TATIANA TOZZI MARTINS SOUZA RODRIGUES

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION, WITH INFERENCE ABOUT RECOMBINATION, FOR *Alternaria* SPECIES RELATED TO EARLY BLIGHT OF POTATO AND TOMATO

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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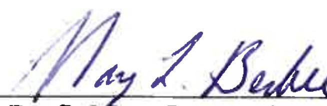
APROVADA: 27 de março de 2009.

Profª. Marisa Vieira de Queiroz
(Co-orientadora)



Profª. Mary Lee Berbee

Prof. Luiz Antonio Maffia

Prof. Robert Weingart Barreto

Prof. Eduardo Seiti Gomide Mizubuti
(Orientador)

Dedico

Ao meu marido Alliekisiei,
meus filhos Paula, Pedro e Maria,
meus pais Edson e Rosa,
meu irmão Gustavo.

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BIOGRAFIA

Tatiana Tozzi Martins Souza Rodrigues é filha de Edson de Souza Martins e Rosa Maria Tozzi Martins, nasceu em Ribeirão Preto no dia 24 de dezembro de 1975.

Em 2002, graduou-se em Agronomia pela Universidade Estadual de Montes Claros (UNIMONTES).

Em 2005, concluiu Mestrado em Fitopatologia pela Universidade Federal de Viçosa (UFV). Em março do mesmo ano iniciou o Doutorado em Fitopatologia na UFV, sob orientação do Professor Eduardo Mizubuti.

ÍNDICE

RESUMO....viii
ABSTRACT....x
INTRODUÇÃO GERAL....1
OBJETIVOS....1
LITERATURA CITADA....3
1- *Alternaria tomatophila* causing early blight on tomato and the occurrence of other species of *Alternaria* associated with early blight on potato....4
ABSTRACT....4
1.1. INTRODUCTION....5
1.2. MATERIALS AND METHODS....8
Isolates....8
Morphological analysis....8
Pathogenicity assays....9
Sequencing....9
Phylogenetic analysis....10
1.3. RESULTS....10
Morphological analysis....10
Pathogenicity assays....11
Phylogenetic analysis....12
1.4. DISCUSSION....13
Tables and Figures....17
1.5. LITERATURE CITED....26
2- Potential for the occurrence of recombination based on mycelial compatibility, the distribution of mating type genes, and analysis of genetic diversity between different species of *Alternaria* related to potato and tomato early blight....28
ABSTRACT....28
2.1. INTRODUCTION....29
2.2. MATERIALS AND METHODS....33
Isolates....33
Mycelial compatibility (MC)....34
Fluorescence and confocal microscopy....34
Nit mutants....35
Mating type idiomorphs....37
AFLP....37
DNA sequence....38
Data Analyses....38
2.3 RESULTS....39
Mycelial compatibility....39
Fluorescence and confocal microscopy....40
Nit mutants....40
Mating type idiomorphs....41
AFLP....41
DNA sequence and phylogenetic analysis....41
2.4. DISCUSSION....42
Tables and Figures....47
2.5. LITERATURE CITED....55
3- Phylogenetic relationships among *Alternaria* species from Solanaceae based on morphological and DNA sequence data....59
ABSTRACT....59
3.1.INTRODUCTION....60

3.2. MATERIALS AND METHODS....62
Isolates and DNA sequencing....62
Morphological characters....63
Phylogenetics Analysis....63
Partitioned Bremer Support (PBS)....64
3.3. RESULTS....................;....64
DNA sequencing....64
Phylogenetic analysis....65
Partitioned Bremer Support (PBS)....65
3.4. DISCUSSION....66
Tables and Figures....69
3.5. LITERATURE CITED....76
GENERAL CONCLUSIONS....78

RESUMO

RODRIGUES, Tatiana Tozzi Martins Souza, D.Sc., Universidade Federal de Viçosa, março de 2009. **Caracterização molecular e morfológica, com inferência sobre recombinação, de espécies de *Alternaria* relacionadas à pinta preta de batateira e tomateiro**. Orientador: Eduardo Seiti Gomide Mizubuti. Co-orientadores: Marisa Vieira de Queiroz e Fabrício Ávila Rodrigues

Alternaria solani sempre foi considerado o agente causal da pinta preta em tomateiro e batateira no Brasil. Entretanto, após o estudo da estrutura genética da população desse patógeno, concluiu-se haver evidências de linhagens clonais associadas às plantas hospedeiras. Dada a possibilidade de ocorrência de mais de uma espécie relacionada à pinta preta em batateira e tomateiro no Brasil, conduziram-se estudos para identificar estes patógenos, com base em morfologia e dados moleculares. De amostras coletadas nas principais regiões produtoras do Brasil, *A. tomatophila* e *A. cretica* foram identificadas nas amostras provenientes de tomateiro, *A grandis* foi identificada em todas as amostras de batateira, enquanto *A. solani* não ocorreu em nenhuma amostra. Filogeneticamente, as espécies dentro de cada hospedeiro são muito próximas e análises de máxima parcimônia, máxima verossimilhança e *neighbor-joining* não permitiram a sua separação. Entretanto, *A. solani* e *A. grandis* são distinguíveis de *A. tomatophila* e *A. cretica*. Em vista da filogenia das espécies de *Alternaria*, conduziram-se trabalhos para verificar evidências de recombinação inter e intraespecífica. A formação de barreira de incompatibilidade entre indivíduos tipo selvagem ocorreu em apenas 21% dos pareamentos, predominantemente entre isolados de *A. tomatophila* e *A. grandis.* Com a utilização de mutantes *nit* foi possível demonstrar o fenômeno de heterocariose entre indivíduos de uma mesma espécie, o que possibilita alta variabilidade genética. Com base no marcador AFLP, comprovou-se alta variabilidade genética (Hd=0,94) entre isolados de *Alternaria* spp. Genes de *mating type* foram amplificados e sua distribuição e frequência na população foram determinadas. Os dois idiomorfos (*MAT1-1* e *MAT1-2*) estão amplamente distribuídos no Brasil. Frequências similares dos dois idiomorfos foram encontradas na população de *A. grandis*. Utilizaram-se sequências dos genes *Alt a 1, Gpd, MAT1-2* para análise filogenética com o objetivo de verificar a ocorrência de híbridos. Dois possíveis híbridos (AS012 e AS248)

foram identificados na população de *A. grandis*. Estudou-se, ainda, o relacionamento de 12 espécies de *Alternaria* provenientes de solanáceas utilizando seqüências dos genes *Alt a 1, Gpd* e *EF.* Grupos foram formados e as espécies de tomateiro e de batateira formaram clados distintos. Dados de características morfológicas e de sequências foram empregados para verificar se espécies morfológicas correspondem a espécies filogenéticas. Valores positivos de suporte de Bremer ocorreram somente nos nós que separam *A. crassa – A. capsici* e *A. grandis – A solani*. Concluiu-se que mais de uma espécie de *Alternaria* é responsável pela doença no Brasil. Há fortes evidências de recombinação na população de *A. grandis*, a qual é prevalente em epidemias de pinta preta em batata.

ABSTRACT

RODRIGUES, Tatiana Tozzi Martins Souza, D.Sc., Universidade Federal de Viçosa, March 2009. **Molecular and morphological characterization, with inference about recombination, for *Alternaria* species related to early blight of potato and tomato.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-advisers: Marisa Vieira de Queiroz and Fabrício Ávila Rodrigues

Alternaria solani has been reported as the causal agent of potato and tomato early blight (EB) in Brazil. However, recent population genetic studies revealed host-associated lineages and there was evidence of more than one *Alternaria* species related to the disease. Studies were conducted to identify, based on morphological and molecular data, the possible species associated with EB on potato and tomato. Samples were collected in the main producing regions of Brazil and based on morphological data; *A. tomatophila* and *A. cretica* were identified in samples from tomato plants and *A. grandis* in samples from potato. *A. solani* was not present in any of the samples. Phylogenetically, species within each host were similar and reconstruction using different methods did not allow their separation. However, *A. solani* and *A. grandis* are distinguishable from *A. tomatophila* and *A. cretica*. Due to the phylogeny of the *Alternaria* species, the evidence of inter and intraspecific recombination was investigated. The barrage zone formation between wild type individuals occurred in only 21% of the pairings, predominantly among isolates of *A. tomatophila* and *A. grandis*. Using *nit* mutants it was possible to demonstrate the occurrence of heterokaryosis between individuals of the same species. High genetic variability (Hd = 0.94) was detected using AFLP marker which is expected in a recombinant population. Additionally, the mating type genes were amplified for both *A. tomatophila* and *A. grandis* and both idiomorphs (*MAT1-1* and *MAT1-2*). The two idiomorphs are widely distributed and occur at equal frequency among isolates of *A. grandis*. When sequences of the *Alt a 1*, *GPD*, *MAT1-2* genes were used for phylogenetic analysis, two putative hybrids (AS012 and AS248) were identified in the *A. grandis* population. Finally, a phylogenetic analysis was conducted to study the relationship of 12 species of *Alternaria* from Solanaceae using sequences of the *Alt a 1*, *GPD*, and *EF* genes and also morphological characters. Species causing EB on tomato and potato were placed in distinct clades. Positive values of Bremer support occurred only in the node that separates *A. crassa* - *A. capsici*, and *A. grandis* and *A. solani*,

but there was no correspondence between DNA sequence and morphological data phylogeny. More than one species cause EB on potato and tomato in Brazil and there is strong evidence of recombination in the population of *A*. *grandis*, the prevalent species causing EB on potato.

INTRODUÇÃO GERAL

A pinta preta é uma doença comum em batateira e tomateiro em todo mundo. No Brasil, a doença tem tomado proporções importantes devido a epidemias de maior intensidade, maiores perdas de produção e relatos de diminuição de sensibilidade do fungo aos fungicidas em condições de campo. A espécie *Alternaria solani* Sorauer tem sido relatada associada à doença em batateira e tomateiro. Entretanto, foram descritas pelo menos mais quatro espécies possivelmente responsáveis por causar pinta preta nestes hospedeiros (Simmons, 2000). As espécies *A. cretica*, *A. subcylindrica* e *A. tomatophila* são responsáveis por causar pinta preta em tomateiro. Esta última parece estar amplamente distribuída em regiões produtoras de tomate em vários países (Simmons, 2000). Em batateira, além de *A. solani, A. grandis* foi relatada como agente causal da pinta preta em algumas regiões nos Estados Unidos (Simmons, 2000).

Um trabalho realizado recentemente no Brasil determinou a estrutura genética da população de *Alternaria* spp. (Lourenço *et al*., *in press*) e apresentou evidências de mais de uma espécie causando pinta preta em batateira e tomateiro. Utilizando seqüências de genes e marcadores RAPD e AFLP concluiu-se haver evidências de linhagens clonais do fitopatógeno associadas às plantas hospedeiras. Resultados semelhantes já haviam sido relatados em outros países como Estados Unidos (Weir *et al*., 1998) e em Cuba (Martínez *et al*., 2004). A determinação das espécies de fitopatógenos associados a determinada doença é de suma importância para adequação de medidas de manejo. Se mais de uma espécie pode causar a doença, estudos sobre a distribuição, freqüência e adaptabilidade dessas espécies são necessários. Trabalhar com um complexo de espécies, ao invés de uma única, interfere diretamente no programas de melhoramento genético visando resistência do hospedeiro ao patógeno e no manejo de aplicação de fungicidas, pois as diferentes espécies podem apresentar diferentes respostas ao mesmo tratamento.

Como a pinta preta ocorre em duas culturas importantes e em vista das evidências de mais de uma espécie de *Alternaria* estarem associadas à doença no Brasil, esse trabalho objetivou:

1- Confirmar a ocorrência de outras espécies de *Alternaria* associadas a tomate e batata, com

base em dados morfológicos e moleculares;

2 - Verificar o potencial de recombinação dentro e entre espécies com base em compatibilidade vegetativa; formação de heterocárion; presença, distribuição e freqüência de genes de *mating type*, diversidade genética por meio de marcador AFLP e identificação de prováveis híbridos;

3 – Determinar o relacionamento filogenético entre 12 espécies de *Alternaria* em Solanaceae e verificar a contribuição de características morfológicas e de seqüências de DNA na determinação das espécies.

LITERATURA CITADA

Lourenço, Jr. V, Moya, A., González-Candelas, F., Carbone, I., Maffia, L.A., Mizubuti, E.S.G. 2009. Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. Phytopathology. Accepted on 4 February 2009.

Martínez, S.P., Snowdon, R., Pons-Kuhnemann, J. 2004. Variability of Cuban and international populations of *Alternaria solani* from different hosts and localities: AFLP genetic analysis. European Jounal of Plant Pathology 110: 99-409.

Simmons, E.G. 2000. *Alternaria* themes and variation (244-286). Species on *Solanaceae*. Mycotaxon 55: 55-163.

Weir, T.L., Huff, D.R., Christ, B.J., Romaine, C.P. 1998. RAPD-PCR Analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato plants. Mycologia 90: 813-821.

1 - *Alternaria tomatophila* and *A. cretica* causing early blight on tomato and the occurrence of other species of *Alternaria* associated with early blight on potato.

ABSTRACT

For many years *Alternaria solani* was reported as the species causing early blight (EB) on potato and tomato in Brazil. In recent years, four species, *A. cretica*, *A. grandis*, *A. subcylindrica*, and *A. tomatophila* were also described associated to EB on both hosts. A previous study conducted to analyze the genetic structure of the Brazilian population of *A. solani* revealed separate lineages associated with hosts. The aim of the present study was to conduct morphological and phylogenetic analyses to identify putative species of *Alternaria* causing EB on potato and tomato. Isolates were sampled from several producing regions and morphological analyses were implemented to compare them with ex-type isolates of *A solani* and *A tomatophila.* At least three species were found causing EB on potato or tomato: *A. cretica* and typical and atypical *A. tomatophila* were recovered from tomato samples; and *A. grandis* was the only species found causing EB on potato. The *A solani* morphospecies was not identified in the present study. Inoculation assays demonstrated that *A. tomatophila* and *A. grandis* were pathogenic to both hosts. DNA sequences of the *Alt a 1*, *Gpd*, *MAT1-1*, and *MAT1-2* genes from ex-type cultures of *A. tomatophila*, *A. solani*, *A. cretica*, and also *A. grandis* were used in phylogenetic analysis. This approach provided strong support to distinguish two groups of species associated to the host plants: *A. cretica* and *A. tomatophila* associated to EB on tomato and *A. solani* and *A. grandis* associated with the disease in potato. The morphospecies *A. tomatophila* and *A. grandis* are the widely distributed species associated to EB in Brazil.

1.1. INTRODUCTION

The mitosporic fungus *Alternaria solani* Sorauer was described in 1896 as the causal agent of early blight on potato (*Solanum tuberosum* L.) (Sorauer, 1896). Subsequently, pathogenicity tests had suggested that this species was also the incitant of early blight on tomato (*Solanum lycopersicum* L.) and of a foliar disease on eggplant (*Solanum melongena* L.) (Rands, 1917). However, morphological and molecular characters varied greatly among isolates of *A. solani* (Bonde, 1927, Bonde, 1929, Rotem, 1966, Weir *et al*., 1998). The lack of a thorough morphological study of isolates from three of the most agronomically-relevant Solanaceae species – potato, tomato, and eggplant – motivated a review of the species of *Alternaria* found on these hosts, especially the pathogenic species that affect potato and tomato (Simmons, 2000). Simmons' (2000) comprehensive monograph of *Alternaria* species provides keys and detailed descriptions for species defined by morphological species concepts (MSC). In addition to *A. solani*, at least seven species were related to foliar diseases on potato and tomato: the six large-spored species, *A. cretica*, *A. grandis*, *A. subcylindrica*, *A. elegans, A. mimicula* and the widely distributed species *A. tomatophila*, the causal agent of tomato early blight; and the small-spored species *A. arborescens*. Importantly for this study, E. G. Simmons maintains and distributes cultures ex type for research use.

The large-spored species found on Solanaceae differ from *A. solani* in their patterns of sporulation and in their conidial shapes, sizes and number of beaks (Simmons, 2000). In the absence of a type specimen of *A. solani*, Simmons (2000) provided a new description for the species based on the ex-type isolate EGS 44-098. The species predominantly produces conidia with a single tapering beak; conidia with two beaks can be formed, and those with three beaks are rare. The conidia are ovoid and variation in development results in long, short, broad, and narrow spores. The

sporulation pattern is characterized by branched and geniculate conidiophores. A similar species, *A. grandis*, also originally isolated from potato, produces solitary and multi-septate conidia, with narrow to ellipsoid body shape (Simmons, 2000). The species recovered from tomato leaf lesions, *A. subcylindrica*, *A. cretica*, and *A. tomatophila* all produce conidia of overlapping size range. However, additional characteristics are used to distinguish these species: sporulation of *A. tomatophila* is scarce in undisturbed medium compared to the other species; *A. cretica* conidiophores are formed in pairs, from the same vegetative hypha, which is uncommon in the other two species; and *A. subcylindrica* rarely produces conidia with three beaks (Simmons, 2000).

Two species, *A. tomatophila* on tomato and *A. solani* on potato, have become the focus of attention in their respective important crops. In addition to morphological differences, *A. tomatophila* was reportedly more virulent than *A. solani* when inoculated on tomato leaves, petioles, and stems (Frazer and Zitter, 2003), and its specific metabolite profile differs from that of *A. solani* (Andersen *et al.*, 2008). Even before the description of other species causing early blight, differences in host preference were inferred in populations identified as *A. solani* (Weir *et al.*, 1998). More recently, two molecular studies supported association of host species with fungal haplotype (Martínez *et al.*, 2004, Lourenço Jr. *et al.*, *in press*). Isolates identified as *A. solani* from potato were separated from isolates from tomato by large genetic distances based on RAPD analysis in the United States (Weir *et al.*, 1998) and by AFLP markers in Cuba (Martínez *et al.*, 2004). In Brazil, Lourenço Jr. *et al.* (*in press*) investigated the population structure of the causal agent of potato and tomato early blight. Three genomic regions were sequenced (*Alt a 1* - Major Allergen Alt a 1 precursor, *Gpd* - Glyceraldehyde-3-phosphate dehydrogenase, and the ITS – Internal transcribed spacer region of rDNA) and molecular population genetics analytical tools revealed strong

evidence for the occurrence of two distinct lineages: one commonly associated with potato and the other with tomato. However, among the Brazilian isolates, host specificity was not absolute and a few isolates of the 'tomato' type were found on potato and vice versa (Lourenço Jr. *et al*., *in press*).

