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Strong Genetic Differentiation between Generalist Populations of *Venturia inaequalis* and Populations from Partially Resistant Apple Cultivars Carrying *Rvi3* or *Rvi5*

David Papp [1,*], Shambhulinga Gangadharappa Harigondra [1], Cristina Paredes [1], Anita Karacs-Végh [2], Károly Penksza [3], Ildikó T.-Járdi [3] and Viktor Papp [3]

[1] Department of Fruit Growing, Hungarian University of Agriculture and Life Sciences, 2100 Budapest, Hungary
[2] Department of Plant Pathology, Hungarian University of Agriculture and Life Sciences, 2100 Budapest, Hungary
[3] Department of Botany, Hungarian University of Agriculture and Life Sciences, 2100 Budapest, Hungary
* Correspondence: papp.david@uni-mate.hu

Abstract: The ascomycete fungus *Venturia inaequalis* causes scab disease, a major problem in apple production. The utilization of resistant cultivars is hindered by emerging new pathogen races, which erode their resistance. Increasing our knowledge on the population genetic processes of the fungus can contribute to the development of resistance gene deployment strategies and more durable resistance. We investigated the effect of four scab race indicator cultivars, 'Gala' (no *R*-gene), 'Golden Delicious' (*Rvi1*), 'Geneva' (*Rvi3*, complex), and OR45t132 (*Rvi5*), on the *V. inaequalis* population genetic structure and diversity. Sixty-six monosporic fungal isolates from the four cultivars were genotyped with seven simple sequence repeat primers. Furthermore, the partial resistance of the indicators and the pathogenicity profile of the conidia from each host were assessed. The genetic diversity and structure of the investigated *V. inaequalis* subpopulations correspond to the partial resistance of the original hosts as well as the subpopulations' pathogenicity profiles. Indicators carrying *Rvi3* and *Rvi5* had strongly diverged and specialized *V. inaequalis* populations on them and fewer symptoms on the field. In line with the complete breakdown of the *Rvi1* gene, the population from 'Golden Delicious' did not segregate from the susceptible 'Gala', and virulence towards *Rvi1* was present in all subpopulations.

Keywords: apple scab; resistance gene; virulence; genetic structure; disease progression

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1. Introduction

Apple scab caused by the ascomycete fungus *Venturia inaequalis* (Cooke) G. Winter is widely regarded as the most relevant disease of the domestic apple (*Malus domestica* Borkh.), especially in humid growing regions where it appears regularly. Symptoms develop on leaves and fruits, but shoots and flowers are also infected sometimes. Diseased apple fruits are not sellable for fresh consumption due to the aesthetically undesirable black velvety spots and later developing brown scabs on the fruits' surfaces. In more severe cases, deformity and fruit cracking result in totally unmarketable fruits [1]. According to various studies thus far, there is a correlation between fruit and leaf scab [2–4], so the latter parameter is more often considered both in disease management practices and scientific research.

With the current technology and market conditions, a large amount of fungicide is required to control scab sufficiently; farmers apply treatments even 15 to 22 times a year [5]. Thus, there is a need to produce scab-resistant apple cultivars and to reduce the negative economic and environmental impact of excessive fungicide usage in the apple industry. The groundbreaking work has been done in the United States (mainly by the PRI program),

where resistant cultivars and breeding materials were developed by the introgression of the *Rvi6* single gene resistance to the cultivated apple from a selection (sel. 821) of the Japanese crabapple *Malus floribunda* Sieb. Ex Van Houtte [6].

The mechanism of single gene resistance vastly utilized by plant breeders is most commonly explained with the GFG (gene-for-gene) model, where a specific receptor of the host (coded by an *R*-gene) recognizes the product of an effector coding avirulence gene of the pathogen (*Avr* gene) and triggers immunity. A mutation on the avirulence locus can make the corresponding *R*-gene ineffective. However, this is hypothesized to infer a fitness cost, as effectors (or other *Avr* products) can have an essential role in the successful infection and development of the pathogen [7]. From the perspective of population genetics, this results in frequency-dependent selection; the resistance gene of the host is selected when the corresponding virulence is frequent in the pathogen population, and selection is towards virulence when the *R*-gene has a high frequency. Various evolutionary forces, such as genetic drift, were implemented in more advanced GFG models, with some excluding virulence cost from the parameters. The different modes of frequency-dependent selection in GFG models and the evolutionary forces affecting polymorphism on the interacting loci have been excessively reviewed recently [8].

Scientific research on the genetics of qualitative scab resistance is intense. So far, twenty *R*-genes have been described against scab (*Rvi* genes) and mapped to the apple genome [4,9]. Moreover, the sequence and functional characterization of *Rvi6*, *Rvi15*, and, most recently, *Rvi12* has been carried out, supported by transgenic experiments [10–13]. Plant-pathogen interactions have been studied to identify new *R*-genes [14] and to profile the distribution of pathogen races in large areas [15,16]. Recently, a long-term study with a broad geographic scope has been published on the practical efficiency of *R*-genes monitored with scab race indicator cultivars [4].

