Molecular Diversity and Evolutionary Processes of *Alternaria solani* in Brazil Inferred Using Genealogical and Coalescent Approaches

Valdir Lourenço, Jr., Andrés Moya, Fernando González-Candelas, Ignazio Carbone,
Luiz A. Maffia, and Eduardo S. G. Mizubuti

First, fifth, and sixth authors: Departamento de Fitopatologia, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil; second and third authors: Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, Apartado Postal 22085, 46071 Valencia, Spain; and fourth author: Center for Integrated Fungal Research, Department of Plant Pathology, North Carolina State University, Raleigh 27695.

Accepted for publication 4 February 2009.

ABSTRACT

Lourenço, V., Jr., Moya, A., González-Candelas, F., Carbone, I., Maffia, L. A., and Mizubuti, E. S. G. 2009. Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. *Phytopathology* 99:765-774.

Alternaria spp. form a heterogeneous group of saprophytic and plant-pathogenic fungi widespread in temperate and tropical regions. However, the relationship between evolutionary processes and genetic diversity with epidemics is unknown for several plant-pathogenic *Alternaria* spp. The interaction of *Alternaria solani* populations with potato and tomato plants is an interesting case study for addressing questions related to molecular evolution of an asexual fungus. Gene genealogies based on the coalescent process were used to infer evolutionary processes that shape the *A. solani* population. Sequences of the rDNA internal transcribed spacer (ITS) region and the genes which encode the allergenic protein alt a 1 (*Alt a 1*) and glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) were used to estimate haplotype and nucleotide diversity as well as for the coalescent analyses. The highest number of parsimony informative sites

($n = 14$), nucleotide diversity (0.007), and the average number of nucleotide differences (3.20) were obtained for *Alt a 1*. Although the highest number of haplotypes ($n = 7$) was generated for ITS, haplotype diversity was the lowest (0.148) for this region. Recombination was not detected. Subdivision was inferred from populations associated with hosts but there was no evidence of geographic subdivision, and gene flow is occurring among subpopulations. In the analysis of the *Alt a 1*, balancing selection and population expansion or purifying selection could have occurred in *A. solani* subpopulations associated with potato and tomato plants, respectively. There is strong evidence that the subpopulation of *A. solani* that causes early blight in potato is genetically distinct from the subpopulation that causes early blight in tomato. The population of *A. solani* is clonal, and gene flow and mutation are the main evolutionary processes shaping its genetic structure.

Additional keywords: molecular epidemiology, population genetics, variability.

The genus *Alternaria* encompasses a complex group of saprophytic and pathogenic fungal species (68). *Alternaria* spp. are frequently reported as allergenic, food spoilers, mycotoxicogenic, opportunistic fungi associated with mycosis in animals and humans, and destructive plant pathogens (60,68). Species of the genus are ubiquitous and are reported to occur in different ecosystems and geographic regions, such as Antarctic soils (39), deserts, and the tropics (27). Many *Alternaria* spp. lack a sexual stage (10). Nevertheless, variability of this asexual species is high (68) and the first assessments of variation date back to the 1920s (14).

Alternaria solani Sorauer is an asexual plant-pathogenic species that causes early blight in potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.). This haploid fungus belongs to the phylum Ascomycota, class Dothideomycetes, order Pleosporales, and to family Pleosporaceae (64). According to morphological characters and phylogenetic analyses using the internal transcribed spacer (ITS) from rDNA region and genes that encode glyceraldehyde-3-phosphate dehydrogenase (*Gpd*), and the aller-

genic protein alt a 1 (*Alt a 1*), *A. solani* is included in the porri-spesies group with large, long-beaked, and noncatenated spores (55,63).

Early blight is spread worldwide (56) and its typical symptoms are necrotic lesions in the aerial plant parts. Severe epidemics can lead to major crop losses in short periods of time (8). In Brazil, early blight is considered one of the most destructive diseases of potato and tomato, the two most important vegetable crops in the country (32,70). Environmental conditions in all areas where potato and tomato are grown are favorable to early blight epidemics but the disease is more severe in the summer, when high temperatures and rainfall prevail (8). Fungicides are regularly and intensively applied to reduce yield losses, because cultivars that combine early blight resistance and good agronomic or commercial characteristics are not available.

The interactions between populations of *A. solani* and potato and tomato crops in tropical and subtropical regions make an interesting case study for addressing questions related to the molecular epidemiology and the evolution of an asexual fungal species. It is well known that variation in populations of plant pathogens directly affects disease management, especially the strategies related to the deployment of resistant cultivars and fungicide usage (38,44,45). Previous studies have focused on the variability in populations of *A. solani* and its implications for early blight epidemics. There is evidence for the existence of *A. solani* populations associated with potato and tomato plants based on the analysis of phenotypic and molecular markers (42,51,

Corresponding author: E. S. G. Mizubuti; E-mail address: mizubuti@ufv.br

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a table showing *Alternaria solani* isolates collected from potato- and tomato-producing regions of Brazil and Figure 1 appears in color online.

doi:10.1094/PHYTO-99-6-0765

© 2009 The American Phytopathological Society

62,72). In Brazil, differences in cultural, pathogenic, serologic, and electrophoretic characteristics between groups of isolates sampled from potato or tomato fields, hereafter referred to as potato and tomato isolates, resulted in two groups according to the host of origin (15,21,62). However, in these studies, the number and size of samples were small and the geographic distribution from where isolates were obtained was restricted.

From an evolutionary perspective, the study of population dynamics of plant-pathogenic fungi in the tropics is an interesting research topic, considering the high number of pathogen life cycles throughout the year and the absence of harsh environmental conditions that could lead to drastic population reduction. The almost continuous cropping systems, the geographic proximity of tomato and potato fields in many areas, and the constant availability of inoculum (8) may contribute to increase the number of pathogen generations in the tropics compared with temperate climate zones. Furthermore, the diversity of wild *Solanum* spp. is higher in the tropics (35,58) and these species can harbor *A. solani* lineages and could serve as reservoirs of inoculum to early blight epidemics. It was hypothesized that the evolution of populations in such an environment is likely to be driven by mutation, selection, and gene flow. There is evidence that these evolutionary processes have influenced *A. solani* populations (42,51,71,72), and there are no reports of the occurrence of sexual or parasexual recombination in this fungus (60,68). However, reliable estimates of parameters related to recombination, mutation, gene flow, and selection have not been obtained yet for *A. solani* populations.

Integrating bioinformatics and molecular population genetics techniques is interesting to study populations of fungi that lack a

known sexual stage, particularly populations that comprise complex groups of species or lineages (67). In the present study, the integrated approach was used to address the following questions. Is molecular diversity among *A. solani* isolates high? Although no sexual structures have been found in *A. solani*, is there any evidence of “cryptic” recombination in the pathogen population? What is the contribution of selection, mutation, recombination, and gene flow in shaping genetic variability in populations of *A. solani*? Pathogen isolates were obtained from samples of both tomato and potato fields, grown in several places in Brazil; three genomic regions were sequenced, and the data were analyzed using descriptive and simulation-based methods.

