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Subdivision and genetic structure of four populations of *Venturia inaequalis* in Switzerland

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Abstract

Analyses of four populations of *Venturia inaequalis* in Switzerland were performed to obtain information about migration and to predict the probable speed of the spread of new pathotypes able to overcome resistance, e.g. *Vf*-resistance, of new cultivars.

Genetic and haplotype diversity was calculated based on allele frequencies of random amplified polymorphic DNA (RAPD) markers and the internal transcribed spacer (ITS)-region of ribosomal DNA, which are regarded to be neutral, and the β-tubulin locus which may be under selection pressure. Within-population diversity was found to be quite similar over all four populations. Normalised haplotype diversity based on RAPD and ITS data was very high with a mean of 0.95. Diversity among populations (G_{ST}) was consistent over all neutral loci with a low mean of 0.04, but reached the high value of 0.26 for the selected β-tubulin locus. Low G_{ST} based on neutral loci may suggest a high level of gene flow.

Considering these results, new pathotypes would be expected soon outside their place of identification. But actual gene flow is easily overestimated because of effects of gene flow in the past. However, naturally occurring gene flow could be increased by human activity. Therefore, it is very difficult to predict durability of the *Vf*-resitance in Switzerland.

Abbreviations: ITS – internal transcribed spacer; rDNA – ribosomal DNA.

Introduction

Apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is the most important disease found in apple (*Malus* × *domestica*) cultivation in Europe. *V. inaequalis* is a heterothallic haploid ascomycete which reproduces sexually and asexually. During the sexual stage which takes place in winter, the fungus grows as a saprophyte in fallen apple leaves and forms pseudothecia (MacHardy, 1996). From the end of March until mid May (Bühler, 1995) ascospores cause primary lesions on young apple leaves, and conidia continue the epidemic. The conidia as well as the ascospores are mainly spread by rain splashing on the leaves (Frey and Keitt, 1925; Wiesmann, 1932). Depending on weather conditions (Mills and Laplante, 1951), six to eight asexual cycles per season may be completed.

By the end of the 19th Century, researchers were becoming aware of the possibility of developing scab-resistant varieties. Besides race-specific resistance genes in *M.* × *domestica* (Sierotzki et al., 1994), a large pool of scab resistances exist in other *Malus* species (crabapples) which were used for the breeding programs. Since 1970 many scab- resistant cultivars carrying the *Vf*-resistance derived from *M. floribunda* 821, have been released (MacHardy, 1996).

The *Vf*-resistance was considered highly durable (Crosby et al., 1992). However, in 1984 the first scab isolates capable of infecting particular *Vf*-resistant cultivars were found in an orchard in Northern Ger-

many (Parisi et al., 1993). The *Vf*-resistance was also overcome in Kent (UK) (Roberts and Crute, 1994). Isolates collected from a naturally infected *M. floribunda* in a house garden caused sporulating lesions on *M. floribunda* 821 as well as on several cultivars carrying the *Vf*-gene. Until now, this virulence has not been found outside the sites of identification even though large orchards of *Vf*-carrying cultivars were planted more than ten years ago in other places. Although the *Vf*-virulence has not yet been observed outside Ahrensburg and Kent, there is growing concern about the possibility of a widespread breakdown of the *Vf*-virulence across Europe due to the spread of the virulence.

The virulence spreads by migration of individuals carrying it into another population. Gene flow occurs when there is also genetic exchange between the invading and the original population and the two populations may merge into one randomly mating population. Differentiation of populations can be caused by random genetic drift or selection and both kinds of differentiation are manifested in differences in allele frequencies among populations (Slatkin, 1985; Slatkin, 1987). Due to the annual numerous recombination events in *V. inaequalis* populations, selection can act on individual loci and still allow gene flow or drift at other loci.