Ideally, the species concept applied to the main *Alternaria* spp. associated with Solanaceae should reflect genetic isolation and barriers to reproductive exchange. Multilocus DNA sequence data can be a valuable resource to support or refute distinctions among species based on morpho-physiological characteristics (Taylor *et al*., 2000, O'Donnell *et al*., 2004, Geiser *et al.*, 2007). For *Alternaria* species the *Alt a 1* and *Gpd* sequences supported the separation of groups of *Alternaria* spp. and related taxa into several species-groups, but closely related species were not fully resolved and other polymorphic genomic regions need to be analyzed (Hong *et al.*, 2005). Sequences of mating type genes (MAT genes) have been demonstrated to mark species boundaries in the *Fusarium graminearum* group and may be useful in phylogenetic studies of other species (O'Donnell *et al*., 2004). Even though MAT genes are related to sexual reproduction, they have been amplified in asexual species of *Alternaria* (Arie *et al*., 2000, Berbee *et al*., 2003). These genes can be of phylogenetic interest because they have a high substitution rate compared with the ITS region, for example, and may contribute to elucidate the evolutionary events that occurred in recently diverged species (Berbee *et al*., 2003). To date, no phylogenetic analysis of *Alternaria* spp. using sequences of the MAT genes was accomplished.

The main objectives of the present study were to identify *Alternaria* species, based on morphological and molecular data and to conduct pathogenicity tests with the isolates in cross-inoculation assays. This represents the first critical morphological and molecular comparison of isolates associated with early blight in Brazil with cultures

from type collections representing known *Alternaria* species from Solanaceae.

1.2. MATERIALS AND METHODS

Isolates. Thirty-two isolates of *Alternaria* spp. were obtained from typical early blight lesions on potato and tomato plants from different areas in Brazil (Table 1). The isolates are part of the culture collection of the Laboratório de Biologia de Populações de Fitopatógenos, at the Universidade Federal de Viçosa. Cultures were stored in filter paper at -80°C. Cultures ex-type and typical cultures of *Alternaria* spp. were provided by Emory G. Simmons (Table 1).

Morphological analysis. Observations were based on three isolates from tomato (AS109, AS232, and AS440), four isolates from potato (AS185, AS216, AS220, and AS248), the ex-type *A. tomatophila* (EGS 42-156), and the ex-type *A. solani* (EGS 44-098). Colonies were grown on V-8 juice agar amended with calcium carbonate (175 ml V8 juice, 3 g $CaCO_3$, 20 g agar, 1 L water) (Simmons, 2007), during 5 days at 25°C in the dark. The colonies were gently wounded and kept under daily fluorescent light/dark cycle of 8/16 h, at 22-23°C (Simmons, 2007). The plates were not sealed with plastic film. After 24 h, the colonies were observed under the microscope at 40X magnification to assess the pattern of sporulation. The conidia harvested from the border of the colony were placed on a drop of lactic acid previously deposited on a microscope slide and the following conidium dimensions were measured under the microscope (40X): body length and width, beak length, and the number of transversal and longitudinal septa (Simmons, 2007) (Table 2). Usually, conidia of tomato isolates had one, two, or three beaks, and 30 spores of each category were measured. For potato isolates, 60 single-beak conidia were measured per isolate. All isolates were sent to E.G. Simmons for species confirmation.

Pathogenicity assays. Two assays were implemented. The first was conducted to check the pathogenicity of tomato isolates on tomato. A suspension of 1×10^4 conidia/ml of each isolate was inoculated in five 60-day-old plants of tomato cv. Santa Clara. Plants were maintained for 16 h in a moist chamber, and then transferred to a greenhouse (Dita Rodriguez *et al*., 2006). The tomato plant was divided into three thirds, and five leaflets of the middle third were assessed counting the number of lesions (NL) and determining the lesion area (LA) six days after inoculation. The lesion was measured in two perpendicular directions, with the aid of a ruler, and the formula of the area of a circle was used to estimate the lesion area.

The second assay assessed the pathogenicity of the isolates on both hosts. A suspension of 5×10^3 conidia/ml of each isolate was inoculated in three 35-day-old plants of tomato cv. Santa Clara, and three of potato cv. Ágata. Plants were maintained for 16 h in a moist chamber, and then transferred to a greenhouse. The NL was determined in five leaflets of the middle third of each plant. To test for the significance of the isolate by host interaction, a linear model was fit to the data from the pathogenicity assay 2 according to a factorial treatment design using the R package version 2.8. (R Development Core Team, 2008).

Sequencing. The DNA of the isolates EGS 46-188 (*A. cretica*), EGS 44-098 (*A. solani*), EGS 42-156 (*A. tomatophila*), EGS 44-106 (*A. grandis*) (Table 1) were extracted using Dneasy Plant Mini Kit (Qiagen), following the manufacturer's instruction. The DNA concentration of each sample was adjusted to 50-100 ng/µl. The primers gpd1 and gpd2 (Berbee *et al.,* 1999), Alt-for and Alt-rev (Hong *et al.,* 2005), Palpha R and ORF 556F, Jen2R and ORF 556F (Inderbitzin *et al*., 2006) were used to amplify the *Alt a 1*, *GPD*, *MAT1-1* and *MAT1-2* genes, respectively. PCR reactions were performed according to Berbee *et al.* (1999). Thermal conditions were initial

denaturation at 95ºC for 5 min followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 52ºC for 1 min, elongation initially at 72ºC for 30s extended by 4 additional seconds per cycle, and a final elongation at 72ºC for 7 min. The PCR products were purified by ethanol precipitation (Okayama and Berg, 1983) and sequenced (Applied Biosystem BigDye v.3.1Terminator Chemistry). The sequencing was performed at NAPS Unit, University of British Columbia, Vancouver-BC, Canada. The sequences were edited and the consensus was built using the AutoAssembler ver. 1.4 software for Macintosh. Sequences of *Alt a 1* and *GPD* for Brazilian isolates were obtained from GenBank database (Lourenço Jr. *et al*., *in press*) (Table 1).

Phylogenetic analysis. The reconstruction of the phylogeny was used to investigate the relationship of the *Alternaria* species from potato and tomato plants. The *Alta a 1*/*GPD* concatenated data set and the *MAT 1-1* and *MAT 1-2* regions were analyzed. Maximum parsimony analysis with heuristic searches for the most parsimonious tree was conducted using closest step-wise addition and tree-bisection-reconnection branch swapping. The majority rule was used to build the consensus tree. Sequence gaps were treated as missing data and 1000 bootstrap replicates were performed. All analyses were implemented in PAUP version 4.0 beta. *A. alternata* was used as an outgroup (GenBank: *GPD* – AF081400, *Alt a 1* – AY563301, *MAT1-1* – AB009451, *MAT1-2* – AB009452).

1.3. RESULTS

Morphological analysis. Among the 16 isolates obtained from tomato, one was classified as *A. tomatophila*, eight as *A. tomatophila cfr* ("compare with"), and two as *A. cretica*. Five isolates did not sporulate. All isolates produced solitary and multi-septate

conidia (Figure 1). Conidia of *A. tomatophila* had narrow-ellipsoid body and narrow-taper beaks. The *A. tomatophila cfr* was not identical to its ex-type culture (EGS 42-156), due to a lower number of three-beak conidia and higher number of one-beak conidia. Conidia of *A. cretica* had narrow-ellipsoid bodies, but the body width was narrower than in *A. tomatophila* and the *A. cretica* spores commonly lacked transverse septa (Figure 1, Table 2). Additionally, *A. cretica* had a characteristic sporulation pattern with pairs of conidiophores developing from the same vegetative hypha. For this species only 6 conidia with three beaks were measured. For all isolates that produced more than one beak, the beaks were shorter in conidia with two or three beaks than in conidia with a single beak.

Among the 16 potato isolates analyzed, *A. grandis* was identified in 15 samples. One isolate did not sporulate. *A. solani* was not found in the samples analyzed in this study (confirmed by E.G.Simmons, *personal communication*). *A. grandis* produced solitary and multi-septate conidia, narrow-ellipsoid body, with a dilation in the middle of the body (maximum width) (Figure 2, D-I). The length of the body and the beak were longer than those observed for *A. solani* (ex-type EGS 44-098) or *A. tomatophila* (ex-type EGS 42-156) conidia (Table 2). However, the body width was narrower than the *A. grandis* ex-type, according to its description (Simmons, 2007).

Pathogenicity assays. In both assays all isolates were pathogenic and infection levels differed depending on age of leaflets. The oldest and the youngest leaflets were more susceptible and resistant to the pathogen, respectively (Figure 3). The first symptoms, small dark-colored spots of dead cells, could be seen 24 h after the inoculation, and five days later well developed lesions were present on all inoculated plants. Symptom severity varied according to the isolate analyzed but isolates from tomato were not consistently more virulent on tomato than on potato and vice versa.

The isolate by host interaction was significant (F=5.98, P-value<0.001) and the means of NL of each isolate were compared by the LSD test in each host. Overall the mean NL caused by tomato isolates (NL-t) was higher than the potato isolates (NL-p). In potato, NL-t was 29.8 and NL-p was 19.2. In tomato these values were 52.2 and 43.8, respectively.

Phylogenetic analysis. The reconstruction of the phylogeny supported two clades. One clade included species from tomato and the other clade was formed by species from potato. The reconstruction based on the *Alt a 1/GPD* concatenated data set resulted in two well-supported clades (bootstrap values higher than 95%). One clade included isolates of *A. tomatophila* (ex-type and Brazilian isolate AS109), *A. tomatophila cfr* and *A. cretica* (ex-type), while the other clade was formed by *A. solani* (ex-type) and the isolates of *A. grandis* (ex-type and Brazilian isolates) (Figure 4). Out of 912 constant characters, 12 were parsimony informative. The score of the best tree was 101 with no homoplasy and a consistency index of 1.0. The *MAT1-2* data set also supported two clades (Figure 5A). One included *A. tomatophila* (ex-type and Brazilian isolate AS109), *A. tomatophila cfr* and *A. cretica* and the other, isolates of *A. grandis*. The *A. cretica* was not distinguishable from *A. tomatophila*. Of the 516 constant characters, 86 characters were variable but not parsimony informative, and 25 were parsimony informative. The score of the best tree was 112, again with a consistency index of 1.0 and no homoplasy.

The *MAT1-1* data set, supported grouping of *Alternaria* spp., but the bootstrap value was not high (<70%) (Figure 5B). A total of 268 characters were constant, and 4 were parsimony informative. The score of the best tree was 574. The consistency index was 0.998 and the homoplasy index was 0.002.

1.4. DISCUSSION

The occurrence of *A. grandis* causing early blight on potato, of *A. tomatophila* and, in low frequency, *A. cretica*, as the causal agents of tomato early blight is an important step towards the understanding of the dynamics of the disease on these hosts. The fact that *A. solani* was not identified in Brazil deserves special attention. Many facts can be raised to explain this: limitations in the sampling scheme; the frequency of this species in the field crops; the suitability of the environmental conditions; and putative differences in aggressiveness among the pathogen species that affect the host plants. The number of samples analyzed was not high, but they were representative of several geographical areas and growing conditions. It is possible that *A. solani* is present in Brazil, but in this case its frequency in the population is low. It is also possible that environmental conditions are not as favorable to *A. solani* as for the other species. Finally, the prevalent species can be more aggressive to the host plants than *A. solani*. This issue is currently being investigated but no data are available yet.

The lack of occurrence of a ubiquitous pathogenic species as *A. solani* has economical and technical implications to the potato production sector in Brazil. If the main species causing early blight on potato is *A. grandis* and if *A. solani* is not present in the country, this fact introduces a crop protection problem related to a non regulated quarantine pest, and procedures to avoid its introduction should be implemented. Another important aspect that is applicable to both host crops is that the Brazilian resistance breeding programs have been screening resistant cultivars to early blight caused by species that differ from *A. solani*. Additionally, the resistance source available in national breeding programs, or in commercial cultivars, are from other countries such as Peru, Netherlands, Germany, where the main pathogen associated with early blight can be distinct from the species occurring in Brazil.

Few studies comparing pathogenicity of *A. tomatophila* and *A. solani* have been conducted and there are important differences between them. In the USA these species were pathogenic on both hosts, but *A. tomatophila* was more virulent than *A. solani* on tomato leaves, petioles, and stems (Frazer and Zitter, 2003). In the present study, although *A. tomatophila* and *A. grandis* caused disease on potato and tomato, there was no difference in isolate aggressiveness according to the inoculated plant. Isolates from potato were not more aggressive on potato than on tomato and vice versa. In fact, the tomato isolates were more aggressive in both tomato and potato. The reasons for this can not be drawn from the data collected in this study. A more thorough investigation assessing more variables (infection frequency, incubation and latent periods) is currently under way. However, *A. tomatophila* and *A. grandis* are close phylogenetic relatives and their two host species are also closely related to one another, and so it seems reasonable that some isolates may be equally aggressive on both hosts (Gilbert and Webbs, 2007), especially under favorable conditions for infection; i.e. high amount of inoculum and favorable temperature and humidity.

Although cross inoculation tests revealed little host-specificity between the species, *A. tomatophila* was only recovered from samples obtained from tomato and the *A. grandis* from samples collected from potato fields. Previous study showed subdivision in populations of *Alternaria* spp. related to early blight in Brazil with most isolates from tomato forming a monophyletic group in this study: the *A. tomatophila* clade. However, host specificity was incomplete and the *A. tomatophila*-like clade also included a few cultures of the same haplotype but isolated from potato (Lourenço Jr. *et al.*, *in press*). Similarly, most cultures from potato formed a monophyletic group corresponding to *A. grandis* in this study, but a few isolates with this same haplotype had been isolated from tomato (Lourenço Jr. *et al.*, *in press*). In many Brazilian regions,

potatoes and tomatoes are cultivated in relatively close proximity and inoculum of *A. tomatophila* would be expected to reach potato leaves while *A. grandis* would be likely to land on tomato leaves. A similar situation occurs with the *Phytophthora infestans* population in Brazil. The BR-1 clonal lineage of *P. infestans* is always associated with potato and it was never found on tomatoes. On the other hand, the US-1 lineage is predominantly associated with tomato and was found causing late blight on potato only in one tomato-producing region (Reis *et al.*, 2003). Differences in aggressiveness between isolates of US-1 and BR-1 clonal lineages contribute to host specificity of *P. infestans*; isolates from a particular clonal lineage were more aggressive on their original host, but can infect the other host (Suassuna *et. al*., 2004). A similar process can occur with populations of *Alternaria* spp., but the population subdivision previously detected was most likely due to the occurrence of the distinct species.

The *Alt a 1*, *GPD* and *MAT1-2* regions allowed strong phylogenetic reconstruction supported by high values of bootstrap, and these regions were not homoplasious. Based on the variable positions for each partition, the *Alt a 1*/*GPD* are more informative than *MAT1-2*, these should be suitable to studies involving phylogenetic species concept (PSC) on *Alternaria* spp., mainly those related to potato and tomato. Applying the PSC clarified the separation of *Alternaria* species by host, i.e. *A. tomatophila* and *A. cretica* are easily distinguishable from *A. solani* and *A grandis*. However, the species from the same host were indistinguishable. There is no sequence variation to support morphological species in this case. Only one variable site is present on sequences of Alt *a 1* and *GPD.* These closely related species probably are species that diverged recently and the sequences did not accumulated mutations yet, or the genes analyzed were not appropriated to demonstrate their evolutionary history. The analysis of others genes is essential and will improve the understanding of relationships among species from both

hosts.

In conclusion, the early blight disease in Brazil is caused by more than one morphospecies of *Alternaria. A. grandis* and *A tomatophila* are the most common causal agents of the disease, and can infect both hosts. Morphological species largely correspond to phylogenetic species when considering *A. cretica*, *A. grandis*, *A. solani*, and *A. tomatophila*. The potential of each species to incite disease on tomato and potato in the field is unknown. Further studies would be required to reveal the importance, real distribution, and putative differences in fitness of the species related to potato and tomato early blight.

Acknowledgements

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TABLE 1. Isolates of Alternaria spp. used in the morphological and molecular analysis.

