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Distribution of mating-type alleles and M13 PCR markers in the black leaf spot fungus *Mycosphaerella fijiensis* of bananas in Brazil

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ABSTRACT. The fungus *Mycosphaerella fijiensis* is the causative agent of black sigatoka, which is one of the most destructive diseases of banana plants. Infection with this pathogen results in underdeveloped fruit, with no commercial value. We analyzed the distribution of the *M. fijiensis* mating-type system and its genetic variability using M13 phage DNA markers. We found a 1:1 distribution of mating-type alleles, indicating *MAT1-1* and *MAT1-2* idiomorphs. A polymorphism analysis using three different primers for M13 markers showed that only the M13 minisatellite primers generated polymorphic products. We then utilized this polymorphism to characterize 40 isolates from various Brazilian states. The largest genetic distances were found between isolates from the

same location and between isolates from different parts of the country. Therefore, there was no correlation between the genetic similarity and the geographic origin of the isolates. The M13 marker was used to generate genetic fingerprints for five isolates; these fingerprints were compared with the band profiles obtained from inter-simple sequence repeat (UBC861) and inter-retrotransposon amplified polymorphism analyses. We found that the M13 marker was more effective than the other two markers for differentiating these isolates.

Key words: *Musa* sp; Polymorphism; Mating-type; ISSR; IRAP

INTRODUCTION

Bananas are one of the most consumed fruits in the world, and Brazil ranks fourth in terms of banana production (FAO, 2010). Biological stress is one of the major limiting factors for the major banana-producing areas. The disease black sigatoka is caused by the fungus *Mycosphaerella fijiensis* Morelet and is particularly harmful to bananas. This fungus causes the premature death of leaves, thus reducing the photosynthetic ability of the plant and causing the production of irregular fruit with no commercial value (Gasparotto et al., 2006). Black sigatoka was detected in Brazil in 1998 in the municipalities of Benjamin Constant and Tabatinga in the State of Amazonas (Pereira et al., 1998). The disease has since spread throughout the North, South, Southeast, and Central-West regions, with the exception of the States of Espírito Santo, Rio de Janeiro, Goiás, and the Federal District (Gasparotto et al., 2006).

Since the introduction of the pathogen in Brazil, research efforts have focused on monitoring the spread of the disease and evaluating the genetic diversity of *M. fijiensis* with regard to its new geographic conditions and host cultivars. Understanding the variations within the pathogen population is essential for directing disease control strategies, especially for host breeding programs focused on resistance (McDonald and Linde, 2002). In addition, these studies allow for the detection of new variants in the population (Markell and Milus, 2008; Milus et al., 2009).

The successful adaptation of exotic phytopathogens is strictly related to the genetic variability of the founding population and the presence of susceptible host genotypes and favorable environments. In novel environments, recombinant pathogenic populations are able to adapt and mutate more rapidly than clonal populations (Bui et al., 2008; Hsueh and Heitman, 2008). In wheat, sexual recombination is important for generating novel allelic combinations in *Mycosphaerella graminicola* that are able to overcome the resistance of the host genotypes (Zhan et al., 2007).

The selection of compatible gametes is essential for successful sexual reproduction in microorganisms. The mating behavior of filamentous Ascomycetes is generally determined by one locus and two functional alleles that are often designated as *MAT-1* and *MAT-2* (Kerenyi et al., 1999). The most intensively studied mating-type system is that of the yeast *Saccharomyces cerevisiae* (Gordon et al., 2011). This system is characterized by only one locus, MAT, and two different alleles, *MAT a* and *MAT α*. Each locus contains two genes that encode homeodomain proteins (a1 and alpha2), which in turn regulate sexual reproduction. Two non-allelic genes, *MAT1.1* and *MAT1.2*, have been reported to enhance the genetic recombination rate in *M. fijiensis* (Conde-Ferráez et al., 2007).

Given the critical role of reproduction in the adaptation and evolution of pathogenic fungi, it is clear that the analysis of the mating-type distribution in *M. fijiensis* populations may increase our understanding of the relatedness of the Brazilian population of this pathogen.

