Microsatellite Primers Indicate the Presence of Asexual Populations of *Venturia inaequalis* in Coastal Israeli Apple Orchards

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This study was initiated to determine whether differences in genotypic diversity among populations of *Venturia inaequalis* (Cke.) Wint., as detected using neutral genetic markers, were related to the ecological conditions in which apples are grown in Israel. Since sexual reproduction in this fungal pathogen has an obligate requirement for sustained low winter temperatures, and since these requirements in Israel are met only on the Golan Heights, we were interested in whether lower elevation populations of this pathogen might be comprised of asexual clonal lineages. Unlike temperate apple growing regions, where the primary spring inoculum is ascosporic derived from overwintered pseudothecia, Israeli apple orchards at lower elevations in the Hula Valley and along the coastal plain rarely if ever experience low winter temperatures and pseudothecia have never been recovered. Two orchards were sampled from the Golan Heights (El Rom and Ortal, n = 38) and three orchards from the Hula Valley and coastal plain (Sede Eliezer, Ginaton and Be'er Tuvia, n = 40). Microsatellite primers were used to analyze population structure and the resulting binary data analyzed by both cluster and parsimony analysis. Populations from the coastal plain were genetically uniform within each of the orchards sampled, whereas populations from the Golan Heights showed levels of genotypic diversity ten times as high. The data support field observations that this pathogen does not reproduce sexually in regions characterized by the absence of low winter temperatures and is instead composed of clonal lineages. This may have bearing on control strategies for the disease in Israel.

KEY WORDS: *Venturia inaequalis*; apple scab; apple orchards; loculoascomycete; population biology; *Spilocea pomi*; *Malus domestica*; fungal DNA.

INTRODUCTION

Apple scab, caused by the loculoascomycete *Venturia inaequalis* (Cke.) Wint. (anamorph *Spilocea pomi* Fr.), is the single most important disease of cultivated apple (*Malus domestica* Borkh.) worldwide. Severe crop losses in susceptible cultivars can result when appropriate control measures are not taken, especially when the spring and summer seasons are moist and temperate (12,16). The pathogen has a pronounced low temperature requirement for the initiation of the sexual or ascigerous stage (29) and, in temperate apple-growing regions, this temperature requirement is usually met only after leaf fall, triggering

Received Nov. 25, 2002; received in final form Jan. 23, 2003; http://www.phytoparasitica.org posting April 21, 2003.

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the development of pseudothecia (4,5,7,10). In temperate regions, the primary inoculum in the spring, responsible for infection of young leaves at the green bud stage and later emergent sepals and fruit, has been demonstrated to be comprised of ascospores derived from overwintered leaves producing pseudothecia (12). To a lesser extent, the pathogen has also been shown to overwinter as wood scab or in bud scales, from which conidia produced asexually can initiate the disease cycle in the spring, but this is considered to be negligible and only a minor component of the primary inoculum when present (12,16).

Control strategies, therefore, have been directed primarily at the ascigerous stage in *V. inaequalis* to prevent primary ascosporic inoculum. Chemical control, both in the autumn using eradicant fungicides to prevent early pseudothecial development (9), and in the spring using protectant or curative fungicides to attenuate ascospore discharge and infection (2,8,12,20,22), are the primary means of control. Proper timing of fungicidal applications is a critical component of disease management and forms the basis for a number of predictive mathematical models based on the monitoring of environmental conditions conducive to ascospore maturation and discharge (3).

In Israel, apple production can be divided into two principal ecological zones, characterized primarily by the presence or absence of low winter temperatures due to elevation: the Upper Galilee and Golan Heights (600–1300 m above sea level), and the Hula Valley (100 m above sea level) and coastal plain. Only the Upper Galilee and Golan regions experience sustained low winter temperatures, whereas such temperatures are rare in apple orchards of the Hula Valley and non-existent along the coastal plain. It is believed that this intrinsic difference in Israeli apple production environments has had a direct impact on the life cycle of the pathogen, attenuating the importance of the ascigerous stage in low-elevation apple production areas (19,23).

The present study was initiated to determine whether differences in genotypic diversity among populations of *V. inaequalis*, as detected using neutral genetic markers, were related to the ecological conditions in which apples are grown in Israel. Since sexual reproduction in this fungal pathogen has an obligate requirement for sustained low winter temperatures (4,5,10,29), and since these requirements in Israel are met only in the Upper Galilee and Golan, we were interested in whether lower elevation populations of this pathogen might be comprised of asexual clonal lineages. This would have a bearing on control strategies for the disease in Israel and may impact on the propensity of this pathogen to develop fungicide resistance in the field (8,22,23). Recently, oligonucleotides specific to microsatellite or simple sequence repeats have proven effective as single primers in conventional polymerase chain reaction (PCR), termed simple sequence repeat (SSR) PCR (30) or random amplified microsatellite (RAMS) PCR (6). Believed to be more robust than conventional randomly amplified polymorphic DNA (RAPD) methods (28), the method has gained increased usage (6,11,13). In the present study, three microsatellite primers were used to assess the relative level of genotypic diversity for *V. inaequalis* isolates originating from five apple orchards situated in the two principal ecological zones of apple production in Israel.

MATERIALS AND METHODS

Collection of isolates Infected apple leaves bearing characteristic lesions of *V. inaequalis* were collected throughout Israeli apple production areas in the autumn of 1994 (Fig. 1; Table 1). A total of 78 isolates were obtained from two climactically distinct growing regions, namely, the Golan Heights and the Hula Valley / coastal plain. Efforts were made

to sample orchards that lay in the geographical center of apple production for each given region. From the Golan Heights, isolates were taken from two commercial orchards: El Rom at 1000 m above sea level (designated orchard G; n = 28) and Ortal at 800 m above sea level (adjacent orchards H and I; n = 10), separated by a distance of 5 km. Isolates obtained from the Hula Valley were taken from only a single orchard in Sede Eliezer (orchard P; n = 7), a site roughly 25 km from the sites in the Golan Heights, and located at a much lower elevation (100 m above sea level). Coastal plain isolates were obtained from two commercial apple orchards located approximately 200 km south of the Sede Eliezer site: Ginaton (adjacent orchards L and M; n = 13) and Be'er Tuvia (orchard R; n = 20), separated by a distance of 38 km. Isolates from the Hula Valley and the coastal plain all originated from the local Israeli apple cultivar 'Anna' (orchards P, L and R) or its pollinator 'Ein Shemer' (orchard M). Isolates collected from the Golan originated from the introduced temperate apple cultivars 'Top Red' (G) and 'Starking Delicious' (H and I) (Table 1).