Isolate - code	Species[a]	Location	Gene		
			Alta1	**GPD**	**MAT1-1/1-2[b]**
Host – *S. tuberosum*					
EGS 44-098[c]	*A. solani*	Washington - USA	x[d]	x	x/nd
EGS 44-106*	*A. grandis*	Pennsylvania - USA	x	x	x/nd
EGS 45-020*	*A. solani*	California - USA	x	x	nd/nd
EGS 45-053*	*A. solani*	New Zealand	x	x	nd/nd
AS012	*A. grandis*	Araxá/MG-BR	nd[e]	nd	nd/nd
AS013	*A. grandis*	Araxá/MG-BR	nd	nd	nd/x
AS033	nd	Mucugê/BA-BR	nd	nd	nd/x
AS088	(-)[f]	Ponta Grossa/PR-BR	nd	nd	nd/nd
AS169	*A. grandis*	B. Brandão/MG-BR	nd	nd	nd/x
AS185	*A. grandis*	Bom Repouso/MG-BR	EU617470.1[g]	EU617527.1	x/nd
AS203	*A. grandis*	Bom Repouso/MG-BR	EU617471.1	EU617528.1	nd/nd
AS216	*A. grandis*	Camanducaia/MG-BR	nd	nd	x/nd
AS220	*A. grandis*	Camanducaia/MG-BR	nd	nd	nd/x
AS248	*A. grandis*	Cons. Lafaiete/MG-BR	nd	nd	nd/nd
AS260	*A. grandis*	Cristalina/GO-BR	EU617476.1	EU617534.1	x/nd
AS263	nd	Cristalina/GO-BR	nd	nd	nd/nd
AS308	*A. grandis*	Contenda/PR-BR	nd	nd	nd/nd
AS313	*A. grandis*	S. Franc. Paula/RS-BR	nd	nd	nd/nd
AS350	nd	Brasília/DF-BR	nd	nd	nd/x
AS352	*A. grandis*	Brasília/DF-BR	EU617422.1	EU617566.1	x/nd
AS394	*A. grandis*	M. Izidro/MG-BR	nd	nd	nd/nd
AS396	nd	M. Izidro/MG-BR	nd	nd	nd/x
BAT5	*A. grandis*	Cons.Lafaiete/MG-BR	nd	nd	nd/nd
VGS1	*A. grandis*	nd	nd	nd	nd/nd
Host – *S. nigrum*					
EGS 46-178*	*A. solani*	California - USA	x	x	nd/nd
Host - *S. lycopersicum*					
EGS 42-156	*A. tomatophila*	Indiana - USA	x	x	nd/x
AS094	*A. tomatophila cfr*	Coimbra/MG-BR	nd	nd	nd/nd
AS095	*A. tomatophila cfr*	Coimbra/MG-BR	nd	nd	nd/nd
AS109	*A. tomatophila*	Vassouras/RJ-BR	EU617462.1	EU617517.1	nd/x
AS113	(-)	Cordeiro/RJ-BR	EU617504.1	EU617518.1	x/nd
AS120	(-)	Itaperuna/RJ-BR	nd	nd	nd/nd
AS160	nd	Taquara/DF-BR	EU617466.1	EU617523.1	nd/nd
AS232	*A. tomatophila cfr*	Cons. Lafaiete/MG-BR	EU617413.1	EU617558.1	nd/x
AS299	nd	Pelotas/RS-BR	EU617431.1	EU617577.1	x/nd

AS304	(-)	Caxias Sul/RS-BR	EU617485.1	EU617579.1	x/nd
AS420	*A. tomatophila cfr*	Jaíba/MG- BR	nd	nd	nd/nd
AS422	*A. tomatophila cfr*	Jaíba/MG- BR	nd	nd	nd/nd
AS423	*A. tomatophila cfr*	Jaíba/MG-BR	EU617498.1	EU617608.1	nd/x
AS440	*A. cretica*	Carandaí/MG-BR	nd	nd	nd/x
AS452	*A. tomatophila cfr*	Planaltina/DF-BR	nd	nd	nd/nd
AS453	nd	Planaltina/DF-BR	nd	nd	nd/x
AS508	(-)	Sumidouro/RJ-BR	nd	nd	nd/nd
S842FV	*A. tomatophila cfr*	Piraúba/MG-BR	nd	nd	nd/nd
TOM4	*A. cretica*	Cons. Lafaiete/MG-BR	nd	nd	nd/nd
UFV1T	(-)	Viçosa/MG-BR	nd	nd	nd/nd

[a] species identification confirmed by E.G.Simmons; [b] MAT1-1/1-2, represents mating type 1-1 and mating type 1-2; [c] EGS code are typical isolates provided by E.G. Simmons; [d] x = determined in the present study; [e] nd=not determined; [f] (-) no sporulation in this study; [g] GenBank accession number. Location refers to municipalities in the Federal District (Distrito Federal – DF) or in the states of Minas Gerais (MG), Rio de Janeiro (RJ), Goiás (GO), Paraná (PR), and Rio Grande do Sul (RS).
* Isolates used only in phylogenetic analysis, availability only of DNA.

TABLE 2. Morphological characteristics of the *Alternaria* spp. isolates collected from infected potato and tomato plants and the ex-type isolates of *A. tomatophila* and *A. solani*.

Isolate	Species	Beaks (%)	Body		Beak	Septa [c]	
		1/2/3*	Length	Width	Length 1/2/3*	Long.	Trans.
Host: *S. lycopersicum*							
EGS 42-156**	*A. tomatophila*	11/65/24[a]	70 - 99	16 - 20	147-216/144-184/97-172 [b]	1 - 4	7 - 12
AS109	*A. tomatophila*	35/52/13	73 - 92	17 - 20	99-197/127-176/102-166	1 - 4	7 - 12
AS232	*A. tomatophila cfr*	59/42/2	77 - 97	12 - 17	161-189/92-144/104-184	1 - 4	8 - 10
AS440	*A. cretica*	62/34/4	70 - 100	12 - 16	133-197/126-199/121-147	0 - 3	7 - 12
Host: *S. tuberosum*							
EGS 44-098	*A. solani*		85 - 100	18 - 22	83 - 110 [d]	1 - 3	8 - 12
AS185	*A. grandis*		132 - 175	14 - 16	135 - 192	0 - 2	11 - 14
AS216	*A grandis*		104 - 161	14 - 17	156 - 206	0 - 3	11 - 14
AS220	*A. grandis*		137 - 184	14 - 16	144 - 202	0 - 2	10 - 14
AS248	*A. grandis*		102 - 152	14 - 16	142 - 189	0 - 2	9 - 11

* Conidium can have 1, 2, or 3 beaks. Measurements were made in all three categories. ** EGS code of ex-type isolates.
[a] Percentage of conidia from tomato isolates with one, two and three beaks, respectively; [b] for tomato isolates, range of beak length (minimum – maximum) of conidia with one, two and three beaks, respectively; [c] trans = transversal, long = longitudinal septa; [d] for potato isolates, range of beak length (minimum – maximum) of conidia with one beak.

TABLE 3. Number of lesions and lesion area on tomato plants inoculated with isolates of *Alternaria* spp. (pathogenicity assay 1).

Isolate - code	Species	Number of lesions (SD)	Lesion area -mm^2 (SD)
EGS 42-156	*A. tomatophila*	9.56 (4.4)	5.2 (5.2)
AS109	*A. tomatophila*	6.8 (3.4)	4.3 (4.2)
AS232	*A. tomatophila cfr*	8.6 (3.9)	5.1 (6.8)
AS420	*A. tomatophila cfr*	8.4 (4.9)	2.7 (3.4)
AS440	*A. cretica*	4.5 (2.9)	9.9 (8.1)

SD = standard deviation

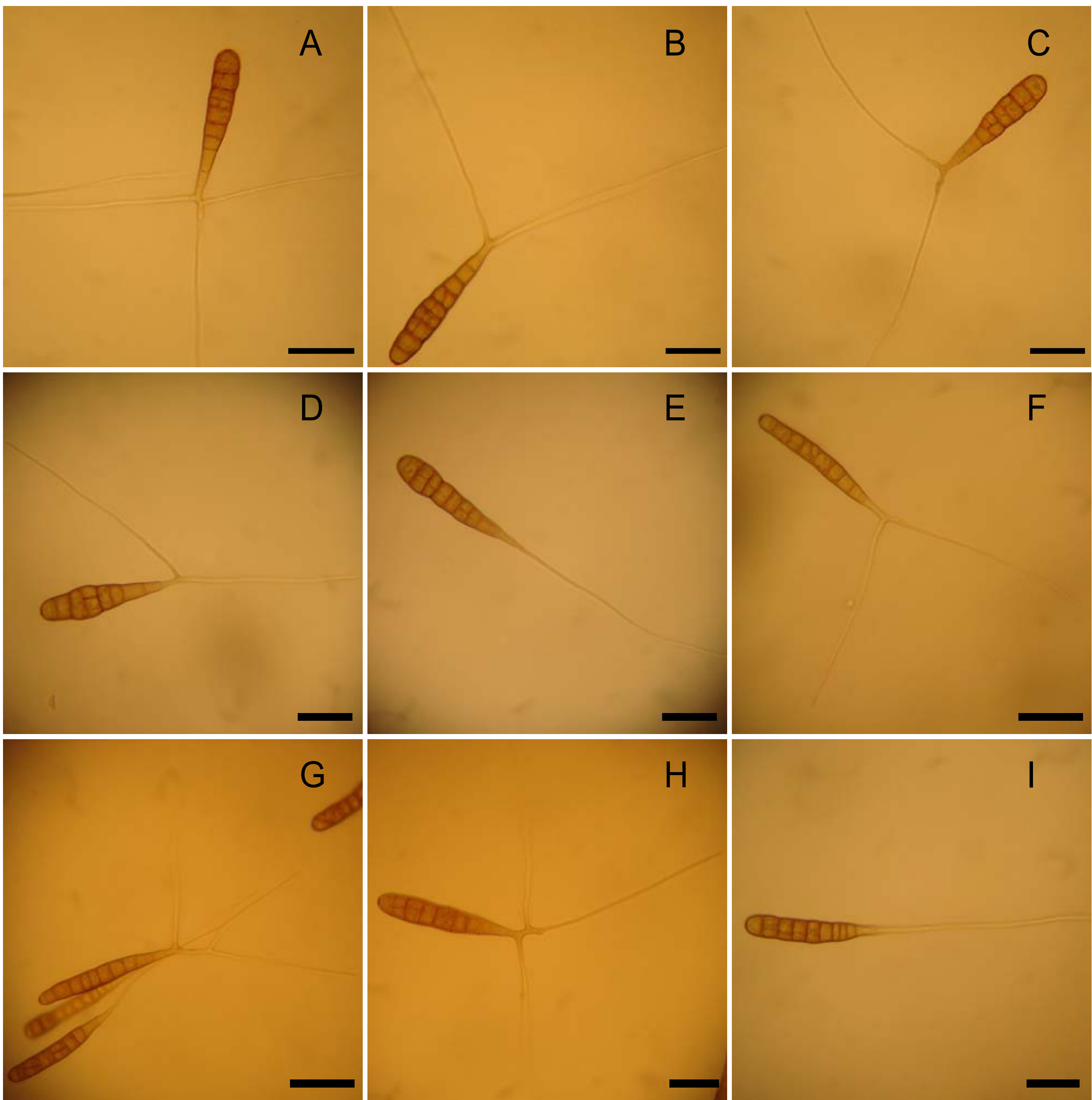


Figure 1. Conidia of *Alternaria* spp. A,B,C - *A. tomatophila* (EGS 42-156); D, E - *A. tomatophila* (AS109); F,G - *A. tomatophila* cfr (AS232), H,I - *A. cretica* (AS440). Bars = 30 µm.

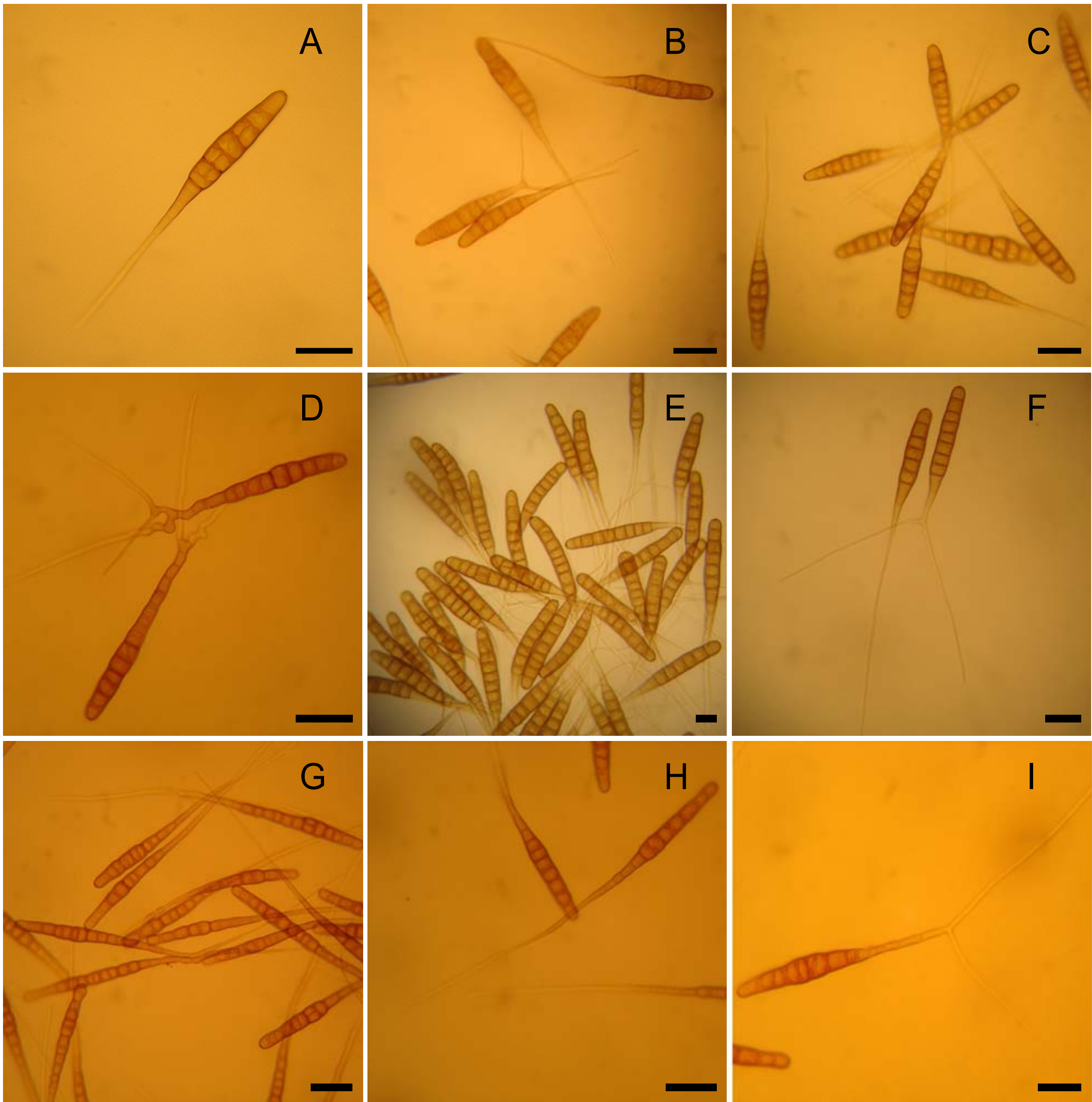


Figure 2. Conidia of *Alternaria* spp. A,B,C - *A. solani* (EGS 44-098); D – I - *A. grandis* (D - AS352; E,F - AS248, G – AS220, H, I - AS185). Bars = 30 µm.

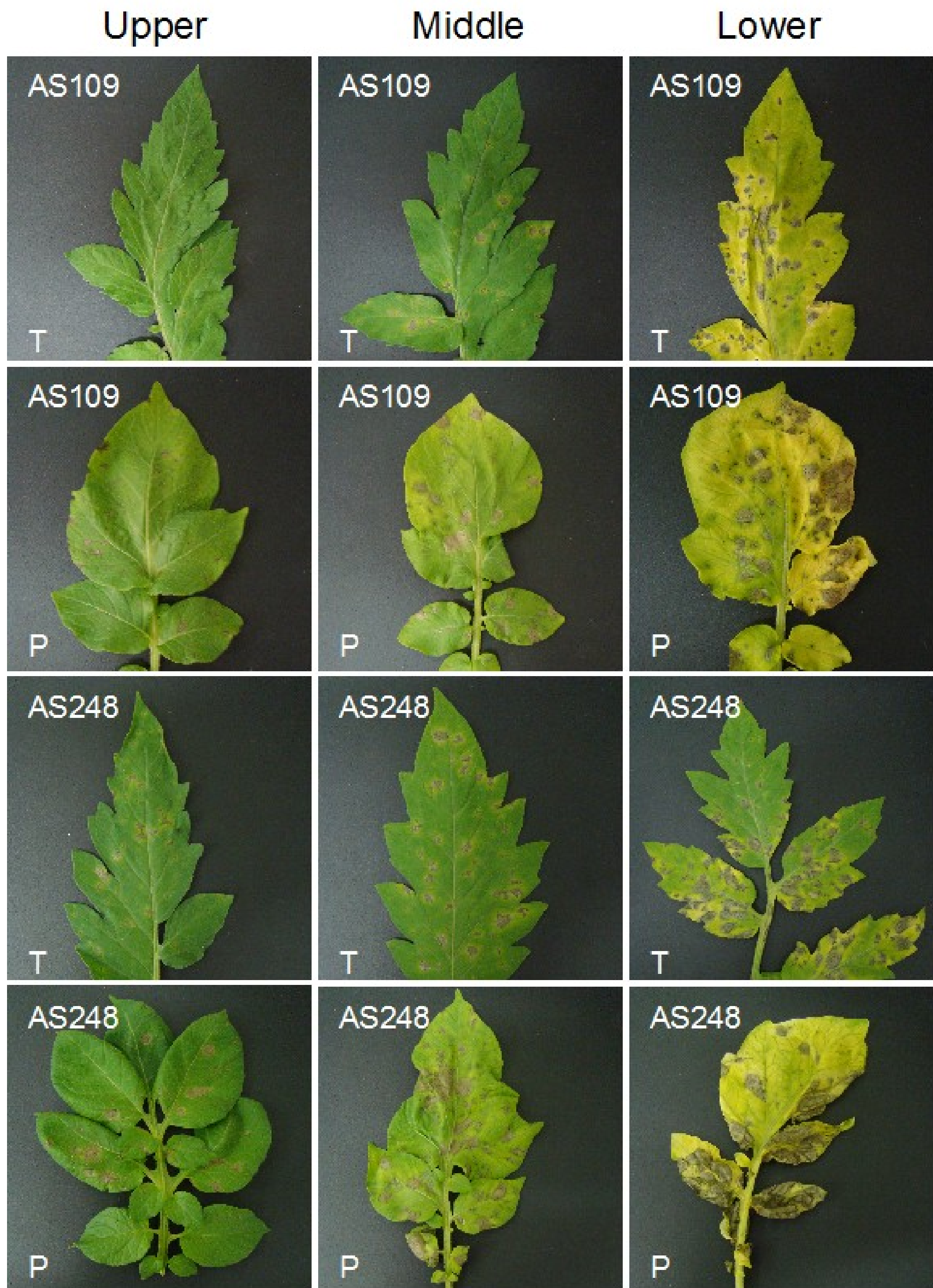


Figure 3. Lesions on potato and tomato plants caused by *A. tomatophila* (AS109) and *A. grandis* (AS248) in the cross inoculation assay (pathogenicity assay 2). Leaflets of different ages correspond to upper, middle and lower thirds of each plant.

