New North American Isolates of *Venturia inaequalis* Can Overcome Apple Scab Resistance of *Malus floribunda* 821

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Abstract

Apple scab, caused by *Venturia inaequalis*, is a destructive fungal disease of major apple cultivars worldwide, most of which are moderately to highly susceptible. Thus, development of scab resistant cultivars is one of the highest priorities of apple breeding programs. The principal source of resistance for breeding programs has been the scab resistance gene *Rvi6* that originated from the Japanese crabapple *Malus floribunda* (Sieb.) sel. 821. Isolates of *V. inaequalis* able to overcome *Rvi6* have been identified in Europe, but have not yet been reported on the American continents. We recently discovered scab infection on *M. floribunda* 821 trees in a research orchard at Geneva, NY, U.S.A., where approximately 10% of the leaves bore profusely sporulating apple scab lesions, many of which had coalesced to cover entire leaves. We observed both chlorosis, typical of *Rvi6*, and pinpoint pitting symptoms typical of failed infections by *V. inaequalis* on hosts bearing the *Rvi7* gene. We assessed genetic diversity and population genetic structure of 11 *V. inaequalis*

isolates in total, of North American and European origin, isolated from *M. floribunda* 821, 'Nova Easygro', 'Golden Delicious', TSR33T239, 'Schone van Boskoop', and 'Prima', using 16,321 genome-wide SNPs. Population genetic structure and PCA separated the isolates into distinct European and U.S. groups. The foregoing suggests that the new *Rvi6* virulent isolates emerged within U.S. populations, rather than being transported from Europe. The complete resistance breakdown in *M. floribunda* 821 but not in descendant cultivars, which kept their field resistance, suggests that durable resistance to apple scab will require a more comprehensive understanding of *Rvi6* mediated resistance in diverse genetic backgrounds.

Keywords: Population genetic structure, virulence, pathogenicity, genome sequencing, disease management, cultivar/resistance, fungi, fruit, tree fruits

Apple scab, caused by the Ascomycete fungus *Venturia inaequalis* (Cke.) Wint., is the economically most important fungal disease of apples. In nearly all apple growing regions of the world, growers rely on fungicide applications each year to limit fruit quality and yield losses due to scab. The decreasing susceptibility of *V. inaequalis* populations to fungicides has led to a need for alternative pest control strategies. Distressingly, current IPM strategies could be counterproductive by expressing heavy selection pressure on fungicide resistance (Beckerman et al. 2015). Recently, *V. inaequalis* isolates were reported to have resistance toward multiple fungicides without having been affected by significant fitness cost (Chapman et al. 2011). Scab-resistant cultivars require fewer fungicide applications, and hence are of high importance to organic production systems and home gardens (Brown and Maloney 2008). However, the commercial success of scab-resistant cultivars has been limited because of their generally low fruit quality. The need to control scab without total reliance upon fungicides has therefore made development of scab resistant cultivars with high fruit quality one of the highest priorities of apple breeding programs.

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The vast majority of the modern scab resistant cultivars derive from a Japanese crabapple clone, *Malus floribunda* (Sieb.) sel. 821, which was found to be completely free of scab in the beginning of the twentieth century. At the University of Illinois, C. S. Crandall built a collection of crabapples and, among other hybridizations, crossed two siblings from a *M. floribunda* 821 × 'Rome Beauty' progeny (Crandall 1926). Two accessions from this cross progeny were later selected for their exceptional scab resistance yet large fruit size, and were used as resistance donors by the PRI (Purdue University, Rutgers University, and the University of Illinois) cooperative breeding program. The first PRI cultivar, 'Prima' was released in 1967, and since then several breeding centers have developed resistant cultivars by using PRI materials (Crosby et al. 1992). Currently, there are more than one hundred modern scab resistant apple cultivars, with approximately 90% of them carrying the resistance derived from *M. floribunda* 821 (Brown and Maloney 2008, 2013).