To estimate gene flow between populations it is important to include both neutral and selected markers because selection on a certain locus could result in a pattern indicating little or no gene flow even if the actual level of gene flow is much larger (Slatkin, 1987). Therefore, supposed neutral loci like RAPD markers and the ITS-region of the ribosomal DNA, as well as the β-tubulin locus which may be under selective pressure were chosen for DNA-analyses.

The purpose of the study was to find out about migration and population differentiation. This was achieved by comparing four different *V. inaequalis* populations of which samples were collected in various parts of Switzerland. The comparisons were then used to estimate the probability of rapid migration of a new virulence such as the *Vf*-virulence.

Material and methods

Production of single spore cultures and DNA extraction. Isolates of *V. inaequalis* were collected in July and August 1993 at four different sites in Switzerland in orchards under various methods of management (Oberwil, Güttingen, Gland and Etoy) (Figure 1).

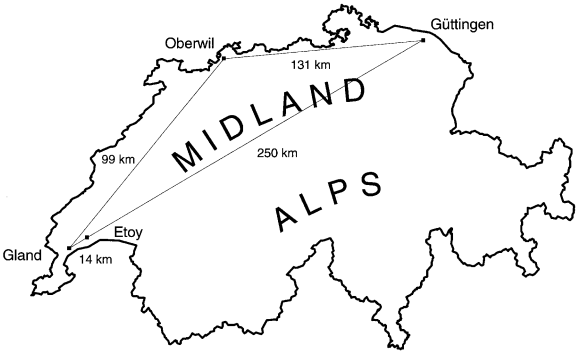

Figure 1. Sampling sites of populations of *Venturia inaequalis* in Switzerland. In Oberwil 43 lesions were collected from 22 trees, in Güttingen 58 lesions were collected from 46 trees, in Gland 46 lesions were collected from 46 trees and in Etoy 24 lesions were collected from 24 trees.

Leaves with well sporulating ascosporic or early secondary lesions were collected from trees chosen at random. In Oberwil four lesions per tree were collected at the most, in Güttingen three lesions per tree, in Gland and Etoy one lesion per tree. If more than one lesion was collected per tree the lesions were taken from different leaves and as far apart from each other as possible to reduce the probability of collecting clones. The lesions were excised, dried in an Eppendorf tube containing silicagel and stored at 3 °C. From each lesion, single spore isolates were produced and grown on terramycin-malt-agar plates (Sierotzki et al., 1994). When the diameter of the culture was about 4 cm, the mycelium was scraped off the agar plate then put into an Eppendorf tube containing glass beads of 0.45–0.5 mm diameter and lyophilised. The lyophilised mycelium and the glass beads were shaken in a cell homogenisor (B. Braun, Melsungen, Germany) for 80 seconds. DNA was extracted after a shortened protocol of the total-DNA mini-preparation of Zolan and Pukkila (1986) with the same modifications as described by Sierotzki et al. (1994).

Selection of markers. The RAPD loci and the ITS-region of the ribosomal DNA were chosen because they are supposed to be selectively neutral. Since neutral markers tell little or nothing about adaptive genetic changes in evolution (Hartl and Clark, 1989), the β-tubulin locus was chosen as a further marker. This locus may be under selective pressure because single-base mutations in this locus are responsible for the resistance of *V. inaequalis* to the fungicide benomyl (Koenraadt et al., 1992). That is why, in addition to the

molecular analysis of the β-tubulin locus, each isolate was tested on benomyl-amended agar plates to test its resistance level.

Polymerase Chain Reaction (PCR). Amplification reaction volumes were 15 μl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany), 0.3 μM Primer for RAPDs and 0.15 μM of each Primer for specific reactions, 5 ng of genomic DNA and 0.1U Super Taq DNA Polymerase (Stehelin, Basel, Switzerland). Amplification was performed in a Perkin Elmer Cetus Gene Amp. PCR System 9600 or a Hybaid OmniGene (Teddington, UK).

