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Variability of Cuban and international populations of *Alternaria solani* from different hosts and localities: AFLP genetic analysis

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Abstract

As causal agent of early blight disease in tomato and potato, *Alternaria solani* is an internationally important horticultural pathogen. Genetic variability was surveyed by amplified fragment length polymorphism analysis in a total of 112 isolates from potato and tomato, representing pathogen populations from different Cuban provinces together with isolates from the USA, Brazil, Turkey, Greece and China. Also included in the analysis were isolates from catenulated *Alternaria* spp. from Brazil, Canada, Greece and Russia, along with single isolates of *Alternaria porri*, *Alternaria alternata* and a *Curvularia* sp. UPGMA clustering revealed a differentiation between the isolates of *A. solani* and all other species with the exception of *A. porri* which could not be distinguished from *A. solani*. Among the isolates of *A. solani*, two distinct subclusters were formed, with high genetic significance revealed by bootstrapping, corresponding to a general subdivision based on the respective solanaceous host. The results are discussed with regard to potential host specificity of *A. solani* on tomato and potato, and in terms of the comparative contributions of regional and international genetic variability in populations of this ubiquitous plant pathogen.

Introduction

Early blight disease caused by *Alternaria solani* is one of the most important fungal diseases of tomato and potato. This pathogen is also the causal agent of collar rot in tomato. *A. solani* is a well-known pathogen of the genus *Alternaria* and is considered a good example for the world-wide distribution of a species (Rotem, 1994). The fungus was described for the first time in potato late in the 19th century (Ellis and Martin, 1882) and attacks on potato and tomato during the first three decades of the last century, on practically all the continents, were described by Neergaard (1945). The first report of *A. solani* in Cuba was in 1918, at which point early blight in tomato was among the most important crop diseases in Havana (Bruner, 1918).

Several studies have shown that *A. solani* isolates differ in morphology, physiology, pathogenicity, genetic makeup and cultural properties. Indeed, isolates can vary so much in their cultural characteristics that it is possible to find almost as many morphotypes as the number of isolates tested (Rotem, 1966). Isolates also vary in the production of phytotoxins. However, no correlation was found between the virulence of specific strains and their ability to produce toxins (Stancheva, 1989). In some cases, strains from leaf and potato tubers differed in their ability to attack potato leaves and tubers, but no correlation was observed between potato isolates and their pathogenicity on leaves and tubers (Bonde, 1929; Mukherji, 1961).

Genetic analysis with isozymes (Petrunak and Christ, 1992), RAPD (Sharma and Tewari, 1998;

Weir et al., 1998; Roberts et al., 2000) and RFLP markers (Adachi et al., 1996; Aradhya et al., 2001) has shown great variability between and within *Alternaria* species. A significant genetic distance was observed between isolates of *A. solani* from tomato and potato (Weir et al., 1998), suggesting the possibility of a pathogenic specialization on solanaceous hosts. Generally, at the species level, the genetic variability corresponds to variation in morphological characteristics (Roberts et al., 2000; Sharma and Tewari, 1998) in *Alternaria* populations of fruits and crucifers, respectively. On the other hand, correlation of genetic markers with host, geographic origin or resistance to fungicides was not always evident (Adachi et al., 1996; Morris et al., 2000; Aradhya et al., 2001). A high level of genetic differentiation by host and/or pathogenicity, consistent with a hypothesis of specialization, has been observed in *Alternaria* spp. from *Citrus* (Peever et al., 1999). In Cuba, the study of the variability of the causal agent of early blight has been limited to tomato, for which 12 races were found in Havana province (Izquierdo, 1981). More recently, we observed pathogenic (Martínez et al., 2002) and genetic diversity in some Cuban isolates using RAPD analysis (unpubl. results).

Amplified fragment length polymorphisms (AFLP; Vos et al., 1995) represent a powerful, highly reproducible, PCR-based DNA-fingerprinting technique for DNA of any origin or complexity. Because a large number of polymorphic loci can be investigated in a single experiment, the AFLP technique has become one of the major methods of choice for studies of genetic diversity, particularly in species where markers requiring genomic sequence information are not available. The robustness of the AFLP procedure on fungal genomic DNA was corroborated by Pei and Ruiz (2000). The highly polymorphic nature of AFLP markers make them especially useful for differentiating clonal lineages of fungi that reproduce asexually (McDonald, 1997). AFLP markers have been used to study genetic diversity in 18 isolates of *Alternaria brassicicola* (Bock et al., 2002). However, a large-scale international survey of genetic variability in *Alternaria* has not been published. The objectives of the present study were to use AFLP analysis to examine genetic differentiation within and between populations from diverse tomato and potato growing regions in Cuba and other countries, and to determine whether the influence of the host could be observed at the DNA level.

Materials and methods

Fungal collection

Isolates of *A. solani* were collected from typical symptoms of tomato and potato early blight plants in different provinces in Cuba (Table 1). Additionally, international isolates were provided by other researchers: Ten isolates originated from Turkey, eight from Brazil, four from Greece, eight from the USA and one from China. One isolate of *Alternaria alternata*, one of *Alternaria porri*, seven of catenulated *Alternaria* spp. and one of *Curvularia* sp. were included in the analysis. Origins and hosts of the isolates are listed in Table 1.