To date, 18 *V. inaequalis* resistance genes (*Rvi* genes) including *Rvi6* and *Rvi7* from *M. floribunda* 821 and genes from other *Malus* accessions have been described. Gene-for-gene relationships are assumed for the majority of them (Broggini et al. 2011; Bus et al. 2011; Khajuria et al. 2018). Based on the segregation, the resistance of *M. floribunda* 821 was also thought to rely on a single dominant gene, previously called *Vf* (syn. *Rvi6*), exhibiting resistance symptoms from hypersensitive reaction to chlorosis (occasionally necrosis or slight sporulation). However, segregation analysis of the resistance symptoms suggested the presence of an additional gene for scab resistance (Parisi et al. 1993; Williams and Kuc 1969). A large-scale pathogenicity study on a 'Golden Delicious' × *M. floribunda* 821 progeny demonstrated the presence of the *Vfh* (syn. *Rvi7*) gene responsible for the hypersensitive reaction in *M. floribunda* 821, which was distinct from the original *Rvi6* (Bénaouf and Parisi 2000). The *Vfh* gene has been lost early on in the breeding process, or has been overcome by the pathogen (Bénaouf and Parisi 2000; Gessler 1989; Parisi and Lespinasse 1996).

Although the resistance conferred by a single major gene can be easily inherited, as preferred by breeders, the breakdown of major genes as a result of the fast evolution of a pathogen has been documented many times in several host-pathogen systems (Pink and Hand 2003). For instance, chlorosis with slight sporulation was observed on 'Prima' (*Rvi6*) in the greenhouse trials even before its release, whereas *Rvi6* cultivars remained resistant under field conditions. Thus, the resistance conferred by *Rvi6* presents as partial resistance when the environmental factors are optimal for disease development, although the 1:1 segregation ratio of resistance expected from a single major gene was only observed if progeny expressing necrotic, slightly sporulating symptoms were still classified resistant (Crosby et al. 1992; MacHardy 1996). It has been demonstrated that the deviation from the expected segregation ratio is caused by sublethal genes (*sl1* and/or *sl2* in case of the presence of a third gene *sl3*) that are linked to the *Rvi6* region (Gao and Van De Weg 2006). In Europe (Ahrensburg, Germany), sporulating lesions were detected in the field on 'Prima' in 1984, and in 1988 on 'Prima' and 'Coop' 7, 9, and 10 in the same orchard, although 'Prima' and 'Coop' 7, 9, and 10 were all resistant in the greenhouse. The inoculum from Ahrensburg and monoconidial isolates were used for testing various *Rvi6* cultivars/selections, all of which were susceptible to the new isolates, but the original source of the resistance, *M. floribunda* 821, remained intact (Parisi et al. 1993). The breakdown of the resistance of *M. floribunda* 821 (carrying both *Rvi6* and *Rvi7*) was reported later in East Malling, England (Roberts and Crute 1994). Interestingly, the isolates overcoming the resistance of *M. floribunda* 821 were avirulent on 'Golden Delicious' and on its resistant descendants (e.g., 'Prima' and 'Florina') harboring the ephemeral *Rvi1* gene. However, it is still unclear whether the isolates were unable to overcome the resistance of these cultivars due to the *Rvi1* gene, or other genetic factors prohibited the adaptation of the pathogen (Gessler and Pertot 2012; Roberts and Crute 1994). It was reported that the background resistance of *M. floribunda* 821 is low, and its severe infection happens when the two major genes *Rvi6* and *Rvi7* have been overcome. This also suggests that the *Rvi6* resistant cultivars infected by less aggressive isolates might possess more complex resistance than those inherited from *M. floribunda* 821 (Caffier et al. 2010; Parisi et al. 2004).

Further population genetic studies suggested that European *Rvi6*-virulent isolates did not emerge from a recent mutation in the *AvrRvi6* gene, but have pre-existed for thousands of years in wild crabapple reservoirs, without relevant gene-flow to populations infecting domesticated apples. The virulent isolates on *Rvi6* cultivars are genetically less variable, but distinct from those collected from susceptible cultivars, suggesting a genetic bottleneck in their recent history (Guérin and Le Cam 2004; Guérin et al. 2007; Lemaire et al. 2016; Michalecka et al. 2018). Although the *Rvi6* virulent and nonvirulent lineages might have been separated for thousands of years, the emergence of *Rvi6* cultivars in Europe allowed gene flow between the two lineages, which might have increased the speed of the pathogen's adaptation, especially in orchards where susceptible and resistant cultivars coexist (Michalecka et al. 2018). Human facilitated transport might represent another factor for the spread of *Rvi6* virulent strains,

and a possible cause of the inconsistency between the geographical origin and genetic polymorphism of isolates in several studies (Gladieux et al. 2010; Guérin et al. 2007; Kaymak et al. 2016; Michalecka et al. 2018).