Random amplified polymorphic DNA (RAPD). After screenings of about 200 decamer primers from Operon Technologies Inc. USA the following four were selected for population analysis: F04 (5′-GGTGATCAGG-3′), R01 (5′-TGCGGGTCCT-3′), T07(5′-GGCAGGCTGT-3′), U19 (5′-GTCAGTGCGG-3′). The amplification of the marker bands was reproducible and the bands were polymorphic among isolates of *V. inaequalis*. Reactions were performed as described by Koller et al. (1993) and the evaluated markers defined with the name of the Operon primer and the size of the band in base pairs (e. g. F04-1300). Electrophoresis was performed on a 1% agarose gel.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). PCR-RFLP was performed for the internal transcribed spacer-region (ITS) of the ribosomal DNA and the β-tubulin-locus. The ITS1, 5.8S rDNA and the ITS2 were amplified with primers provided by MWG-Biotech (Synthesis Lab, Münchenstein, Switzerland) ITS5 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990). A 1190 base pair fragment of the β-tubulin gene (Koenraadt et al., 1992) was amplified using the primers P1 5′-CAAACCATCTCTGGCGAACACG-3′ and P2 5′-CGTGGTCTTAAGATGTCCTCCA-3′ (Wisconsin sequence Analysis Package, Version 8, Genetic computer group, Madison, USA). Amplification conditions for the ITS region were 40 s at 94 °C, 15 s at 64 °C, 2 min at 72 °C, 20 s at 94 °C, 20 s at 62 degC, 2 min at 72 °C, 20 s at 94 °C, 20 s at 60 °C, 2 min at 72 °C. Then 30 cycles with 20 s at 94 °C, 20 s at 60 °C and 2 min at 72 °C followed. The last amplification was at 72 °C for 10 min. Amplification conditions for the β-tubulin gene were 90 s at 94 °C then 40 cycles with 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C. Amplified DNA was electrophoresed on a 1% agarose gel. Digestion of the amplified fragments was performed for two hours in a final volume of 10 μl containing 1U enzyme, 1x reaction buffer (Boehringer Mannheim, Germany) and 100 ng amplification product. A 2% agarose gel was used for the electrophoresis.

ITS region. For further analysis of the ITS region, the amplified fragments were purified (NucleotraP®CR, Macherey-Nagel GmbH&Co. KG, Düren) and cloned into the vector pUC18 (Pharmacia Biotech Sure Clone Ligation Kit). Competent *E. coli* (strain DH5α) cells were used for the transformation following a standard protocol (Sambrook et al., 1989). Plasmids were extracted (Wizard™ midipreps, DNA Purification System, Promega, Madison, Wisconsin, USA) and the ITS region was sequenced (Microsynth GmbH, Balgach, Switzerland). The position of the rDNA and ITS was determined by sequence comparison using Wisconsin sequence Analysis Package (Version 8, Genetic computer group, Madison, USA). Using the same package, restriction sites within the sequence could be found.

In vitro *test for benomyl resistance.* Each isolate was grown on potato dextrose agar (3.9% w/v PDA, Difco, USA) amended with 6 different concentrations (0, 0.005%, 0.05%, 0.125%, 0.5% 1% w/v) of benomyl (Benlate, 50% benomyl, du Pont de Nemours & Co., Wilmington, DE, USA). After three weeks, the isolates were classified as susceptible (S, growth on < 0.005% benomyl), low resistant (LR, growth on < 0.05% benomyl), medium resistant (MR, growth on < 0.125% benomyl), high resistant (HR, growth on < 0.5% benomyl) and very high resistant (VHR, growth on 1% benomyl) (Stanis and Jones, 1984).

Data analysis. Presence or absence of polymorphic bands was scored. Frequencies of the alleles at each locus were calculated and the contingency χ^2 test of Workman and Niswander (1970) for significant differences in allele frequencies among populations was performed.

