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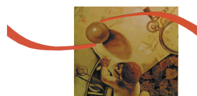

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Characterization of race 65 of *Colletotrichum lindemuthianum* by sequencing ITS regions

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ABSTRACT. The present work aimed characterize isolates of *C. lindemuthianum* race 65 from different regions in Brazil by ITS sequencing. A total of 17 isolates of race 65, collected in the states of Mato Grosso, Minas Gerais, Paraná, Santa Catarina and São Paulo, were studied. Analysis of the sequences of isolates 8, 9, 12, 14 and 15 revealed the presence of two single nucleotide polymorphisms (SNPs) in the ITS1 region at the same positions. These isolates, when analyzed together with the sequence of isolate 17, revealed a SNP in the ITS2 region. The highest genetic dissimilarity, observed between isolates 11 and 3 and between isolates 11 and 10, was 0.772. In turn, isolates 7 and 2 were the most similar, with a value of 0.002 for genetic distance. The phylogenetic tree obtained based on the sequences of the ITS1 and ITS2 regions revealed the formation of two groups, one with a subgroup. The results reveal high molecular variability among isolates of race 65 of *C. lindemuthianum*.

Keywords: anthracnose, genetic variability, pathogens, dissimilarity.

Caracterização da raça 65 de *Colletotrichum lindemuthianum* utilizando sequenciamento das regiões ITS

RESUMO. O presente trabalho teve como objetivo caracterizar isolados de *C. lindemuthianum* da raça 65 provenientes de diversas regiões do Brasil, por meio de sequenciamento de regiões ITS. Um total de 17 isolados da raça 65, coletados nos estados do Mato Grosso, Minas Gerais, Paraná, Santa Catarina e São Paulo, foram estudados. As análises das sequências dos isolados 8, 9, 12, 14 e 15 revelaram a presença de dois SNPs na região ITS1 nas mesmas posições. Estes mesmos isolados quando analisados juntamente com a sequência do isolado 17 apresentaram um SNP na região ITS2. A maior dissimilaridade genética foi de 0,772 observada entre os isolados 11 e 3 e entre os isolados 11 e 10. Por sua vez, os isolados 7 e 2 foram os mais similares, com valor de distância genética de 0,002. A árvore filogenética obtida com base nas sequências das regiões ITS1 e ITS2 revelou a formação de dois grupos, sendo um com a divisão de um subgrupo. Estes resultados revelam uma elevada variabilidade molecular entre isolados da raça 65 de *C. lindemuthianum*.

Palavras-chave: antracnose, variabilidade genética, patógenos, dissimilaridade.

Introduction

The common bean (*Phaseolus vulgaris* L.) is admittedly an important source of protein and energy (Vieira, Vieira, Euclydes, & Silva, 1983; Borém & Carneiro, 1998) and is a legume of great economic significance (Broughton et al., 2003) and agronomic interest across the world (Angioi et al., 2010). Moreover, it is the most important legume for human consumption and an important source of proteins, vitamins and minerals (Ca, Cu, Fe, Mg, Mn, and Zn) in the human diet, particularly in developing countries (Beebe, 2012).

This crop is cultivated throughout the year under different temperature, light intensity, relative humidity and water availability conditions. These conditions favor the development of several pathogens, such as fungi (Paula Júnior & Zambolin, 2006).

The common bean anthracnose is one of the main fungal diseases affecting this crop, especially if the environmental conditions favor the pathogen's development, leading to yield losses of up to 100% when susceptible cultivars are used (Talamini et al., 2006; Damasceno e Silva, Souza, & Ishikawa, 2007). *Colletotrichum lindemuthianum* is a cosmopolitan

pathogen and is therefore widely distributed in common bean-producing regions (Bianchini, Maringoni, & Carneiro, 2005).

Anthracnose occurs more severely in places where relative humidity conditions above 91% and temperatures ranging from 18° and 22°C predominate. The pathogen's development limits infection at temperatures higher than 24ºC and lower than 18ºC (Cháves, 1980; Kelly, Afanador, & Cameron, 1994). The control of anthracnose in common bean is greatly impaired due to the survival capacity of the fungus for several months in both the soil and infected crop residues (Sutton, 1992; Dillard & Cobb, 1993). Integrated measures are recommended for the efficient control of anthracnose, including the use of pathogen-free and fungicide-treated seeds, crop rotation with non-host plants for a period of 2 to 3 years, varietal resistance and chemical control (Rava, Purchio, & Sartorato, 1994).

