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Genetic diversity of *Venturia inaequalis* across Europe

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Abstract

The genetic diversity of eleven populations of *Venturia inaequalis* from five European countries was calculated based on the allele frequencies of 18 random amplified polymorphic DNA markers and the internal transcribed spacer region of the ribosomal DNA. Diversity within each population (H_S) was high with values ranging from 0.26 to 0.33. Average differentiation among populations (G_{ST}) was 0.11 and populations were isolated by distance ($r^2 = 0.50$, $P < 0.01$). This indicates that extensive short-distance gene flow occurs in Europe and that dispersal over longer distances also appears to occur frequently enough to prevent differentiation due to genetic drift. We had expected more pronounced differentiation between populations north and south of the Alps, because *V. inaequalis* is primarily dispersed by rain splashing and wind distribution of leaf litter, and because the Alps might form a barrier to dispersal. However, our results indicate that human-mediated gene flow due to transport of spores or infected plant material has probably occurred.

Introduction

Apple scab, caused by the ascomycete *Venturia inaequalis* (Cke.) Wint., is the most important disease in apple (*Malus domestica*) cultivation in Europe. In spring, ascospores start the epidemic and cause primary lesions on young apple leaves. Conidia are then produced and continue the epidemic by infecting leaves and fruits. Up to eight cycles may be completed in a growing season (MacHardy, 1996). Although ascospores and conidia are primarily spread by rain splashing on infected leaves (Frey and Keitt, 1925; Wiesmann, 1932), wind distribution of infected leaves and aerosol ascospores can contribute to migration of the fungus (MacHardy, 1996).

Principal control of *V. inaequalis* is by fungicides. However, breeding and cultivation of less sensitive cultivars could be a step towards more ecological production of high-quality apples with reduced fungicide input. Several breeding programs include other *Malus* species (crab apples), such as *M. baccata* and *M. floribunda*, as resistance sources (Gessler, 1997). The *Vf*-resistance originating from *M. floribunda* 821 has become the most important, and many cultivars carrying this resistance have been released since 1970 (Gessler, 1997; MacHardy, 1996). Although this resistance was considered highly durable originally (Crosby et al., 1992), scab isolates capable of infecting particular *Vf*-resistant cultivars were found on such cultivars at or near the breeding stations Ahrensburg (Germany) in 1984 (Parisi et al., 1993), Kent (Great Britain) in 1994 (Roberts and Crute, 1994) and Wilheminadorp (the Netherlands) in 1997 (Kemp and Schouten, 1998). Since cultivars carrying the *Vf*-resistance are also planted in other regions, there is growing concern about the possible migration of *Vf*-virulent scab from these orchards.

In an earlier study of the genetic structure of four populations of *V. inaequalis* in Switzerland (Tenzer and Gessler, 1997), we found high genetic diversity

within populations [H_S (Nei, 1973) between 0.24 and 0.62], but low differentiation among populations [average G_{ST} (Nei, 1973) was 0.04 for RAPD loci, 0.05 for the ITS locus and 0.28 for the β-tubulin locus], suggesting a homogeneous distribution of *V. inaequalis* populations within Switzerland and a high level of gene flow among them. However, current levels of gene flow might be overestimated due to effects of gene flow in the past, when populations were not in migration/drift equilibrium (Slatkin, 1987). We interpreted the fact that the frequency of four markers out of fourteen was significantly different among the four populations, as an indication that differentiation among populations due to genetic drift might be in progress. This information, combined with the knowledge that *V. inaequalis* is spread by rain splashing or wind distribution of infected leaf litter, suggested that it might take many years before *Vf*-virulent scab isolates could spread over the whole of Europe.

Since it is difficult to extrapolate data from Switzerland to a broader geographic range, eleven *V. inaequalis* populations were sampled in 1995 in five European countries. They were examined for diversity and differentiation based on allele frequencies of 18 random amplified polymorphic DNA (RAPD) markers and the internal transcribed spacer (ITS) region of the ribosomal DNA. We were interested in the level of differentiation among the European populations, but we also analysed the importance of the Alps as a probable natural barrier inhibiting the dispersal of the fungus. We tested for isolation by distance because the biology of the fungus restricts the likelihood of long-distance dispersal.

