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GENETIC VARIABILITY WITHIN *FUSARIUM SOLANI* SPECIE AS REVEALED BY PCR-FINGERPRINTING BASED ON PCR MARKERS

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ABSTRACT

Fusarium solani fungus (teleomorph *Haematonectria haematococca*) is of relevance for agriculture, producing a disease that causes significant losses for many cultivars. Moreover, *F. solani* is an opportunistic pathogen to animals and humans. The complexity associated to its correct identification by traditional methods justifies the efforts of using molecular markers for isolates characterization. In this work, three PCR-based methods (one PCR-ribotyping and two PCR-fingerprinting) were used to investigate the molecular variability of eighteen *F. solani* isolates from four Brazilian States, collected from different substrates. Genetic analysis revealed the intraspecific variability within the *F. solani* isolates, without any correlation to their geographical origin and substrate. Its polymorphism was observed even in the very conserved sequence of rDNA locus, and the SPAR marker $(GTG)_5$ showed the highest polymorphism. Together, those results may contribute to understand the relation between fungal genetic variability and cultivars resistance phenotypes to fungal-caused diseases, helping plant-breeding programs.

Key words: intron splice site, *Fusarium solani,* microsatellite, PCR-fingerprinting, ribosomal DNA

INTRODUCTION

The fungus *Fusarium solani* (teleomorph *Haematonectria haematococca*) is widely found in soil and constitutes one of the most important phytopatogen in agriculture. It infects cultivars like soybean (*Glycine max*), bean (*Phaseolus vulgaris*), cassava (*Manihot esculenta*), potato (*Solanum tuberosum*), among others (11,12), causing rotteness of roots and fruits, wilting of the plant upper parts. As an opportunist pathogen, it can cause superficial mycoses in humans and animals (5,18).

F. solani is sub-classified into *formae specialis* (*phaseoli, pisi, cucurbitae, batatas, radicicola, robiniae, mori, piperis, eumartii, xanthoxyli, hibisci, lycopersici* and *phaseoli*) based on host specificity (19,20). Variations in the degree of virulence in *formae specialis*, as well as genetic diversity in isolates of different origins revealed that the *Nectria haematococca-F. solani* complex is composed by several phylogenetic species responsible for biologically distinct phytopathologies (10). Therefore, the knowledge of the genetic diversity within this pathogen specie should help to understand the causes of different disease manifestations.

In this sense, molecular tools based on DNA analysis are being currently used as an alternative to conventional morphological and biochemical tests for biotyping variants of many fungi species. The cluster of ribosomal DNA (Fig. 1), consisting of a tandem repeat of three coding (18S, 5.8S and 28S) and two non-coding (Internal Transcribed Sequences-ITS and Intergenic Sequences-IGS) spacer regions (9), is a very informative locus for this kind of analysis. Due to its highly

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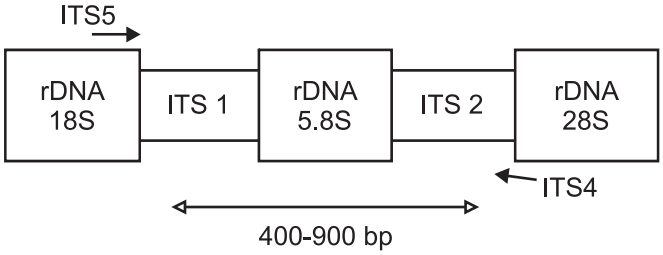

Figure 1. Schematic diagram of the fungal rDNA gene cluster. Genes encoding 18S, 5.8S and 28S ribosomal RNA subunits are separated by the internal transcribed sequences 1 (ITS1) and 2 (ITS2) that are spliced after transcription. The primers ITS4 and ITS5 (single-headed arrows) have been used to amplify this rDNA cluster from all fungi species so far, producing a species-specific fragment ranging from 400 to 900 bp (double-headed arrow).

repetitive characteristic, but relative slow evolving rate, rDNA clusters are also subjected to inter-specific internal length and nucleotide variation (17,21). Furthermore, restriction analysis of ITS amplicons enhances the potential of this systematic tool.