MATERIALS AND METHODS

Sampling and isolate maintenance. Major potato- and tomato-producing areas of Brazil were sampled (Fig. 1). A larger number of fields were sampled in the central and southeastern regions, the main producing areas (57). According to the intensity of early blight epidemics, 1 to 20 samples (diseased potato leaves and tomato leaves or fruit) from each field were collected and taken to the laboratory, where direct or indirect pathogen isolation was conducted (19). For direct isolation, plant material was incubated for 24 h in moist chambers. Under a dissecting microscope ($\times 80$), conidia were transferred to water-agar (WA) medium in petri plates, incubating at 25°C. After 12 h, one germinated conidium was transferred to potato dextrose agar (PDA) medium in petri plates. For nonsporulating lesions, the fungus was indirectly isolated. Fragments from the lesion margins were transferred to 70% ethanol for 30 s, disinfested with 2% sodium hypochlorite

Fig. 1. Distribution of haplotypes (H) from analyses of *Alt a 1* (Alta1) and *Gpd* genes (Gpd), and the internal transcribed spacer (ITS) region in the sampled areas (black dots) located in potato- and tomato-producing regions of Brazil. Numbers next to pie charts represent the frequency of *Alternaria solani* isolates from each haplotype.

for 2 min, washed twice with sterilized distilled water for 2 min, air dried under the flow hood on sterilized filter paper, and then transferred to PDA in petri plates, incubating at 25°C with a 12-h photoperiod. After colony development, mycelial plugs were transferred to V8 medium in Erlenmeyer flasks that were continuously agitated at 110 rpm at 26°C. After 7 days, the mycelium was triturated and transferred to PDA in petri plates that were kept uncovered at 25°C under near-UV light for 12 h to induce sporulation. After 3 days, a conidial suspension was prepared, diluted, and pipetted to slides with WA. The slides were kept in petri plates at 25°C and, after 12 h, checked under a compound microscope (×400). To obtain monoconidial cultures, germinated and individualized conidia were transferred to PDA medium in petri plates. Cultures were maintained in mineral oil at 8°C or in colonized sterilized filter paper at –80°C.

DNA extraction. Isolates were grown in liquid medium (10 g of sucrose, 2 g of L-asparagin, 2 g of yeast extract, 15 mM KH₂PO₄, 0.4 mM MgSO₄ · 7H₂O, 1.5 μM ZnSO₄ · 7H₂O, 1.8 μM FeCl₃ · 6 H₂O, and 2.5 μM MnCl₂ · H₂O) in Erlenmeyer flasks under continuous agitation at 26°C. After 7 days, the mycelium was washed with distilled water, transferred to filter paper to dry, macerated in a mortar with liquid nitrogen, and DNA was extracted using a cetyltrimethylammonium bromide-based protocol (46). Depending on the size of the DNA pellet, 30 to 50 μl of Tris-EDTA buffer with RNAase were added and resuspended at 37°C for 2 h.

DNA amplification and sequencing. Partial sequences of the ITS region and *Gpd* and *Alt a 1* genes were polymerase chain reaction (PCR) amplified using fungal specific primers ITS4 and ITS5 (73), *gpd1* and *gpd2* (11), and *Alt*-for and *Alt*-rev (29), respectively. All amplification reactions were performed in a 50-μl reaction volume. PCR conditions for each gene were according to the references provided above. After electrophoresis in 0.8% agarose gels stained with ethidium bromide, PCR products were purified using minicolumns, purification resin, and buffer according to the manufacturer's protocols (High Pure PCR Product Purification Kit; Roche Mannheim, Germany).

Purified amplicons were sequenced using Big Dye Terminator (v3.1; Applied Biosystems, Foster City, CA) or Dyanamic ET Dye Terminator (GE Life Sciences, Piscataway, NJ). All sequencing was performed at the Sección de Secuenciación de ADN y Proteínas of the Universidad de Valencia and at the Laboratório de Genômica of the Instituto de Biotecnologia Aplicada à Agricultura of the Universidade Federal de Viçosa.

For each isolate, sequences were manually edited using The Staden Package, ver. 1.6.0 (65) to generate a consensus sequence. Sequences were aligned with ClustalW (69).

Molecular population genetic analyses. Several analyses were performed to quantify the amount and distribution of genetic diversity in the populations. Isolates were analyzed either as belonging to a single population or grouped into potato and tomato subpopulations. Statistics of DNA polymorphism within and between subpopulations were carried out with DnaSP 4.0 (61). Analysis of molecular variance using Arlequin 3.11 (20) was conducted to assess population structure. The Kimura 2-parameter distance (36) was set for the analyses, and 1,000 permutations were performed. The Mantel correlation coefficient (40) between matrices of geographic and genetic distances was estimated using Arlequin. The significance of the Mantel correlation was assessed using 1,000 random permutations of matrices.

Estimating parameters of evolutionary mechanisms. Parameter estimates of selection, mutation, gene flow (migration), and recombination were obtained using different software packages. The analyses started with SNAP Workbench, which integrates and manages a series of programs used in evolutionary population genetics (54). SNAP Map was used to collapse nucleotide sequences with phenotype (host of origin of each isolate) into haplotypes using the options for recoding indels and excluding infinite-sites violation (5). Compatibility matrices were generated for haplotypes that share a common ancestry in the strict consensus tree to examine the overall support or conflict among variable sites in a DNA sequence alignment (33). These compatibility matrices, useful for visual identification of recombinant blocks, were generated using SNAP Clade (16). The Recombination Detection Program (RDP2, ver. 2.0) (41) was used to identify recombinant sequences and recombination breakpoints.

Neutrality tests, Tajima's (66), Fu and Li (23), and Fu's Fs statistics (22) were conducted with DnaSP for *Alt a 1* and *Gpd* genes.

Nonparametric tests of population subdivision were conducted with SNAP Workbench after analyzing the results from the compatibility matrices and neutrality tests. The nucleotide sequence files were converted by seqtomatrix, recoding indels and excluding infinite-sites violations (30,31). Hudson's permutation tests using Permtest (nonparametric permutation method based on Monte Carlo simulations) were used to analyze population subdivision according to the host plant (potato and tomato) (30,31).