Materials and methods

Production of single-spore cultures and DNA extraction

Scab infected leaves were collected in May and June 1995 at eleven sites in five European countries in orchards managed according to organic farming guidelines: one in France (F), two in Germany (D1, D2), four in Italy (South Tyrol, I1–4), one in The Netherlands (NL) and three in Switzerland (two in Southern Switzerland, CH1, CH2, and one in Northern Switzerland, CH3) (Figure 1).

Trees were chosen at random and leaves with well sporulating primary lesions (assumed to be derived from ascospore infection) or early secondary lesions were collected. Only one leaf per tree was sampled. The isolates were collected from the cultivar Golden Delicious except for the populations from Ahrensburg, Wageningen and Oberwil. The isolates from Ahrensburg were collected from Golden Delicious as well as from cultivars carrying the *Vf*-gene such as Florina, Freedom and Prima, but also from progenies of two crosses, which were selected for *Vf*-resistance (Parisi et al., 1993). Isolates from the orchard in Wageningen were collected from different cultivars and progenies of diverse crossings (Centrum voor Plantenveredelings en Reprodukieonderzoek, Wageningen, the Netherlands, J. Janse, pers. comm.), whereas the isolates from Oberwil were sampled from Boskoop, Champagner Reinette, Glocken-Apfel, Maigold and Spartan (Table 1).

Lesions were excised, dried in a microcentrifuge 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). The mycelium was then grown in Erlenmeyer flasks containing 50 ml of potato dextrose broth (2.4% w/v, Difco, USA). After three weeks the mycelium was centrifuged to pellet, washed twice with 20 ml ice-cold water and then lyophilised. About 20 mg of freeze-dried mycelium were placed into a microcentrifuge tube containing glass beads of 0.45–0.5 mm diameter and shaken in a cell homogeniser (B. Braun, Melsungen, Germany) for 20 s. DNA was extracted after a shortened protocol of Zolan and Pukkila (1986) with the same modifications as described in Sierotzki et al. (1994) or with the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland).

Polymerase chain reaction (PCR)

Amplification reaction volumes were 15 µl containing 1× reaction buffer (Pharmacia Biotech, Uppsala, Sweden), 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 ITS reactions, 5 ng of genomic DNA and 1.05 U Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). Amplification was performed in a Perkin Elmer Cetus Gene Amp. PCR System 9600.

After screening approximately 200 decamer primers (Operon Technologies Inc., USA) the following six were selected for population analysis because they showed clear reproducible bands and because polymorphisms were found: D07 (5′-TTGGCACGGG-3′), E15

Figure 1. Origin of the eleven *V. inaequalis* populations collected in Europe in 1995.

Table 1. Origin of the eleven *V. inaequalis* populations collected in Europe in 1995

Country	Orchard[a]	Abbreviation	*N*[b]	Cultivar
France	Angers	F	44	Golden Delicious
Germany	Ahrensburg	D1	28	Florina[c], Freedom[c], Golden Delicious, Prima[c], Progenies of two crossings selected for *Vf*- resistance[d]
Italy (South Tyrol)	Algund	I1	36	Golden Delicious
Italy (South Tyrol)	Auer	I2	31	Golden Delicious
Italy (South Tyrol)	Margreid	I3	21	Golden Delicious
Italy (South Tyrol)	Neumarkt	I4	30	Golden Delicious
The Netherlands	Wageningen	NL	46	Diverse cultivars, and progenies of diverse crossings[e]
Switzerland (Southern Switzerland)	Ascona	CH1	26	Golden Delicious
Switzerland (Southern Switzerland)	San Antonino	CH2	14	Golden Delicious
Switzerland (Northern Switzerland)	Oberwil	CH3	23	Ananas Reinette, Boskoop, Champagner Reinette, Glocken-Apfel, Maigold, Spartan

[a] All orchards were managed according to the guidelines of organic farming (no input of artificial fertilisers or pesticides).
[b] Sample size.
[c] Cultivars carrying the *Vf*-gene.
[d] Prima × A143/24 and Prima × Fiesta (9).
[e] Centrum voor Plantenveredelings en Reprodukieonderzoek (CPRO-DLO), Wageningen, the Netherlands, (J. Janse, pers. comm.).

