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RESEARCH ARTICLE

Genetic variability of *Bipolaris sorokiniana* isolates using URP-PCR

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ABSTRACT

Spot blotch disease, caused by *Bipolaris sorokiniana*, is one of the major diseases of wheat and is responsible for large losses of wheat crops worldwide. We used polymerase chain reaction (PCR) with universal rice primers (URP) for molecular characterization of 60 monosporic *B. sorokiniana* isolates from Brazil and other countries, and evaluated the diversity of the samples. PCR amplification generated 232 different DNA fragments ranging in size from 100 to 2018 bp. The primers URP-4R, URP-2R, and URP-1F generated greater numbers of amplified fragments (36, 30, and 25, respectively) from the single-spore isolates, and some diversity was observed among the isolates generated using these primers. The primers URP-2F, URP-6R, URP-17R, URP-30F, and URP-38F produced a pattern of monomorphic fragments and 73% of the isolates showed an average of 44 different DNA-amplified fragments. Primer URP-2F generated a 578-bp fragment that was common to 83.7% of the isolates; primer URP-6R generated a 548-bp fragment and primer URP-38F generated a 650-bp product that was common to 89.1% and 80% of the isolates, respectively. The URP-PCR primers provided important information about the genetic profiles of the monosporic cultures, which showed intraspecific variability among the monoconidial isolates and among the monosporic cultures that originated from the same polysporic strain. Our results indicate that URP's are sensitive and give reproducible results for assaying the genetic variability of *B. sorokiniana*.
Key words: *Triticum aestivun*, spot blotch disease, wheat.

INTRODUCTION

Wheat (*Triticum aestivun* L.) is one of the most important cereal grasses, used extensively as a raw material for food and beverages and on a smaller scale for animal fodder (Cunha & Bacaltchuk, 2000). Although Brazil is a large producer of grains, it is not self-sufficient in wheat production and depends on imports to fully supply the domestic demand (USDA, 2013). In addition, several environmental factors affect wheat production in Brazil, including warm, humid winters that promote the development of fungal diseases that can result in up to 50% loss of winter crops.

Brazil and other warm humid countries are affected by spot blotch, which is caused by the fungus *Bipolaris sorokiniana* (Sacc. in Sorok) Shoemaker, 1959, (teleomorph): *Cochliobolus sativus* (Ito & Kuribayashi) Drechsl. Ex Dastur. It is the causal agent of common root rot, leaf spot, seedling blight, head blight of wheat and barley and black point of grains and affects all parts of the plant. Sources of primary inoculum include infected seeds, remains of infected crops, volunteer plants, secondary hosts, and non-germinated conidia (Reis et al., 2001). The use of pathogen-free seeds is one strategy for reducing sources of inoculum and controlling the spread of disease.

Considering the damage caused by this fungus and the demonstrated variability in its genome, molecular characterization of *B. sorokiniana* is required to better understand its genetic variability. Molecular techniques including random amplified polymorphic DNA (RAPD) analysis (Oliveira et al., 2002; Müller et al., 2005; Jaiswal et al., 2007) and cleaved amplified polymorphic sequence (CAPS) analysis of the ITS regions have been used to characterize this pathogen (Nascimento & Van Der Sand, 2008). Data reported in the literature show a high degree of intra- and inter-specific groupings (Müller et al., 2005). Studies of *B. sorokiniana* polymorphisms among isolates from different geographic origins have revealed a high degree of variability.

Polymerase chain reaction with universal rice primers (URP-PCR) has been used to study inter- and intraspecific genetic variability among microorganisms and has been shown to produce quick and effective results (Kang et al., 2002). Kang et al. (2003) used 20 URPs to study 25 isolates of six different *Alternaria* species that produced host-specific toxins; their results showed that eight primers could be used to reveal polymorphisms of

Alternaria isolates at the intra- and inter-species levels. Cho et al. (2012), studying *Pseudomonas* pv. strains, found that the pathovar could be divided into four clusters based on 63% similarity by URP-PCR using 2F, 9F, and 17R primers. The *P. syringae* pv. *tomato* cluster was also well separated from 30 other *P. syringae* pathovars.

Aggarwal et al. (2008) successfully characterized isolates of *Chaetomiun* spp. using URP-PCR. In contrast, in a study of *B. sorokiniana* isolates from India, Aggarwal et al. (2010) observed a polymorphic profile among the isolates and only two URP-PCR primers were able to amplify monomorphic bands in this phytopathogen. The aim of the present study was to assess the genetic diversity of monoconidial isolates of *B. sorokiniana* using URP-PCR assays.

MATERIALS AND METHODS

Fungal cultures

Twenty *B. sorokiniana* isolates used in this study were isolated from wheat seeds supplied by Embrapa Trigo (Brazil) and International Maize and Wheat Improvement Center (CIMMYT) (Mexico) (Table 1).

