VALDIR LOURENÇO JÚNIOR

MOLECULAR POPULATION GENETICS of *Alternaria solani* IN BRAZIL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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BIOGRAFIA

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RESUMO

LOURENÇO JÚNIOR, Valdir, D.Sc., Universidade Federal de Viçosa, março de 2008. **Genética molecular de populações de *Alternaria solani* no Brasil.** Orientador: Eduardo Seiti Gomide Mizubuti. Co-orientadores: Luiz Antonio dos Santos Dias e Luiz Antonio Maffia.

A determinação da estrutura genética da população de *Alternaria solani* é um componente importante para estudos de epidemiologia molecular da pinta preta da batateira (Ba) e tomateiro (To). Coletaram-se isolados de AS nas regiões Nordeste, Centro-Oeste, Sudeste e Sul do Brasil. Inicialmente, utilizaram-se os marcadores AFLP e RAPD para estimar a diversidade genética. Obtiveram-se 123 haplótipos com 62 locos, a partir da combinação de marcadores AFLP e RAPD (92% polimorfismo). A frequência de alelos nas duas populações de AS (em Ba e To) variou de 0,015 (RAPD45) a 1,000 (RAPD47, RAPD56, RAPD57, RAPD59, RAPD61, RAPD62). A estimativa teta (Θ) e o valor médio de Gst foram 0,29 (P= 0,001) e 0,18, respectivamente. No entanto, houve maior variação genética dentro (70,6%) do que entre (29,4%) populações. Na análise de agrupamento, detectaram-se haplótipos associados com Ba. A diversidade gênica (h) da população AS foi 0,36 (± 0,38). Os valores da diversidade genotípica quantificados pelos índices de Shannon-Wiener (*H'*) e Stoddart & Taylor's *G* para as populações Ba e To foram, 1,01 e 1,00, 0,99 e 0,98, respectivamente. Não houve evidência de recombinação (I_A = 7,25, P<0,001). Os mecanismos evolutivos foram parametrizados a partir de análises de genealogia de genes por processos coalescentes. Utilizaram-se seqüências parciais dos genes *Alt a 1* (471 pb) e gliceraldeído-3-fosfato desidrogenase (*Gpd*) (584 pb), e a região dos espaçador interno transcrito do rDNA (ITS) (578 pb) para estimar a diversidade molecular e análises de coalescência. Seqüências de *Alt a 1*, *Gpd* (584 bp) e ITS foram obtidas para 111, 110 e 104 isolados, respectivamente. Maior número de sítios parcimônios informativos (14), diversidade de nucleotídeos (0,007) e número médio de diferenças de nucleotídeos (3,202) foi registrado para *Alt a 1*. Apesar de o maior número de haplótipos (7) ter sido registrado para ITS, a diversidade de haplótipos (0,15) foi a mais baixa. Detectou-se diferenciação genética entre populações AS na análise do loco *Alt a 1*. Eventos de recombinação não foram

detectados. Não há evidência de subdivisão geográfica, e o fluxo gênico está ocorrendo entre populações de AS de Ba e To. Conduziram-se análises filogenéticas a partir das seqüências *Alt a 1, Gpd*, e ITS. Além disso, o marcador RAPD foi combinado com as seqüências *Alt a 1* e *Gpd* em análises de parcimônia. Detectou-se associação de AS com Ba e To nas análises dos locos *Alt a 1* e *Gpd* com valores significativos de bootstrap (>70) e de probabilidade Bayesiana *a posteriori* (>95). Apesar da incongruência entre o conjunto de dados concatenados (*Alt a 1*, *Gpd* e RAPD) (P=0,01), estimaram-se valores positivos de Suporte de Bremer para um nó que agrupou linhagens AS associadas com Ba e To. Concluiu-se haver evidências de linhagens clonais do fitopatógeno associadas a plantas hospedeiras, e que o fluxo gênico e a mutação são os principais mecanismos evolutivos que atuam nas populações de *A. solani* no Brasil.

ABSTRACT

LOURENÇO JÚNIOR, Valdir, D.Sc., Universidade Federal de Viçosa, March, 2008. **Molecular population genetics of *Alternaria solani* in Brazil.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-advisers: Luiz Antonio dos Santos Dias and Luiz Antonio Maffia.

The analysis of the genetic structure of the population of *Alternaria solani* is an important component of the molecular epidemiology potato (Po) and tomato (To) early blight. Isolates were sampled from the Northeast, Central-West, Southeast, and South regions of Brazil. AFLP and RAPD markers were used to estimate genetic diversity. A multilocus combined dataset using AFLP and RAPD matrices generated 123 AS haplotypes with 62 loci (92% polymorphism). The allele frequency for AS populations from To and Po ranged from 0.015 (RAPD45) to 1.000 (RAPD47, RAPD56, RAPD57, RAPD59, RAPD61, RAPD62). The theta estimate (Θ) and the Gst average values were 0.29 (P= 0.001), and 0.18, respectively. However, there was greater genetic variation within (70.6%) than between (29.4%) AS populations. There was evidence of AS haplotypes associated with Po. High gene diversity was detected over all AS population (h=0.36, S.D. ± 0.38). The genotypic diversity measured by both Shannon-Wiener (H') and Stoddart & Taylor's G for Po and To populations were 1.01 and 1.00, and 0.99 and 0.98, respectively. There was no evidence of recombination (I_A = 7.24728, $P<0.001$). In addition, gene genealogies based on the coalescent process were used to infer about evolutionary mechanisms. Sequences of the *Alt a 1* and glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) genes, and rDNA internal transcribed spacer region (ITS) were used to assess molecular diversity and for the coalescent analyses. Sequences from 111, 110, and 104 isolates were obtained for the *Alt a 1* (471 bp) and *Gpd* (584 bp) genes, and for the ITS region (578 bp), respectively. The highest number of parsimony informative sites (14), nucleotide diversity (0.007) and the average number of nucleotide differences (3.202) were obtained for *Alt a 1*. Although the highest number of haplotypes (7) was generated for ITS, haplotype diversity was the lowest (0.15) for this region. The

genetic differentiation between AS populations was detected for *Alt a 1* sequences. Recombination was not detected, however, subdivision and recent divergence time were inferred from populations associated with hosts. There is no evidence of geographic subdivision, and gene flow is occurring among AS populations. Phylogenetic analyses were conducted using *Alt a 1, Gpd*, and ITS sequences. In addition, RAPD marker was combined with *Alt a 1* and *Gpd* sequences for parsimony analysis. Association of AS with Po and To was detected when analyzing *Alt a 1* and *Gpd* loci with significant bootstrap (>70) and Bayesian Posterior Probability (>95) values. Despite significant incongruence for the combined dataset (P=0.01), positive values of Partitioned Bremer Support were obtained for a node which clustered AS lineages associated with potato and tomato plants. Thus, there was evidence of pathogen clonal lineages associated with hosts. Gene flow and mutation are the main evolutionary mechanisms affecting AS population.

I. General introduction

Tomato and potato, the two most important vegetable crops in Brazil, are threatened by various diseases. Early blight, caused by *Alternaria solani*, is one of the most destructive foliar diseases in both crops and significant loss can occur due to early blight epidemics. Consequently, many and frequent fungicide sprays are used in both tomato or potato crops. Reducing fungicide usage in these crops through effective management strategies is the major objective of a research program we started a few years ago.

Effective plant disease control is achieved only when management strategies are established on an epidemiological basis. Plant disease epidemics result from interaction of populations: population of host interacts with population of pathogen under the effects of the environment (Zadoks and Schein, 1979). Epidemiology, as a science of populations (Van der Plank, 1963), has the objective of understanding the interactions among the three components of the disease triangle: the host, the environment, and the pathogen. Usually, aspects of the populations of the host plants are relatively well known: tomato and potato populations are comprised of hybrids and/or cultivars bred for high yields but susceptible to pathogens. The environmental conditions in which host and pathogen interact can be easily monitored with modern and accessible electronic devices such as dataloggers. In general, environmental conditions favor fungal diseases in most areas were these crops are grown in Brazil. Thus, reasonable knowledge is available about two of the components of the interaction. However, pathogen populations are not commonly investigated and very little is known about Brazilian populations of *A. solani*.

Complete epidemiological studies should start by generating information about pathogen population. As pointed out by Jeger & Chan (1995) “for epidemiology to be seen as a mature science rather than an ad hoc empirical tool, its contribution must be based on an understanding of biological processes, and processes occurring very much at the population level, involving interacting populations of host plants and their pathogens". Thus, plant disease epidemiology will necessarily address questions at the

population level, and studies of population biology of plant pathogens should be the starting point for research programs aiming at establishing effective management strategies to disease control.

Pathogen population biology encompasses both ecological and genetic aspects of populations (Milgroom and Peever, 2003). Ecological studies have been one of the focuses of classical epidemiology over the past years. Nevertheless, genetics of pathogen population has not been an integral part of botanical epidemiology. Probably, the reduced availability of suitable genetic markers limited advancement in this field. Nowadays, we are experiencing ready access to molecular tools, especially those that allow the development of markers useful for studying variability in populations. This has substantially improved the amount of information about the genetic aspects of pathogen populations.

Knowledge about genetic variability is essential to determine strategies of disease management, especially those related to the deployment of host resistance and chemical control. Plant breeding programs aiming at disease resistance and deployment of resistant cultivars benefit from information of the population biology of plant pathogens. In some countries, breeding programs that consider information about genetic variability of pathogen populations are becoming a reality (McDonald and Linde, 2002b). Evaluating the effectiveness and durability of resistance genes is possible if the amount and distribution of genetic variation in populations of plant pathogens – the population structure - is known (Vera Cruz et al., 2000). As a logical next step, the strategic use of resistant cultivars for disease management can be better established if guided by information of pathogen population structure. Some interesting examples of usage of the population biology approach include: (i) the system that recommends wheat cultivars to be planted in particular areas of the United States based on the distribution of the rust pathogen *Puccinia graminis* f.sp. *tritici* (Frey et al., 1973); (ii) the deployment of rice resistance to manage bacterial blight caused by *Xanthomonas oryza*e pv. *oryzae* according to prevalent pathogen population in a given region (Ahmed et al., 1997); and (iii) the use of cultivar mixtures to control rice blast caused by *Magnaporthe grisea* in China (Zhu et al., 2000).

Fungicide usage can be rationalized based on knowledge of pathogen population structure. The frequency of monitoring insensitive populations of plant pathogenic fungi and the sampling procedures to be adopted could be optimized based on the information about the mechanisms that shape the evolution of populations, the amount of genetic variability, and how genetic variation is geographically distributed.

One of the most powerful techniques that allow analysis of sequencing data and making inferences on the evolutionary processes that shape populations is based on gene genealogies through the coalescent process (Hudson, 1991; Rosenberg and Nordborg, 2002). This technique is of recent usage in plant pathology where examples are becoming available and already revealed its potential contribution (Carbone and Kohn, 2001; Banke et al., 2004; Carbone et al., 2004). The coalescent process is advantageous because: (i) it is a sample-based theory. Since the study of a population usually relies on a sample of individuals from that population, a theory that describes the properties of a sample is more relevant than the classical population genetics theory that describes the properties of the entire population; (ii) It is a highly efficient approach with efficient algorithms for simulating population samples under various population genetic models. Coalescent theory is particularly suitable for molecular data, such as DNA sequence samples, which contain rich information about the ancestral relationships among the individuals sampled (Fu and Li, 1999).

The population biology approach forms the basis of a research program for management of early blight of tomato and potato in Brazil. Recently, a study was conducted to assess the population structure of *Phytophthora infestans* using molecular markers such as isozymes and RFLP as well as classical population genetics analyses (Reis et al., 2003). On the other hand, no aspects have been properly quantified for populations of *A. solani*. The present work was oriented towards generating knowledge about Brazilian populations of *A. solani*. The main approach was to characterize the population of *A. solani* with a set of markers and perform detailed population genetics analyses.

II. Literature review

The disease

Early blight is a fungal disease caused by *Alternaria solani* Sor. which affects aerial parts of tomato (*Solanum lycopersicum* L. = *Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.), the two most important vegetable crops in Brazil (IBGE, 2005). Epidemics of early blight occur with high intensity in almost all areas where these crops are grown and is an important disease worldwide (Pscheidt and Stevenson, 1986; Rotem, 1994). Yield loss due to early blight can be high when disease control measures are not properly adopted (Shtienberg et al., 1996; Dillard et al., 1997). Ideally, cultural, biological, physical, resistance, and chemical control measures should be integrated to manage early blight. However, several of these practices, especially the cultural and physical measures, are of low efficacy, some are difficult to implement in large acreages, and are of high costs. Furthermore, resistant cultivars with good agronomic traits are not available and because early blight epidemics are polycyclic, growers heavily rely on fungicide application; which results in environmental pollution and increased production costs (Shtienberg and Fry, 1990; Shtienberg et al., 1996). Reducing the amount of chemicals used without crop losses is the final goal of most plant disease research. Therefore, one needs to establish the most effective control strategies to be implemented.

Population biology and its interface with disease management

Plant disease management strategies must be established on an epidemiological basis. Epidemiology is the study of the spatial and temporal dynamics of epidemics (Campbell and Madden, 1990), which in turn are the result of the interaction of host and pathogen populations (Zadoks and Schein, 1979). Thus, the study of plant diseases epidemics must take the population approach. Population biology of plant pathogens is the study of the ecological and genetic aspects of populations and is the "synthesis of plant disease epidemiology and population genetics" (Milgroom and Peever, 2003). Under this paradigm, defining the genetic structure of populations is the first logical step to study plant pathogen population, because the genetic structure reflects the evolutionary history and the potential of a population to evolve (Leung et al., 1993; McDonald and McDermott, 1993).

The genetic structure of plant pathogen populations refers to the determination of the amount of genetic variability and its distribution within and between subpopulations (Leung et al., 1993; McDonald and McDermott, 1993). Genetic variation is a product of evolutionary processes acting upon the population, thus understanding the evolutionary mechanisms that shape pathogen populations is a common goal in population biology studies. From the evolutionary point of view, genetic variability of populations is important because it determines the adaptation potential of an organism to the biotic and abiotic environment. From the epidemiological perspective, pathogenic variability directly affects disease management. For example, gene flow events can result in the introduction and distribution of more aggressive isolates for which higher amounts of fungicides need to be employed to achieve satisfactory disease control (Kato et al., 1997); mutation has implication in the risks associated with resistance of pathogen populations to fungicides (Oshima et al., 2002); and selection can strongly affect the effectiveness – durability of resistant cultivars (McDonald and Linde, 2002b).

Sensitivity to fungicides and resistance deployment are practices largely influenced by the evolutionary processes that affect populations of plant pathogens (Wolfe and Caten, 1987; Milgroom and Fry, 1997; Gisi et al., 2002; Milgroom and Peever, 2003). The life-time of a fungicide is greatly

influenced by the evolutionary rate of pathogen populations. Higher genotypic diversity in panmictic populations increases the chances of existing fungicide insensitive isolates in relation to clonal populations (Bateman et al., 1995). In addition to the mode of reproduction, mutation and migration are also of great concern. Mutation can alter individual sensitivity to fungicides which, under selection, could contribute to shift sensitivity of the population as whole, and disease control becomes difficult when insensitive populations prevail. Migration can also interfere with the efficacy of fungicides by introducing insensitive individuals into an area and thus reducing the performance of these products (Goodwin et al., 1996; Gisi et al., 2002).

Durability of resistant varieties can be estimated based on knowledge of the pathogen population structure (McDonald and Linde, 2002a). Bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) is an important foliar disease of rice in Southeast Asia. Resistance in rice is conferred by many genes, but durability of the *Xa7* gene is presumably long lasting based on the fitness cost associated with mutation in the corresponding avirulence gene in the pathogen (Vera Cruz et al., 2000). Deployment of resistant cultivars and/or extending their efficacy must be based on the amount and distribution of genetic variability. A classic example is the management of wheat stem rust by planting resistant cultivars in areas where certain pathotypes are more likely to occur (Frey et al., 1973). Rice bacterial blight management was also improved when distribution of pathogen population in each region became known (Ahmed et al., 1997).

Understanding dynamics of genetic variability of plant pathogen populations is required to understand how populations evolve and the implications to durability of disease control measures (McDonald et al., 1999). For most purposes, population genetics provides the most suitable tools to estimate genetic variability of pathogen populations. The major evolutionary mechanisms that affect variability of populations are: selection, random genetic drift, migration, mutation, and recombination (Hartl and Clark, 2007). Quantifying the contribution of each mechanism is important and constitutes the ultimate goal of many studies of population biology of plant pathogens (Zhan and McDonald, 2004).

The pathogen

A. solani is a mitosporic fungus, class Hyphomycetes, order Hyphomycetales, and family Dematiaceae (Thomma, 2003). Fungal mycelium is septate, branched, melanized, velvety, and the color of the hyphae can be gray, black, or olive. Septate conidiophores are brown colored and occur individually or in groups (Rotem, 1994). Conidia are individual and beaked with transverse and longitudinal septa, and their dimensions vary from 12-20 µm width to 139-441 µm length (Rotem, 1994).

Morphological and physiological variability of *A. solani* has been studied since the 1920's (Bonde, 1929), and populations of the fungus are regarded as highly variable (Bonde, 1929; Rotem, 1994; Martinez et al., 2004; van der Waals et al., 2004). All phenotypic characteristics assessed so far are variable. Assessment of genetic variability in *A. solani* was first attempted using isozymes (Petrunak and Christ, 1992). Twenty isozymes were screened among 54 isolates of *A. solani*. There was no clear association of isozyme profile with host of origin or geographical location. Later, 35 isolates of *A. solani* were analyzed by RAPD markers. Higher genetic variation was due to differences between populations of *A. solani* in potato and tomato crops, which suggests the occurrence of host-specificity in this system (Weir et al., 1998).

In two recent studies, AFLP and RAMS (random amplified microsatellites) were used to assess genetic variability among isolates of *A. solani* (Martinez et al., 2004; van der Waals et al., 2004). Genetic diversity was high when 46 isolates from potatoes were compared by RAMS (van der Waals et al., 2004). By using AFLPs to characterize 112 isolates from six countries, including Brazil, there was an association between genetic marker and host of origin (Martinez et al., 2004). Most of the genetic variability was due to differences among countries. Unfortunately, all eight Brazilian isolates were sampled from a single location and only one host (potato), thus no inference on host specificity could be made. A group of 45 isolates of *A. solani* was characterized, sampled from different geographical areas, from potato or tomato crops. Using RAPD markers, there was strong evidence of

host specificity in the Brazilian populations of *A. solani* (Scheuermann et al., 2004).

To this point, many questions remain unanswered regarding the high variability observed among *A. solani* isolates, and to the consequences this have brought to epidemiology and evolution of populations. The high variability in populations of *A. solani* is an interesting feature considering a strictly asexual fungus for which no sexual stage and reproductive morphology are known (Rotem, 1994; Seifert and Gams, 2001). Besides recombination (meiotic or mitotic), other evolutionary forces such as genetic drift, gene flow, and mutation can contribute to the observed high variability. Nevertheless, no study has been conducted to quantify the contribution of these mechanisms to the variability of *A. solani*.

Reliable inferences about the evolution of pathogen populations require quantification of the contribution of each of the evolutionary mechanisms. This process is sometimes called “parameterization” of evolutionary forces (Zhan and McDonald, 2004) . Regarding the procedural aspects required for this purpose, they vary according to the mechanism. Selection has been estimated through assessments of fitness components and methods are available for this purpose (Antonovics and Alexander, 1989). Genetic drift can only be inferred to have occurred, but there is on parameter that directly assess the magnitude of this mechanism. The experimental procedures for determining and quantifying the effects of genetic drift are very difficult to be implemented and the reliability of the results has been questioned (Wright, 1969). Thus, among the five mechanisms, estimates of migration, mutation, and recombination are more widely applied to the population and, as opposed to selection, less dependent on particular situations. Furthermore, parameters can be estimated through detailed genetic analyses. Many studies were conducted to understand the role of migration, mutation, and recombination on the genetic structure of plant pathogen populations (McDermott and McDonald, 1993; Milgroom, 1996; Roossinck, 1997; Taylor et al., 1999; Brown, 2000; Vera Cruz et al., 2000; Zhan and McDonald, 2004).

Techniques for quantifying genetic variability in pathogen populations

Variation can be assessed through a large array of traits. Among the several markers used to study populations of plant pathogens, morphological and pathological markers were the first generally employed. Phenotypic markers such as virulence and fungicide resistance, biochemical (enzyme production), and molecular markers (DNA-based markers) have been commonly employed (Brown, 1996). Analyses using high resolution markers will contribute to answer more complex questions associated with genetic variability. Neutral genetic markers such as RAPD, RFLP, AFLP, microsatellites, and SNPs (single nucleotide polymorphism) can be used to study populations of plant pathogens (Brown, 1996; McDonald, 1997; Zhang and Hewitt, 2003; van der Waals et al., 2004). However, with easier access to genomic data, genetic variation can also be investigated by sequencing and analyzing specific genes. For plant pathogens, examples of commonly sequenced regions are the internal transcribed spacer of nuclear ribosomal DNA (rDNA ITS1 of the 5.8S RNA gene), mitochondrial small sub-unit rDNA genes (mtSSU) (Pryor and Gilbertson, 2000), β-tubulin (Banke et al., 2004), and the translation elongation factor 1α (EF-1α) (Couch and Kohn, 2000). Sequencing of genes directly linked to a trait such as resistance or virulence could also be done and used in analyses. The bottom line is that the possibility of obtaining detailed information about the genealogy of sampled genes contribute to enhance the understanding of particular events (Hudson, 1991).