(5′-ACGCACAACC-3′), F01 (5′-ACGGATCCTG-3′), F04 (5′-GGTGATCAGG-3′), U10 (5′-ACCTCGG-CAC-3′) and U19 (5′-GTCAGTGCGG-3′). Reactions were performed as described by Koller et al. (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., D07-900). Electrophoresis was performed on a 1% agarose gel in 0.5× Tris-borate/EDTA buffer (Sambrook et al., 1989). The bands that were polymorphic among isolates of *V. inaequalis* were tested for reproducibility by repeating the reaction two or three times with a set of eight isolates.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was performed for the ITS of the ribosomal DNA, and the five alleles A, B, C, D and E described in Tenzer and Gessler (1997) were analysed.

Data analyses

Polymorphic bands were scored and interpreted as the two alleles [presence or absence of a band (Williams et al., 1993; Peever and Milgroom, 1994; McDonald, 1997; Sicard et al., 1997)] at a locus. Allele frequency at each locus was calculated and the contingency χ^2 test (Workman and Niswander, 1970) was performed to test for heterogeneity of allele frequencies among the eleven European *V. inaequalis* populations.

Genetic diversity within (H_S, Equation 1) and among populations (G_{ST}, Equation 3) was calculated using the following formulae (Nei, 1973):

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

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

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

where h is the number of alleles and x_{ij} is the frequency of the ith 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 eleven populations. $\bar{H}_S$ is the average genetic diversity of all populations.

Isolation by distance was tested as described by Slatkin (1993). Gene flow estimates ($M' = (1/G_{ST} - 1)/2$) (Slatkin, 1993) and geographic distance between each pair of populations were determined. The denominator for the calculation of M' was 2 instead of 4 because *V. inaequalis* is haploid. The logarithm of M' will be negatively correlated with the logarithm of the geographic distance if populations are in migration/drift equilibrium (Slatkin, 1993). All computations were performed with Microsoft Excel 5.0a.

Results

The 11 European *V. inaequalis* populations sampled were genetically differentiated and the allele frequencies of 12 out of 19 markers were significantly different (χ^2 test, $P < 0.05$) among all populations (Table 2). At the ITS locus, the alleles B and E were the most frequent, whereas the alleles A, C and D were either very rare or absent (Table 2). Allele E was found in 61% or more of the isolates from north of the Alps, whereas in the populations from south of the Alps the frequency of allele E was 31% or less. Allele B was found in 54% or more of all isolates in populations south of the Alps and in up to 32% in populations north of the Alps (Table 2).

The average genetic diversity within each population (H_S) over all loci was high with values ranging from 0.26 to 0.33 (maximum possible value of H_S for these markers is 0.52, lowest is 0). Diversity within populations D2, I2, I4 and NL is equal to or higher than 0.3 (Table 2). The lowest diversity was found within populations F ($H_S = 0.26$), D1 ($H_S = 0.27$) and CH2 ($H_S = 0.26$). The average genetic differentiation among all sampled European populations (G_{ST}) was 0.11, even though the value at single loci varied from 0.03 to 0.28 (Table 2). Pairwise genetic differentiation values (G_{ST}) among all loci were low (Table 3). Values higher than 0.1 were only found for the comparisons of D1 with particular populations from south of the Alps (I2, I3, CH1, CH2) and CH3 (Table 3).