New isolates that can overcome the resistance of *M. floribunda* 821 have not yet been reported on the American continent, but the breakdown of the resistance was presumed by Beckerman from 2007, after seeing scab symptoms on *M. floribunda* 821, but never confirmed. Later in 2008 and 2009, scab-like symptoms were documented on 'Pristine' (*Vf*) and other *Vf* accessions ('Pixie Crunch', 'Jonafree', 'Enterprise') (<https://www.extension.purdue.edu/extmedia/BP/BP-76-W.pdf>). To monitor the race comparison of *V. inaequalis* in North America, a 33-year-long assay was carried out in the Secrest Arboretum (Ohio), where heavy sporulation was observed on a Japanese crabapple tree in 2003 and 2005, although its resistance was effective for decades previously. The authors, however, did not confirm whether the accession was identical to *M. floribunda* 821 (Beckerman et al. 2009). In this study, we report new *V. inaequalis* isolates collected from the infected *M. floribunda* 821 trees grown in an apple orchard, their origin and genetic diversity, and their pathogenicity on *Rvi6* resistant 'Macfree'.

Materials and Methods

Field assessment of apple scab symptoms. In June 2019, apple scab infection was observed on approximately 20-year-old trees of *M. floribunda* 821 and scab resistant apple cultivars 'Prima' and 'Nova Easygro' grown in the research orchard Darrow Farm (Geneva, U.S.A.). In addition, four *Rvi6* resistant PRI cultivars, 'Dayton', 'Jonafree', 'Priscilla', 'Redfree', as well as 'Gala', 'Macintosh', 'Golden Delicious', and the French resistant cultivar 'Florina' were present within the same orchard. The trees from each genotype were present in four replications in a randomly distributed pattern across the entire orchard space. The orchard was maintained without any fungicide spray since 2017 (Table 1).

Apple scab incidence was assessed using the 0–9 grade scale of Lateur and Populer (1994) as follows: 0 - no observation (missing plant), 1 - no visible lesions, 2 - one or very few lesions detectable on close scrutiny of the tree (0 to 1%), 3 - immediately apparent lesions in general clustered in few parts of the tree (1 to 5%), 4 - intermediate, 5 - numerous lesions widespread over a large part of the tree ($\pm 25\%$), 6 - intermediate, 7 - severe infection with half of the leaves badly infected by multiple lesions ($\pm 50\%$), 8 - intermediate ($\pm 75\%$), or 9 - tree completely affected with (nearly) all the leaves badly infected by multiple lesions ($>90\%$). Reaction type was determined according to the scale used by the PRI breeding program (Crosby et al. 1992) as follows: 0 - no symptom, 1 - pin point pits, 2 - irregular chlorotic or necrotic lesions, 3 - few restricted sporulation (M) mixture of necrotic and chlorotic and sparsely sporulating lesions, or 4 - abundant sporulation.

Sample collection. Scab infected leaves were collected from *M. floribunda* 821 'Nova Easygro' trees located in Darrow Farm (Geneva, NY) (Table 2). Monospore isolates were prepared by sticking infected leaves to the inner top side of petri dishes containing 1.2%

Table 1. Apple scab symptoms and incidence on *Rvi6* cultivars and susceptible controls. Note: PRI stands for Purdue, Rutgers, Illinois apple breeding program.

PI number	Cultivar name	<i>Rvi</i> genes	Symptom class (0-4)	Incidence (0-9)	Source
392303	Gala	None	4	5	-
590186	Wijcik McIntosh	None	4	8	-
588998	Marshall McIntosh	None	4	8	-
590184	Golden Delicious	<i>Rvi1</i>	4	7	-
589827	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	4	4	-
588747	Florina	<i>Rvi1</i> , <i>Rvi6</i>	0	1	EU (French)
588838	Nova Easygro	<i>Rvi6</i>	M	2	PRI (Canadian)
589181	Prima	<i>Rvi1</i> , <i>Rvi6</i>	3	2	PRI
589962	Jonafree	<i>Rvi6</i>	0	1	PRI
590183	Dayton	<i>Rvi6</i>	0	1	PRI
594111	Redfree	<i>Rvi6</i>	0	1	PRI
589965	Priscilla	<i>Rvi6</i>	0	1	PRI

water agar. The dishes were sealed and incubated overnight to let the spores fall on the agar and germinate. Single germinating spores were observed under a microscope and were carefully transferred to new PDA plates under sterile conditions to avoid the transfer of any contaminant. Scab isolates ‘VI-1797-9’ and ‘VI-NY05-GD’ were previously collected from another orchard in Geneva, NY, and TSR33T239 (carrying *Rvi4*) was collected from Ohio, U.S.A. (Schnabel et al. 1999). The well-characterized reference *V. inaequalis* isolates of European origin EU-B05 and EU-NL24 (Caffier et al. 2015) were obtained from INRA, France.