Analysis of gene genealogy based on the coalescent process is a powerful technique and high resolution tool used in population genetics studies of microorganisms. When sequences from distinct individuals of the same species are available, genealogical information can be obtained and gene trees can be inferred (Hudson, 1991). It is possible to determine which sampled sequences are more closely related to each other based on gene tree analysis (Hudson, 1991). The coalescent is a mathematical approximation (model) of the actual ancestral structure of a population (Carbone and Kohn, 2004). It is a stochastic process capable of modeling

the genealogy of a DNA sequence taking into account the results of the unique history of mutation, recombination, and coalescence of lineages in the ancestry of the sample (Rosenberg and Nordborg, 2002). The stochastic properties of gene genealogies can be used to estimate population parameters such as rates of mutation, migration, recombination, and selection (Carbone and Kohn, 2004).

Gene genealogies and the coalescent process have only recently been introduced in the analysis of plant pathogen populations. A study was conducted to estimate migration of *Cryphonectria hypovirus 1* (CHV-1), a double-stranded RNA virus, between genotypes of the fungus *Cryphonectria parasitica* (Carbone et al., 2004). This is important because infection by CHV-1 can result in biological control of the fungus. Examples with fungal pathogens are also available. Gene genealogy analysis revealed a global phylogeographic structure and reproductive isolation of *Fusarium graminearum* (O'Donnell et al., 2000). This conclusion was reached after analyzing the phylogenetic relationships which indicated restricted gene flow among populations and low levels of recombination. A similar approach can be used to study populations of *A. solani* in Brazil.

As contradictory as it may sound, understanding the role of recombination in asexual fungi such as *A. solani* has become an important issue in population genetics studies. Fine-scale genetic analysis using multilocus variability has been conducted to gain more information on the role of recombination, since there has been cumulative evidence of cryptic sex in many fungi that have no known sexual stage (Burt et al., 1996; Taylor et al., 1999). *Coccidioides immitis*, a human fungal pathogen that causes Valley Fever, was considered an asexual organism. Using phylogenetic and gene genealogies analyses, the high variability observed in populations of *C. immitis* was attributed to the occurrence of recombination (Burt et al., 1996). Although populations are primarily clonal, evidence for recombination suggests that sexual reproduction or some other form of genetic exchange occurs in this species. Similar processes can occur in populations of plant pathogenic fungi.

Detailed analyses revealed the involvement of some kind of recombination in “classic” asexual plant pathogenic fungi such as *Sclerotium*

cepivorum, *Fusarium oxysporum*, and *Alternaria* spp. *S. cepivorum* causes onion and garlic white rot. The only survival/reproductive structures formed by this organism are sclerotia which are responsible for clonal spread in the field. Using gene genealogy analysis, it was possible to conclude that there were genetic exchange and recombination among isolates (Couch and Kohn, 2000). *F. oxysporum* is another interesting example. Data generated in one population genetic study of *F. oxysporum* f. sp. *cubense* (Koenig et al., 1997) were re-analyzed, and the results do not support the claim that this species is exclusively clonal in nature (Taylor et al., 1999). Isolates of the 'asexual' *Alternaria* species, *A. brassicae*, *A. brassicicola*, and the *A. tenuissima* group were subjected to phylogenetic analyses to check for any evidence of recombination. Using different relative substitution rates, it was concluded that sexual or parasexual reproduction was involved in the exchanging substitutions (Berbee et al., 2003). Unfortunately, no gene genealogy analysis using the coalescent process was conducted to determine the contribution of recombination or when it occurred, and no isolate of *A. solani* was included in the study. These examples corroborate the idea that wholly clonal fungi appear to be rare.

The information gathered from population biology studies may contribute to solve important practical epidemiological problems. For pathogens that can reproduce sexually and asexually, the contribution of each type of inoculum can differentially affect disease management (Milgroom and Fry, 1997). Oospores are sexual spores formed by *P. infestans* and even though there is strong evidence of oospore formation in a field, its contribution to late blight epidemics is not completely ascertained. By analyzing population structure, it is possible to infer about the involvement of a particular kind of inoculum. Consequently, management strategies directed to those propagules can be set. Analysis of migration events can aid tracking the introduction pathways of new genotypes into an area and, more importantly, to find out which substrates (seeds, transplants, soil, containers, etc.) may harbor pathogen propagules. Particular knowledge of the source of an introduced or invading species is useful for: (i) inferring evolutionary history and predicting adaptive range, (ii) locating sources of host plant resistance, (iii) deciphering the history of introductions and pattern of human

transport, (iv) locating sources of biological control agents, and (v) facilitating quarantine efforts (Downie, 2002). Finally, quantifying mutation rate is important to predict durability of resistance and to set strategies of fungicide usage to avoid rapid selection of insensitive populations (Gisi et al., 2002). These are a few examples of the potential usefulness of the population biology approach to disease management. Many other application can be derived and applied to answer epidemiological questions in a given pathosystem.

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1. Genetic structure of the population of *Alternaria solani* in Brazil

1.1. Abstract

The knowledge of the genetic structure of *Alternaria solani* (AS) is important to the establishment of suitable control strategies of early blight on potato (Po) and tomato (To) plants. However, there is no information about the amount and distribution of genetic variability of AS in Brazil. AFLP and RAPD markers were used to estimate the AS genetic diversity. A multilocus dataset from AFLP and RAPD matrices generated 123 haplotypes with 62 loci (92% polymorphism). The allele frequency for AS populations from To and Po ranged from 0.015 (RAPD45) to 1.000 (RAPD47, RAPD56, RAPD57, RAPD59, RAPD61, RAPD62). All alleles were present in AS populations, but frequencies of 32 loci differed. The theta estimate (Θ) and the Gst average values were 0.29 (P=0.001) and 0.18, respectively. However, there was greater genetic variation within (70.6%) than between (29.4%) AS populations. From cluster analyses, we found evidence of AS haplotypes associated with Po. High gene diversity was detected over all AS population (*h*=0.36, S.D. ± 0.38). Gene diversity values detected for Po and To populations were *h*=0.35 (S.D. ± 0.18) and *h*=0.24 (S.D. ± 0.19), respectively. The genotypic diversity measured by both Shannon-Wiener (*H'*) and Stoddart & Taylor's *G for* Po and To populations were, 1.01 and 1.00, and 0.99 and 0.98, respectively. There is no evidence of recombination (*I*A=7.25, *P<0.001*). Moderate gene flow is occurring between AS populations (*Nm*=2). There was no correlation between genetic distance and geographic origin of AS haplotypes (-0.084, P=0.86). Therefore, the population of *A. solani* is clonal with high genetic variability and haplotypes are widely distributed along potato and tomato producing regions.

1.2. Introduction

The amount and distribution of genetic variability of plant pathogen populations affect the efficacy of control strategies of plant diseases (McDonald & Linde, 2002). Two control measures are directly impacted by genetic variability: the efficacy of host resistance and the use of fungicides (Milgroom & Peever, 2003). Therefore, knowledge of the genetic structure of pathogen populations should be one of the first steps for the delineation of disease management programs.

Alternaria solani Sorauer is a mitosporic fungus that causes early blight on potato and tomato plants. Epidemics of early blight occur worldwide wherever potato and tomato are grown (Pscheidt & Stevenson, 1986; Rotem, 1994), and the epidemics are more severe in tropical countries during warm and wet summer seasons (Batista *et al.*, 2006; Mantecón, 2007). Wild *Solanum* species were shown to be a natural reservoir of resistance genes to early blight (Jansky & Rouse, 2003; Chaerani *et al.*, 2007). Nevertheless, there are no tomato or potato early blight resistant cultivars with good agronomic/commercial characteristics. The integrated management of early blight on potato and tomato crops is desirable, however, the limited information regarding the genetic variability of *A. solani* in a region limits the implementation of these control strategies. Combining all reasons listed above, in the tropics, the main control measure to reduce crop losses due to early blight remains the intensive use of fungicides (Batista *et al.*, 2006).

There are reports of high genetic variability of *A. solani* in some countries (Petrunak & Christ, 1992; Weir *et al.*, 1998; Martínez *et al.*, 2004; van der Waals *et al.*, 2004). The degree of genetic variation among *A. solani* isolates has been evaluated using isozymes (Petrunak & Christ, 1992) and RAPD in the USA (Weir *et al.*, 1998). There was high genetic diversity between isolates from potato and tomato plants as well as significant differences between isolates from other countries (Weir *et al.*, 1998). Similarly, the use of random amplified microsatellites (RAMS) identified high genetic variability among *A.* isolates from potato collected in South Africa (van der Waals *et al.*, 2004). Genetic differentiation and evidence of host

specificity of *A. solani* isolates from Cuba, Turkey, Russia, USA, Greece and Brazil were detected using AFLP marker (Martínez *et al.*, 2004). Unfortunately, the number of isolates from Brazil was small (8) and all isolates were sampled from a single host (potato) in one location (Minas Gerais State). The genetic structure of the population of *A*. *solani* in Brazil remains largely unknown.

During the 1990's few studies were conducted to assess variation of *A*. *solani* in Brazil, using classical markers. Isolates from potato and tomato plants (hereafter referred to as “potato isolates” and “tomato isolates”, respectively) were compared based on pathogenicity tests, isozyme analysis, and fungicide sensitivity tests (Fancelli, 1991). Differences in aggressiveness when inoculated on tomato plants, and physiological and genetic variability were detected in isolates from different host species. However, only 10 isolates, collected in a single region (São Paulo State) were analyzed (Fancelli, 1991). Using RAPD markers, in a previous work, there were evidences of genetic differentiation between *A. solani* associated with potato and tomato plants, when analyzing isolates collected from different regions of Brazil (Scheuermann *et al.*, 2004). This motivated an expanded investigation aimed at quantifying the amount of genetic variation in the population of *A*. *solani* and its distribution countrywide.

To allow for reliable inferences, the genetic structure should be determined based on more than one marker. Despite of the low reproducibility of RAPD, this marker is commonly used to gather preliminary information about genetic variation (Schlotterer, 2004). Combined with AFLP, a more reproducible marker, wide genome regions can be scanned and a more accurate assessment of genetic variation can be accomplished (Schlotterer, 2004). In the present study, we estimated the genetic diversity within and between populations of *A*. *solani* associated with potato and tomato plants sampled from a wide geographical range in Brazil, using both RAPD and AFLP markers. Our purposes were: (i) to quantify genic and genotypic diversity of *A*. *solani*; (ii) to determine how genetic variation between populations is spatially distributed and whether genetic variation is related to the geographical origin of isolates; and (iii) to investigate the evidence of specificity of *A*. *solani* isolates to potato and tomato crops.

1.3. Materials and Methods

Sampling, isolation and DNA extraction

Infected plant organs were collected in several producing regions (Fig.1). Pure monoconidial cultures of *A. solani* were obtained after direct or indirect isolation from lesioned potato and tomato leaves (Dhingra & Sinclair, 1995). The monoconidial isolates were preserved using four methods: filter paper stored at -20°C (Borromeu *et al.,*1993), mineral oil, sterilized distilled water, and silica gel. The last three were maintained in 10 mL flasks that were stored at 20°C (Dhingra & Sinclair, 1995).

For DNA extraction, isolates were grown in liquid medium (10 g sucrose, 2 g L-asparagin, 2 g yeast extract, 1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6\ H_2O$, and 0.36 mg $MnCl_2.\ H_2O$) in Erlenmeyer flasks under continuous agitation at 26°C (Alfenas *et al.*, 1998). After 7 days, the mycelium was washed with distilled water, transferred to filter paper to dry, and macerated in a mortar with liquid nitrogen. DNA extraction was conducted using a CTAB-based protocol (Murray & Thompson, 1980). Depending on the size of the DNA pellet, 30 to 50 µL of TE buffer with RNAse were added, and resuspended at 37°C for 2 h. Sample integrity was checked by visual inspection after electrophoresis in agarose gel at 1% with 0.15 µg/mL of ethidium bromide. The quantity of DNA was measured using the lambda phage as molecular weight.

RAPD and AFLP analyses

For RAPD analysis, PCR was performed in a final volume of 15 µL with 20 ng of DNA, 1.5 mM of $MgCl_2$, 1 U of Taq DNA polymerase, 0.5 mM of primer, and 0.2 mM of dATP, dCTP, dGTP, and dTTP. Primers P203 (5'CACGGCGAGT 3'), P284 (5'CAGGCGCACA 3') (Weir *et al.*, 1998), OPC15 (5'GACGGATCAG 3'), OPE14 (5'TGCGGCTGAG 3'), and OPE20 (5'AACGGTGACC 3') (Scheuermann *et al.*, 2004) were selected for diversity analyses. Each amplification reaction consisted of 40 cycles at 94°C for 30s (denaturation), 35°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 7 min (Scheuermann *et al.*, 2004). Amplified fragments were separated by agarose gel electrophoresis for 7-8 h.

For AFLP analyses, the genomic DNA was digested with restriction endonucleases *Eco*RI and *Mse*I, and adapters of double strand were linked at the ends of the fragments (AFLP Microorganism Primer Kit, Invitrogen). The PCR products from the pre-amplification reaction were diluted and used as templates to the selective amplification using primers E+AC/+AA and M+A/+G/+T/+C available in the AFLP kit. Amplified fragments were separated by polyacrilamide gel electrophoresis silver nitrate (Creste *et al.*, 2001).

The pattern of both RAPD and AFLP polymorphic bands was visually analyzed. Binary matrices were constructed by attributing the presence (1) or absence (0) of amplified fragments. Both RAPD and AFLP bands were treated as loci, and the presence or absence of amplified fragments as alleles.

Data analyses

A multilocus dataset was constructed combining AFLP and RAPD matrices using SNAP Combine (Aylor *et al.* 2006). Genetic variability was assessed by quantifying gene and genotypic diversity, genetic distance and genetic differentiation between *A. solani* populations from potato and tomato. The maximum likelihood ratio test (G^2) and gene diversity were estimated using POPGENE (Yeh *et al.,* 1997). Multilocus genotypic diversity was estimated with the Shannon-Wiener's *H'* and Stoddart and Taylor's *G* indices (HIll, 1973; Stoddart & Taylor, 1988). To compare the diversity indices calculated for populations with different sample sizes, Shannon-Wiener's *H'* and Stoddart and Taylor's *G* indices were scaled by the expected number of genotypes for the smallest sample size being compared (Grünwald *et al.*, 2003). The expected number of genotypes was estimated based on rarefaction curves using the R package version 2.6.1 (R Development Core Team, 2007). A dendrogram was constructed based on Nei-Li genetic distance matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as the clustering algorithm (Nei, 1987).

The analysis of molecular variance (AMOVA), implemented in the Arlequin 3.11, was used to estimate the subdivision of the population (Φ_{ST}) (Excoffier *et al.*, 2005). The coefficient of genetic differentiation (G_{ST}) between *A. solani* populations was calculated using POPGENE. In addition, genetic differentiation between populations was tested using theta (Θ) estimate of Weir & Cockerham, with the generation of 1000 randomized datasets as described in the program Multilocus 1.3. (Agapow & Burt, 2001).

The relationship between genetic diversity and geographic distance was estimated by Mantel's test (Mantel, 1967) implemented in Arlequin 3.11. The Mantel test was carried out using 1000 random permutation tests.

The index of association (I_A), was used to infer about random mating in the population.The test was performed with Multilocus 1.3.

1.4. Results

Genetic diversity was assessed from 130 isolates of *A*. *solani* collected from the main potato and tomato producing regions of Brazil (Fig. 1, Table 1). A high percentage of polymorphic loci was estimated for RAPD, AFLP, and the multilocus dataset. For RAPD, the number of scored loci was 34, and 85% were polymorphic. For AFLP, 28 loci were scored and all were polymorphic. For the combined dataset, 62 loci were scored and 92% were polymorphic. The scaled indices of genotypic diversity measured by Shannon-Wiener (*H'*) and Stoddart & Taylor's *G* for potato population had the same value: (1.01). For the tomato population the indices were 0.99 and 0.98, respectively.

For each locus, the allele frequency for *A. solani* populations from tomato and potato plants ranged from 0.015 (RAPD45) to 1.000 (RAPD47, RAPD56, RAPD57, RAPD59, RAPD61, and RAPD62) (Table 2). All alleles were present in populations sampled from potato and tomato plants, but frequencies on 32 loci differed (Table 2). High gene diversity was detected over all populations (h = 0.36, S.D. ± 0.38). Gene diversity values for potato and tomato populations were h = 0.35 (S.D. ± 0.18) and h = 0.24 (S.D. ± 0.19), respectively.

Considering the combined dataset with 62 loci, 123 haplotypes were observed. There was evidence of association of *A. solani* haplotypes with potato and tomato plants (Fig.2). Cluster 1 had mostly haplotypes associated with potato plants (29 isolates), and 13 haplotypes from tomato plants. Distribution of haplotypes according to host plants was more equally distributed in cluster 2: 48 haplotypes were sampled from tomato plants and 41 haplotypes from potato.

Genetic differentiation estimated by the average Gst was moderate (G_{ST} = 0.18). The ✈ statistic was estimated to be 0.29 (P = 0.001). There was evidence of genetic differentiation between populations. Nevertheless, there was greater genetic variation within (70.6%) than between (29.4%) populations (Table 3).

There was no evidence of recombination in *A. solani* populations. The null hypothesis of random association of alleles was rejected (I_A = 7.25, *P<0.001*). Moderate gene flow is occurring in *A. solani* populations with the average number of migrants estimated around 2 individuals per generation (Table 3). Consequently, there was no correlation between genetic distance and geographic origin of *A. solani* haplotypes (r = -0.084, P = 0.857).

1.5. Discussion

The Brazilian population of *A*. *solani* is variable, partially associated with host plants, and is not randomly mating. Due to the fact that the pathogen lacks a known sexual stage, we expected to find strong evidence for a clonal population structure. Two evolutionary mechanisms seem to play an important role in shaping population variability: mutation and gene flow.

High mutation rates determine the variation in the population of *A*. *solani*. In general, high genetic diversity is expected for fungal populations with sexual reproduction (Milgroom, 1996). Although recombination was inferred in populations of some *Alternaria* species (Berbee *et al.*, 2003), and low values of I_A were calculated for *A. brassicicola* using AFLP markers (Bock *et al.*, 2005), there is no evidence of recombination occurring in the population of *A*. *solani* in Brazil. No sexual structures are known to be formed by *A*. *solani* under Brazilian conditions, neither has parasexual recombination been reported. Additionally, according to the genetic analyses carried out in the present study, the null hypothesis of a randomly mating population was rejected by the I_A test. All lines of evidences suggest that mutation plays an important role in generating a diverse population. However, quantification of the mutation rate would be more properly accomplished using DNA sequence data. Another important issue is the involvement of transposable elements in *A. solani,* that are responsible for high mutation rates detected in several fungal species (Daboussi & Capy, 2003). Further studies should be conducted to identify the presence of these elements and their importance regarding to genetic variability and evolution of *A. solani*.

Differences in fitness components when associated with host species can explain the host association of *A*. *solani* (van der Waals et al., 2004). In Brazil, *A. solani* isolates from tomato were more aggressive in their host of origin than potato isolates inoculated in tomato plants (Fancelli, 1991). This fact corroborates the evidence of association of *A. solani* haplotypes to potato and tomato plants detected in our study. In addition, ecological factors (e.g. temperature, solar radiation, humidity) effect can contribute to host specificity of *A. solani* in Brazil. However, at this point, we do not have enough information to infer about the mechanisms that affect the association

of *A. solani* on potato and tomato plants.

Although *A. solani* haplotypes were found associated with potato or tomato plants, low genetic differentiation among populations from each host species was estimated in our study. Gene flow is occurring among *A. solani* populations preventing geographic subdivision, as well as a putative adaptive genetic differentiation between isolates from potato and tomato plants. Aerial conidia are the main type of inoculum for early blight epidemics in both potato and tomato fields, and the close proximity between fields of these two crops in Brazil favor the exchange of *A. solani* haplotypes (Batista, 2006).

Based on our results, early blight management strategies based on fungicide and host resistance should be carefully evaluated in order to incorporate the rational use of chemicals and quantitative host resistance. For a plant pathogen with high genetic variability and with evidence of inoculum movement among growing areas (gene flow), resistance to site specific fungicides is a common problem, including for *A*. solani (Pasche *et al.*, 2005). The use of decision support systems (DSS) should be incorporated to the management of early blight on potato and tomato crops to rationalize fungicide spraying, avoiding the environmental contamination and the selection of resistant isolates. Finally, preventive control with protectant fungicides must be always conducted.

The knowledge of genetic diversity of plant pathogens is important in selecting the appropriated type of resistance and in selecting resistance genes in breeding programs (McDonald & Linde, 2002). Sources of resistance to early blight can be found in wild species of potato and tomato (Jansky & Rouse, 2003; Chaerani *et al.*, 2007). According to a decision diagram prepared to aid breeders in setting up programs aimed at disease resistance (McDonald & Linde, 2002b), the selection of quantitative resistance on potato and tomato plants appears more appropriate to reduce yield losses caused by *A. solani* in Brazil.

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1.7. Figures legends

Fig.1. Sampled areas (dots) located in potato and tomato producing regions of Brazil. From each area, leaves (potato and tomato) or fruits (tomato) infected with *Alternaria solani* were collected. The sampling sites were georeferenced with a portable GPS device. States from which samples were collected are shaded.

Fig.2. Dendrogram generated by the unweighted pair group method with arithmetic average analysis based on Nei-Li genetic distance matrix among *Alternaria solani* populations from potato and tomato plants.

1.8. Tables and Figures

Table 1 - Description of *A. solani* isolates collected from potato and tomato producing regions of Brazil.

