	
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)

<sup>a</sup>Number of scab isolates genotyped

<sup>b</sup>Range: smallest allele size – mode allele size (two numbers indicates two alleles present in equal numbers) – largest allele size

<sup>c</sup>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	<i>P</i> -value <sup>a</sup>
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

<sup>a</sup>based on 999 permutations

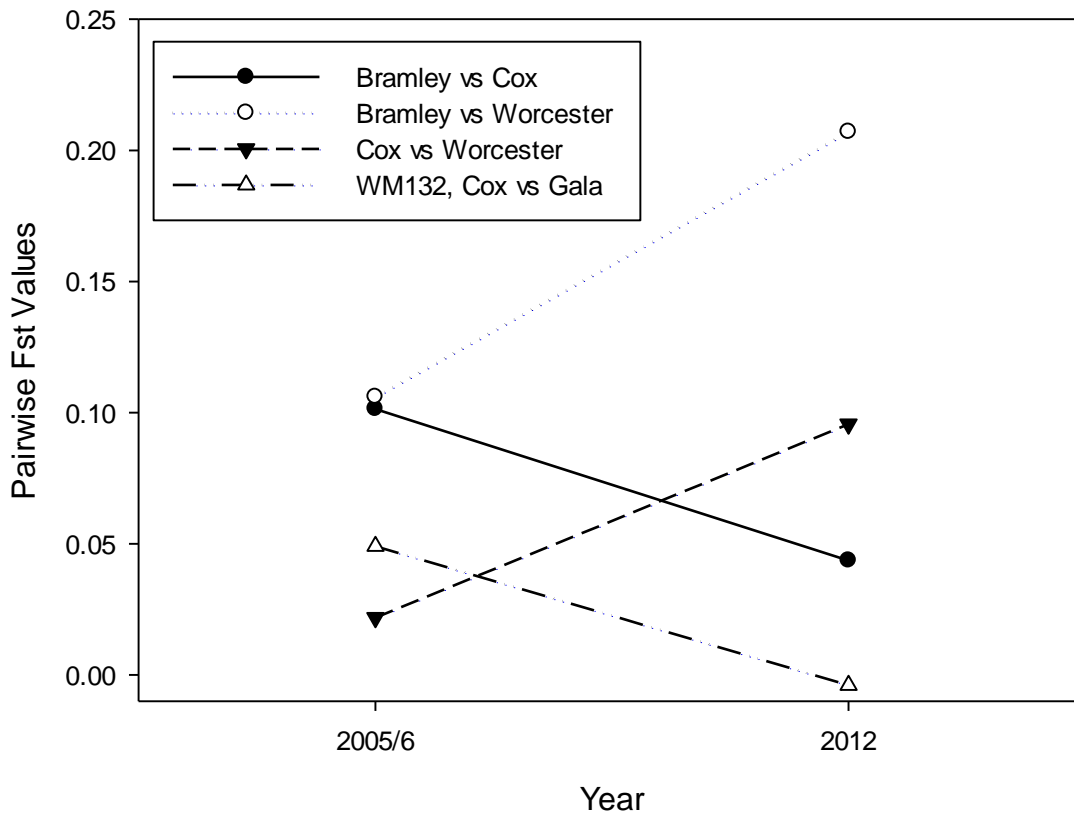
**Table 5** Multi-locus linkage disequilibrium test run on two orchard populations of apple scab and scab population on individual cultivars in two different years. Null hypothesis is the population is in linkage equilibrium

Orchard	Cultivar(s)	Year	<i>P</i> -value <sup>a</sup>	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 <sup>b</sup>
		2012	<0.01	8
	Worcester	2005	0.04	6 <sup>b</sup>
		2012	<0.01	6 <sup>b</sup>
WM132	Whole Orchard	2006	1.00	8
		2012	0.17	8
	Cox	2006	1.00	7 <sup>c</sup>
		2012	1.00	7 <sup>c</sup>
	Gala	2006	1.00	8
		2012	0.11	8

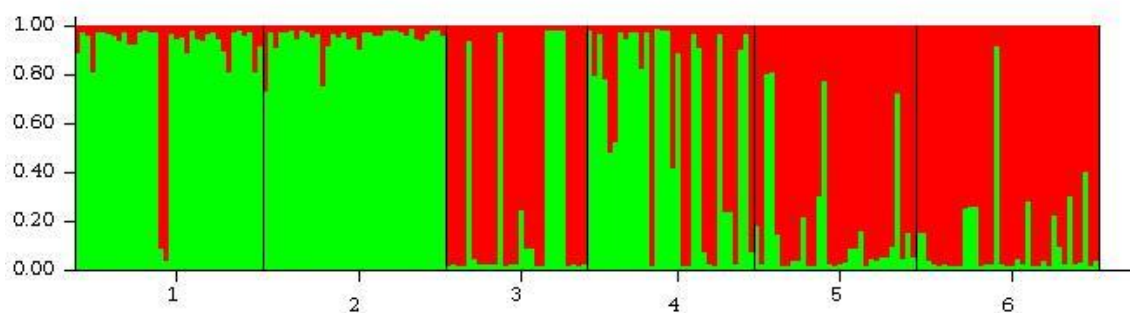
<sup>a</sup>Tests run on 1000 permutations;

<sup>b</sup>Full 8 loci could not be run due to lack of polymorphism in Vict1/130 and Vitc2/16;

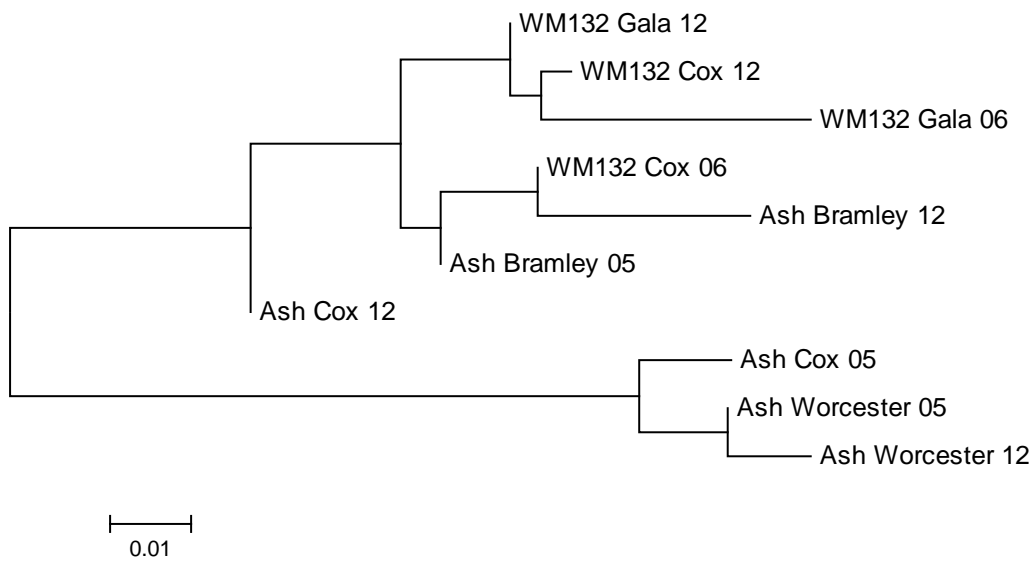
<sup>c</sup>Full 8 loci could not be run due to lack of polymorphism in Vitc2/16



**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)