The migration with division (MDIV) program was used to simultaneously estimate time of divergence (T) and migration rate (M) between two subdivided populations. MDIV was used to distinguish between equilibrium migration and subdivision due to shared ancestral polymorphism between the two subpopulations (48). Analyses were repeated using five independent simulation runs with a different random number seed for each run. The

TABLE 1. Frequencies of *Alternaria solani* haplotypes (H) obtained from potato and tomato producing regions of Brazil^a

Locus ^b	Region/state ^c										
	NE/CE	NE/BA	CW/GO	CW/DF	SE/MG	SE/RJ	SE/SP	SE/ES	S/PR	S/SC	S/RS
<i>Alt a 1</i>	H1 (1P); H5 (2P)	H1 (2P)	H1 (5P); H5 (1T)	H1 (1P); H2 (1T); H4 (1P); H5 (5P, 6T)	H1 (21P, 1T); H3 (1T); H4 (1T); H5 (7P, 14T)	H4 (1T); H5 (4T)	H1 (1P); H5 (6T)	H1 (1T); H5 (1P, 4T)	H1 (7P, 1T); H5 (3P, 3T)	H4 (1T); H5 (4T)	H4 (1T); H5 (3T)
<i>Gpd</i>	H1 (1P); H3 (1P); H4 (1P)	H1 (2P)	H1 (5P); H3 (1T)	H1 (1P); H3 (5P, 3T); H4 (1P, 3T); H5 (1T)	H1 (19P, 1T); H3 (5P, 9T); H4 (2P, 5T); H5 (1T)	H3 (3T); H4 (2T)	H1 (2P); H3 (4T); H4 (3T)	H1 (1T); H3 (2T); H4 (1T)	H1 (4P, 1T); H3 (3T); H4 (4P, 1T); H6 (1P)	H3 (3T); H4 (1P, 2T)	H1 (1P); H2 (1T); H3 (2T); H4 (1T)
ITS	H1 (2P)	H1 (3P)	H1 (4P)	H1 (5P, 5T); H2 (1T)	H1 (24P, 16T); H2 (1T); H5 (1P)	H1 (5T)	H1 (1P, 7T)	H1 (1P, 5T)	H1 (8P, 4T); H4 (1P, 1T)	H1 (2T); H3 (1T), H4 (1T)	H1 (1P, 4T)

^a Numbers and letters in parentheses designate, respectively, the frequency of occurrence and the host (P = potato or T = tomato) from which each haplotype was sampled.

^b *Alt a 1*: *Alternaria* allergen gene; *Gpd*: glyceraldehyde-3-phosphate dehydrogenase; ITS1, 5.8S, and ITS2: rDNA from the internal transcribed spacer (ITS) region.

^c States: PR (Paraná), RJ (Rio de Janeiro), MG (Minas Gerais), BA (Bahia), SP (São Paulo), ES (Espírito Santo), GO (Goiás), SC (Santa Catarina), RS (Rio Grande do Sul), CE (Ceará), and DF (Distrito Federal). Regions: S (south), SE (southeast), NE (northeast), and CW (central-west).

length of the Markov chain was set to 2 million and the burn-in time was 500,000. Migration rates were also estimated using the program MIGRATE (9). Simulations were carried out with 20 short chains with 5,000 sampled genealogies each (10× the default) and 3 long chains with 50,000 sampled genealogies each (10× the default). This process was repeated five times with different random seed number.

Simulations of the genealogies of each gene under the coalescent process were performed using GENETREE (6). In all cases, constant population sizes were assumed and the genealogy with the highest root probability was chosen to represent the process. Genealogies were inferred using five simulations (1 million runs each) with different random seed numbers for *Alt a 1*, *Gpd*, and ITS loci. The genealogy of a concatenated data set comprising *Alt a 1* and *Gpd* sequences was also simulated (five simulations) with 10 million runs each. The ages of mutations were estimated for the rooted tree with the highest likelihood, and a graph of the tree was generated with coalescent unit times using Treepic (26).

RESULTS

Summary statistics of the molecular population genetic analyses. The total number of sequences analyzed for *Alt a 1*, *Gpd*, and ITS loci was variable, although each had approximately equal numbers of sequences for tomato and potato isolates (Table 1). Sequences were obtained for 111, 110, and 104 isolates of *A. solani* for the *Alt a 1*, *Gpd*, and ITS loci, respectively. A concatenated data set for the three loci (1,612 bp in length) was constructed for 88 isolates of *A. solani*. It was not possible to obtain sequences from all isolates used in this study due to problems with amplification or sequencing of some samples.

The variability in the three sequences was 1.5 to 3% (Table 2). The estimated mutation rate per site (θ_w) was 0.002 to 0.008. The highest number of haplotypes ($n = 7$) was generated when analyzing ITS sequences; however, haplotype diversity was the lowest for this region (0.148) (Table 2). The highest values of nucleotide diversity ($\Pi = 0.007$) and of the average number of nucleotide differences ($k = 3.20$) were obtained for *Alt a 1*.

The highest Π and k were recorded for the potato subpopulation when analyzing the *Alt a 1* gene, whereas the lowest values of these statistics were estimated for the potato subpopulation for the ITS region. For the *Gpd* locus, Π and k of the potato subpopulation were also higher than in the tomato subpopulation. Nevertheless, when analyzing the ITS region, Π and k of the tomato subpopulation were higher than the estimated values of the potato subpopulation (Table 2).

The haplotype distribution and base substitutions for the *Alt a 1* and *Gpd* genes and the ITS region are summarized in Table 3. Variable positions which violated the infinite sites model were excluded from the coalescent analyses. Thus, fewer haplotypes were generated in the analysis of ITS region (Table 3).

The partitioning of genetic variation between tomato and potato subpopulations was investigated with four data sets: single locus (*Alt a 1*, *Gpd*, and ITS) and the three-sequence concatenated data set (Table 4). For the *Alt a 1* data set, there was greater variation between than within subpopulations. The opposite was found for the *Gpd* gene, ITS region, and concatenated data sets.

There was no evidence of geographic differentiation and no correlation between geographic location and genetic distance. The correlation coefficient estimated with the Mantel's test for *Alt a 1*, *Gpd*, ITS, and the concatenated sequences was 0.11 ($P = 0.08$), -0.06 ($P = 0.77$), 0.04 ($P = 0.27$), and -0.02 ($P = 0.61$), respectively.

TABLE 2. Summary statistics of DNA polymorphisms in populations of *Alternaria solani* from potato and tomato plants based on sequences of the *Alt a 1* and *Gpd* genes, and internal transcribed spacer (ITS) region

Statistic	<i>Alt a 1</i>			<i>Gpd</i>			ITS		
	Potato	Tomato	Total	Potato	Tomato	Total	Potato	Tomato	Total
No. of sequences (length) ^a	57	54	111 (471)	56	54	110 (577)	51	53	104 (564)
No. of invar/var/PIS ^b	464/7/6	455/16/14	455/16/14	568/9/5	563/14/13	559/18/13	547/5/2	523/14/8	514/15/09
θ_w (per site/per sequence)	0.003/1.52	0.008/3.51	0.006/3.03	0.003/1.96	0.005/3.07	0.006/3.41	0.002/1.11	0.006/3.09	0.005/2.8
No. of haplotypes	3	5	5	4	5	6	4	5	7
Haplotype diversity (Hd)	0.464	0.302	0.544	0.553	0.594	0.687	0.115	0.181	0.148
Nucleotide diversity (Π)	0.006	0.003	0.007	0.004	0.003	0.005	0.001	0.002	0.001
Nucleotide differences (k) ^c	2.75	1.43	3.20	2.36	1.68	2.72	0.31	0.82	0.57
Total no. of mutations	7	16	16	9	14	18	6	14	17

^a Number of sequences (sequence length).

