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

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

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Running head: Non-random mating of apple scab fungus

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. Clustered GSA were observed in almost all contigs. This indicates population differentiation across the whole genome, presumably due to lack of crossing-over events between Bramley and Worcester isolates. This lack 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 from contact between independently infected leaves in leaf litter on the orchard floor. This would especially be the case if sexual reproduction is initiated before leaf-fall.

Keywords: Apple scab; apple black spot; ascospore production; super-race; host mixture

Introduction

The ascomycete *Venturia inaequalis* is the causal agent of apple scab, one of the most important diseases of apples worldwide (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 (Wolfe, 1985; Mundt, 2002). The potential for cultivar mixtures 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 cv. 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 combination 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 (Sierotzki *et al.*, 1994; Koch *et al.*, 2000). 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 genetic distances among the populations did not reduce over about a decade, indicating lack of recombination between isolates from different cultivars (Passey *et al.*, 2016). Several hypotheses could explain this. First, sexual reproduction could occur entirely or primarily between isolates infecting the same leaf, because they will be both physically close and having a long time for hyphae to meet and mate. Second, the mating between strains on fallen leaves is possibly more likely among leaves from neighbouring trees in the same row (i.e. same genotypes) than between leave s from different rows.. Third, but less likely, interactions between nearby genetic elements could render recombinants unfit. Finally, chromosomal rearrangements could prevent viable crossing over between certain genotypes, associated by chance with host specialisation.

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 identified those which had the same allele among isolates from a particular host cultivar, but had a different allele in all isolates from another cultivar; we refer to the alleles at these SNP loci as genotype (cultivar) specific alleles (GSA). We tested whether these GSA loci on each contig were randomly distributed in respect to isolate origin (i.e. cultivars) to infer the nature of mating among isolates from different cultivars.

Materials and methods

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 a non-dwarfing rootstock. 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 with no two isolates from the same shoot (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).

Library preparation and sequencing

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 9 nM before pooling and then diluted to 4 nM (libraries of 5 isolates). Denatured, pooled libraries at 20 pM were sequenced using 300 bp reads on an Illumina MiSeq.

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). Read depth was calculated per bp using aligned sequence reads using the SAMtools v.1.3.1 depth function, and Median read coverage determined from these values. 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 & 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, biallelic SNPs using the vcffilter function from vcflib and setting the following filtering options: Minimum quality (phred-scaled probability of a SNP occurring at the site) of 40, minimum MQ (phred-scaled probability that the read is mapped to the correct location) of 30, minimum depth of 10 and minimum GQ (phred-scaled probability that the sample genotype being called is correct, given that there is a SNP at that site) of 30. VCFTools (Danecek et al. 2011) was used to remove indels and missing data for genetic analyses.

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, based on all SNPs after removal of missing data but without quality filtering. A neighbour joining (NJ) tree based on 1000 bootstrap replicates was produced using the ape package in R and visualised using Figtree ver.1.4.3, to show unrooted phylogeny of the isolates after both removal of missing data and quality filtering.

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. 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.

Aggregation of GSA loci

Those remaining GSA loci after removal of missing data and quality filtering were analysed for aggregation. The number of GSA loci between Bramley and Cox, and between Cox and Bramley, was small. Thus subsequent aggregation analysis of GSA loci was only applied to the GSA loci distinguishing Bramley and Worcester isolates.

Number of GSAs within a contig: We wanted to assess whether the GSA loci in the Bramley and Worcester isolates were randomly distributed among and within the contigs, given the number of total SNP loci in each contig.

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 the distribution of 999 simulated variances to estimate a p-value.

***Distribution of GSA loci within a contig*:** If GSA loci have inter-dependent functions - whether to do with host specificity or not - we would expect selection to maintain them as blocks. The selection required would be weaker in relation to a given degree of crossing if the loci were close together (namely crossing-over events among these loci are less

frequently, hence more progeny ascospores will inherit the full set of loci). 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 & 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.

Results

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 (Supplementary Table A). 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). Due to stringent filtering of SNPs to allow analyses with only the best quality data, the NJ tree (Figure 2) shows some very closely

199 related isolates as identical, however when looking at unfiltered SNPs there are no isolates
200 that have an identical set of SNPs.