Table 1. Hosts and origins of fungal isolates used in AFLP analysis

Species	ID[1]	Host	Year	Origin
A. alternata (1)	*A. alternata*[*]	Air	1994	Cuba[H]
A. porri (1)	*A. porri*	Onion	1994	Cuba[H]
A. solani (112)	C-5[**]	Tomato	1993	Cuba[H]
	C-6[**]	Tomato	1993	Cuba[H]
	C-7[**]	Tomato	1993	Cuba[H]
	C-12[**]	Tomato	1993	Cuba[H]
	C-16[**]	Tomato	1993	Cuba[H]
	C-24	Tomato	1993	Cuba[H]
	C-25	Tomato	1993	Cuba[V]
	C-26	Tomato	1993	Cuba[H]
	C-27	Tomato	1993	Cuba[H]
	C-28[**]	Tomato	1994	Cuba[H]
	C-36[**]	Tomato	1993	Cuba[H]
	C-38[**]	Tomato	Unknown	Cuba[H]
	C-42[**]	Tomato	1994	Cuba[H]
	C-44[**]	Tomato	1994	Cuba[H]
	C-45[**]	Tomato	1994	Cuba[H]
	C-48[**]	Potato	1994	Cuba[H]
	C-49[**]	Tomato	1995	Cuba[H]
	C-50[**]	Tomato	1995	Cuba[H]
	C-54[**]	Tomato	1995	Cuba[H]
	C-58[**]	Tomato	1996	Cuba[H]
	C-64	Tomato	Unknown	Cuba[H]
	C-74[**]	Tomato	1997	Cuba[H]
	C-78[**]	Tomato	1997	Cuba[H]
	C-79[**]	Tomato	1997	Cuba[H]
	C-81[**]	Tomato	1997	Cuba[H]
	C-82[**]	Tomato	1997	Cuba[H]
	C-83[**]	Tomato	1997	Cuba[H]
	C-90[**]	Tomato	1997	Cuba[H]
	C-91[**]	Tomato	1997	Cuba[H]
	C-92[**]	Tomato	1997	Cuba[H]
	C-98[**]	Tomato	1997	Cuba[H]
	C-99[**]	Tomato	1997	Cuba[H]
	C-114	Potato	1997	Cuba[P]

Table 1. (Continued)

Species	ID[1]	Host	Year	Origin
	C-115**	Potato	1997	Cuba[H]
	C-119**	Potato	1997	Cuba[H]
	C-120**	Potato	1997	Cuba[H]
	C-126**	Potato	1997	Cuba[V]
	C-128**	Potato	1997	Cuba[V]
	C-131**	Tomato	1997	Cuba[V]
	C-132**	Tomato	1997	Cuba[V]
	C-135**	Tomato	1997	Cuba[F]
	C-136**	Tomato	1997	Cuba[F]
	C-138	Tomato	1998	Cuba[G]
	C-139	Tomato	1998	Cuba[G]
	C-141*	Tomato	1998	Cuba[O]
	C-142	Tomato	1998	Cuba[G]
	C-150	Potato	1998	Cuba[T]
	C-154*	Potato	1998	Cuba[T]
	C-156	Potato	1998	Cuba[T]
	C-159*	Tomato	1998	Cuba[T]
	C-162*	Tomato	1998	Cuba[T]
	C-165*	Tomato	1998	Cuba[T]
	C-171*	Tomato	1998	Cuba[T]
	C-179	Tomato	1998	Cuba[O]
	C-181	Tomato	1998	Cuba[O]
	C-182*	Tomato	1998	Cuba[G]
	C-183*	Tomato	1998	Cuba[G]
	C-187	Potato	1998	Cuba[T]
	C-188*	Tomato	1998	Cuba[T]
	C-189*	Tomato	1998	Cuba[T]
	C-190*	Tomato	1998	Cuba[O]
	C-193	Tomato	1998	Cuba[T]
	C-201	Tomato	1998	Cuba[C]
	C-203*	Tomato	1998	Cuba[F]
	C-213*	Tomato	1998	Cuba[C]
	C-219**	Tomato	1999	Cuba[G]
	C-223	Tomato	1999	Cuba[G]
	C-227	Tomato	1999	Cuba[G]
	C-230	Tomato	1999	Cuba[G]
	C-232	Tomato	1999	Cuba[O]
	C-235	Tomato	1999	Cuba[O]
	C-240	Tomato	1999	Cuba[O]
	C-246	Tomato	1999	Cuba[O]
	C-248	Tomato	1999	Cuba[O]
	C-249	Tomato	1999	Cuba[O]
	C-250	Tomato	1999	Cuba[F]
	C-251	Potato	1999	Cuba[F]
	C-254	Tomato	1999	Cuba[C]
	C-255	Potato	1999	Cuba[C]
	C-258	Potato	1999	Cuba[C]
	C-260	Tomato	1999	Cuba[H]
	B-3	Potato	Unknown	Brazil, Lavras
	B-4	Potato	Unknown	Brazil, Lavras
	B-6	Potato	1999	Brazil, Lavras
	B-8**	Potato	2000	Brazil, Lavras
	B-9**	Potato	2000	Brazil, Lavras
	B-12**	Potato	2000	Brazil, Lavras
	B-13**	Potato	2000	Brazil, Lavras
	B-14**	Potato	2000	Brazil, Lavras
	Ch-1	Tomato	1992	China, Jiangsu
	G-1**	Tomato	1997	Greece, Tragano Ilias
	G-2**	Tomato	1997	Greece, Tragano Ilias
	G-3**	Potato	1999	Greece, Central Peloponnese
	G-4**	Potato	1999	Greece, Central Peloponnese
	T-1	Potato	1999	Turkey, Torul-G
	T-2	Potato	1999	Turkey, Torul-G
	T-3	Potato	1999	Turkey, Torul-G
	T-4	Tomato	1999	Turkey, Torul-G
	T-5	Tomato	1999	Turkey, Igdir
	T-6	Tomato	1999	Turkey, Orul-E
	T-7	Tomato	1990	Turkey, Balikesir
	T-8	Tomato	1991	Turkey, Kumluca-Antalya
	T-9	Tomato	1991	Turkey, Kumluca-Antalya
	T-10	Tomato	1991	Turkey, Fethiye
	USA-1*	Potato	1998	USA, SD
	USA-2*	Potato	1998	USA, WI
	USA-3*	Potato	1999	USA, NE
	USA-4*	Potato	1999	USA, CO
	USA-5*	Potato	1999	USA, MN
	USA-6*	Potato	2000	USA, ND
	USA-7*	Potato	2000	USA, ND
	USA-8*	Potato	2000	USA, ND
Alternaria spp. (7)	B-1*	Potato	Unknown	Brazil, Lavras
	Can-1*	Tomato	2000	Canada, Harrow
	Can-2*	Tomato	2000	Canada, Harrow
	G-5*	Tomato	Unknown	Greece, Gythios
	R-1*	Tomato	1995	Russia, St. Petersburg
	R-2*	Tomato	1999	Russia, St. Petersburg
	R-3*	Tomato	Unknown	Russia, St. Petersburg
Curvularia sp. (1)	*Curvularia***	Tomato	1999	Cuba