Region /State[a]	County Collection date[a]	Isolate code[b]	Date collected[c]	Haplotypes[d]
SE/MG	Araxá	AS012P, AS013P	01/2005	H1, H121
	Bom Repouso	AS299T, AS194P, AS185P, AS203P, AS189P, AS187P	01/2006	H13, H32, H38, H87, H108, H122
	Bueno Brandão	AS076P	06/2004	H95
	Bueno Brandão	AS168P, AS180P, AS169P, AS170P	01/2006	H35, H119, H120, H123
	Cajuri	AS130T, AS131T	07/2000	H52, H86
	Camanducaia	AS221P, AS216P	01/2006	H4, H82
	Carandaí	AS440T, AS107P, AS108P	03/2005	H28, H116, H118
	Coimbra	AS094T, AS095T	05/2000	H47, H115
	Conselheiro Lafaiete	AS255P, AS243T, AS532P, AS242T, AS248P, AS250P, AS229T, AS247P, AS232T	05/2005	H9, H37, H43, H49, H50, H50, H51, H53, H60
	Ibiá	AS021P, AS027P, AS016P	01/2005	H2, H101, H123
	Igarapé			
	Ipuiúna	AS373P, AS372P, AS360P, AS361P	01/2006	H44, H81, H97, H114
	Ituverava	AS409T	05/2005	H111
	Jaíba	AS422T, AS423T, AS420T	07/2005	H22, H24, H26

	Monsenhor Izidro	AS394P, AS401P	05/2005	H61, H66
	Poços de Caldas	AS093P	07/2000	H117
	Prudente de Morais	AS139T	N/A	H99
	Ressaquinha	AS489T, AS482T, AS480T	03/2005	H27, H29, H85
	Teixeiras	AS080P	N/A	H3
	Tocantins	AS124T	07/2000	H84
	Viçosa	AS096T, AS098P, AS091T	05/2000	H89, H100, H103
	N/A	AS083T	01/1996	H102
SE/RJ				
	Bom Jardim	AS111T	06/2000	H30
	Cordeiro	AS113T	06/2000	H98
	Itaperuna	AS120T	06/2000	H12
	Sumidouro	AS508T, AS495T, AS505T	02/2005	H27, H63, H100
	Vassouras	AS109T	06/2000	H92
SE/SP				
	Capão Bonito	AS090T	04/2000	H107
	Ibiúna	AS063T, AS069T	01/2005	H76, H91
	Itapetininga	AS337P, AS338P, AS331T, AS329T, AS332T, AS336P	10/2005	H11, H14, H19, H36, H41, H113
	Marília	AS132T	N/A	H39
SE/ES				

	Castelo	AS226T	01/2005	H48
	Domingos Martins	AS272T, AS270T, AS264P	01/2005	H17, H23, H49
	Marechal Floriano	AS451T	01/2005	H62
	Venda Nova do Imigrante	AS517T	01/2005	H105
S/PR				
	Araucária	AS301T, AS319T	04/2005	H25, H110
	Colombo	AS317T	04/2005	H96
	Contenda	AS307P, AS305P, AS309P, AS308P, AS311P	04/2005	H31, H33, H68, H69, H109
	Ponta Grossa	AS087P, AS088P, AS086T, AS088P	04/2000	H83, H88, H98, H104
	São Mateus do Sul	AS084P, AS085P	04/2000	H46, H123
S/RS				
	Caxias do Sul	AS304T	04/2005	H15
	Pelotas	AS300T, AS298	05/2005	H54, H77
	São Francisco de Paula	AS315P, AS316P, AS313P	04/2005	H6, H34, H73
	Taquara	AS160T	N/A	H57
S/SC				
	Alfredo Wagner	AS294T, AS296T	03/2005	H21, H65
	Caçador	AS293T	03/2005	H8
	Lages do Régis	AS297T	03/2005	H59
	Rancho Queimado	AS550T, AS553T	03/2006	H79, H80

CW/DF				
	Brasília	AS357P, AS347P, AS349P, AS353P, AS350P, AS352P, AS358P	07/2005	H42, H45, H55, H56, H58, H72, H78
	Ponte Alta	AS288T	N/A	H93
	Planaltina	AS460T, AS456T, AS453T, AS458T, AS464T	07/2005	H16, H20, H40, H70, H112
CW/GO				
	Cristalina	AS263P, AS259P, AS260P, AS261P, AS258P	07/2005	H5, H18, H64, H67, H74
	Planaltina de Goiás	AS079T	N/A	H90
TO/CW	Colméia	AS339T	11/2005	H54
NE/BA				
	Mucugê	AS033P, AS034P	N/A	H75, H106
NE/CE				
	Guaraciaba do Norte	AS325P, AS323P, AS326P	08/2005	H7, H71, H94

[a] 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), TO (Tocantins); Regions: S (South), SE (Southeast), NE (Northeast), and CW (Central-West); N/A: geographic origin is not available;

[b] Collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves;

[c] N/A: date collected not available;

[d] Haplotypes obtained after concatenating AFLP with RAPD bands.

Table 2 – Allele frequencies, population genetic differentiation, and number of migrants (*Nm*) estimated for multilocus dataset among *Alternaria solani* populations from potato and tomato plants.

Locus	Allele frequency			G^2	*Gst*	*Nm*
	Tomato	Potato	Overall			
AFLP01	0.6190	0.6912	0.6565	0.75ns	0.0058	86.36
AFLP02	0.4921	0.5588	0.5267	0.59ns	0.0045	111.40
AFLP03	0.6825	0.3529	0.5115	14.50*	0.1088	4.10
AFLP04	0.3810	0.3676	0.3740	0.02ns	0.0002	2000.00
AFLP05	0.2381	0.2794	0.2595	0.29ns	0.0022	224.21
AFLP06	0.4444	0.5588	0.5038	1.72ns	0.0131	37.72
AFLP07	0.5714	0.5735	0.5725	0.01ns	0.0000	2000.00
AFLP08	0.6190	0.7059	0.6641	1.11ns	0.0084	58.81
AFLP09	0.6349	0.7206	0.6794	1.10ns	0.0084	59.02
AFLP10	0.2540	0.3676	0.3130	1.98ns	0.0151	32.65
AFLP11	0.6984	0.6912	0.6947	0.01ns	0.0001	2000.00
AFLP12	0.1587	0.5588	0.3664	23.68*	0.1740	2.37
AFLP13	0.4762	0.5294	0.5038	0.37ns	0.0028	176.02
AFLP14	0.7302	0.7941	0.7634	0.74ns	0.0056	88.13
AFLP15	0.3968	0.6029	0.5038	5.60*	0.0425	11.27
AFLP16	0.1587	0.3971	0.2824	9.46*	0.0708	6.57

AFLP17	0.3175	0.5882	0.4580	9.80*	0.0740	6.2589
AFLP18	0.2222	0.3971	0.3130	4.72*	0.0357	13.49
AFLP19	0.6190	0.4118	0.5115	5.67*	0.0430	11.13
AFLP20	0.1270	0.5294	0.3359	25.24*	0.1836	2.23
AFLP21	0.2063	0.5000	0.3588	12.60*	0.0944	4.7984
AFLP22	0.6032	0.4118	0.5038	4.82*	0.0366	13.1441
AFLP23	0.4762	0.3676	0.4198	1.58ns	0.0121	40.90
AFLP24	0.3810	0.4559	0.4198	0.76ns	0.0058	86.18
AFLP25	0.2540	0.2500	0.2519	0.01ns	0.0000	2000.00
AFLP26	0.7937	0.7353	0.7634	0.62ns	0.0047	105.24
AFLP27	0.4921	0.3529	0.4198	2.61ns	0.0198	24.71
AFLP28	0.4286	0.4559	0.4427	0.10ns	0.0008	660.89
RAPD29	0.9841	1.0000	0.9924	1.47ns	0.0080	62.00
RAPD30	0.9841	0.2794	0.6183	83.36*	0.5337	0.44
RAPD31	0.0317	0.7206	0.3893	76.83*	0.5055	0.49
RAPD32	0.9841	1.0000	0.9924	1.47ns	0.0080	62.00
RAPD33	0.9841	1.0000	0.9924	1.47ns	0.0080	62.00
RAPD34	0.9841	1.0000	0.9924	1.47ns	0.0080	62.00
RAPD35	0.8571	0.3382	0.5878	38.85*	0.2800	1.29
RAPD36	0.8571	0.3382	0.5878	38.85*	0.2800	1.29
RAPD37	0.9841	0.9853	0.9847	0.01ns	0.0000	2000.00

RAPD38	0.1270	0.6471	0.3969	39.74*	0.2850	1.25
RAPD39	0.1270	0.6471	0.3969	39.74*	0.2850	1.25
RAPD40	0.1270	0.6471	0.3969	39.74*	0.2850	1.25
RAPD41	0.1429	0.6618	0.4122	38.85*	0.2800	1.29
RAPD42	0.1429	0.6618	0.4122	38.85*	0.2800	1.29
RAPD43	0.8730	0.3529	0.6031	39.74*	0.2850	1.25
RAPD44	0.1270	0.6471	0.3969	39.74*	0.2850	1.25
RAPD45	0.0159	0.0147	0.0153	0.01ns	0.0000	2000.00
RAPD46	0.9841	0.9853	0.9847	0.01ns	0.0000	2000.00
RAPD47	1.0000	1.0000	1.0000	0.00ns	-	-
RAPD48	0.9524	0.2941	0.6107	68.62*	0.4613	0.58
RAPD49	1.0000	0.9412	0.9695	5.36*	0.0303	16.00
RAPD50	0.0476	0.6471	0.3588	58.59*	0.3963	0.76
RAPD51	0.9524	0.2941	0.6107	68.62*	0.4613	0.58
RAPD52	0.0000	0.0588	0.0305	5.36*	0.0303	16.00
RAPD53	0.9524	0.3529	0.6412	58.59*	0.3963	0.76
RAPD54	0.0476	0.6471	0.3588	58.59*	0.3963	0.76
RAPD55	0.9683	0.2941	0.6183	74.07*	0.4881	0.52
RAPD56	1.0000	1.0000	1.0000	0.00ns	-	-
RAPD57	1.0000	1.0000	1.0000	0.00ns	-	-
RAPD58	0.9524	0.2647	0.5954	74.08*	0.4963	0.51

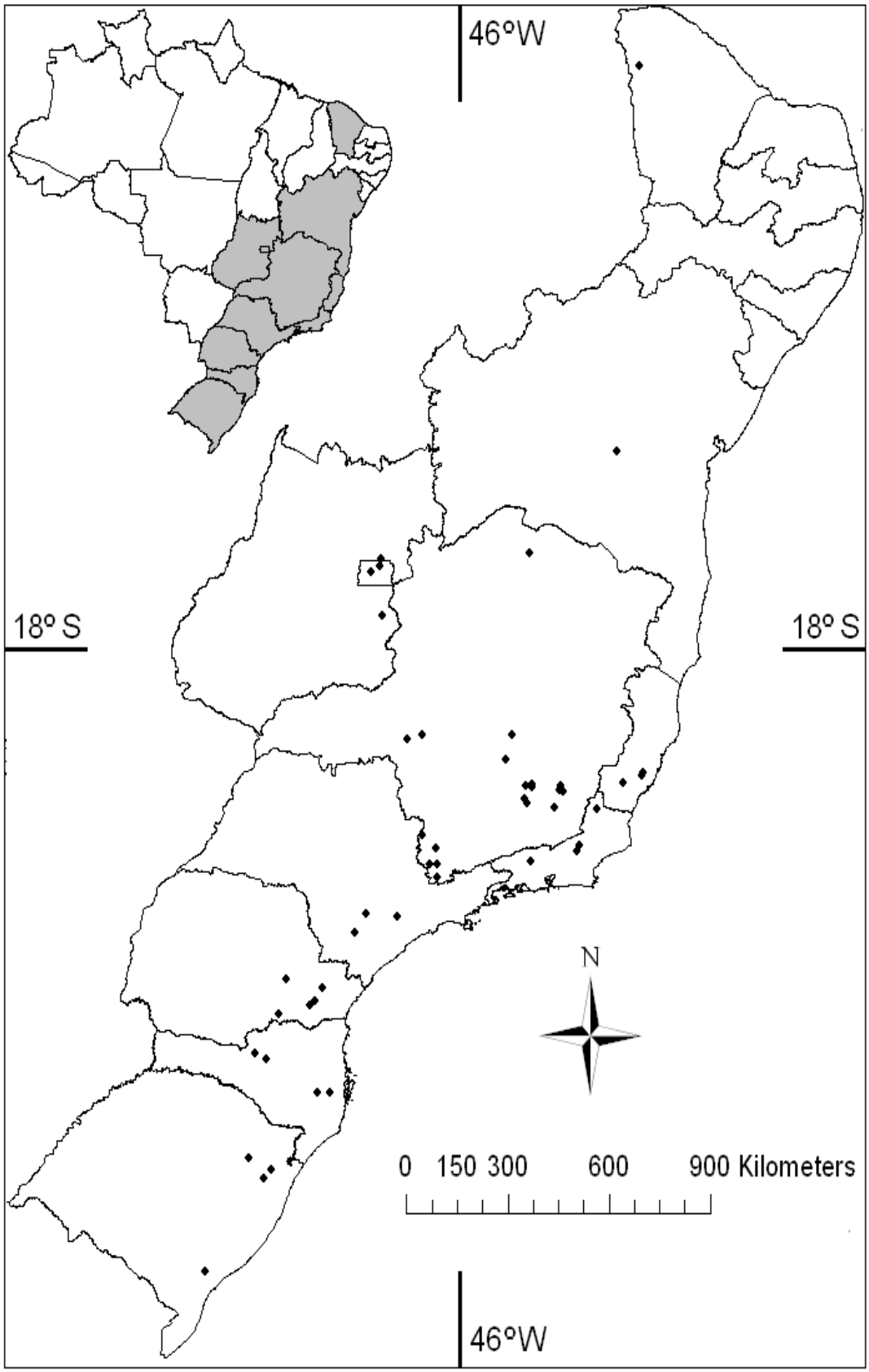
RAPD59	0.0476	0.7353	0.4046	74.08*	0.4963	0.51
RAPD60	0.9524	0.2647	0.5954	74.08*	0.4963	0.51
RAPD61	1.0000	1.0000	1.0000	0.00ns	-	-
RAPD62	1.0000	1.0000	1.0000	0.00ns	-	-
Average	-	-	-		0.1809	2.26

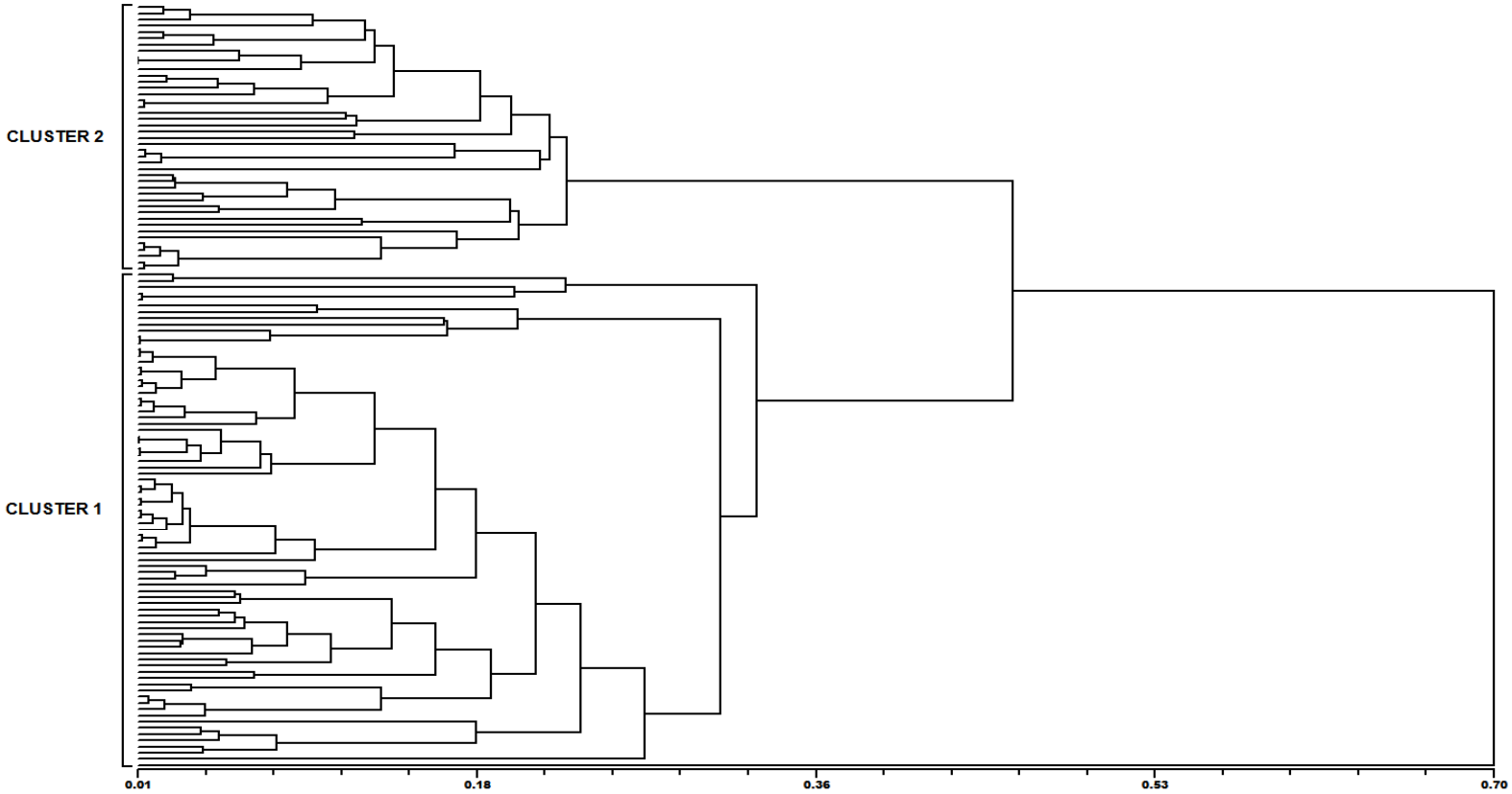
ns = not significant; * = Significant (P<0.05).

Table 3. Analysis of molecular variance (AMOVA) of *Alternaria solani* populations for multilocus combined dataset (RAPD and AFLP).

Locus	**Source of variation**	**D.F.***	**Sum of squares**	**Components of variance**	**Percentage of variation**	**Φst (P value)**
Multilocus combined dataset	Between Populations	1	265.75	3.92	29.41	
	Within Populations	129	1213.34	9.41	70.59	
	Total	130	1479.08	13.33		0.29 (P<0.0001)

*Degrees of freedom


46°W
18° S
18° S
N
0 150 300 600 900 Kilometers
46°W



CLUSTER 1: H3, H8, H9, H10, H12, H13, H14, H16, H17, H18, H19, H20, H21, H22, H23, H24, H25, H26, H27, H28, H29, H31, H32, H37, H38, H41, H42, H44, H46, H48, H49, H50, H51, H52, H53, H54, H55, H56, H57, H58, H59, H60, H61, H63, H64, H66, H67, H71, H75, H77, H78, H79, H80, H81, H84, H85, H86, H87, H89, H90, H91, H92, H93, H94, H95, H98, H99, H100, H101, H103, H104, H105, H106, H108, H111, H112, H116, H117, and H123.

CLUSTER 2: H1, H2, H4, H5, H6, H7, H11, H15, H30, H33, H34, H35, H36, H39, H40, H43, H45, H47, H62, H65, H68, H69, H70, H72, H73, H74, H75, H82, H83, H88, H96, H97, H102, H107, H109, H110, H113, H114, H115, H118, H119, H120, H121, and H122.

2. Molecular diversity of *Alternaria solani* and evolutionary mechanisms estimated using genealogical and coalescent approaches.

2.1. Abstract

Alternaria spp. is a heterogeneous group of saprophytic and plant pathogenic fungi widespread in temperate and tropical regions. However, the relationship between evolutionary forces and genetic diversity with plant disease epidemics is unknown for several *Alternaria* species associated with host species. The interactions of *Alternaria solani* (AS) populations with potato and tomato plants is an interesting case study for addressing questions related to molecular evolution of asexual fungi populations. Thus, gene genealogies based on the coalescent process were used to infer about evolutionary mechanisms that shape the AS population. Sequences of the *Alt a 1* and glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) genes, and rDNA internal transcribed spacer region (ITS) were used to estimate haplotype and nucleotide diversity as well as for the coalescent analyses. The highest number of parsimony informative sites (14), nucleotide diversity (0.007) and the average number of nucleotide differences (3.202) were obtained for *Alt a 1*. Although the highest number of haplotypes (7) was generated for ITS, haplotype diversity was the lowest (0.15) for this region. The genetic differentiation between AS populations was detected for *Alt a 1* sequences. Recombination and selection were not detected, however, subdivision and recent divergence time were inferred from populations associated with hosts. There is no evidence of geographic subdivision, and gene flow is occurring among AS populations. Thus, there is evidence of pathogen clonal lineages associated with hosts, in which gene flow and mutation are the main evolutionary mechanisms affecting AS population.

2.2. Introduction

The genus *Alternaria* encompasses a complex group of saprophytic and pathogenic fungal species (Thomma, 2003). *Alternaria* spp. are frequently reported as allergenic, food spoilers, mycotoxicogenic, opportunistic fungi associated with mycosis in animals and humans, and destructive plant pathogens (Rotem, 1994; Thomma, 2003). The genus is cosmopolitan and species are reported to occur in different ecosystems and geographical regions, from the Antartic soils (Malosso *et al.* 2006) to deserts and the tropics (Grishkan *et al.* 2008). Many *Alternaria* spp. lack a sexual stage (Berbee *et al.* 2003). Nevertheless, variability of the asexual species is high, and the first assessments of variation date back to the 1920´s (Bonde, 1929).

Alternaria solani Sorauer is an asexual plant pathogenic species that causes early blight in potatoes (*Solanum tuberosum* L.) and tomatoes (*Solanum lycopersicum* L.). The disease occurs worldwide (Pscheidt & Stevenson, 1986) and its characteristic symptoms are necrotic lesions in the aerial plant parts. Severe epidemics can lead to major crop losses in short periods of time (Batista *et al.*, 2006). In Brazil, early blight is considered one of the most destructive diseases of potato and tomato, the two most widely/intensively cultivated vegetable crops in the country (Vale *et al.* 1992; IBGE, 2005). 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 (Batista *et al.* 2006). Fungicides are regularly and intensively applied to reduce yield losses, since cultivars that combine early blight resistance and good agronomic/commercial characteristics are not available.