C. lindemuthianum exhibits wide genetic variability, as indicated by the high number of races in the common bean-producing regions, which impairs the durability of cultivar resistance (Souza, Souza, & Mendes-Costa, 2007). The high number of existing physiological races and the complexity in the use of genetic resistance of the *C. lindemuthianum* fungus are evidence of wide virulence diversity (Pinto, Pereira, Mota, Ishikawa, & Souza, 2012). Brazil is notably the country with the highest pathogenic variability, with 73 pathogenic races distributed in 15 states; race 65 is present in 12 states (Nunes, Gonçalves-Vidigal, Lacanallo, & Coimbra, 2013).

Race 65 has been reported as one of the most frequent and widely distributed races in several countries, including Brazil, the United States, Mexico, Argentina, Equator, Africa and India, with increased prominence in the past 30 years anos (Pastor-Corrales, Erazo, Estrada, & Singh, 1994; Rava et al., 1994; Balardin, Jarosz, & Kelly, 1997; Balardin & Kelly, 1998; Sharma, Kumar, Sharma, Sud, & Tyagi, 1999; Thomazella et al., 2002; Alzate-Marin & Sartorato, 2004; Mahuku & Riascos, 2004; Talamini et al., 2004; Bonett, Schewe, & Silva, 2008; Gonçalves-Vidigal, Thomazella, Vidigal Filho, Kvitschal, & Elias, 2008; Gonçalves-Vidigal et al., 2009). Consequently, breeding programs have focused their attention on this race with the goal of controlling anthracnose (Davide & Souza, 2009).

Several studies have shown that the race 65 isolate previously known as Epsilon (Alpha Group) is noteworthy due to its high frequency and wide geographic distribution (Rava et al., 1994; Carbonell et al., 1999; Alzate-Marin & Sartorato, 2004; Talamini et al., 2004).

Molecular tools have been widely used in the study of genetic diversity in many organisms, including fungi. One of those molecular tools is the sequencing of conserved regions, such as the internal transcribed spacer (ITS) in ribosomal DNA (rDNA), which has been used to establish molecular phylogenetic relationships within many groups of fungi (Stewart, Zaowei, Crous, & Szabo, 1999). The ITS region is divided into ITS1, located between genes 18S and 5.8S, and ITS2, which separates genes 5.8S and 28S; the ITS1 and ITS2 regions are transcribed and processed to yield ribosomal RNA (Hillis & Dixon, 1991; Schlotterer, Hauser, Huesler, & Tautz, 1994). Given the importance of *C. lindemuthianum* race 65, this work aimed to characterize isolates of *C. lindemuthianum* race 65 from several regions in Brazil by ITS sequencing.

Material and methods

Biological material

The present work was conducted under greenhouse conditions and at Laboratório de Melhoramento de Feijão Comum e de Biologia Molecular of the Núcleo de Pesquisa Aplicada à Agricultura (Nupagri), Universidade Estadual de Maringá, Paraná State, Brazil. A total of 17 isolates of race 65, collected in the states of Mato Grosso, Minas Gerais, Paraná, Santa Catarina and São Paulo, were studied.

Twelve isolates were obtained from the mycology collection of the Núcleo de Pesquisa Aplicada à Agricultura at the Universidade Estadual de Maringá and five were kindly provided by Ms. Tamires Ribeiro of the Instituto Agronômico de Campinas, Campinas, São Paulo State, Brazil.

Growth of mycelial mass

The monosporic cultures of *C. lindemuthianum* were first replicated to Petri dishes with potato dextrose agar (PDA) medium (Menezes & Silva-Hanlin, 1997), and spore discs (ascospores) were then replicated and transferred to beakers containing liquid potato dextrose (PD) medium, according to the methodology proposed by Kado and Hesket (1970).

Subsequently, the mycelial mass was filtered, washed with distilled water and dried using autoclaved filter paper. After drying, the mycelium was wrapped in aluminum foil, properly labeled, and stored at a temperature of -20ºC.

DNA extraction, quantification and amplification of ITS regions

The genomic DNA extraction was performed from the mycelial mass previously stored at -20ºC, according to the modified SDS protocol proposed by Cárdenas, Galván, Barrera, and Carmona (2012). DNA quality was estimated by 0.8% agarose gel electrophoresis. DNA quantification was performed using a Quant-iT^{TM} fluorometer. The ITS1-5.8S-ITS2 regions of the rDNA were amplified by PCR using the primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes & Bruns, 1993) and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White, Bruns, Lee, & Taylor, 1990).