Letter codes of the Cuban isolates are explained in the key to Figure 2. Total numbers of isolates are given in brackets. A double asterisk indicates monosporic isolates, whereas single asterisks show isolates that were not monosporic but where spores were observed during the experiments. No spore production was observed in isolates without asterisks.

Isolation and identity of fungal cultures

Surface-sterilized sections from the leading edges of lesions were placed in water agar. Small tufts of mycelia that emerged around lesion borders were transferred to potato dextrose agar and finally multiple-conidial isolates were purified. Single-conidial isolates were dissected directly from seeding spores from the infected leaf tissue, under a stereo-microscope. Criteria for morphological identification of *Alternaria* isolates were based on Ellis (1971). *Alternaria* spp. isolates from international sources were received as purified cultures. All isolates were stored in the culture collection at CENSA (Havana, Cuba).

Growth of mycelium and DNA extraction

Mycelia were produced in 250 ml Erlenmeyer flasks filled with 40 ml of potato dextrose broth. Flasks were inoculated with tufts of mycelium and incubated in the dark at $27 \pm 0.5\,^{\circ}$C for 4 days in still culture. Mycelium was harvested, vacuum-filtered and freezed at $-80\,^{\circ}$C until use. DNA from each isolate was extracted from 0.3–0.6 g of freeze-dried mycelium based on the extraction method of Sambrook et al. (1989). The DNA samples were re-extracted once to reduce discolouration and RNA was degraded by addition of 1 μl RNAse ($10\,\mathrm{mg\,ml^{-1}}$). After quantification using a fluorometer, samples were diluted to $50\,\mathrm{ng\,\mu l^{-1}}$ in TE buffer and stored at $4\,^{\circ}$C.

AFLP analysis

Due to the small genome of *A. solani*, a modified AFLP technique was applied to ensure amplification of sufficient polymorphic bands. Genomic DNA from each isolate was restricted with *Eco*RI and *Mse*I and ligated to PCR adapters using the AFLP kit of Gibco, BRL (Gaithersburg, MD, USA). A two-step PCR procedure was adopted for selective amplification. In the first step (pre-amplification), primers with a single selective nucleotide extension (A) were used for both the *Mse*I and the *Eco*RI fragment ends. In the second amplification, *Mse*I and IRD800-labelled *Eco*RI primers with one and two selective nucleotides, respectively, were used in combination (*M*-A and *E*-AT/-AC/-AG). AFLP products were separated in polyacrylamide gels with 1 × TBE buffer on a LI-COR 4200 fluorescent DNA analyser (LI-COR Inc., Nebraska, USA) using standard electrophoretic conditions suggested by the manufacturer.

A 50–350 bp DNA length standard (LI-COR. Inc., Nebraska, USA) was used as molecular weight marker for allele size calling. DNA from the isolate C-74 was included as a reference marker in all gels, and all fragments were scored against the two standards to ensure accurate designation of bands to their respective loci over all genotypes. Isolate genotypes were screened for presence or absence (1–0) of all AFLP fragments between 50 and 350 bp using the electrophoresis analysis software RFLPscan v2.1 (Scananalitics-CSPI, Billerica, MA, USA). Lanes and bands were tracked manually and a binary data matrix was generated describing the presence or absence of bands at all scored loci for the three primer pairs.