201 No GSA loci were observed between populations from Cox (7 samples) and Worcester (8
202 samples), while 160 GSA loci (0.03% of all SNP loci) were found between Cox and Bramley (6
203 samples) populations, and 7168 (1.15% of all SNP loci) between Bramley and Worcester
204 (Table 1). Of the GSA loci between populations from Bramley and Worcester, 3821 (53%)
205 were in the regions of predicted genes, of which 1019 (27%) were nonsynonymous. These
206 proportions are similar to those found among all SNPs: 50% in genes with 28% of these non-
207 synonymous. The breakdown of total SNP and GSA loci for individual contigs is given in
208 Supplementary table B.

209 **Aggregation of GSAs**

210 ***Number of GSAs within a contig between Bramley and Worcester*****:** After removal of contigs
211 with less than 100 SNP loci, 99.96% of SNP loci remained, across 92 contigs and covering
212 90.4% of the total genome length. The GSA loci were extremely aggregated in particular
213 contigs (Permutation test $P < 0.001$; Fig. 3).

214 ***Distribution of GSAs within a contig*****:** In all contigs with more than 100 SNP loci, the
215 observed number of consecutive GSA loci was much greater than the maximum of the
216 corresponding values in all the 1000 permutations (Supplementary Table C). For example, for
217 contig 47 (Supplementary information D) in 1000 permutations, two consecutive GSAs
218 occurred a maximum 17 times (122 observed), three consecutive GSAs a maximum three
219 times (77 observed) and just once four consecutive GSA loci (53 observed); but the observed
220 contig had a single run of 52 consecutive GSA loci.

221 Across all contigs, the most consecutive GSA loci observed in 1000 permutations was six,
222 on contig 65. In the data, the longest consecutive run of GSAs loci observed was on contig 8,

223 with 121 consecutive GSA loci. Contig 8 was 1.5 Mb long (2.0% of the genome) and has a
224 total of 13,278 SNPs within the combined Bramley and Worcester populations (2.1% of all
225 SNPs across the genome), of which 339 were GSA loci (4.7% of all GSAs across the genome).
226 However, 70% of contig 8 GSA loci were between positions 770024 and 781706, comprising
227 only 0.8% of the total contig length.

228

229 ## Discussion

230 We compared the DNA sequences of isolates of *V. inaequalis* taken from three different
231 cultivars within a single 50 year-old mixed orchard. Isolates from Bramley and Worcester
232 were clearly distinguishable by a large number of SNPs found on many contigs. This supports
233 previous evidence for separation between isolates derived from Bramley and Worcester,
234 based on artificial inoculation studies (Barbara *et al.*, 2008) and molecular comparisons using
235 SSRs (Xu *et al.*, 2013). However, few GSAs separated Cox from either Worcester or Bramley.
236 This also supports previously published evidence. Although the sample sizes in this study
237 were small and therefore some loci with apparent GSA will not actually host GSAs, the
238 permutation tests allow for this, and the lack of GSA between Cox and the other varieties
239 acts as a control for the level of this effect.

240 The mixed orchard where scab isolates were sampled has not received any sanitation or
241 leaf degradation management. Thus, there have been plenty of fallen leaves from all
242 cultivars on which sexual processes of *V. inaequalis* are believed to take place (MacHardy,
243 1996). Any explanation for the observed genetic structure needs to be consistent with the
244 fact that the populations studied here have been in spatial proximity for at least 50 rounds
245 of sexual reproduction. There are two broad classes of explanation. First, host specificity

could act as a barrier to gene flow between the populations by selecting for specific alleles and combinations of alleles. Second, the populations on each host may not in practice interbreed with populations from other hosts, preserving both chance founder effects and GSA not necessarily associated with host specificity. This could be for physical or genetic reasons. These mechanisms for intra-species differentiation as observed in the present study are similar to speciation in the *Venturia* genus on different host species (Le Cam *et al.*, 2002; Giraud *et al.*, 2010)**.**

We consider first whether host specificity can explain the results. 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). *In vitro* crossing has shown that 1)there are no physiological barriers to sexual recombination between isolates from Worcester and Bramley; and 2) that recombination of virulence factors producing isolates virulent on both Worcester and Bramley did take place (Barbara *et al.*, 2008).