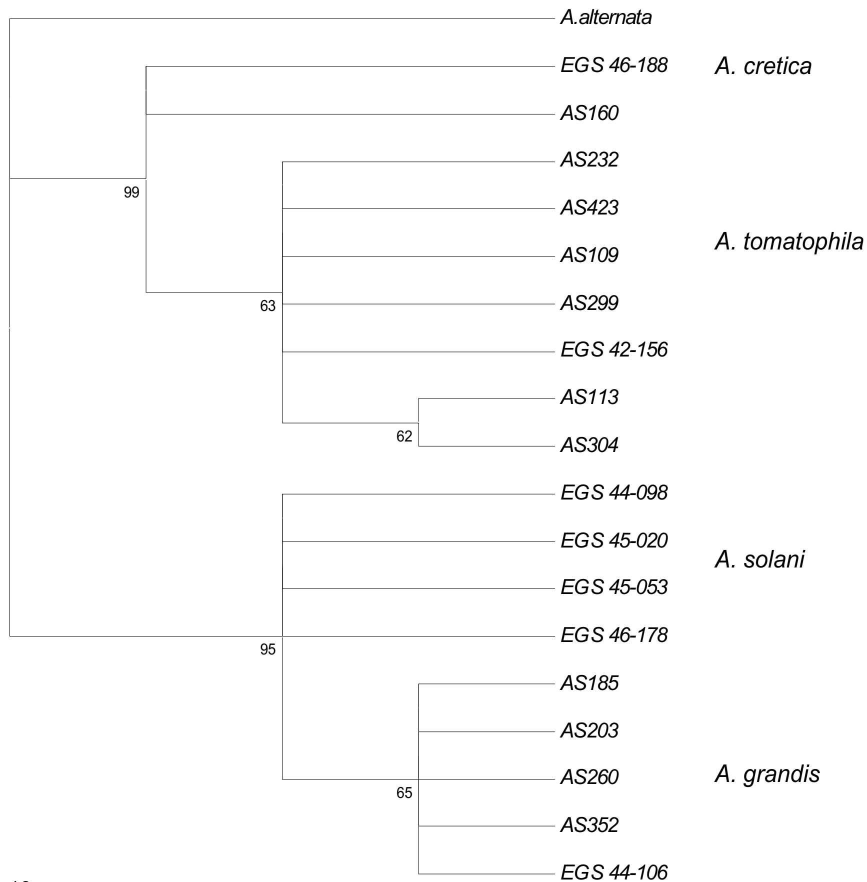
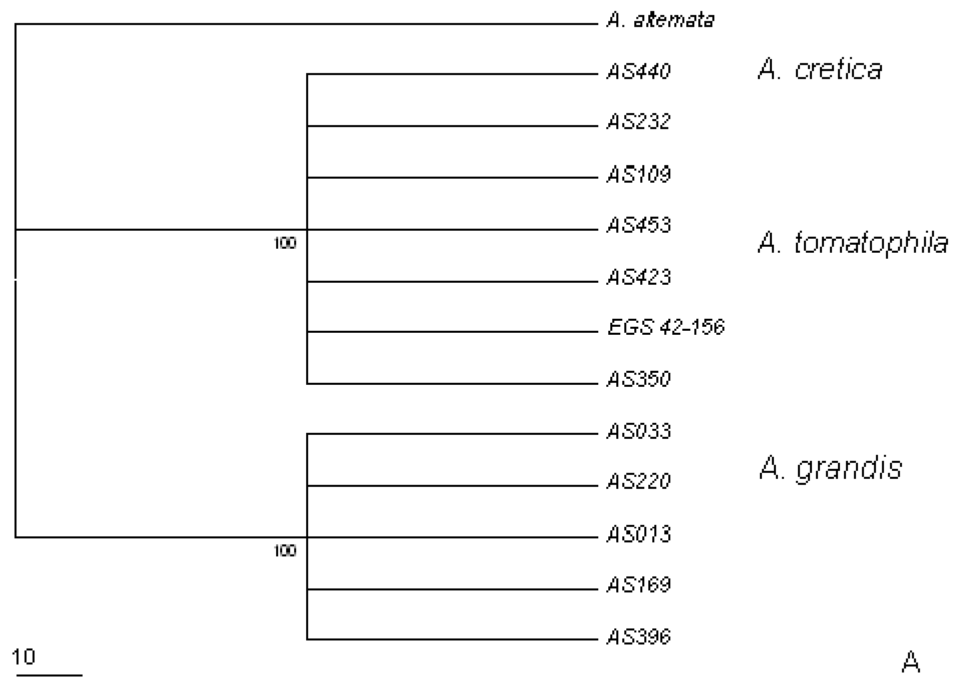


Figure 4. One of 10 most-parsimonious phylogenetic trees inferred from the combined dataset, Alta1/GPD, illustrating the relationship of *Alternaria* spp. obtained from potato and tomato. The tree was rooted to *A. alternata* (GenBank: *Alt a 1* – AY563301, *GPD* – AF081400). Bootstrap values are indicated below nodes.



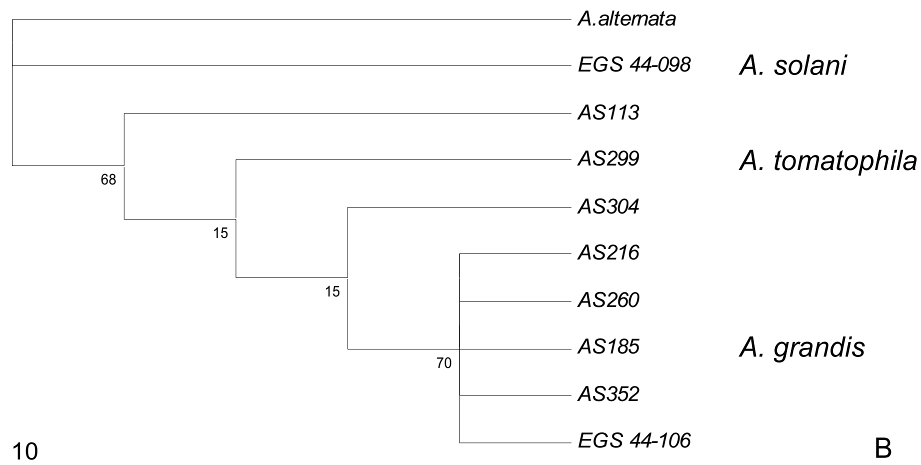


Figure 5. One of 10 most-parsimonious phylogenetic trees inferred from the MAT1-2 (A) and MAT1-1 (B) data set, illustrating the relationship of *Alternaria* spp. obtained from potato and tomato. The tree was rooted to *A. alternata* (GenBank *MAT1-1* – AB009451, *MAT1-2* – AB009452). Bootstrap values are indicated below nodes.

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2- Potential for the occurrence of recombination based on mycelial compatibility, the distribution of mating type genes, and analysis of genetic diversity between different species of *Alternaria* associated with potato and tomato early blight.

ABSTRACT

An important requirement to heterokaryon formation and consequently recombination events is the mycelial compatibility (MC) to allow hyphal anastomosis. The MC was assessed in isolates of *Alternaria tomatophila* and *Alternaria grandis*, causal agents of tomato and potato early blight in Brazil, respectively, by pairing wild type isolates and *nit* mutants. The formation of a barrage zone was observed mostly between isolates from different species, even though most interactions were compatible within and between species. Heterokaryosis was confirmed when pairing *nit* mutants from isolates of the same species. Pairings involving NitM and *nit1/3* were most successfully to recover heterokaryons. The mating type genes were amplified to determine its frequency and distribution in the Brazilian populations of *A. tomatophila* and *A. grandis*. Both mating types were widely distributed in different regions and were present in both species, but no isolate had the two idiomorphs. The equal frequency of the *MAT1-1* and *MAT1-2* in *A. grandis* population supports the potential occurrence of recombination in this species. High genetic diversity within and between species was demonstrated by analysis of AFLP marker. The sequences of *Alt a 1*, *GPD* and *MAT1-2* genes were polymorphic in populations of *A. tomatophila* and *A. grandis* and these species are phylogenetically separated. The analysis of the three loci revealed two putative hybrids on the *A. grandis* population. The putative hybrids shared sequences from *A. tomatophila* and *A. grandis*. There was strong evidence for the occurrence of recombination in *Alternaria* spp. related to early blight in potato and tomato crops.

2.1. INTRODUCTION

In several parts of the world, a single species of the genus *Alternaria, A. solani* Sorauer, has been commonly reported as the causal agent of potato and tomato early blight (Rotem, 1994). However, after reviewing the *Alternaria* spp. associated with the Solanaceae, Simmons (2000) described *A. cretica*, *A. subcylindrica*, and the widely distributed species, *A. tomatophila,* as the causal agents of early blight on tomato. In addition to *A. solani*, *A. grandis* Simmons has also been described as a causal agent of early blight on potato. Although there have been few studies about the occurrence and distribution of those new species, *A. tomatophila* and *A. solani* seem to be the most common species associated to early blight disease on tomato and potato (Simmons, 2007).

An understanding of the occurrence and distribution of species of plant pathogenic fungi is crucial to plan proper control actions and minimize crop damage. Recently, a study reported variability in *Alternaria* species associated with potato and tomato from different producing areas in Brazil (Rodrigues *et al*., unpublished data). Using both morphological and molecular data, this study reported: 1. The first occurrence of *A. tomatophila* and *A. cretica* on tomato; 2. The wide distribution of *A. grandis* as incitant of potato early blight; 3. The capability of conidia of *A. tomatophila* and *A. grandis* to infect both hosts; and 4. The close phylogenetic relationship among the *Alternaria* species causing early blight on potato and tomato.

Given this situation, studies aimed at understanding the biology, ecology, and pathogenic aspects of *Alternaria* spp. need to be conducted to develop strategies to control the disease. Due to the geographic proximity of several potato and tomato fields, an important consideration is the concomitant occurrence of different fungal species in a given area causing early blight.. Host specificity is not strict and so nearby potato and

tomato fields offer an opportunity for different fungal species to grow near one another. In Brazil conditions favorable to inoculum dispersal are present year-round and airborne conidia of *Alternaria* spp. have been captured throughout the year (Batista, 2006). Under these conditions, could individuals of different pathogenic species exchange genetic material? Is there any evidence of recombination within and between species?

A first step to recombination via either the sexual or parasexual cycle is the anastomosis between hyphae of different individuals (Leslie, 1993). Anastomosis allows the formation of a stable heterokaryon in a compatible interaction. On the other hand, an incompatible interaction can result in cell death (Glass *et al*., 2003, Glass *et al*., 2004). Multinucleate cells have been reported in hyphae, conidiophores, conidia, and germ tubes of *A. solani* (Stall, 1958, King and Alexander, 1969). The authors observed hyphae anastomosis and suggested that heterokaryosis may have generated the multinucleate state. Evidence of heterokaryosis was found using auxotrophic mutants in *A. dauci* (Netzer and Kenneth, 1970) and *A. brassicicola* (Craven *et. al*., 2008), but apparently, no study has been done with *A. solani* and the species associated with Solanaceae.

The stable heterokaryon is permitted between individuals that have vegetative compatibility (VC). Vegetative compatibility is governed by numerous loci, thus incompatible allelic and nonallelic interactions between those loci can result in vegetative incompatibility (Glass *et al*., 2000). It is important in maintaining the integrity of fungal individuals and preventing the spread of parasitic elements such as viruses that would otherwise be transmitted in the cytoplasm (Jacobson *et al*., 1998). Because VC has a phenotype, compatible individuals can be placed in the same vegetative compatibility group (VCG). Hence, this marker has been used in studies of the population biology of several plant pathogenic fungi, such as *Cryphonectria*

parasitica (Cortesi *et al*., 1996, Liu and Milgroom, 2007), *Fusarium graminearum* (McCallum *et al*., 2004, Akinsanmi *et al*., 2008), *Sclerotinia homoeocarpa* (Powell and Vargas Jr, 2001), and *Cercospora coffeicola* (Martins *et al*., 2008).

The VC can be assessed using mycelial interaction (mycelial compatibility – MC) or by pairing complementary nitrate nonutilizing mutants (*nit* mutants) to test for the formation of VCG. For MC, the presence or absence of a barrage zone in incompatible and compatible interactions, respectively, are used to score a pairing. In *A. solani*, the barrage zone is characterized by a brown line, visible from underneath, at the interface where incompatible mycelia of the two isolates have met (van der Walls *et al*., 2004). Compatible isolates produced no detectable barrage zone, and the mycelia mix freely (van der Walls *et al*., 2004). In a study conducted in Brazil using vegetative compatibility, the MC groups (MCG) of *A. solani* isolates was used to investigate differences between potato and tomato isolates, but results were unclear (Oliveira, 2007). Three aspects possibly affected the conclusions: 1. Failure in recognizing that more than one species of *Alternaria* can be associated with early blight in Brazil, as mentioned above; 2. The low number of isolates from tomato; and 3. The inherent variability associated with MCG.

Since the barrage zone differs between species and varies according to environmental conditions such as temperature, lighting, and culture medium (McCallum *et al*., 2004), an additional technique to assess the interaction involves microscopy. Transmission electron microscopy has been used to recognize cell death after anastomosis in incompatible interactions between *Neurospora crassa* individuals (Jacobson *et al*., 1998). Although electron microscopy does reveal ultrastructural changes in dying cells, preparing hyphae for observation is time consuming. Detecting the autofluorescence emitted by dead cells using the fluorescence microscopy seems to

be a good alternative since it requires no special tissue preparation. One of the first reports of autofluorescence in dead cells of fungi described the relationship between stressed cells and fluorescence in 15 species of fungi including *Alternaria* spp. (Wu and Warren, 1984). A more recent report used autofluorescence to detect nonviable cells of an arbuscular mycorrhizal (AM) fungus in association with palm roots (Dreyer *et al.*, 2006). In this study of AM fungus, the source of autofluorescence was located in the fungal cell wall although its exact nature was not determined. In other studies of AM fungi, the fluorescence was concentrated in the cytoplasm, and some kind of phenolic material may have been involved (Vierheilig *et al.*, 1999, Vierheilig *et al.*, 2001).

A more definitive analysis of heterokaryon formation, and consequently of VC, is based on pairings between complementary nitrate nonutilizing mutants (*nit* mutants). Chlorate and nitrate assimilation follow the same pathway, but once in the cell, chlorate is reduced to the toxic chlorite, reducing hyphal growth. The *nit* mutants emerge spontaneously as chlorate resistant sectors. Based on the growth in different sources of nitrogen, the mutants' phenotypes can be assigned as *nit1*, *nit3,* or NitM (Correll *et al.*, 1987). Compatible pairings between *nit1/3* and NitM, and between NitM, result in strong complementation, which is easily visualized when dense, aerial, prototrophic hyphal growth in the contact zone between two mutant colonies (Hawthorne and Rees-George, 1996). Having an easy way to screen for the heterokaryon formation is important in selecting for auxotrophic mutants. However, obtaining *nit* mutants is not always easy (Kohn *et al.*, 1990). For the fungal species where *nit* mutants cannot be selected, use of the MC to analyze VC, offers a feasible alternative, even though strict correspondence between VCG and MCG is not always observed (Ford *et al.*, 1995).

The process of sexual reproduction in ascomycetes involves a mating system that is controlled by mating type genes. Mating compatibility is regulated by a mating

type locus (MAT) composed by a single locus in two different forms, or idiomorphs: *MAT1-1* and *MAT1-2* (Metzenberg and Glass, 1990, Turgeon and Yoder, 2000). The occurrence of both or of one idiomorph in a single organism determines the mating pattern as self-fertilization or obligate out-crossing, respectively (Coppin *et al*., 1997). In general, the MAT genes regulate expression of sex pheromones and pheromone receptors, which promote morphogenic changes, allowing for sexual reproduction. In *Neurospora crassa,* MAT genes control not only mating, but also vegetative incompatibility (Coppin *et al*., 1997). Little is known about the MAT genes and the potential additional functions they may exert on fungal life cycle. Involvement in the maintenance of cell wall integrity (Barbour and Xiao, 2005), and pathogenicity (Zhan *et al*., 2007) have been postulated to be associated with MAT genes.

The mating type idiomorphs were found in supposedly asexual species of *Alternaria*, which supports a history of recombination, or a current non-characterized recombination process (es) (Berbee *et al*., 2003). *In vitro* and *in vivo* observations failed to detect sexual structures, probably because specific conditions to induce fruiting bodies are required (O'Gorman *et al*., 2009).

Despite the wide distribution of *A. tomatophila* and *A. grandis*, no previous studies addressed the potential exchange of genetic material between species. Thus, the main objectives of this study were to characterize the vegetative compatibility, to detect and quantify the frequency and distribution of mating type idiomorphs, and to infer about recombination within and between the species related to early blight on potato and tomato.

2.2. MATERIALS AND METHODS

Isolates. Monoconidial cultures of *Alternaria* spp. were obtained from typical

early blight lesions developed on infected potato and tomato plants. Different fields were sampled in producing regions of Brazil (Table 1). Samples were taken to the laboratory where direct or indirect pathogen isolation was conducted (Lourenço Jr et al., *in press*). The isolates are part of the culture collection of the Laboratório de Biologia de Populações de Fitopatógenos of the Universidade Federal de Viçosa. Cultures were stored in filter paper at -80°C. The ex-type isolate of *A. cretica* (EGS 46-188), *A. grandis* (EGS 44-108), *A. solani* (EGS 44-098) and *A. tomatophila* (EGS 42-156) were provided by E. G. Simmons.