Fig. 1. Map of Israel indicating the five collection sites for *Venturia inaequalis*. El Rom (G; n=28) and Ortal (H/I; n=10) isolates originated from introduced temperate apple cultivars grown on the Golan Heights at an elevation of 800–1000 m above sea level (large oval, upper right). The Sede Eliezer (P; n=7) isolates were from the Hula Valley at an elevation of 100 m above sea level (small oval), whereas the Ginaton (L/M; n=13) and Be'er Tuvia (R; n=20) isolates originated from the coastal plain (sea level). Isolates from the latter three sites originated from the local apple cultivar Anna and its pollinator Ein Shemer. Distances between sites are indicated in the text.

TABLE 1. List of Israeli *Venturia inaequalis* isolates used in this study

Isolate[z]	Location/cultivar	Tree	Leaf	Lesion	Spore
G2.1.1	[a]El Rom/Top Red	2	1	1	1
G2.1.2	El Rom/Top Red	2	1	1	2
G2.1.3	El Rom/Top Red	2	1	1	3
G2.2.1	El Rom/Top Red	2	1	2	1
G2.2.2	El Rom/Top Red	2	1	2	2
G2.2.3	El Rom/Top Red	2	1	2	3
G2.5.1	El Rom/Top Red	2	1	5	1
G2.5.2	El Rom/Top Red	2	1	5	2
G2.5.3	El Rom/Top Red	2	1	5	3
G2.6.1	El Rom/Top Red	2	1	6	1
G2.6.2	El Rom/Top Red	2	1	6	2
G2.6.3	El Rom/Top Red	2	1	6	3
G2.7.1	El Rom/Top Red	2	1	7	1
G2.7.2	El Rom/Top Red	2	1	7	2
G2.7.3	El Rom/Top Red	2	1	7	3
G2.12.1	El Rom/Top Red	2	1	12	1
G2.12.2	El Rom/Top Red	2	1	12	2
G2.12.3	El Rom/Top Red	2	1	12	3
G4	El Rom/Top Red	4	1	1	1
G5	El Rom/Top Red	5	1	1	1
G7	El Rom/Top Red	7	1	1	1
G8	El Rom/Top Red	8	1	1	1
G9	El Rom/Top Red	9	1	1	1
G12	El Rom/Top Red	12	1	1	1
G13	El Rom/Top Red	13	1	1	1
G20	El Rom/Top Red	20	1	1	1
G22	El Rom/Top Red	22	1	1	1
H1	[b]Ortal/Starking	1	1	1	1
H3	Ortal/Starking	3	1	1	1
H4	Ortal/Starking	4	1	1	1
H5	Ortal/Starking	5	1	1	1
I1	Ortal/Starking	1	1	1	1
I3	Ortal/Starking	3	1	1	1
I5	Ortal/Starking	5	1	1	1
I6	Ortal/Starking	6	1	1	1
I9	Ortal/Starking	9	1	1	1
I10	Ortal/Starking	10	1	1	1
P1	[c]Sede Eliezer/Anna	1	1	1	1
P8	Sede Eliezer/Anna	8	1	1	1
P9	Sede Eliezer/Anna	9	1	1	1
P11	Sede Eliezer/Anna	11	1	1	1
P12	Sede Eliezer/Anna	12	1	1	1
P14	Sede Eliezer/Anna	14	1	1	1

[z]Isolates from presumptive sexual populations (*i.e.*, orchards with recovered pseudothecia) from the Golan Heights: [a]El Rom (orchard G) and [b]Ortal (orchards H and I). Isolates from presumptive asexual populations (*i.e.*, pseudothecia never recovered): [c]Sede Eliezer (Hula Valley, orchard P), [d]Ginaton (orchards L and M) and [e]Be'er Tuvia (orchard R).

Table 1. (cont'd.)

Isolate[z]	Location/cultivar	Tree	Leaf	Lesion	Spore
P16	Sede Eliezer/Anna	16	1	1	1
L7	[d]Ginaton/Anna	7	1	1	1
L8	Ginaton/Anna	8	1	1	1
L11	Ginaton/Anna	11	1	1	1
L12	Ginaton/Anna	12	1	1	1
L17	Ginaton/Anna	17	1	1	1
L19	Ginaton/Anna	19	1	1	1
L28	Ginaton/Anna	28	1	1	1
M1	Ginaton/Ein Shemer	1	1	1	1
M2	Ginaton/Ein Shemer	2	1	1	1
M5	Ginaton/Ein Shemer	5	1	1	1
M7	Ginaton/Ein Shemer	7	1	1	1
M8	Ginaton/Ein Shemer	8	1	1	1
M9	Ginaton/Ein Shemer	9	1	1	1
R1.1.1	[e]Be'er Tuvia/Anna	1	1	1	1
R1.1.2	Be'er Tuvia/Anna	1	1	1	2
R1.1.3	Be'er Tuvia/Anna	1	1	1	3
R1.1.4	Be'er Tuvia/Anna	1	1	1	4
R1.1.5	Be'er Tuvia/Anna	1	1	1	5
R2.1.1	Be'er Tuvia/Anna	2	1	1	1
R2.1.2	Be'er Tuvia/Anna	2	1	1	2
R2.1.3	Be'er Tuvia/Anna	2	1	1	3
R2.1.4	Be'er Tuvia/Anna	2	1	1	4
R2.1.5	Be'er Tuvia/Anna	2	1	1	5
R6.1.1	Be'er Tuvia/Anna	6	1	1	1
R6.1.2	Be'er Tuvia/Anna	6	1	1	2
R6.1.3	Be'er Tuvia/Anna	6	1	1	3
R6.1.4	Be'er Tuvia/Anna	6	1	1	4
R6.1.5	Be'er Tuvia/Anna	6	1	1	5
R7.1.1	Be'er Tuvia/Anna	7	1	1	1
R7.1.2	Be'er Tuvia/Anna	7	1	1	2
R7.1.3	Be'er Tuvia/Anna	7	1	1	3
R7.1.4	Be'er Tuvia/Anna	7	1	1	4
R7.1.5	Be'er Tuvia/Anna	7	1	1	5

[z]Isolates from presumptive sexual populations (*i.e.*, orchards with recovered pseudothecia) from the Golan Heights: [a]El Rom (orchard G) and [b]Ortal (orchards H and I). Isolates from presumptive asexual populations (*i.e.*, pseudothecia never recovered): [c]Sede Eliezer (Hula Valley, orchard P), [d]Ginaton (orchards L and M) and [e]Be'er Tuvia (orchard R).