The interactions of the *A. solani* x potato/tomato pathosystems in tropical countries make an interesting case study for addressing questions related to molecular epidemiology and evolution of populations of an asexual fungal species. From an epidemiological perspective, the occurrence of early blight and late blight, caused by the oomycete *Phytophthora infestans* (Mont.) De Bary, another destructive disease of potatoes and tomatoes, must be accounted for in management programs. Therefore, information about the

genetic structure of *A. solani* populations must be gathered to help predicting changes that can affect sensitivity to chemicals, durability of host resistance genes, and differential ecological responses. Understanding the adaptation process and its implications to epidemics may allow preventive actions. The integration of bioinformatics and molecular population genetics can help elucidate the interactions among ecology, genetics and evolution of plant pathogen populations (Carbone & Kohn, 2004).

It is well known that variation in populations of plant pathogens directly affects disease management, especially the use of strategies related to the deployment of resistant varieties and fungicide usage (Milgroom & Fry, 1997; MacDonald *et al.* 2002; Milgroom & Peever, 2003). For late blight, the implications of genetic variation for disease management have been well demonstrated (Kato *et al.* 1997; Grünwald *et al.* 2001; Fernández-Pavía *et al.* 2004). Population biology studies revealed the occurrence of two host-specific clonal lineages of *P. infestans* in Brazil (Reis *et al.* 2003). Each lineage has ecological requirements that affect important stages of the epidemics to the point that forecast systems previously developed had to be modified to provide reliable predictions (Batista *et al.* 2006). To date, no information is available for populations of *A. solani* in Brazil.

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 geographical proximity of tomato and potato fields in many areas, and the constant availability of inoculum (Batista *et al.* 2006) may contribute to increase the number of pathogen generations as compared to temperate climate zones. Furthermore the diversity of wild hosts is also expected to be higher in the tropics. The evolution of populations in such an environment is likely to be largely driven by mutation, selection and gene flow. As mentioned, no sexual structures have been reported for *A. solani,* and large populations reduce genetic drift effects, which could be caused by host unavailability or adverse environmental conditions (severe winter or extremely dry seasons).

Over the years, many studies aimed at investigating variability in

populations of *A*. *solani* and its implications for early blight epidemics. There is evidence for the existence of host-specific populations based on data from studies that analyzed phenotypic and molecular markers (Petrunak & Christ, 1992; Weir *et al.*, 1998; Castro *et al.* 2000; Martínez *et al.*, 2004; Scheuermann *et al.*, 2004; van der Waals *et al.*, 2004). In Brazil, differences in cultural, pathogenic, serologic and electrophoretic characteristics between groups of isolates sampled from potato and tomato, hereafter referred to as potato and tomato isolates, resulted in two groups according to the host of origin (Bóveda, 1986; Fancelli, 1991; Scheuermann *et al.* 2004). However, in these studies the number and size of samples were small and the geographic distribution from where isolates were obtained was restricted. Reliable estimates of parameters related to evolutionary mechanisms have never been estimated for populations of *Alternaria* spp.

The analysis of gene sequences is a suitable source of information to understand the evolutionary history of a pathogen. Despite the importance of early blight, no detailed analysis using gene sequences has been carried out to assess the genetic structure of populations of *A*. *solani* so far. Furthermore, no gene genealogy based in the coalescent process has been conducted to elucidate the evolutionary mechanisms that shape the population structure of *A. solani*. These approaches are helpful to study populations of plant pathogenic fungi composed by complex groups of species or lineages with unknown sexual stage (Taylor *et al.*, 1999). Using this approach, some questions of interest are: is there high molecular diversity between *A. solani* isolates from potatoes and tomatoes? Are these two putative populations under selection? Although no sexual structures have been found associated with *A*. *solani*, is there any evidence of “cryptic” recombination events in the population? What is the contribution of selection, mutation, recombination, and gene flow in shaping genetic variability in the population of *A*. *solani* in Brazil? To address these questions, we sampled isolates from both tomato and potato, grown in several places in Brazil. The data analysis framework included several approaches: molecular population genetics techniques, gene genealogy analyses based on the coalescent, and specific tests for estimating parameters of evolutionary mechanisms in populations of *A*. *solani*.

2.3. Material and Methods

2.3.1. Sampling and isolate maintenance

Major potato and tomato producing areas of Brazil were sampled (Fig. 1). Higher number of fields were sampled in the central and southeastern regions, the main producing areas (Reis *et al.* 2003). According to the early blight epidemics, between one to 20 samples (diseased potato and tomato leaves or fruits) from each crop field were collected and taken to the laboratory where direct or indirect pathogen isolation was conducted (Dhingra & Sinclair, 1995). From colonies grown in V8-agar medium, pure cultures were obtained by either hyphal tip technique or single conidium isolation (Dhingra & Sinclair, 1995). Cultures were maintained in mineral oil at 8^{o}C or in colonized sterilized filter paper at -80^{o}C.

2.3.2. DNA extraction

Isolates were grown in liquid medium (10 g sucrose, 2 g L-asparagin , yeast extract 2 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.1 g, $ZnSO_4.7H_2O$ 0.44 mg, $FeCl_3.6\ H_2O$ 0.48 mg, and $MnCl_2.\ H_2O$ 0.36 mg) 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 CTAB-based protocol (Murray & Thompson, 1980). Depending on the size of the DNA pellet, 30 to 50 µL of TE buffer with RNAase were added, and resuspended at 37°C for 2 h. The integrity and quantity of DNA samples were assessed in agarose gels (1%) stained with ethidium bromide (0.15 µg/mL) using lambda phage as molecular weight.

2.3.3. DNA amplification and sequencing

Partial sequences of the ITS rDNA region (ITS1, 5.8S, ITS2), *Gpd* (glyceraldehyde-3-phosphate dehydrogenase) and *Alt a 1* genes were PCR-amplified using fungal specific primers ITS4 and ITS5 (White *et al.* 1990), gpd1 and gpd2 (Berbee *et al.* 1999), and Alt-for and Alt-rev (Hong *et al.* 2005), respectively. All amplification reactions were performed in a 50 µl reaction volume. PCR conditions for each gene were according to the references provided above. Once checked on 0.8% agarose gels stained with ethidium bromide, PCR products were purified using minicolumns, purification resin, and buffer according to the manufacturer's protocols (Roche - High Pure PCR Product Purification Kit).

The sequencing reactions of amplicons were carried out using Big Dye Terminator v3.1 (Applied Biosystems) or Dyenamic ET Dye Terminator (GE Life Sciences). Primers used to amplify the fragments were used for direct sequencing. Automated sequencing was accomplished at the Seción 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 – BIOAGRO – at the Universidade Federal de Viçosa. Both strands of each fragment were determined for sequence confirmation.

For each isolate, sequences were manually edited using The Staden Package, ver 1.6.0 (Staden, 1996) to generate a consensus sequence. Sequences were aligned with ClustalW (Thompson *et al.* 1994). Alignments were visually checked to ascertain proper matches and to minimize gaps.

2.3.4. Molecular population genetic analyses

Several analyses were carried out to investigate the amount and distribution of genetic diversity in the populations. Isolates were analyzed either as pertaining to a single population or grouped into two populations defined on a host basis: potato and tomato populations. Haplotype diversity, nucleotide diversity, and statistics based on distances to quantify genetic variability within and between populations were carried out with DnaSP 4.0 (Rozas *et al.* 2003). Analysis of molecular variance (AMOVA) using Arlequin 3.11 (Excoffier *et al.* 2005) was conducted to assess population structure. The Kimura 2-parameter distance was set for the analyses and 1000 permutations were executed. The Mantel correlation coefficient (Mantel, 1967) between matrices of geographical and genetic distances was estimated using Arlequin. The significance of the Mantel correlation was assessed using 1000 random permutations of matrices.

2.3.5. Estimating evolutionary mechanisms parameters

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 (Price & Carbone, 2005). 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 (Aylor *et al.* 2006). Compatibility matrices were generated for haplotypes that share a common ancestral to examine the overall support or conflict among variable sites in a DNA sequence alignment. These compatibility matrices, useful for visual identification of recombinant blocks, were generated using SNAP Clade (Bowden *et al.* 2008). The Recombination Detection Program (RDP2, version 2.0) (Martin *et al.* 2005) was used to identify recombinant sequences and recombination breakpoints.

Neutrality tests, Tajima's (Tajima, 1989), Fu and Li (Fu *et al.* 1993),

and Fu's Fs statistics (Fu, 1997) were conducted with DnaSP for *Alt a 1* and *Gpd* genes.

Non-parametric 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 to seqtomatrix using the option recoding indels and excluding infinite-sites violations (Hudson *et al.* 1992a; Hudson *et al.* 1992b). Permutation tests using Permtest were used to analyze population subdivision according to the host plant (potato and tomato) (Hudson *et al.* 1992a; Hudson *et al.* 1992b).

The migration with division (MDIV) analysis was conducted to estimate time of divergence (*T*) and migration rate (*M*) between two subdivided populations. MDIV was used to detect equilibrium migration or subdivision due to shared ancestral polymorphism between the two populations (Nielsen & Wakeley, 2001). Analyses were conducted using five simulation runs with distinct random number seed in each run. The length of the Markov chain was set to 2 million and the burn-in time was 500000.

Simulations of the genealogies of each gene under the coalescent process were executed using GENETREE (Bahlo & Griffiths, 2000). In all situations constant population sizes were assumed, and the genealogy with the highest root probability was chosen to represent the process. Genealogies were inferred using 5 simulations (1 million runs each) with different random seed numbers. 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.

2.4. Results

The total number of sequences analyzed for *Alt a 1* (111), *Gpd* (110), and ITS loci (104) was variable, although each had approximately equal number of sequences for tomato and potato isolates (Table 1). A concatenated data set for the three loci (1612 bp length) was constructed for 88 isolates of *A*. *solani*.

The number of parsimony informative sites (PIS) was small (Table 2) and the highest number was recorded for the *Alt a 1* gene. The estimated mutation rate per site (Theta-W) varied from 0.003 to 0.008. The highest number of haplotypes (7) was generated when analyzing ITS sequences however haplotype diversity was the lowest for this region (0.15) (Table 2). The highest nucleotide diversity (Pi = 0.007) and the average number of nucleotide differences (k = 3.202) were obtained for *Alt a 1*.

The potato population had higher Pi and k than the tomato population. Highest Pi and k were recorded for the potato population when analyzing the *Alt a 1* gene, whereas the lowest Pi and k values were estimated for the ITS region (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. Indels and 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 partition of molecular variation was investigated with four datasets: individual locus (*Alt a 1*, *Gpd*, and ITS) and the three-gene concatenated dataset (Table 4). Variance partition differed according to the gene analyzed: for *Alt a 1* there was greater variation between (50.8%) than within (49.2%) populations. For the *Gpd* gene and the ITS region, variation was greater within population (59.3% and 99.97%, respectively) than between populations (40.7% and 0.03%, respectively). For the concatenated dataset, 42.4% of the variation was due differences between and 57.6% to variation within populations.

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. Although the neutral theory was not rejected in most neutrality tests, the null hypothesis of Tajima's D test was rejected for the *Alt a 1* (potato and tomato populations) (Table 5). Negative and significant D value indicates that population size expansion and/or purifying selection have occurred in *A. solani* populations in the analysis of the *Alt a 1*.

There was evidence for population subdivision of *A. solani* for *Alt a 1* and *Gpd*. Kst values from Hudson's permutation test 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 and divergence time were assessed using *Alt a 1* and *Gpd* loci since no subdivision was detected when analyzing the ITS region. There is evidence of intermediate gene flow between *A. solani* populations on potato and tomato plants for both *Alt a 1* and *Gpd*. The migration rate was estimated to be about one migrant exchanged for each generation between potato and tomato populations (Figures 2 A and C). The subdivision of *A. solani* populations on potato and tomato plants is a recent event, whose divergence time was estimated to be close to zero for *Alt a 1* and *Gpd* (Figures 2 B and D).

There was no evidence of population subdivision based on geographical locations. There was no correlation between geographical 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.

Genealogies for the *Alt a 1, Gpd,* and ITS loci were simulated under the coalescent process with mutation and migration (Figure 3). For the *Alt a 1*, 16 mutations defined five haplotypes of *A*. *solani* (Figure 3). Haplotypes H4 and H5 are comprised mainly of tomato isolates, with three mutations associated with this host in their evolutionary history. Most potato isolates were of haplotype H1, in which eight mutations associated with potato plants were mapped. Haplotypes H2 and H3 are associated with tomato and comprise of a single isolate each.

The pattern of the genealogy tree generated in the coalescent analysis of the *Gpd* locus was similar to the pattern for *Alt a 1*. Six haplotypes were

defined based on the sequences of *Gpd,* and 18 mutations occurred (Figure 3). Haplotypes H2, H3, H4 and H5 are composed mainly of tomato isolates. Haplotypes H1 and H6 are found associated with potato plants. Six mutations were detected in haplotypes exclusively associated with tomato plants (H2 and H5) whereas 10 mutations were simulated for the H1 haplotype, associated exclusively with potato plants (Figure 3).

For the ITS region 13 mutations were estimated to have occurred along the evolutionary history (Figure 3). However, it was not possible to establish a clear relationship between haplotype mutations and host of origin. Most isolates had haplotype H1 (97). Haplotypes H4 and H2 were assigned to three and two isolates, respectively, and each of the other two haplotypes were assigned to a single isolate.

2.5. Discussion

We are not aware of any molecular population genetics studies with genealogical approaches based on the coalescent theory to estimate evolutionary mechanisms in population of any species of the genus *Alternaria*. Also, no characterization of molecular diversity has been conducted using multilocus sequece typing data (MLST) in *A*. *solani*. Using the approaches mentioned above, low genetic diversity was detected in populations of *A. solani* in Brazil. We expected to find high polymorphisms and mutation rates, considering several previous reports of high morphological, physiological, pathogenic and genetic variability in *A*. *solani* populations. In other countries, *A. solani* populations have been described as highly variable, by assessing genetic variation through RAPDs, Random Amplified Microsatellites, and AFLP markers (Weir *et al.* 1998; Martínez *et al.* 2004; van der Waals *et al.* 2004). Although molecular markers are advantageous for scanning wide genomic regions, they are not recommended in genealogical and coalescent analyses. The MLST approach is more suitable to study long-time evolutionary history of populations whereas molecular markers are more adequate to detect recent genetic changes (Melles *et al.* 2007). Differences in the rate of evolution of gene sequences and restriction sites may have contributed to the apparent discrepant results.

A single species, *A. solani*, causes early blight in potato and tomato plants in Brazil, but individuals associated with either host are members of populations that started to diverge recently. Although the highest number of haplotypes was related to ITS sequences, haplotype diversity for this region was the lowest. The high number of isolates with a single haplotype (97 of 104 isolates) contributed to the low diversity. There was not enough fixed variation in the ITS region for the Brazilian isolates of *A*. *solani* that affect potato or tomato. The relatively low number of mutations found in *Alt a 1* and *Gpd* can be due to a recent event of genetic divergence between populations of *A. solani*. This evidence is supported by the time of divergence close to zero estimated in the MDIV test.

Mutation is the main evolutionary mechanism that generates

polymorphisms and its implications to disease management is noticeable (Anderson, 2005; Jolley *et al.* 2005; Hartl & Clark, 2007). For *A*. *solani,* point-mutations in the sequence of the *cyt b* gene resulted in loss of sensitivity to the fungicide azoxystrobin (Pasche *et al.* 2005; Grasso *et al.* 2006). 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 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 (Perez-Losada *et al.* 2006). The evolution of other genes or genomic regions should be quantified to allow better inferences about the mutation rate in populations of *A*. *solani*.

Isolates from geographically distant fields were not more genetically divergent. Brazilian population of *A*. *solani* is comprised of haplotypes widely distributed throughout the main producing regions of potato and tomato. A clonal population structure is strengthened because few haplotypes are widely distributed (Anderson & Kohn, 1995; Avila-Adame *et al.* 2006; Bolton *et al.* 2006; Nebavi *et al.* 2006; Reis *et al.* 2003). In several Brazilian regions, potatoes and tomatoes are grown all year round, not rarely in contiguous fields (Batista *et al.* 2006). Conidia of *A. solani* produced on infected tissues can be dispersed by wind and rainfall (Batista *et al.* 2006) and long distance dispersal is facilitated by the movement of diseased tomato fruits and potato tubers. However, given trade market characteristics, long distance pathogen dispersal is not very likely, since symptomatic infected plant material is eliminated and growers do not regularly use either seeds from diseased tomato fruits or diseased potato tubers as planting materials. 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 geographical subdivision regarding haplotypes.

Population subdivision according to the host of origin was detected when both *Alt a 1* and *Gpd* were analyzed. Separation was more evident when analyzing *Alt a 1* sequences. Even though *Alt a 1* is not widely common among fungal species (Bowyer & Denning, 2006), it was appropriate gene for studying intraspecific variation in *A*. *solani* populations.

In our study, sequences of the *Alt a 1* and *Gpd* genes had more parsimony informative polymorphisms than the ITS region. *Gpd* was reported to evolve at high rates, although *Alt a 1* was shown to evolve at higher rates (Hong *et al.* 2005). The use of MLST was efficient to detect population subdivision even with low polymorphism in sequences of *Alt a 1* and *Gpd*. The robustness of MLST analysis for discriminating fungal populations with low genetic differentiation at the molecular level has been reported previously (Lan & Xu, 2006; Bain *et al.* 2007). The main advantage of this method is the ability to identify specific mutations associated with evolutionary changes in fungal species (Taylor & Fisher, 2003).

Host specificity of *A. solani* on potato and tomato plants were detected in studies of genetic diversity (Petrunak & Christ, 1992; Weir *et al.* 1998; Martínez *et al.*, 2004; Scheuermann *et al.* 2004). Strong evidence of pathogenic specialization was inferred based on large genetic distance between potato and tomato isolates (Martínez *et al.* 2004). However, ecological and genetic factors involved in the association of *A. solani* to potato and tomato plants are unknown. Host specificity of many plant pathogens that reproduce mainly by asexual processes is determined by selection (Anderson & Kohn, 1995; Jimenez-Gasco *et al.* 2004; Wichmann *et al.* 2005). Under highly favorable conditions for pathogen population growth, where climatic variables are adequate and hosts are available almost year-round can contribute to host specificity, since many cycles of pathogen can take place (Sicard *et al.* 2007). In this study, the genetic differentiation between populations was more evident when analyzing the *Alt a 1*. Evidences of purifying selection or population growth was detected in the *Alt a 1*, which may suggest an incipient host specialization process. There are some evidences that this gene can be involved in the pathogenicity of *Alternaria* species to host plants (Cramer & Lawrence, 2003). However, no data are available yet.

Geographic separation and restricted gene flow did not affect subdivision of *A. solani* populations in Brazil. In many regions, potato and tomato fields are physically closely planted, which reduces the plausibility of geographical isolation affecting subdivision. Furthermore, there was no association between geographical and genetic distances. According to the

migration rates detected in the coalescent simulations, gene flow between populations is not very high, but enough to prevent accentuated genetic separation. Thus, the distribution of ancestral polymorphisms associated with a clonal population of *A*. *solani* adapted to each host crop is likely to be the most important factor determining the observed genetic differences that resulted in population subdivision.

Genetic analyses allow inferences about the occurrence of recombination. They can be useful to optimize the prospection of sexual structures and contribute to elucidate the reproductive biology of a pathogen. Prospecting sexual structures of many plant pathogenic fungi is laborious and has low success rate (Taylor *et al.* 1999; Taylor *et al*. 2004). For presumably asexual fungi, such as *A*. *solani*, the search for sexual structures, if any, should be accomplished in different substrates and edaphoclimatic conditions. There was no strong evidence of recombination in the *A. solani* population, and its structure is typically clonal. As mentioned before, continuous availability of host crops and conducive environmental conditions favor asexual reproduction, and detecting a clonal population structure was expected.

The sexual stage reported in some species of *Alternaria* is commonly classified as *Lewia* spp., however, sexual structures are seldom formed (Thomma, 2003). Evidence of some sort of recombination was reported in populations of *A*. *brassicicola* but no sexual structures were found (Bock *et al.* 2005). However, the authors used genetic analyses with lower resolution than the ones used in the present study. We used several techniques to enhance the chances of detecting recombination. Therefore, we consider it safe to conclude that no recombination occurred. Our results support the hypothesis that asexual reproduction is the main process of spore production in *A*. *solani* populations. Epidemiologically, conidia remain the main type of inoculum for early blight epidemics to both potato and tomato fields and these propagules are promptly exchanged in an area-wide pattern.

There are two divergent lineages of *A. solani* associated with host plants, but the number of mutations was not enough to detect a speciation event. The oldest lineage was presumably originated from an ancestral found associated with tomato plants. The sequencing and identification of genomic

regions associated with aggressiveness, host specificity, and adaptation to ecologic niches will help to elucidate the biological aspects of this plant pathogen and its relationship with early blight epidemics on potato and tomato plants. The efficiency of early blight control can be improved with the selection of appropriate types of resistance in breeding programs, and the integration of cultural control practices and a fungicide spray program suitable to *A. solani* populations in Brazil.

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2.7. Figure legends

Fig.1. Sampled areas (dots) located in potato and tomato producing regions of Brazil. From each area leaves or fruits (tomato) with early blight were collected. The sampling sites were georeferenced with a portable GPS device. States from which samples were collected are shaded.

Fig.2. The migration (A and C) and divergence time (B and D) posterior probability distributions between *Alternaria solani* populations on potato and tomato plants estimated for *Alt a 1* (A and B) and *Gpd* (C and D) genes. The data were simulated assuming an infinite sites model: two 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 number seeds 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 while the X axes represent time measured in 2*N* generations. The results were plotted using gnuplot (Williams, T. and Kelley, C., 2004; available online).