PCR amplification was carried out in a total volume of 50 μL, containing 40 ng of total DNA, 0,2 mM dNTP, 3.0 mM $MgCl_2$, 1X PCR buffer, 5 mM each primer and 1 unit of *Taq* DNA polymerase (Invitrogen). The amplification was programmed as follows: Preheating for 5 min. at 94°C; 30 cycles, each for 30 s at 94°C (denaturation), 30 s at the annealing temperature of 55 - 58°C, and 30 s at 72°C (extension) and a final extension at 94°C for 7 min., followed by cooling at 4°C for infinite period. All the amplification reactions were performed in a thermal cycler model TC-412 (M.J. Research Inc., Waltham, M.A.). The amplicons were then analyzed via 1.2% agarose gel electrophoresis. The DNA bands were visualized under ultraviolet light, and digital images were recorded with an L-PIX Image EX model (Loccus Biotecnologia - Loccus do Brasil, Cotia, São Paulo State, Brazil).

Purification of amplified fragments and sequencing

The amplicons corresponding to the ITS regions of the rDNA from isolates of *C. lindemuthianum* were purified using the PureLink PCR Purification Kit (Invitrogen), according to the manufacturer's recommendations, for subsequent sequencing. The Big Dye® Terminator v3.1 Cycle Sequencing Kit was used for sequencing the ITS regions of the rDNA.

The samples were sent for sequencing to the Centro de Estudos do Genoma Humano e Células-Tronco CEHG-CEL of the Universidade de São Paulo – USP, São Paulo State, Brazil.

Analysis of the sequences of ITS region

For the construction comparing the consensus sequence, genetic distance and phylogenetic tree, was used 15 sequences obtained from GenBank database NCBI -BLAST (Altschul et al., 1997). These sequences were selected because they were highly similar (99 to 100%) to the race 65 isolates used in this study.

After sequencing, the BioEdit Sequence Alignment Editor version 7.2.5 program (Hall, 1999), was used for comparing the sequence data, which enabled the localization and identification of the differences as single nucleotide polymorphisms (SNPs). Subsequently, the Mega 5.2.2 program (Tamura et al., 2011) was used to construction of the phylogenetic tree, with a bootstrap of 10,000 replicates with the Neighbor-Joining method (Saitou & Nei, 1987). The method described by de Tamura et al. (2011) was used for generating the distance matrix.

Results and discussion

The PCR products obtained in the amplification of the ITS1 region, gene 5.8S and ITS2 region of the rDNA from *C. lindemuthianum* isolates had an approximate size of 600 base pairs (bp) (Figure 1).

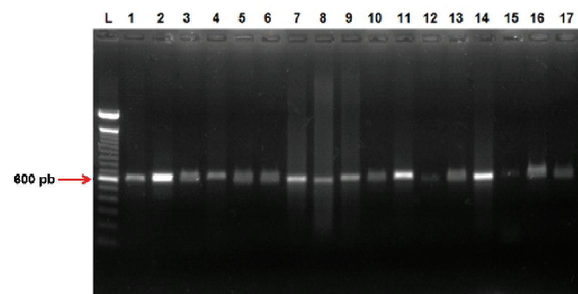

Figure 1. PCR amplicons of the ITS1, gene 5.8S and ITS2 regions of 17 isolates of *C. lindemuthianum* race 65. Isolates 1 to 3: State of Mato Grosso; isolate 4: State of Minas Gerais; isolate 5: State of Paraná; isolates 6 to 12 and 17: State of Santa Catarina; isolates 13 to 16: State of São Paulo; L: Ladder.

Identification changes in nucleotides (SNPs – Single nucleotide polymorphisms)

As illustrated in Figure 2 the comparison of the DNA sequences of the isolates of race 65 were compared with the sequences obtained from GenBank. The sequence analysis revealed that the race 23 obtained from the database showed 100% similarity with five isolates of the race 65 and 99% of similarity with the other 12 isolates of this race.

The genetic variability of the isolates of race 65 collected in several regions of Brazil indicates divergence between and within race 65, according to studies conducted to characterize isolates collected in the State of Santa Catarina, race 65 was among the races identified (Balardin, Pastor-Corrales, & Otoya, 1990; Gonçalves-Vidigal et al., 2008).