Phenetic analysis

Similarity matrices (DICE, JACCARD and SIMPLE) produced using the WINDIST software (I.W. Yap, University of Washington, USA) were used to cluster the data with three algorithms (Single linkage, UPGMA and Complete linkage) using the SAHN module of NTSYSpc (v2.01, Exeter Software, Setauket, NY, USA). Cophenetic values were calculated using the MXCOMP module. Dendrograms were constructed with the SAHN module using the similarity matrices from the three primer combinations considering all 542 polymorphic fragments, and alternatively with a reduced data set of 317 fragments in which loci for which all isolates were identical were pooled.

Population analysis

Population analysis was performed with the software AMOVA (v1.5, Excoffier, University of Geneva, Switzerland) using the reduced data set of 317 fragments. AMOVA input files were prepared using AMOVA-PREP v1.01 (Mark P. Miller, University of Northern Arizona, USA). Pair-wise comparisons of the variation between populations were analysed by calculating Φ_{st} values, which represent the proportion of the total variance that is partitioned between populations and measure the genetic differentiation of subpopulations. Significance levels for Φ_{st} were computed by non-parametric permutation procedures (Excoffier et al., 1992). The robustness of the phenogram was determined by bootstrap analysis of 1000 bootstrapped samples using WINBOOT (Yap and Nelson, 1996).

A 70% bootstrapping frequency was used as the lower limit for recognition of robust clusters.

Results

AFLP polymorphism

An example of typical AFLP variation is shown in Figure 1. A total of 542 fragments were observed in all isolates using the three selected primer combinations (Table 2). All primer combinations showed 100% polymorphism for the complete set of isolates, whereas the degree of polymorphism ranged from 89.2% to 98.6% in different isolate subsets. The primer combination *E*-AT detected more fragments for all subsets of isolates, but was less polymorphic than *E*-AC and *E*-AG in the isolate subsets.

Phenetic analysis

Dendrograms constructed using three similarity coefficients (DICE, JACCARD and SIMPLE) and three clustering methods (UPGMA, Single linkage and Complete linkage) were examined and compared to evaluate the goodness of fit of the resulting phylogenetic trees with respect to the reliability and stability of the inferred relationships (Table 3). High cophenetic values (ranging from 0.94 to 0.99) were found with all combinations, both with all 542 fragments and also with the reduced data set of 317 independent loci. In general $r > 0.9$ indicates a very good fit, indicating no major variation among the dendrogram patterns or the three distance calculation methods.

To assess the usefulness of AFLPs as phenetic markers, a similarity matrix based on the SIMPLE coefficient and UPGMA cluster method was constructed to estimate the level of relatedness among the *A. solani*, *A. porri*, *Alternaria* spp. and *Curvularia* sp. isolates. The resulting dendrogram (Figure 2) formed two main clusters with extremely high bootstrap values that clearly distinguished the species, with the exception of the single *A. porri* isolate which cosegregated with the *A. solani* isolates. All *A. solani* isolates grouped together in the first cluster (Cluster 1), whereas the other cluster grouped all *Alternaria* spp. together with the *A. alternata* and *Curvularia* sp. isolates (Cluster 2).

Within Cluster 1, two distinct branches were consistently formed in 100% of the 1000 bootstrapped trees. These subclusters revealed a general clustering by host, with most *A. solani* isolates from tomato being assigned to subcluster 1.1 and the majority of the potato isolates grouping in subcluster 1.2, with a similarity coefficient of around 0.76 between the two host-associated clusters. Subcluster 1.1 represents a large branch containing 87 genetically similar isolates

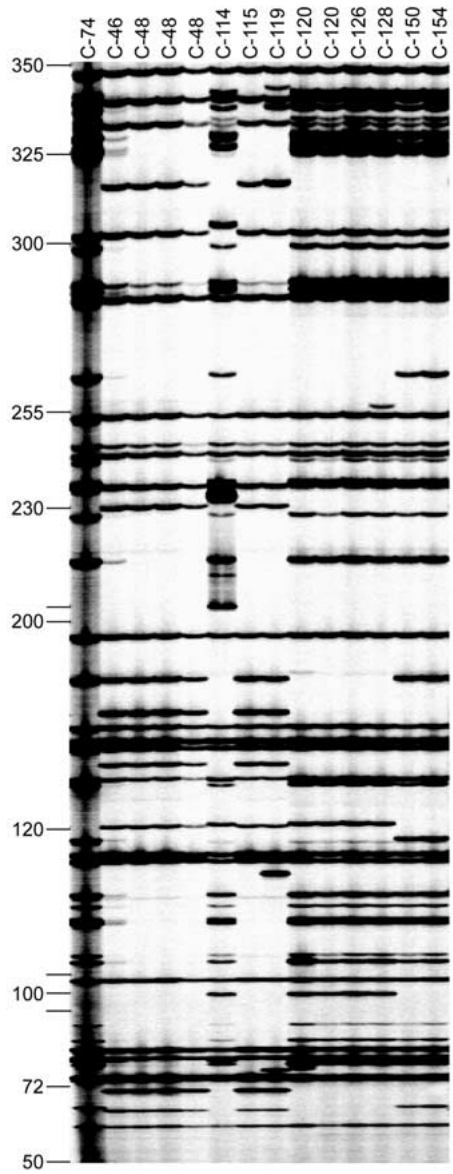

Figure 1. Example of AFLP fingerprints in 14 isolates of *Alternaria solani* using the primer combination *M*-A/*E*-AT with one selective *Mse*I nucleotide (A) and two selective *Eco*RI nucleotides (AT), respectively. Details of isolates are given in Table 1.