A hierarchical sampling strategy was used to collect *V. inaequalis* isolates such that the collection would provide data on both inter- and intra-populational differences and be representative for the country as a whole. From two sites representing the two environmental extremes in this study, namely, El Rom (G) on the Golan Heights and Be'er Tuvia (R) on the coastal plain, a number of isolates were taken from single, non-coalesced lesions and leaves to provide information on microgeographic population structure (Table 1). The remaining isolates in this study were each collected from a single leaf originating from different apple trees. All isolates used in this study were derived

from single conidiospores of the anamorph and thus each represented a genetically uniform culture. Spore suspensions derived from single leaf lesions were suspended in 500 μl sterile water and spread onto fresh potato dextrose agar (PDA) plates using a bent glass rod. Colonies derived from single conidiospores were removed under a dissecting microscope and transferred to fresh PDA slants using a sterile needle.

TABLE 2. Arithmetic means of genetic distance[z] within (*italicized*) and between geographic groupings of Israeli *Venturia inaequalis* populations

	G[y] n=10	H/I n=10	P n=7	L/M n=13	R[y] n=4
G: El Rom	*0.3477*				
H/I: Ortal	0.4246	*0.1579*			
P: Sede Eliezer	0.3930	0.4213	*0.0114*		
L/M: Ginaton	0.4174	0.4090	0.2149	*0.0*	
R: Be'er Tuvia	0.4840	0.4979	0.3428	0.2175	*0.0*

[z]Genetic similarities were computed for all pairs of isolates using the formula given by Nei (14): $S = 2b_{ij} / (b_i + b_j)$, where b_{ij} is the number of amplicons shared by two isolates, $_i$ and $_j$, and b_i and b_j are the total number of amplicons found in isolates $_i$ and $_j$, respectively. Genetic distances (dissimilarities) were calculated as 1 - S.

[y]Multiple isolates originating from single leaves and lesions from El Rom (G) and Be'er Tuvia (R) were omitted from the analysis, such that the data set contained only one fungal isolate per tree (see Materials and Methods).

DNA extraction and PCR Inoculum plugs from agar slants were used to inoculate PDA liquid shake cultures (200 rpm), which were grown for 4 to 6 weeks at 25°C. Fungal material was collected by filtration, rinsed in sterile water, snap frozen in liquid N_2 and lyophilized until dry. Using a motorized pestle and sterile white quartz sand, the material was dry-ground to talc-like consistency. Pre-warmed (65°C) extraction buffer (1% cetyltrimethylammonium bromide, 0.7 M NaCl, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA and 1% β-mercaptoethanol) was then added and the tubes were incubated for 120 min as described in Boehm *et al.* (1). An equal volume of chloroform:isoamyl alcohol (24:1) was added to emulsify, and nucleic acids were precipitated from the recovered supernatant by adding an equal volume of isopropanol. The final nucleic acid pellets were dissolved in TE (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) and RNase (20 μg ml^{-1}), incubated for 60 min at 37°C, re-extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by two rounds of chloroform alone and finally ethanol-precipitated. Relative concentrations were estimated by running aliquots with known amounts of size standards.

PCR reactions were performed in 25 μl volumes and included 50 ng genomic template, 50 mM KCl, 10 mM Tris-Cl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U of *Taq*-DNA polymerase and 1 μM of primer. Microsatellite primers used in this study were $(CAG)_5$, $(GACAC)_3$ and $(GACA)_4$ (6,11,13). Reactions were performed using a Hybaid thermal cycler (Hybaid Ltd., Ashford, UK) with the following run parameters: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealment at either 60°C for $(CAG)_5$ or 48°C for $(GACAC)_3$ and $(GACA)_4$ for 30 sec, and extension at 74°CC for 90 sec. All isolates were run at least twice. Amplicons were separated in 1.8% agarose gels, stained, and photographed.

Data analysis Each isolate was scored visually for the presence or absence of each detectable amplified fragment (amplicon) and data for all three microsatellite primers were combined into a single binary data set. Relationships within and between the collections of Israeli isolates of *V. inaequalis*, obtained from five geographic sites (G, H/I, P, L/M and R) and representing two ecological zones (Golan and Hula Valley / coastal plain), were determined by a comparison of genetic distance, cluster and parsimony analysis. For the calculation of genetic distance or dissimilarity (Table 2), a subset of the isolates listed in Table 1 was used. In the case of isolates originating from El Rom (G) on the Golan and from Be'er Tuvia (R) on the coastal plain, where multiple isolates were collected from individual leaves and lesions, only a single, arbitrarily chosen isolate per tree was considered. This resulted in a reduced data set for the El Rom (G, n=10) and Be'er Tuvia (R, n=4) isolates and limited the calculation to 44 isolates (Table 2). Genetic similarity is given by Nei (14) as: $S = 2b_{ij} / (b_i + b_j)$, where b_{ij} is the number of amplicons shared by two isolates, $_i$ and $_j$, and b_i and b_j are the number of amplicons found in isolates $_i$ and $_j$, respectively, and converted to genetic distance or dissimilarity as 1 – S.

In contrast to the calculation of genetic distance, both cluster and parsimony analysis considered the data set as a whole (n=78). Cluster analysis is a rapid method of hierarchically grouping taxa on the basis of similarity or distance, whereas parsimony is a method that operates on the principle of minimizing the number of events needed to produce the optimal hierarchical grouping (25). Phenograms were generated using the unweighted pair-group method of arithmetic averages (UPGMA) found in the programs SIMQUAL and SAHN in NTSYS-pc Version 2.1 (Exeter Software, Setauket, NY, USA). Cluster analysis was based on Jaccard's similarity coefficient, which measures the proportion of band mismatches between pairs of isolates, given as: $S_J = a / n - d$, where *a* is the number of positive matches, *n* the total sample size and *d* the number of negative matches. A cophenetic value matrix from the tree matrix produced by SAHN was used to test the goodness of fit of the cluster analysis to the similarity matrix on which the tree was based. The NJOIN program, based on the unweighted neighbor joining parsimony method of generating trees found in NTSYS-pc, was also included in the analysis to see if differences in tree topology might result.