GSA distinguishing Worcester and Bramley were spatially aggregated within all contigs. This would be consistent with recombination in the presence of selection for particular alleles at loci required for infection of a particular variety; aggregation would arise from linkage drag around these alleles. However, an over-whelming majority of contigs, covering >90% of the genome length, contain multiple GSA between Bramley and Worcester. If these were all caused by strong selection against hybrids - on both Worcester and Bramley but not Cox - there need to be multiple regions involved in host specialisation on all chromosomes. The nature of the specialisation would also have to be such as to prevent the offspring of Cox-Worcester or Cox-Bramley mating from infecting Cox, so that little or no indirect gene flow occurred between scab populations on Worcester and Bramley. Although all three

cultivars are commonly regarded as susceptible to scab, partial resistance evidently exists in all three. However, in the same orchard, seven out of 53 viable ascospores from three crosses between isolates from Cox, Bramley and Worcester could already infect both Bramley and Worcester with no obvious difference in lesion development (Barbara *et al.*, 2008). It is hard to reconcile this with a considerable fitness cost across ca. 90% of the whole genome.

A small proportion of overwintering inoculum could be conidia (Passey et al., 2017). However, this would not maintain GSA if sexual reproduction were able to move alleles from one variety to another. In addition, it is unlikely that asexual overwintering is restricted to a completely separate sub-group of the *V. inaequalis* population. It is more likely that some individuals overwinter as mycelia due to largely chance factors.

A final possibility is that chromosomal rearrangements, such as translocations or inversions, have led to lethality of some recombinants. If several epistatic loci are required for virulence on Worcester, rearrangements holding them together would be favoured. This is hard to test without a fully assembled genome. It would mean that the results here are not general, but depend on specific chromosomal rearrangements in these *V. inaequalis* populations.

Taken together, these arguments suggest that rather than being caused by selection in the presence of gene-flow, the genetic structure is consistent with an absence of (or very infrequent) recombination between isolates on different host cultivars. This is possible if sexual mating is more likely to succeed between isolates infecting the same leaf than between isolates from different leaves after leaf-fall. If sex is initiated before leaf-fall, only isolates with similar genetic backgrounds for virulence have the opportunity to mate. Even if sexual mating is initiated on fallen leaves, the chance of successful mating is still likely to be

much higher between isolates on the same leaf than isolates on different leaves, since sufficient close contact time to initiate the mating process will be much more likely. Further research is necessary to investigate when mating is initiated relative to leaf fall and the minimum contact time between isolates from different leaves required to initiate the mating process. If the hypothesis of minimal sexual mating among isolates that infected different leaves is correct, there are significant implications for understanding pathogen evolution and practical disease management, such as the timing of end-of-season control measures to reduce 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 was very low. This is consistent with the isolates of Cox being a mixture, with some isolates grouping more closely with isolates from Bramley and some more closely with isolates from Worcester (Fig 2). This hypothesis is supported both by artificial inoculation studies (Barbara *et al.*, 2008) and by population comparisons based on SSR markers (Xu *et al.*, 2013; Passey *et al.*, 2016). However, it means that, unless these two sub-populations are separated in some way, isolates virulent on both Bramley and Worcester via the Cox isolates as a mating partner and with the alternate allele at the genome specific allele should arise by recombination, giving rise to “super-race” isolates virulent on all the cultivars in the mixture. It is an important consideration in establishing a mixed cultivar orchard that the cultivars have differing resistance factors. Although we have no evidence for a breakdown in the difference between Bramley and Worcester in this orchard, this may be due to specific factors due to the particular varieties mixed. In particular, the maintenance of separate Cox-Bramley and Cox-Worcester groupings in the Cox population could be explained by chromosomal rearrangements, with the loci conferring virulence lying within an inverted or translocated region, which would give rise to lethal recombinants in crosses heterogeneous

318 for the rearranged regions. The proportion of the genome involved in, or affected by,
319 rearrangements would be extensive but not unprecedented (Raeside *et al.*, 2014; Shi-Kunne
320 *et al.*, 2018; Olarte *et al.*, 2019). As pointed out above, chromosomal rearrangements would,
321 of course, also be another explanation for the aggregation of GSAs. In general, therefore,
322 despite the present results, it would be prudent not to have a potential intermediate
323 cultivar.