Fig.3. Coalescent-based gene genealogies, inferred from GENETREE, depicting the distribution of mutations in *Alt a 1*, *Gpd*, and ITS loci from *A. solani*. For each locus the rooted genealogy was inferred based on 5 different starting random number seeds with 1 million simulations of the coalescent for each run, assuming population subdivision and equal sample size. 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 a1* and *Gpd* genes, and ITS region. The numbers below the genealogy trees designate each distinct haplotype and its frequency associated with potato (P) and tomato (T) plants.

2.8. Tables and Figures

Table 1. Description of *A. solani* isolates collected from potato and tomato producing regions of Brazil.

Region/ State	County	Isolate code[a]	Date collected[c]	GenBank accession[d]		
				Alt a 1	*Gpd*	ITS
SE/MG	Araxá	AS012P, AS013P	2005	N/A, x	x, x	x, x
	Bom Repouso	AS185P, AS187P, AS189P, AS194P, AS203P	2006	x, x, N/A, x, x	x, x, x, x, x	x, x, N/A, x, x
	Bueno Brandão	AS168P, AS169P, AS170P, AS180P	2006	x, x, x, x	x, x, x, x	x, x, N/A, x
	Cajuri	AS130T, AS131T	2000	x, x	x, x	x, x
	Camanducaia	AS216P, AS220P, AS221P	2006	x, x, x	x, N/A, N/A	x, x, x
	Carandaí	AS432T	2005	N/A	x	x
	Conselheiro Lafaiete	AS229T, AS232T, AS242T, AS247P, AS248P, AS250P, AS252T, AS255P, AS532P	2005	x, x, x, x, x, x, x, x, x	x, x, x, x, x, x, x, x, x	x, x, x, x, x, x, x, x, x
	Ibiá	AS016P, AS020P, AS023P,	2005	x, x, x, x	x, x, x, x	x, x, x, x

		AS027P				
	Igarapé	AS158T	N/A	x	x	x
	Ipuiúna	AS360P, AS361P, AS381P	2006	x, x, x	x, x, x	x, x, N/A
	Ituverava	AS409T	2005	x	x	x
	Jaíba	AS420T, AS422T, AS423T	2005	x, x, x	x, N/A, x	x, N/A, x
	Monsenhor Izidro	AS396P	2005	x	x	x
	Poços de Caldas	AS093P	2000	x	N/A	N/A
	Prudente de Morais	AS139T	N/A	x	x	x
	Ressaquinha	AS482T, AS488T, AS489T	2005	x, x, x	N/A, x, x	x, x, x
	Teixeiras	AS080P	1999	x	N/A	N/A
	Tocantins	AS124T	2000	x	x	x
	Viçosa	AS096T, AS098P	2000	x, x	x, x	x, x
SE/RJ						
	Bom Jardim	AS111T	2000	x	x	x
	Cordeiro	AS113T	2000	x	x	x
	Itaperuna	AS120T	2000	x	x	x
	Vassouras	AS005T, AS109T	2000	x, x	x, x	x, x
SE/SP						
	Capão Bonito	AS090T	2000	x	x	x
	Ibiúna	AS052T, AS063T, AS069T	2005	x, x, x	x, x, x	x, x, x
	Itapetininga	AS328T, AS329T, AS331T,	2005	N/A, x, x, x, N/A	x, x, x, x, x	x, x, x, N/A, x

		AS336P, AS337P				
SE/ES						
	Castelo	AS226T, AS554T	2005	x, x	x, x	x, x
	Domingos Martins	AS264P, AS270T, AS272T	2005	x, x, x	N/A, x, N/A	x, x, x
	Marechal Floriano	AS451T	2005	x	x	x
S/PR						
	Araucária	AS301T, AS303T, AS319T	2005	x, x, x	x, x, x	x, x, x
	Colombo	AS317T	2005	N/A	x	x
	Contenda	AS305P, AS307P, AS308P, AS309P, AS310P, AS311P	2005	x, x, x, x, x, x	x, x, N/A, x, x, x	x, N/A, x, x, x, x
	Ponta Grossa	AS002P AS086T, AS087P, AS088P	2000	x, x, x, N/A	x, x, x, x	x, x, x, N/A
	São Mateus do Sul	AS001P, AS084P	2000	X, X	N/A, X	X, X
S/RS						
	Caxias do Sul	AS304T	2005	x	x	x
	Pelotas	AS299T, AS300T	2005	x, x	x, x	x, x
	São Francisco de Paula	AS313P	2005	N/A	x	x
	Taquara	AS160T	N/A	x	x	x
S/SC						
	Alfredo Wagner	AS294T, AS296T	2005	x, x	x, x	x, x
	Caçador	AS293T	N/A	x	x	x

	Lages do Régis	AS297T	2005	x	x	x
	Rancho Queimado	AS550T, AS558P	2006	X, N/A	x, x	N/A, N/A
CW/DF						
	Brasília	AS320T	N/A	x, x, x, x, x, x, x, x	x, x, x, x, x, x, x	x, x, N/A, x, N/A,
		AS347P, AS348P, AS349P, AS350P, AS352P, AS353P, AS358P	2005			x, x
	Ponte Alta	AS288T	N/A	x	x	N/A
	Planaltina	AS453T, AS460T, AS464T, AS465T, AS468T	2005	x, x, x, x, x, x	x, x, x, x, x, x	x, x, x, x, x, N/A
		AS079T	2006			
CW/GO						
		AS258P, AS260P, AS261P, AS263P, AS555P	2005	x, x, x, x, x	x, x, x, x, x	x, x, N/A, x, x
NE/BA						
	Mucugê	AS033P, AS034, AS565P	2005	x, x, N/A	x, x, N/A	x, x, x
NE/CE						
	Guaraciaba do Norte	AS322P, AS323P, AS325P	2005	x, x, x	x, x, x	x, N/A, x

[a] Collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves;

[b] 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).

[c] N/A: date collected not available.

[d] *Alt a 1*: *Alternaria* allergen gene; *Gpd*: glyceraldehyde-3-phosphate dehydrogenase; ITS1, 5.8S and ITS2: rDNA from the internal transcribed spacer region; N/A: sequence not available.

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 ITS region.

Statistic	***Alt a 1***			***Gpd***			**ITS**		
	Total	**Potato**	**Tomato**	**Total**	**Potato**	**Tomato**	**Total**	**Potato**	**Tomato**
Number of sequences (sequence length)	111 (471)	57	54	110 (577)	56	54	104 (564)	51	53
Number of invariable / variable / and parsimony informative sites (PIS)	455/16/14	464/7/6	455/16/14	559/18/13	568/9/5	563/14/13	514/15/9	547/5/2	523/14/8
Theta-W (per site / per sequence)	0.006/3.03	0.003/1.52	0.008/3.51	0.006/3.41	0.003/1.96	0.005/3.07	0.005/2.88	0.002/1.11	0.006/3.09
Total number of mutations	16	7	16	18	9	14	17	6	14
Number of	5	3	5	6	4	5	7	4	5

	Alt a 1			***Gpd***			**ITS**		
haplotypes									
Haplotype diversity (Hd)	0.544	0.464	0.302	0.687	0.553	0.594	0.148	0.115	0.181
Nucleotide diversity (Pi)	0.007	0.006	0.003	0.005	0.004	0.003	0.001	0.001	0.002
Average number of nucleotide differences (k)	3.20	2.75	1.43	2.72	2.36	1.68	0.57	0.31	0.82

Table 3. Distribution of haplotypes and base substitutions in *Alt a 1* and *Gpd* genes, and ITS region of *Alternaria solani*.

Locus	*Alt a 1*																*Gpd*																ITS											
Position	1	1	2	2	3	3	3	3	3	3	3	3	3	4	4		1	1	1	1	1	1	2	2	4		1	1	1	5	5	5	5	5	5	5	5	5					
	6	2	4	1	7	0	3	3	3	4	4	6	7	9	6	6	1	3	3	3	5	5	7	8	9	0	0	0	0	9	9	1	3	5	6	5	6	9	0	1	4	4	4	4	5	5	5
	3	9	7	4	0	5	3	5	7	6	8	1	4	9	1	2	9	1	2	6	3	6	6	8	7	0	1	2	3	0	6	7	4	6	1	0	6	1	4	8	0	3	4	5	0	2	4
Site number										1	1	1	1	1	1	1										1	1	1	1	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	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	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	t	v	v	t	v	t	v	t	t	v	t	v	t	t	t	v	v	t	v	t	t	t	t	t	t	t	t	t	v	t	v	v	v	t	v	v	v
Character type[b]	i	i	-	-	-	i	-	i	-	i	-	i	i	-	-	i	-	i	-	-	i	i	-	i	i	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	i	i	-
H1	.	.	.	.	T	.	.	.	A	.	C	.	.	T	A	.	H1	.	C	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	H1	.	.	.	.	.	.	.	.	.	.	.	.	.
H2	G	T	.	.	.	G	.	.	.	G	.	G	T	.	.	C	H2	T	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	H2	T	T	T	C	A	C	.	.	.	.	.	.	.
H3	G	T	.	A	.	G	T	.	.	G	.	G	T	.	.	C	H3	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	H3	.	.	.	.	.	.	C	G	T	A	.	.	.
H4	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	H4	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	H4	.	.	.	.	.	.	.	.	.	.	C	A	.
H5	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	H5	.	C	T	A	.	.	C	.	.	.	.	.	.	T	C	T	C	T	H5	.	.	.	.	.	.	.	.	.	.	C	A	T
																	H6	.	C	.	.	C	.	.	.	T	G	T	C	A	.	.	.	.	.												

[a] t: transitions; v: transversions.

[b] i: informative sites; -: uninformative sites.

[c] H: Haplotype.

Table 4. Analysis of molecular variance (AMOVA) of *Alternaria solani* populations using Kimura 2-parameter distance for three loci (*Alt a 1*, *Gpd1* and ITS) and a concatenated data set (*Alt a 1* + *Gpd1* + ITS).

Locus	**Source of variation**	**D.F.***	**Sum of squares**	**Components of variance**	**Percentage of variation**	**Φst (P value)**
Alt a 1						
	Between Populations	1	62.06	1.10	50.78	
	Within Populations	109	116.19	1.07	49.22	
	Total	110	178.25	2.17		0.51 (P<0.0001)
Gpd1						
	Between Populations	1	39.45	0.70	40.67	
	Within Populations	108	110.11	1.02	59.33	
	Total	109	149.56	1.72		0.41

						(P<0.0001)
ITS						
	Between Populations	1	0,29	0.00008	0.03	
	Within Populations	102	29.22	0.2865	99.97	
	Total	103	29.51	0.2866		0.00029 (P=0.55)
Concatenated						
	Between Populations	1	72.41	1.60	42.41	
	Within Populations	86	186.78	2.17	57.59	
	Total	87	259.19	3.77		0.42 (P=0.0001)

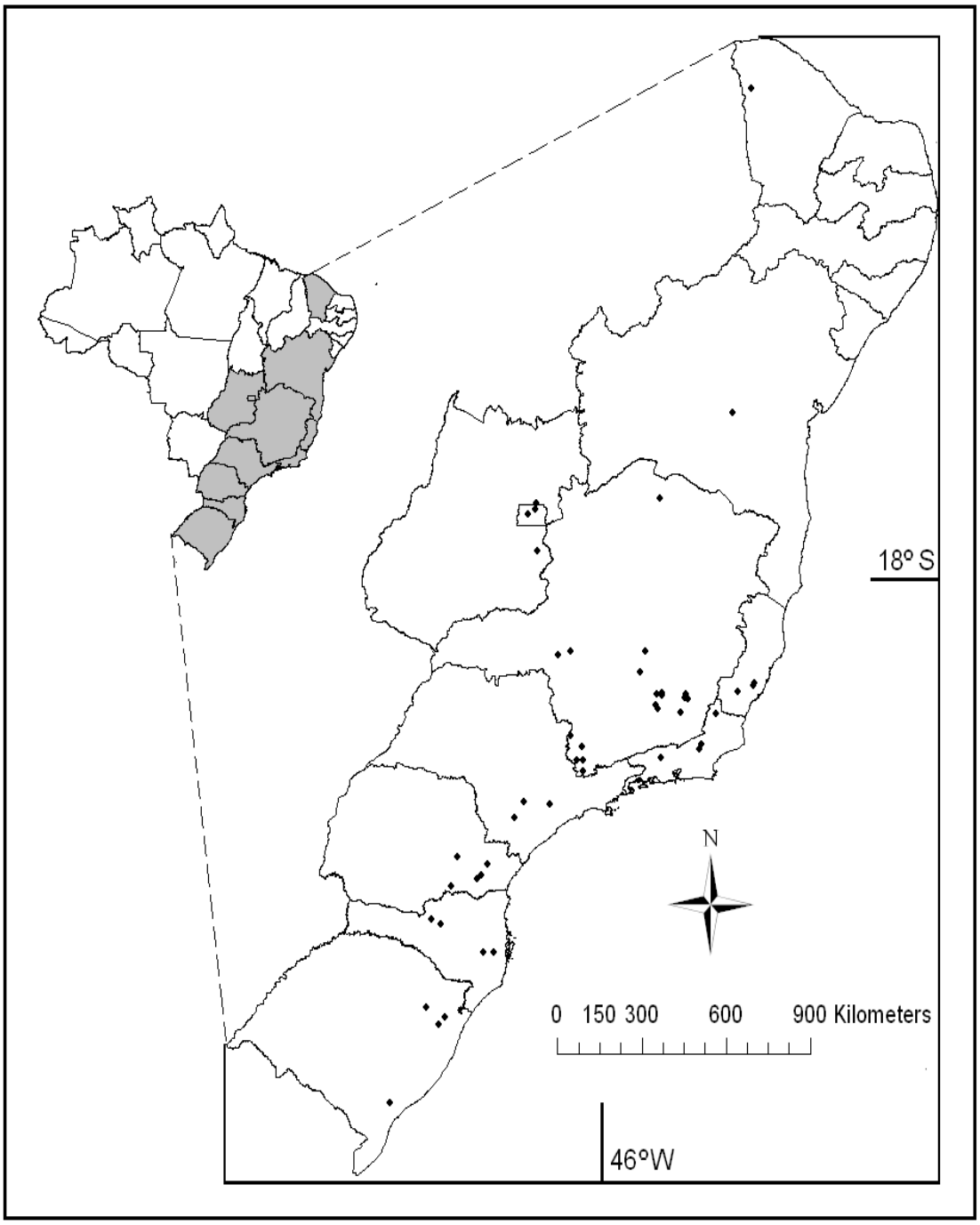
*Degrees of freedom

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.

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

NS = not significant

NA= not applicable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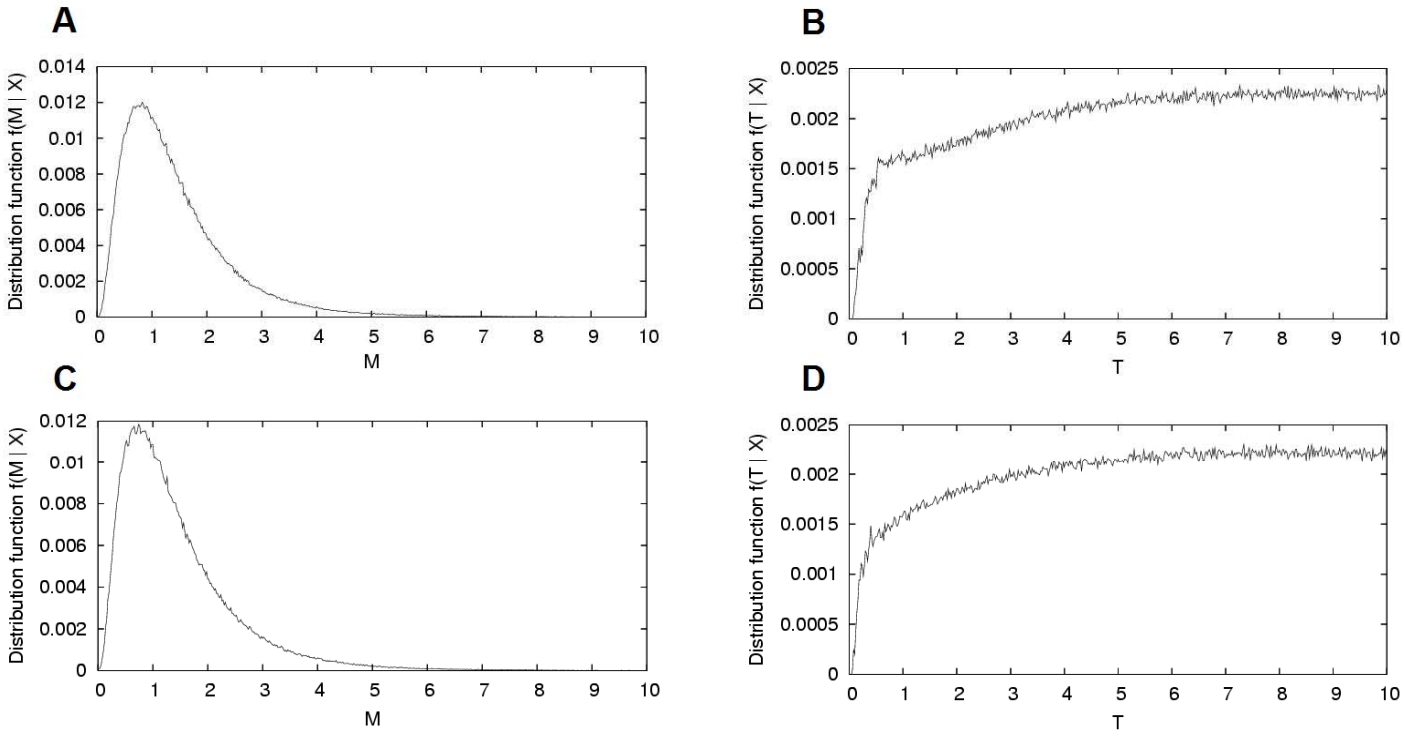
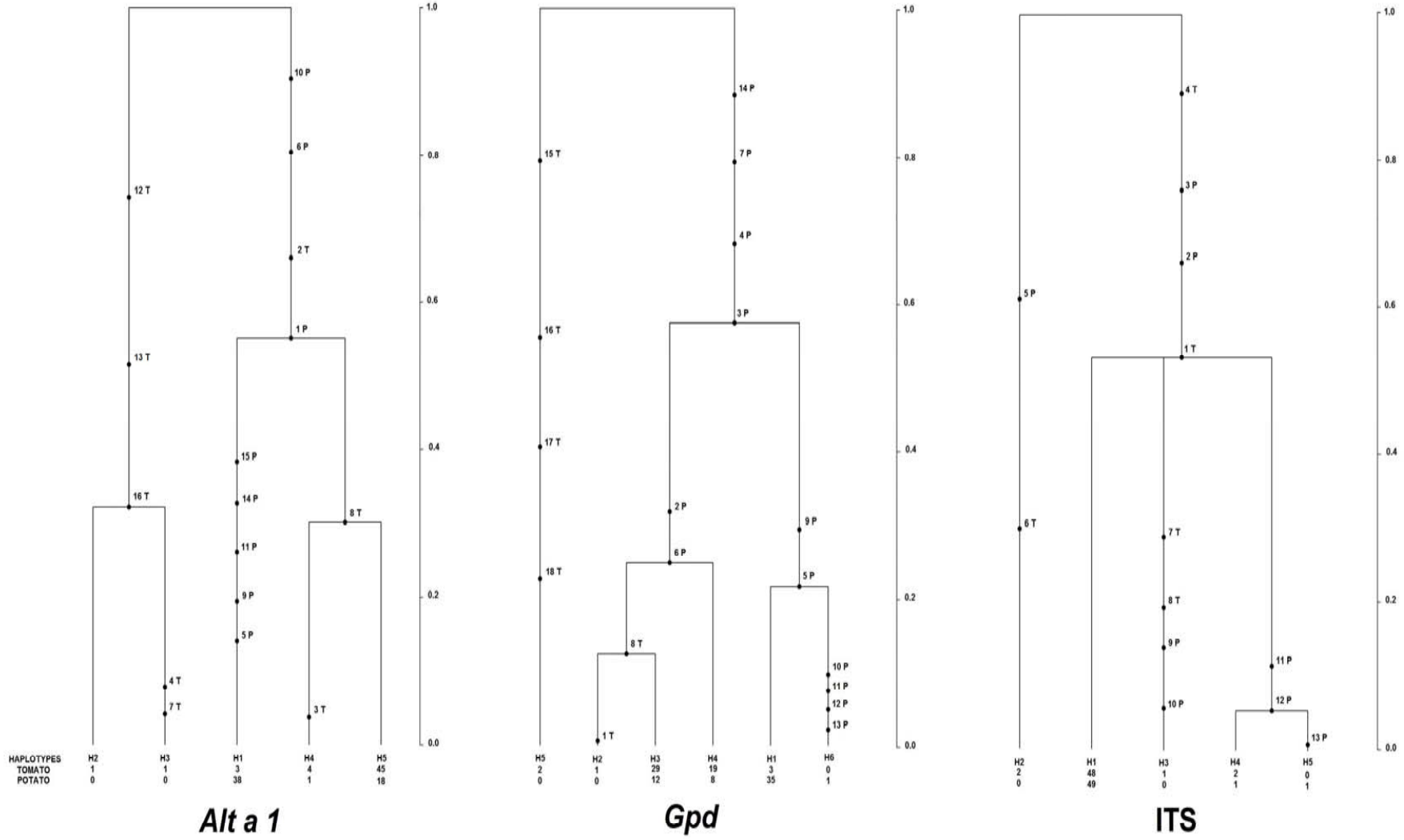
0 150 300 600 900 Kilometers
46ºW


A
Distribution function f(M | X)
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0 1 2 3 4 5 6 7 8 9 10
M
B
Distribution function f(T | X)
0.0025
0.002
0.0015
0.001
0.0005
0
0 1 2 3 4 5 6 7 8 9 10
T
C
Distribution function f(M | X)
0.012
0.01
0.008
0.006
0.004
0.002
0
0 1 2 3 4 5 6 7 8 9 10
M
D
Distribution function f(T | X)
0.0025
0.002
0.0015
0.001
0.0005
0
0 1 2 3 4 5 6 7 8 9 10
T


HAPLOTYPES H2 H3 H1 H4 H5
TOMATO 1 1 3 4 45
POTATO 0 0 38 1 18
12 T
13 T
16 T
4 T
7 T
10 P
6 P
2 T
1 P
15 P
14 P
11 P
9 P
5 P
8 T
3 T
1.0
0.8
0.6
0.4
0.2
0.0
Alt a 1
H5 H2 H3 H4 H1 H6
2 1 29 19 3 0
0 0 12 8 35 1
15 T
16 T
17 T
18 T
1 T
14 P
7 P
4 P
3 P
2 P
6 P
8 T
9 P
5 P
10 P
11 P
12 P
13 P
Gpd
H2 H1 H3 H4 H5
2 48 1 2 0
0 49 0 1 1
4 T
3 P
2 P
5 P
1 T
6 T
7 T
8 T
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11 P
12 P
13 P
ITS

3. A phylogenetic-based approach to investigate the association of Alternaria solani to potato and tomato plants.

3.1. Abstract

The evolutionary history of *Alternaria solani* with its main hosts, potato and tomato was studied with the objective of seeking a better understanding of the host-pathogen interaction and its implications for early blight epidemics. Isolates were sampled from the Northwest, Central-West, Southeast, and South regions of Brazil. Phylogenetic analyses were conducted using partial sequences of the *Alt a 1* and glyceraldehyde-3-phosphate dehydrogenase genes (*Gpd*), and the internal transcribed spacer region (ITS). In addition, RAPD marker profile was combined with *Alt a 1* and *Gpd* sequences for parsimony analysis. The congruence of the combined dataset (*Alt a 1*, *Gpd*, and RAPD) was assessed by the incongruence length difference test (ILD test) and the partitioned Bremer support (PBS). Association of AS with potato and tomato plants was detected when analyzing *Alt a 1* and *Gpd* loci with significant bootstrap (>70) and Bayesian Posterior Probability (>95) values. Although the ILD test was significant (P=0.01) for the combined dataset, positive values of PBS were obtained for a node which clustered AS lineages associated with potato and tomato plants. There is evidence of association of lineages of *A. solani* with hosts plants.