Globally, the genetic diversity of *M. fijiensis* populations has been studied using restriction fragment length polymorphism (Carlier et al., 1996; Hayden et al., 2003) and simple sequence repeat (SSR) (Müller et al., 1997; Rieux et al., 2011) analyses. Carlier et al. (1996) reported a high degree of divergence in populations originating from Southeast Asia. However, populations from South America, Africa and the Pacific Islands have a low extent of variability compared to those from Southeast Asia (Hayden et al., 2003; Fahleson et al., 2009). Müller et al. (1997) used SSR analysis to demonstrate that the genetic variability of *M. fijiensis* isolates depends on the lesion, plant, cultivar, and location where the phytopathogen was collected. Each technique is unique and demonstrates a different discriminatory power; thus, analyses using various markers may increase our understanding of the genetic diversity in fungal populations.

The genetic variability of pathogenic populations has been analyzed using several different molecular markers (Zein et al., 2010; Nusaibah et al., 2011). The most commonly used marker techniques include inter-simple sequence repeat (ISSR) analysis, which has been widely used to study fungal variability (Neal et al., 2011), and the analysis of inter-retrotransposon amplified polymorphisms (IRAPs), which is a readily accessible method owed to the availability of sequencing databases and the abundance of retrotransposons in the genomes of many organisms (Zein et al., 2010).

Genetic fingerprinting is a robust tool for detecting the presence of pathogens and is useful for molecular screening and diagnosis. Ryskov et al. (1988) first proposed the use of the M13 phage as a universal marker because it can be used to rapidly generate a DNA fingerprint from organisms belonging to distinct taxonomic groups (Degen et al., 1995). This technique has been called M13 fingerprinting (Ulrich et al., 2009), M13 random amplified polymorphic DNA (RAPD) analysis (Rossetti and Giraff, 2005) and M13 minisatellite analysis (Zamponi et al., 2007). In fungi, M13 fingerprinting has been used to analyze genetic instability in *Cryptococcus neoformans* during the infection stage (Ulrich et al., 2009) and to genetically characterize both the *Histoplasma capsulatum* (Muniz et al., 2010) and *Sporothrix schenckii* populations (Reis et al., 2009). M13 fingerprinting has also been used to identify different *Trichophyton* varieties (Gräser et al., 1998) and to distinguish between *Penicillium commune* and *P. palitans* (Kure et al., 2002).

Because knowledge of the behavior of *M. fijiensis* under Brazilian conditions is necessary for guiding black sigatoka control strategies, the goal of this study was to analyze the mating-type distribution and genetic variability of *M. fijiensis* isolates using M13 markers.

MATERIAL AND METHODS

Isolation and culture conditions

These studies were performed at the Laboratórios de Fitopatologia e Biologia Molecular da Embrapa da Amazônia Ocidental, Amazonas, Brazil. The *M. fijiensis* isolates were obtained from banana leaves showing disease symptoms and maintained using potato dextrose agar media at 27°C. Mycelial masses were cultured in potato dextrose medium at 25°C and 120 rpm for 2 weeks.

DNA extraction

DNA was extracted from *M. fijiensis* mycelia that were ground in liquid nitrogen according to the method described by Specht et al. (1982). The extracted DNA was quantified using a spectrophotometer (NanoDrop) and confirmed by electrophoresis with a 0.8% agarose gel.

Mating-type analysis

The *MAT1-1-1* and *MAT1-2-1* target genes from 131 isolates were analyzed by PCR. The primer sequences were described by Arzanlou et al. (2010): MAT1.1F - 5'-CATGAGCACGCTGCAGCAAG-3', MAT1.1R - 5'-GTAGCAGTGGTTGACCAGGTCA T-3', MAT1.2F - 5'-GGCGCTCCGGCAAATCTTC-3', and MAT1.2R - 5'-CTTCTCGGATG GCTTGCGTG-3'. The reactions were performed in a final volume of 15 µL using 50 ng DNA, 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, and 1% Triton X-100), 2 mM $MgCl_2$, 0.15 mM of each dNTP, 0.25 µM of each primer and 0.4 U Taq-DNA polymerase (Phoneutria). The temperature cycles used for the amplifications were as follows: an initial 2-min denaturation at 94°C, 40 cycles of 94°C for 1 min, 70°C for 30 s and 72°C for 1 min, and a final 10-min extension at 72°C.