Further genetic diversity within (H_S, equation 1) and among populations (G_{ST}, equation 3) was calculated with the following formulae (Nei, 1973):

$$H_S = 1 - \sum_{i=1}^{h} x_{ij}^2 \qquad (1)$$

$$H_T = 1 - \sum_{i=1}^{k} \bar{x}_{ij}^2 \quad (2)$$

$$G_{ST} = \frac{H_T - \bar{H}_S}{T_T} \quad (3)$$

where h is the number of alleles and x_{ij} is the frequency of the i^{th} allele in population j. H_T (equation 2) is the total genetic diversity over all populations where k is the number of populations and $\bar{x}_{ij}$ is the frequency of allele i averaged over all populations. $\bar{H}_S$ is the average genetic diversity of all populations.

Haplotype diversity (Shannon's index of diversity) is $SI_j = -\sum_{i=1}^{k} p_{ij} \times \ln p_{ij}$ (Krebs, 1985) where p_{ij} is the frequency of the i^{th} haplotype in population j. Because SI_j is dependent on sample size, normalised haplotype diversity was calculated as $SI'_j = SI_j/\ln k$ where k is the sample size (Sheldon, 1969).

Results

Twelve polymorphic RAPD bands were sufficiently clear and constantly amplified to be scored easily (Table 1). With primers ITS4 and ITS5 either a fragment of 955bp or 573bp was obtained. Restriction fragment polymorphisms were found only within the intron of the 18S rDNA (Figure 2). Five alleles (A–E) were identified in this region with the restriction enzymes *Cfo*I and *Sal*I. While alleles A, B and E were present in all four populations, the allele C was found only in the population from Etoy and the allele D only in the population from Güttingen (Table 2).

Four alleles were found at the β-tubulin locus (Table 2). Except for allele 4 which was only present in benomyl-susceptible isolates, no allele was correlated with a resistance class. In the samples from Gland and Güttingen all resistance classes as well as susceptible isolates were present, whereas in the population from Oberwil only susceptible isolates were detected and in the population from Etoy only very highly resistant isolates were found. However, all isolates from Etoy possessed only allele 3 whereas all four alleles were found in the other populations (Figure 3).

Allele freqencies of the loci T07-550, U19-450, ITS and β-tubulin were significantly different (χ^2 test) among the four populations (Tables 1 and 2).

The within-population diversities (H_S) were very similar in all four locations and genetic diversity across all populations (H_T) did not differ much from the means

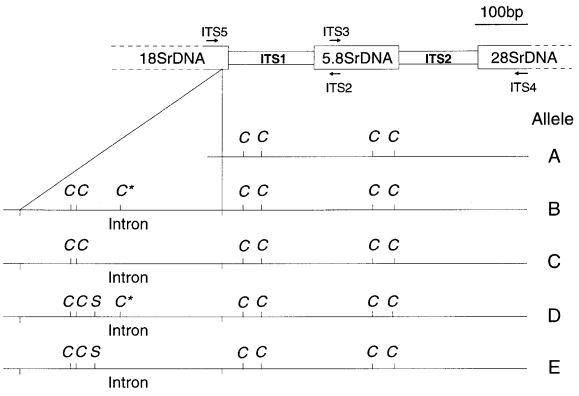

Figure 2. Internal transcribed spacer (ITS) region with the five alleles (A-E) found within isolates of *Venturia inaequalis*. Restriction sites of *Cfo*I and *Sal*I are indicated as C and S, respectively. C* is the only polymorphic restriction site recognised by *Cfo*I.

($\bar{H}_S$). Diversity indices of the RAPD loci varied from very low (H_S, $\bar{H}_S$, H_T <0.1) to very high (H_S, $\bar{H}_S$, H_T >0.4) values within populations whereas genetic diversity among populations (G_{ST}) of *V. inaequalis* in Switzerland was consistent over presumed neutral loci (RAPD and ITS) with a low mean of 0.04 (Tables 1 and 2). In contrast, G_{ST} for the β-tubulin locus was 0.26 (Table 2).