Artificial inoculation of the *Rvi6* resistant commercial cultivar ‘Macfree’. Conidial suspension was prepared by collecting abundantly sporulating leaves from the field (Darrow Farm), from each of the four *M. floribunda* 821 trees. The fresh lesions were cut, placed into a beaker containing distilled water and 0.5 µl/ml Tween 20, and shaken for 20 min. The suspension was filtered by two layers of sterile cheesecloth, and the conidia concentration was estimated by a hemocytometer. To set the concentration to at least 3×10^4 conidia/ml, the suspension was centrifuged at 3,000 rpm for 10 min, and the supernatant was discarded to retain the precipitate. The resulting precipitate was resuspended in a minimal amount of distilled water and the final concentration was measured by hemocytometer.

Four ‘Macfree’ scions were grafted on ‘M9’ rootstocks and maintained in a greenhouse under controlled conditions, where the 1-year-old grafted plants were pruned to acquire new growth and young leaves. The spore inoculum was used to spray the first 4 to 6 actively growing intact leaves from the top. The inoculated plants were held in a moist chamber under 18°C and 12-h photoperiod. To maintain 100% humidity, the sprayed leaves were covered first by wet paper towels, and then by resealable plastic bags. The incubated shoot part was tagged by marking the shoots below the bags to ease future evaluation. After 48 h of incubation, the bags were removed, and the plants were further maintained under low light, 18°C temperature, and 70% humidity with no irrigation for 2–3 weeks (Peil et al. 2018). As for the field assessment, reaction types were classed using the PRI program (Crosby et al. 1992). Light microscopy was used to evaluate the intensity of sporulation on the leaves. Spores from the infected leaves were suspended in a minimal amount of water and their morphology was observed under the microscope to confirm the identity of *V. inaequalis*. Images were taken using Touptview software with a digital camera for microscope (OMAX, Gyeonggi-do, South Korea).

DNA extraction and genome sequencing of *V. inaequalis* isolates. DNA was extracted from eight *V. inaequalis* monospore isolates, six of which were obtained from *M. floribunda* 821, one from ‘Golden Delicious’, and one from ‘Nova Easygro’. In case of *M. floribunda* 821, one, two, or three samples were collected from different parts of each of three different trees. In addition, DNA was extracted from two European isolates, EU-NL24 and EU-B05, and U.S. isolate VI-1797-9 from Purdue University (Table 2). Cultures were grown on potato dextrose agar for 4 weeks. No more than 0.2 g wet mycelia was cut and cleared from the agar by a sterile scalpel. Samples were ground in Eppendorf tubes with a disposable

homogenizer pestle under liquid nitrogen. DNA was isolated with the Wizard Genomic DNA Purification Kit (Promega) as described by Singh and Khan (2019). The quality of DNA was checked using agarose gel (1%) electrophoresis and quantified with Nanodrop and UV-Vis Spectrophotometer (Thermo Fisher Scientific, U.S.A.).

A total of 50 ng DNA for each sample was used to prepare genome sequencing libraries for Illumina Nextera skim sequencing at Institute of Biotechnology, Cornell University, Ithaca, NY. Agilent Bioanalyzer (Agilent; <https://www.agilent.com>) was used to assess the quality and quantity of genomic libraries. Individual *V. inaequalis* samples were barcoded and sequenced in a single lane of Illumina Mi-Seq platform to generate 2×250 base pair paired-end read data.

Sequence analysis and variant detection. Raw sequences were separated into sample-specific reads with the barcode information and used to assess the sequence quality with fastqc program (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The program Trimmomatic (Bolger et al. 2014) was used to remove the sequencing adaptors and low-quality reads with quality scores below 20. The genome assembly of *V. inaequalis* strain EU-B04 VeinUTG001 (Le Cam et al. 2019) was retrieved from the National Center for Biotechnology Information (NCBI) genome database and used to align the high-quality reads from the previous step. The alignments were filtered to eliminate PCR duplicated sequences and a sorted binary alignment format (BAM) was obtained for each sample using SAMtools (Li et al. 2009).