Mycelial compatibility (MC). Sixty-nine isolates (35 of tomato, 34 of potato) were paired in all combinations, including the self-pairings to serve as controls. A total of 2,415 pairings were done. Fifteen 5 mm-diameter mycelial plugs were transferred to potato-dextrose-agar medium (PDA) in a Petri dish (90 mm diameter). Plugs were placed approximately 20 mm apart from each other (Figure 1A). Plates were kept at 25°C in the dark for 14 days, and then scored for reactions. Isolates were scored as compatible when no barrage was formed between two colonies. Incompatible interactions were characterized by a barrage zone; a brown line formed at the site where the mycelia of the two isolates met (Figure 1A) (van der Waals *et al.*, 2004). Plates were kept under the same incubation conditions and compatible pairings were inspected at weekly intervals for the presence of sexual structures up to 60 days of incubation. Each pairing was performed at least twice.

Fluorescence and confocal microscopy. Sixteen compatible and incompatible interactions were observed at the cellular level under the fluorescence and confocal microscopes. Preliminary fluorescence microscopic analysis allowed establishment of the appropriate wavelengths of excitation and emission to capture the autofluorescence in hyphae of *Alternaria* spp. The pairings were observed under UV excitation using the

filters to Rhodamine (λ excitation = 540 ηm, λ emission = 625 ηm), DAPI (λ excitation = 350 ηm, λ emission = 470 ηm), and FITC (λ excitation = 490 ηm, λ emission = 525 ηm). The pairings for microscopic analysis were performed as follows: a thin layer of PDA was placed on a Petri dish. A microscope slide was placed on the medium and covered with another thin layer of PDA. Two plugs of different isolates were placed 20 mm apart so that the contact zone between the colonies formed over the microscope slide. After 10 days at 25°C in the dark, the microscope slide was removed and observed. Three treatments were assessed under the fluorescence microscope (Zeiss Stemi DV4): self-pairing (positive control), incompatible pairing, and compatible pairing submitted to heat treatment to kill fungal cells (negative control). The fungus was dead after 1 min in the microwave at maximum power. For the confocal analysis, pairings between the isolates AS440 (*A.cretica*), AS260 (*A.grandis*), AS109 (*A.tomatophila*), and EGS 44-098 (*A.solani*) were performed. The images were obtained using the Zeiss LSM 510 inverted META system, helium-neon laser and a 100X oil immersion objective. The auto-fluorescence was detected with excitation of 524 nm and its emission was collected from 530 to 600 ηm. The pinhole was fixed providing an optical band from 0.8 to 1 ηm. The images were processed using the LSM image BROWER 4 software (Zeiss). The analyses were conduced at the Núcleo de Microscopia e Microanálise of the Universidade Federal de Viçosa.

***Nit* mutants.** The same isolates used in the MC experiment were used to generate nitrate non-utilizing mutants. Isolates grew in Czapek Dox Agar medium (CDA) at 25°C in the dark, and after 7 days transferred to CDA amended with Bengal Rose-BR (10 ml/ L CDA, stock solution 5 mg BR/ml ethanol 95%), and 4% potassium chlorate (Elias and Cotty, 1995). The plates were examined periodically for the appearance of fast-growing sectors, which usually appeared around 25 days of incubation. To verify the mutation,

and to optimize the detection of NitM, 15 plugs of each sector were transferred to CDA. The plugs were arranged in three rows, five plugs per row. After five days colonies that grew to be thin, expansive, and without aerial mycelial were considered *nit* mutants. The Nit M mutants were detected by the heterokaryon formation with adjacent mutants (Nit M, *nit1* or *nit3*). Complementation, as a result of heterokaryon formation, was observed as dense, aerial, prototrophic growth in the contact zone between two mutant colonies (Fig. 1E, F) (Hawthorne and Rees-George, 1996). The phenotypes of mutants were confirmed based on their growth on minimal agar medium (MM) (10 g glucose; 1 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 10 mg $FeSO_4.7H_2O$; 20 g agar; 2 g $NaNO_3$; 1 L distilled water), amended with one of several nitrogen sources: sodium nitrate 20 g/L (MMNA), sodium nitrite 0.5 g/L (MMNI), ammonium-tartrate 1g/L (MMAT) and hypoxanthine 0.2 g/L (MMHY) (Correll et al., 1987). For all *nit1* and *nit3* mutants, one duplicate per isolate was preserved in cultures tubes with MMAT at 5°C in the dark (Martins *et al*., 2008). All Nit M mutants obtained were preserved. After preservation from 2 to 12 months, the mutants were checked for stability of mutation and phenotype. Colonies of stable mutants retained the thin mycelial growth on MMNA described previously. Mutants were considered unstable if the wild type growing pattern was observed on MMNA, or if a different phenotype from that previously characterized was observed. Each stable mutant was subcultured using the hyphal tip technique at least three times to confirm stability. Twenty-eight isolates were paired in all combinations. For each isolate, two different phenotypes were paired (Nit M and *nit1* or *nit 3*). Fifteen 5 mm-diameter mycelial plugs were placed in 3 rows, with five plugs each, on CDA medium in a Petri dish (130 mm diameter). Plugs were placed approximately 20 mm apart. Plates were kept at 25°C in the dark for 14 days, and then scored for reactions. Isolates were scored as compatible when a heterokaryon was formed. Each pairing was

performed twice.

Mating type idiomorphs. The fungus was grown in liquid medium (Alfenas *et al*., 1998) and the mycelium was filtered to extract DNA. The extraction was performed using a CTAB protocol (Scheuermann *et al*., 2004). The DNA concentration of each sample was adjusted to 50-100 ηg DNA/µl. PCR reactions were done using Pure *Taq* ReadyToGo PCR Beads (GE Healthcare) according to the manufacturer's instruction. A combination of specific primers ORF556 forward (5'-GTG GTC AAG GTC CGT GAC GG-3') - Jen2 reverse (5'-CGA AGG TGC TCT TCT TTT GC-3'), and ORF556 forward - PaAlpha reverse (5'-GGA GAG CTT CTT CAT GGG CC-3') was used to detect the HMG box of the *MAT1-2* gene, and the Alpha box of the *MAT 1-1* gene, respectively (Inderbitzin *et al*., 2006). To obtain the mating type fragments 10 µl of the product of the first amplification was separated in a 2% NuSieve GT agarose gel. The band with the expected size was excised from the gel and melted in 100 µl of water at 65°C for 15 min. The sample was diluted 10x and used as a template for the second amplification with the same primers. Thermal conditions were initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, elongation at 72°C for 30s and extended by 4 additional seconds per cycle, and a final elongation at 72°C for 7 min. The same thermal conditions were used for the first and second PCR, except the annealing temperature of the second PCR was raised to 55°C. The PCR products were purified by ethanol precipitation (Okayama and Berg, 1983) and sequenced (Applied Biosystems BigDye v.3.1 Terminator Chemistry). The sequencing was performed at NAPS Unit, University of British Columbia, Vancouver BC, Canada. The sequences were edited and the consensus sequence was built using the AutoAssembler Ver. 1.4 software for Macintosh.

AFLP. For AFLP analyses, the genomic DNA was digested with restriction

endonucleases *Eco*RI and *Mse*I following the manufacturer's instructions (Invitrogen KIT AFLP – AFLP® Analysis System I). The selective amplification was done using the combination of primers E+AC - M+A and E+AT - M+A . The PCR products were visualized in 6% polyacrylamide gel after stained with silver nitrate (Creste *et al.*, 2001).

DNA sequence. Based on the results from the MC, *nit* mutants interactions, and AFLP analysis, formation of interspecific hybrids was demonstrated to be feasible. Thus, sequences of the *Alt a 1*, *GPD*, and mating type genes were used in phylogenetic analysis to address this issue. Haplotypes of the *Alt a 1* and *GPD* regions from a previous study were used to construct clone-corrected data sets (Lourenço Júnior *et al., in press*). Different data sets were constructed according to the species and gene under consideration.

Data Analyses. For the AFLP data each genotype was analyzed for the presence (1) or absence (0) of bands in all polymorphic informative AFLP loci. A binary matrix was constructed to create multilocus haplotypes. The number and diversity of haplotypes were calculated using the Arlequin 3.0 program (Excoffier *et al.*, 2005). To assess the recombination between species, two populations were defined (potato and tomato), and the Index Association (I_A) was calculated by Multilocus 1.3 program (Agapow & Burt, 2001). The phylogeny was also inferred using AFLP haplotypes data. The dendrogram was constructed with unweighted pair group method of averages (UPGMA) and the distances were computed using the Nei method. The bootstrap consensus tree was inferred from 1000 pseudoreplicates. The analysis was implemented on Treecon for Windows version 1.3b.

A $\chi 2$ test was applied to determine whether the frequencies of the two mating types departed from the null hypothesis of a 1:1 ratio (Groenewald *et al.*, 2008). Using

Alta a 1, *GPD* and *MAT1-2* sequences the evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree was inferred from 10,000 pseudoreplicates. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Codon positions included were 1st+2nd+3rd +Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

2.3. RESULTS

Mycelial compatibility. Of the 2,415 total pairings, compatible and incompatible reactions were observed for 1,654 and 761 pairings, respectively. Typical and atypical compatible and incompatible interactions were observed. Atypical compatible interactions showed no barrage zone, but the mycelia did not mix freely (Figure 1A). Atypical incompatible interactions were characterized by the formation of a weak barrage zone (less intense and clearer than the typical reaction). In other cases where reactions were atypical, colonies were sharply delimited with no mix of mycelia of adjacent colonies, resembling pairings where secretion of inhibitory compounds limited growth (Figure 1A, B). Most of the incompatible pairings involved isolates from different species/host. Of the incompatible reactions, 618 resulted when *A. grandis* isolates were paired with *A. tomatophila* isolates and the single *A. cretica* isolate (AS440). One hundred and twenty-nine incompatible reactions resulted from pairings between *A. grandis* isolates, and only 14 between *A. tomatophila* isolates (Table 1). All isolates were self-compatible. Most isolates within each species revealed compatibility irrespective of their geographical origin. The majority of the interactions were compatible, but each isolate represented a MCG, because it was not possible to cluster

compatible isolates in a group where all of them were incompatible with all of the isolates in another group. No sexual structures were observed in compatible interactions between isolates of the same or different species.

Fluorescence and confocal microscopy. The fluorescence emitted by dead cells was observed under UV excitation using the filter to Rhodamine. The number of cells with fluorescence in the negative control and in the incompatible interactions was higher than in the positive control (data not shown). In the confocal microscopical analysis the auto-fluorescence was observed in the cytoplasm of the cells in all types of incompatible interactions (typical or not). Smaller numbers of fluorescent cells were observed in compatible interactions (*A. cretica* – AS440 x AS440), than in the incompatible ones (*A. grandis* x *A. cretica* – AS260 x AS440 and *A. solani* x *A. tomatophila* – EGS 44-098 x AS109) (Figure 2).

***Nit* mutants.** Of the 69 isolates tested, 742 *nit* mutants were obtained for 55 isolates (31 from tomato and 24 from potato). No mutants were obtained for 14 isolates either because they did not grow on CDA+BR or failed to produce characteristic mutant sectors (Figure 1C, D). Usually, the recovery of mutant sectors is around 100%, but in this study only 80% of the isolates produced *nit* mutants. The *nit3* was the most frequent phenotype (45.6%), followed by *nit1* (31.7%), and Nit M (10.9%). Another phenotype, corresponding to inability to use the nitrogen sources available in this study, was detected and designated *nit-* (11.8%). Three different phenotypes, including the Nit M, were obtained for 28 isolates. Out of 1,596 pairings, only 40 were compatible. Auto incompatibility occurred in 46% of the isolates. Compatible interactions occurred between isolates of the same species (AS016 x AS080, AS110 x AS124, AS298 x AS482). An exception was the compatible pairing between AS440 (*A. cretica*) and AS349 (*A. grandis*). All complementation occurred between Nit M and *nit1* or *3*. Out of

the 84 mutants, only 15 were stable after three subcultures (10 of tomato, 5 of potato). The reason for the instability was not determined.

Mating type idiomorphs. The mating type of 58 isolates and the ex-type culture of *A. tomatophila* (EGS 42-156) were determined and PCR amplification of both idiomorphs was attempted for each species. Twelve and 32 sequences were obtained for the *MAT1-1* and *MAT1-2*, respectively (Table 1). The sizes of the amplified fragments were 760 bp for *MAT1-1* and 629 bp for *MAT1-2*. Among the isolates sampled from potato fields in Camanducaia-MG, Brasília-DF, Cristalina-GO, and Pelotas-RS there were isolates of *MAT1-1* and isolates of *MAT1-2* (Table 1). Regardless of the host crop, no sampled isolates had both idiomorphs in the same individual. When testing the null hypothesis of an equal ratio of MAT idiomorphs in the groups of isolates from potato or tomato, an unequal ratio was observed for the tomato population, but *MAT1-1* and *MAT1-2* occurred in a 1:1 ratio in the group of potato isolates (Table 2).

AFLP. A total of 28 polymorphic loci were scored using the E+AC – M+A primer combination, and the haplotype diversity (Hd) was 1.0. Using the E+AT - M+A primers, 24 loci were scored, some haplotypes were shared among populations, and haplotype diversity was lower than for the first combination (E+AC – M+A) (Tables 1 and 2). Clustering according to the UPGMA analysis resulted in two clades (Figure 4): One with isolates of *A. grandis* only and another with isolates of *A. tomatophila*, *A. cretica*, and *A. grandis*. In the second clade, at least three groups were formed and one group (H10, H14, H24, H27, H1, H15) comprised of predominantly *A. tomatophila* haplotypes, but the H10 is represented by one isolate of *A. grandis* (AS248). For the index of association test, the average I_A value was 2.75 ($P< 0.001$) indicating that random mating was not occurring in the population.

DNA sequence and phylogenetic analysis. The reconstruction of the phylogeny

supported two clades. One clade included *A. tomatophila* and *A. cretica* and the other clade was formed by *A. grandis* and *A. solani*. The *A. grandis* isolates AS248 and AS012 were placed on different clades according to the gene analyzed (Figure 3). For *Alt a 1* and *GPD* sequences the isolate AS248 was placed in the *A. tomatophila* clade. However, for the *MAT1-2* sequences, the isolate was placed in the *A. grandis* clade. The isolate AS012 was placed in the *A. tomatophila* and *A. grandis* clade when analyzed for the *GPD* and *MAT1-2* genes, respectively. The sequence of the *Alt a 1* gene was not determined for isolate AS012.

2.4. DISCUSSION

Different species of *Alternaria* are associated with early blight on potato and tomato, and incompatibility supports the results of morphological and molecular analysis gathered in previous reports (Lourenço Jr. *et al., in press*; Rodrigues *et al.*, unpublished data). Incompatibility was more frequently observed when pairing isolates from different host species. However, high compatibility was also observed between *A. tomatophila* and *A. grandis*. One possible reason is a recent speciation process and the incomplete genetic isolation. Presumably, some isolates of these species still retain ancestral alleles enabling them to interact with each other (Akinsanmi *et al.*, 2008). Nevertheless, the high number of MC groups even within population makes interpretation of the MC data not trivial. For *A. tomatophila* population, there were several compatible reactions and mutually compatible isolates showed different patterns of compatibility with other isolates. Additionally, in general, compatibility was uncorrelated with geographical origin of the isolates at the state or region levels. The only significant correlation was between compatible isolates from the same and nearby municipalities, even though they were from different host species (Viçosa-MG,

Itapetininga-MG, Ponta Grossa-PR). Few exceptions were noted for isolates of *A. grandis* (Brasília-DF, Planaltina-GO, Cristalina-GO, Contenda-PR, Ponta Grossa-PR) for which incompatibility occurred between isolates of the same municipalities. One possible reason for such variation is high allelic diversity at the loci controlling MC (Akinsanmi *et al*., 2008). The lack of correlation between MC and AFLP also supports high allelic diversity. In South Africa, MC was also independent of geographical origin and populations of *A. solani* were not genetically isolated (van der Walls, *et al.*, 2004).

Based on the present MC analysis exchange of genetic material within and between species is possible. However, despite the high genetic diversity, no sexual structures and evidence of recombination were ever detected in populations of *Alternaria* species from Solanaceae, or in closely related species. The evolutionary consequences of mycelial compatibility between distinct isolates remain to be fully understood. One possible reason is the occurrence of cryptic recombination events. When the sexual stage is lost, parasexual cycle and heterokaryosis become the main mechanisms to generate variability (Leslie, 1993). However, the real potential of these processes in generating recombinants is still unclear, since they have been known to occur only in laboratory conditions (McGuire *et al.*, 2005, Noguchi *et al*., 2006). In the present study, using the *nit* mutants, the phenomenon of the heterokaryosis was demonstrated in *Alternaria* spp., confirming that two different nuclei are able to exist in the same cell.

The microscopical analysis approach improved the understanding of MC in *Alternaria* spp. The absence of proper knowledge about genetic regulation of VC in *Alternaria* spp. prevents clear characterization of compatible and incompatible reactions and the use of autofluorescence was helpful to confirm the way reactions were assessed. Reactions were easily distinguished, including the atypical ones. Another advantageous

aspect of the technique is the observation *in vivo* without staining. However, more information about the mechanism involved in autofluorescence and its relation with viability in fungal MC reactions need to be explored.

In spite of the widespread occurrence of both mating type idiomorphs in all regions, mating and formation of sexual structures were not observed in compatible pairings. The two idiomorphs were found in both populations of the species assessed, but apparently the individuals were unable to reproduce by typical recombination process, under the conditions crosses were made in this study. However, it is possible that recombination is taking place in the population because of the vegetative compatibility data, the maintenance of both idiomorphs, and the high genotypic diversity of the isolates revealed by AFLP markers. For the *A. grandis* population, this hypothesis is more plausible since *MAT1-1* and *MAT1-2* were found in an approximately 1:1 ratio, as expected for a recombinant population (Milgroom, 1996, Groenewald *et al*., 2008) and the Hd values were high. However, using the sequences of *Alt a 1*, *GPD* and *MAT1-2* formal recombination tests implemented in the RDP3 software were performed but there was no evidence of recombinants in the population (data not shown). The low number of polymorphisms may have prevented the detection of recombination events in the sequences and other genomic regions need to be analyzed.