Despite all of this, the vast majority of the modern resistant cultivars available for farmers still carry the *Rvi6* gene as the source of their resistance, which has become an increasing problem since a new *V. inaequalis* race overcame this *R*-gene and started to spread a few decades ago [17]. Cultivars carrying *Rv6* seem to react differently to the spread of the new pathogen race. Within the same orchard, symptoms on different *Rvi6* cultivars can vary from immunity to partial resistance and even to high levels of susceptibility comparable to that of non-resistant widespread modern apple cultivars [18].

After the breakdown of *Rvi6* resistance, many breeding centers developed gene pyramids (accumulating *R* genes and/or quantitative trait loci in a single cultivar) for more durable resistance. Scab-resistant elite lines with multiple sources of resistance are already available. Field trials have positive results, but not all *R*-gene pyramids are successful, and combining functionally different resistant factors, e.g., quantitative trait loci with *R*-genes, has been recommended [19–21]. Still, we have no direct empirical evidence whether scab resistance gene pyramids are indeed durable and will remain efficient if used on a geographically wide scale for a long time, which is not likely to happen until their commercial production.

In a disturbing report, an isolate of *V. inaequalis* was identified with extremely wide virulence spectra; nine of the twenty known *Rvi* genes were ineffective against it [22]. Furthermore, the pathogen has already overcome the resistance of some apple cultivars with naturally occurring complex single gene resistance, such as in 'Geneva' (*Rvi3*, complex), which carries at least five different *R*-genes controlling GFG interactions [4,23].

In general, growing cultivars with gene pyramids is not always the most efficient resistance deployment strategy [24]. Other deployment strategies, including the concurrent use of resistance genes within orchards in the form of cultivar mixtures, as well as the regional or sequential deployment of *R*-genes, have potential when it comes to controlling the evolution and population dynamics of *V. inaequalis* to our advantage [25]. Decreasing the effective population size of pathogens or the gene-flow between populations was concluded to increase the efficiency of resistance, for which we need a better understanding

of the population dynamics of the pathogen in connection to the resistance factors and the way of their deployment [26].

The main focus of our current research is to investigate the effect of scab resistance coded by *Rvi1*, *Rvi3*, and *Rvi5* on the genetic diversity and structure of the local *V. inaequalis* population of an apple cultivar collection (Budapest, Hungary). Scab infection was observed on race indicator cultivars for over a decade in the collection (Papp et al., unpublished data). In this period, the cultivars 'Gala' (no *R*-gene), 'Golden Delicious' (*Rvi1*), and 'Geneva' (*Rvi3*, complex) showed scab symptoms every year, while symptoms of sel. OR45t132 (*Rvi5*) occurred occasionally. Although the pathogen has overcome the resistance of the studied *R*-genes in the collection, the nature of their partial resistance remains a question. Thus, in relation to the genetic data, we also aimed to precisely quantify the resistance capacity of the four investigated race indicators on the field by following the progression of the disease on them in two seasons. Furthermore, an assay was carried out to clarify the virulence spectra of the *V. inaequalis* populations defined by their hosts. Our data support that *R*-genes of host cultivars can have a strong effect on *V. inaequalis* population structure even after the resistance breakdown, and this might be in relation to the *de facto* partial resistance of the once were resistant cultivars.

2. Materials and Methods

2.1. Field Resistance of Indicator Cultivars

The field research was carried out in the apple cultivar collection of Soroksár (Budapest, Hungary, lat. 47.49801, long. 19.03991). Apple scab development was evaluated on four race indicator cultivars: 'Gala' (no *R* gene), 'Golden Delicious' (*Rvi1*), 'Geneva' (*Rvi3*, complex), and OR45t132 (*Rvi5*). Each cultivar in the collection had been planted continuously in four repetitions. Trees of the investigated cultivars were in close proximity to each other (150 m maximum distance). No fungicide treatment was applied to the trees. One branch of each cultivar was marked at 100 leaves from the top. The selected branches were assessed once per week for eight weeks from 19 May 2017 and 11 May 2018. Scab severity was defined as the percentage of infected leaves (infected leaves/all leaves). A leaf was considered infected if there were matte, olive green-to-black colored lesions on it, indicating active sporulation. The area under the disease progression curve (AUDPC) was calculated with the trapezoid method [27]. To estimate disease pressure, Mills periods [28] were calculated using the data of a local meteorological station (iMetos, Pessl Instruments, Austria).

2.2. Pathogenicity Testing by Detached Leaf Assay

Conidia suspensions were obtained by harvesting conidia directly from naturally occurring scab lesions. Eight infected leaves were collected randomly from each of the four investigated cultivars in June 2022. Eight leaf discs (one per leaf per cultivar) with one sporulating lesion on each disc were cut using a 6 mm wide cork borer. To suspend the conidia, the discs were placed into an Eppendorf, and after adding 500 µL of tap water, the samples were vortexed for 5 min. The spore concentration of the suspensions was set to approx. 5.6×10^4 conidia/mL using a hemocytometer. The viability of the conidia was checked by dropping 20 µL of suspension to a 2% water agar plate and counting the number of germinated conidia/100 conidia after 24 h of incubation at room temperature.