324 Conidia are likely to have a role as part of the primary inoculum in some regions (Holb *et*
325 *al.*, 2004, 2005; Passey *et al.*, 2017). If climate of the study orchard does not favour
326 ascospore production and so only asexual clonal races of the pathogen are present, this
327 could also explain the differentiation between Worcester and Bramley, even with an
328 intermediate cultivar, Cox, present. However, this is highly unlikely. Although no
329 pseudothecia have ever been recovered in areas of Israel that lack the necessary lower
330 winter temperatures (Boehm *et al.*, 2003), there is no indication that this happens in
331 orchards in temperate regions and all the evidence is that ascospores are, at least, an
332 important source of inoculum in these areas.

333 If mating among scab isolates within an orchard is not random because the opposite mating
334 types have to be able to infect the same host, this would reduce the rate at which virulence
335 factors against different resistance genes can recombine. This could explain how populations
336 on different cultivars within the same orchard remain different after ca. 50 years of sexual
337 reproduction, though less obviously how the Cox population remains sub-divided.

338 The implementation of orchards with mixed cultivars of differing resistance, shown to
339 decrease levels of apple scab compared to monoculture (Didelot *et al.*, 2007; Parisi *et al.*,
340 2013), is a more attractive option if the risk of “super-races”, combining virulence on all
341 components of a mixture, emerging is much less than predicted on the assumption of

342 random mating across cultivars. The risk is further reduced if orchards are replaced after
343 short periods: current commercial apple orchards are replaced after only 15-20 years.

344 In summary, the findings presented here add to the evidence that isolates from specific
345 cultivars within a mixed orchard do not mate at random. We suggest that the most likely
346 explanation is a low frequency of mating between isolates initially infecting different leaves,
347 though other explanations are possible. This separation of sub-populations suggests that the
348 risk of super-races in mixed orchards may be low enough for mixtures that reduce apple scab
349 to remain viable for the lifetime of commercial orchards (15-20 years). A similar conclusion
350 would follow for other pathosystems requiring hyphal mating on living tissue.

351

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357

358 ## Data availability

359 The data that support the findings of this study are available from the corresponding author
360 upon reasonable request.

361

362 ## References

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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 platform.

Supplementary table B. The whole genome sequence (WGS) of 14 *V. inaequalis* isolates (6 from Bramley, 8 from Worcester) 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.

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.

Supplementary information D. An example 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.

Figure 1. Heatmap 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). Due to rounding percentage values, several isolates showed "100%" IBS; examining unfiltered SNPs showed that there are no isolates that have an identical set of SNPs.

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. Plots of standard deviation of (A) the number of Bramley-Worcester genotype specific allele (GSA) loci and (B) the number of Bramley-Worcester GSA as the percentage of total number of Cox-Bramley-Worcester GSA on each contig under the assumption of random distribution of all the GSA loci across all contigs. The 1000 values consisted of 999 from permutations and the observed values.

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)	Coverage[c]
05/007	Bramley	2	13136208	31
05/024	Bramley	4	12453319	33
05/025	Bramley	3	14340023	33
05/030	Bramley	4	11430665	28
05/036	Bramley	5	2029822	3[d]
05/044	Bramley	2	19637369	45
05/049	Worcester[b]	1	13700931	39
05/057	Bramley	1	13336096	10[d]
05/083	Cox	2	12003095	29

05/096	Cox	3	13063352	46[34]
05/097	Cox	5	12123271	27
05/098	Cox	1	17982080	52
05/101	Cox	4	13983601	37
05/106	Cox	4	9259589	24
05/118	Cox	3	11758642	9[d]
05/119	Cox	1	14438772	38
05/172[a]	Worcester	2	12672586	32
05/173	Worcester	5	19115153	48
05/182	Worcester	2	15752464	40
05/190	Worcester	5	13003150	33
05/196	Worcester	3	11826771	33
05/197	Worcester	4	13898993	36
05/199	Bramley[b]	1	21335557	54
05/202	Worcester	3	14365407	37

[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*.