^b Number of invariable/variable/parsimony informative sites (PIS).

^c Average number of nucleotide differences (k).

TABLE 3. Distribution of haplotypes and base substitutions in *Alt a 1* and *Gpd* genes and the internal transcribed spacer (ITS) region of *Alternaria solani*

Locus	<i>Alt a 1</i>	<i>Gpd</i>	ITS
Position	1 1 2 2 3 3 3 3 3 3 3 3 4 4 6 2 4 1 7 0 3 3 3 4 4 6 7 9 6 6 3 9 7 4 0 5 3 5 7 6 8 1 4 9 1 2	1 1 1 1 1 1 2 2 4 1 3 3 3 5 5 7 8 9 0 0 0 0 9 1 3 5 9 1 2 6 3 6 6 8 7 0 1 2 3 0 6 7 4 6	1 1 1 5 5 5 5 5 5 5 5 5 6 5 6 9 0 1 4 4 4 4 5 5 5 1 0 6 1 4 8 0 3 4 5 0 2 4
Site number	1 1 1 1 1 1 1 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6	1 1 1 1 1 1 1 1 1 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8	1 1 1 1 1 2 3 4 5 6 7 8 9 0 1 2 3
Consensus	A C C G C A C T G A T C G C C T	G T C T T G T C C T G T C C T C T C	C C C T C T A T A G A C G
Site type ^a	t t t t t t t t t t v v t v t	v t t v t v t t t v v t t t t t	t t t t v t v v t v v v
Character type ^b	i i - - i - i - i - i - i	- i - - i i - i - - - - -	- - - - - - - - i i -
H1 (41) ^c	... T ... A . C . . T A .	H1 (38) . C . . C . . T	H1 (97) . . . . .
H2 (01)	G T . . . G . . . G . G T . . C	H2 (01) T . . . . C . T	H2 (02) T T T C A C . . . . .
H3 (01)	G T . A . G T . . G . G T . . C	H3 (41) . . . . . C . T	H3 (01) . . . . . C G T A . . .
H4 (05)	.. T . . . . C	H4 (27) . . . . . C	H4 (03) . . . . . C A .
H5 (63)	..... C	H5 (02) . C T A . . C . . . . T C T C T	H5 (01) . . . . . C A T
		H6 (01) . C . . C . . T G T C A	

^a t: Transitions and v: transversions.

^b i: Informative sites and -: uninformative sites.

^c H: haplotype.

Estimating evolutionary mechanisms parameters. No recombinant sequences or recombination breakpoints in the ITS region and in both *Gpd* and *Alt a 1* genes, either individually or in the concatenated sequences, were detected in compatibility matrices and analyses implemented in the RDP2 program (data not shown). Overall, most neutrality tests were not significant. However, the null hypothesis of Tajima's D test was rejected for the *Alt a 1* when analyzing both potato and tomato subpopulations (Table 5). For the potato population, the D value was positive, which suggests the occurrence of balancing selection. For the tomato subpopulation, D value was negative, suggesting that population size expansion or purifying selection could have occurred.

There was evidence of population subdivision according to the host plant. For *Alt a 1* and *Gpd*, the Kst values were 0.34 ($P = 0.03$) and 0.26 ($P = 0.01$), respectively. No significant population subdivision was detected when analyzing ITS sequences (Kst = 0.001, $P = 0.98$).

Migration rates and divergence times were assessed using *Alt a 1* and *Gpd* loci individually or concatenated (*Alt a 1* and *Gpd*) because no subdivision was detected when analyzing the ITS region. There is evidence of gene flow between subpopulations of *A. solani* from potato and tomato plants for both *Alt a 1* and *Gpd*. The migration rate was estimated to be approximately one migrant exchanged for each generation between potato and tomato subpopulations (Fig. 2A, C, and E). The migration rates estimated using MDIV and MIGRATE tests were similar (data not shown). MDIV results are consistent with an isolation model with moderate migration and long divergence times (Fig. 2B, D, and F).

In the genealogy of the *Alt a 1* gene, 16 mutations were associated with five haplotypes of *A. solani* (Fig. 3). Haplotypes H4 and H5 comprised mainly tomato isolates, with three mutations showing the highest likelihood of arising in this host in their evolutionary history. Most potato isolates belonged to haplotype H1, in which eight mutations were more likely associated with potato plants. Haplotypes H2 and H3 were both isolated from tomato and represented once in the sample.

The coalescent-rooted genealogy inferred for the *Gpd* locus was similar to *Alt a 1*. Six haplotypes were inferred based on 18 mutations (Fig. 3). Haplotypes H2, H3, H4, and H5 were predominantly tomato isolates whereas haplotypes H1 and H6 were potato isolates. In all, 5 mutations were mapped in haplotypes exclusively associated with tomato (H2 and H5) whereas, for haplotypes associated mainly with potato plants, 6 and 10 mutations were mapped for the H1 and H6, respectively (Fig. 3).

For the ITS region, 13 mutations were available for genealogical inference (Fig. 3). However, it was not possible to establish a clear relationship between haplotype mutations and host of origin. Most isolates belonged to haplotype H1 ($n = 97$). Haplotypes H2 and H4 were assigned to two and three isolates, respectively, and each of the other two haplotypes were assigned to a single isolate.

Population subdivision was detected for *Alt a 1* and *Gpd* genes, and these loci were concatenated to increase the resolution of the genealogical tree (Fig. 3). A total of 25 mutations defined seven haplotypes. Haplotypes H1, H2, H3, H4, and H5 were composed mainly of tomato isolates. Haplotypes H6 and H7 were predominantly potato isolates. In all, 9 mutations were mapped in haplotypes associated with potato plants whereas 16 mutations were simulated for haplotypes associated mainly with tomato plants (Fig. 3).