Differentiation between the pooled populations from north of the Alps and the pooled populations from south of the Alps was 0.05. Differentiation among populations from the same region was 0.03 for South Tyrol (I1–4), 0.04 for Northern Germany (D1, D2) and 0.04 for Southern Switzerland (CH1, CH2). Diversity among all populations from south of the Alps (CH1, CH2, I1–4) was 0.05 (data not shown).

Isolation by distance among the populations could be demonstrated using the relationship of the logarithm of gene flow and the logarithm of the geographic distance introduced by Slatkin (Slatkin, 1993). The regression analysis was highly significant ($P < 0.01$); 50% ($r^2 = 0.50$) of the variation among populations could be explained by geographic distance (Figure 2).

Table 2. Allele frequencies of the random amplified polymorphic DNA (RAPD) and internal transcribed spacer (ITS) loci in each *V. inaequalis* population collected in Europe in 1995. Average genetic diversity within (H_S) and among (G_{ST}) populations as well as significant differences in allele frequency among all populations (χ^2 test) are shown

Locus	Population											G_{ST}
	F[a]	D1	D2	I1	I2	I3	I4	NL	CH1	CH2	CH3	
D07-900***	0.84[b]	0.61	0.84	0.31	0.42	0.38	0.42	0.63	0.54	0.36	0.35	0.13
D07-1350	0.91	1.00	0.86	0.94	0.94	1.00	1.00	0.96	1.00	0.86	0.87	0.05
E15-1000***	0.34	0.46	0.56	0.31	0.55	0.52	0.52	0.15	0.46	0.57	0.43	0.06
E15-1800	0.55	0.71	0.49	0.44	0.35	0.38	0.48	0.50	0.50	0.71	0.52	0.05
F01-390*	0.66	0.25	0.44	0.44	0.58	0.62	0.52	0.57	0.62	0.05	0.70	0.06
F01-800	0.02	0.00	0.07	0.06	0.06	0.05	0.03	0.04	0.04	0.14	0.13	0.03
F01-900***	0.73	0.14	0.47	0.72	0.77	0.52	0.58	0.65	0.73	0.86	0.78	0.16
F04-1000***	0.07	0.07	0.21	0.00	0.07	0.00	0.03	0.00	0.00	0.07	0.13	0.07
F04-1300	0.02	0.00	0.02	0.06	0.07	0.00	0.10	0.00	0.34	0.00	0.00	0.04
F04-1500***	0.95	1.00	1.00	0.92	0.67	0.56	0.84	1.00	0.88	0.93	1.00	0.12
F04-1800***	0.35	0.21	0.37	0.47	0.87	0.67	0.52	0.41	0.58	0.79	0.22	0.17
U10-1250	0.95	0.68	0.86	0.86	0.87	0.86	0.81	0.85	0.92	0.86	0.96	0.04
U10-1400	1.00	1.00	1.00	1.00	1.00	0.90	0.97	0.93	1.00	0.93	0.96	0.04
U10-1700	0.41	0.43	0.58	0.58	0.61	0.52	0.58	0.57	0.42	0.71	0.48	0.03
U10-1800***	0.50	0.71	0.70	0.42	0.39	0.10	0.29	0.54	0.15	0.00	0.52	0.21
U19-400***	0.02	0.36	0.40	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.17	0.28
U19-450***	0.93	0.89	0.86	0.64	0.74	0.71	0.84	0.96	0.88	1.00	0.87	0.08
U19-500***	0.81	0.14	0.44	0.25	0.32	0.29	0.19	0.43	0.54	0.36	0.96	0.24
ITS***												
A	0.09	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	
B	0.23	0.32	0.28	0.78	0.73	0.76	0.73	0.28	0.54	0.67	0.22	
C	0.00	0.00	0.00	0.00	0.03	0.00	0.07	0.00	0.15	0.04	0.00	0.19
D	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	
E	0.68	0.68	0.70	0.22	0.23	0.14	0.20	0.72	0.31	0.29	0.61	
Average	0.26	0.27	0.33	0.29	0.31	0.29	0.31	0.30	0.28	0.26	0.29	0.11

*, *** χ^2 values were significant at $P < 0.05$ and 0.005, respectively.