Parsimony analysis employed the combined binary data matrix to construct phylograms using the computer program PAUP Version 4.0b4a (Illinois Natural History Survey, Champaign, IL, USA). The heuristic algorithm with branch swapping was used to generate a set of the most parsimonious trees and a 50% majority-rule consensus tree was generated by 1000 repetitions of the bootstrap algorithm to determine branch strengths. Data were analyzed using both Wagner parsimony and Dollo parsimony methods. The former assumes that all characters can change from one state to another with equal probability, whereas Dollo parsimony allows characters to change to the derived state only once, in this case priming site present, but allows unlimited reversals. RAPD data sets presumably fall between these two extremes, with the priming site more likely to be lost than gained (25).

RESULTS

A total of 78 single-spore *V. inaequalis* isolates were obtained from five geographical locations in Israel, representing two climatically distinct zones that differ primarily by the presence or absence of sustained low winter temperatures due to elevation (Fig. 1, Table 1). The El Rom (G) and Ortal (H/I) isolates from the Golan Heights were obtained from

confirmed sexual populations (*i.e.*, frequent recovery of pseudothecia from orchard leaf litter) present on introduced temperate apple cultivars (Top Red and Golden Delicious, respectively). The Sede Eliezer (P), Ginaton (L/M) and Be'er Tuvia (R) isolates were obtained from the local apple cultivars Anna and Ein Shemer grown at lower elevations and represent presumptive asexual populations, since pseudothecia have never been recovered from these orchards (Fig. 1, Table 1).

The three microsatellite primers produced a total of 48 scorable amplicons, of which 38 (79%) were polymorphic among the isolate set as a whole. The percentage of polymorphic bands varied depending on the primer, although all three primers were informative. Primers $(CAG)_5$, $(GACAC)_3$ and $(GACA)_4$ had a frequency of polymorphic bands at 70.5%, 75.0% and 89.4%, respectively, for the sample set as a whole. The percentage of polymorphic bands within each of the five populations was indicative of the amount of genotypic diversity present. Isolates originating from known sexual populations collected from the Golan, namely, El Rom (G; n = 28) and Ortal (H/I; n = 10), had respectively 50% and 14.6% of their bands polymorphic. In sharp contrast, isolates originating from Ginaton (L/M; n = 13) and Be'er Tuvia (R; n = 20), along the coastal plain, displayed no band polymorphisms among isolates collected from the same orchard. Although differences were observed between orchards, within each site all sampled isolates were identical and therefore comprised a single clonal lineage. Isolates originating from the Anna orchard in Sede Eliezer (P; n = 7), located in the Hula Valley, were not so uniform as the Ginaton (L/M) and Be'er Tuvia (R) isolates, but still only 4.2% of their bands were polymorphic. An example using primer $(GACAC)_3$ is presented in Figure 2. As is evident, the El Rom and Ortal isolates from the Golan display abundant band polymorphisms, whereas the isolates originating from the Hula Valley (Sede Eliezer) and the coastal plain (Ginaton and Be'er Tuvia), within an orchard, display no polymorphism (Fig. 2). Similar results were encountered using the two other primers.

Calculated genetic distance within and between populations for each orchard sampled considered only a subset of the isolates in Table 1. Specifically, the sample sizes from the El Rom (G) and Be'er Tuvia (R) orchards were reduced to exclude isolates derived from the same leaf or tree (see Materials and Methods). Genetic distance within populations averaged 0.3477 for the El Rom (n = 10) and 0.1579 for the Ortal (n = 10) isolates, both from the Golan Heights (Table 2). In sharp contrast, calculated genetic distances within the Sede Eliezer isolates collected from the Hula Valley (n = 7) were 0.0114 and zero for both the Ginaton (n = 13) and Be'er Tuvia isolates (n = 4), collected from the coastal plain (Table 2). Thus, more than a tenfold difference in calculated genetic distance was found within populations originating from the Golan Heights (n = 20), as compared with those from lower elevation apple production areas (n = 24) (Table 2).

Variation within a single lesion, leaf or tree was based on two sites representing the two environmental extremes in this study, namely, El Rom (G) from the Golan and Be'er Tuvia (R) from the coastal plain (Fig. 2). From El Rom, 18 isolates were collected from a single leaf, representing six individual lesions, whereas from Be'er Tuvia, 20 isolates were collected from four leaves, each originating from four different, widely spaced trees in the orchard (Table 1). The difference in hierarchical sampling strategy between these two sites was premised on the expectation of greater genotypic diversity from a known sexual population (G) as compared with a presumptive asexual population (R). The 18 Golan isolates from El Rom showed a high level of polymorphism, considering the fact