Besides rDNA markers, another PCR-based markers have shown to be very informative in discriminating fungal isolates. The introns have potentially high rates of sequence evolution and their analysis has become an important tool in studies of genome relatedness (4). Introns can be sorted out into four major categories (group I, group II, nuclear mRNA and nuclear tRNA) based on the splicing mechanisms (3). Group I introns in the small subunit rDNA have been found in a number of fungi (7) and the presence or absence of these introns caused length polymorphism of the small subunit rDNA 3' region of *Fusarium solani* (20). Additionally, another PCR-fingerprinting marker takes advantage on the use of microsatellite oligonucleotides that amplify genomic segments different from the repeat region itself. This approach, named Single Primer Amplification Reaction (SPAR), uses a single primer consisting of the core motif of microsatellites with repeat motifs, such as (CA)n, (CT)n, (GT)n, (GAC)n, (GTG)n, (GACA)n, (GATA)n, (TGTC)n, etc. These primers trigger site-specific annealing and initiates PCR amplification of genomic segments, which are flanked by inversely orientated and closely spaced repeat sequences (8). This method has been used to discriminate isolates of different fungi species, such as *S. cerevisiae* (8), *F. oxysporum* (1), *Cenococcum geophilum* (13), *Phytophthora capsici* (22) and *Exophiala* species (23).

The presence of different *formae speciales* associated to different degrees of pathogenicity and the complex taxonomy of *F. solani* justify efforts to genetically characterise this species in order to further develop an effective biocontrol strategy. The present report aimed to analyse different unrelated Brazilian isolates of *F. solani* based on PCR-fingerprinting methods.

MATERIALS AND METHODS

Fungal strains

Eighteen isolates of *F. solani* (Table 1) were provided by the mycological collection of the Department of Mycology, Federal University of Pernambuco (URM-UFPE). All strains were chosen according to their pathogen characteristics to different plants. The isolates were maintained in potato-dextrose-agar slants at 4ºC.

DNA extraction

Flasks containing 100 ml Czapeck medium were inoculated with 3 ml of *F. solani* conidial suspensions (10^6 conidia/ml) and incubated at 250 rpm and 30ºC for 96 h. The mycelia were harvested by filtration, washed with sterile-distilled water and stored at -20ºC until use. For total genomic DNA was extracted, the mycelium was ground into the fine powder under liquid nitrogen and suspended in 800 mðl extraction buffer (200 mM Tris-HCl pH 8.0; 250 mM NaCl; 25 mM EDTA; 1% SDS). Upon homogenisation, the tubes were incubated for 15 min at 65ºC. DNA samples were purified with equal volumes of saturated phenol (1x), phenol:chloroform (1:1) mixture (1x) and chloroform:isoamyl alcohol (24:1) mixture (1x), and precipitated with 0.3 M NaCl and 2 volumes ethanol at -20ºC for 30 min. The tubes were centrifuged at 12000 rpm (SS4 rotor, Kubota, Japan) for 15 min and DNA pellets were rinsed with 70% ethanol, air-dried, suspended in TE buffer (pH8.0) and stored at 4ºC until use (14).

PCR-ribotyping

Amplification reactions were prepared to final volume of 25 mðl containing 1x *Taq* buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 50 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 12.5 pmols of each ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAA-3') and 1.25 U *Taq* DNA polymerase (Operon Technologies, CA), as described by White *et al*. (21). Thermal cycling consisted of initial denaturation of 4 minutes at 95ºC, followed by 40 cycles of 1 minute at 92ºC, 1 minute at 55ºC and 2 minutes at 72ºC, with 5 minutes at 72ºC for final extension. Amplification products were visualized in 1% (w/v) agarose gel at 3 V/cm^{-1} in TBE buffer (pH8.0) after ethidium bromide staining.

Aliquots of 4 µl of the amplicons were subjected to enzymatic digestion with *Dra*I, *Eco*RI, *Hae*III or *Msp*I, according to manufacturer instructions. Fragments were separated in 1.4% (w/v) agarose gels and their molecular weights were determined using to 50-bp ladder marker (Invitrogen). Polyacrilamyde gel electrophoresis was performed according to Sambrook *et al.* (16).

PCR-Fingerprinting

Fingerprinting analysis were performed with EI1 Type I Intron Splice Site primer (5'-CTGGCTTGGTGTATGT-3') (4) and

Table 1. Geographical origin, sampling substrate and ribotyping of *Fusarium solani* isolates.