Table 2. AFLP fragments amplified in isolates of *A. solani* and related species with different *Eco*RI primer selective nucleotides. In all cases the *Mse*I primer included one selective nucleotide (A)

*Eco*RI primer selective nucleotides	Overall isolates ($n = 122$)		*A. solani* ($n = 112$)		*Alternaria* spp. ($n = 8$)		*A. porri* ($n = 1$)	*Curvularia* sp. ($n = 1$)
	Total[a]	% pol.[b]	Total[a]	% pol.[b]	Total[a]	% pol.[b]	Total	Total
E-AC	164	100	87	91.9	74	98.6	19	44
E-AG	169	100	88	94.3	80	96.3	24	46
E-AT	209	100	120	89.2	106	93.4	48	58
Total	542	100	295	91.5	260	95.8	91	148

[a]Total number of fragments observed.
[b]Percentage of total fragments in a given species that were polymorphic.

Table 3. Comparison of cophenetic values obtained from three similarity coefficients and three clustering used for analysing the AFLP data

Clustering method	Similarity coefficient					
	DICE		JACCARD		SIMPLE	
	All[a]	Reduced[b]	All[a]	Reduced[b]	All[a]	Reduced[b]
UPGMA	0.9910	0.9846	0.9455	0.9818	0.9895	0.9772
Single linkage	0.9612	0.9434	0.9455	0.9818	0.9828	0.9724
Complete linkage	0.9822	0.9715	0.9705	0.9572	0.9838	0.9799

[a]Cophenetic values considering all 542 detected fragments.
[b]Cophenetic values considering only the 317 unequal fragments.

(similarity coefficient >0.86), including 74 isolates from tomato and 12 from potato. Statistical support for minor subgroups containing isolates of different geographic origin was poor (bootstrap values <60%), however the *A. solani* isolates from more exotic sources (China, Greece, Turkey) generally tended to group separately from the Cuban and Brazilian *A. solani* isolates, with some exceptions. The Cuban isolate C-240 appears to be genetically distinct from the rest of subcluster 1.1, as it grouped separately from all other isolates with 93% confidence. The isolates forming subcluster 1.1 remained almost unchanged when the phenogram was generated using other similarity indices. The *A. porri* isolate from onion also clustered within this branch.

The other major branch within Cluster 1 (subcluster 1.2) comprises 26 *A. solani* isolates, including 21 from potato and 5 from tomato. Of all 34 isolates originating from potato, 65% grouped within this subcluster (Figure 2). Isolates from different origins within the USA, Greece and Turkey clustered separately to isolates originating from Cuba and Brazil, with a similarity coefficient of 0.80. All isolates from the USA clustered together in a single subgroup within subcluster 1.2 at confidence level of 100%, although they originated from different states. On the other hand, not all isolates from Brazil clustered together, although they all came from the Lavras region. Three Turkish isolates (T-4, T-5 and T-6) from tomato formed a separate subgroup in subcluster 1.2 with a higher genetic distance to the other isolates, albeit with a lower statistical support of only 63%.

Cluster 2 is comprised of nine isolates from tomato, from different substrates and with different geographical origins. These included the airborne isolate of *A. alternata*, six isolates of *Alternaria* spp. with small catenulated spores and the *Curvularia* sp. isolate. The dendrogram revealed great genetic dissimilarity within this cluster, with high statistical support for the subgroups. The three isolates from Russia were almost identical, as were the two isolates from Canada. The *Curvularia* and *A. alternata* isolates were genetically distinct from all other isolates.

Population analysis

AMOVA was used to estimate and partition the total genetic variance into within- and between-subgroup (population) components. Pair-wise comparisons were made separately between different populations, considering the species, hosts and countries of origin.