Monoconidial cultures

The monoconidial cultures were obtained from the aerial mycelia of the 20 polyconidial cultures grown on plates with potato dextrose agar (PDA). A 0.85% saline solution was poured over the plated colonies, and the conidia were transferred to microcentrifuge tubes. The tubes were vortexed thoroughly to achieve complete conidia release. The suspension was transferred to a Petri dish with PDA and incubated at room temperature for 2 h. Using a stereomicroscope with optical magnification of ×40, the conidia were transferred using a needle to slant tubes with PDA media. The tubes were maintained at 24 ± 2ºC until the complete colonies had developed, and then were stored at 4ºC. Each spore culture was designated with a letter (A, B, and C); 60 monoconidial cultures comprised the isolates used in this work.

Morphological characteristics of the colonies

Morphological groupings were made based on colonial characteristics and mycelial growth (Jaiswal et al., 2007). For this purpose, a 0.5 cm disc containing mycelium of each isolate was inoculated in the center of a Petri dish with PDA medium and incubated at 25ºC for 5 d with a 12 h photoperiod. The morphological analysis consisted of observation of mycelial color and texture, formation or lack of formation of sectors, and type of mycelial growth.

Genomic DNA extraction

Genomic DNA was extracted following the protocol developed by Ashktorab & Cohen (1992) with some modifications. The isolates were grown in Erlenmeyer flasks containing 300 mL potato-dextrose broth (PD). After inoculation, incubation took place at room temperature under orbital agitation (Oxylab) of 120 rpm for 7-10 days in order to obtain 300 mg of mycelia (wet weight). After incubation, the mycelium of each isolate was strained and washed three times with sterile distilled water, and excess water was removed using filter paper. Weighed mycelium was ground to fine powder using liquid nitrogen. The powder was transferred to a tube, and 1 mL of extraction-lysis buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, 2% SDS) per gram of mycelium was added along with 10 µL/mL β-mercaptoethanol (Sigma) and 50 µg/mL proteinase K (Promega). The tubes were incubated

TABLE 1 - Origin of *Bipolaris sorokiniana* isolated from wheat seeds.

Number	Isolate code	City, State, Country
1	98030	Cruz Alta, Rio Grande do Sul, Brazil
2	98011	Lagoa Vermelha, Rio Grande do Sul, Brazil
3	98012	Lagoa Vermelha, Rio Grande do Sul, Brazil
4	98043	Pelotas, Rio Grande do Sul, Brazil
5	98026	Piratini, Rio Grande do Sul, Brazil
6	98032	Engenheiro Beltrão, Paraná, Brazil
7	98017	Samambaia, Paraná, Brazil
8	98013	União da Vitória, Paraná, Brazil
9	98023	União da Vitória, Paraná, Brazil
10	98034	União da Vitória, Paraná, Brazil
11	98041	União da Vitória, Paraná, Brazil
12	BS15M2	Delicias, Chihuahua, Mexico
13	BS16M1	Delicias, Chihuahua, Mexico
14	BS18M2	Poza Rica, Veracruz, Mexico
15	CM0105	Poza Rica, Mexico
16	BS52M1	Monterrey, Nuevo León, Mexico
17	CS1004	Hanoi, Vietnam
18	NRRL 5851	South Africa
19	CF0201	South Africa
20	A 20	Saskatoon, Saskatchewan, Canada

in a water bath at 65ºC for 1 h, shaken every 15 min, and then centrifuged at 2795 ×*g* for 20 min. The supernatant was transferred to clean centrifuge tubes and DNA was purified twice with 1 volume of phenol (pH 8.0) and three times with 1 volume of phenol-chloroform (1:1 v/v). Finally, 1 volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the supernatant. Pancreatic RNAse I (50 µg/mL) was added to the aqueous phase resulting from the centrifugation and the mixture was incubated at 37ºC for 20 min. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of isopropanol at -20ºC. DNA was collected with a glass rod, transferred to a falcon tube, washed with 70% ethanol (v/v), and centrifuged at 2795 × *g* for 10 min. DNA was allowed to dry at room temperature and was then resuspended in Milli-Q water and stored at -20ºC.

DNA amplification by URP-PCR

DNA from the 60 single spore isolates was amplified using 12 URP primers (Table 2), as described by Kang et al. (2002) with modifications. The reactions were performed in a 25 µL volume containing 50 ng of genomic DNA, 1 U *Taq* DNA polymerase, 20 pmoL primer, 0.2 mM dNTP, 1× reaction buffer, 4 mM $MgCl_2$, 200 ng/µL bovine serum albumin, and sterile Milli-Q water. The amplification conditions were adapted accordingly to the samples used in this work to obtain the optimal products. All amplifications were performed in an Eppendorf Mastercycler Personal thermal cycler under the following conditions: one cycle of denaturation at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and at 72ºC for 2 min, and a final extension at 72ºC for 7 min. Amplification products were separated by electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide, and photographed under UV light using a Vilber Lourmat Doc-Print II. Digital photos of the gels were taken using a Kodak DC 120 digital camera and Kodak 1D (version 3.5.2) software. All amplifications were repeated at least twice for each isolate in separate experiments.