[c]Coverage showing median read coverage per bp.

[d]Because of low coverage, these isolates were in population analysis.

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

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Supplementary table C. The top row for each contig 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; the main entry in bold (second row for each contig) is the observed number of consecutive GSA on each contig. Only contigs with any GSA loci presented

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

19	133	5	1	0	0	0	0	0	0	0	0
		44	***19***	***14***	***8***	***6***	***4***	***4***	***3***	***2***	***1***
20	529	37	6	1	1	0	0	0	0	0	0
		189	***90***	***55***	***35***	***26***	***18***	***13***	***10***	***9***	***7***
21	12	1	0	0	0	0	0	0	0	0	0
		2	***1***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
22	94	5	1	0	0	0	0	0	0	0	0
		25	***11***	***8***	***4***	***2***	***2***	***2***	***1***	***1***	***1***
23	61	3	1	0	0	0	0	0	0	0	0
		20	***11***	***6***	***3***	***3***	***2***	***2***	***2***	***1***	***1***
24	2	0	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
25	87	5	1	1	1	0	0	0	0	0	0
		32	***18***	***12***	***9***	***6***	***5***	***4***	***4***	***3***	***2***
26	11	1	0	0	0	0	0	0	0	0	0
		3	***1***	***1***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
27	45	4	0	0	0	0	0	0	0	0	0
		17	***8***	***6***	***4***	***1***	***1***	***1***	***0***	***0***	***0***
28	59	4	1	0	0	0	0	0	0	0	0
		23	***12***	***8***	***6***	***4***	***2***	***2***	***2***	***2***	***2***
29	49	3	1	0	0	0	0	0	0	0	0
		17	***8***	***7***	***4***	***2***	***2***	***2***	***1***	***1***	***10***
31	42	4	1	0	0	0	0	0	0	0	0
		10	***3***	***1***	***1***	***1***	***1***	***0***	***0***	***0***	***0***
32	62	5	1	0	0	0	0	0	0	0	0
		25	***12***	***7***	***6***	***5***	***2***	***0***	***0***	***0***	***0***
33	46	3	1	0	0	0	0	0	0	0	0
		19	***12***	***9***	***6***	***4***	***3***	***3***	***2***	***2***	***2***
34	10	1	0	0	0	0	0	0	0	0	0
		2	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
35	14	1	0	0	0	0	0	0	0	0	0
		2	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
37	170	13	3	1	0	0	0	0	0	0	0
		54	***25***	***14***	***6***	***5***	***3***	***3***	***1***	***0***	***0***
38	3	0	0	0	0	0	0	0	0	0	0
		1	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
39	399	30	4	2	1	0	0	0	0	0	0
		148	***88***	***54***	***35***	***27***	***21***	***17***	***15***	***13***	***9***
40	8	1	0	0	0	0	0	0	0	0	0
		3	***2***	***1***	***1***	***1***	***0***	***0***	***0***	***0***	***0***
43	25	2	0	0	0	0	0	0	0	0	0
		8	***3***	***1***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
44	81	5	1	0	0	0	0	0	0	0	0
		24	***11***	***4***	***3***	***2***	***2***	***1***	***1***	***1***	***0***
45	237	15	3	1	1	0	0	0	0	0	0
		99	***59***	***43***	***32***	***24***	***20***	***18***	***13***	***12***	***10***