In the ITS1 region, isolate 3 from the State of Mato Grosso showed a SNP at position 79, where C was replaced by T. Isolate 8 from Santa Catarina showed 3 SNPs, a change from C to A at position 83, a change from G to T at position 119,

and a change from G to A at position 199. Isolate 4 from Paraná showed a SNP at position 120, where G was replaced by C, and isolate 14 from São Paulo had 3 SNPs, a change from G to T at position 119, a change from A to G at position 156, and a change from G to A at position 199. Carneiro (1999) identified 13 races of *C. lindemuthianum,* among which races 81, 65 and 73 were the most frequent in a study of pathogen variability conducted with 70 isolates collected in the State of Paraná.

Carbonell et al. (1999) conducted a study focusing on the reaction of cultivars recommended for the State of São Paulo and on the identification of pathogen races. Nine races were identified, and races 65, 81 and 89 were predominant. However, when two isolates of race 31, two of race 65 and three of race 81 were inoculated into cultivars recommended for cultivation in the state, differences among races were observed. Such differences suggested that the set of differential cultivars of *C. lindemuthianum* was not sufficient to differentiate the pathogenicity diversity of the isolates evaluated due to possible interactions and existing gene interactions between the genes for pathogen resistance.

The sequences of isolates 9, 12, 15, and 17 showed a SNP in the ITS1 region, at position 119, where G was changed to T. Considering position 199 in Figure 2, it is observed that isolates 9, 12, 13 and 15 showed a SNP in the ITS1 region with a change from G to A. In turn, in the ITS2 region, there was a change from C to A at position 501 in the sequences of isolates 8, 9, 12, 14, 15, and 17.

Conversely, the isolate 4 from Paraná showed a change from G to A at position 480, and isolate 16 from São Paulo had 3 SNPs, a change from C to G at position 435, a change from G to A at position 436, and a change from A to C at position 470. Isolates 1 and 2 of race 65 from the State of Mato Grosso were similar to each other, whereas isolate 3 had one SNP in the ITS1 region that was distinct from the other race 65 isolates. Isolates 1, 2 and 3 originated from the same locality, which demonstrates the occurrence of genetic variability in the *C. lindemuthianum* fungus. Sherriff et al. (1994) and Sherriff, Whelan, Arnold, and Bailey (1995) obtained similar results for the ITS2 region when they compared *Colletotrichum* species.