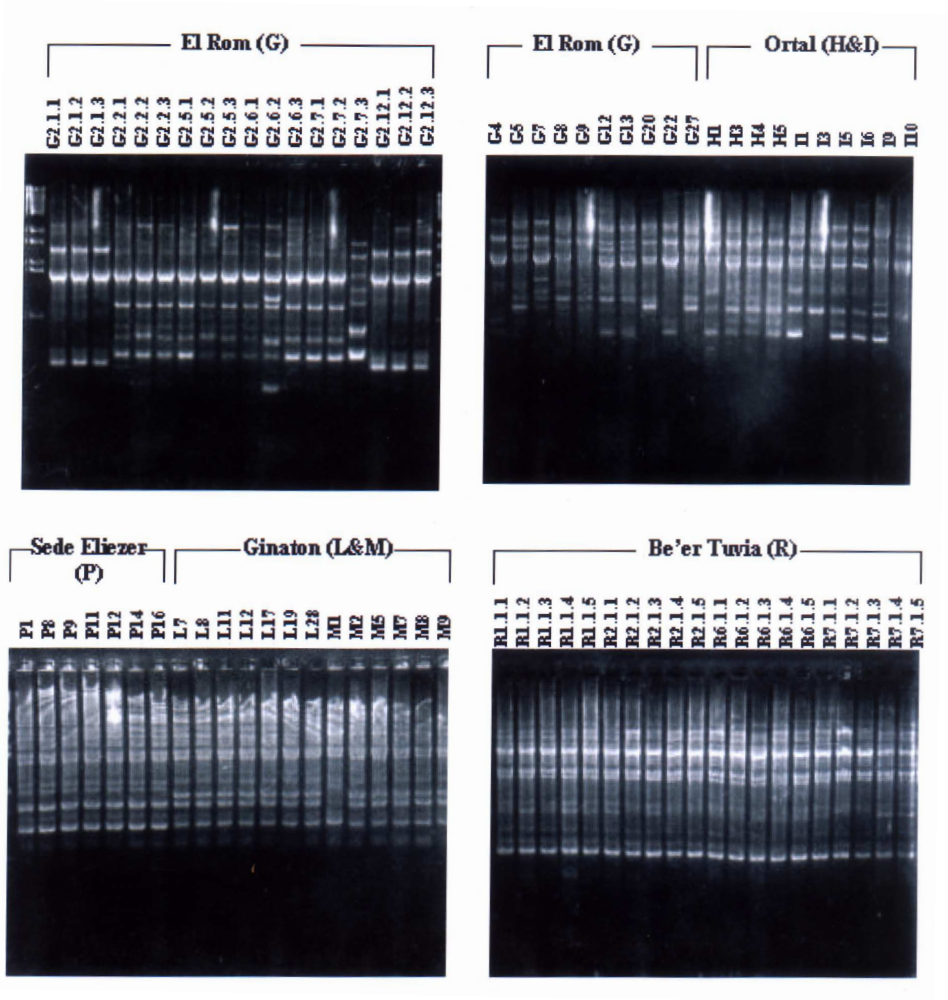

Fig. 2. Ethidium bromide-stained gels of the Israeli *Venturia inaequalis* isolates (n=78) collected from the Golan Heights (G, El Rom and H&I, Ortal), the Hula Valley (P, Sede Eliezer) and the coastal plain (L&M, Ginaton and R, Be'er Tuvia), amplified with the microsatellite primer $(GACAC)_3$. The El Rom G isolates in the top left panel originated from six lesions, three isolates each, along the same leaf, whereas the Be'er Tuvia R isolates in the bottom right panel originated from four trees, in replicates of five isolates per leaf. All other isolates were each collected from a different tree (see Table 1).

that they all were collected from a single leaf. Variation was detected even from spores originating from the same lesion (*e.g.* isolate G2.6.2 *vs* G2.6.1 and G2.6.3, or G2.7.3 *vs* G2.7.1 and G2.7.2 in Fig. 2). In sharp contrast, all 20 isolates sampled from Be'er Tuvia were genetically uniform even though they were collected from different trees (Fig. 2).

Both cluster and parsimony analysis yielded similar hierarchical groupings of isolates. The population structure of Israeli *V. inaequalis*, as determined from phenograms generated from the various cluster analysis packages found in NTSYS-pc, without exception grouped

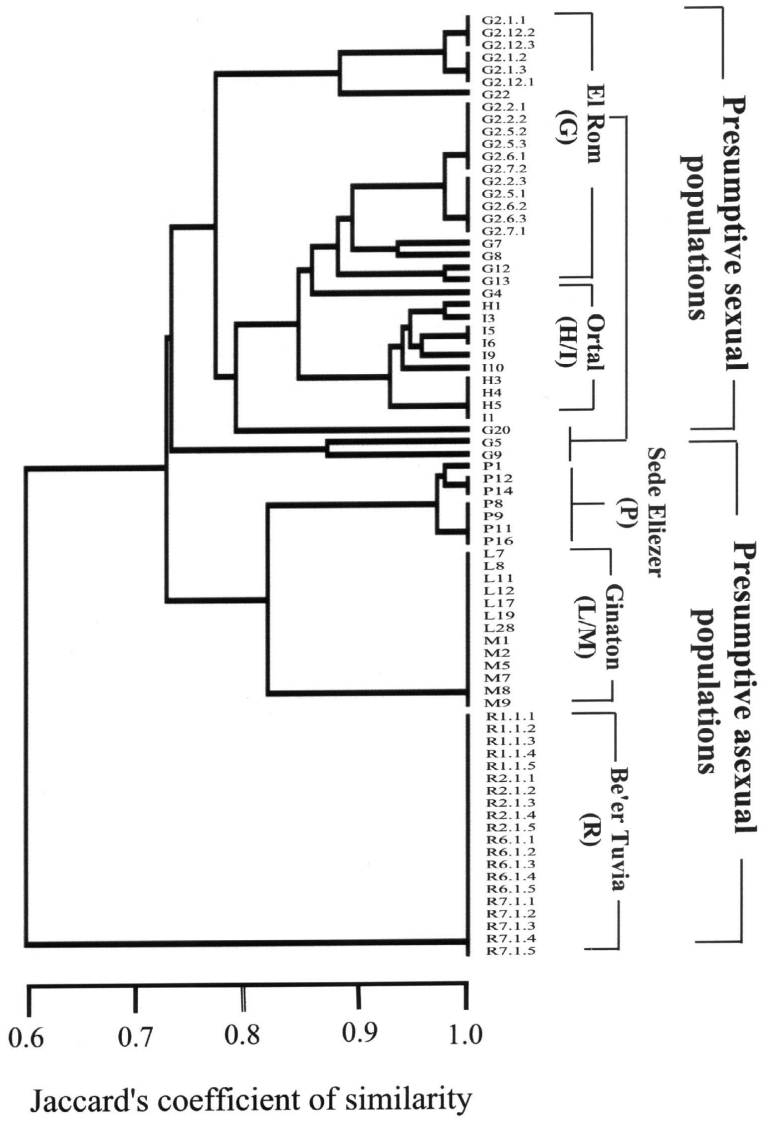

Fig. 3. Phenogram generated by cluster analysis and UPGMA found in the SAHN package of NTSYS-pc (see Materials and Methods). *Venturia inaequalis* isolates (n=78) from presumptive sexual populations in the Golan Heights: G (El Rom) and H/I (Ortal); and from presumptive asexual populations in the Hula Valley: P (Sede Eliezer) and along the coastal plain: L/M (Ginaton) and R (Be'er Tuvia). The scale is the percentage of similarity as given by Jaccard's coefficient; cophenetic correlation $r = 0.96$.

isolates according to their geographic origin (Fig. 3). Three groups were discernable: the first contained both of the sexual Golan populations (G and H/I), the second grouped the Hula Valley (P) and Ginaton (L/M) isolates together, whereas the third group contained