Isolates[a]	Access number[b]	Geographical origin	Substrate	ITS (bp)	ITS-RFLP (bp)		
					*Eco*RI	*Msp*I	*Hae*III
1	1709	Pernambuco	Air	620	310(x2)	380+240	250(x2)+120
2	2143	Santa Catarina	*Araucaria excelsa*	600	300(x2)	460+140	340+150+110
3	2391	Pernambuco	*Arachis hypogaea*	620	310(x2)	380+240	250(x2)+120
4	2429	Pernambuco	Foliage	620	310(x2)	380+240	250(x2)+120
5	2490	Pernambuco	Foliage	620	310(x2)	380+240	250(x2)+120
6	2696	Pernambuco	*Lycopersicom esculentum*	620	310(x2)	380+240	250(x2)+120
7	3088	São Paulo	Albertia myrcifolia leaves	620	310(x2)	380+240	250(x2)+120
8	3105	São Paulo	Rudigea gardenoides leaves	620	310(x2)	380+240	250(x2)+120
9	3338	São Paulo	Vermonia herbacea rhyzosphere	620	310(x2)	380+240	250(x2)+120
10	3472	São Paulo	Vermonia herbacea rhyzosphere	620	310(x2)	380+240	250(x2)+120
11	3821	Pernambuco	Soil	620	310(x2)	380+240	250(x2)+120
12	3838	Pernambuco	Soil	620	310(x2)	380+240	250(x2)+120
13	4050	Pernambuco	Interdigital feet scale	620	310(x2)	380+240	250(x2)+120
14	4054	Pernambuco	Interdigital feet scale	620	310(x2)	380+240	250(x2)+120
15	4055	Pernambuco	Interdigital feet scale	620	310(x2)	380+240	250(x2)+120
16	4059	Pernambuco	Interdigital feet scale	620	310(x2)	380+240	250(x2)+120
17	4098	Bahia	Passiflora edulis seeds	620	310(x2)	380+240	250(x2)+120
18	2121	Santa Catarina	*Allium cepa*	620	310(x2)	380+240	250(x2)+120

[a]Isolate number considered in this paper; [b]Access number according to Mycological collection Micoteca-URM (Recife, Brazil).

the $(GTG)_5$ single primer (8) by amplification reactions improved in our laboratory. For the first primer, the amplification reactions contained 1x *Taq* buffer, 50 ng of template DNA, 3 mM $MgCl_2$, 0.25 mM dNTP, 25 pmols of EI1 primer and 1.25 U *Taq* DNA polymerase (Operon Technologies, CA) into final volume of 25 µl. Thermal cycling consisted of an initial denaturation of 3 minutes at 94ºC, followed by 40 cycles of 1 minute at 94ºC, 2 minute at 45ºC and 90 seconds at 74ºC, with to final extension of 5 minutes at 74ºC. For the second primer, the amplification reactions contained 1x *Taq* buffer, 50 ng of template DNA, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 25 pmols of $(GTG)_5$ primer and 1.25 U of *Taq* DNA polymerase (Operon Technologies, CA) to 25 µl final volume. Thermal cycling consisted of an initial denaturation of 5 minutes at 93ºC, followed by 40 cycles of 20 seconds at 93ºC, 45 seconds at 55ºC and 90 seconds at 72ºC, with 6 minutes at 72ºC for final extension. The Amplicons were visualised in 1% (w/v) agarose gel at 3 V/cm^{-1} in TBE buffer (pH8.0) after ethidium bromide staining.

Genetic analysis

The variable binary similarity matrix was prepared using Jaccard coefficient by the NTSYS (Numerical Taxonomy System of multivariate program) computer program version pc2.1 (15). Dendrograms were prepared by UPGMA (Unweighted Pair Group Method with Arithmetical average) analysis.