Variants across the *V. inaequalis* genome were identified using Genome Analysis Toolkit (GATK version 3.8.0; McKenna et al. 2010) with sample-specific BAM files as input. The HaplotypeCaller plugin in GATK was used to obtain genotype variant call format (gVCF), and individual sample gVCF files were merged into a single variant file with the GenotypeGVCF plugin. The raw variants were processed through “hard filtering” criteria as per GATK best practices. The resulting variants were used for base recalibration to remove false positives. The variant dataset was separated into single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs).

Genetic diversity and population structure analysis. Genetic diversity and population genetic analysis was conducted with SNP data only. The SNPs were filtered using the minimum read depth criterion of four. Furthermore, we kept SNPs with no missing data even in a single sample. Remaining high-quality SNPs were used to calculate the genome-wide statistics of diversity, population structure, and selection indices in *V. inaequalis*. The population level nucleotide variation was examined through the number of variants, nucleotide diversity (π), and principal component analysis (PCA) in Tassel v5 (Bradbury et al. 2007). The structure in the *V. inaequalis* population was further examined using the BYM admixture model and the haploid genome option in the TESS software (Caye et al. 2016) with default settings. In addition, spatial coordinates were provided for the 11 isolates to infer the accurate structure in *V. inaequalis* isolates. Convergence in the population structure results was checked by running 20 independent runs for individual K values ranging from two to eight. DIC and Kmax were plotted to identify the number of clusters/groups in the samples. Figures showing assignment of isolates to different clusters were generated

Table 2. The single spore isolates of *Venturia inaequalis* originating from Europe and Darrow farm (U.S.A.) from *Malus floribunda* 821, and apple cultivars ‘Golden Delicious’ and ‘Nova Easygro’

Code	Origin	Host	Host R genes	Reference
VI-NY01-NE	New York, U.S.A.	Nova Easygro	<i>Rvi6</i>	-
VI-NY02-MFa	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY02-MFb	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY02-MFc	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY03-MF	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY04-MFa	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY04-MFb	New York, U.S.A.	<i>M. floribunda</i> 821	<i>Rvi6</i> , <i>Rvi7</i>	-
VI-NY05-GD	New York, U.S.A.	Golden Delicious	<i>Rvi1</i>	-
VI-1797-9	Ohio, U.S.A.	TSR33T239	<i>Rvi4</i>	Schnabel et al. 1999
EU-NL24	The Netherlands	Prima	<i>Rvi1</i> , <i>Rvi6</i>	Caffier et al. 2015
EU-B05	Belgium	Schone van Boskoop	None	Caffier et al. 2015

using Clumpak - Cluster Markov Packager Across K (Kopelman et al. 2015).

Results

Apple scab infection in the orchard and under controlled conditions. In the beginning of June, scab symptoms were observed on *M. floribunda* 821 in the orchard at Darrow Farm, Geneva, NY on each of the four replicated trees. The initial sparse symptoms spread to approximately 10% (incidence grade 4) of the leaves later in July and August (Table 1). The heavy infection resulted in abundant sporulation (class 4), which often covered the whole leaf surface area (Fig. 1). Chlorosis and pin point pit type resistance reactions typical of the *Rvi6* and *Rvi7* genes, respectively, which are hypothetically the result of the large amount of inoculum from avirulent strains, were also frequent on the leaves.

The scab-susceptible commercial cultivars, ‘Gala’, ‘Wijcik McIntosh’, ‘Marshall McIntosh’, and ‘Golden Delicious’, all showed severe scab symptoms (class 4). Scab symptoms were more severe on ‘Golden Delicious’ than on ‘Gala’ (incidence 7 and 5, respectively). Many *Rvi6* cultivars originating from *M. floribunda* 821, such as ‘Dayton’, ‘Florina’, ‘Jonafree’, ‘Priscilla’, and ‘Redfree’, were totally free of scab during the whole season. Weak symptoms were also detected on the descendants of *M. floribunda* 821, ‘Prima’, and ‘Nova Easygro’; however, on these cultivars the infection was barely detectable. No more than one or two leaves per tree were infected in these cultivars (grade 1). In case of ‘Nova Easygro’, both chlorosis and necrosis were detected around the slight sporulation as the sign of an induced defense response (M), while the weak

sporulating lesions on ‘Prima’ were all surrounded by unusually strong chlorosis (3).