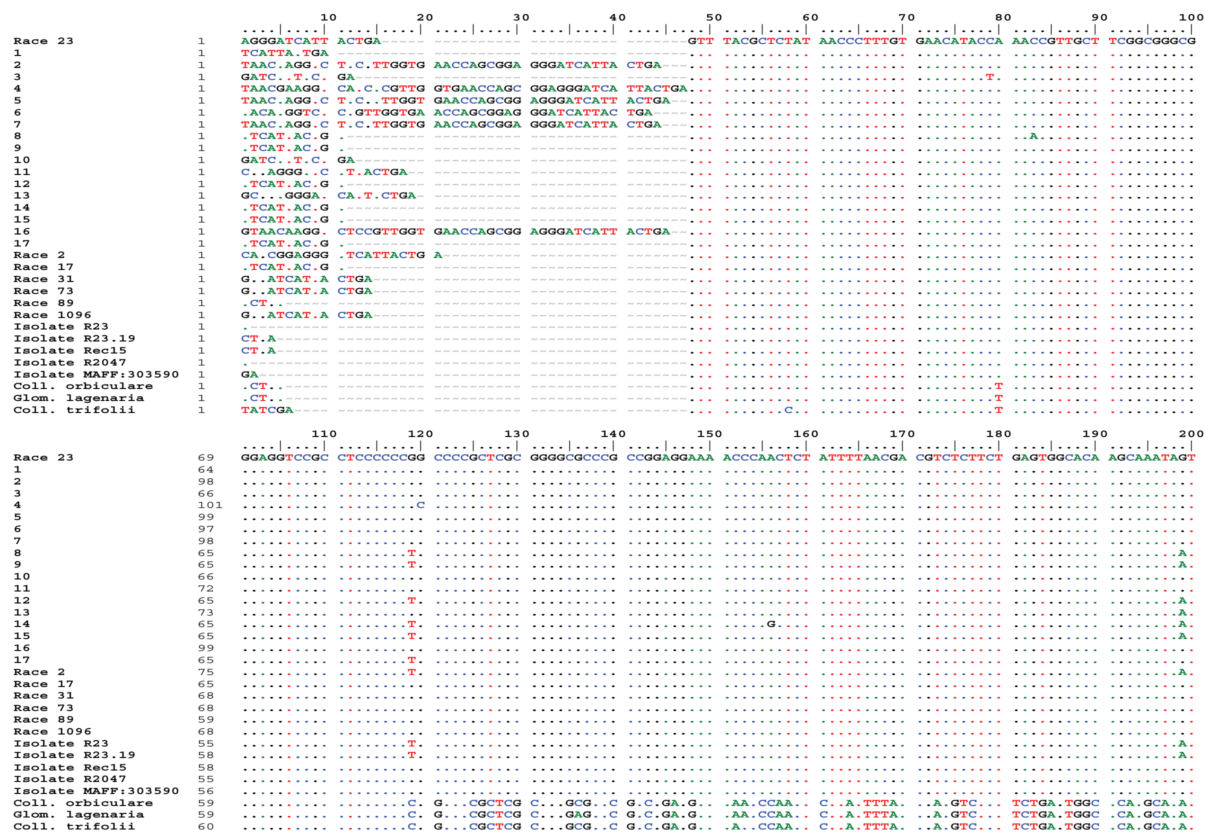

Figure 2. Alignment of the sequences of ITS region (5'-3'direction) isolates of C. lindemuthianum. (.) Indicates similarity to isolate 1; (~) Indicates introduction gap. The sequences of the ITS1, ITS2 and 5.8S gene is found at position: 48-201, 202-360 and 361-519, respectively. (continue ...)

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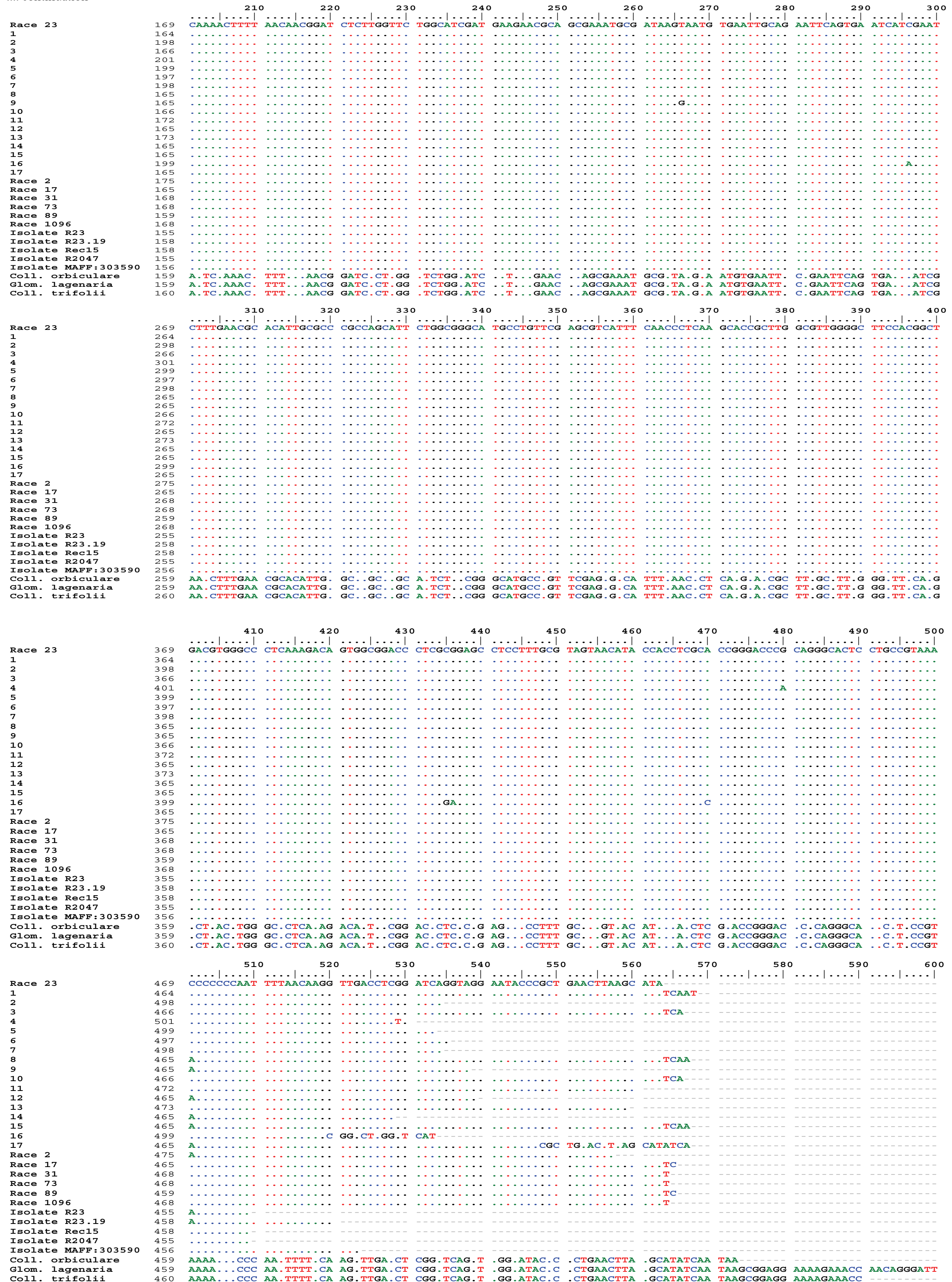