RESULTS AND DISCUSSION

Amplification of the small subunit ribosomal DNA ITS1-5.8S-ITS2 (ITS) produced one fragment of 620-bp for all *F. solani* isolates (ITS-type I), except for the isolates 2143 and 4098, for which a single band of approximately 600-bp (ITS-type II) was obtained (Table 1). These results were later confirmed by running the amplicons in 6% polyacrilamide gel that allows higher definition between fragments that differs by few base pairs. These results suggested a possible length polymorphism for that sequence among different isolates of this species, which was further corroborated by digesting the amplification products with restriction enzymes (Table 1). No digestion at all was observed for *Dra*I, while *Eco*RI produced a double monomorphic fragment of approximately 310-bp for ITS-type I and a 300-bp for ITS-type II isolates. Digestion with *Msp*I produced DNA fragments of 380-bp and 240-bp for all isolates ITS-type I, and DNA fragments of 460-bp and 140-bp for ITS-type II isolates. These two isolates also produced *Hae*III restriction fragments different from the standard profile of 250-bp and 120-bp fragments. Restrictions with the last two enzymes showed polymorphism in both number and length of the resulting fragments and support the idea of an intraspecific genetic diversity among different isolates of *F. solani*.

These results are supported by previous studies on the genetic diversity found for this species complex, as observed by Edel *et al.* (6) after digesting ITS amplicons of different isolates of *F. solani* with *Msp*I. On the other hand, other reports failed to detect genetic polymorphism after digesting ITS amplicons of different isolates of *F. solani* with *Hae*III (2,10). Nevertheless, rDNA intergenic spacer (IGS) digested with *Msp*I also showed fragment polymorphism among different *F. solani* isolates (9). Taking it into account, all these information reinforce the genetic complexity of the specie *F. solani* complex.

Further investigations on the intraspecific polymorphism used both low-variable intron splice site marker and high-variable SPAR marker. The Fig. 2 shows the amplification profile of the *F. solani* isolates using the intron splice site, which varied from 7 to 13 fragments ranging from 250-bp to 3500-bp. Clustering analysis (data not shown) showed that isolates 3088, 3105, 3472, 3821 and 3838 presented the same amplification profile with 100% similarity, comprising the EI1-group 1, which could represent a clonal lineage. The isolates 3338 and 4054 shared 80-85% similarity with the EI1-group 1 pattern, while isolates 2391 and 4050 showed only 50% of similarity. The level of similarity lied between 50 to 60% for the other isolates. Isolates 2143 and 4098 showed the highest genetic divergence for the EI1 primer, as detected by the ITS analysis.

The type I intron sequences were detected in the 3' region of the small subunit rDNA (18S gene) of different *formae speciales* of *F. solani* (20). These authors reported length polymorphism of the intron sequences inside the 18S gene, although it has been a consensus that this region may evolve slowly among distantly related organisms. Therefore, it suggests that isolates 2143 and 4098 contain significant genomic differences that highlight the genetic complexity of this species. To our knowledge, this is the first report on the use of the primer EI1 for fingerprinting analysis of *Fusarium*.

The amplification with $(GTG)_5$ primer showed fingerprinting patterns containing 7 to 16 reproducible fragments, ranging from 500-bp to 3500-bp (Fig. 3). This primer was able to discriminate all *F. solani* isolates analyzed, including those of