46	257	18	3	1	0	0	0	0	0	0	0
		103	***58***	***41***	***26***	***21***	***18***	***14***	***10***	***9***	***9***
47	270	17	3	1	0	0	0	0	0	0	0
		122	***77***	***53***	***39***	***33***	***24***	***21***	***19***	***16***	***11***
48	231	20	5	1	1	0	0	0	0	0	0
		87	***47***	***30***	***23***	***18***	***12***	***7***	***5***	***5***	***4***
49	42	2	1	0	0	0	0	0	0	0	0
		8	***3***	***1***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
51	2	1	0	0	0	0	0	0	0	0	0
		1	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
54	175	14	3	1	0	0	0	0	0	0	0
		70	***45***	***28***	***21***	***16***	***14***	***12***	***11***	***8***	***7***
55	1	0	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
56	18	2	1	0	0	0	0	0	0	0	0
		2	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
58	107	9	2	1	0	0	0	0	0	0	0
		47	***30***	***21***	***16***	***13***	***10***	***9***	***6***	***6***	***6***
63	4	1	0	0	0	0	0	0	0	0	0
		1	***1***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
65	235	31	7	3	1	1	0	0	0	0	0
		65	***24***	***15***	***10***	***4***	***3***	***3***	***1***	***1***	***1***
66	2	0	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
67	77	10	2	1	0	0	0	0	0	0	0
		17	***4***	***2***	***2***	***1***	***1***	***0***	***0***	***0***	***0***
68	112	13	3	1	1	0	0	0	0	0	0
		51	***30***	***21***	***16***	***12***	***9***	***8***	***6***	***5***	***4***
71	2	0	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
72	53	6	1	1	0	0	0	0	0	0	0
		15	***6***	***4***	***2***	***2***	***0***	***0***	***0***	***0***	***0***
74	2	1	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
76	29	4	1	1	0	0	0	0	0	0	0
		13	***9***	***6***	***4***	***4***	***2***	***2***	***2***	***1***	***1***
77	2	1	0	0	0	0	0	0	0	0	0
		1	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
82	4	1	0	0	0	0	0	0	0	0	0
		0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
83	21	3	1	0	0	0	0	0	0	0	0
		3	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***
91	6	1	0	0	0	0	0	0	0	0	0
		2	***1***	***1***	***1***	***0***	***0***	***0***	***0***	***0***	***0***
92	69	9	2	1	0	0	0	0	0	0	0
		24	***13***	***7***	***4***	***4***	***3***	***3***	***3***	***1***	***1***

120	16	3	1	0	0	0	0	0	0	0	0
		4	***2***	***1***	***1***	***0***	***0***	***0***	***0***	***0***	***0***

Supplementary information D. An example 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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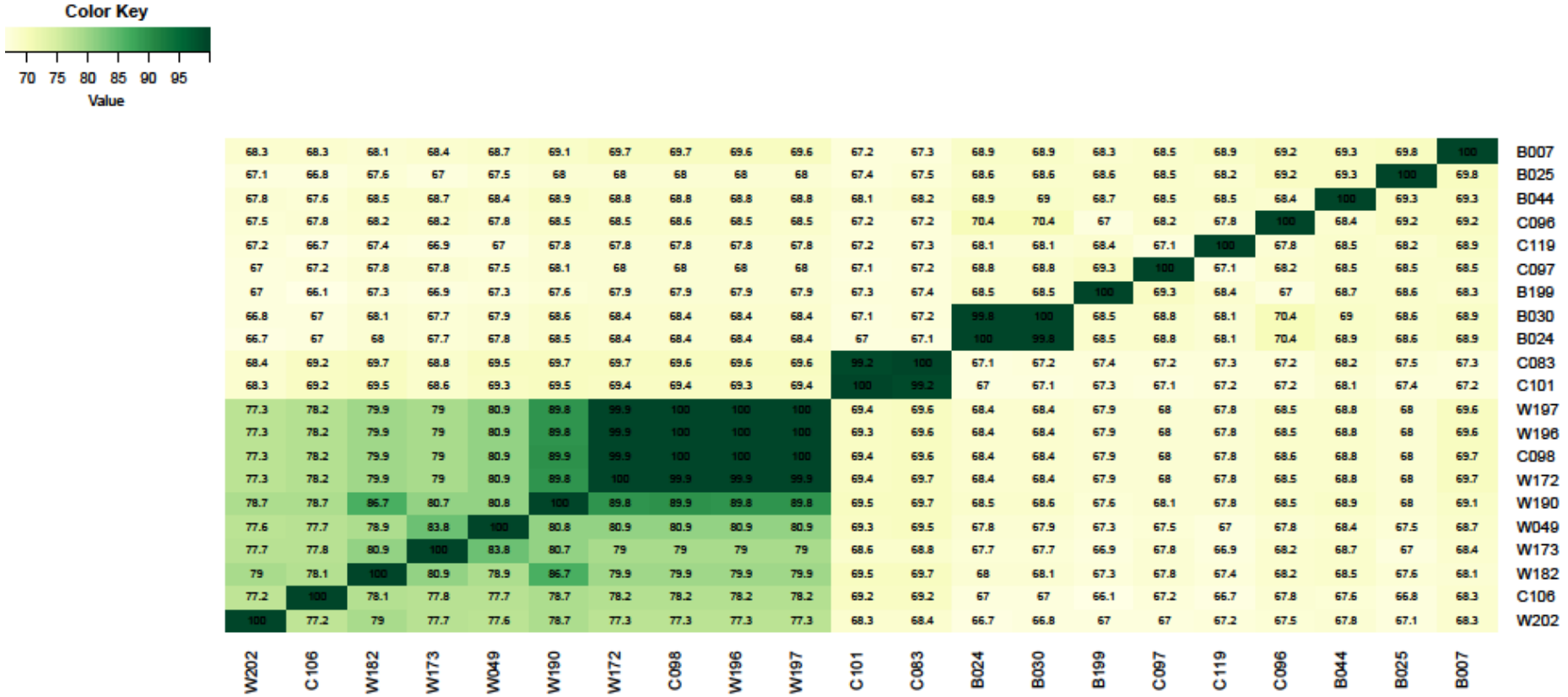