M13 analysis

The M13 minisatellite marker was used to analyze the genetic variability of 40 *M. fijiensis* isolates from the States of Amazonas, Acre, Rondônia, Roraima, Pará, Mato Grosso, and São Paulo (Table 1).

The PCR protocol was optimized using one randomly selected sample. The tested DNA concentrations ranged from 25 to 100 ng, the primer concentrations ranged from 0.2 to 0.6 µM, and the $MgCl_2$ concentrations ranged from 1.5 to 4.0 mM. The following primers were used: M13mp18F - GTACTGGTGACGAAACTC, M13mp18R - ATCGATAGCAGCACCGTA (Degen et al., 1995), M13 - TTATGTAAAACGGCCAGT, and M13 minisatellite - GAGGGTGGCGGTGGTTCT (Vassart et al., 1987). The reactions were performed in a final volume of 20 µL that contained 0.6 mM of each dNTP, 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, and 1% Triton X-100) and 1 U Taq DNA polymerase (Phoneutria). The amplification cycle was as follows: an initial 4-min denaturation at 94°C, 35 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. The ideal annealing temperature for the primers was determined by varying the temperature from 46° to 61°C. The PCR optimization tests indicated that 0.6 mM primer, an annealing temperature of 46°C and 25 ng DNA maximized the amplicon quality. The $MgCl_2$ concentrations varied depending on the primer used, with 2.5, 3.0, and 2.0 mM required for M13mp, M13 minisatellite and M13, respectively.

ISSR and IRAP analysis

Five isolates (Mf141, Mf195, Mf205, Mf217, and Mf218) were selected for comparisons of their M13, ISSR and IRAP fingerprints.

Table 1. *Mycosphaerella fijiensis* isolates and their corresponding geographical information.

Isolate	Host	Geographic information	
		State/municipality	GPS coordinates
		Amazonas	
Mf02	Thap Maeo	Presidente Figueiredo	S 02° 03 346 W 59° 40 047
Mf 05	Thap Maeo	Presidente Figueiredo	S 02° 03 329 W 59° 34 650
Mf 07	Thap Maeo	Manacapuru	S 03° 16 252 W 60° 30 539
Mf 37	Maçã	Rio Preto da Eva	S 02° 42 956 W 59° 41 802
Mf 41	Maçã	Rio Preto da Eva	S 02° 51 594 W 59° 24 414
Mf 44	Prata comum	Manaus	S 02° 59 042 W 60° 05 587
Mf 62	Maçã	Manacapuru	S 03° 16 511 W 60° 38 280
Mf 63	Prata comum	Manacapuru	S 03° 16 509 W 60° 38 279
Mf 68	IAC 2001	Manacapuru	S 03° 16 509 W 60°38 284
Mf 99	D'Angola	Iranduba	S 03°11 633 W 60° 08 392
Mf 100	Prata comum	Iranduba	S 03° 10 035 W 60° 06 341
Mf 102	Maçã	Iranduba	S 03° 08 372 W 60° 14 286
Mf 127	Caru Roxa	Presidente Figueiredo	-
Mf 134	Prata comum	Atalaia do Norte	S 04° 22 598 W 70° 10 356
Mf 136	SH 3640	Tabatinga	S 04° 13 210 W 69° 55 067
Mf 138	D'Angola	Tabatinga	S 04° 08 236 W 69° 56 767
Mf 141	Maçã	Autazes	S 03° 34 790 W 59° 41 560
Mf 150	Prata comum	Itacoatiara	S 03° 03 520 W 58° 50 140
Mf 158	D'Angola	Autazes	S 03° 36 153 W 59° 30 813
Mf 160	Nanica	Careiro Castanho	-
Mf 188	Maçã	Itacoatiara	-
Mf 195	D'Angola	Itacoatiara	S 03° 07 088 W 59° 05 945
Mf 224	Nanica	Iranduba	S 03° 13 933 W 60° 06 836
Mf 225	Nanica	Iranduba	S 03° 16 456 W 60° 11 647
		Roraima	
Mf 118	Maçã	Caroebe	-
Mf 119	Pacovan	Caroebe	S 00° 47 820 W 59° 25 749
Mf 120	Maçã	Caroebe	S 00° 48 268 W 59° 25 838
Mf 121	Pacovan	Caroebe	-
Mf 130	Pacovan	Caroebe	S 00° 53 040 W 59° 41 864
Mf 131	Pacovan	Caroebe	S 00° 47 875 W 59° 25 837
		Pará	
Mf 139	Caipira	Marituba	-
		Rondônia	
Mf 175	Caru roxa	Porto Velho	S 08° 40 367 W 63° 49 810
		Acre	
Mf 196	ST 1231	Rio Branco	S 10° 01 591 W 67° 42 394
		Mato Grosso	
Mf 82	IAC 2001	Cáceres	S 16° 09 147 W 57° 37 914
		São Paulo	
Mf 201	Nanicão	Pedro de Toledo	S 24° 16 819 W 47° 13 139
Mf 205	Prata anã	Miracatu	S 24° 18 239 W 47° 29 349
Mf 217	Prata anã	Jacupiranga	S 24° 43 328 W 48O04 210
Mf 218	.	Jacupiranga	S 24° 42 185 W 48O 00 607
Mf 219	Maçã	Pariquera-Açu	S 24° 37 070 W 47O53 106
Mf 220	Nanicão	Pariquera-Açu	S 24° 36 498 W 47O 53 568