DISCUSSION

This is one of the first molecular population genetics studies applying genealogical approaches based on the coalescent theory to estimate population processes of any species of the genus *Alternaria*. Also, no characterization of molecular diversity has been yet conducted using multilocus sequence typing data (MLST) in *A. solani*. Using the approaches described above, there is evidence of genetic diversity and differentiation between the potato and tomato populations of *A. solani* in Brazil, although the number of fixed mutations in each population is not high. Higher levels of polymorphism were expected to be found considering previously published reports of high morphological, physiological, pathogenic, and genetic variability in *A. solani* popula-

TABLE 4. Analysis of molecular variance using Kimura 2-parameter distance for three loci (*Alt a 1*, *Gpd*, and internal transcribed spacer [ITS]) and a concatenated data set (*Alt a 1* + *Gpd* + ITS) of *Alternaria solani* subpopulations sampled from potato and tomato plants

Locus, source of variation	df	Sum of squares	Component of variance	Variation (%)	Φ_{st} (P value)
<i>Alt a 1</i>					
Between subpopulations	1	62.06	1.10	50.78	...
Within subpopulations	109	116.19	1.07	49.22	...
Total	110	178.25	2.17	...	0.51 (<0.0001)
<i>Gpd</i>					
Between subpopulations	1	39.45	0.70	40.67	...
Within subpopulations	108	110.11	1.02	59.33	...
Total	109	149.56	1.72	...	0.41 (<0.0001)
ITS					
Between subpopulations	1	0.29	0.00008	0.03	...
Within subpopulations	102	29.22	0.2865	99.97	...
Total	103	29.51	0.2866	...	0.00029 (0.55)
Concatenated					
Between subpopulations	1	72.41	1.60	42.41	...
Within subpopulations	86	186.78	2.17	57.59	...
Total	87	259.19	3.77	...	0.42 (0.0001)

TABLE 5. Neutrality tests using Tajima's D, Fu and Li's D*, Fu and Li's F*, and Fu's F methods in *Alt a 1* and *Gpd* sequences from *Alternaria solani* isolates^a

Population	Locus	Tajima's D	Fu and Li's D*	Fu and Li's F*	Fu's F statistic
Total	<i>Alt a 1</i>	0.1579 (NS)	0.5610 (NS)	0.4910 (NS)	6.337 (NS)
	<i>Gpd</i>	-0.5697 (NS)	-0.7603 (NS)	-0.8233 (NS)	3.853 (NS)
Potato	<i>Alt a 1</i>	2.0931 ($P < 0.05$)	0.4350 (NS)	1.1421 (NS)	7.055 (NS)
	<i>Gpd</i>	0.5551 (NS)	-1.3546 (NS)	-0.8550 (NS)	4.264 (NS)
Tomato	<i>Alt a 1</i>	-1.8139 ($P < 0.05$)	0.7065 (NS)	-0.1897 (NS)	1.008 (NS)
	<i>Gpd</i>	-1.3598 (NS)	1.0514 (NS)	0.2735 (NS)	1.544 (NS)

^a NS = nonsignificant.

tions. For Brazilian populations in previous studies based on phenotypic and molecular markers (21,62), there was high variability and evidence of population subdivision. However, the number of isolates assessed was small and the analyses were of low resolution. In other countries, *A. solani* populations have been described as highly variable by assessing genetic variation through random amplified polymorphism DNA (RAPD), random amplified microsatellites, and amplified fragment length polymorphism (AFLP) markers (37,42,71,72). Although these molecular phenotypic markers are advantageous for genotyping and provide a snapshot of genome-wide variation, they are not appropriate for reconstructing patterns of descent in genealogical analyses (17). The MLST approach is more suitable to study long-time evolutionary history of populations whereas molecular fingerprints are more adequate to detect recent genetic changes (43). Differences in the rate of evolution of gene sequences and restriction sites may have contributed to the apparently discordant inferences.

Mutation is the main evolutionary mechanism that generates polymorphisms, and its implications to disease management are noticeable (2,28,34). For *A. solani*, point mutations in the sequence of the *cyt b* gene resulted in loss of sensitivity to the fungicide azoxystrobin (25,49). Furthermore, new alleles introduced in the population increase the chances of breakdown of resistance genes in either potato or tomato cultivars. The relatively low mutation rate estimated for the three loci does not necessarily indicate that the population as a whole had low mutation rate. After analyzing 91 housekeeping genes, estimates

of population parameters varied according to the gene under study (50). The evolution of other genes or genomic regions should be quantified for more precise estimates of the mutation rate in populations of *A. solani*.

Although population subdivision according to the host of origin was detected when both *Alt a 1* and *Gpd* were analyzed, the number of mutations that were estimated and mapped in the genealogies of these sequences was not high enough to solve the question of a putative new species causing early blight. To further investigate the host-association issue, haplotypes comprising only one or two isolates were removed from the coalescent analyses to map mutations exclusively associated with host species. For the tomato and potato subpopulations, the estimated number of mutations in the *Alt a 1* and *Gpd* genes was three and four, and four and two, respectively (data not shown). Due to a relatively low number of mutations, a putative ancestral host-specific lineage could not be safely inferred. However, there is evidence of host-associated subpopulations of *A. solani*, as reported in other studies (42,51,62,72). The use of molecular markers distributed in more than one region of the genome would have facilitated the detection of polymorphisms associated with host crops in these studies. Additional genes are being examined to clarify this issue.

Polymorphisms in the ITS region were not associated with potato or tomato. The DNA sequencing variation in the ITS region has been used in molecular systematic studies of *Alternaria* spp. (55). The degree of nucleotide difference in the ITS region in the porri species-group, which includes *A. solani*, is low (55).

Fig. 2. A, C, and E, Migration and B, D, and F, divergence time posterior probability distributions between *Alternaria solani* populations on potato and tomato plants estimated for A and B, *Alt a 1* and C and D, *Gpd* individually or for E and F, a concatenated data set. The data were simulated under the infinite sites model: 2 million steps in the chain for the estimation of the posterior distribution and an initial 500,000 steps to ensure that genealogies were simulated before approximating the posterior distribution. Five independent replicates using different starting random seed numbers were simulated under the same model and parameters. The results were similar between different runs. Likelihood function for migration $f(M|X)$ and time of divergence $f(T|X)$ are presented in the Y axes and the estimated migration and divergence time measured in $2N$ generations in the x axis.

One possible reason is the action of concerted evolution in this region and the homogenizing effect it causes (24). Thus, little or no variation is expected within populations associated with host species or closely related *Alternaria* spp. when analyzing this genomic region.

Isolates from geographically distant fields were not more genetically divergent. The Brazilian population of *A. solani* comprises haplotypes widely distributed throughout the main producing regions of potato and tomato. A clonal population structure is strengthened because few haplotypes are widely distributed (3,4,13,47,57). In several Brazilian regions, potato and tomato are grown all year round and, in some cases, in contiguous fields (8). The detection of shared haplotypes between potato and tomato subpopulations suggests the occurrence of migration. The migra-

tion rates estimated using coalescent simulations between potato and tomato subpopulations were enough to prevent geographic isolation and accentuated genetic separation. Conidia of *A. solani* produced in diseased tissues can be dispersed by wind and rainfall (7), and long-distance dispersal is facilitated by the movement of diseased tomato fruit and potato tubers. However, given trade market characteristics, long-distance pathogen dispersal is not very likely, because diseased plant material is eliminated and growers do not regularly plant seed from either diseased tomato fruit or diseased potato tubers. Therefore, aerial dispersal and widespread distribution of host crops are likely to be the major factors that lead to intense inoculum movement and the lack of geographic subdivision regarding inferred haplotypes. Additionally, mutations shared between populations might be due to the