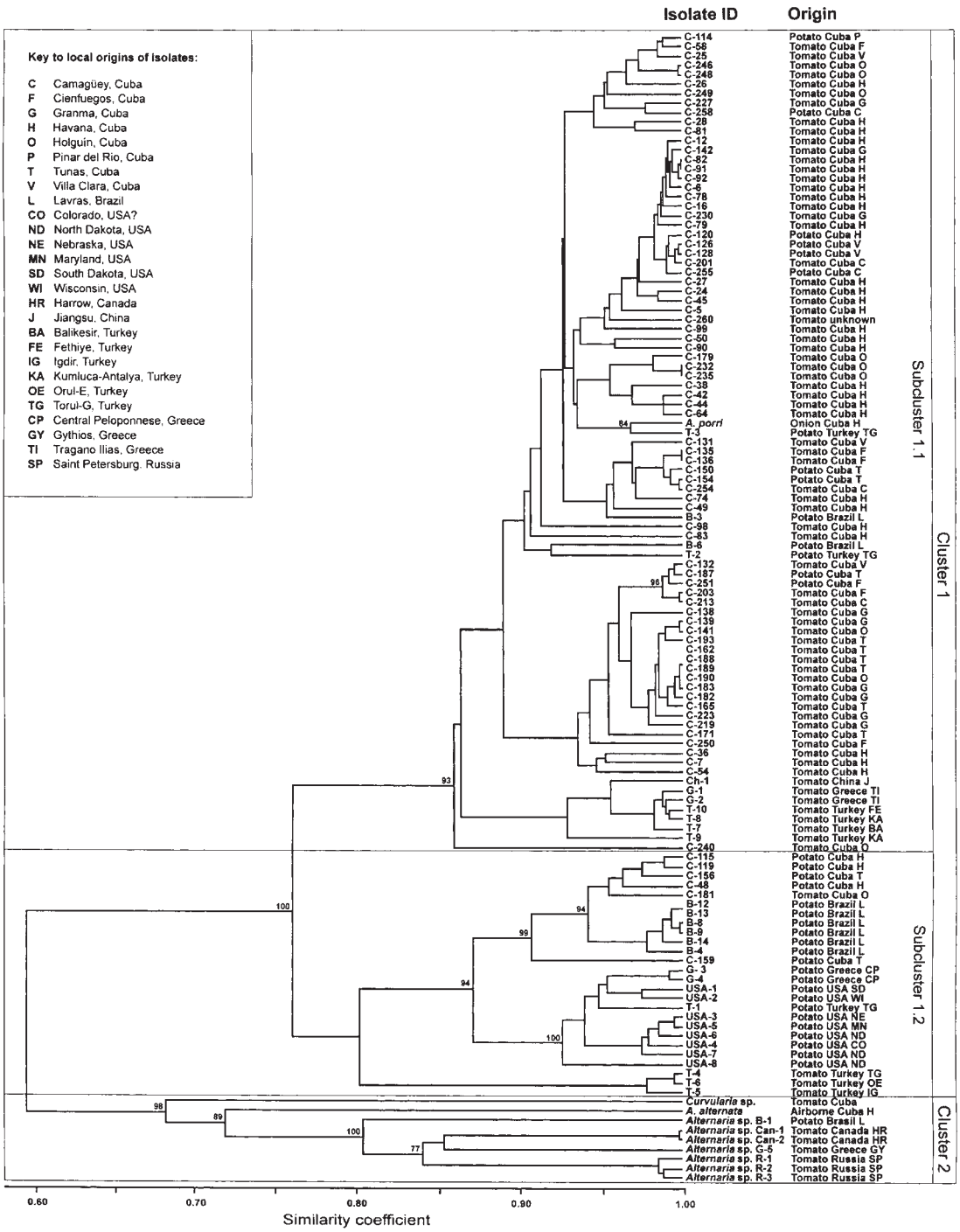

Figure 2. Phenogram showing genetic similarity among 112 isolates of *Alternaria solani*, seven isolates of catenulated *Alternaria* spp. and one isolate each of *A. porri*, *A. alternata* and *Curvularia* sp., revealed by UPGMA cluster analysis based on AFLP genetic fingerprints obtained with three primer combinations. Numbers shown above branches represent percentage confidence limits obtained by bootstrap analysis with 1000 bootstrap repetitions. Confidence limits below 70% are not shown. The isolates are separated into two highly significant clusters with a similarity coefficient of around 0.60, and the larger cluster is further divided into two subclusters with a similarity coefficient of less than 0.80.

Of the 112 *A. solani* isolates, 105 formed different haplotypes with the three primer combinations tested, indicating a great genetic diversity. Considering *A. solani* (together with the *A. porri* isolate), all catenulated *Alternaria* isolates and *Curvularia* sp. as separated populations, most of the total variability resided among populations with a $\Phi_{st} = 0.5415$ (which represent 54.15% of the total variability) and the rest within populations. After re-configuring the data to represent six different populations consisting of *A. solani* isolates from Cuba, Brazil, USA, Greece, Turkey and China, 46.35% of the total genetic variability was found to reside between countries ($\Phi_{st} = 0.4635$). Considering *A. solani* isolates from tomato and potato as two different populations, the genetic distance among them was 0.330 with high statistical significance ($P < 0.0001$).

Table 4 shows genetic differences explained by geographical origins of populations from tomato and potato, respectively. The greatest genetic distances among populations from tomato were observed between the isolates from Greece and China ($\Phi_{st} = 0.6923$, $P < 0.0001$), and between the populations from Cuba and those from Greece and Turkey ($\Phi_{st} = 0.4593$ and $\Phi_{st} = 0.4560$, respectively; $P < 0.0001$). Among potato populations, the greatest genetic distances were found between the USA population and those from Cuba and Brazil. All genetic distances within this group showed highly significant differences, with the exception of the populations from Cuba and Turkey. The Φ_{st} values resulting from the pair-wise populations comparison between different hosts revealed a great genetic differentiation. The greatest genetic distances (with Φ_{st} values over 0.5196) were found between the population representing USA isolates and seven of the other eight populations analysed. The Cuban populations from tomato and potato showed a moderate but significant genetic difference; the same was observed between Turkish isolates, whereas between the isolates from Greece the differences were quite large ($\Phi_{st} = 0.9591$, $P < 0.0001$). A pair-wise comparison between the domestic Cuban and foreign tomato and potato isolates, respectively, revealed highly significant ($P < 0.0001$) genetic differences between the Cuban isolate populations and the international isolates (Table 5).

Genetic variability among the Cuban provincial populations ($\Phi_{st} = 0.1289$) was considerably lower than among populations from different countries ($\Phi_{st} = 0.4635$). This was consistent with the low

Table 5. Genetic distances among the Cuban and foreign isolates of *A. solani* from tomato and potato, respectively, calculated using three AFLP primer combinations

ID	Tomato		Potato	
	Cuba ($n = 70$) 1	Foreign ($n = 10$) 2	Cuba ($n = 14$) 3	Foreign ($n = 21$) 4
1	—	0.0000	0.0000	0.0000
2	0.4353	—	0.0000	0.0000
3	0.1544	0.3585	—	0.0000
4	0.5336	0.4748	0.2791	—

Numbers below the diagonal are measures of inter-population genetic distance (Φ_{st}). Numbers above the diagonal are the probabilities that a random Φ_{st} value will be greater than the observed value, and represent the significance of the observed Φ_{st} value.