Figure 2. Alignment of the sequences of ITS region (5'-3'direction) isolates of C. lindemuthianum. (.) Indicates similarity to isolate 1; (~) Indicates introduction gap. The sequences of the ITS1, ITS2 and 5.8S gene is found at position: 48-201, 202-360 and 361-519, respectively.

The results presented in Figure 2 are similar to the ones reported by Balardin, Smith, and Kelly (1999), who detected variability in the ITS2 region when they analyzed the sequences of 14 isolates of *C. lindemuthianum*. Those authors observed that isolates of *C. lindemuthianum* with the same virulence phenotype differed in terms of molecular variability, for example, races 7, 17, 31 and 73 collected from different locations in Argentina, Brazil, Canada, Colombia, Costa Rica, Dominican Republic, Honduras, Mexico, the Netherlands, Peru and the United States showed polymorphic bands.

Isolate 16 from São Paulo state had only one SNP in the ITS2 region, which showed it to be divergent from all race 65 isolates and the sequences obtained in BLAST. On the other hand, the isolate 4 from the State of Minas Gerais revealed the presence of one SNP in the ITS1 region and one in the ITS2 region, both are different from the SNPs observed in the other isolates. Studies conducted by Talamini et al. (2006) noted the presence of molecular variability between and within isolates belonging to race 65 from the State of Minas Gerais.

Davide and Souza (2009) conducted a study to evaluate pathogenic variation within race 65. Six isolates collected in the State of Minas Gerais were inoculated into the twelve differential cultivars and into seven commercial cultivars; these cultivars were either resistant or susceptible to race 65 depending on the isolate inoculated, showing the presence of pathogenic variation within race 65. Pinto et al. (2012) analyzed 74 isolates of *C. lindemuthianum* in two regions of the State of Minas Gerais and observed the occurrence of six pathogenic races, with predominance of race 65 in the two regions.

The race 65 isolates 13, 14, 15 and 16 from the State of São Paulo used in this study showed differences between them and from all the other race 65 isolates, isolate 16 was the most dissimilar because it had SNPs in the region of the 5.8S gene and the ITS2 region that differed from all the other isolates of this race, a finding that suggests high genetic variability between and within race 65.

Similarity and dissimilarity measures

The results of the sequence analysis based on the methodology proposed by Tamura et al. (2011) using the Mega software indicated that the highest genetic distance occurred between isolates 10 and 11 of race 65 from Santa Catarina, with a value of 0.772 (Table 1). High dissimilarity was also observed between isolates 3 and 11 from Mato Grosso and Santa Catarina, respectively, with the same value of 0.772. In turn, the most similar isolates were 2 and 7, collected in Mato Grosso and in Santa Catarina, which had a genetic distance of 0.002 (Table 1).

Isolates 15 from the State of São Paulo and 9 from the State of Santa Catarina showed a genetic distance of 0.004. The isolates of race 65 from the State of Santa Catarina used in this study showed high dissimilarity, as observed in Table 1. According to Alzate-Marin et al. (2001), wide of polymorphism were identified between isolates in studies of genetic variability in pathotypes of C. lindemuthianum and can be used in studies of genetic diversity between pathotypes. Thomazella et al. (2002) used random amplified polymorphic DNA (RAPD) markers to identify genetic variability in different isolates of race 65 collected in the State of Paraná, showing molecular variability within that race.

Isolates classified as belonging to race 65 differ in their reactions to several common bean genotypes. Isolates 15 and 8, collected in São Paulo and in Santa Catarina respectively, and isolates 15 and 14, collected in São Paulo, had genetic distances of 0.006. Three isolates of race 65 collected in the State of Santa Catarina (8, 9 and 12) and two in São Paulo (14 and 15) had genetic distances of 0.009 between them.