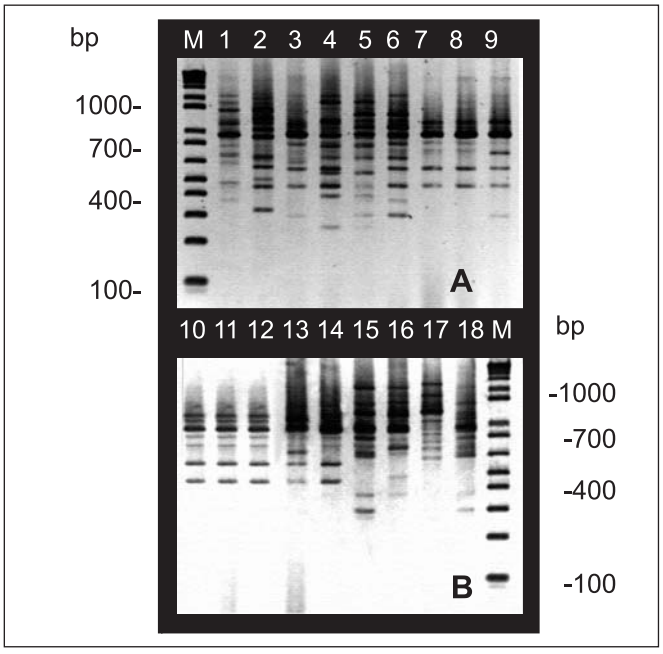

Figure 2. DNA fingerprinting of *Fusarium solani* isolates by using intron splice site primer EI1. (A) Lanes 1-9 represent the amplification products of the isolates 1709, 2143, 2391, 2429, 2490, 2696, 3088, 3105 and 3338, respectively. (B) Lanes 10-17 represent the amplification products of the isolates 3472, 3821, 3838, 4050, 4054, 4055, 4059 and 4098, respectively. Lane 18: isolate 2121 (*F. solani* var. *minus*). M: 1-Kb ladder.

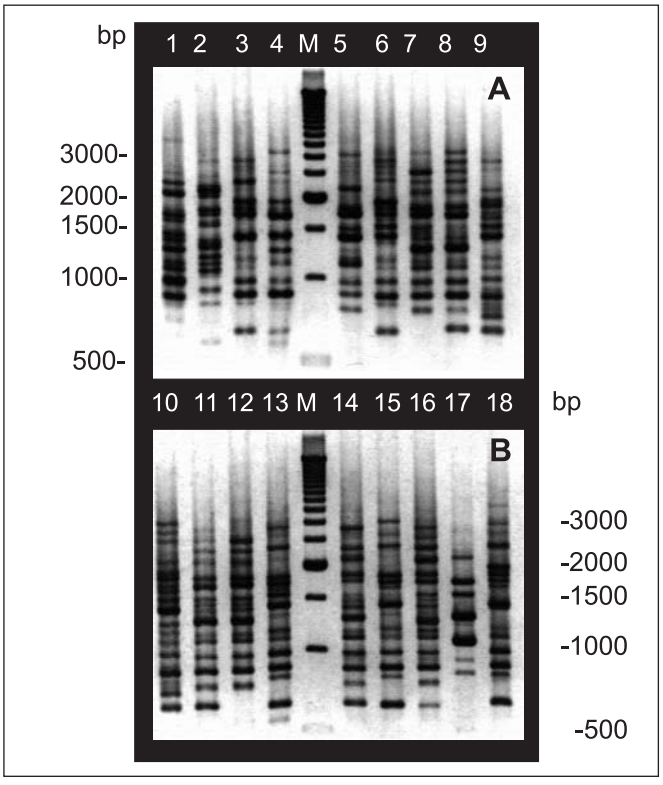

Figure 3. DNA fingerprinting of *Fusarium solani* isolates by using the SPAR $(GTG)_5$. (A) Lanes 1-9 represent the amplification products of the isolates 1709, 2143, 2391, 2429, 2490, 2696, 3088, 3105 and 3338, respectively. (B) Lanes 10-17 represent the amplification products of the isolates 3472, 3821, 3838, 4050, 4054, 4055, 4059 and 4098, respectively. Lane 18: isolate 2121 (*F. solani* var. *minus*). M: 500-pb ladder.

the EI1-group 1. Again, isolates 2143 and 4098, together with isolates 3821 and 3838, showed the highest genetic diversity compared to the others isolates (data not shown), thus emphasizing their genomic divergence. Barve *et al.* (1) reported that $(AGT)_5$, (ATC)5 and $(GATA)_4$, among 13 other SPARs tested, were able to discriminate isolates of four different races of *F. oxysporum* f. sp. c*iceri*. The present paper describes the first report on the use of the $(GTG)_5$ to analyse intraspecific genetic diversity of the *F. solani*. Together, both intron splice site and SPAR analysis may contribute to understand the genetic complexity of this species.

A combinatory clustering analysis used all three PCR markers and revealed the genetic relatedness among the isolates (Fig. 4). Indeed, as shown for PCR-ribotyping, isolates 2143 and 4098 were the most divergent isolates in our analysis producing two phenetic groups. The third group was composed by the isolate 2121 and 15 other isolates, although it presented similarity level below 70% for most of isolates. The fact that 2121 has been originally classified as *F. solani* var. *minus* suggests that our analysis can be used for *F. solani* variety and/or *formae specialis* discrimination. SPAR primers have been postulated for variability studies within the *F. oxysporum* f. sp. *ciceri* races, where the race 3 represented the most distinct group of the taxon with 26.7% of similarity to others races (1). Therefore, it is plausible to speculate that the isolates 2143 and 4098, showing only 30% similarity between them and 25% similarity to other isolates analyzed here, may compose two subspecies group (races or varieties) of *F. solani*. Unfortunately, no correlation was found with geographical origin of the isolates and their genetic relatedness in this study.