Table 4. Genetic distances among nine populations representing different geographic origins and hosts of 112 *A. solani* isolates, calculated using three AFLP primer combinations

ID	Tomato				Potato				
	Cuba ($n = 67$) 1	Greece ($n = 2$) 2	Turkey ($n = 7$) 3	China ($n = 1$) 4	Cuba ($n = 14$) 5	Brazil ($n = 8$) 6	USA ($n = 8$) 7	Greece ($n = 2$) 8	Turkey ($n = 3$) 9
1	—	0.0000	0.0000	0.0190	0.0000	0.0000	0.0000	0.0000	0.0190
2	0.4593	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3	0.4560	0.0591	—	0.4835	0.0000	0.0000	0.0000	0.0000	0.0000
4	0.2889	0.6923	−0.0067	—	0.5744	0.2557	0.0000	0.0000	0.0000
5	0.1544	0.3588	0.3467	0.1351	—	0.0000	0.0000	0.0000	0.1668
6	0.5486	0.6170	0.5068	0.5220	0.2557	—	0.0000	0.0000	0.0000
7	0.7002	0.8479	0.6600	0.8338	0.5363	0.5196	—	0.0000	0.0000
8	0.6551	0.9591	0.5985	0.9645	0.3990	0.3693	0.3473	—	0.0000
9	0.2183	0.3176	0.2717	−0.1169	−0.0161	0.2294	0.5434	0.2849	—

Numbers below the diagonal are measures of inter-population genetic distance (Φ_{st}). Numbers above the diagonal are the probabilities that a random Φ_{st} value will be greater than the observed value, and represent the significance of the observed Φ_{st} value.

Table 6. Genetic distances among eight populations comprising 80 *A. solani* isolates from different provinces of Cuba, measured using three AFLP primer combinations

ID	Pinar del Río ($n = 1$) 1	Havanna ($n = 33$) 2	Cienfuegos ($n = 6$) 3	Villa Clara ($n = 5$) 4	Camagüey ($n = 6$) 5	Tunas ($n = 10$) 6	Holguín ($n = 11$) 7	Granma ($n = 8$) 8
1	—	0.1908	0.3526	0.3297	0.0000	0.2218	0.3776	0.0000
2	0.1485	—	0.0000	0.0789	0.0400	0.0509	0.0480	0.0000
3	0.0600	0.1741	—	0.4106	0.1928	0.3916	0.0000	0.0000
4	−0.0342	0.0208	−0.0130	—	0.6793	0.0000	0.6024	0.0000
5	0.2201	0.0394	0.0485	−0.1215	—	0.0000	0.0000	0.0000
6	0.1137	0.1828	0.0326	0.1115	0.1517	—	0.0000	0.2438
7	−0.0648	0.0758	0.0958	0.0524	0.1007	0.0757	—	0.0000
8	0.5113	0.2640	0.1635	0.2758	0.3352	0.0340	0.1478	—

Numbers below the diagonal are measures of inter-population genetic distance (Φ_{st}). Numbers above the diagonal are the probabilities that a random Φ_{st} values will be greater than the observed value, and represent the significance of the observed Φ_{st} value.

bootstrap confidence levels for subcluster 1.1 in the UPGMA cluster analysis (Figure 2). Results of a pair-wise comparison between the eight *A. solani* populations from different Cuban provinces are shown in Table 6. The greatest genetic distance among populations was observed between the samples from Pinar del Rio and Granma ($\Phi_{st} = 0.5113$; $P < 0.0001$), however the Pinar del Rio sample consists of only one isolate. Considering only the significant distances at the $P = 0.01$ level, Granma is the most distinct population from the others with six significant distances ($\Phi_{st} = 0.1478$–0.5113; $P < 0.0001$), followed by Camagüey ($\Phi_{st} = 0.1007$–0.2201; $P < 0.0001$) and Holguín ($\Phi_{st} = 0.0757$–0.1478; $P < 0.0001$) with four significant distances each. Tunas showed three significant distances ($\Phi_{st} = 0.0757$–0.1517; $P < 0.0001$) and the rest of the populations only two.

Discussion

Alternaria solani is distributed all over the world where tomato and potato are cultivated (Rotem, 1994). In this paper, we describe a large-scale survey of genetic variability in 122 isolates of *A. solani* and related species, from tomato and potato hosts, using AFLP genetic fingerprinting. A modified AFLP protocol was applied, optimized for the small size of the *Alternaria* genome by the use of PCR primers with only one and two selective nucleotides, respectively.

The AFLP data corroborated the high genetic variability reported previously for *A. solani* populations using isozymes and RAPD markers (Petrunak and Christ, 1992; Weir et al., 1998). Petrunak and Christ (1992) estimated a genetic distance within a population of *A. solani* of 0.28, whereas Weir et al. (1998) revealed a distance of 0.23. A similar level of diversity was observed in the present study, with a minimal genetic similarity coefficient of around 0.76 at polymorphic AFLP loci for *A. solani* isolates from Cuban and international sources.