3.1. Introduction

The elucidation of the evolutionary history of plant pathogens associated with host species is crucial to understand the interaction between pathogens and hosts, and their relationships with plant disease epidemics (Roy, 2001). Such interaction drives the co-evolution among virulence and aggressiveness factors of plant pathogens and multi-defense mechanisms of host plants, with the potential to shape the evolutionary dynamics of host-pathogens populations in the agroecosystem (Chisholm *et al.* 2006). Despite being ubiquitous fungi that cause infections and allergenic disorders in humans (Denis *et al.* 2007) and affects economically important crops worldwide (Rotem, 1994), no detailed intraspecific study has been conducted with *Alternaria* species. Most phylogenetic studies conducted aimed at elucidating the systematic and evolutionary history of different species of *Alternaria* (Pryor & Bigelow, 2003; Hong *et al.* 2005). None of these studies used sequence data to investigate the genealogical processes and the differences among individuals of a given species and, in the case of plant pathogenic species, its implications for epidemics in host plants.

Host specific lineages of some *Alternaria* species have been detected in phylogenetic studies (Peever *et al.* 2002; Peever *et al.* 2005). In a phylogeographical study, the distribution of lineages allowed inferences about the chronological pattern of citrus brown spot spreading into citrus-growing areas of the world (Peever *et al.* 2002). In lieu of the progress in understanding host specificity of *Alternaria* spp. on citrus, there is insufficient information about the association of other *Alternaria* species with their host plants, particularly in the *Alternaria solani* x potato – tomato pathosystems.

A. solani causes early blight on potato and tomato in growing regions located in the tropics and in temperate zones. Host-specific clonal populations of *A. solani* have been commonly reported. In the United States, RAPD markers were used to quantify the genetic variation among isolates of *A. alternata* and *A. solani* (Weir *et al.* 1998). There was high genetic diversity among isolates, but pathogenic specialization was inferred based on large genetic distances between isolates of *A. solani* obtained from potato and tomato plants (Weir *et al.* 1998). Similar results were obtained when

analyzing isolates from several countries (Brazil, Cuba, Greece, Russia, Turkey, and USA) through AFLPs markers (Martínez *et al.* 2004). Preliminary analysis of Brazilian isolates of *A*. *solani* using RAPD markers also revealed differentiation between isolates collected from potato and tomato fields (Scheuermann *et al.* 2004). Despite the availability of information about the genetic variability of *A. solani*, no thorough intraspecific phylogenetic analyses based on DNA sequence data has been conducted so far, and the genealogy of this pathogen remains largely unknown.

The acquisition of large and diverse molecular data sets is recommended if reliable inferences are to be drawn from phylogenetic analyses (Libkind *et al.* 2007). Nowadays, several studies oriented towards the understanding of evolutionary history of microorganisms are being carried out using a multilocus sequence typing (MLST) approach (Taylor & Fisher, 2003; Melles *et al.* 2007). However, low levels of polymorphism or parsimony informative sites (PIS) in some genes can limit inferences regarding the evolutionary relationships between lineages within species (Lan & Xu, 2006). Particularly to *A*. *solani*, variation in the genes sequenced and available so far is relatively low, with limited polymorphisms and PIS. Thus, for some phylogenetic questions, at the intraspecific level, it would be necessary to either sequence variable genomic regions or to complement sequence data with polymorphic markers.

Morphological characters (Lambkin, 2004) as well as molecular markers and DNA sequence data can be combined and analyzed to address evolutionary questions. Molecular markers can complement phylogenetic studies (Sharma *et al.* 1996). When combined with MLST, these markers can increase the resolution of phylogenetic trees (Sola *et al.* 2001), because MLST data generally provide information about long term evolutionary processes, while molecular markers (such as RAPDs and AFLPs) reflect more recent changes (Melles *et al.* 2007) Furthermore, the combined use of MLST and molecular markers is an alternative method to study intraspecific evolutionary history of fungal species without information about their genomes.

Although the use of a diverse data set is desirable for phylogenetic inferences, the main limitation is to measure the support from each data

partition to a given node of interest in the tree generated from the combined data matrices (Creer *et al.* 2003; Lambkin, 2004). Bootstrapping and jack-knifing are often used for this purpose. However, the statistical interpretation and confidence limits of these resampling methods can be prone to errors (Bremer, 1994). A different method named partitioned Bremer support (PBS) has been suggested for measuring the consistency of each data partition in branching patterns of parsimonious trees (Bremer, 1994; Baker & DeSalle, 1997). PBS allows the identification and location of congruence or disagreement between data partitions in nodes (clades), whereas tests such as ILD (Incongruence Length Differences) estimate overall levels of conflict among the dataset considering the whole phylogenetic tree (Bremer, 1994; Lambkin *et al.*, 2002). This approach has been implemented mainly in phylogenetic studies of animals such as viper (Creer *et al.* 2003), bee flies (Lambkin, 2004) and stick insects (Whiting *et al.* 2003). Few studies have been conducted using PBS in phylogenetic analyses of plant pathogens (Feau *et al.* 2006).

Phylogenetic analyses of *A*. *solani* isolates using MLST individually and combined with molecular markers can help elucidating two important points: 1. An evolutionary question about the causing agent of early blight on host plants, and 2. The potential existence of lineages associated with potato and tomato crops. A new species of *Alternaria, A*. *tomatophila*, was recently reported associated with early blight on tomatoes (Simmons, 2000). The author suggested that *A*. *tomatophila* be considered the causal agent of tomato early blight, whereas *A*. *solani* was associated with early blight on potato. These species were distinguished based mainly on morphological characteristics of conidia produced in culture medium. *A*. *tomatophila* has been detected in USA, Australia, New Zealand, and Venezuela (Simmons, 2000), but there is no information about its occurrence in Brazil. A sequence of the ITS1, 5.8S, ITS2 region (ITS) from a purportedly *A. tomatophila* specimen is available (GenBank AM237289), but we are not aware of any phylogenetic analysis of this species. The molecular phylogenetic approach can thus be used to assess the occurrence of *A. tomatophila* in Brazil; the relatedness of this species with *A*. *solani*; and to test a putative association of *A. solani* and *A. tomatophila* with potato and tomato host plants.

Based on previous phylogenetic studies with *Alternaria* spp., we chose the ITS1, 5.8S, ITS2 region (ITS), and the *Gpd* (Pryor & Bigelow, 2003), and the *Alt a 1* (Hong *et al.* 2005) genes to study the molecular phylogeny of *A. solani* isolates collected from the main producing regions of potato and tomato in Brazil. Additionally, we used RAPD marker data based on primers previously screened to generate the fingerprinting of *A. solani* isolates (Scheuermann *et al.* 2004). The RAPD data was combined with MLST for parsimony analysis to evaluate evidence of association between *A. solani* lineages on potato and tomato plants.

3.2. Material and Methods

3.2.1. Sampling, isolation, and preservation of isolates

A. solani isolates were obtained from samples collected in the main tomato and potato producing regions of Brazil (Lourenço Jr. *et al.* manuscript in preparation). Diseased potato or tomato leaflets were collected, taken to the laboratory, and incubated for 24 h in moist chambers. Under a dissecting microscope (80X), conidia were transferred to water-agar (WA) in Petri dishes. After 12 h at 25°C, individualized germinated conidium was transferred to Potato-Dextrose-Agar medium (PDA) in Petri dishes. For non-sporulating lesions, indirect isolation was as follows: fragments from the lesions margins were transferred to 70% ethanol for 30 s, disinfested with 2% sodium hypochlorite for 2 min, washed twice in sterilized distilled water for 2 min, air-dried under a flow hood on sterilized filter paper, and transferred to PDA in Petri dishes. Plates were maintained at 25°C with a 12 h photoperiod. After colony development, monoconidial isolates were obtained by transferring mycelial plugs to V8-agar in Erlenmeyer flasks that were continuously shaken at 110 rpm at 26°C. After 7 days, mycelium was triturated and transferred to PDA medium in Petri dishes that were kept uncovered at 25°C under near-UV light for 12 h. After 3 days, a conidial suspension was prepared, diluted, and pipetted to slides with WA. The slides were kept inside Petri dishes at 25°C. After 12 h, the slides were removed and inspected under a compound microscope (400X). Germinated and individualized conidia were transferred to PDA medium in Petri dishes to allow monoconidial colony development (Rodrigues, 2005).

For preservation purposes, mycelial plugs from monoconidial colonies were transferred to PDA medium covered with fragments of sterilized filter paper in Petri dishes. Colonized filter paper fragments were removed from PDA and transferred to sterilized envelopes that were stored at -20°C (Borromeu *et al.* 1993).

3.2.2. DNA extraction and sequencing

Isolates were grown in liquid medium (10 g sucrose, 2 g L-asparagin, 2 g yeast extract, 1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6\ H_2O$, and 0.36 mg $MnCl_2.\ H_2O$) in Erlenmeyer flasks under continuous agitation at 26°C (Alfenas *et al.* 1998). After 5-7 days, mycelia were washed with distilled water, set on filter paper to dry, and macerated in mortar with liquid nitrogen until formation of a fine powder. Genomic DNA was extracted from 50 mg of mycelium powder (Murray & Thompson, 1980). The integrity and quantity of DNA samples were assessed in agarose gels (1%) stained with ethidium bromide (0.15 µg/mL) using lambda phage as molecular weight.

Partial sequences of the ITS region and of the *Alt a 1* and *Gpd* genes were amplified using fungal specific primers ITS4 and ITS5 (White *et al.* 1990), *Gpd*1 and *Gpd*2 (Berbee *et al.* 1999), and Alt-for and Alt-rev (Hong *et al.* 2005), respectively. All amplification reactions were performed according to the references provided above. PCR fragments were purified using minicolumns, purification resin, and buffers according the manufacturer's protocols (Roche - High Pure PCR Product Purification Kit).

The sequencing reactions were carried out directly from PCR-amplified fragments using Big Dye Terminator v3.1 (Applied Biosystems) using the same primers used for PCR amplification. Automated sequencing was accomplished at the Seción 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 – BIOAGRO – at the Universidade Federal de Viçosa. Both strands of each fragment were determined for sequence confirmation. Novel sequences generated in this study were submitted to GenBank (Table 1).

For each isolate, sequences obtained were manually edited to generate a consensus sequence using The Staden Package, ver 1.6.0 (Staden, 1996). Sequences were aligned with ClustalW (Thompson *et al.* 1994), and alignments were visually checked to ascertain proper matches

and minimize gaps.

3.2.3. RAPD analysis

Reactions were adjusted to final volume of 15 µL with 20 ng of DNA, 1.5 mM of $MgCl_2$, 0.15 U of Taq DNA polymerase, 0.5 mM of primer, and 0.2 mM of dATP, dCTP, dGTP, and dTTP (Scheuermann *et al.* 2004). Primers P203 (5'CACGGCGAGT 3'), P284 (5'CAGGCGCACA 3') (Weir *et al.* 1998), OPC15 (5'GACGGATCAG 3'), OPC20 (5'ACTTCGCCAC 3'), and OPE14 (5'TGCGGCTGAG 3') (Scheuermann *et al.* 2004) were selected. The reaction conditions were 94°C for 30s (denaturation), 35°C for 1 min (annealing), 72°C for 1 min (extension), and the final extension at 72°C for 7 min (Scheuermann *et al.* 2004). The amplification was carried out with 40 cycles, and fragments were separated in agarose gel for 7-8 h. Polymorphic bands were scored manually.

3.2.4. Phylogenetic analysis of ITS region, and Alt a 1 and Gpd genes

Because the presence of recombinant sequence can influence the branching pattern of phylogenetic trees (Martin *et al.* 2005), analyses to detect recombination events were carried out before phylogenetic reconstruction. The Recombination Detection Program (RDP2, version 2.0) (Martin *et al.* 2005) was used to identify recombinant sequences and recombination breakpoints.

Neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference were compared to evaluate phylogenetic relationships among *A. solani* isolates. For NJ, the evolutionary distance was calculated using the Kimura's 2-parameter model (Kimura, 1980) and trees were constructed using Molecular Evolutionary Genetics Analysis program (MEGA, version 4) (Tamura *et al.* 2007). The pairwise-deletion option was chosen when reconstructing phylogeny with NJ. Support of phylogenetic trees was estimated with 1000 bootstrap replicates in all phylogeny tests. Phylogenies were reconstructed based on datasets comprised of single and

concatenated partial sequences of the ITS region, *Gpd* and *Alt a 1* genes. SNAP Combine (Aylor *et al.* 2006) was used for concatenating multiple aligned ITS, *Gpd*, and *Alt a 1* sequences. The congruence between ITS region, *Alt a 1* and *Gpd* genes was evaluated by the incongruence length difference (ILD) test implemented in Phylogenetic Analysis Using Parsimony Software (PAUP* version 4.0b10, Sinauer Associates Inc., Sunderland, Massachusetts) (Swofford, 2003).

MP and ML analyses were performed with PAUP and Phylogenies by Maximum Likelihood (PhyML) (Guindon & Gascuel, 2003), respectively. MP was through a heuristic search with 10 random sequence repetitions and tree-bisection-reconnection (TBR) branch-swapping. Alignment gaps were treated as missing information. For ML phylogeny reconstruction, the DNA substitution model was chosen based on the Akaike information criterion (AIC) and the likelihood ration test (lnL) implemented in the Modeltest 3.7 (Posada & Crandall, 2001).

Bayesian analyses were performed using MrBayes 3.1.2 (Bayesian Inference of Phylogeny) (Ronquist & Huelsenbeck, 2003). The DNA substitution model was chosen based on the AIC criterion of MrModelTest (Nylander, 2004). Markov chains were initiated from a random tree with 2,000,000 generation runs. Each run consisted of four simultaneously incrementally heated Markov chains. The 50% majority rule consensus tree was used to obtain the Bayesian posterior probabilities (BPP). Clades supported by BPP greater than or equal to 0.80 were considered significant.

Gpd, *Alt a 1,* and ITS sequences from *Stemphylium vesicarium*, *A. alternata*, and *A. solani*, available in GenBank, were included in the phylogenetic analyses (Table 1). The sequences of *S. vesicarium* were used as outgroup in all analyses. A partial sequence of ITS from *A. tomatophila* was also included when necessary (Table 1).

3.2.5. Neutrality tests

Statistical methods to detect natural selection as a factor leading to the association of *A. solani* isolates with potato and tomato were conducted for the ITS region, *Alt a 1* and *Gpd* genes. Tests of neutrality based on Tajima's D (Tajima, 1989), Fu and Li's D* and F* (Fu & Li, 1993), and Fu's Fs methods (Fu, 1997) were conducted using DnaSP (Rozas *et al.* 2003). Significant results obtained from these tests, except of Fu's method, suggest that balancing selection has affected the pattern of parsimony informative sites detected for the *Alt a 1* and *Gpd* genes. For the ITS region, only Tajima's method was used in the neutrality test.

3.2.6. Partitioned Bremer Support (PBS) analyses for MLST and RAPD

PBS analyses were conducted using the SNAP Workbench (Price & Carbone, 2005). MLST and binary matrices from RAPD were combined in a multilocus dataset using SNAP Combine (Aylor *et al.* 2006). The multilocus dataset was converted to haplotypes excluding indels and sites that violated the infinite sites model. MP analyses were implemented in PAUP, as described above.

PBS values were tabulated for each node in the combined phylogenetic tree using TreeRot (Sorenson & Franzosa, 2007). These values reflect the consistency or conflict from each data partition with a particular node (Bremer, 1994). If PBS is positive for a given partition, it means that a node is supported by that partition, whereas negative PBS values suggest evidence for an alternative node not present in the current (combined) tree. Conflicting PBS values, i.e. negative for one partition and positive for another at a single node imply that characters in the different data partitions disagree relative to the formation of that node (Lambkin, 2004).

Bootstrap test was performed with 1000 replicates in the combined phylogenetic tree. The results were compared to those from the PBS analysis. *S. solani* was used as outgroup in the parsimony analysis using MLST combined with RAPD.

3.3. Results

No recombinant sequences or recombination breakpoints in the ITS region, and in the *Gpd* and *Alt a 1* genes, either individually or in the concatenated sequences, were detected (data not shown).

3.3.1. Phylogenetic analysis of the ITS region, Alt a 1 and Gpd genes

Isolates were chosen based on geographical origin and host plant (Table 1). Sequences from 111, 110, and 104 isolates were obtained for *Alt a 1* (471 bp), *Gpd* (584 bp) and the ITS region (578 bp), respectively.

Similar tree topologies were obtained with all phylogenetic reconstruction methods. MP trees were chosen to depict the evolutionary relationships among *A. solani* isolates (Figs. 1, 2, 3, and 4). MP analyses produced similar results with *Alt a 1*, *Gpd*, ITS, and concatenated sequences. The alignment of Alt a 1 sequences from *A. solani*, *A. alternata*, and *S. vesicarium* resulted in 471 characters with 20 parsimony-informative and 81 parsimony-uninformative characters. The total number of rearrangements was 260766 and the score of the best tree was 107. The consistency (CI), homoplasy (HI), retention (RI), and rescaled consistency (RC) indices were 0.9907, 0.0093, 0.9962, and 0.9869, respectively. Gpd sequences resulted in 584 characters with 28 parsimony-informative and 102 parsimony-uninformative characters. The total number of rearrangements was 1687848, and the score of the best tree was 149. The CI, HI, RI, and RC values were, 0.9866, 0.0134, 0.9911, and 0.9778, respectively. The *A. tomatophila* sequence was included in the alignment of ITS, which resulted in 578 characters with 15 parsimony-informative and 53 parsimony-uninformative characters. The total number of rearrangements was 20781448 and the score of the best tree was 81. The CI, HI, RI, and RC values were 0.9630, 0.0370, 0.8846, and 0.8519, respectively. The analyses with the concatenated dataset resulted in 1633 characters with 63 parsimony-informative and 230 parsimony-uninformative characters. The total number of rearrangements was 165320, and the score of the best tree was 335. The CI,

HI, RI, and RC values were, 0.9701, 0.0299, 0.9733, and 0.9442, respectively. However, the ILD test was significant (P=0.01).

Two well-supported clades with significant bootstrap and probability values were detected for *Alt a 1* sequences (Fig. 1). Clade 1 consists of 38 *A. solani* isolates from potato and only three isolates from tomato. Most isolates from tomato (49 individuals) were grouped in clade 2, which includes 19 isolates from potato. The *A. solani* sequence obtained from GenBank (AY563299), a tomato isolate collected in Mexico (ATCC 58177), clustered in clade 2. A subclade with isolates AS113T, AS131T, AS293T, AS304T, and AS348P was formed in clade 2. Additionally, a paraphyletic clade with clade 2 comprises two isolates from tomato (AS252T and AS320T).

The phylogenetic analysis of *Gpd* sequences resulted in the formation of two clades with high BPP and bootstrap values similar to *Alt a 1* tree (Fig. 2). Clade 1 was mostly comprised of *A. solani* isolates from potato (36 individuals), with only 3 isolates from tomato. Clade 2 had 49 isolates from tomato and 20 isolates from potato. The *A. solani* sequence obtained from GenBank (AY278807), a tomato isolate (ATCC 58177), clustered in a subclade generated at clade 2. Isolates AS320T and AS252T are paraphyletic with clades 1 and 2.