Detached leaf assay was carried out following previous works [29,30], but with slight modifications. Young, undamaged leaves of the four studied cultivars were collected from the field. After picking them, the leaves were instantly put into water and then washed for 15 min under running tap water. The petioles were cut back, and each leaf was placed on a 2% water agar plate. Plates were prepared in two repetitions for the 16 combinations (four cultivars × four inoculums). Conidia suspension (20 µL) was pipetted to the four edges of each leaf, and the plates were sealed with parafilm and placed to 19 °C with constant fluorescent light. After 4 days, the leaves were washed again with tap water and dried under laminar flow to get rid of the initial conidia and any contaminating epiphyte. Symptoms were analyzed 14 days post-inoculation under an Olympus BX41

(Olympus Corporation, Tokyo, Japan) or a Zeiss Axio Imager A2 (Carl Zeiss Microscopy, Munich, Germany) compound microscope. For making photos, we used a Zeiss AxioCam HRc camera (Carl Zeiss Microscopy, Munich, Germany). Development of fresh conidia on conidiophores as well as running hyphae or stroma development were considered successful infections.

2.3. Culturing Monosporic Isolates of V. inaequalis

Altogether, 66 monosporic *V. inaequalis* isolates were prepared from four differential hosts with a population size ranging from 10–23 per subpopulation. Sporulating leaves were collected randomly from the trees planted in four repetitions per cultivar. The leaves were attached to the inner upper side of Petri dishes containing 2% water agar. The dishes were incubated for 24 h to let the conidia fall onto the agar and germinate. Isolation of single spores to new PDA (potato dextrose agar, Carl Roth GmbH, Karlsruhe, Germany) plates was done under laminar flow using a stereomicroscope (Olympus SZ51, Olympus Corporation, Tokyo, Japan) and a sterilized needle [31].

2.4. DNA Extraction and SSR Marker Analyses

Mycelia of monosporic *V. inaequalis* isolates (n = 66) grown on solid PDA were cut and stored at −80 °C. Frozen samples were ground in a mortar, and DNA was extracted using the E.Z.N.A. plant DNA kit (Norcoss, GA, USA), according to the manufacturer's recommendations. Concentrations were verified by staining and running the extracted DNA on 1% agarose gel (BioReagent, Sigma-Aldrich, St. Louis, MO, USA).

The PCR (polymerase chain reaction) was carried out using a Thermal Cycler 2720 PCR machine (Applied Biosystems, Foster City, MA, USA). The PCR mixture with a final volume of 16 µL was prepared with the DreamTaq Green PCR Master Mix (Fermentas, Waltham, MA, USA). Primer pairs for seven SSR (simple-sequence repeat) loci labeled with 6-FAM (6-Carboxyfluorescein) were used for the amplification: 1tc1a, 1tc1b, 1tc1g, and 1aac3b [32], as well as Vitg11/70, Vicacg8/42, and Vica10/154 [33]. The PCR program was set as suggested [33], but all primers ran with 60 °C annealing temperature except 1tc1g, for which the program was set to 58 °C. Fragments were sized by capillary electrophoresis using an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, MA, USA) with Genescan 500 LIZ inner size standard (Applied Biosystems, Foster City, MA, USA). The lengths of the fragments were determined with the GeneMapper 4.0 software (Applied Biosystems, Foster City, MA, USA).

2.5. Statistical Analyses of the Genetic Data

GenAlEx 6.5 [34,35] was used to calculate various diversity indices, such as the mean number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), and haploid diversity (h). Polymorphic informative content (PIC) values were computed for the populations with the Molkin v. 3.0 software [36].

The underlying genetic populations among the isolates were detected using the Structure 2.3.4 software [37]. The simulation was run with a burning period length of 50,000. The number of MCMC (Markov Chain Monte Carlo) reps after burn-in was set to 100,000. For each K (1–10) the simulation was run 20 times. We used the StructureSelector software [38] to summarize the results and to estimate the optimal number of genetic clusters (K value).

AMOVA (analyses of molecular variance) was carried out using GenAlEx 6.5 [34,35]. Pairwise population PhiPT values were calculated by applying 999 permutations. In addition, PCoA (principal coordinate analyses) was created using Nei's genetic distance matrix.

3. Results

3.1. Field Scab Severity

The development of scab symptoms on four scab race indicator cultivars, 'Gala' (no *R*-gene), 'Geneva' (*Rvi3*, complex), 'Golden Delicious' (*Rvi1*), and OR45t132 (*Rvi5*), was evaluated for two years in 2017 and 2018 in a cultivar collection (Budapest, Hungary).

Severity was scored from mid-May on a weekly basis eight times a year, and AUDPC values were calculated (Figure 1). More substantial disease pressure was observed in 2017, especially in the more determinative early stage of disease development. In the first 21 days of the study in 2017, there were 6 Mills periods, compared to the 2 Mills periods in 2018. Therefore, in 2017, scab severity was considerably higher on all cultivars. Due to the less favorable conditions of 2018, no scab was found on OR45t132. This year's effect was the least strong on the scab severity of 'Gala', resulting in 19% lower AUDPC in 2018 compared to 2017 (43% reduction of AUDPC for 'Golden Delicious' and 63% for 'Geneva').