Phenetic and population analysis of the AFLP fingerprint data revealed an association between genetic background and host origin. A highly significant genetic differentiation ($\Phi_{st} > 0.1544$; $P < 0.0001$) was revealed among Cuban, Greek and Turkish isolates from tomato and potato (Tables 4 and 5). Evidence for host specialization of *A. solani* was apparent from the phenogram of 112 isolates revealed by UPGMA cluster analysis. Sixty-two percent of the isolates originating from potato and 86.7% from tomato clustered clearly in two separate branches in the dendrogram with a maximum bootstrapping confidence level (100%). AMOVA analysis with no consideration of geographic origin indicated a considerable genetic differentiation ($\Phi_{st} = 0.289$) with high statistical significance ($P < 0.0001$) between the isolates representing populations from tomato and potato. Franco et al. (2001) considered Φ_{st} values between 0.15 and 0.25 and above 0.25 to represent moderately high and high genetic differentiation between groups, respectively.

To date, evidence of pathogenic specialization of *A. solani* on tomato and potato has not been reported. However, the AFLP evidence presented here is consistent with preliminary observations of pathogenicity in some of the *A. solani* isolates used in this study. Isolates from potato were less aggressive on different tomato genotypes than the tomato isolates (data not presented). Information on the specialization of pathogen populations and detailed monitoring of

their pathogenicity are essential for the development of effective disease control strategies.

In regard to pathogenic specialization, Simmons (2000) proposed a new species, *Alternaria tomatophila*, within the well-known *A. solani*, reporting that *A. tomatophila* is 'the common and widely distributed incitant of early blight of tomato'. All isolates but one from potato examined in that study were *A. solani*. Host specialization in *A. solani* was indicated by the general separation of tomato and potato isolates into subclusters 1.1 and 1.2 with a genetic similarity coefficient of 0.7 (Figure 2). However, some isolates were not consistent with a general host specialization hypothesis. Nine potato isolates from Cuba, two from Turkey and two from Brazil were grouped among tomato isolates in subcluster 1.1, whereas one Cuban and three Turkish tomato isolates grouped within the potato isolates in subcluster 1.2. We have therefore classified all potato and tomato isolates in *A. solani*, according to Ellis (1971), until detailed microscopic and cultural examination according to Simmons (2000) can be performed.

The results of the present study revealed an influence of geographic origin on genetic variability among the populations of *A. solani*. Isolates from the USA, Brazil, Cuba and Turkey were genetically distant from each other with $\Phi_{st} = 0.4635$, but regional populations within Cuba showed little significant genetic diversity (Table 6). This lack of significant geographic differentiation among regional *Alternaria* spp. populations suggests (1) widespread dispersal of fungal spores and (2) weak host selection pressure within and between populations (Sharma and Tewari, 1998; Aradhya et al., 2001).

A high degree of morphological and DNA similarity among *A. solani* and *A. porri* has been observed (Neergaard, 1945; Pryor and Gilbertson, 2000). The members of the porri species-group (*A. porri*, *A. solani*, *Alternaria dauci*, *Alternaria macrospora* and *Alternaria crassa*) exhibit a high degree of rDNA similarity, with no differences or only minor variation in mitochondrial small subunit or nuclear internal transcribed spacer sequences. Based on this evidence, an extremely close relationship among species in this group has been suggested (Pryor and Gilbertson, 2000), and this was clearly reflected by the inability of the AFLP analysis to distinguish the *A. porri* isolate from *A. solani* in the present study.

The origin of the variability present in *A. solani* is unknown. It is commonly assumed that sexual reproduction is essential to the long-term viability of a species, because it provides phenotypic variation on which selection may act (Gordon and Martyn, 1997). With the exception of a single report (Esquivel, 1984) the sexual stage in *A. solani* has been reported neither in nature nor *in vitro* (Simmons, 1992). The genetic variability may therefore arise from a parasexual cycle, as is the case in other imperfect fungi. However, evidence of natural parasexualism has not been obtained to date in *A. solani*, or in the well-known *A. alternata* pathogens (Akamatsu et al., 1999; Salamiah et al., 2001). Heterokaryons (Tsuge et al., 1987) and stable fusants have been purified (Salamiah et al., 2001).

Because of the low number of catenulated *Alternaria* spp. isolates studied it is difficult to make conclusions regarding their genetic similarity to other isolates. However, our results are consistent with earlier studies that found high variability within catenulated isolates (Morris et al., 2000; Roberts et al., 2000; Andersen et al., 2001). Moreover, in our experiment the catenulated isolates showed a low genetic similarity (0.61) in relation to the *A. solani* isolates. In other studies *A. solani* and *A. alternata* were also clearly distinguishable from each other (Petrunak and Christ, 1992; Weir et al., 1998). Three *Alternaria* species pathogenic to crucifers with small and large spores were also distinguishable using RAPD analysis (Sharma and Tewari, 1998).

This study demonstrates the suitability of the AFLP technique for detailed analysis of genetic variation in *A. solani* and related pathogens. Cluster analysis indicated a level of host specificity within *A. solani*. This may have important implications for effective early blight disease management in tomato and potato. It appears that the use of reproducible, highly polymorphic AFLP markers has the potential to play a major role in the accurate taxonomic identification of *A. solani* subgroups.

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