Abundantly sporulating scab symptoms were observed under the microscope on the leaves of ‘Macfree’ 15 days after inoculation in controlled environment (Fig. 2). The sporulation covered a large part of the leaf surface area without any trace of a chlorotic or necrotic response, hence we consider this reaction to represent the full compatibility infection class (4).

Genomic diversity in the *V. inaequalis* isolates. To understand the genetic diversity, population structure, and admixture, we sequenced and analyzed the 11 *V. inaequalis* genomes in this study (Table 2). A total of 34.5 million raw sequencing reads were obtained, out of which 0.38% low quality reads were discarded from further analysis (Table 3). The filtered sequence data constituted about 3.1 million average reads per sample, and provided 9.2× coverage of the *V. inaequalis* genome in NCBI. The read alignment rate against the *V. inaequalis* genome varied from 97.7 to 99.2%, with an average of 98.7% (Table 3).

After variant detection and GATK hard filtration, a total of 199,607 SNPs were identified with no missing data and representing an average of one SNP of every 360 base pairs of the *V. inaequalis* genome. About 38.1% ($n = 76,130$) SNPs were detected in European isolates and 25.2% ($n = 50,292$) were present in the two isolates collected from U.S. locations other than Darrow farm (Supplementary Fig. S1). The seven isolates from Darrow farm constituted about 68.3% ($n = 136,406$) of the total SNPs identified. Approximately 4.4% of SNPs were shared among these three groups of *V. inaequalis* isolates (Supplementary Fig. S1). The six isolates from *M. floribunda*

Fig. 1. Symptoms of apple scab infection detected on *Malus floribunda* 821, ‘Nova Easygro’ and ‘Prima’: heavy sporulation (A) alongside separate chlorotic and pin point type resistance symptoms (B) on *M. floribunda* 821; apparently intact crown of ‘Nova Easygro’ (C); weak sporulation surrounded by chlorosis and necrosis on ‘Nova Easygro’ (D) and by strong chlorosis on ‘Prima’ (E).

821 had 119,109 SNPs, representing about 59.6% of the total genetic diversity in the population. There were 55,130 SNPs identified in the three isolates collected from the same *M. floribunda* 821 tree. We further observed about 0.29 nucleotide diversity (π) in this *V. inaequalis* collection. The seven isolates collected from Darrow farm had a π value of 0.39 and six isolates from *M. floribunda* 821 exhibited a π value of 0.43, thus representing slightly higher nucleotide diversity than the entire collection. The nucleotide diversity of the two U.S. isolates outside of Darrow farm and the two European isolates was not estimated due to limited sample size.

Population genetic structure analysis. The population structure was examined using 16,321 high-quality SNPs to perform principal component analysis (PCA) and BYM admixture estimation of *V. inaequalis* genomes. PCA clearly separated the 11 isolates into two distinct European and U.S. groups (Fig. 3). The two European isolates from Belgium and Netherlands also showed clear distinction from each other. The U.S. groups also exhibited a slightly dispersed pattern (Fig. 3). For instance, two *V. inaequalis* isolates from non-*Rvi6* hosts showed slightly distinct clustering from the five isolates from *Rvi6* hosts, which exhibited much closer grouping among themselves on the PCA biplot. These results might reflect that host preference and isolate origin is in line with the genetic dissimilarities observed between these isolates. Furthermore, the remaining two isolates from *Rvi6* hosts had a distinct placement in the PCA biplot. These two isolates represent two of the three samples that were collected from the same tree of *M. floribunda* 821, suggesting the existence of both clonal and nonclonal isolates on the same tree. The remaining isolate colocalized with the other isolates from *M. floribunda* 821. The other subgroup of 5 U.S. isolates from Darrow farm had a diverse host range including ‘Golden Delicious’, ‘Nova Easygro’, and *M. floribunda* 821.