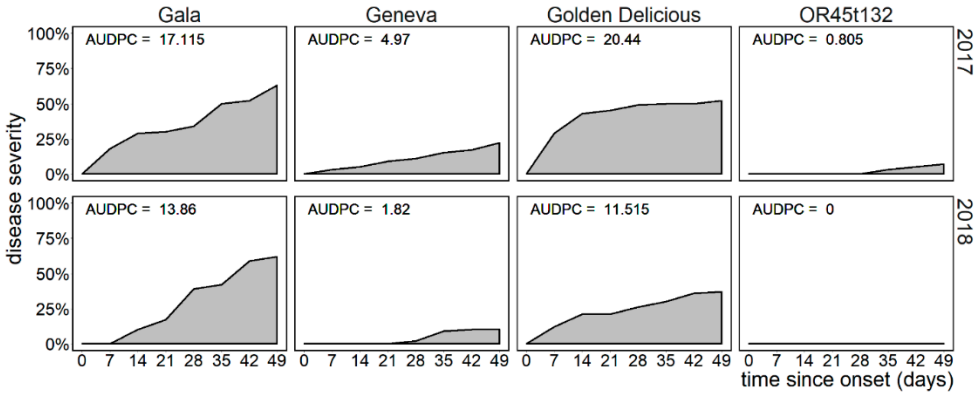

Figure 1. Progression of apple scab on four indicator cultivars in 2017 and 2018 with the area under disease progression curve (AUDPC) calculated; disease severity is defined by the proportion of infected leaves; symptoms began on 19 May 2017 and 11 May 2018.

The two years' average AUDPC of 'Gala' (15.48) and 'Golden Delicious' (15.97) did not differ significantly. However, in both years, 'Golden Delicious' developed symptoms faster in the early season, while the scab on 'Gala' reached considerably higher severity at the end (63% maximum compared to 52%). AUDPC values were considerably lower for 'Geneva' (3.39 on average) and OR45t132 (0.4), with a maximum leaf scab severity of 22% on 'Geneva' and 7% on OR45t132. The latter is close to the threshold level, below which fungicide treatment is not required by integrated plant protection standards [3].

3.2. Detached Leaf Assay

Four conidia suspensions were obtained by harvesting *V. inaequalis* conidia from naturally infected leaves of 'Gala', 'Geneva', 'Golden Delicious', and OR45t132, and then, detached leaves of the same four cultivars were drop inoculated with each suspension (Figure 2). All conidia suspensions caused obvious scab symptoms on their original host cultivars, fulfilling Koch's postulates.

All conidia suspensions caused infections on 'Gala' and 'Golden Delicious' (*Rvi1*), which is in line with the GFG model where virulent pathogens are also capable of infecting non-resistant hosts [39]. Thus, all conidia suspensions can be designated to race (1). In contrast to this, no successful infection was detected on 'Geneva' (*Rvi3*) or OR45t132 (*Rvi5*) when the inoculum had a different host of origin. Only the conidia collected from the same cultivar caused symptoms on either 'Geneva' or OR45t132. Therefore, the 4 conidia suspensions represent 3 different pathogenicity profiles: the conidia from 'Gala' and 'Golden Delicious' belong to race (1); the conidia from 'Geneva' belongs to race (1, 3); and the conidia from OR45t132 to race (1, 5).