Statistical analysis

The amplifications of all the isolates obtained with the 12 URP primers were used to construct a binary matrix by scoring the presence and absence of fragments as 1 as 0, respectively. The matrix was analyzed using PAST using Jaccard´s coefficient of similarity. The dendrogram was produced using the unweighted pair-group method with arithmetic averages (UPMGA). The data sets were considered clustered if they had a similarity coefficient above 70%. All amplifications were repeated at least twice for each isolate in separate experiments.

RESULTS

The single-spore isolates grown in PDA medium showed phenotypic variation in mycelia color, from gray to black and beige. This variability was observed among 75% of the isolates. Among single-spore isolates that originated from the same polysporic culture this variability was also observed.

Each of the 12 tested primers was efficient in producing different levels of polymorphism. The 232 polymorphic fragments produced by the primers varied in size from 100 to 2018 bp. The 12 primers amplified 84% of the isolates. The primer URP-30F amplified 95% of the isolates and primer URP-13R showed amplification products in 70% of the isolates. Primers URP-1F, URP-2R, and URP-4R produced a greater number of polymorphic fragments than primers URP-2F, URP-6R, URP-9F, URP-13R, URP-17R, URP-25F, URP-30F, URP-32F, and URP-38F (Table 3).

Primer URP-2F was more specific to amplify isolates from Brazil while primer URP-6R was able to distinguish isolates from international collections, producing distinct amplification profiles among isolates. However, none of the other primers was able to discriminate the geographic origins of the isolates. Polymorphic fragments from single-spore isolates that originated from the same polysporic strain were observed with all the primers. This polymorphism is shown in Figure 1 for some of the isolates amplified with primer URP-1F. Primers URP-2F, URP-6R, and URP-38F showed some monomorphic fragments among the isolates they amplified. Primer URP-2F generated a 578 bp fragment in 83.7% of the isolates, primer URP-6R produced a fragment of 548 bp in 89.1% of the isolates, and primer URP-38F showed a 650 bp amplification product in 80% of the isolates. Figure 2 shows the profile of the isolates amplified using primer URP-38F. The dendrogram constructed from these data revealed 23 groups, assuming a similarity coefficient of >70%. Using primer URP-38F it was possible to group some of the single-spore isolates that originated from the same polysporic strain from Brazil and also with some from international strains. Conidia A, B, and C of isolate 98043 from Pelotas (Brazil), and conidia A and B of isolate 98030 from Cruz Alta (Brazil) clustered together with a similarity coefficient of 1.000. Conidia B and C of isolate 98011 clustered together with a similarity coefficient of 1.000. In contrast, isolates 98013A, 98013C, 98034B, 98034C, 98023A, 98023B, 98023C, 98041A, 98041B, 98041C and 98032B and C from Paraná (Brazil) showed a very low similarity coefficient. Isolate 98017C did not group with any other isolate. Isolates 98032A, 98034A, 98012B, 98011A, BS52M1A, and BS52M1B did not amplify with primer URP-38F.

The data obtained with isolates from international collections using primer URP-38F showed that isolates BS18M2, BS16M1, A20, and their conidia A, B, and C clustered together with a similarity coefficient of 1.000. On the other hand, conidia A, B, and C of isolates NRRL5851 and CF0201 from South Africa did not cluster together, and showed a very low similarity coefficient (Figure 2). This primer can be used to study variability among

TABLE 2 - Sequences of primers used to amplify *Bipolaris sorokiniana* DNA, and number of generated fragments.

Primer	Sequence (5′–3′)	No. of polymorphic fragments	No. of isolates with a common fragment
URP 1F	ATCCAAGGTCCGAGACAACC	25	33
URP 2F	GTGTGCGATCAGTTGCTGGG	19	41
URP 2R	CCCAGCAACTGATCGCACAC	30	29
URP 4R	AGGACTCGATAACAGGCTCC	36	29
URP 6R	GCAAGCTGGTGGGAGGTAC	17	49
URP 9F	ATGTGTGCGATCAGTTGCTG	11	39
URP 13R	TACATCGCAAGTGACACAGG	11	33
URP 17R	AATGTGGGCAAGCTGGTGGT	14	41
URP 25F	GATGTGTTCTTGGAGCCTGT	12	27
URP 30F	GGACAAGAAGAGGATGTGGA	15	45
URP 32F	TACACGTCTCGATCTACAGG	19	26
URP 38F	AAGAGGCATTCTACCACCAC	15	43

TABLE 3 - Amplification profile of monomorphic *Bipolaris sorokiniana* isolates obtained by URP-PCR assay.