The ISSR amplification reactions were performed in a final volume of 20 µL containing 50 ng DNA, 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, and 1% Triton X-100), 1.5 mM $MgCl_2$, 0.15 mM of each dNTP, 0.75 µM UBC 861 - $(ACC)_6$ primer and 1 U Taq DNA polymerase (Phoneutria). The following amplification conditions were used: 94°C for 3 min for the initial denaturation followed by 40 cycles of 94°C for 30 s, 60.6°C for 1 min and 72°C for 2 min, and a final 7-min extension at 72°C.

The IRAP amplification reactions were performed in a final volume of 20 µL containing 50 ng DNA, 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, and 1% Triton X-100), 2.0 mM $MgCl_2$, 0.05 mM of each dNTP, 0.5 µM of each primer and 1 U Taq DNA polymerase (Phoneutria). The primers were designed using the *M. fijiensis* retrotransposon: LTRMf-F - 5'-GCGCTTAGCGTTAGGCTAACT-3' and LTRMf-R - 5'-CGTGTAGCCTCTTTGGCCCTA-3'. The amplification conditions were as follows: 95°C for 15 s for the initial denaturation, 95°C for 15 s, 60°C for 1 min, 68°C for 2 min, and a final 5-min extension at 68°C.

Data analysis

The band profiles generated from the reactions were visualized using a 1.5% agarose gel. A binary matrix was then generated based on the absence (0) or presence (1) of bands and analyzed using the NTSYS 2.1 program.

The unweighted pair group method with arithmetic mean (UPGMA) and the sequential, agglomerative, hierarchical, and non-overlapping (SAHN) methods were used to construct a dendrogram based on Dice similarity coefficients.

RESULTS

M. fijiensis mating-type

The 129 isolates analyzed contained or lacked a fragment with an expected size of approximately 700 bp for both *MAT1-1-1* and *MAT1-2-1*. A complete population consisting of isolates from six states exhibited an equal 1:1 distribution of mating-types, indicating that sexual recombination occurs in the Brazilian *M. fijiensis* population. A ratio close to 1 was also observed for the groups of isolates from the States of Amazonas, Roraima and São Paulo (Table 2).