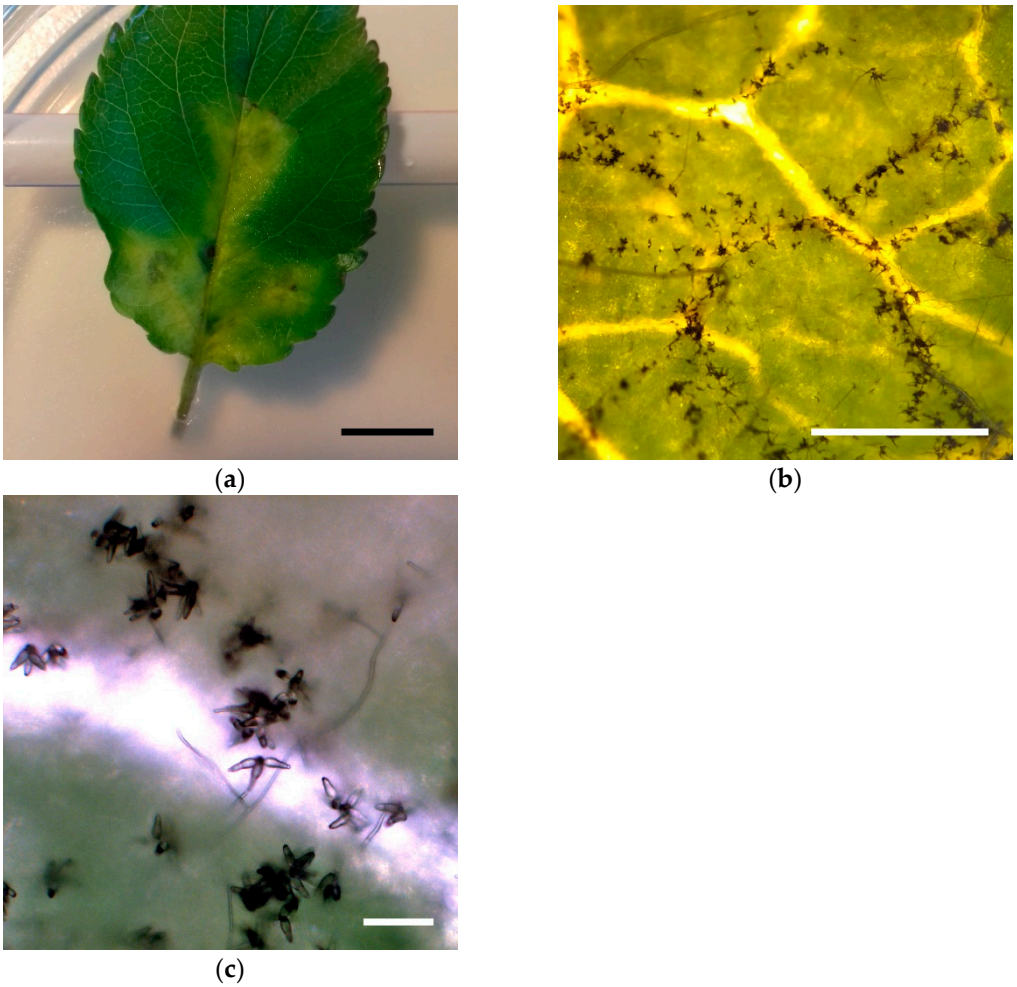

Figure 2. Symptoms caused by *V. inaequalis* on the detached leaves of 'Gala', 14 days post-inoculation: (**a**) Sporulating lesions surrounded by chlorosis, scale = 5 mm; (**b**) Conidia alongside running hyphae, forming a characteristic net-like or "crisscross" shape, scale = 0.5 mm; (**c**) conidia developing on conidiophores in clusters, scale = 50 µm.

3.3. Polymorphism of SSR Markers

In total, 66 monosporic *V. inaequalis* isolates from different host cultivars but from identical geographic locations were genotyped with 7 SSR markers (Table 1). Although all SSR markers were polymorphic and produced multiple alleles, 1aac3b showed a considerably lower level of polymorphism than the other markers. Altogether, 77 different alleles (Na) were amplified by the 7 markers, ranging by loci from 2 (1aac3b) to 22 (1tc1g). All indices showed 1aac3b as the least and 1tc1g as the most polymorphic marker. The number of effective alleles (Ne) ranged from 0.09 to 14.9, and the haploid diversity (h) from 0.08 to 0.93. Besides haploid diversity and the number of different alleles, Shannon's information index (I, ranging from 0.18 to 2.87) showed a large gap between the least (1aac3b, I = 0.18) and the next least informative marker (1tc1b, I = 1.18), with the latter being close to the average of all markers (I = 1.64).

Table 1. Summary statistics of seven SSR markers used for genotyping 66 monosporic *V. inaequalis* isolates.

Marker	Na	Ne	I	h
1tc1a	14	6.55	2.20	0.84
1tc1b	8	2.12	1.18	0.52
1tc1g	22	14.97	2.87	0.93
1aac3b	2	1.09	0.18	0.08
Vitg11/70	7	3.69	1.48	0.72
Vicacg8/42	10	4.51	1.74	0.77
Vica10/154	14	3.79	1.81	0.73

Na (no. of different alleles), Ne (no. of effective alleles), I (Shannon's information index), and h (haploid diversity).

3.4. Population Genetic Diversity

Subpopulations of the *V. inaequalis* isolates (n = 66) were defined by their host cultivars of origin (Table 2). Genetic diversity estimators calculated for each population were consistent with each other. In line with the other estimators, the Vi-Gala population had the highest haploid diversity index (h = 0.70), followed by Vi-GD (h = 0.64), Vi-Geneva (h = 0.44), and Vi-OR as the least diverse (h = 0.27). Clonal isolates with identical SSR profiles were the most frequent in the Vi-OR population but were also present in the Vi-Geneva and Vi-Gala populations. The SSR profile of haplotypes is available in Supplementary Material Table S1. No clonal isolates belonged to different subpopulations.

Table 2. Genetic diversity estimators of four *V. inaequalis* subpopulations from different host cultivars (data obtained by genotyping 66 isolates with seven SSR markers).