The phylogenetic trees constructed based on ITS sequences resulted in the formation of a single clade that is paraphyletic with AS320T and AS252T isolates (Fig. 3). ITS sequences of *A. solani* (AF229475; ATCC 58177) and *A. tomatophila* (AM237289) obtained from GenBank clustered together.

The resulting phylogenetic trees for the concatenated data set had two well-supported clades, similar to the ones observed for the *Alt a 1* and *Gpd* based trees (Fig. 4). Clade 1 consisted mainly of *A. solani* isolates from potato (25 individuals), with 2 isolates from tomato. Clade 2 had mostly *A. solani* isolates from tomato (43 individuals), plus 16 isolates from potato. A subclade was formed in clade 2 constituted by 26 isolates from tomato and 9 from potato. Concatenated sequences of *A. solani* obtained from GenBank (ATCC 58177) clustered in this subclade. Isolates AS320T and AS252T are paraphyletic with clades 1 and 2.

3.3.2. Neutrality tests

Neutrality was rejected when assessed by Tajima's statistic method for *Alt a 1* (potato and tomato populations) and the ITS region (total, potato, and tomato populations) (Table 2). As most neutrality tests for *Alt a 1* and *Gpd* were not significant, there was no strong evidence that selection has affected the pattern of parsimony informative sites detected in these loci. For the ITS region, selection has affected the pattern of PIS throughout the evolution of *A. solani*. However, the possible occurrence of selection in this region had not influenced on the association of *A. solani* with host species.

3.3.3. Partitioned Bremer Support (PBS) analyses for MLST and RAPD

A total of 21 haplotypes were generated after concatenating *Alt a 1* and *Gpd* sequences (MLST) with RAPD markers from 90 *A. solani* isolates (Table 1). As no association was detected for ITS sequences, and this is a non-coding region, we did not include it in the combined analyses with MLST and RAPD.

The concatenation of MLST with RAPD markers resulted in 1130 characters with 44 parsimony-informative and 176 parsimony-uninformative characters. The total number of rearrangements was 21208, and the score of the best tree was 286. The CI, HI, RI, and RC values were, 0.7867, 0.2133, 0.6980, and 0.5491, respectively.

The ILD test result was significant (P = 0.01), and two distinct clades with significant PBS and bootstrap values were formed for the concatenated dataset (Fig. 5). Clade 1 is predominantly constituted by potato isolates (27). Only six individuals in clade 1 were obtained from tomato plants. Clade 2 is constituted mainly by isolates from tomato. This clade had 40 tomato and 16 potato isolates.

Although nodes 7 and 14 have positive PBS values for the concatenated dataset, there are conflicts between these partitions in four nodes (nodes 6, 9, 10, 13), with negative and positive PBS values for MLST

and RAPD, respectively (Fig.5). Negative PBS values estimated for MLST indicate that this data partition supports alternative resolutions available on trees without those nodes. Nodes 3, 5, and 8 are supported only by RAPD markers, whereas PBS values for nodes 1, 2, 4, and 12 were zero for all data partitions (Fig.5). When PBS values are zero for one or all data partitions, the node is not supported in a phylogenetic tree due to lack of phylogenetic information or within dataset incongruence (Lambkin *et al.* 2002).

3.4. Discussion

There is evidence of association of *A. solani* with potato and tomato plants in Brazil. The use of phylogenetic approaches using MLST individually and combined with RAPD marker allowed the distinction of two lineages, one associated with tomato and another with potato plants. Compared to the concatenated multilocus dataset, phylogenetic trees with MLST data had higher resolution and support by both bootstrap and BPP values. Despite the differences regarding tree support and resolution, the parsimonious tree obtained with MLST and RAPD marker were congruent with the separation of *A. solani* lineages from potato and tomato plants.

There was incongruence among data partitions used in the reconstruction of phylogenetic trees. The level of homoplasy (HI = 0.2133) and the incongruence among data partitions contributed to the low overall support of the tree generated from multilocus data set compared with MLST data. Homoplasy, rate heterogeneity among nucleotide sites, and differences in evolutionary rates increase the conflict among data partitions (Felsenstein, 2004). In this study, we estimated different models of DNA substitution for *Alt a 1*, *Gpd*, and ITS sequences which suggest that these loci are differentially affected by evolutionary mechanisms and subjected to distinct rates of change (Nylander, 2004; Posada & Crandall, 2001). These differences could have accounted for or contributed to incongruence when analyzing the concatenated sequences. The disagreement among data partitions was also detected when the dataset of MLST was combined with RAPD. Although the genomic origin of RAPD bands amplified from *A. solani* is unknown, differences in mutation rates in these genomic regions compared to the *Alt a 1* and *Gpd* loci can be involved in the incongruence detected in the ILD test.

The use of the PBS method allowed proper assessment of the contribution of concatenating MLST with RAPD marker for the study of evolutionary relationships between *A. solani* lineages. Despite the overall incongruence of data partitions and the disagreement of MLST and RAPD data for some nodes of the phylogenetic tree, the PBS analysis was efficient to identify a node supported by both datasets in which the two lineages

associated with potato and tomato plants were clustered.

Two hypotheses can be derived from our study: *i*. differences in fitness contribute to host specificity, and *ii*. predominance of individuals adapted to certain ecological conditions – ecotypes, mainly temperature, affects the association of isolates with either potato or tomato. Host specificity is commonly reported in plant pathogens in which differences in aggressiveness and environmental conditions are the main factors involved in the association of lineages with host species (Antonovics & Alexander, 1989; Sicard *et al.* 2007). Differences in the aggressiveness on tomato plants and mycelial growth in culture medium under different temperature levels have been reported for *A*. *solani* isolates from potato and tomato plants in Brazil (Fancelli, 1991). However, a low number of isolates and lack of rigorous quantification of fitness components prevent further conclusions.

Selection can occur due to physiological and pathogenic variability of *A. solani* isolates. An important component of the physiology of fungal plant pathogens is toxin production (Rotem, 1994). *A*. *solani* produces non-specific toxins such as alternaric acid and zinniol, which can affect infection and colonization of host plant tissues (Rotem, 1994). However, there is no information about the synthesis and biological function of these toxins in *A. solani* and their relationship with pathogenicity to potato and tomato. *A. solani* produces toxins and enzymes that may be involved in pathogenicity that could lead to host-specificity, but this aspect has not been elucidated yet. To date, there is no report of quantitative assessments of epidemiological components of *A*. *solani* from potato and tomato plants that support the involvement of toxin with aggressiveness. These analyses will be critical for better understanding the association of *A*. *solani* isolates with host plants, and more complete experiments with better assessments of fitness components and larger sample sizes are needed.

The null hypothesis of most neutrality tests was not rejected. Therefore selection was not strong enough in the loci analyzed and no signature could be detected that supports association with host plants. In addition to selection, patterns of genetic variation between lineages or species have been subjected to other evolutionary mechanisms including neutral mutations and genetic drift (Macpherson *et al.* 2007). Some genomic

regions have high levels of polymorphism due to neutral mutations whereas others have elevated non-synonymous divergence (MacPherson *et al.* 2007). Thus, additional studies should be performed to assess whether the pattern of polymorphism in other genomic regions has been affected by natural selection. The asexual reproduction of *A. solani* could allow for a hitchhike effect (Taylor *et al.* 1999) and other regions could be under selection.

Clusters were not mutually exclusive regarding host of origin of the isolates. *A. solani* isolates from potato and tomato plants were found in the same clade. As gene flow has a great influence in shaping *A. solani* population in Brazil (Lourenço Jr. *et al.* manuscript in preparation), migration allows movement of lineages between potato and tomato fields. Two isolates, AS248P and AS232T, collected from different hosts in the same region (Table 1) were grouped in the same cluster when the *Alt a 1* phylogenetic tree was constructed (Fig. 2). In many producing regions, potato and tomato are grown in contiguous fields, which favors exchange of individuals between populations. Furthermore, the recent population subdivision and low genetic differentiation between *A. solani* lineages from potato and tomato plants (Lourenço Jr. *et al.* manuscript in preparation) can explain the low number of PIS estimated for the sequence data and multilocus dataset which contributed to the clustering of some isolates from different host species in the same clade. Nevertheless, the isolates AS320T and AS252T did not cluster in any of the two major clades. Although both isolates were collected from tomato plants, they are genetically distinct and have different evolutionary history from other lineages identified in this study.

In contrast to the topologies obtained with *Alt a 1*, *Gpd*, and the concatenated multilocus dataset, no well defined groups were observed for the ITS region. Furthermore, there was no clear separation between *A. tomatophila* and *A. solani* isolates collected from potato and tomato plants in the analysis of ITS sequences. Based on ITS sequences, minor differences between these species were detected, but these differences may not be large enough to support the presence of two separate species. In another study, phylogenetic analysis based on the *Alternaria* spp. was of low resolution, suggesting that the ITS region of the rDNA is highly conserved in the genus *Alternaria* (Kusaba & Tsuge, 1995). Other genes need to be

sequenced for a more complete analysis of the evolutionary history of *A. tomatophila*. A genome scan approach (Oetjen & Reusch, 2007) to detect divergent selection in genomic regions of *A. solani* and *A. tomatophila* could help elucidating the evolutionary biology and systematics of these pathogens.

Host association can be determined by differences in pathogen population in response to environmental factors, particularly temperature. Ecotypes of microorganisms can play a role on the distribution of genetic variation which in turn may be reflected in phylogenetic reconstruction (Cohan, 2002; Wedén *et al.*, 2004). No ecotypes of *A. solani* have been reported, but these are likely to occur in Brazil due to different environmental conditions where both major host crops are cultivated. In addition, the clonal nature of the pathogen population, the almost continuous availability of host tissue, and the short reproduction cycle of *A. solani* would allow the development and predominance of variants more adapted to local environmental conditions. Potatoes are grown mainly in regions with mild to low temperatures, usually at altitudes higher than 800 m above sea level. Tomato plants are grown countrywide, predominantly in areas with mean temperatures higher than those of potato producing zones (Reis *et al.* 2003). Consequently, differences in aggressiveness between *A. solani* isolates more adapted to lower or higher temperatures could lead to differential association with host. Temperature driven association was also postulated to be involved with host specialization of clonal lineages of *Phytophthora infestans* to tomato and potato plants in Brazil (Mizubuti & Fry, 2006). Isolates of lineages commonly found in the USA also had differential response to temperature when sporangial germination was assessed (Mizubuti & Fry, 1998). Detailed measurements of epidemiological components of potato and tomato early blight at different temperatures need to be performed to properly address this issue.

Chemical control of early blight does not play a role in host association. The same groups of chemicals are used in both crops to control early blight (Batista *et al.* 2006). Furthermore, we have assessed the sensitivity of almost 200 *A. solani* isolates to chlorothalonil, mancozeb, and azoxystrobin fungicides and found no evidence of association of fungicide

resistance to a specific population of *A*. *solani* (Lourenço Jr. and Mizubuti, unpublished results).

The detection of host-adapted lineages can be relevant to disease control, mainly as support information for breeders and to enhance fungicide usage in crops. Control strategies for early blight are not based on the use of resistance, since there are no commercial cultivars resistant to the disease. However, sources of resistance to early blight can be found in wild species and hybrids of tomato and potato (Jansky & Rouse, 2003; Chaerani *et al.* 2007). Selection of the appropriate types of resistance and the use of resistance genes in breeding programs can be based on the *A. solani* lineages identified in the present study. Regarding fungicide application, the efficiency of early blight control can be improved with the integration of cultural control practices and a fungicide spray program adjusted to *A. solani* lineages. If lineages respond differentially to weather variables, then modifications to the existing decision support systems could be required to improve the efficacy of control measures. If mutation rates differ between lineages, then evolution of fungicide resistance needs to assessed to prevent loss of control efficiency in a short time span.

Further investigation on the causes of host specificity of *A. solani* is the next logical step to pursue. To accomplish this, fitness components at different temperatures, disease intensity on potato and tomato plants and survival analysis in crop debris, soil, and other hosts plants need to be assessed. Also, it will be important to analyze the effects of environmental conditions (e.g. temperature, solar radiation, and humidity), using epidemiological approaches to gain a more complete body of information about the interaction between *A. solani* clonal lineages with potato and tomato plants.

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3.6. Figure legends

Fig.1 Phylogenetic tree for *Alt a 1* gene obtained from the maximum parsimony (MP) analysis of *Alternaria solani* in Brazil. All topoplogies obtained from neighbor-joining (NJ), Maximum Likelihood (ML) and Bayesian inferences of phylogenetic were congruent with the MP tree. ML and Markov Chain Monte Carlo analyses were based on Tamura-Nei (TrN) and Hasegawa-Kishino-Yano models of DNA substitution (HKY), respectively. The topology was rooted with a sequence from *Stemphylium vesicarium* (GenBank AY563275). In addition, sequences from *A. alternata* (AY563301) and *A*. *solani* (AY563299) were included in the analyses. Bayesian posterior probabilities (BPP) and bootstrap values from NJ, MP and ML are shown above the nodes. AS is the collection code of *A*. *solani* isolates obtained from potato (P) and tomato (T) leaves.
Ajustar o texto das legendas seguintes em função do texto corrigido da Fig.2.

Fig.2 Phylogenetic tree for *Gpd* gene obtained from the maximum parsimony (MP) analysis of *Alternaria solani* in Brazil. All topoplogies obtained from neighbor-joining (NJ), Maximum Likelihood (ML) and Bayesian inferences of phylogenetic were congruent with the MP tree. MP tree. ML and Markov Chain Monte Carlo analyses were based on Transition with invariable sites (TIM+I) and general time reversible plus invariable sites (GTR+I) models of DNA substitution, respectively. The topology was rooted with a sequence from *Stemphylium vesicarium* (GenBank AY563275). In addition, sequences from *A. alternata* (AY563301) and *A. solani* (AY563299) were included in the analyses. Bayesian posterior probabilities (BPP) and bootstrap values from NJ, MP and ML are shown above the nodes. AS is the collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves.

Fig.3 Phylogenetic tree for ITS region obtained from the maximum parsimony (MP) analysis of *Alternaria solani* in Brazil. All topoplogies obtained from neighbor-joining (NJ), Maximum Likelihood (ML) and Bayesian

inferences of phylogenetic were congruent with the MP tree. ML and Markov Chain Monte Carlo analyses were based on two transversion with unequal base frequencies (F81uf) and general time reversible plus invariable sites (GTR+I) models of DNA substitution, respectively. The topology was rooted with a sequence from *Stemphylium vesicarium* (GenBank AY563275). In addition, sequences from *A. alternata* (AY563301) and *A. solani* (AY563299) were included in the analyses. Bayesian posterior probabilities (BPP) and bootstrap values from NJ, MP and ML are shown above the nodes. AS is the collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves.

Fig.4 Phylogenetic tree for concatenated sequences (ITS1, 5.8S, ITS2, *Gpd*, and *Alt a 1*) obtained from the maximum parsimony (MP) analysis of *Alternaria solani* in Brazil. All topoplogies obtained from neighbor-joining (NJ), Maximum Likelihood (ML) and Bayesian inferences of phylogenetic were congruent with the MP tree. ML and Markov Chain Monte Carlo analyses were based on transition with invariable sites (TIM+I) and general time reversible plus invariable sites (GTR+I) models of DNA substitution, respectively. The topology was rooted with a sequence from *Stemphylium vesicarium* (GenBank AY563275). In addition, sequences from *A. alternata* (AY563301) and *A. solani* (AY563299) were included in the analyses. Bayesian posterior probabilities (BPP) and bootstrap values from NJ, MP and ML are shown above the nodes. AS is the collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves. *Branch not supported in the ML tree.

Fig.5 Maximum parsimony tree with bootstrap (above) and PBS (below) values shown to corresponding nodes for the combined dataset MLST (*Alt a 1* and *Gpd* genes) and RAPD markers in the phylogenetic analysis of *Alternaria solani* on potato and tomato plants. Haplotypes (H) were generated after collapsing the multilocus dataset (MLST and RAPD).

3.7. Tables and Figures

Table 1. Description of Alternaria solani isolates collected from potato and tomato producing regions of Brazil.

Region/ State	County Collection date	Isolate code[a]	Date collected[c]	GenBank accession[d]			Haplotypes[e]
				Alt a 1	*Gpd*	ITS	
SE/MG	Araxá	AS012P, AS013P	2005	N/A, x	x, x	x, x	N/A, H6
	Bom Repouso	AS185P, AS187P, AS189P, AS194P, AS203P	2006	x, x, N/A, x, x	x, x, x, x, x	x, x, N/A, x, x	H6, H10, N/A, H6, H6
	Bueno Brandão	AS168P, AS169P, AS170P, AS180P	2006	x, x, x, x	x, x, x, x	x, x, N/A, x	H6, H6, H6, H6
	Cajuri	AS130T, AS131T	2000	x, x	x, x	x, x	H12, H4
	Camanducaia	AS216P, AS220P, AS221P	2006	x, x, x	x, N/A, N/A	x, x, x	H6, N/A, N/A
	Carandaí	AS432T	2005	N/A	x	x	N/A
	Conselheiro Lafaiete	AS229T, AS232T, AS242T, AS247P, AS248P, AS250P,	2005	x, x, x, x, x, x, x, x,	x, x, x, x, x, x, x, x,	x, x, x, x, x, x, x, x,	H15, H2, H2, H12, H2, H2, N/A, H12, H2

		AS252P, AS255P, AS532		x	x	x	
	Ibiá	AS016P, AS020P, AS023P, AS027P	2005	x, x, x, x	x, x, x, x	x, x, x, x	H6, N/A, N/A, H6
	Igarapé	AS158T	N/A	x	x	x	H12
	Ipuiúna	AS360P, AS361P, AS381P	2006	x, x, x	x, x, x	x, x, N/A	H21, H6, H6
	Ituverava	AS409T	2005	x	x	x	H12
	Jaíba	AS420T, AS422T, AS423T	2005	x, x, x	x, N/A, x	x, N/A, x	H2, N/A, H2
	Monsenhor Izidro	AS396P	2005	x	x	x	N/A
	Poços de Caldas	AS093P	2000	x	N/A	N/A	N/A
	Prudente de Morais	AS139T	N/A	x	x	x	H2
	Ressaquinha	AS482T, AS488T, AS489T	2005	x, x, x	N/A, x, x	x, x, x	N/A, H2, H2
	Teixeiras	AS080P	1999	x	N/A	N/A	N/A
	Tocantins	AS124T	2000	x	x	x	H12
	Viçosa	AS096T, AS098P	2000	x, x	x, x	x, x	H9, H2
SE/RJ							
	Bom Jardim	AS111T	2000	x	x	x	H2
	Cordeiro	AS113T	2000	x	x	x	H4
	Itaperuna	AS120T	2000	x	x	x	H15
	Vassouras	AS005T, AS109T	2000	x	x	x	H12, H19
SE/SP							

	Capão Bonito	AS090T	2000	x	x	x	H17
	Ibiúna	AS052T, AS063T, AS069T	2005	x, x, x	x, x, x	x, x, x	N/A, H2, H2
	Itapetininga	AS328T, AS329T, AS331T, AS336P, AS337P	2005	N/A, x, x, x, N/A	x, x, x, x, x	x, x, x, N/A, x	N/A, H12, H12, H6, N/A
SE/ES							
	Castelo	AS226T, AS554T	2005	x, x	x, x	x, x	H2, N/A
	Domingos Martins	AS264P, AS270T, AS272T	2005	x, x, x	N/A, x, N/A	x, x, x	N/A, H12, N/A
	Marechal Floriano	AS451T	2005	x	x	x	H2
S/PR							
	Araucária	AS301T, AS303T, AS319T	2005	x, x	x, x	x, x	H12, N/A, H2
	Colombo	AS317T	2005	N/A	x	x	N/A
	Contenda	AS305P, AS307P, AS308P, AS309P, AS310P, AS311P	2005	x, x, x, x, x, x	x, x, N/A, x, x, x	x, N/A, x, x, x, x	H6, H7, N/A, H6, N/A, H6
	Ponta Grossa	AS002P AS086T, AS087P, AS088P	2000	x, x, N/A	x, x, x	x, x, N/A	N/A, H2, H12, N/A
	São Mateus do Sul	AS001P, AS084P	2000	X, X	N/A, X	X, X	N/A, H14
S/RS							
	Caxias do Sul	AS304T	2005	x	x	x	H4
	Pelotas	AS299T, AS300T	2005	x, x	x, x	x, x	H2, H11

	São Francisco de Paula	AS313P	2005	N/A	x	x	N/A
	Taquara	AS160T	N/A	x	x	x	H12
S/SC							
	Alfredo Wagner	AS294T, AS296T	2005	x, x	x, x	x, x	H20, H12
	Caçador	AS293T	N/A	x	x	x	H5
	Lages do Régis	AS297T	2005	x	x	x	H2
	Rancho Queimado	AS550T, AS558P	2006	X, N/A	x, x	N/A, N/A	H15, N/A
CW/DF							
	Brasília	AS320T AS347P, AS348P, AS349P, AS350P, AS352P, AS353P, AS358P	N/A 2005	x, x, x, x, x, x, x	x, x, x, x, x, x, x	x, N/A, x, N/A, x, x, x	H3, H2, H4, H2, H2, H8, H13, H2
	Ponte Alta	AS288T	N/A	x	x	N/A	H19
	Planaltina	AS453T, AS460T, AS464T, AS465T, AS468T AS079T	2005 2006	x, x, x, x, x	x, x, x, x, x	x, x, x, x, x	H12, H2, H2, H12, H16
CW/GO							
	Cristalina	AS258P, AS260P, AS261P, AS263P, AS555P	2005	x, x, x, x, x	x, x, x, x, x	x, x, N/A, x, x	H10, H6, H6, H6, N/A
	Planaltina de Goiás			x	x	N/A	H2

NE/BA							
	Mucugê	AS033P, AS034, AS565P	2005	x, x, N/A	x, x, N/A	x, x, x	H6, H6, N/A
NE/CE							
	Guaraciaba do Norte	AS322P, AS323P, AS325P	2005	x, x, x	x, x, x	x, N/A, x	N/A, H6, H18
N/A							
	A. solani	N/A	N/A	AY563299	AY278807	AF229475	N/A
	A. tomatophila	N/A	N/A	N/A	N/A	AM237289	N/A
	A. alternata	N/A	N/A	AY563301	AF081400	AF347031	N/A
	Stemphylium vesicarium	N/A	N/A	AY563275	AY278821	AF229484	N/A
	S. solani	N/A	N/A	x	x	x	H1

[e] Collection code of *A. solani* isolates obtained from potato (P) and tomato (T) leaves; *A. solani, A. tomatophila, A. alternata, and S. vesicarium* sequences obtained from GenBank.