Table 2. Chi-squared (χ^2) test results and the ratio, total number of isolates (N) and number of isolates of each mating-type for each state.

State	N	*MAT1.1*	*MAT1.2*	Ratio	χ^2
Acre	5	0	5	0	5
Amazonas	78	40	38	1.05	0.05
Mato Grosso	11	4	7	0.57	0.8
Rondônia	3	1	2	0.5	0.33
Roraima	15	8	7	1.14	0.06
São Paulo	17	10	7	1.4	0.53

M13 marker analysis

Of the three primer pairs that were evaluated, only the M13 minisatellite pair exhibited polymorphisms, which allowed it to be used for the genetic discrimination of the isolates analyzed.

As shown in the dendrogram, genetic similarity ranged from 0.57 to 1.00 with the

majority of the isolates sharing the maximum extent of similarity independent of geographic origin (Figure 1). The isolates were divided into two major groups that exhibited a similarity of approximately 0.70 and were interconnected by a single isolate from the State of Roraima. The first group could be divided into three subgroups: the first subgroup contained 85% of the isolates from various geographic areas, while the two other subgroups primarily contained isolates from the State of Amazonas. The second group comprised two Amazonian isolates and three isolates from São Paulo.

The high levels of genetic relationship between the isolates analyzed may partially be due to the small sample size and the historically recent expansion of the pathogen in the country. However, we should emphasize that there were distinct genetic differences between the major groups identified in the dendrogram, especially for the three isolates from São Paulo (*Mf205*, *Mf217* and *Mf218*) and the three isolates that originated near or at the location where the pathogen first entered the country, i.e., the municipality of Tabatinga in the State of Amazonas (*Mf134*, *Mf136* and *Mf138*). Another factor that should be considered is the potential detection of genetic diversity resulting from sexual reproduction and the wide variety of host genotypes that are cultivated in Brazil.

The fingerprinting results obtained using the M13 marker for five isolates were compared to the band profiles obtained using ISSR (UBC861) or IRAP. The M13 fingerprinting method showed the greatest discriminatory power despite the greater number of bands generated by the ISSR and IRAP markers (Figure 2).

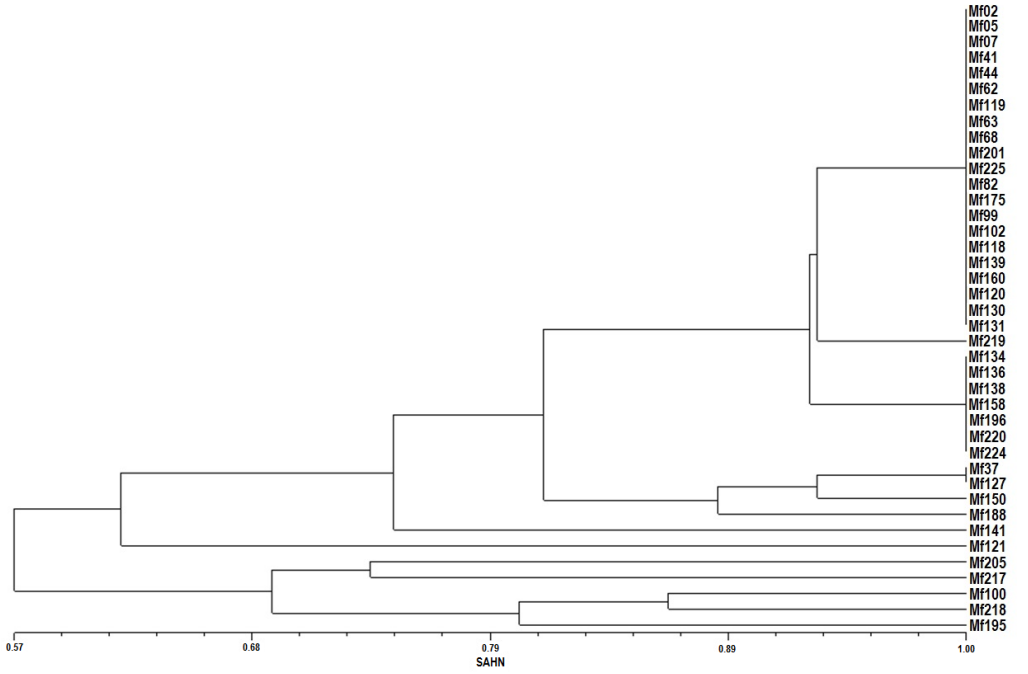

Figure 1. Dendrogram generated using the unweighted pair group method with arithmetic mean based on Dice similarity coefficient. SAHN = sequential, agglomerative, hierarchical, and non-overlapping methods.