Fig. 3. Coalescent-based gene genealogies inferred using GENETREE, showing the distribution of mutations in the internal transcribed spacer (ITS) region and *Alt a 1* and *Gpd* genes (individually or concatenated) from *Alternaria solani*. Rooted genealogies were inferred based on five different starting random seed numbers assuming population subdivision by host (potato and tomato plants) with equal population size and symmetrical migration rate (one migrant exchanged for each generation between potato and tomato populations) estimated using migration with division and MIGRATE tests. One million runs of the coalescent were performed for *Alt a 1* and *Gpd* loci individually. Ten million runs of the coalescent were carried out for the concatenated (*Alt a 1* + *Gpd*). For the ITS region, the rooted genealogy was inferred based on five different starting random seed numbers, with 1 million simulations of the coalescent for each run assuming equal sample size and nonpopulation subdivision. The results were similar for each run. The time scale is in coalescent units of effective population size. The direction of divergence is from the top of the genealogy (past) to the bottom (present). Solid circles represent the distribution of the mutations in the genealogy for *Alt a 1* and *Gpd* genes (individually and concatenated) and the ITS region. The numbers below the gene genealogies designate each distinct haplotype and its frequency associated with potato (P) and tomato (T) plants.

distribution of ancestral polymorphisms associated with a clonal population of *A. solani*. Recently, ancestral polymorphisms were demonstrated to be associated with the genetic differences between *Neurospora* spp. (53). Additional studies to assess other genomic regions of *A. solani* with ancestral polymorphisms associated with genetic differentiation between populations are needed.

Evidence of selection was detected in the partial sequence of the *Alt a 1* gene. For the potato subpopulation, the occurrence of balancing selection can be attributed to the presence of intermediate-frequency variants (66), as expected under a model of population subdivision within this group. Because some isolates were from contiguous potato and tomato fields, it is possible that isolates obtained from potato plants were originated from a tomato field infected with an adapted lineage to this host. For instance, *A. solani* isolates were collected from different hosts in the same region in Minas Gerais State (Table 1) and had the same haplotype (H5) in the *Alt a 1* gene tree (Fig. 2). For the tomato subpopulation, the presence of haplotypes H2 and H3, which are rare variants, influenced the neutrality tests and purifying selection was detected. When these haplotypes were removed, background selection was inferred in the subtomato population (data not shown). However, H2 and H3 were kept in the analysis because they have been evolutionarily separated from all other haplotypes.

The population size expansion may also have contributed to the significance of the neutrality test for the *Alt a 1* gene in the tomato subpopulation. This expansion cannot be completely ruled out mostly due to three aspects. (i) It is likely that the populations of *A. solani* were recently established in Brazil, but there is no reliable information on when the pathogen was introduced. (ii) Possible population expansion events could have occurred after the introduction of the pathogen in Brazil and also due to the recent establishment of production fields in new geographic areas. Early blight epidemics in Brazil were already reported in 1898 (52), and new planting areas of both hosts started to be established in the last 20 years. Traditionally, potato and tomato crops have been cultivated mainly in the south and southeast of Brazil. The new producing areas were established in the northeast and central-west regions, and population expansion could have occurred. Nevertheless, these two events can be considered recent in evolutionary grounds. (iii) Finally, potato tubers are often imported from Chile, Argentina, Canada, the United States, Netherlands, France, and Germany; therefore, a recent genotype could have been introduced. However, given the high quality of seed tubers Brazilian growers import, the likelihood of a tuber-borne mediated introduction is low. Therefore, the most likely factor associated with the occurrence of selection in the population is an incipient host specialization process, mainly when assessing the pattern of genetic subdivision based on the *Alt a 1* gene. There are reports that this gene can be involved in the pathogenicity of *Alternaria* spp. to host plants but no experimental data are still available (18). The existence of more than one species of *Alternaria* seems to be the most likely reason for the significant neutrality test.

A distinct species of *Alternaria*, *A. tomatophila*, is associated with early blight in tomato in several countries but it has not yet been reported in Brazil. *A. tomatophila* has been detected in the United States, Australia, New Zealand, and Venezuela (63). Interestingly, in these countries, *A. tomatophila* causes early blight in tomato and *A. solani* is associated with the disease in potato. In addition to differences in morphological variables, there are physiological differences between these species, such as the profile of host-specific metabolites detected in high-performance liquid chromatography analysis (1). However, the function and chemical structure of these compounds are unknown. In the present study, the haplotype association with host species can be evidence of an ongoing speciation process. Furthermore, isolates in haplotypes H2 and H3 (*Alt a 1*) and H5 (*Gpd*) appear as ancient

tomato lineages that could be a cryptic species. Further analyses of morphological characters and fitness components of these haplotypes are currently being conducted to identify the putative species of *Alternaria* associated with tomato and potato early blight in Brazil.

Genetic analyses that provide inferences on the frequency of recombination can also provide indirect evidence of sexual structures and, thus, contribute significantly to understand the reproductive biology of a pathogen. Identifying sexual structures of many plant-pathogenic fungi is laborious and has a low success rate (67). For presumably asexual fungi, such as *A. solani*, the search for sexual structures, if any, should be conducted using different substrates and edaphoclimatic conditions. There was no strong evidence of recombination in the *A. solani* population, and its structure is typically clonal. The continuous availability of host crops and conducive environmental conditions would favor asexual reproduction.

The sexual stage reported in some *Alternaria* spp. is commonly classified as *Lewia* spp.; however, sexual structures are seldom formed (68). Evidence of recombination was reported in populations of *A. brassicicola* but no sexual structures were found (12). These authors used genetic analyses with lower resolution than the ones used in the present study. Several techniques were used to increase the chance of detecting recombination but, overall, there was very little conflict in the data. Furthermore, evidence of recombination in *A. solani* was not detected in other studies using molecular markers. Therefore, it is reasonable to conclude that no recombination occurred and that asexual reproduction is the main process of spore production in *A. solani* populations. Epidemiologically, conidia are widely distributed and are the main type of inoculum for early blight epidemics in both potato and tomato fields.

Based on the genomic regions analyzed in the present study, two genetically distinct groups of isolates could be detected and they may correspond to either host-specific populations or even different phylogenetic species. This argument is supported by the results and also by the data from the analyses of RAPD and AFLP markers, done with the same set of isolates used in the present study (V. Lourenço, Jr., and E. S. G. Mizubuti, *unpublished data*). Other genomic regions are being currently investigated, and preliminary results also support this claim (59). Additionally, the sequencing and identification of genomic regions associated with aggressiveness on host species and adaptation to ecologic niches are also being assessed. This will help to elucidate important biological aspects of this plant pathogen and their relationships with early blight epidemics on potato and tomato plants.