Another important aspect that support the occurrence of recombination in populations of *A. tomatophila* and *A. grandis* is the presence of putative hybrids. When the phylogenetic relationship between *A. tomatophila* and *A. grandis* was reconstructed using sequences from two protein-coding and the *MAT1-2* genes, two putative hybrids (AS012, AS248) were detected. Assuming that reciprocal monophyly and fixation of parsimony-informative sites are indicative of an advanced state of biological speciation

(O'Donnell *et al*., 2000), isolates that shared sequences of *A. tomatophila* and *A. grandis* can support the occurrence of event(s) of hybridization between them. In addition to the evidence gathered after the phylogenetic analysis, the pattern of the AFLP profile for the hybrids was similar to that of *A. tomatophila.* The recovery of hybrids in nature is a rare phenomenon, due to the difficulty to identify the parents and gain or loss of fitness. In controlled conditions interspecific hybrids between *Fusarium oxysporum* f.sp. *lycopersici* and *Fusarium graminearum* demonstrated significant differences in pathogenicity to tomato and wheat from that expressed by the parents (Madhosingh, 2008). The hybrids identified in this study should be subjected to further studies related to pathogenicity, secondary metabolites profile and amplification of other genes to certify the hybrid state.

Detection of sexual structures will formally prove the occurrence of recombination in the population, but this will be a challenging task. More than six months were necessary to observe mature cleistothecia of *Aspergillus fumigatus* under controlled conditions (O'Gorman *et al*., 2009). In the present study, the time, nutritional conditions, and environmental variables may have been inadequate to allow formation of sexual structures and further studies need to be designed to search for the formation of fruiting bodies.

The present study supports the possibility of recombination in populations of *Alternaria* spp. associated with potato and tomato early blight in Brazil. The vegetative compatibility allows the formation of the heterokaryon, an important condition for recombination to take place. The *MAT1-1* and *MAT1-2* genes are present in the population, and an equal ratio of the two idiomorphs in *A. grandis* populations is consistent with recombination. Putative interspecific hybrids were discovered, but detailed epidemiological studies need to be conducted to understand their relationships

with early blight in the field.

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TABLE 1. Description of the *Alternaria* spp. isolates used in this study according to the location from where they were obtained, the isolate code, the morphospecies classification, the mycelial incompatibility reaction, the mating-type idiomorph (MAT) amplified, and the AFLP haplotype.

Location – state [a]	Isolate code [b]	Species	Mycelial incompatibility	MAT	AFLP haplot.
Araxá-MG	AS012P	*A. grandis*	14T,1P (AS349) [c]	1-2	H16
	AS013P	*A. grandis*	15T, 3P(AS098,349,350)	1-2	H17
Bom Repouso-MG	AS185P	*A. grandis*	11T, 2P(AS087,349)	1-1	H21
	AS187P	nd	12T, 2P(AS088,264)	1-1	H16
	AS203P	*A. grandis*	8T, 1P(AS098)	1-1	H17
Bueno Brandão-MG	AS169P	*A. grandis*	14T, 6P(AS080,087,088,098,261,349)	1-2	H17
	AS170P	nd	5T, 1P(AS087)	1-2	H16
Cajuri-MG	AS131T	nd	14P	1-1	H9
Camanducaia-MG	AS216P	*A. grandis*	15T, 4P(AS087,264,349,353)	1-1	H22
	AS220P	*A. grandis*	17T, 6P(AS087,088,248,264,349,350)	1-2	H23
Carandaí-MG	AS440T	*A. cretica*	14P	1-2	H28
Coimbra-MG	AS095T	*A. tomatophila*	9P, 1T(AS120)	1-2	H4
Conselh. Lafaiete-MG	AS232T	*A. tomatophila*	6P	1-2	H10
	AS242T	nd	13P, 2T(AS296,300)	1-2	H10
	AS248P	*A. grandis*	5T, 3P(AS220,263,394)	1-2	H10
Ibiá-MG	AS016P	nd	15T,7P(AS080,087,088,098,248,264, 349)	1-2	H17
Jaíba-MG	AS420T	*A. tomatophila*	15P	1-2	H15
	AS422T	*A. tomatophila*	14P	1-2	H15
	AS423T	*A. tomatophila*	5P	1-2	H15
Monsenhor Izidro-MG	AS394P	*A.grandis*	19T, 2P(AS248,349)	nd [d]	H16
	AS396P	nd	16T, 3P(AS80,264,349)	1-2	H25
Poços de Caldas-MG	AS093P	nd	1T	1-1	H17
Ressaquinha-MG	AS482T	nd	6P	1-2	H15
	AS489T	nd	18P, 1T(AS550)	1-2	H15
Teixeira-MG	AS080P	nd	1T, 5P(AS088,169,337,396,565)	1-1	H19
Tocantins-MG	AS124T	nd	3P	1-2	H4
Viçosa-MG	AS096T	nd	3P, 1T(AS319)	nd	H4
	AS098P	nd	2P (AS169,203)	1-2	H20
Bom Jardim-RJ	AS111T	nd	5P	1-2	H6
Cordeiro-RJ	AS113T	nd	17P	1-1	H7
Itaperuna-RJ	AS120T	nd	10P, 1T(AS095)	1-2	H8
Vassouras-RJ	AS109T	nd	2P	1-2	H5
	AS110T	nd	4P	1-2	H4

Itapetininga-SP	AS329T	nd	6P	1-2	H15
	AS331T	nd	6P	1-2	H15
	AS337P	nd	13T, 5P(AS80,87,349,350,353)	1-1	H26
Capão Bonito-SP	AS090T	nd	9P, 1T(AS296)	1-2	H3
Domingos Martins-ES	AS264P	nd	5T, 1P(AS120)	1-2	H24
Venda N. Imigrante-ES	AS513T	nd	5P	nd	H15
Brasília-DF	AS349P	nd	4T,15P(AS084,169,185,216,220,260, 261,263,309,313,337,352,394,396,565)	1-2	H15
	AS350P	nd	8P(AS220,260,261,308,309,315,317, 352)	1-2	H27
	AS352P	nd	12T, 2P(AS349,350)	1-1	H28
	AS353P	nd	1T, 3P(AS216,313,317)	1-2	H15
Taquara-DF	AS160T	nd	4P	nd	H1
Cristalina-GO	AS260P	*A. grandis*	13T, 3P(AS087,349,350)	1-1	H23
	AS261P	nd	12T, 3P(AS169,349,350)	nd	H23
	AS263P	nd	16T, 3P(AS087,248,349)	1-2	H23
Planaltina-GO	AS079T	nd	5P	nd	H1
	AS452T	*A.tomatophila*	10P, 1T(AS300)	1-2	H1
	AS453T	nd	11P	1-2	H15
Colméia-TO	AS339T	nd	8P	1-1	H15
Mucugê-BA	AS033P	nd	3T	1-2	H18
	AS565P	nd	16T, 3P(AS080,087,349)	1-2	H26
Araucária-PN	AS319T	nd	12P, 1T(AS096)	nd	H14
Colombo-PN	AS317T	nd	6P	1-2	H13
Contenda-PN	AS308P	*A.grandis*	16T, 2P(AS313,350)	1-1	H25
	AS309P	nd	8T, 4P(AS087,088,349,350)	nd	H16
Ponta Grossa-PN	AS086T	nd	7P	1-2	H2
	AS087P	nd	1T,10P(AS169,170,185,216,220,260, 263,309,337,565)	1-2	H19
	AS088P	*A.grandis*	5P(AS080,169,187,220,309)	1-2	H19
São Mateus do Sul-PN	AS084P	nd	17T, 2P(AS264,349)	1-1	H16
Alfredo Wagner-SC	AS296T	nd	2P, 2T(AS090,242)	nd	H5
Rancho Queimado-SC	AS550T	nd	12P, 1T(AS489)	1-2	H15
	AS553T	nd	12P	nd	H35
Pelotas-RS	AS298T	nd	3P	1-2	H10
	AS299T	nd	12P	1-1	H11
	AS300T	nd	3P, 2T(AS242,452)	nd	H12
São F. de Paula-RS	AS313P	*A.grandis*	13T, 3P(AS308,349,353)	1-2	H23
	AS315P	nd	9T, 1P(AS350)	1-2	H16

[a] Location refers to municipalities in the Federal District (Distrito Federal – DF) and in the states of Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP), Espírito Santo (ES), Goiás (GO), Tocantins (TO), Bahia (BA), Ceará (CE), Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS).
[b] Letters and numbers refer to the isolate code and an indication of the host plant from which the isolate was obtained: tomato (T), potato (P).
[c] Number of incompatible isolates and their host. Incompatible isolates from the same host are indicated in parenthesis.
[d] nd: not determined.

TABLE 2. Mating type frequencies and AFLP haplotype diversity of *Alternaria* spp. populations.

		Frequencies			AFLP [b]	
Species	n	MAT1-1	MAT1-2	χ^2 value [a]	h	Hd
Tomato	27	0,15	0,85	13,370*	15	0,8739
Potato	31	0,36	0,64	2,613	15	0,9162
Total	58	0,26	0,74		28	0,9373

[a] Value based on 1:1 ratio and 1 degree of freedom. *Indicates mating type frequency is significantly different at $P<0.05$. n.s. = non significant.
[b] Primers E+AT - M+A
h: Number of haplotypes, Hd: Haplotype diversity

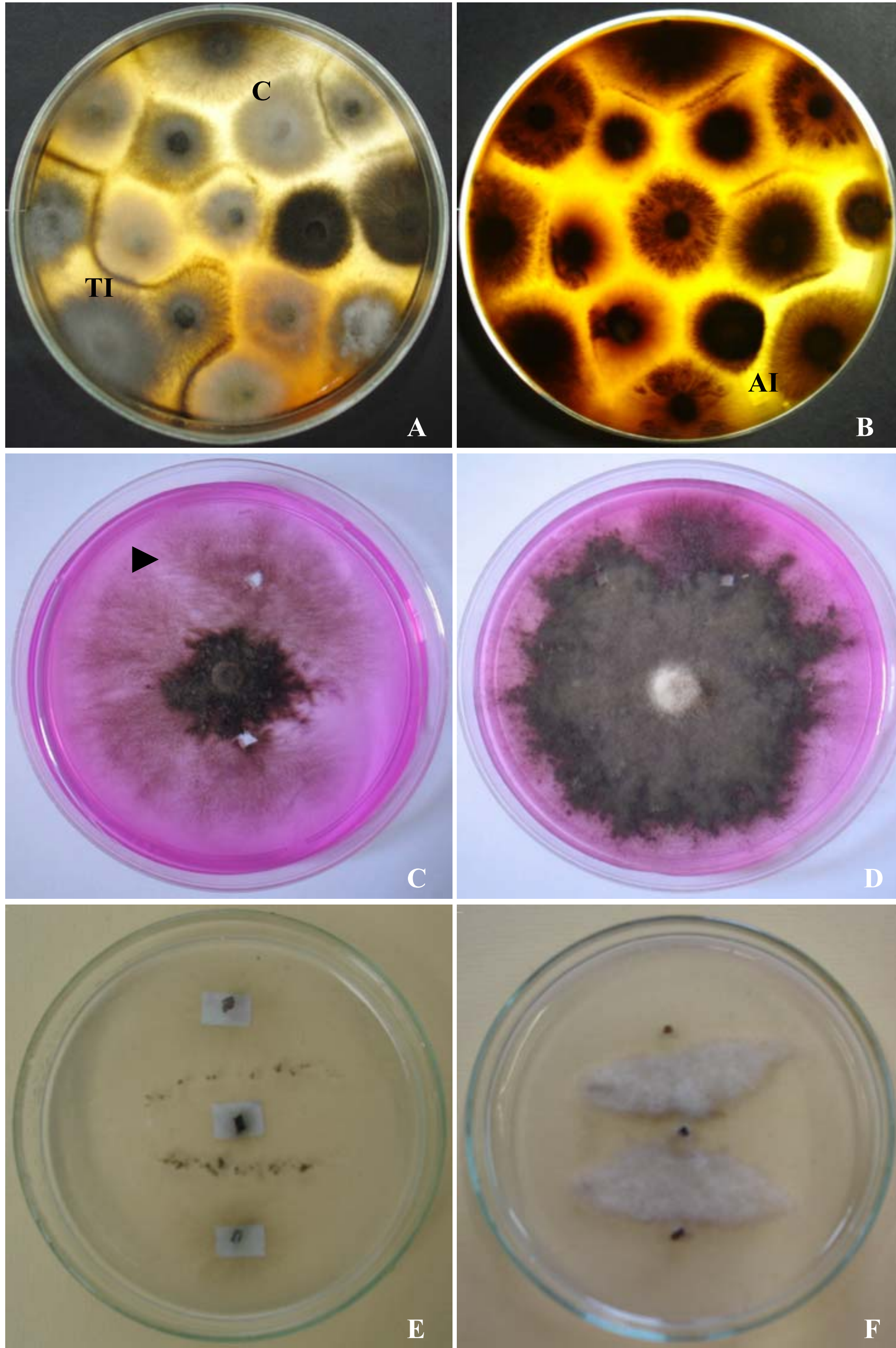

Figure 1. A- Arrangement of the *Alternaria* spp. mycelial plugs in pairings to assess mycelial compatibility. TI = typical incompatible interaction, C = compatible interaction. B- AI = atypical incompatible interaction. C- Arrowhead indicates the fast-growing sector as a consequence of resistance to potassium chlorate. D- Isolate growing on CDA+RB (4% potassium chlorate) without a mutant sector. E, F- Heterokaryon formation between *nit1*/*nit3*, and NitM/NitM mutants, respectively.

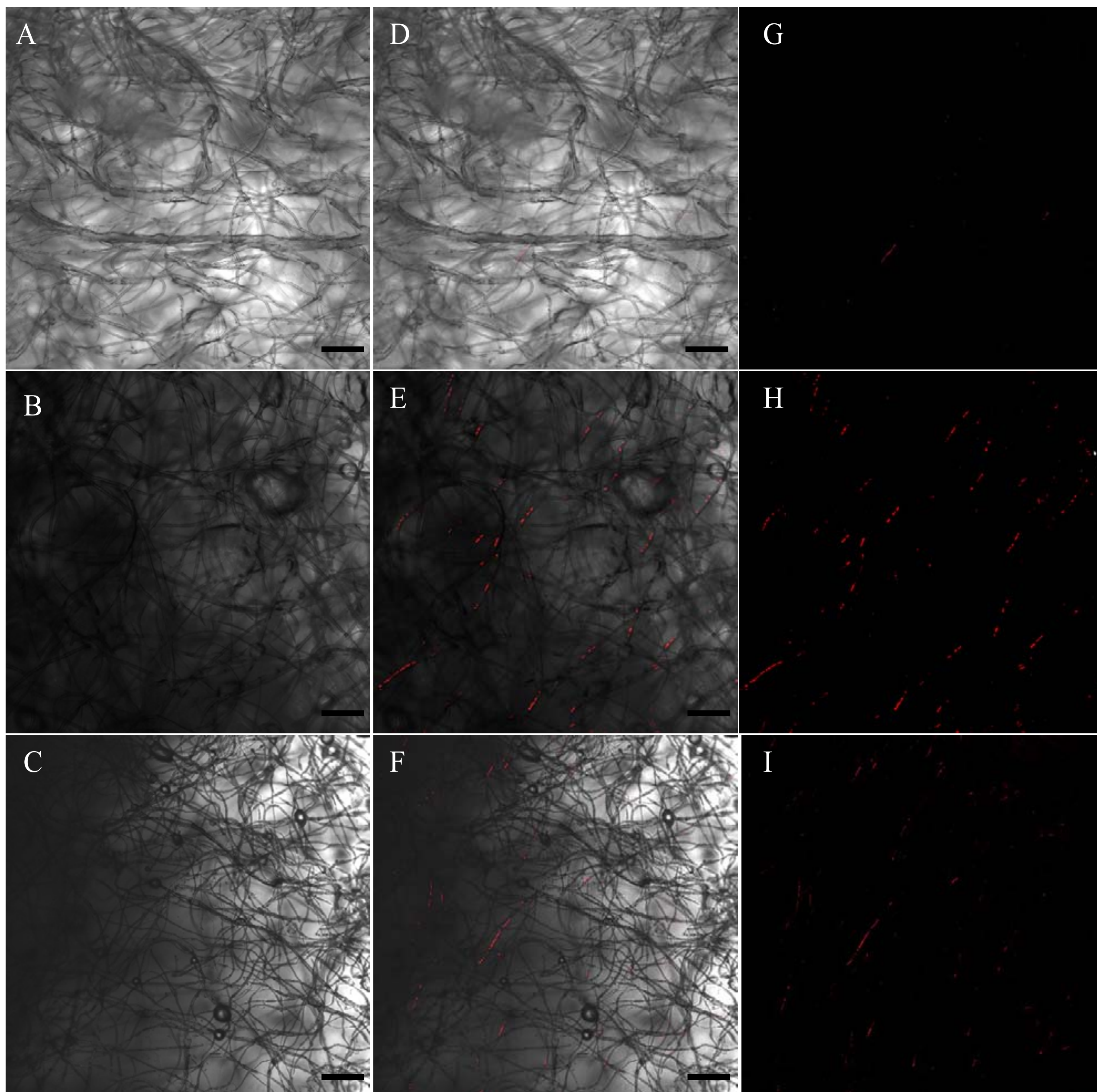

Figure 2. Pairings showing compatible (CI) and incompatible (II) interactions visualized by confocal microscopy. CI: A,D,G: AS440xAS440; II: B,E,H and C,F,I: AS440 x AS260, EGS44-098 x AS109, respectively. Auto fluorescence in red. A-C: light, D-F: merged, E-I: auto fluorescence. Bars = 100µm.