There was very low clonality within the collected populations indicated by high normalised haplotype diversities (SI'_j) based on RAPD and ITS data. SI'_j was 0.87 in Oberwil (24 isolates tested), 0.97 in Güttingen (37 isolates tested), 0.97 in Gland (15 isolates tested) and 0.98 in Etoy (21 isolates tested), whereas the highest possible value for SI'_j would be 1.00 if all isolates had a unique haplotype.

Discussion

The estimates of the parameters H_S, H_T, G_{ST} and SI'_j and the low differentiation (χ^2 test) among populations suggest that the *V. inaequalis* population is highly diverse, but distributed homogeneously throughout Switzerland.

The polymorphisms within the 18S rDNA of *V. inaequalis* were located in the middle of the intron. In addition to the alleles A, B and E, present in all populations, alleles C and D were found in low frequencies in the populations from Etoy and Güttingen, respectively. This might be due to recombination within the locus, but nevertheless it could be that the different alleles evolved independently in the different populations.

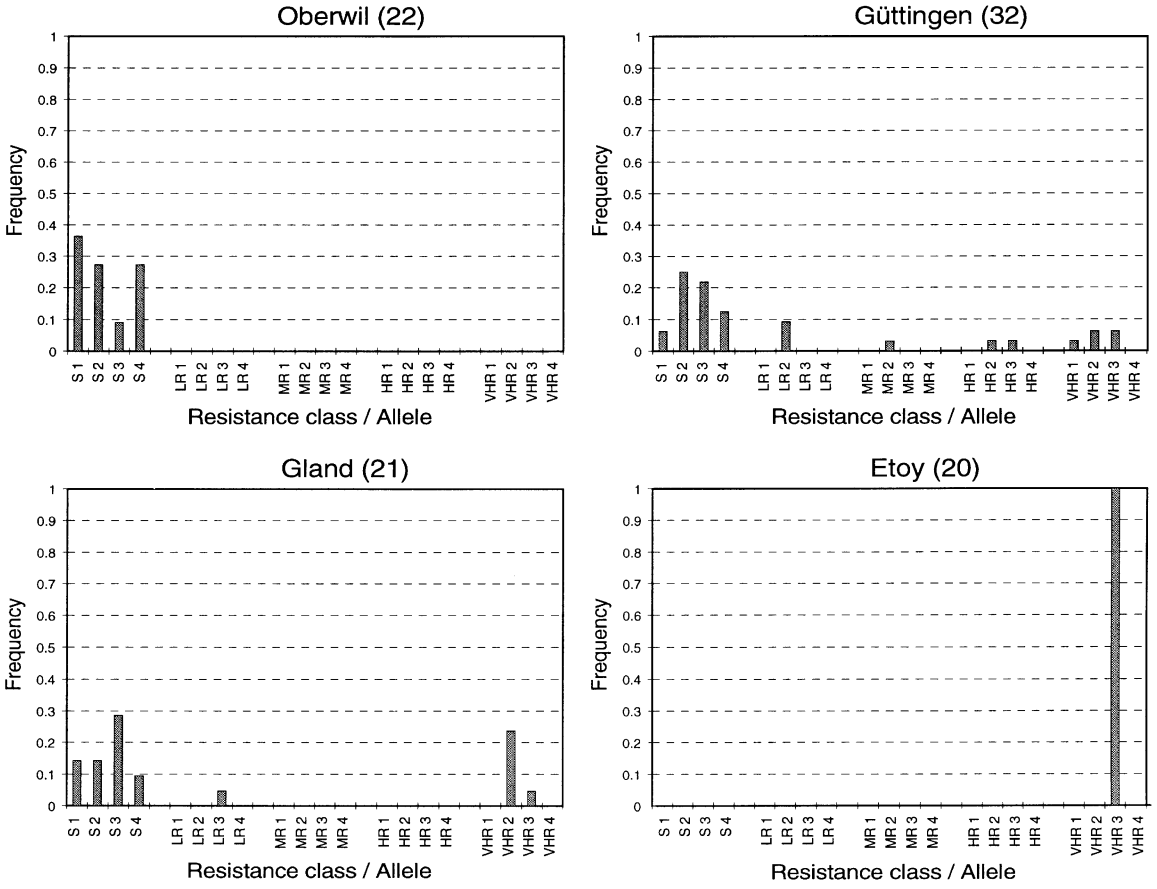

Figure 3. Distribution of the *Venturia inaequalis* isolates concerning their sensitivity towards the fungicide benomyl and the allele of the β-tubulin locus. Shown is the frequency (y-axis) of the isolates for each combination of resistance class (S, LR, MR, HR, VHR) / allele (1–4) (x-axis) in each population. The number of isolates analysed per population is given in parentheses.

With respect to the selected β-tubulin locus it is very probable that no correlation exists between an allele and a resistance class. This might be either because sample size is too small in each resistance class / allele combination or because restriction polymorphisms are due to neutral mutations within the β-tubulin gene. Distribution of the resistance classes within the four populations has to be discussed with knowledge about benomyl-treatments of the orchards. In Oberwil, only susceptible isolates were found and this orchard was never treated with benomyl. In all other orchards benomyl was used as a fungicide until the problem of building up of resistance occurred and this fungicide was replaced by others by the mid 1980s. Remarkable is the conservation of only resistant isolates in the population from Etoy even though selection pressure had been stopped.