ACKNOWLEDGMENTS

This research was partially funded by FAPEMIG. We thank A. Reis for providing several isolates used in this study; R. B. Martins for preparing Figure 1; and CAPES and CNPq for providing fellowships to V. Lourenço, Jr., E. S. G. Mizubuti, and L. A. Maffia during this study.

LITERATURE CITED

1. Andersen, B., Dongo, A., and Pryor, B. M. 2008. Secondary metabolite profiling of *Alternaria dauci*, *A. porri*, *A. solani*, and *A. tomatophila*. *Mycol. Res.* 112:241-250.
2. Anderson, J. B. 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat. Rev. Microbiol.* 3:547-556.
3. Anderson, J. B., and Kohn, L. M. 1995. Clonality in soilborne, plant pathogenic fungi. *Annu. Rev. Phytopathol.* 33:369-391.
4. Avila-Adame, C., Gomez-Alpizar, L., Zismann, V., Jones, K. M., Buell, C. R., and Ristaino, J. B. 2006. Mitochondrial genome sequences and molecular evolution of the Irish potato famine pathogen, *Phytophthora infestans*. *Curr. Genet.* 49:39-46.
5. Aylor, D. L., Price, E. W., and Carbone, I. 2006. SNAP: Combine and Map modules for multilocus population genetic analysis. *Bioinformatics* 22:1399-1401.

6. Bahlo, M., and Griffiths, R. C. 2000. Inference from gene trees in a subdivided population. *Theor. Popul. Biol.* 57:79-95.
7. Batista, D. C. 2006. Dinâmica de inóculo de *Alternaria solani*, efeito da densidade de plantio na intensidade da pinta preta e requeima e previsão dessas doenças em tomateiro e batateira. Ph.D. thesis, Universidade Federal de Viçosa, Minas Gerais, Brazil.
8. Batista, D. C., Lima, M. A., Haddad, F., Maffia, L. A., and Mizubuti, E. S. G. 2006. Validation of decision support systems for tomato early blight and potato late blight, under Brazilian conditions. *Crop Prot.* 25:664-670.
9. Beerli, P., and Felsenstein, J. 2001. Maximum likelihood estimation of a migration matrix and effective population sizes in subpopulations by using a coalescent approach. *Proc. Natl. Acad. Sci. USA* 98:4563-4568.
10. Berbee, M. L., Payne, B. P., Zhang, G., Roberts, R. G., and Turgeon, B. G. 2003. Shared ITS DNA substitutions in isolates of opposite mating type reveal a recombining history for three presumed asexual species in the filamentous ascomycete genus *Alternaria*. *Mycol. Res.* 107:169-182.
11. Berbee, M. L., Pirseyedi, M., and Hubbard, S. 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* 91:964-977.
12. Bock, C. H., Thrall, P. H., and Burdon, J. J. 2005. Genetic structure of populations of *Alternaria brassicicola* suggests the occurrence of sexual recombination. *Mycol. Res.* 109:227-236.
13. Bolton, M. D., Thomma, B. P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum* (Lib.) De Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* 7:1-16.
14. Bonde, R. 1929. Physiological strains of *Alternaria solani*. *Phytopathology* 19:533-548.
15. Bóveda, R. R. V. 1986. Morfologia, patogenicidade, esporulação e sensibilidade a fungicidas "in vitro" de *Alternaria solani* (Ellis & Martin) Jones & Grout e *Alternaria alternata* (Fr) Keissler de solanáceas. Ph.D. thesis, Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, Brazil.
16. Bowden, L. C., Price, E. W., and Carbone, I. 2008. SNAP Clade and Matrix, Version2. Distributed over the Internet, <http://www.cals.ncsu.edu/plantpath/faculty/carbone/home.html>, Department of Plant Pathology, North Carolina State University.
17. Carbone, I., Anderson, J. B., and Kohn, L. M. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. *Evolution* 53:11-21.
18. Cramer, R. A., and Lawrence, C. B. 2003. Cloning of a gene encoding an *Alt a 1* isoallergen differentially expressed by the necrotrophic fungus *Alternaria brassicicola*. *Appl. Environ. Microbiol.* 69:2361-2364.
19. Dhingra, O. D., and Sinclair, J. B. 1995. *Basic Plant Pathology Methods*, second ed. CRC Lewis, Boca Raton, FL.
20. Excoffier, L., Laval, G., and Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinf. Online* 1:47-50.
21. Fancelli, M. I. 1991. Comparação patogênica, cultural, serológica e eletroforética entre isolados de *Alternaria solani* do tomate e da batata e variabilidade patogênica de *A. solani* f. sp. *lycopersici* N. F. Ph.D. thesis, Escola Superior de Agronomia "Luiz de Queiroz", Piracicaba, Brazil.
22. Fu, Y. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147:915-925.
23. Fu, Y., and Li, W. 1993. Statistical tests of neutrality of mutations. *Genetics* 133:693-709.
24. Ganley, A. R. D., and Kobayashi, T. 2007. Highly efficient concerted evolution in the ribosomal DNA repeats: Total rDNA repeat variation revealed by whole-genome shotgun sequence data. *Genome Res.* 17:184-191.
25. Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manage. Sci.* 62:465-472.
26. Griffiths, R. C., and Tavaré, S. 1994. Ancestral inference in population genetics. *Stat. Sci.* 9:307-319.
27. Grishkan, I., Beharav, A., Kirzhener, V., and Nevo, E. 2007. Adaptive spatiotemporal distribution of soil microfungi in 'Evolution Canyon' III, Nahal Shaharut, extreme southern Negev desert, Israel. *Biol. J. Linn. Soc. Lond.* 90:263-277.
28. Hartl, D. L., and Clark, A. G. 2007. *Principles of Population Genetics*. Sinauer Associated, Sunderland, MA.
29. Hong, S. G., Cramer, R. A., Lawrence, C. B., and Pryor, B. M. 2005. *Alt a 1* allergen homologs from *Alternaria* and related taxa: Analysis of phylogenetic content and secondary structure. *Fungal Genet. Biol.* 42:119-129.
30. Hudson, R. R., Boos, D. D., and Kaplan, N. L. 1992. A statistical test for detecting geographic subdivision. *Mol. Biol. Evol.* 9:138-151.
31. Hudson, R. R., Slatkin, M., and Maddison, W. P. 1992. Estimation of levels of gene flow from DNA sequence data. *Genetics* 132:583-589.
32. IBGE 2005. Produção Agrícola Municipal. Sistema IBGE de Recuperação Automática—SIDRA. www.sidra.ibge.gov.br
33. Jakobsen, I. B., Wilson, S. R., and Easteal, S. 1997. The partition matrix: Exploring variable phylogenetic signals along nucleotide sequence alignments. *Mol. Biol. Evol.* 14:474-484.
34. Jolley, K. A., Wilson, D. J., Kriz, P., McVean, G., and Maiden, M. C. J. 2005. The influence of mutation, recombination, population history, and selection on patterns of genetic diversity in *Neisseria meningitidis*. *Mol. Biol. Evol.* 22:562-569.
35. Karasawa, M., Rodrigues, R., Sudré, C. P., Silva, M. P., Riva, E. M., and Amaral Júnior, A. T. 2005. Aplicação de métodos de agrupamento na quantificação da divergência genética entre acessos de tomateiro. *Hortic. Bras.* 23:1000-1005.
36. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-112.
37. Kumar, V., Haldar, S., Pandey, K. K., Singh, R. P., Singh, A. K., and Singh, P. C. 2008. Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. *World J. Microbiol. Biotechnol.* 24:1003-1009.
38. MacDonald, E., Millward, L., Ravishanker, J. P., and Money, N. P. 2002. Biomechanical interaction between hyphae of two *Pythium* species (Oomycota) and host tissues. *Fungal Genet. Biol.* 37:245-249.
39. Malosso, E., Waite, I. S., English, L., Hopkins, D. W., and O'Donnell, A. G. 2006. Fungal diversity in maritime Antarctic soils determined using a combination of culture isolation, molecular fingerprinting and cloning techniques. *Polar Biol.* 29:552-561.
40. Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27:209-220.
41. Martin, D. P., Williamson, C., and Posada, D. 2005. RDP2: Recombination detection and analysis from sequence alignments. *Bioinformatics* 21:260-262.
42. Martínez, S. P., Snowdon, R., and Pons-Kühnemann, J. 2004. Variability of Cuban and international populations of *Alternaria solani* from different hosts and localities: AFLP genetic analysis. *Eur. J. Plant Pathol.* 110:399-409.
43. Melles, D. C., van Leeuwen, W. B., Snijders, S. V., Horst-Kreft, D., Peeters, J. K., Verbrugh, H. A., and van Belkum, A. 2007. Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. *J. Microbiol. Methods* 69:371-375.
44. Milgroom, M. G., and Fry, W. E., 1997. Contributions of population genetics to plant disease management. *Adv. Bot. Res.* 24:1-30.
45. Milgroom, M. G., and Peever, T. L. 2003. Population biology of plant pathogens. The synthesis of plant disease epidemiology and population genetics. *Plant Dis.* 87:608-617.
46. Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Res.* 8:4321-4325.
47. Nebavi, F., Ayala, F. J., Renaud, F., Bertout, S., Eholie, S., Moussa, K., Mallie, M., and de Meeus, T. 2006. Clonal population structure and genetic diversity of *Candida albicans* in AIDS patients from Abidjan (Cote d'Ivoire). *Proc. Natl. Acad. Sci. USA* 103:3663-3668.
48. Nielsen, R., and Wakeley, J. 2001. Distinguishing migration from isolation: a Markov chain Monte Carlo approach. *Genetics* 158:885-896.
49. Pasche, J. S. M., Piche, L., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis.* 89:269-278.
50. Perez-Lozada, M., Browne, E. B., Madsen, A., Wirth, T., Viscidi, R., and Crandall, K. A. 2006. Population genetics of microbial pathogen estimated from multilocus sequence typing (MLST) data. *Infect. Genet. Evol.* 6:97-112.
51. Petrunak, D. M., and Christ, B. J. 1992. Isozyme variability in *Alternaria solani* and *A. alternata*. *Phytopathology* 82:1343-1347.
52. Potel, H. 1898. As moléstias cryptogâmicas da batataINGLEZA (*Solanum tuberosum*) e seu tratamento. *Bol. Inst. Agron.* 10:795-799.
53. Powell, A. J., Jacobson, D. J., and Natvig, D. O. 2007. Ancestral polymorphism and linkage disequilibrium at the het-6 region in pseudo-homothallic *Neurospora tetrasperma*. *Fungal Genet. Biol.* 44:896-904.
54. Price, E. W., and Carbone, I. 2005. SNAP: Workbench management tool for evolutionary population genetic analysis. *Bioinformatics* 21:402-404.
55. Pryor, B., and Gilbertson, R. 2000. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycol. Res.* 11:1312-1321.
56. Pscheidt, J. W., and Stevenson, W. R. 1986. Early Blight of Potato and Tomato: A Literature Review. College of Agriculture & Life Sciences, Madison, WI.
57. Reis, A., Smart, C. D., Fry, W. E., Maffia, L. A., and Mizubuti, E. S. G. 2003. Characterization of *Phytophthora infestans* isolates from Southern and Southeastern Brazil from 1998 to 2000. *Plant Dis.* 87:896-900.
58. Rocha, B. H. G., Augustin, E., Silva, J. B., and Viégas, J. 2001. Isoenzymatic variability in wild potatoes. *Pesqui. Agropecu. Bras.* 36:781-791.
59. Rodrigues, T. T. M. S., Lourenço, V., Jr., Silva, M. B., Mary, B., and Mizubuti, E. S. G. 2008. Mycelial compatibility, AFLP marker and