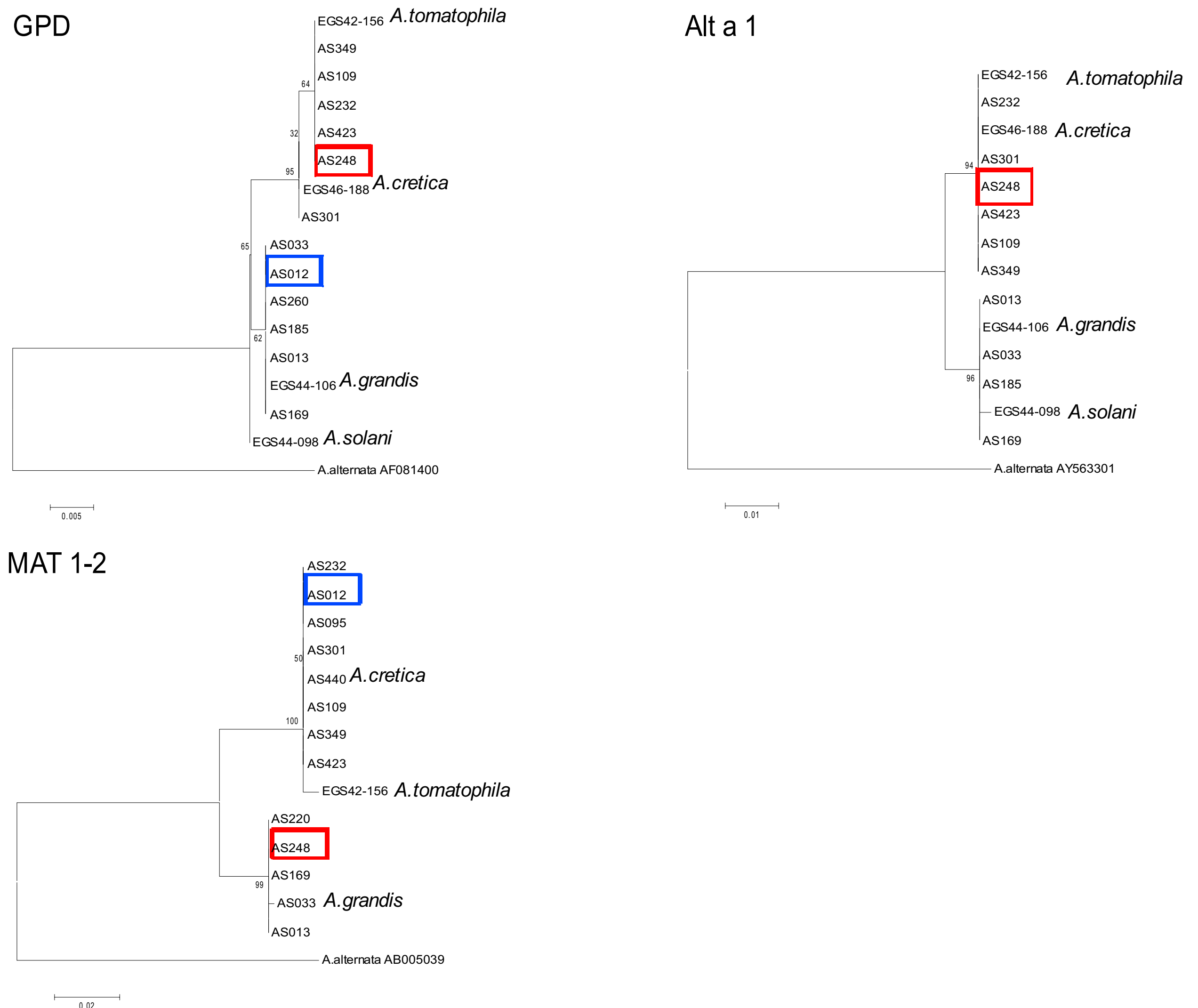

Figure 3. Neighbor-joining phylogenetic tree based on the sequences of the *Alta a 1*, *GPD* and MAT1-2 genes of *Alternaria* spp. Bootstrap values are shown at the nodes. Red and blue boxes represent the variation in grouping the putative hybrids, AS012 and AS248, in different clades according to the gene analyzed.
Alta1 and *GPD* squences GenBank accession:
AS013:EU617450/EU617506,AS033:EU617406/EU617551,
AS109:EU617462/EU617517,AS169:EU617469/EU617525,
AS185:EU617470/EU617527,AS232:EU617413/EU617558,
AS248:EU617414/EU617559,AS260:EU617476/EU617534,
AS301:EU617432/EU617614,AS349:EU617489/EU617599,
AS423: EU617498/EU617608

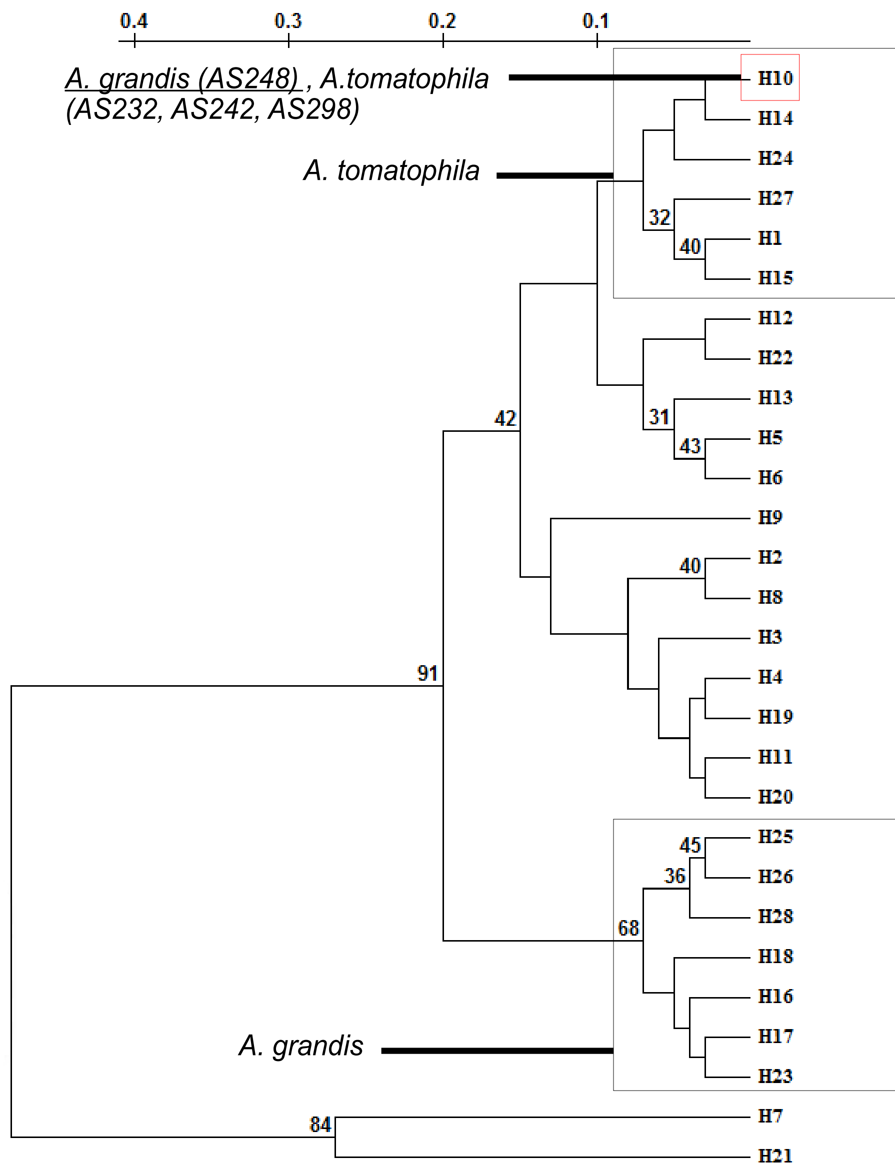

Figure 4. Graphical representation of the genetic distance generated by the UPGMA clustering of AFLP haplotypes of *Alternaria* spp. The scale bar at the top represents the genetic distance. Bootstrap support values are shown at the nodes. Internal boxes delimit a group formed by isolates of *A. grandis* or by isolates of *A. tomatophila*, except the H10 (red box) which is represented by one isolate of *A. grandis.*

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3- Phylogenetic relationships among *Alternaria* species from Solanaceae based on morphological and DNA sequence data

ABSTRACT

Five species of *Alternaria* are reported to be associated with early blight on potato and tomato crops: *A. solani*, *A. grandis*, *A. tomatophila*, *A. cretica*, and *A. subcylindrica*, but no study using morphological and molecular data was conducted to assess their phylogenetic relationships. Understanding these relations is important for diagnosis and to provide support to breeding programs aimed at developing resistant varieties. Morphological variables used in the taxonomy of the genus were assessed and the DNA sequences of three protein-coding regions were determined for 12 species of *Alternaria* from Solanaceae: the *Alt a 1* (major allergen Alt a 1 precursor), *GPD* (glyceraldehyde-3-phosphate dehydrogenase), and the *EF* (elongation factor alpha) genes. The phylogeny reconstruction was accomplished using three methods: distance (Neighbor-Joining), maximum parsimony, and maximum likelihood. Additionally, analysis of partitioned Bremer support was implemented to evaluate the contribution of morphological and DNA sequence data to assess if the morphospecies correspond to phylogenetic species. *Alta a 1*, *GPD* and *EF* sequences did not resolve the morphospecies of *Alternaria* spp. that affect Solanaceae. However, species as *A. solani* and *A. grandis* are separated from *A. tomatophila*, *A. cretica* and *A subcylindrica.* These genes are appropriated to distinguish the *Alternaria* species associated to EB on potato and tomato. In most cases the molecular data did not support morphological data, according to the Partitioned Bremer Support.

3.1. INTRODUCTION

Alternaria is an ubiquitous genus of mitosporic fungi that includes many important plant and human pathogens. The morphology of the conidium, conidiophores, and the pattern of sporulation has been the primary variables assessed to identify *Alternaria* species (Simmons, 2007). The species that affect Solanaceae are morphologically diverse and relatively easily identified. However, the classification of *Alternaria* species related to foliar diseases on potato and tomato can be subjective, due to a reduced set of variables that are used to separate these individuals. In fact, the most important morphological character that distinguishes species from both hosts is the number of beaks of the conidia (Simmons, 2007).

The correct classification of the species of *Alternaria*, the causal agent of early blight disease (EB) on potato and tomato, is required for the implementation of proper disease management actions. Several species of *Alternaria* can cause EB, for example, *A. solani* has been traditionally reported to cause EB on both potato and tomato; however, *A. tomatophila* was also described as the causal agent of EB on tomato and is apparently widely distributed (Simmons, 2000, Rodrigues *et al.*, *unpublished data*). Additionally, *A. cretica* and *A. subcylindrica* were also related to EB on tomato, and *A. grandis* to EB on potato (Simmons, 2000). To date, no epidemiological studies were conducted to assess whether EB epidemics in the field vary according to the causing species, but preliminary data indicate important differential responses in trials under controlled conditions (Frazer and Zitter, 2004; Cardoso *et al.*, *personnal communication*). Therefore, management actions can be dependent on the prevalent species of the pathogen and correct classification is a crucial step. The most important morphological character to distinguish the *Alternaria* spp. that affects potato and tomato is the number of beaks of the conidia (Simmons, 2007). But, to improve the resolution

of the analyses of species classification within each host, other characteristics are required to reduce dubious interpretation caused by the overlapping of the measured variables.

Alternatively and, in many cases, complementary to the morphological characterization, the molecular taxonomy can solve the problems of classification and misidentification of fungal species. The molecular taxonomy and the phylogenetic relationships among *Alternaria* species have been extensively reported (Pryor and Gilbert, 2000, Hoog and Horré, 2002, Pryor and Bigelow, 2003, Hong *et al*., 2005, Andrew *et al*., 2009) and the correct choice of the genes to report the evolutionary history of each species is an important step. Overall, the ITS and SSU rDNA regions are designated as low to moderately variable among species and in the case of the *Alternaria* genus these regions poorly resolved the *Alternaria* species into groups (Pryor and Gilbert, 2000). On the other hand, the *GPD* sequences contained almost twice the number of variable and parsimony-informative sites as the ITS sequences, which in turn contained approximately twice the number of variable sites and parsimony-informative sites as the mtSSU region (Pryor and Bigelow, 2003). However, one of the most informative genes to study *Alternaria* spp. is the *Alt a 1* gene, a precursor of an allergenic protein (Hong *et al*., 2005). The analysis of *Alt a 1* strongly supported groupings of *Alternaria* spp., especially the infectoria, alternata, porri, brassicicola, and radicina species-groups (Hong *et al*., 2005). Nevertheless, the relationships among closely related species of the same species-group were not fully resolved. High resolution could be obtained by using combined data sets of the *Alt a 1* and *GPD* sequences (Lourenço Jr. et al. *in press*). In a recent study, using different genomic regions, this approach was used to resolve morphological species within the small-spored *Alternaria* species complex, *A. arborescens*, *A. alternata*, *A. tenuissima* groups

(Andrew *et al.*, 2009). No support for differentiation was observed to separate *A. tenuissima* and *A. alternata* groups in an evolutionary context and many other species were assigned as intermediate between the two groups. A study to address a similar question needs to be conducted with the species that affect Solanaceae. Thus, the main objective of the present study is to understand the evolutionary relationships among *Alternaria* spp. from Solanaceae, especially the species that cause EB on potato and tomato, by analyzing the DNA sequences of three loci and to investigate how this information can complement the morphospecies classification.

3.2. MATERIALS AND METHODS

Isolates and DNA sequencing. The DNA of 12 ex-type isolates of *Alternaria* species from seven different plants of the Solanaceae family (Table 1) were extracted using Dneasy Plant Mini Kit (Quiagen), following the manufacturer's instructions. The DNA concentration of each sample was adjusted to 50-100 ng/µl. The primers gpd1 and gpd2 (Berbee *et al.,* 1999), Alt-for and Alt-rev (Hong *et al.,* 2005), EF451F, EF3R and EF1473R (Inderbitzin *et al*., 2006) were used to amplify the *Alt a 1*, *GPD*, and the elongation factor alpha (*EF*) genes, respectively. To obtain the EF fragments 10 µl of the product of the first amplification using the primers EF451F and EF1473R was separated in a 2% NuSieve GT agarose gel. The band with the expected size was excised from the gel and melted in 100 µl of water at 65ºC for 15 min. The sample was diluted 10x and used as a template to the second amplification with the primers EF451F and EF3R. Thermal conditions were initial denaturation at 95ºC for 5 min followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 52ºC for 1 min, elongation at 72ºC for 30s and extended by 4 additional seconds per cycle, and a final elongation at

72ºC for 7 min. The same thermal conditions were used for the first and second PCR, except for the annealing temperature of the second PCR which was 55ºC. PCR reactions to amplify *Alt a 1* and *GPD* were the same as described above, but 55ºC was set as the annealing temperature. The PCR products were purified by ethanol precipitation (Okayama & Berg, 1983) and sequenced (Applied Biosystem BigDye v.3.1Terminator Chemistry). The sequencing was performed at NAPS Unit, University of British Columbia, Vancouver BC, Canada. The sequences were edited and the consensus was built using the AutoAssembler ver. 1.4 software for Macintosh.

Morphological characters. The key available in *Alternaria*: An Identification Manual (Simmons, 2007) was used to create a list with 41 taxonomical characteristics that identify the species of *Alternaria* associated with plants of the Solanaceae family (Table 2). A binary matrix was constructed with data comprised of the presence (1) or absence (0) of a given character for each species.

Phylogenetics Analysis. Three methods were used to reconstruct the phylogeny: distance-based approach using the neighbor-joining algorithm (NJ), maximum parsimony (MP), and maximum likelihood (ML). For NJ, the distance between any two isolates was calculated using the Kimura's 2-parameter model (Kimura, 1980). The option pairwise-deletion was chosen to treat gaps and missing data and 1000 bootstrap pseudoreplicates were performed. The analysis was implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4) (Tamura *et al.,* 2007). The MP analysis was conducted using the heuristic search for the most parsimonious tree with closest step-wise addition and tree-bisection-reconnection (TBR) branch-swapping. The majority rule was used to build the consensus tree. Sequence gaps were treated as missing data and 1000 bootstrap pseudoreplicates were performed. For ML phylogeny reconstruction, the DNA substitution model was chosen based on the Akaike

information criterion and the likelihood ration test (lnL) implemented in the Modeltest 3.7 (Posada & Crandall, 1998) and then used in PAUP version 4.0 beta (Swofford, 2003) to compute ML bootstrap percentages after 100 pseudoreplications with Neighbor- Joining starting trees and NNI parameter. The MP and ML analyses were conducted in PAUP version 4.0 beta.

Partitioned Bremer Support (PBS) – The PBS analyses were implemented in a combined morphological and molecular data set. The main purpose of this analysis was to identify if morphological characteristics agree with DNA sequences allowing more robust reconstruction of the phylogenetic relationships among *Alternaria* spp. The PBS analysis was conducted in PAUP and in the SNAP Workbench program (Price and Carbone, 2005). The *Alt a 1* and *GPD* concatenated sequences and binary matrices from morphological characters 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 (Lourenço Júnior *et al*., *in press*). MP analyses were implemented in PAUP, as described above. PBS values were tabulated for each node in the combined phylogenetic tree. These values reflect the consistency or conflict from each data partition with a particular node (Bremer, 1994).

3.3. RESULTS

DNA sequencing. Sequences of *Alt a 1* and *GPD* were obtained for all species analyzed. The amplified *Alt a 1* and *GPD* gene fragments had 466 and 570 bp, respectively. The primers used to amplify the EF gene did not allow the amplification to *A. capsici, A crassa*, *A. cyphomandrae*, and *A. nitrimali.* The amplified fragments of the EF gene for the other species had 899 bp.

Phylogenetic analysis. The phylogenies reconstructed by the NJ, MP, and ML methods had the same tree topology (Figures 1 and 2). The *Alt a 1/GPD* concatenated data set resulted in two well supported clades (bootstrap values 100%). One clade included all species from tomato and potato with one exception, the species *A. cyphomandrae*. The other clade was formed by *A. capsici, A. crassa*, and *A. nitrimali.* Out of 744 constant characters, 111 were parsimony-informative. The score of the best tree was 403. The consistency index (CI) was 0.84 and the homoplasy index (HI) was 0.16. For the EF sequences, the score of the best tree was 240, CI=0.88, HI=0.12. A total of 714 constants characters, 92 variable parsimony-uninformative, and 83 parsimony-informative.