[a] Population abbreviations are in Table 1.

[b] Frequency of the RAPD band (plus allele) and the alleles at the ITS locus, respectively.

[c] G_{ST} was calculated over all 11 populations before rounding off.

Discussion

The eleven scab populations sampled in Europe in 1995 were isolated by distance under geographically limited gene flow. Fifty percent of the variation among populations could be explained by isolation resulting from geographic distance. The other 50% might be due to other factors such as the loci analysed, identity of the cultivars sampled, local climatic conditions, fungicide treatments, selection, or other unmeasured factors.

Loci with G_{ST} values higher than 0.1 might be under selective pressure or linked to a locus under selection in some populations. However, *V. inaequalis* undergoes an obligate annual sexual stage. Thus, crossovers recombine allele combinations generating new haplotypes. So selection can act on an individual locus without affecting drift or gene flow at unlinked loci. Looking at the differentiation between each pair of populations, high G_{ST} values ($G_{ST} > 0.1$) were only observed between population D1 and two populations from Italy (I2, I3) and D1 and the three Swiss populations (CH1–3). However, between populations with small geographic distance, such as the four Italian (I1–4), the two Southern Swiss (CH1 and CH2) and the two German populations (D1 and D2), differentiation was always very low ($G_{ST} \leq 0.04$). This leads to the conclusion that in Europe very frequent short distance gene flow happens, but also dispersal over longer distances occurs frequently enough to counteract genetic differentiation due to genetic drift or local selection.

Genetic drift due to random changes in allele frequencies is mainly important in small populations

Table 3. Genetic differentiation (G_{ST}, in lower triangle) and geographic distance (km, in upper triangle) between *V. inaequalis* populations collected in Europe in 1995

Population	F[a]	D1	D2	I1	I2	I3	I4	NL	CH1	CH2	CH3
F	—	1010[c]	990	890	890	890	890	660	750	740	610
D1	0.09[b]	—	20	770	810	810	810	380	830	830	700
D2	0.04	0.04	—	750	790	790	790	360	810	800	670
I1	0.06	0.08	0.06	—	40	40	40	760	190	170	290
I2	0.07	0.11	0.07	0.03	—	10	1	800	190	170	310
I3	0.07	0.10	0.08	0.03	0.03	—	1	800	190	170	310
I4	0.06	0.07	0.06	0.02	0.02	0.01	—	800	190	170	310
NL	0.04	0.06	0.03	0.05	0.07	0.07	0.05	—	730	740	560
CH1	0.03	0.10	0.07	0.03	0.03	0.02	0.02	0.05	—	20	180
CH2	0.08	0.13	0.09	0.06	0.05	0.04	0.04	0.08	0.04	—	190
CH3	0.03	0.11	0.06	0.06	0.08	0.08	0.07	0.05	0.05	0.09	—

[a] Population abbreviations are in Table 1.
[b] Pairwise genetic differentiation between populations (G_{ST}).
[c] Distance in kilometres between populations.

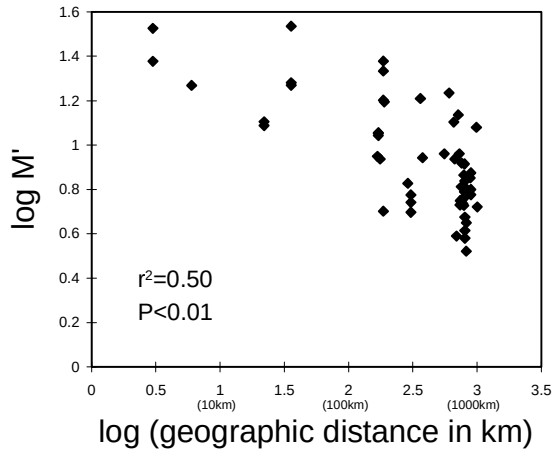

Figure 2. Correlation between the logarithm of the estimator of gene flow (M') and the logarithm of the geographic distance between each pair of *V. inaequalis* populations collected in Europe in 1995. Estimation of gene flow is based on data of 18 random amplified polymorphic DNA loci and the internal transcribed spacer locus.