- mating type genes suggest that Brazilian population of *Alternaria solani* is clonal. MSA Annual Meeting. Abstract online.
60. Rotem, J. 1994. The Genus *Alternaria*. Biology, Epidemiology, and Pathogenicity. American Phytopathological Society Press, St. Paul, MN.
  61. Rozas, J., Sánchez-DelBarrio, J. C., Messeguer, X., and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496-2497.
  62. Scheuermann, K. K., Falleiro, B. A. S., Reis, A., Brommonschenkel, S. H., and Mizubuti, E. S. G. 2004. Seleção de marcadores RAPD para estudo de diversidade genética de *Alternaria solani*. (Abstr.) *Fitopatol. Bras.* 29:49-50.
  63. Simmons, E. G. 2000. *Alternaria* themes and variations (244-286) species on Solanaceae. *Mycotaxon* 75:1-115.
  64. Simmons, E. G. 2007. *Alternaria: An Identification Manual*. CBS Fungal Biodiversity Centre.
  65. Staden, R. 1996. The staden sequence analysis package. *Mol. Biotechnol.* 5:233-241.
  66. Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585-595.
  67. Taylor, J., Jacobson, D., and Fisher, M. 1999. The evolution of asexual fungi: Reproduction, speciation and classification. *Annu. Rev. Phytopathol.* 37:197-246.
  68. Thomma, B. P. H. J. 2003. *Alternaria* spp.: From general saprophyte to specific parasite. *Mol. Plant Pathol.* 4:225-236.
  69. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res.* 22:4673-4680.
  70. Vale, F. X. R., Zambolim, L., Chaves, G. M., and Correia, L. G. 1992. Avaliação fitossanitária da cultura do tomateiro em regiões produtoras de Minas Gerais e Espírito Santo. *Fitopatol. Bras.* 17:211.
  71. van der Waals, J. E., Korsten, L., and Slippers, B. 2004. Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. *Plant Dis.* 88:959-964.
  72. Weir, T. L., Huff, D. R., Christ, B. J., and Romaine, C. P. 1998. RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. *Mycologia* 90:813-821.
  73. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages: 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.