Population	Host Cultivar	N	Nh	Na	Ne	I	h	PIC
Vi-Gala	Gala	23	22	7.42	4.79	1.61	0.70	8.89
Vi-Geneva	Geneva	14	12	3.00	2.16	0.78	0.44	8.53
Vi-GD	Golden Delicious	10	10	5.00	4.00	1.34	0.64	8.86
Vi-OR	OR45T132	19	12	2.28	1.72	0.48	0.27	8.23

N (no. of samples), Nh (no. of haplotypes), Na (no. of different alleles), Ne (no. of effective alleles), I (Shannon's information index), h (haploid diversity), and PIC (polymorphic informative content).

3.5. Population Structure

According to the structure analyses, the number of ancestral populations (K value) was predicted to be between 2 to 4 by different statistics, with most suggesting K = 3. With the Evanno method, the delta K plot suggested K = 2, and the LnP(K) plot suggested K = 4 [40]. If LOCPRIOR was set (prior information on sampling location was set to assist the clustering), K = 3 was suggested by both plots. All plots of the Puechmaille method [41] (MedMeaK, MaxMeaK, MedMedK, and MaxMedK) suggested K = 3 independently from the LOCPRIOR function.

At K = 3, populations Vi-GD and Vi-Gala belong to the same genetic cluster (Figure 3). Without LOCPRIOR, four individuals (6.06% of the whole population) showed a lower cluster membership coefficient (Q) than 0.7 and were considered admixed. One of these isolates is from the Vi-Gala population and three are from the Vi-Geneva population, and they represent admixtures in all combinations of the three genetic clusters. One individual of Vi-Geneva was considered a migrant, as it has shown membership with more than 0.7 probability to another genetic cluster, the combined cluster of Vi-GD and Vi-Gala. Similar results were suggested at K = 4, except that the Vi-GD and Vi-Gala populations were grouped into two separate but completely admixed genetic clusters. At K = 2, the Vi-Geneva isolates were grouped against the Vi-OR isolates together with all the other samples. With the LOCPRIOR option, no admixture was shown except between the Vi-GD and Vi-Gala populations.

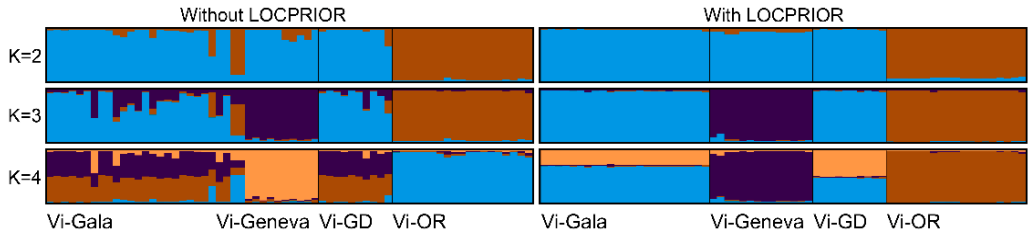

Figure 3. Structure bar plot showing ancestry coefficients of *V. inaequalis* isolates of four populations at K = 2 to 4, with and without adding the information on the sampling location (LOCPRIOR) to the model (different colors represent the different ancestral populations).

3.6. AMOVA and PCoA

The AMOVA revealed high variation (78%) within populations; only a lower proportion of the total variation was detected among the populations (22%). Lower population variation suggests substantial gene-flow between the populations.

Pairwise PhiPT values were calculated to express the relative contribution of the variance between populations to the whole genetic variation within the data (Table 3). PhiPT values ranged from 0.02 to 0.41 between the populations, and the difference was significant ($p < 0.01$) in all pairwise comparisons except for Vi-Gala and Vi-Golden (PhiPT = 0.02, $p = 0.15$). The highest difference was found between Vi-Geneva and Vi-OR (0.41). The most divergent population was Vi-OR followed by Vi-Geneva. Based on Wright's interpretation on the analogue FST values [42], the Vi-OR population differs greatly from all the other populations (PhiPT values larger than 0.25), while moderate differentiations (values of 0.15 to 0.25) were observed between Vi-Geneva and the rest of the populations.

The principal coordinate analysis (PCoA) based on Nei's genetic distance matrix supported the results obtained by the AMOVA and STRUCTURE analyses (Figure 4). The most divergent population was Vi-OR, followed by Vi-Geneva. Furthermore, the 95% confidence ellipses demonstrate an almost complete overlap between the Vi-Gala and Vi-Golden populations.