(Nei et al., 1975). In practice, population size of *V. inaequalis* becomes reduced when primary inoculum is lowered by rotting and collecting of fallen infected apple leaves in winter and during the season when fungicides are applied. These measures are very effective in reducing disease incidence and might cause genetic bottlenecks which decrease diversity and reduce the number of alleles (Nei et al., 1975). Since we always found very high diversity within each of the eleven populations, we suspect that reduction in population size is not strong enough that genetic drift could differentiate populations.

Gene flow, which counteracts genetic drift, can be due to the migration of individuals between existing populations, but also due to recolonisation after a complete local extinction (Slatkin, 1977). For every kind of gene flow, it is difficult to distinguish between current and historical effects (Slatkin, 1987). Both aspects of gene flow and their relation to drift must be considered in respect to apple growing systems in Europe, and to the biology of the fungus. Until 50 years ago, apples were grown in the meadow system where trees were planted throughout the landscape often with wide spaces between trees. Splash dispersal of conidia of *V. inaequalis* is mainly important for migration within or between neighbouring trees, whereas long-distance dispersal of *V. inaequalis* is primarily due to wind distribution of airborne ascospores or infected leaf litter. The more tightly trees are planted, the higher the probability that potential inoculum reaches a new host. With today's intensive orchard growing system inoculum reaches a new host within the orchard very easily. Large gaps without apple trees between orchards make it difficult for spores to naturally migrate among orchards. However, low-input apple cultivation in house gardens or on meadows could serve as reservoirs for *V. inaequalis*, preventing effects of genetic drift. This could also be a reason why recolonisation of newly planted apple trees apparently occurs quite quickly, since there has never been a scab-free (not fungicide treated) orchard reported in Europe. However, newly planted single trees in isolated sites may stay scab free for several years (C. Gessler, pers. obs.). In Western

Australia, where apples are grown in widely separated locations, orchards with susceptible cultivars free of *V. inaequalis* still existed until about 1990. The chronological occurrence of newly infected orchards in this region has been reported in detail (MacHardy, 1996). New infections can be traced back to the introduction of infected apple trees from a nursery or transport of conidia of *V. inaequalis* on the body or clothing of humans travelling among heavily infected orchards and previously disease-free orchards (MacHardy, 1996). With this example, the importance of humans in influencing the population dynamics of plant pathogenic fungi was shown. The transport of infected plant material might be a reason why only moderate differentiation among European populations was found, and why geographically close populations are only slightly differentiated.

If the Alps were an important barrier to gene flow, a very high differentiation among northern and southern populations would be expected. However, differentiation among populations from south and north of the Alps was low. This finding let us conclude that the Alps are not a barrier sufficient to clearly differentiate populations from north and south. It cannot be determined whether *V. inaequalis* bypasses the Alps by natural migration or by human intervention.

Geographic distance seems to be the most important factor in reducing gene flow, as it explains 50% of the variation among the European *V. inaequalis* populations. It reflects the process of natural gene flow and does not contradict the human caused gene flow (as plant material is distributed more frequently locally and less frequently over long distances). A slow, radial, natural distribution of the *Vf*-virulence with Ahrensburg or Kent as centres should be expected. The importance of human-mediated migration of scab due to the transport of spores or infected plant material must not be neglected, and could lead to the establishment of secondary foci of the new virulence. The ability of *Vf*-virulent scab to infect susceptible cultivars such as Golden Delicious (the most common cultivar in Europe) makes the detection of an eventual migration very problematic. Orchards with *Vf*-resistant cultivars could serve as spore traps for the identification and monitoring of the dispersal of *Vf*-virulent individuals.

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