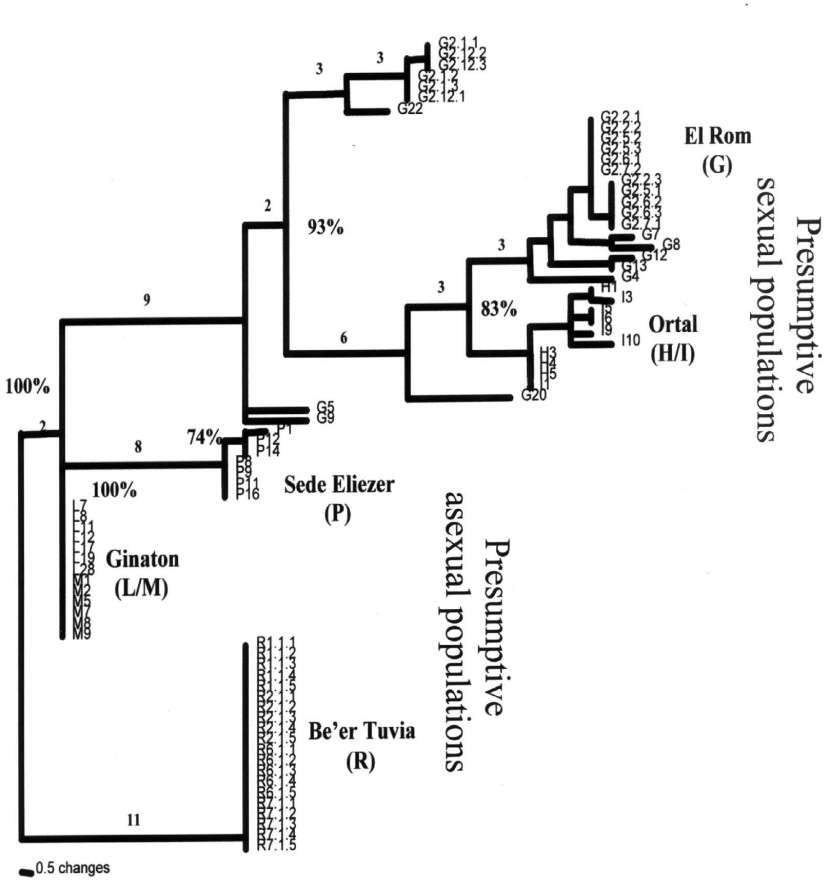

Fig. 4. Phylogram of the 50% majority-rule consensus of one of the nine most parsimonious trees for Israeli *Venturia inaequalis* isolates (n=78). Groupings were determined from the heuristic search in PAUP version 4.0b4a using Wagner parsimony (see Materials and Methods). Branch strengths were tested by 1000 repetitions of the bootstrap algorithm with branch swapping in PAUP, indicated as percentages along major branch points. Branch lengths are given above the line. Isolates originated from presumptive sexual populations in the Golan Heights: G (El Rom) and H/I (Ortal); and from presumptive asexual populations in the Hula Valley: P (Sede Eliezer) and along the coastal plain: L/M (Ginaton) and R (Be'er Tuvia).

the R isolates from Be'er Tuvia (Fig. 3). The Be'er Tuvia isolates were as different from the sexual Golan isolates (G and H/I) as they were from the Sede Eliezer (P) and Ginaton (L/M) populations, having the principal branch dichotomy at only 60%. The P and L/M populations, separated by a greater geographical distance as compared with the R *vs* L/M (Fig. 1), however, were more than 80% similar (Fig. 3). Groupings for the G isolates from El Rom indicated a range of genetic distances, in line with the presence of an actively recombining sexual population; nonetheless, within this group, the H/I isolates

from Ortal constituted a distinct cluster (Fig. 3). Neighbor joining trees derived from NJOIN in NTSYS-pc produced trees identical to those generated by the SAHN method (data not presented).

Phylograms derived from parsimony analysis performed using PAUP were very similar to those produced by cluster analysis (Fig. 4). Three groups, corresponding to geographic origin, were again evident, with the isolates collected from the Golan clearly having greater genotypic diversity than the lower elevation isolates. Bootstrap analysis supported the principal branch dichotomies at the 100% level for 1000 repetitions for all three isolate subgroupings (Fig. 4). Nine equally parsimonious trees were generated using Wagner parsimony with a tree length of 68 steps (consistency index = 0.73; retention index = 0.95). Parsimony with Dollo yielded a longer tree but with identical topology (data not presented).

DISCUSSION

The population structure of a representative sample of Israeli *V. inaequalis* isolates, originating from two contrasting ecological zones, characterized primarily by the presence or absence of low winter temperatures due to elevation, was determined using three microsatellite primers, and the binary data analyzed using both cluster and parsimony analysis. The study was initiated to determine whether levels of genotypic diversity, as measured using neutral genetic markers, could be correlated with either geographic origin of the isolates or with the climatological conditions under which apples are grown in Israel. The Golan Heights is a volcanic plateau rising 1000–1300 m above sea level in the northern corner of the country, overlooking the Hula Valley, and is typified by sustained low winter temperatures and occasional snow cover. This region is typical of other temperate apple-producing regions and cultivars are predominantly introduced varieties, such as Top Red and Starking Delicious, that break bud dormancy through late March and April. In these Golan orchards, the primary spring inoculum has been demonstrated to be ascosporic, derived from overwintered apple leaves on the orchard floor which produce pseudothecia (19,20).

In sharp contrast, Israeli apple production areas along the coastal plain, and to a lesser extent in the Hula Valley, are characterized by local apple cultivars such as Anna and its pollinator Ein Shemer. Most importantly, pseudothecia have never been recovered from these lower elevation orchards, despite repeated yearly orchard leaf litter surveys. In contrast to temperate apple cultivars, Anna has a low chilling requirement and breaks bud dormancy very early, from late January to early February, coinciding with the rainy season and the cool temperatures required for infection. These coastal orchards never experience the severe winter conditions typical of most temperate apple-growing regions, conditions that usually do not allow for the survival of the pathogen in overwintered lesions on fruit and leaves. The primary inoculum in Anna orchards is believed to originate from non-abscised, overwintered apple leaves producing asexual conidiospores. This is because Anna never loses all of its leaves and non-abscised, infected leaves from the previous season are readily available to serve as inoculum sources for newly emergent leaves.