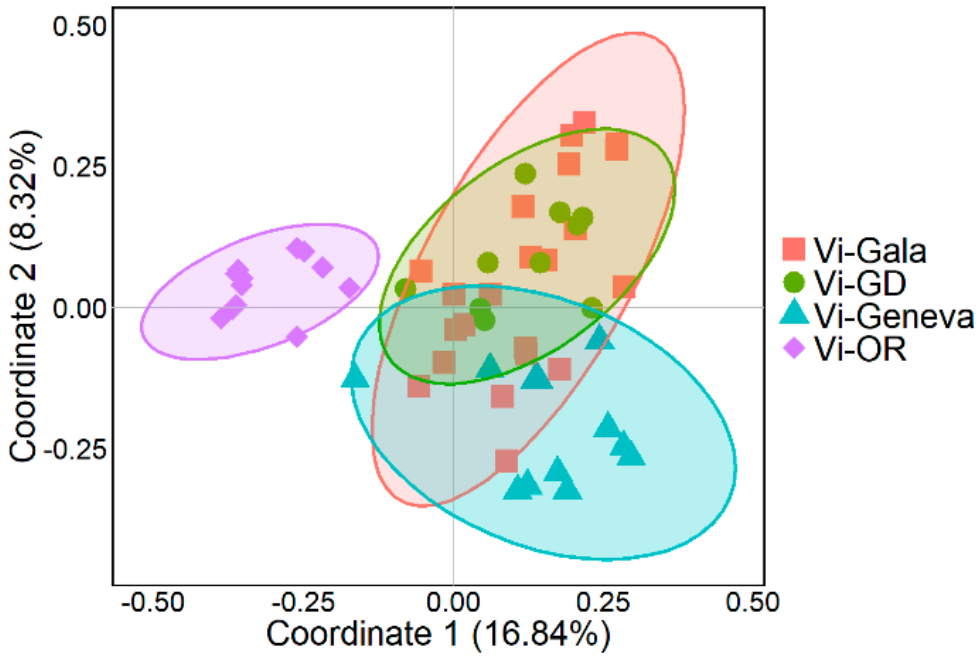

Figure 4. Results of the principal coordinate analysis (PCoA) of the 66 *V. inaequalis* isolates based on seven SSR markers (the ellipses represent 95% confidence ellipses; colors and point shapes indicate subpopulations defined by different original host cultivars; numbers in parenthesis represent the proportion of variation explained by the coordinate).

Table 3. Pairwise genetic distance (PhiPT) between the four investigated *V. inaequalis* populations.

Vi-Gala	Vi-Geneva	Vi-GD	Vi-OR	
-	0.001	0.152 *	0.001	Vi-Gala
0.104	-	0.001	0.001	Vi-Geneva
0.020	0.162	-	0.001	Vi-GD
0.286	0.410	0.347	-	Vi-OR

PhiPT values below diagonal, statistical significance above diagonal (* no significant difference at $p < 0.01$).

4. Discussion

Gaining a better understanding of how cultivar use affects the evolution of *V. inaequalis* is crucial for efficient breeding and for managing scab in an environmentally conscious manner. Many papers have been published on the population genetics of *V. inaequalis* in the last decades, increasing our knowledge of its evolutionary processes and history. There is genetic evidence that the population dynamics of *V. inaequalis* tracked that of its host during the domestication of the apple due to the strong coevolutionary relationship between the two species [43]. Later research suggested that *V. inaequalis* had been present in Iran before the domestication of the apple; thus, Iran might be the center of origin of the pathogen species [44].

Most studies suggested weak differentiation between *V. inaequalis* populations despite being separated by large geographic distances [44–50], with a few exceptions where mountainous or coastal regions with vastly differing climate conditions were also included in the study [51,52]. Besides environmental factors, host-specialization is among the major evolutionary forces guiding population differentiation. Susceptible hosts, in most cases, had little or no effect on the genetic clustering of *V. inaequalis* isolates [44,49]. Adaptation to susceptible cultivars with no identified *R*-genes but unknown qualitative partial resistance factors ('Bramley' and 'Worchester'), however, caused divergence in a local *V. inaequalis* population, and the genetic distance even grew with time [53–55]. Strong genetic differentiation has been observed between *Rvi6* infecting and avirulent isolates, supported by relatively large data [31,48,56,57]. It has been suggested that the *Rvi6* virulent population diverged from non-virulent isolates 8000 to 50,000 years ago and hybridization is only happening recently in commercial orchards [56]. It has also been proposed that *Rvi1* host cultivars might have caused a similar split in the pathogen population in Kashmir, India [45]. Still, unlike *Rvi6*, neither *Rvi1* nor *Rvi17* in the host seem to affect the *V. inaequalis* population structure [48].

In the current study, field research was carried out in a Hungarian apple cultivar collection (Soroksár, Budapest). Following the progression of apple scab in two consecutive years revealed strong partial resistance of the *Rvi3* ('Geneva') and *Rvi5* (sel. OR45t132) cultivars after their resistance had long been overcome by *V. inaequalis*. Race-specific partial apple scab resistance (syn. residual resistance) granted by qualitative *R*-genes had been documented before for traditional apple cultivars, such as 'Bramley' [53] or 'Président Roulin' [58], and for several *Rvi6* cultivars [18,59]. Interestingly, the partial resistance of *Rvi6* cultivars was genetically associated with the *R*-gene and not with other genetic factors [59].

Like in most studies the *Rvi1* gene in 'Golden Delicious' did not express a noticeable degree of resistance [4,16], although the disease progression curve was slightly different of the non-resistant 'Gala' and the *Rvi1* cultivar 'Golden Delicious'. The disease progressed faster on 'Golden Delicious' than on 'Gala' but reached lower severity by the end of the season. This can be explained by its later budbreak [60], resulting in a lower degree of ontogenic resistance during primer infection, a significant factor of *de facto* scab resistance [1].