The very low genetic diversity among populations (G_{ST} = 0.04) across the presumed neutral loci (RAPD and ITS) can be explained by the history of apple growing in Switzerland. Until about 50 years ago apple trees covered the whole midland not in the form of formal orchards but as meadow growing trees with little or no scab control. With the assumption that the scab population was continous at that time, constant migration over the whole area could have been possible. Short-distance transport of the inoculum due to natural forces such as splash dispersal of conidia during the rainy periods of summer and leaf litter distribution of infected plant material during winter storms brought scab with a high probability to a new host. Eventually, inoculum was also spread over large distances through the exchange of scab-infected plant material by human activity. Since then, planting methods of apple trees have changed. Orchards with intensive

Table 1. Allele frequencies of the RAPD markers (x_{ij}) in populations of *Venturia inaequalis* collected at four locations in Switzerland. χ^2 test for significant differences in allele frequencies among populations was performed and genetic diversity within populations (H_S), among populations (G_{ST}) and within the total collected population (H_T) was calculated. The number of isolates analysed per population is given in parentheses

Marker	Population										
	Oberwil (43)		Güttingen (58)		Gland (46)		Etoy (24)		Switzerland (171)		
	x_{ij}	H_S	x_{ij}	H_S	x_{ij}	H_S	x_{ij}	H_s	$\bar{H}_S$	H_T	G_{ST}
F04-1300	0.03[a]	0.05	0.11	0.19	0.03	0.06	0.00	0.00	0.08	0.08	0.04
F04-1500	0.95	0.10	0.98	0.04	0.94	0.12	0.96	0.08	0.08	0.08	0.01
F04-1800	0.66	0.45	0.42	0.49	0.41	0.48	0.46	0.50	0.48	0.50	0.04
R01-750	0.95	0.09	0.95	0.10	0.96	0.08	0.96	0.08	0.09	0.09	0.00
R01-850	0.95	0.10	0.93	0.13	0.80	0.31	0.83	0.28	0.20	0.21	0.04
R01-1000	0.45	0.50	0.30	0.41	0.35	0.45	0.30	0.41	0.44	0.45	0.02
R01-1150	0.07	0.13	0.17	0.29	0.20	0.31	0.21	0.33	0.27	0.17	0.02
T07-500	1.00	0.00	0.88	0.21	0.93	0.13	0.96	0.08	0.11	0.11	0.04
T07-550***	0.13	0.22	0.43	0.49	0.19	0.31	0.13	0.22	0.31	0.34	0.10
U19-410	0.41	0.49	0.28	0.40	0.19	0.31	0.42	0.49	0.42	0.44	0.04
U19-450***	0.71	0.41	0.97	0.07	0.69	0.43	0.96	0.08	0.25	0.28	0.12
U19-500	0.46	0.50	0.41	0.49	0.34	0.45	0.21	0.33	0.44	0.46	0.04
Average		*0.25*		*0.28*		*0.29*		*0.24*	*0.26*	*0.28*	*0.04*