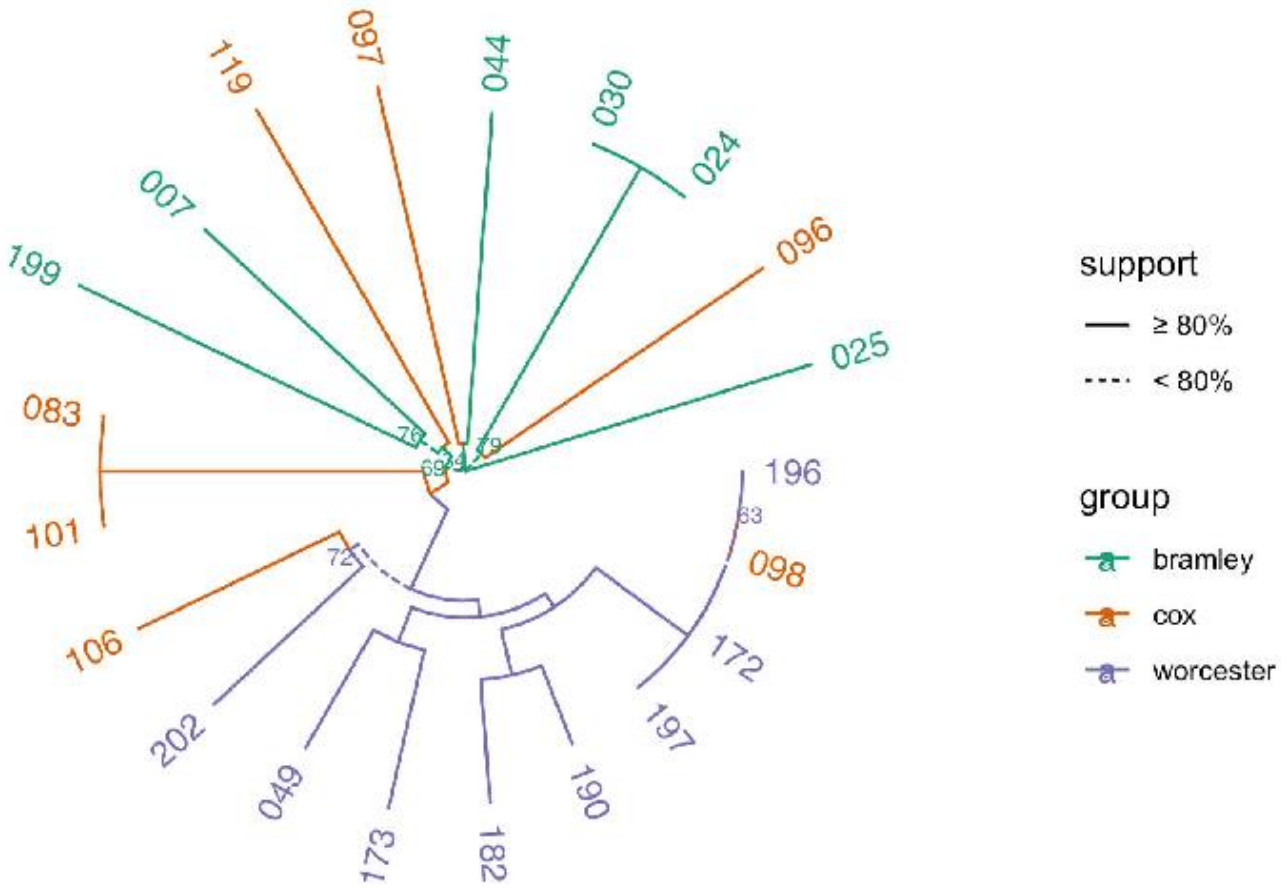
573

574 **Figure 1.** Heatmap to represent clustering of 21 *V. inaequalis* isolates from three different apple cultivars, Bramley (B), Cox (C) and Worcester

575 (W), present in the same orchard. Data from identity-by-state (IBS) calculated on the percentage similarity of shared alleles between samples

576 to produce a SNP matrix (the darker the shading the more alike the isolates). Due to rounding percentage values, several isolates showed

577 “100%” IBS; examining unfiltered SNPs showed that there are no isolates that have an identical set of SNPs.



578