Detached leaf assay showed that virulence towards *Rvi1* is common in the local *V. inaequalis* population. This is in accordance with other studies where 87% [16] and 71% [15] of *V. inaequalis* isolates were virulent on 'Golden Delicious' (*Rvi1*), in Europe and India, respectively. In contrast, no conidia from other hosts caused an infection on the leaves of *Rvi3* or *Rvi5* cultivars. It should be noted that repetition numbers and thus the statistical

support of this data is limited, so it cannot be excluded that there might be pathogen strains in the orchard with multiple virulence factors, including *avrRvi3* or *avrRvi5*.

We conducted a genetic marker analysis using 7 SSR markers on 66 monosporic *V. inaequalis* isolates originating from the 4 investigated race indicator apple cultivars. Most studies on the population genetics of *Venturia* species work with a higher number of markers, e.g., in a recent study, 30 markers were used [61]. A recent statistical analysis of marker numbers and inferred information revealed, however, that seven markers are sufficient to detect population genetic diversity and structure [62]. In accordance with this, our genetic analyses using seven polymorphic SSR markers demonstrated three well-distinguishable genetic clusters with no or minimal admixture.

Our data on the genetic diversity and structure of the *V. inaequals* population is in line with the scab severity and pathogenicity data. Vi-OR (subpopulation collected from the *Rvi5*-resistant OR45t132) was the genetically most distinct and least diverse group. The conidia suspension from OR45t132 was specific to its host, based on our detached leaf assay. In addition, scab severity was the lowest on the indicator cultivar OR45t132 (*Rvi5*). The same tendency is followed in all respects by the Vi-Geneva population and the *Rvi3* cultivar 'Geneva'. Vi-Geneva is genetically less distinct from the rest of the population than Vi-OR, and the host cultivar is considerably more susceptible on the field. However, the race is similarly specific to its host. Isolates from 'Gala' and 'Golden Delicious' did not show significant genetic differences and had identical pathogenicity profiles. However, *Rvi1* infecting isolates were less diverse, and 'Golden Delicious' had slightly different disease progression curves than 'Gala'.

It varies by pathosystem whether resistance in hosts causes a genetic split in the pathogen populations. For example, the genetic divergence of rust populations (*Melampsora larici-populina* Kleb.) was induced by *R*-genes carried by the hosts [63], but in other studies on wheat powdery mildew (*Blumeriella graminis* (DC.) Speer), virulence frequencies did not tend to correspond to population structure [64,65]. The latter case was explained by the low levels of resistance and thus expressed evolutionary pressure by the host population. In the case of the *Malus-Venturia* pathosystem, the strong gene-flow barrier between *Rvi6* virulent and non-virulent populations has been previously hypothesized. However, it is still unclear if the fitness cost of virulence alone was sufficient enough to cause the strong divergence or if other factors have resulted in highly diverged populations, later maintaining their structure due to pre-zygotic genetic barriers [57].

In summary, our results indicate that the genetic diversity and structure of the *V. inaequalis* pathogen are closely related to the *de facto* resistance of apple cultivars carrying qualitative *R*-genes. Host diversity is a major factor in maintaining resistance at the population level [66], and according to our data, this diversifying selection pressure expressed by apple cultivars on *V. inaequalis* populations seems to be primarily influenced by *R*-genes and is weak or negligible in case of susceptible cultivars having no *R*-genes [44,49]. Based on our results, different *R*-gene deployment strategies alongside gene pyramids might have strong potential in the apple industry and should be investigated. Promoting ecological consciousness, we would like to encourage further work on the diversity of economically important plant pathogens in strong agreement with the recently proposed motto of Stam and McDonald: "know your enemy, and its diversity" [28].

5. Conclusions

Scab race indicator apple cultivars with the *Rvi3* or *Rvi5* resistance genes expressed strong partial resistance on the field in a Hungarian cultivar collection. In contrast to this, the *Rvi1* resistance gene did not express a significant level of scab resistance. A detached leaf assay with inoculum originating from four race indicator cultivars (carrying *Rvi1*, *Rvi3*, or *Rvi5*, or no *R*-gene) revealed specificity towards *Rvi3* and *Rvi5* but widespread virulence towards *Rvi1* in the local *V. inaequalis* population. We have genotyped 66 monosporic *V. inaequalis* isolates from the four investigated indicator cultivars with seven SSR primers, which indicated a structured population with three genetic clusters with limited gene-

flow between them. Based on the AMOVA and supported by the PCoA, the pathogen populations from *Rvi3* and *Rvi5* cultivars showed moderate and strong differentiation from the other populations, respectively. Meanwhile, no significant genetic differentiation was detected between the populations from the *Rvi1* and the susceptible 'Gala' cultivars. The study suggests a connection between the genetic structure of the pathogen population and the partial resistance expressed by *R*-genes after they have been overcome by the pathogen. The authors emphasize the importance of further research work on the topic and the development of new *R*-gene deployment strategies in apple growing.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121050/s1, Table S1: SSR data on monosporic *V. inaequalis* isolates.

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