According to the results presented here, the isolates from the states of Santa Catarina and São Paulo showed wide genetic variability among them. In a survey of races, Alzate-Marin & Sartorato (2004) identified races 65, 73 and 81 as the most frequent and widely distributed in Brazil, and these races are commonly found in the States of Paraná, Santa Catarina, Goiás and Distrito Federal.

The phylogenetic tree based on the sequences of the ITS1 and ITS2 regions revealed the separation of the isolates in two groups, but within the group where 5 isolated from the State of Santa Catarina belonging to race 65, there was a formation of a subgroup with the races 23, 31, 73, and 1096 obtained from the database together with the isolated 11 from Santa Catarina.

Figure 3 illustrates that isolates 3 (Mato Grosso), 10 and 11 (Santa Catarina) belong to the same group, but isolates 3 and 10 are very close to, but distant from the isolated 11 belonging to another subgroup. Isolated 2 (Mato Grosso) and 7 (Santa Catarina) are very close within the same group.

Table 1. Genetic distance (%) between pairs of Colletotrichum lindemuthianum isolates. Isolates 1 to 3: State of Mato Grosso; isolate 4: State of Minas Gerais; isolate 5: State of Paraná; isolates 6 to 12 and 17: State of Santa Catarina; isolates 13 to 16: State of São Paulo; isolates 18 to 32: GenBank sequences.

	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
1																																
2	0.736																															
3	0.721	0.745																														
4	0.711	0.677	0.743																													
5	0.745	0.634	0.751	0.668																												
6	0.755	0.696	0.726	0.704	0.721																											
7	0.734	0.002	0.745	0.674	0.636	0.694																										
8	0.702	0.753	0.698	0.747	0.738	0.745	0.753																									
9	0.698	0.749	0.696	0.747	0.734	0.743	0.749	0.009																								
10	0.719	0.745	0.009	0.740	0.751	0.730	0.745	0.696	0.691																							
11	0.719	0.734	0.772	0.736	0.747	0.762	0.734	0.717	0.717	0.772																						
12	0.691	0.753	0.704	0.749	0.738	0.749	0.753	0.011	0.011	0.700	0.715																					
13	0.753	0.755	0.711	0.764	0.728	0.762	0.757	0.723	0.721	0.713	0.711	0.723																				
14	0.700	0.753	0.702	0.749	0.738	0.745	0.753	0.009	0.009	0.700	0.717	0.013	0.726																			
15	0.696	0.749	0.700	0.745	0.734	0.745	0.749	0.006	0.004	0.696	0.717	0.009	0.726	0.006																		
16	0.745	0.666	0.755	0.670	0.040	0.715	0.664	0.732	0.728	0.755	0.753	0.732	0.732	0.732	0.728																	
17	0.685	0.753	0.700	0.745	0.736	0.743	0.751	0.026	0.023	0.700	0.711	0.028	0.728	0.026	0.019	0.728																
18	0.740	0.798	0.745	0.732	0.766	0.766	0.798	0.730	0.734	0.745	0.713	0.732	0.721	0.732	0.732	0.764	0.736															
19	0.698	0.757	0.700	0.749	0.736	0.747	0.757	0.009	0.009	0.698	0.717	0.015	0.726	0.011	0.009	0.730	0.023	0.730														
20	0.736	0.730	0.713	0.755	0.764	0.779	0.730	0.709	0.709	0.713	0.715	0.706	0.704	0.709	0.706	0.779	0.706	0.774	0.711													
21	0.715	0.781	0.723	0.762	0.745	0.734	0.779	0.711	0.715	0.721	0.706	0.715	0.734	0.713	0.715	0.732	0.719	0.713	0.715	0.700												
22	0.715	0.781	0.723	0.762	0.745	0.734	0.779	0.711	0.715	0.721	0.706	0.715	0.734	0.713	0.715	0.732	0.719	0.713	0.715	0.700	0.000											
23	0.738	0.762	0.711	0.779	0.757	0.723	0.762	0.770	0.762	0.711	0.757	0.762	0.745	0.768	0.766	0.753	0.770	0.728	0.766	0.726	0.743	0.743										
24	0.715	0.781	0.723	0.762	0.745	0.734	0.779	0.711	0.715	0.721	0.706	0.715	0.734	0.713	0.715	0.732	0.719	0.713	0.715	0.700	0.000	0.000	0.743									
25	0.730	0.706	0.734	0.751	0.732	0.774	0.709	0.772	0.768	0.732	0.723	0.768	0.717	0.768	0.766	0.734	0.764	0.749	0.770	0.738	0.753	0.753	0.755	0.753								
26	0.728	0.747	0.755	0.732	0.738	0.738	0.747	0.734	0.738	0.757	0.757	0.738	0.760	0.734	0.736	0.740	0.736	0.755	0.732	0.753	0.732	0.732	0.736	0.732	0.749							
27	0.726	0.747	0.751	0.732	0.743	0.743	0.747	0.734	0.738	0.753	0.757	0.738	0.757	0.734	0.736	0.745	0.736	0.753	0.732	0.751	0.732	0.732	0.738	0.732	0.749	0.004						
28	0.732	0.709	0.734	0.749	0.732	0.774	0.711	0.768	0.764	0.732	0.721	0.764	0.721	0.764	0.762	0.734	0.760	0.747	0.766	0.734	0.757	0.757	0.753	0.757	0.006	0.747	0.747					
29	0.755	0.764	0.719	0.751	0.772	0.755	0.764	0.753	0.755	0.719	0.770	0.757	0.715	0.749	0.753	0.783	0.757	0.698	0.755	0.751	0.755	0.755	0.768	0.755	0.770	0.736	0.734	0.768				
30	0.734	0.755	0.736	0.772	0.726	0.753	0.755	0.734	0.730	0.736	0.747	0.726	0.745	0.732	0.730	0.730	0.736	0.736	0.732	0.706	0.757	0.757	0.557	0.757	0.749	0.740	0.738	0.747	0.755			
31	0.721	0.762	0.715	0.764	0.785	0.745	0.760	0.738	0.736	0.713	0.743	0.734	0.774	0.736	0.736	0.777	0.732	0.772	0.734	0.711	0.732	0.732	0.772	0.732	0.791	0.700	0.702	0.791	0.728	0.743		
32	0.779	0.764	0.755	0.762	0.753	0.747	0.764	0.743	0.743	0.755	0.794	0.745	0.768	0.745	0.745	0.743	0.740	0.785	0.736	0.783	0.747	0.747	0.734	0.747	0.798	0.760	0.762	0.802	0.751	0.736	0.755	