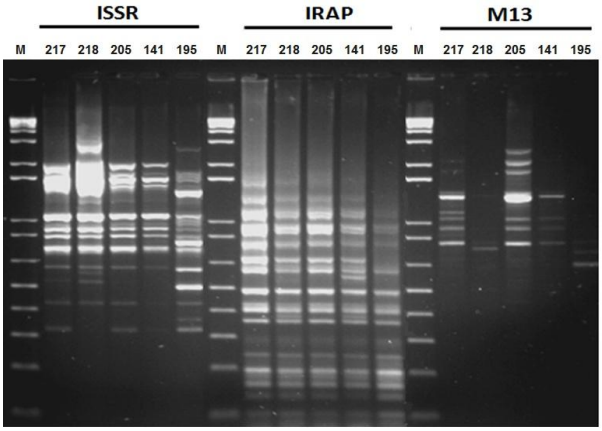

Figure 2. Band profiles derived from inter-simple sequence repeat (ISSR) (UBC861 primer), inter-retrotransposon amplified polymorphism (IRAP) and M13 fingerprinting analyses of five *Mycosphaerella fijiensis* isolates are shown on a 1.5% agarose gel. *Lane M* = DNA molecular marker.

DISCUSSION

Mating-type

One would expect sexual reproduction to be an adaptation strategy employed by heterothallic fungal species because the resulting ascospores are highly relevant to pathogenic infections and the pathogen life cycle. The equal distribution of the mating-type alleles observed in this study suggests that sexual reproduction occurs randomly in the Brazilian *M. fijiensis* population.

The distribution of the mating-type alleles does not specifically imply the existence of a sexual stage, although the presence of two idiomorphs at equal frequencies is indicative of sexual recombination in a given population (Linde et al., 2003). Sexual reproduction promotes gene flow between individuals, and rearrangements resulting from crossovers generate new allelic combinations, thereby increasing the amount of genetic variability within the pathogen. In Mexican *M. fijiensis* populations, the equal distribution of mating-type alleles confirms the Mendelian inheritance of these genes (Conde-Ferráez et al., 2010).

In certain fungi, the mechanisms associated with sexual compatibility and development suggest that the mating-type genes are linked to pathogen virulence (Hsueh and Heitman, 2008; Lee et al., 2010). The populations that maintain sexual reproduction are able to generate genetic combinations capable of overcoming the host plant's resistance (Lee et al., 2010). In wheat, significant differences in pathogenicity have been found between the *MAT1-1* and *MAT1-2* groups of *M. graminicola* (Zhan et al., 2007).

M13 markers

A comparison between the results using M13, ISSR and IRAP markers showed that the M13 marker could potentially be applied for *M. fijiensis* fingerprinting. The M13 minisatellite marker has also been successfully used in *Cryptococcus gattii* (Ulrich et al.,

2009), *C. neoformans* (Liaw et al., 2010) and *Lactobacillus* spp (Mercanti et al., 2011).

The genetic variability of the Brazilian isolates was low and did not show a geographic pattern, which partially corroborates the results from analyses involving populations representative of the pathogen's worldwide distribution. High levels of variation have been observed in the populations from Southeast Asia, which is thought to be the origin of *M. fijiensis* (Carlier et al., 1994). In the continental regions where the pathogen populations are thought to be derived from founding events (South America, Africa and the Pacific Islands), diversity has been shown to be lower (Hayden et al., 2003; Rivas et al., 2004).

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