[a] Frequency of the allele that is shown by presence of the band.
*, **, *** χ^2 values were significant at $P<0.05$, 0.01 and 0.005, respectively.

Table 2. Allele frequencies of the ITS locus (A–E) and the β-tubulin locus (1–4) of populations of *Venturia inaequalis* collected at four locations in Switzerland. χ^2 test for significant differences in allele frequencies among populations was performed and genetic diversity within populations (H_S), among populations (G_{ST}) and within the total collected population (H_T) was calculated

Population	ITS***							β-tubulin***					
	N[a]	A	B	C	D	E	H_S	N	1	2	3	4	H_S
Oberwil	25	0.08	0.28	0.00	0.00	0.64	0.51	22	0.36	0.27	0.09	0.27	0.71
Güttingen	39	0.10	0.36	0.00	0.10	0.44	0.66	37	0.11	0.51	0.27	0.11	0.64
Gland	32	0.38	0.22	0.00	0.00	0.41	0.65	23	0.13	0.39	0.39	0.09	0.67
Etoy	19	0.05	0.32	0.16	0.00	0.47	0.65	17	0.00	0.00	1.00	0.00	0.00
Switzerland	115	0.17	0.30	0.03	0.03	0.48	0.62[b]	99	0.15	0.34	0.38	0.12	0.51[b]
							0.65[c]						0.69[c]
							0.05[d]						0.26[d]

*, **, *** χ^2 values were significant at $P<0.05$, 0.01 and 0.005, respectively.
[a] Sample size.
[b] $\bar{H}_S$.
[c] H_T.
[d] G_{ST}.

scab control have replaced the old system almost completely, and large regions without apple trees have appeared. Therefore, the natural spread of scab may be more limited and due to the fact that planting material originates from nurseries with almost complete scab control, eventual spread through human activity might have almost been eliminated. This theory leads to the assumption that, even if data show only little differentiation, migration between the populations might be limited. Gene flow easily could be overestimated because of the effects of gene flow in the past. Therefore, the effects of colonisation events cannot be distinguished from current gene flow when differentiation among populations is analysed (Slatkin, 1985; Slatkin, 1987).

The fact that four particular markers, three assumed to be neutral, appear to a significant different frequency among the populations, may be interpreted as a first indication for subdivision of the scab populations in Switzerland.

The question arises why scab on *Vf*-resistant trees has so far only been found in Ahrensburg (Germany) and Kent (UK) but not in any other country where orchards with *Vf*-resistant cultivars are also planted. It is a possibility that the *Vf*-virulence has not had enough time to spread from Ahrensburg and Kent to Switzerland and it could be for this reason that to date *Vf*-virulence has not been recorded. Nevertheless, its spread could go on without being recognised through the ability of *Vf*-virulent scab to also infect non-*Vf*-carrying cultivars such as the most important cultivar in Europe, Golden Delicious.

If only natural gene flow occurs, that means splash dispersal of conidia or distribution of scab-infected leaf litter, it may take many years for the *Vf*-virulent scab to migrate to Switzerland and other countries. The speed of migration might be increased however due to artificial gene flow through exchange of scab-infected plant material that should not be neglected. The fact that gene flow occurred in the past, and natural as well as artificial gene flow are hard to distinguish, makes it very difficult to predict durability of the *Vf*-resistance in Switzerland.

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