The most striking finding to emerge from the data was that *V. inaequalis* isolates collected from lower elevation apple orchards along the coastal plain (Ginaton and Be'er Tuvia) were genetically identical within an orchard and thus were each comprised of a single clonal lineage, despite the fact that efforts were made to collect isolates from widely spaced trees within each orchard. Ginaton isolates were genetically uniform, despite

their origin from two different apple cultivars (Anna and Ein Shemer), sampled from two adjacent orchards (L and M). Conversely, isolates collected from introduced temperate apple cultivars on the Golan Heights (El Rom and Ortal) showed extensive levels of genotypic diversity using the same primers and, most importantly, similar sample sizes. Isolates obtained from Sede Eliezer in the Hula Valley, a region adjacent to the Golan Heights, but at a much lower elevation and experiencing mild Mediterranean winters typical of the coastal plain, showed limited genotypic diversity, resembling that found along the coastal plain. Additionally, both cluster and parsimony analysis grouped the Israeli *V. inaequalis* isolates according to geographic origin, as was especially evident among the Hula Valley and coastal plain isolates. The highly polymorphic Golan isolates from Ortal grouped within the El Rom isolates, the two northern populations forming a distinct clade, separate from the lower elevation isolates.

Diversity levels were reflected in the percentage of polymorphic bands present and the calculation of genetic distances within each of the five populations sampled. Isolates collected from the Golan were more than ten times as diverse as those collected from the lower elevation apple orchards. In the El Rom population from the Golan, this high level of genotypic diversity extended to include multiple isolates obtained from the same leaf and even from the same lesion. In some cases, isolates obtained from within a single lesion were more similar to those obtained from other lesions along the same leaf, suggesting that more than one ascospore had initiated the lesion. Conversely, multiple isolates from single trees in Be'er Tuvia along the coastal plain were identical, despite their origin among widely spaced trees in the orchard.

The microsatellite primers clearly demonstrated the overrepresentation of identical genotypes for *V. inaequalis* populations sampled from coastal plain orchards, as compared with similar sample sizes originating from the Golan Heights. A number of explanations could account for the observed high genotypic diversity associated with *V. inaequalis* isolates collected from orchards in the Golan Heights *vs* the lack of polymorphism detected from the coastal plain. Some populations could be more diverse simply as a result of a founder effect associated with the relative time of introduction of a particular pathogen population into a given area (15,24). However, Israeli apple production along the coastal plain predates apple production on the Golan Heights by several decades. Alternatively, diversity differences could be due to a sampling bias, whereby orchards located peripherally to central production areas may reflect recent pathogen introductions. However, care was taken to sample only orchards centrally located within each of the respective growing regions and trees sampled from these orchards were centrally located as well. Differences could also be associated with host selection, since in this study fungal isolates were recovered from four different apple cultivars. Yet, no apple cultivar bias was apparent in the Ginaton orchards, where samples were derived from two different apple cultivars (Anna and Ein Shemer) and, in the Golan Heights, cluster and parsimony analysis did not indicate isolate sub-groupings based on different cultivars.

Lastly, an argument could be made that the effective population size in coastal apple orchards is minimized each year, leading to genetic bottlenecks (15) each spring, due to the inability of the pathogen to survive winter conditions effectively. Conversely, in the Golan, due to the sexual stage, a successful overwintering strategy has been developed, thus maintaining a diverse population. We do not believe this to be the case, as populations of *V. inaequalis* present in apple production areas along the coastal plain in Israel have

been demonstrated to overwinter as viable vegetative mycelia in lesions on fruit and leaves from the previous production season. Unlike the introduced temperate apple varieties grown in the Golan, Anna was specifically developed for coastal production in Israel and infected leaves from the previous season are readily available to serve as inoculum sources. Furthermore, Anna has a low chilling requirement and breaks bud dormancy early, coinciding with the rainy season and the cool temperatures required for infection. Given these circumstances and the absence of sustained low winter temperatures, the pathogen is under no constraint to overwinter.

Rather, the observed differences in the relative levels of genotypic diversity may relate to the different climatological conditions under which apples are grown in Israel, which in turn may influence the importance of the ascigerous stage in this fungal pathogen. Wilson (29) was the first to demonstrate that, given enough free moisture, low winter temperatures were a prerequisite to pseudothecial development in *V. inaequalis*. Subsequent workers have confirmed and extended these findings and have shown that low temperatures have a marked effect on pseudothecial ontogeny (7), the numbers of pseudothecia produced per unit of infected leaf (17) and the rate of ascus and ascospore maturation (4,10,29). These environmental conditions are characteristic of temperate apple-producing regions throughout the world, but are found in Israeli apple orchards only in the Upper Galilee and along the Golan Heights.

The high degree of genetic uniformity found in this study for coastal Israeli *V. inaequalis* isolates has not been reported before in the literature and, until now, the pathogen has been presumed to have an obligate sexual cycle (12). The high degree of homogeneity found among coastal isolates, from widely spaced trees in a single orchard, contrasts sharply with the high levels of genetic polymorphism found within single lesions from higher elevation populations, despite similar sampling strategies and methods of analysis. The data indicate the presence of non-recombining, clonal lineages for this pathogen on the Anna and Ein Shemer apple cultivars along the coastal plain. Tenzer and Gessler (27) found a high degree of genotypic diversity among European (France, Germany, Switzerland, The Netherlands and northern Italy) populations of *V. inaequalis* based on the allele frequencies of 18 polymorphic RAPD markers and the internal transcribed spacer region of the ribosomal DNA. Similar results were found within orchards in Switzerland (26). Likewise, Schnabel *et al.* (18) demonstrated considerable polymorphism among world populations of *V. inaequalis* in sequence variation of the internal transcribed spacer and for the presence or absence of an optional Group I intron in the 18 rDNA. These studies, however, utilized populations of the pathogen subject to sustained low winter temperatures and therefore resemble the levels of genotypic diversity found in this study for the Golan populations. The present study is the first, as far as we know, to consider the population structure of *V. inaequalis* in areas devoid of sustained low winter temperatures and, as such, presents evidence for an alternative life cycle developed by this pathogen in a Mediterranean climate not suitable for the development of the telomorph. In this regard, it would be interesting to expand this study to consider *V. inaequalis* populations from other warm climate apple production areas, such as in Central Italy and Western Australia.

Disease management strategies for controlling apple scab in Israel would be expected to differ, given the different life cycles of the pathogen in different parts of the country. For instance, in Anna apple orchards located along the coastal plain, control practices such as

destruction of orchard leaf litter to eradicate the ascigerous stage would not be so effective as on the Golan Heights, where the primary inoculum in the spring is ascosporic. This is because Anna cultivars retain a portion of their leaves after the harvest season. Additionally, the timing of fungicide applications in the spring would be expected to differ for the two regions, given cultivar differences in the breaking of bud dormancy. Lastly, the presence of asexual populations comprised of clonal lineages in coastal Anna apple orchards would have bearing on the ability of the pathogen to generate fungicide resistance (8,20). Indeed, it has recently been demonstrated that *V. inaequalis* isolates collected from the Golan Heights have a greater propensity to develop fungicide resistance to demethylation inhibitor fungicides than do isolates collected from Anna orchards along the coastal plain (23).