[f] 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); N/A: geographic origin is not available.

[g] N/A: date collected not available

[h] *Alt a 1*: *Alternaria* major allergen gene; *Gpd*: glyceraldehyde-3-phosphate dehydrogenase; ITS1, 5.8S and ITS2: rDNA from

the internal transcribed spacer region; N/A: sequence not available.

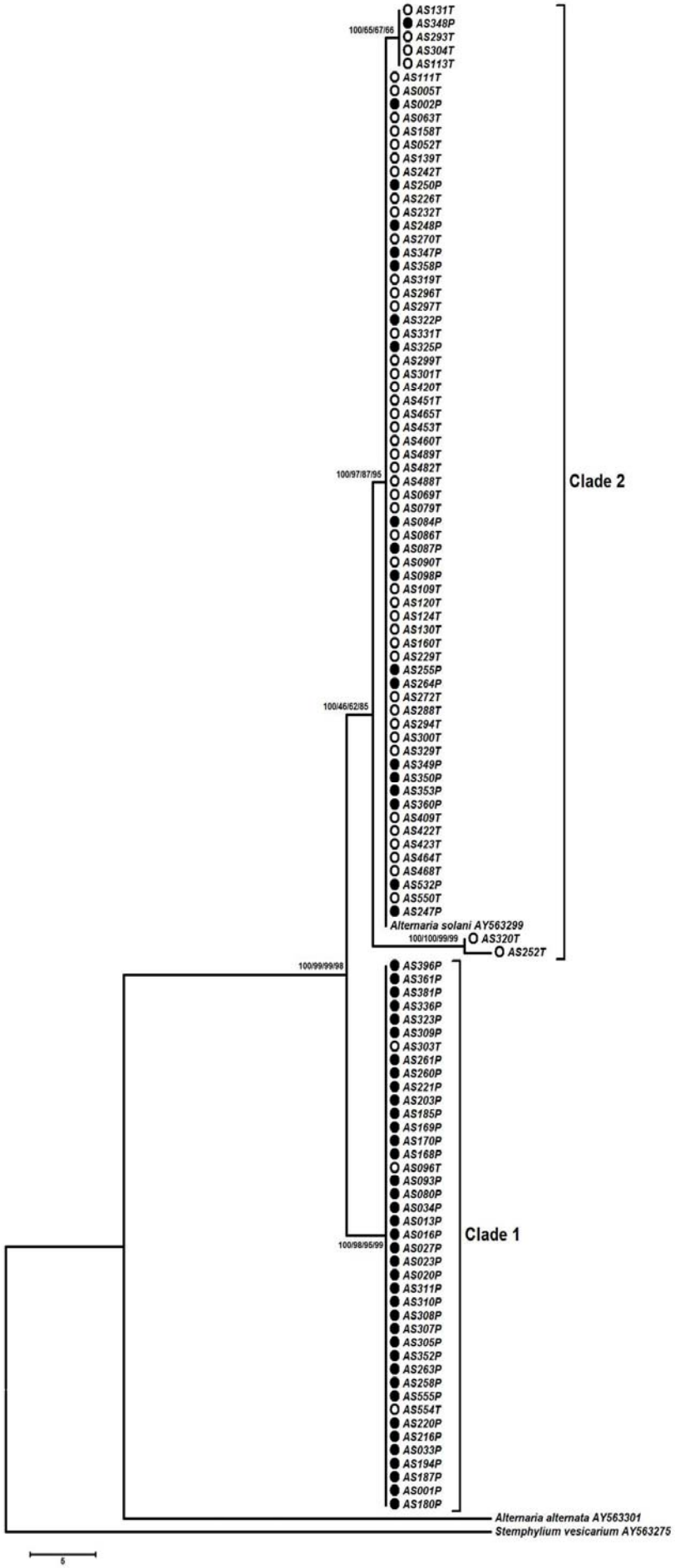
[i] Haplotypes obtained after concatenating *Alt a 1* and *Gpd* sequences with RAPD bands; N/A: haplotypes not generated.

Table 2. 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*, *Gpd1* and ITS sequences from *Alternaria solani* isolates.

Population	**Locus**	**Tajima´s D**	**Fu and Li´s D***	**Fu and Li´s F***	**Fu´s F statistic**
Total	*Alt A1*	0.1579 (NS)	0.5610 (NS)	0.4910 (NS)	6.337 (NS)
	Gpd1	-0.5697(NS)	-0.7603 (NS)	-0.8233(NS)	3.853 (NS)
	ITS	-2.32024 (P<0.01)	NA	NA	NA
Potato	*Alt A1*	2.0931 (P<0.05)	0.4350 (NS)	1.1421 (NS)	7.055 (NS)
	Gpd1	0.5551 (NS)	-1.3546 (NS)	-0.8550 (NS)	4.264 (NS)
	ITS	-1.94666 (P<0.05)	NA	NA	NA
Tomato	*Alt A1*	-1.8139 (P<0.05)	0.7065 (NS)	-0.1897 (NS)	1.008 (NS)
	Gpd1	-1.3598 (NS)	1.0514 (NS)	0.2735(NS)	1.544 (NS)
	ITS	-2.20774 (P<0.0\t1)	NA	NA	NA

NS = not significant

NA= not applicable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AS350P
AS353P
AS360P
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AS422T
AS423T
AS464T
AS468T
AS532P
AS550T
AS247P
Clade 2
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AS323P
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AS260P
AS221P
AS203P
AS185P
AS169P
AS170P
AS168P
AS096T
AS093P
AS080P
AS034P
AS013P
AS016P
100/98/95/99
AS027P
AS023P
AS020P
AS311P
AS310P
AS308P
AS307P
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AS352P
AS263P
AS258P
AS555P
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AS220P
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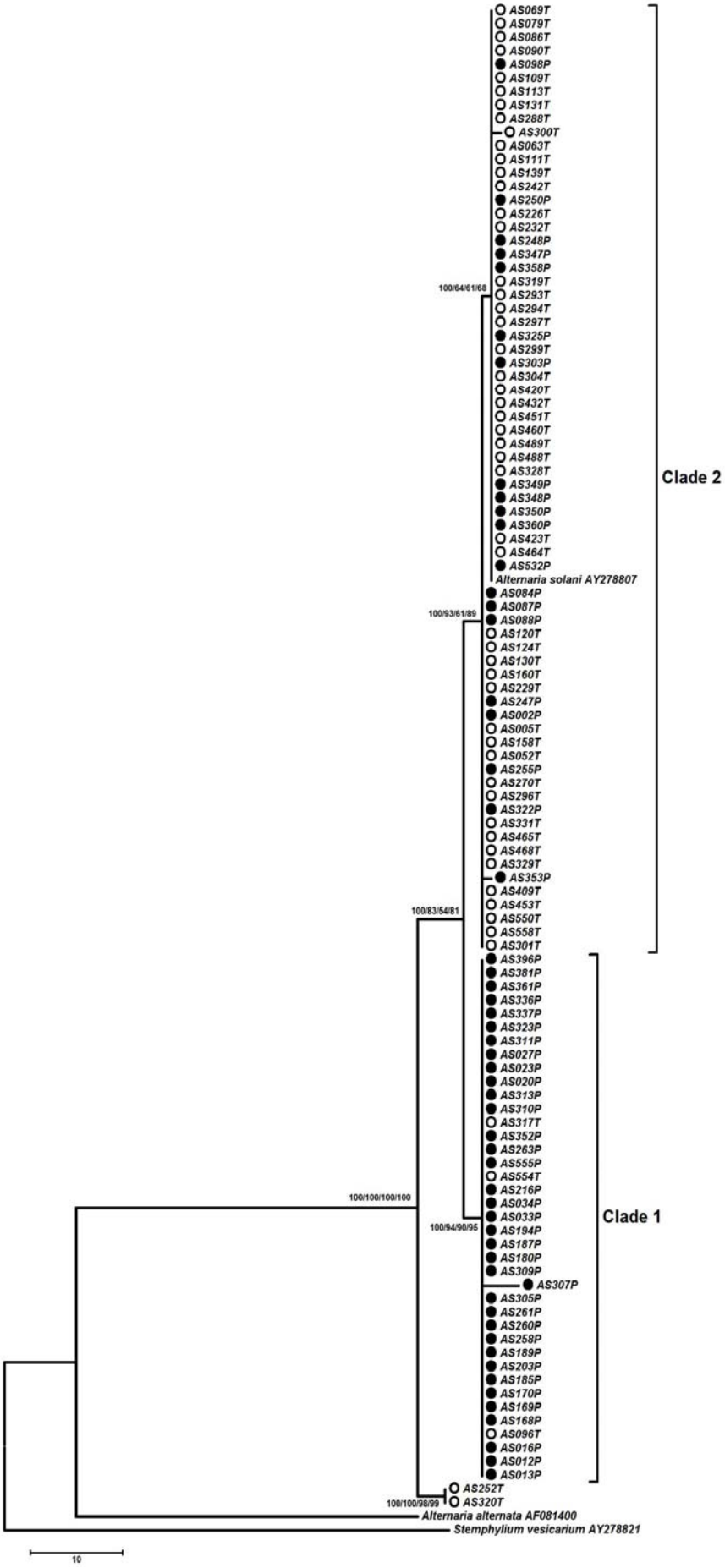
Stemphylium vesicarium AY563275
5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AS248P
AS347P
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AS319T
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AS294T
AS297T
AS325P
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AS304T
AS420T
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AS489T
AS488T
AS328T
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AS423T
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Clade 2
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AS453T
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AS554T
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AS034P
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AS033P
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AS194P
AS187P
AS180P
AS309P
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AS305P
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AS168P
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Stemphylium vesicarium AY278821
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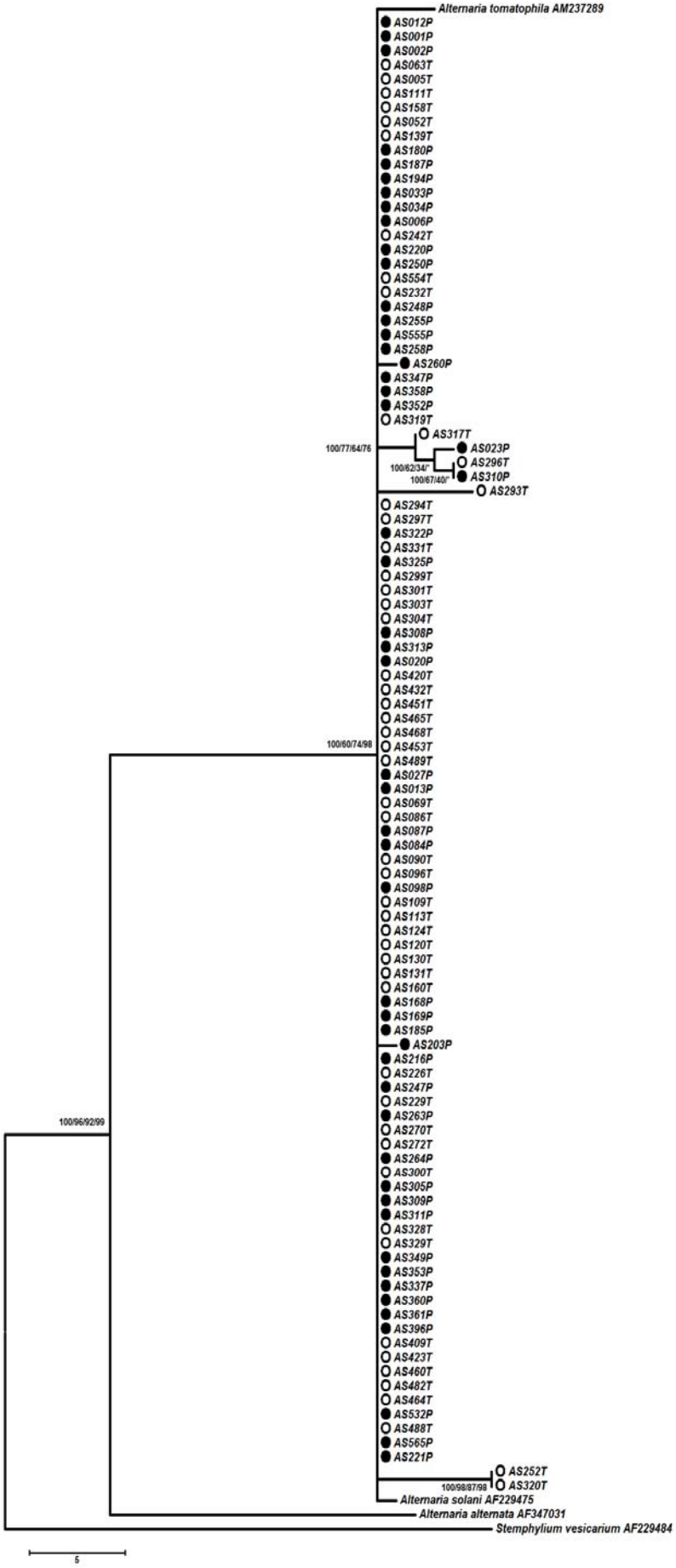
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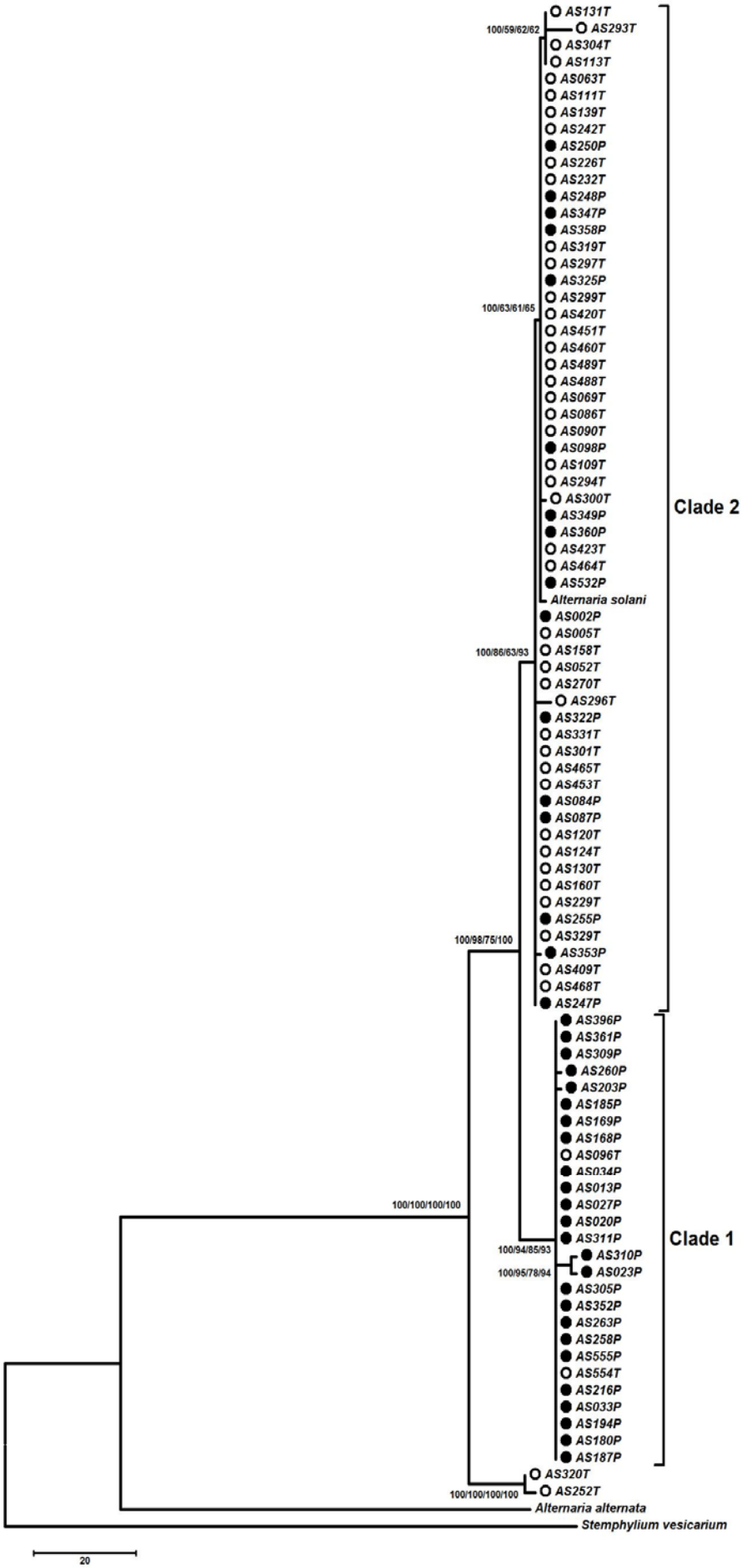
5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AS555P
AS554T
AS216P
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Clade 1
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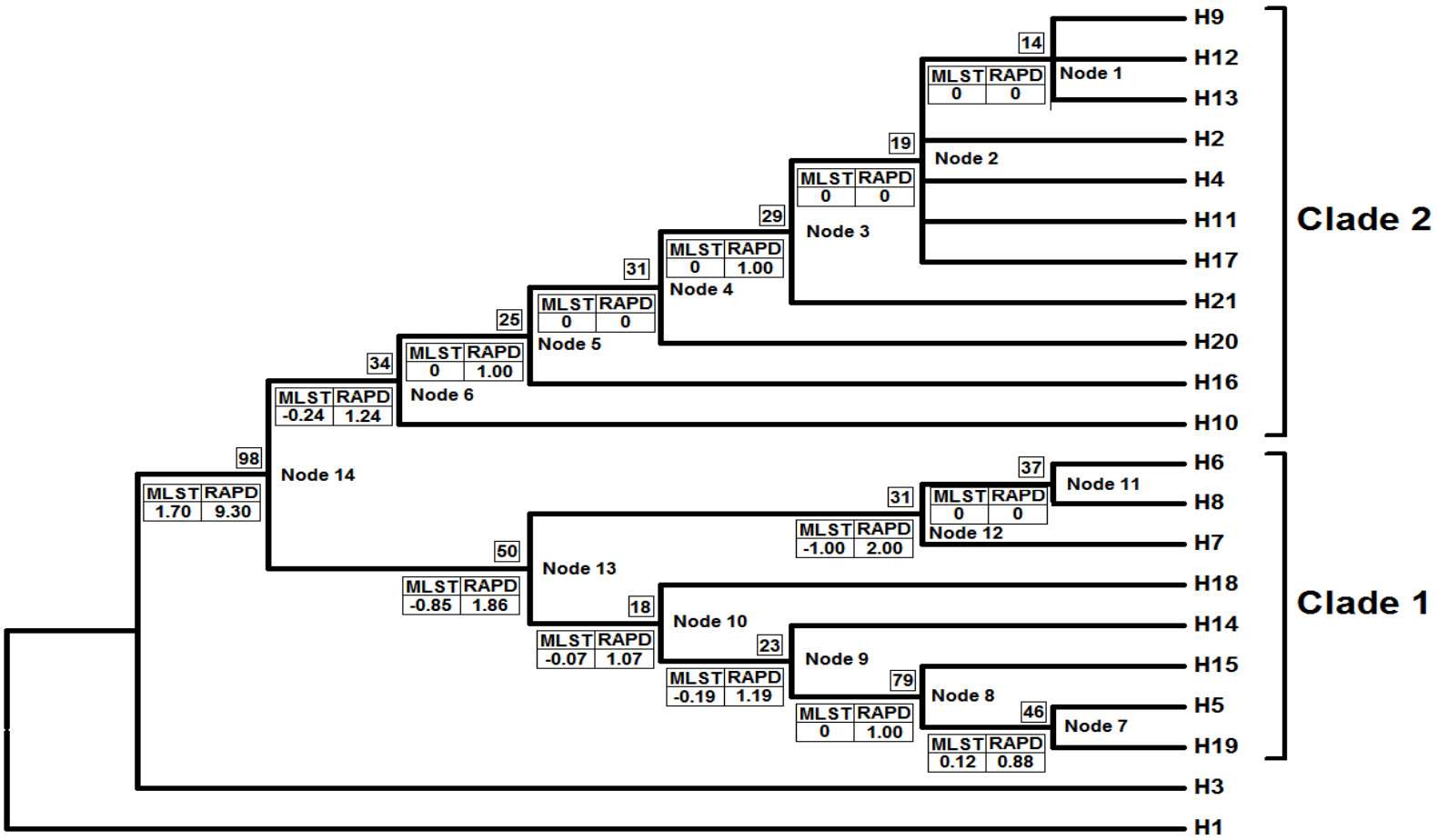
100/100/100/100
AS252T
Alternaria alternata
Stemphylium vesicarium
20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H2
H4
H11
H17
H21
H20
H16
H10
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H8
H7
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H14
H15
H5
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H1
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CONCLUSIONS

- Gene flow and mutation are the main evolutionary mechanisms that shape *Alternaria solani* populations;
- There is no evidence of recombination in *A. solani* populations;
- There is evidence of *A. solani* clonal lineages associated with potato and tomato plants;
- The divergence time of *A. solani* populations is recent;
- *A. tomatophila* was not found associated with tomato early blight in Brazil.