The ML analyses for the *Alt a 1/GPD* concatenated data set and *EF* sequences were based on general time reversible (GTR+G) model of DNA substitution. Bootstrap support for ML topology was similar to that estimated for the MP and NJ topology (Figures 1 and 2).

Partitioned Bremer Support (PBS). A total of 12 haplotypes, each with 1077 characters, were generated after concatenating *Alt a 1* and *GPD* sequences with morphological data for the 12 *Alternaria* species under study. The positive PBS values for a given partition mean that a node is supported by that partition, whereas negative PBS values suggest an alternative node not present in the combined tree. Conflicting PBS values at a single node imply that characters in the different data partitions disagree relative to the formation of that node (Lambkin, 2004). Only the nodes 2 (*A. crassa* and *A. capsici*) and 4 (*A grandis* and *A solani*) had positive PBS values for the two partitions; thus, the groups were support by both morphological and DNA sequence data. The node 1 (*A. tomatophila* and *A. cretica*) had positive PBS value to the morphological partition and negative value to the sequences partition. The node 3 had

negative PBS value to the morphological partition and positive value to the sequences partition. The PBS values for nodes 5, 6, and 7 were zero for one of the data partitions (Figure 3).

3.4. DISCUSSION

The molecular phylogenetic relationships among *Alternaria* species from Solanaceae were not properly resolved using the *Alt a 1* and *GPD* sequences. Previous studies using these sequences examined the relationships among *Alternaria* species from several hosts with good resolution (Pryor and Bigelow, 2003, Hong *et al*., 2005). In the present study the *A. arborescens* (alternata species-group), *A. mimicula* (brassicicola species- group) (Hong *et al*., 2005) and *A. elegans* formed a distinct clade from the porri species-group, which contains the species causing leaf spot on Solanaceae. *A. nitrimali* and *A. cyphomandrae*, also are part of the porri species-group defined by Hong *et al*., (2005). Within the porri species-group, the clade of tomato-related species differed from the clade comprised of species from potato. This was the highest resolution achieved the analysis of DNA sequence data. Separation of *A. tomatophila* and *A. subcylindrica*, *A. crassa* and *A. capsici* as well as *A. solani* and *A. grandis* were not possible based on the *Alt a 1* and *GPD* concatenated sequences. The limited number of polymorphisms in the sequences of more closely related species, i.e. the causal agents of EB on tomato and potato prevented proper separation in groups that correspond to the morphospecies classification.

Despite the lack of *EF* sequences for some *Alternaria* spp., all species from potato and tomato were represented and could be analyzed. The phylogeny reconstructed based on the *EF* sequences separated species from potato and tomato, but again the species within each host were indistinguishable. For small-spored *Alternaria*

species the host-specificity among clades was not evident, confirming that host is not a useful character for *Alternaria* classification (Andrew *et al*., 2009). Based on the result of the present study host is important to the classification of large-spored *Alternaria* species on tomato and potato. Probably, the species related to tomato diverged recently and the fixation of mutation on the sequences analyzed were not enough to allow clear separation at the species level. The same applies to *Alternaria* species related to potato.

Although the close species are indistinguishable phylogenetically, the morphospecies present specific characteristics that allow its separation. The correspondence of morphological characteristics and DNA sequences were used to predict if morphospecies comprise phylogenetics species. Strict agreement measured by PBS between molecular and morphological data occurred only for the *A. grandis* and *A. solani*, and for *A. capsici* and *A. crassa*. In most cases, the nodes had PBS value equal zero for the morphological data partition. Thus the node is not supported in a phylogenetic tree due to the lack of phylogenetic information or within dataset incongruence for morphological characters (Lambkin, 2002). A revision of the characters given its phylogenetic information content can be suggested, but it is anticipated to be difficult to be implemented.

DNA sequence data used in the present study did not resolve the morphospecies of *Alternaria* spp. that affect Solanaceae. Other genes, with higher number of fixed polymorphisms need to be analyzed. The prospection of candidate genes should be prioritized as a next step to improve the classification of *Alternaria* spp. that affect potato and tomato as well as to better understand the apparently recent divergence of these species.

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TABLE 1. Description of the *Alternaria* spp. ex-type isolates used in this study.

Isolate - code*	Species	Host	Location
EGS 01-056	*A. mimicula*	*Solanum lycopersicum*	Georgia - USA
EGS 39-128	*A. arborescens*	*S. lycopersicum*	California - USA
EGS 40-058	*A. cyphomandrae*	*Cyphomandra betacea*	New Zealand
EGS 42-156	*A. tomatophila*	*S. lycopersicum*	Indiana - USA
EGS 44-071	*A. crassa*	*Datura stramonium*	Indiana - USA
EGS 44-098	*A. solani*	*S. tuberosum*	Washington - USA
EGS 44-106	*A. grandis*	*S. tuberosum*	Pennsylvania - USA
EGS 45-072	*A. elegans*	*S. lycopersicum*	Burkina Faso
EGS 45-075	*A. capsici*	*Capsicum annuum*	Australia
EGS 45-113	*A. subcylindrica*	*S. lycopersicum var. cerasiforme*	Lousisiana - USA
EGS 46-151	*A. nitrimali*	*S. viarum*	Puerto Rico - USA
EGS 46-188	*A. cretica*	*S. lycopersicum*	Crete - Greece

* Isolates provided by E.G. Simmons

TABLE 2. List of morphological characteristics that classify each species of *Alternaria* in this study, as used by Simmons (2007).

Number	Morphological characteristic
1	Conidium beak narrow-taper long (>100 μm), body long (>100 μm)
2	Conidia in surface clumps of branching chains or of unbranched chains arising at close intervals near a primary conidiophore apex; secondary conidiophores not conspicuous or dominant elements of bushy architecture
3	Conidia in aerial portions of the colony are borne mostly in openly branching tufts near apex of long, erect, solitary conidiogenous axes *ca* 1 mm tall
4	Conidium apex produces a filamentous beak 1.5-2.5(-3.2) μm wide throughout its length.
5	Conidium beak filamentous, long (>100 μm); body medium (50-100 μm), rarely short at maturity (<50 μm)
6	Conidium chains (secondary conidia) abundant; conspicuous in culture
7	Conidium body usually erostrate; long filamentous beak present on low % conidia
8	Erostrate conidium body to *ca* 36-53 x 10-13 μm, 5-7 transeptate
9	Erostrate conidium body to *ca* 70-87 x 14-19 μm, 7-11 transeptate
10	Beak 1 (more than one not known)
11	Beaks 2 common (even if mostly 1-beak or rarely 3-4 beaks)
12	Beaks 3 or more present or abundant (even if mostly 1-2 beaks)
13	Conidium width max. *ca* 20 μm
14	Conidium width commonly in 20-30 μm range
15	Conidia solitary and commonly catenate
16	Length erostrate conidia 45-75 μm; total body + beak 300-375 μm
17	Conidia seldom remain beakless except as terminal element in chain
18	Beak length max. commonly in 180-275 μm range
19	Conidium body up to 12-16 transeptate; sturdy beak conspicuously to 17-transeptate
20	Conidium body rel. narrow; seldom more than 20 μm wide
21	Conidium body becoming broader; max. width range above 20 μm
22	Beak length seldom/never up to 200 μm
23	Secondary conidiophores not found in V-8 culture
24	Beak length range above 200 μm (200-250 μm)
25	Beak length range above 175 μm
26	Beak length range lower 175 μm
27	Beak length into range 100-120 μm
28	Conidium body length range reaches 150-200 μm
29	Conidium body length range lower than 150 μm
30	Conidium body width max. less (<24 μm) in V-8 culture
31	Conidia catenate, secondary conidiophores conspicuous
32	Conidium width mostly (90%) in 16-19 μm range; also to 24 μm
33	Conidiophores with angular branching, 3-9 conidiougenous extentions
34	Sporulation abundant in undisturbed V-8 colony; high % of conidia remain relatively narrow (14-17 μm)

	ellipsoid
35	Sporulation scanty in undisturbed V-8 colony; abundant after colony scarification; high % of conidia become broad (16-27 µm)
36	Conidia at max. size long-ellipsoid, almost cylindrical before production of longisepta, commonly constricted at transepta; longisepta lacking in most conidia; secondary conidiophores usually a single short cell
37	Conida at max. size mostly ovoid
38	Largest conidia (usally primary in chain) 35-40 x 7-10 µm; others 8-18 x 5-8 µm or smaller
39	Conidia in a size range to 25-40 x 5-25 µm
40	Conidia mostly ovoid, 7-25(-40) x 5-12 µm; or ellipsoid, 25-35(-40) x 5-9 µm
41	Primary conidia 15-30(-42) x 7-11 µm, others about the same size, tan to brown, punctate to verrucose

TABLE 3. Binary matrix constructed with morphological data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
AB	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
CA	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CE	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
CR	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CY	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
EL	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GR	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
MI	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
NI	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SO	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
SU	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TO	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

* Numbers on the first row represent the characteristic described in Table 2.
The numbers 1 and 0 represent the presence and absence of the specific characteristic, respectively.
AB – *A. arborescens*; CA – *A. capsici*; CE – *A. cretica*; CR – *A. crassa*; CY – *A. cyphomandrae*; EL – *A. elegans*; GR – *A. grandis*; MI – *A. mimicula;* NI – *A. nitrimali*; SO – *A. solani*; SU – *A. subcylindrica*; TO – *A. tomatophila.*

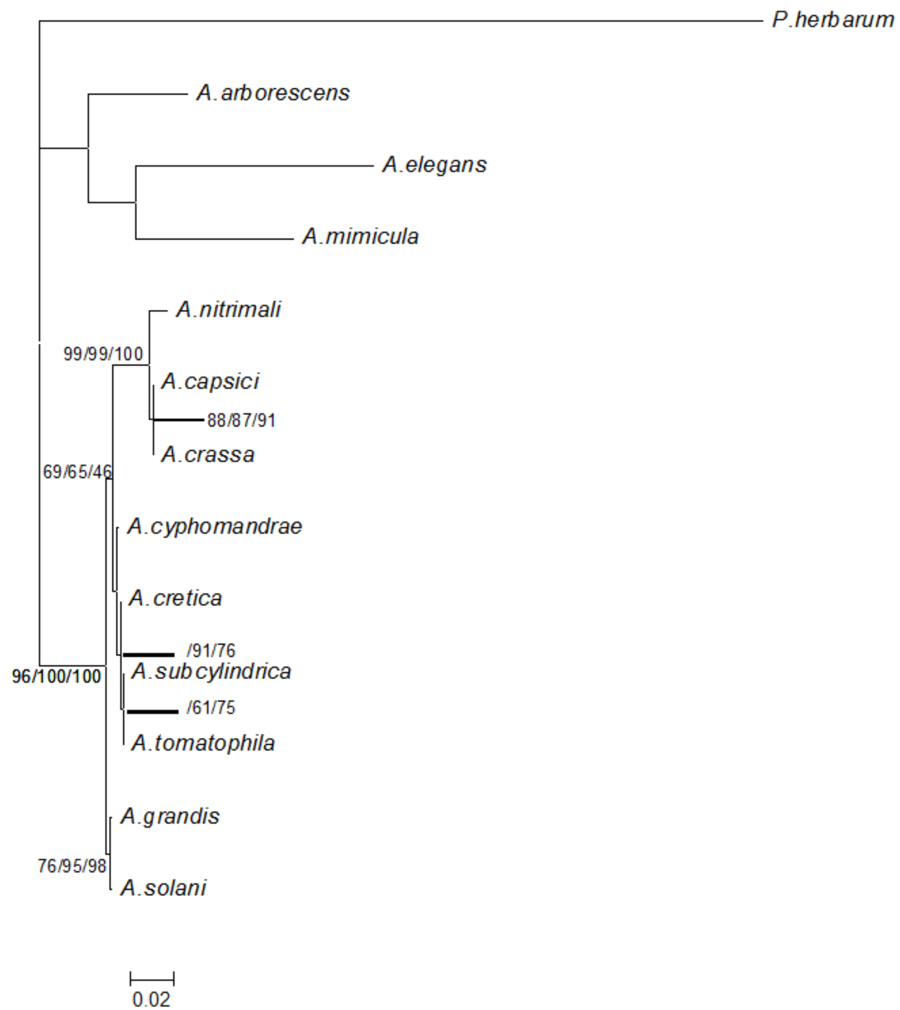

Figure 1. Inferred maximum likelihood (ML) tree based on the *Alt a 1/GPD* concatenated sequences data set. Numbers between slashes indicate bootstrap values in ML, maximum parsimony and neighbor-joining analysis. For the ML analysis the bootstrap values lower than 50% were not indicated. The topology was rooted with sequences of *P. herbarum* (GenBank accession: *Alt a 1*:AY563277, *GPD*: AF443885).

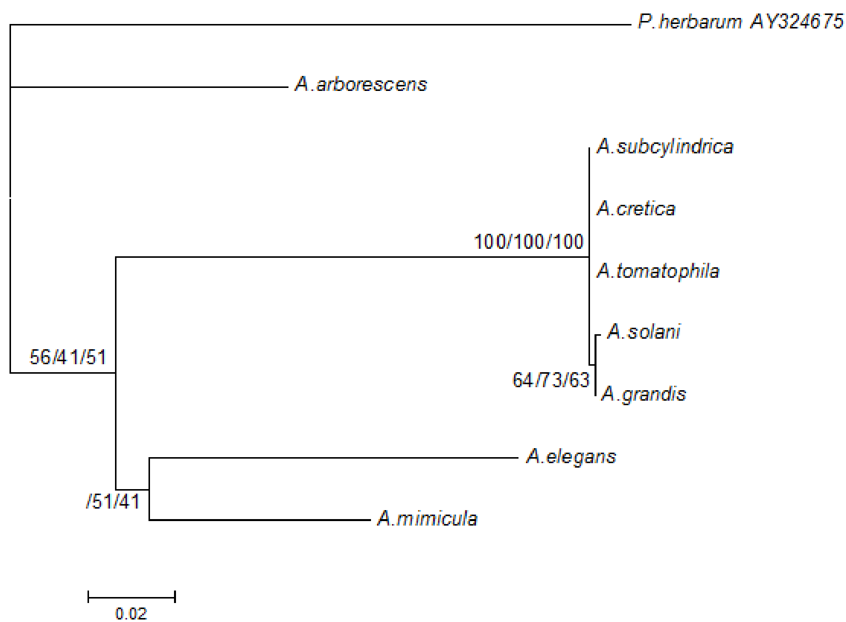

Figure 2. Inferred maximum likelihood (ML) tree based on the sequences of the elongation factor alpha (EF) gene. Numbers between slashes indicate bootstrap values in ML, maximum parsimony and neighbor-joining analysis. For the ML analysis the bootstrap values lower than 50% were not indicated. The topology was rooted with sequences of *P. herbarum* (GenBank accession: AY324675). AY324675)

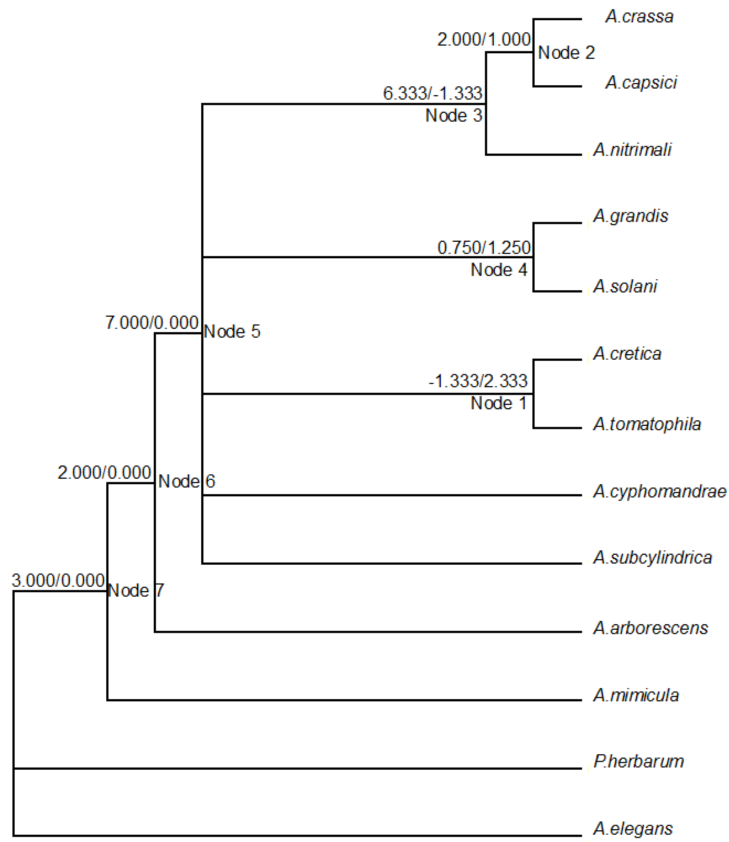

Figure 3. Maximum parsimony tree for the combined dataset of DNA sequences (*Alt a1* and *Gpd* genes) and morphological characteristics with positive and negative partition Bremer support (PBS) values for to corresponding nodes of the phylogenetic analysis of *Alternaria* spp. from Solanaceae. The numbers before and after the slash are the PBS value for the sequence and morphological data, respectively. The topology was rooted with sequences of *P. herbarum* (GenBank accession: *Alt a 1*:AY563277, *GPD*: AF443885).

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4- GENERAL CONCLUSIONS

1- Early blight disease in Brazil is caused by more than one morphospecies of *Alternaria. A. grandis* and *A tomatophila* are the most common causal agents of the disease;

2- Recombination is possible in populations of *Alternaria* spp. due to the high vegetative compatibility between isolates, heterokaryon formation, widely distribution of MAT1-*1* and *MAT1-2* genes, and the occurrence of putative interspecific hybrids;

3- *Alta a 1*, *GPD* and *EF* sequences used in the present study did not resolve the morphospecies of *Alternaria* spp. that affect Solanaceae. However, species as *A. solani* and *A. grandis* are separated from *A. tomatophila*, *A. cretica* and *A. subcylindrica.* This demonstrated that those genes are appropriated to distinguish the *Alternaria* species associated to EB on potato and tomato.