A genome admixture analysis reflected four main clusters across the 11 *V. inaequalis* isolates (Fig. 4). Two of the clusters represent the two European isolates and the other two clusters were specific to the U.S. isolates. A major proportion of genomic composition was different between the two European isolates, which also differed from the remaining isolates from the U.S. regions. A U.S. isolate VI-NY05-GD obtained from ‘Golden Delicious’ exhibited a partially different genomic composition from the other U.S. isolates from within and outside Darrow farm. The six isolates from *M. floribunda* 821 mostly showed similar genome composition except VI-NY03-MF, which had some admixture from European genomes (Fig. 4). The main differences observed in genomic composition at $K = 4$ remained consistent as K value changed from 3 to 5. At $K = 5$, the few U.S. isolates and two European isolates showed a small proportion of distinct genome other than the four groups observed at $K = 4$.

Discussion

We observed severe apple scab symptoms on trees of the Japanese crabapple *M. floribunda* 821, which carries the scab resistance genes *Rvi6* and *Rvi7*, in a research orchard at Geneva, NY in 2019. The breakdown of *Rvi6* resistance was reported within a decade of its deployment from several European locations (Bénaouf and Parisi 1997; Parisi et al. 1993), but until now resistance has remained effective

elsewhere. We have confirmed the loss of the resistance of *M. floribunda* 821 in North America. Seven cultivars descended from *M. floribunda* 821 (‘Dayton’, ‘Jonafree’, ‘Prima’, ‘Priscilla’, ‘Redfree’, ‘Florina’, and ‘Nova Easygro’) within the same orchard developed levels of scab much lower than observed on Japanese crabapple,

Table 3. Summary statistics of number of sequence reads generated, quality filtering, and percent alignment (mapping) against the *Venturia inaequalis* reference genome (Le Cam et al. 2019)

Sample ID	Raw reads	Cleaned reads	Mapping (%)
VI-NY05-GD	3549542	3549542	99.08
VI-NY01-NE	3223354	3223354	98.91
VI-NY03-MF	2850184	2850184	99.1
VI-NY04-MFa	3342636	3342636	99.2
VI-NY04-MFb	2453088	2453088	98.74
VI-NY02-MFa	3128934	3128934	98.93
VI-NY02-MFb	2770586	2770586	97.69
VI-NY02-MFc	2602972	2602972	99.27
VI-1797-9	4555028	4509802	98.75
EU-NL24	3015184	2973782	98.41
EU-B05	2990158	2945712	98.54
Total	34481666	34350592	98.7
Coverage		9.21

Fig. 3. Principal Component Analysis of 11 *Venturia inaequalis* isolates using 16,321 SNPs supported by a minimum of four sequencing reads. The original variant dataset was filtered to retain SNPs, supported by minimum four sequencing reads. The color of points on PCA biplot represent different groups of isolates, as indicated. Overlapping of data points is shown by darker color.

Fig. 2. Susceptible apple scab reaction of ‘Macfree’ (*Rvi6*) in a controlled environment to the inoculum of *Venturia inaequalis* isolates collected from the field from *Malus floribunda* 821. Sporulating leaf surface at 16 $\times$ magnification (A), macroscopic lesions (B), and suspended conidiospores at 40 $\times$ magnification (C).

despite their common ancestry with Japanese crabapple and possession of *Rvi6* (Table 1). The high incidence of scab across a range of known susceptible cultivars, and the uniform infection among all replicate trees of *M. floribunda* 821, would indicate that low scab incidence among these remaining resistant cultivars was not due to escape, but because of the presence of *Rvi6* in a genetic background still contributing additional resistance to *V. inaequalis*. Yet unidentified *R* genes deriving from cultivars in the pedigree of *Rvi6* cultivars might also contribute to their resistance. The foregoing would be consistent with the report by Roberts and Crute (1994) that many *Rvi6* cultivars remained resistant to scab in Europe after the resistance of *M. floribunda* 821 had eroded there. Parisi et al. (2004) also reported that ‘Prima’ remained more resistant to scab than *M. floribunda* 821 when challenged by isolates able to overcome *Rvi6*. It has been proposed that *Rvi1*, derived from ‘Golden Delicious’, or other ephemeral genes might contribute additional resistance when paired with *Rvi6*. We observed continued resistance to scab among many *Rvi6* cultivars that to our knowledge do not carry *Rvi1* (e.g., ‘Priscilla’ is used as differential host 6 as it is considered solely carrying *Rvi6*, Bus et al. 2011), meanwhile slight sporulation was observed on ‘Prima’ carrying *Rvi1* alongside *Rvi6* (Table 1). Gessler and Pertot (2012) speculated that *Rvi1* might occasionally act indirectly against *Rvi6* virulent isolates, and Caffier et al. (2010) hypothesized a fitness cost to virulence toward *Rvi6* conferred by loss of *avrRvi6*. However, the ability to overcome a larger number of *R* genes (excluding *Rvi6*) has not correlated with low fitness in past studies (Parisi et al. 2004; Peil et al. 2018).