579 **Figure 2.** A Neighbour joining (NJ) tree, in polar format, showing clustering of *Venturia inaequalis* isolates from three different apple cultivars,

580 Bramley, Cox and Worcester, present in the same orchard.

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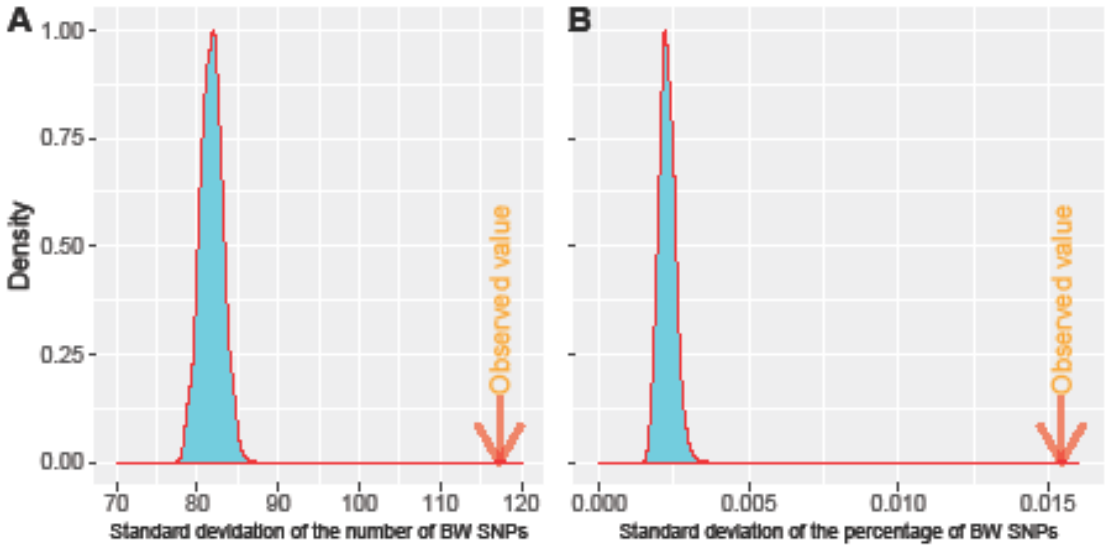

582

583 **Figure 3.** Plots of standard deviation of (A) the number of Bramley-Worcester genotype specific allele (GSA) loci and (B) the number of

584 Bramley-Worcester GSA as the percentage of total number of Cox-Bramley-Worcester GSA on each contig under the assumption of random

585 distribution of all the GSA loci across all contigs. The 1000 values consisted of 999 from permutations and the observed values.