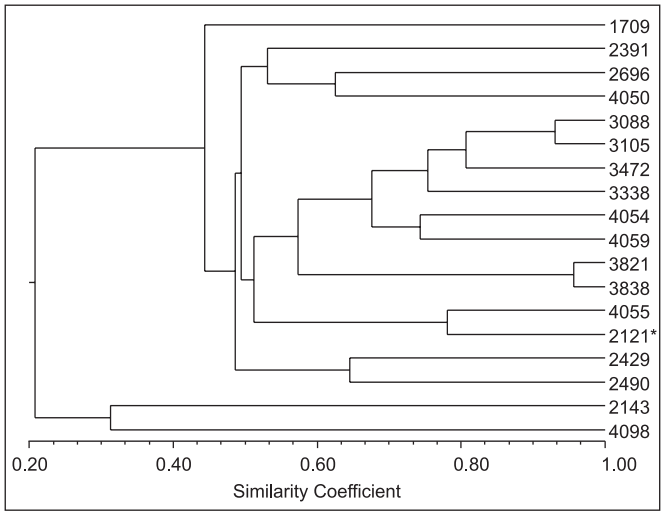

Figure 4. Combinatory clustering analysis of the 18 *F. solani* isolates using all the PCR markers used in this work. The asterisk represents the strain 2121 of *F. solani* var. *minus*. Similarity matrix was prepared using Jaccard coefficient to generate a dendrogram by UPGM.

The results reported here point out $(GTG)_5$ primer as a reliable method for detecting genetic differences between isolates of *F. solani*, as it has been postulated for other fungi species (8,13,23), while ITS and EI1 primers are more useful for clonal analysis. Similarly, results of our laboratory on the fingerprinting of *S. cerevisiae* isolates showed that EI1 primer was poorly informative for yeast strain identification, whereas $(GTG)_5$ primer unequivocally discriminate genetic strains (data not shown).

Our study concluded that *F. solani* isolates might compose a highly genetically variable species that could be related to its wide range of hosts. Therefore, understanding the relation between fungal variability and plant resistance phenotype may help driving the progress of breeding programs or the use of recombinant DNA technology towards producing resistant cultivars.

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RESUMO

Variabilidade genética em espécies de *Fusarium solani* revelada pela técnica de impressão genética baseada em marcadores PCR

O fungo *Fusarium solani* (teleomorfo *Haematonectria haematococca*) apresenta uma expressiva importância na agricultura por ser considerado patógeno para várias culturas de interesse econômico causando doença conhecida por podridão das raízes, além de ser patógeno aos animais e ao homem, provocando nestes últimos, micoses superficiais e sistêmicas. A complexidade associada a sua identificação correta através de métodos tradicionais justifica os esforços de usar marcadores moleculares para caracterização dos isolados. Neste trabalho, três métodos baseados na tecnologia da PCR (um por ribotipagem por PCR e dois por impressão genética por PCR) foram utilizados para investigar a variabilidade molecular de dezoito isolados de *F. solani* de quatro Estados brasileiros, coletados de diferentes substratos. A análise genética revelou a variabilidade intraespecífica dos isolados de *F. solani,* sem qualquer correlação para a origem geográfica e substrato. Seu polimorfismo foi observado até mesmo na seqüência conservada do locus do rDNA, e o marcador SPAR $(GTG)_5$ mostrou o mais alto polimorfismo. Em conjunto, estes resultados poderão auxiliar nos estudos da relação entre variabilidade do

perfil genético de isolados e os fenótipos de resistência de determinados cultivares às doenças provocadas pelo fungo, orientando programas de melhoramento vegetal.

Palavras-chave: DNA ribossomal, intron splice site, *Fusarium solani,* impressão genética por PCR, microssatélite.

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