It is important to distinguish between mild chlorotic flecking typical of greenhouse inoculations and the loss of field resistance to apple scab, where profusely sporulating lesions are macroscopically visible in orchard assessments. The chlorotic and necrotic symptoms seen on the field on ‘Prima’ and ‘Nova Easygro’ cannot be considered as evidence that their field resistance has been overcome. Likewise, chlorotic lesions supporting sparse sporulation have been reported on ‘Prima’ following greenhouse inoculations (Crosby et al. 1992). Even in cases of compatible interactions between the host and the pathogen, from a practical standpoint, whether resistance breakdown can be considered significant might depend on the severity and economic impact (Delmotte et al. 2016). Sparse chlorotic sporulation on commercial *Rvi6* cultivars is not generally considered a sign of resistance breakdown (Bus et al. 2011). On the other hand, *Rvi6* cultivars including ‘Macfree’ often appear to be susceptible under greenhouse conditions, indicating the breakdown of *Rvi6* resistance, but remains highly resistant in field plantings (Crosby et al. 1992; Roberts and Crute 1994). We observed similar mild and chlorotic symptoms on several scab resistant cultivars in the field (Table 1), but these are easily distinguished both qualitatively and quantitatively from the symptoms recorded on *M. floribunda* 821.

Fig. 4. Plot showing population structure and admixture levels in the genomes of 11 *Venturia inaequalis* isolates using 16,321 SNPs supported by minimum four sequencing reads. The analysis was conducted with the TESS software (Caye et al. 2016) using BYM admixture model and haploid genome with default settings. Convergence in the population structure results was checked by running 20 independent runs for individual *K* values. The best population structure was represented by *K* = 4.

The earlier discovery of *Rvi6*- and *Rvi7*-virulent isolates in Europe creates the possibility of transportation of such isolates to North America and subsequent spread. However, our very limited comparison of genetic polymorphism of the European and American virulent isolates suggested that both evolved or were selected from genetically distinct and separate populations. In Europe, the *Rvi6*-virulent lineage has existed separately in nonagricultural areas for thousands of years (Lemaire et al. 2016), which could also be true for North America. While there is considerable genetic distinction between the isolate from ‘Golden Delicious’ and the new virulent isolates, we do not have sufficient data to entirely exclude the possibility of gene flow and human transportation of isolates.

The considerable genetic polymorphism among the *M. floribunda* 821 virulent American isolates makes them less likely to be clonal variants. Interbreeding between *Rvi6* virulent and nonvirulent isolates has already been documented in mixed orchards (Michalecka et al. 2018). Even though our isolates were collected within an unsprayed experimental orchard comprised of several cultivars, the genetic dissimilarity detected between the groups suggests that intense hybridization has not yet occurred between the *M. floribunda* 821 virulent and avirulent isolates, although a larger number of isolates should be analyzed to draw a conclusion.

To successfully manage the disease resistance of cultivars, the distribution and evolutionary processes of pathogen isolates must be understood. Despite the breakdown of resistance in *M. floribunda* 821, many of its descendants were uninfected in the field, or showed a scarcely noticeable amount of sporulation, hence remained field resistant. This suggests that the overall genetic background into which a single major *R* gene is incorporated can substantially affect outcomes with respect to durability of resistance over time. Pyramiding major *R* genes or involving partial polygenic resistance is considered a durable alternative to utilizing single major resistance genes (Corwin and Kliebenstein 2017; Khan and Chao 2017; Robinson 1996). Breeders prefer to choose the most promising disease resistance genes and gene combinations to achieve commercially successful cultivars with durable disease resistance. However, due to the time needed to select and breed for polygenic resistance and the possibility of linkage drag of undesirable alleles, in the past breeding programs have prioritized major monogenic resistances. In-depth understanding of the factors resulting in the emergence of *M. floribunda* 821 resistance-breaking isolates, and the breakdown of resistance from *M. floribunda* 821 but not in descendant cultivars, may ultimately reduce the risk of eroding the resistance of *Rvi6* cultivars and enhance their durability.

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