ACKNOWLEDGMENTS

This research was supported by the United States - Israel Binational Agricultural Research and Development (BARD) fund as Postdoctoral Award No. FU-0203-94 to the senior author. We gratefully acknowledge the cooperation of Dr. Talma Katan (Department of Plant Pathology, ARO, The Volcani Center, Bet Dagan, Israel) in allowing this project to be developed in her laboratory.

REFERENCES

1. Boehm, E.W.A., Ma, Z. and Michailides, T.J. (2001) Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. *Phytopathology* 91: 428-439.
2. Ellis, M.A., Ferree, D.C., Funt, R.C. and Madden, L.V. (1998) Effects of an apple scab-resistant cultivar on use patterns of inorganic and organic fungicides and economics of disease control. *Plant Dis.* 82:428-433.
3. Ellis, M.A., Madden, L.V. and Wilson, L.L. (1984) Evaluation of an electronic apple scab predictor for scheduling fungicides with curative activity. *Plant Dis.* 68:1055-1057.
4. Gadoury, D.M. and MacHardy, W.E. (1982) Effects of temperature on the development of pseudothecia of *Venturia inaequalis*. *Plant Dis.* 66:464-468.
5. Gadoury, D.M., MacHardy, W.E. and Hu, C. (1984) Effects of temperature during ascus formation and frequency of ascospore discharge on pseudothecial development of *Venturia inaequalis*. *Plant Dis.* 68:223-225.
6. Hantula, J., Dusabenyagasani, M. and Hamelin, R. (1996) Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation with fungi. *Eur. J. For. Pathol.* 26:159-166.
7. James, J.R. and Sutton, T.B. (1982) Environmental factors influencing pseudothecial development and ascospore maturation of *Venturia inaequalis*. *Phytopathology* 72:1073-1080.
8. Katan, T., Shabi, E. and Gilpatrick, J.D. (1983) Genetics of resistance to benomyl in *Venturia inaequalis* isolates from Israel and New York. *Phytopathology* 73: 600-603.
9. Keitt, G.W., Clayton, C.N. and Langford, M.H. (1941) Experiments with eradicant fungicides for combating apple scab. *Phytopathology* 31:296-322.
10. Keitt, G.W. and Langford, M.H. (1941) *Venturia inaequalis* (Cke.) Wint. I. A groundwork for genetic studies. *Am. J. Bot.* 28:805-820.
11. Ma, Z., Boehm, E.W.A., Luo, Y. and Michailides, T.J. (2001) Population structure of *Botryosphaeria dothidea* from pistachio and other hosts in California. *Phytopathology* 91:665-672.
12. MacHardy, W.E. (1996) Apple Scab: Biology, Epidemiology and Management. APS Press, St. Paul, MN, USA.
13. Meyer, W., Mitchell, T.G., Freedman, E.Z. and Vilgalys, R. (1993) Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol*. 31:2274-2280.
14. Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, NY.
15. Nei, M., Maruyama, T. and Chakraborty, R. (1975) The bottleneck effect and genetic variability in populations. *Evolution* 29:1-10.
16. Ogawa, J.M. and English, H. (1991) Diseases of Temperate Zone Tree Fruit and Nut Crops. Publ. #3345. University of California Press, Berkeley, CA, USA.
17. Ross, R.G. and Hamlin, S.A. (1962) Production of perithecia of *Venturia inaequalis* (Cke.) Wint. on sterile apple leaf disks. *Can. J. Bot.* 40:629-635.

18. Schnabel, G., Schnabel, E.L. and Jones, A.L. (1999) Characterization of ribosomal DNA from *Venturia inaequalis* and its phylogenetic relationship to rDNA from other tree-fruit *Venturia* species. *Phytopathology* 89:100-108.
19. Shabi, E. (1999) Scab and fire blight on pear and apple in Israel. *5th Workshop on Integrated Control of Pome Fruit Diseases* (Fontevraud-l'Abbaye, France), p. 15.
20. Shabi, E. and Elisha, S. (1995) Strobilurins and DMI fungicides against scab and powdery mildew. *Fungicide and Nematicide Tests* 51:25-26.
21. Shabi, E., Elisha, S. and Zelig, Y. (1981) Control of pear and apple diseases in Israel with sterol-inhibiting fungicides. *Plant Dis.* 65:992-994.
22. Shabi, E., Katan, T. and Marton, K. (1983) Inheritance of resistance to benomyl in isolates of *Venturia inaequalis* from Israel. *Plant Pathol.* 32:207-212.
23. Shabi, E., Roberts, A.L., Crute, I.R., Boehm, E.W.A., Oppenheim, D., Elisha, S. *et al.* (1997) Variation in sensitivity of *Venturia inaequalis* to DMI and strobilurin fungicides. *Phytoparasitica* 25:260-261 (abstr.).
24. Slatkin, M. (1977) Gene flow and genetic drift in a species subject to frequent local extinctions. *Theor. Pop. Biol.* 12:253-262.
25. Swofford, D.L., Olsen, G.J., Waddell, P.J. and Hillis, D.M. (1996) Phylogenetic inference. *in:* Hillis, D.M., Moritz, C. and Mable, B.K. [Eds.] Molecular Systematics. Sinauer Associates, Inc., Sunderland, MA, USA. pp. 407-514.
26. Tenzer, I. and Gessler, C. (1997) Subdivision and genetic structure of four populations of *Venturia inaequalis* in Switzerland. *Eur. J. Plant Pathol.* 103:565-571.
27. Tenzer, I. and Gessler, C. (1999) Genetic diversity of *Venturia inaequalis* across Europe. *Eur. J. Plant Pathol.* 105:545-552.
28. Weising, K., Atkinson, R.G. and Gardner, R.C. (1995) Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *PCR Methods Appl.* 4:249-255.
29. Wilson, E.E. (1928) Studies of the ascigerous stage of *Venturia inaequalis* (Cke.) Wint. in relation to certain factors of the environment. *Phytopathology* 18:375-418.
30. Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:178-183.