Identification of groups within the Phylogenetic Tree.

Figure 3 shows the formation of two distinct groups. Five isolates of race 65 are restricted to group I and twelve isolates to group II, with one (1) elonging to the subgroup, which demonstrates the high genetic variability of this race.

Figure 3. Phylogenetic tree of the 32 isolates of *Colletotrichum* based on the DNA sequences in the IST1 region, gene 5.8S and the ITS2 region.

Group I consists of five isolates of race 65, the isolates of races R23, R2047, race 89 and *C. orbiculare* and *C. trifolii*. In turn, Group II consists of twelve isolates of race 65, the isolates of race 17, race 2, MAFF 305390, R23.19, Rec15, the subgroup with isolate 11 from the State of Santa Catarina belonging to race 65, and races 23, 31, 73 and 1096. Gonçalves-Vidigal and Kelly (2006) used an isolate of race 65 that overcame resistance in cultivar BAT93 that differed from the one used by Alzate-Marin et al. (2007) for which BAT93 showed resistance. In the present study, two groups within race 65 were observed in the phylogenetic tree.

Likewise, Ishikawa, Souza, and Davide (2008) worked with 13 isolates of race 65 from the State of Minas Gerais from different regions and years and used RAPD analysis to identify 11 groups with the phylogenetic tree, which demonstrates the presence of variability within race 65. Santos, Antunes, Rey, and Rosseto (2008) reported pathogenic variability within isolates of race 65, 73 and 81, and two isolates of the same race were inoculated into 37 cultivars used in this study.

These results demonstrate the high variability of the pathogen, suggesting the existence of new races or sub-races that cannot be detected through the current series of differential cultivars.

Conclusion

The highest variability for the 17 isolates of *C. lindemuthianum* race 65 was observed in the ITS1 region. From a total of 11 SNPs detected, six SNPs occurred in

the ITS1 region, whereas five nucleotide changes were found in the ITS2 region. The results obtained in this study revealed high molecular variability between isolates of race 65 of *C. lindemuthianum*.

We also emphasize the importance of the ITS regions for the characterization of isolates of *C. lindemuthianum.* The characterization of these ITS regions may be used in the differentiation of isolates at the molecular level.

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