	PRIMER											
	URP 1F	URP 2F	URP 2R	URP 4R	URP 6R	URP 9F	URP 13R	URP 17R	URP 25F	URP 30F	URP 32F	URP 38F
No. of amplified fragments	25	19	30	36	19	11	15	15	12	15	19	16
No. of isolates amplified with each primer	88.34%	81.70%	80%	88.34%	91.70%	83.34%	70%	91.67%	71.67%	95%	71.67%	90%
No. of isolates that did not amplify	11.66%	18.30%	20%	11.66%	8.30%	16.66%	30%	8.33%	28.33%	5%	28.33%	10%
No. of isolates with a common fragment	33	41	29	29	49	39	33	41	27	45	26	43

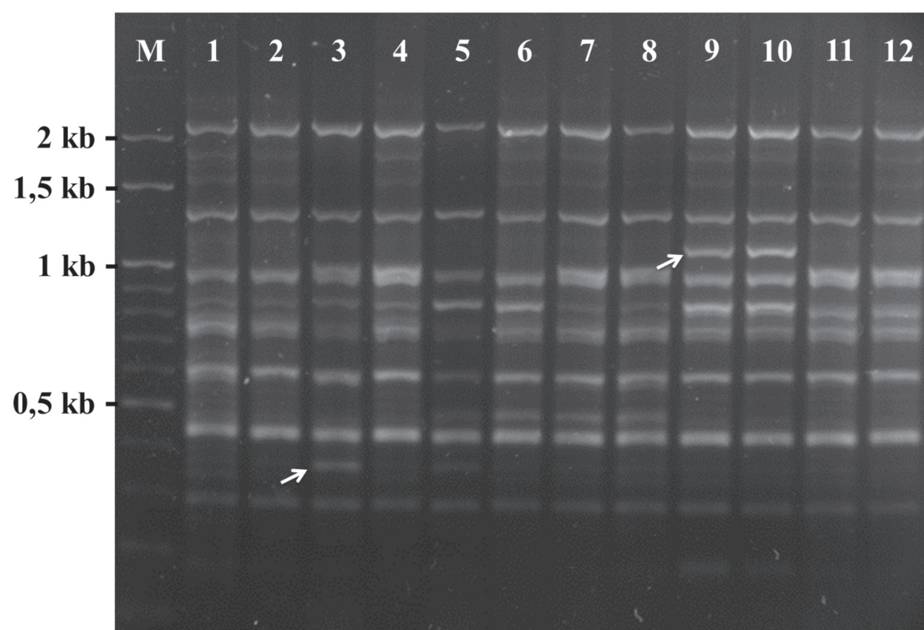

FIGURE 1 - Genomic DNA amplification product of *Bipolaris sorokiniana* monosporic isolates obtained using primer URP-1F. M, DNA Marker Ladder 100 bp; 1, 98013A; 2, 98013B; 3, 98026B; 4, 98026C; 5, 98032B; 6, 98032C; 7, 98034B; 8, 98034C; 9, 98041A; 10, 98041C; 11, BS16M1A; 12, BS16M1C. → indicates polymorphism among the isolates.

the isolates however it is not a good primer to study the geographic origin of the isolates. Primer URP-4R generated the highest number of polymorphic fragments among the amplified isolates. The dendrogram (Figure 3) show the polymorphisms that resulted from this amplification, where few isolates showed a similarity coefficient of 1.000. The dendrogram constructed with the amplification data obtained with primer URP-4R showed 34 groups with a similarity coefficient >70%. The 98043A-B isolates from Brazil confirmed the previous grouping of URP-38F isolates and BS15M2A isolates (Delicias, Chihuahua, Mexico) with URP-4R isolates in the dendrogram. Isolates 98011A, 98012B, 98012C, 98030A, 98032B, and 98034A-C were not amplified with primer URP-4R.

DISCUSSION

The molecular characterization of single-spore isolates of *B. sorokiniana* revealed wide diversity in their genetic profiles. This work demonstrates the applicability of URP-PCR primers to the study of *B. sorokiniana* isolates; URP-PCR can be used for characterization and evaluation of intraspecific polymorphisms of monosporic fungal cultures.

Poloni et al. (2008) evaluated morphological variability of monosporic and polysporic *B. sorokiniana* cultures grown in different media, and observed high rates of morphological variability in the replicates of polysporic cultures but few differences among monoconidial cultures. In the present study we observed high genetic diversity and some morphological variability among isolates: in most groups containing the three monosporic isolates, at least one of the isolates showed high variability and consequently a low similarity coefficient.

Christensen (1925) working with *B. sorokiniana* isolates also observed very important morphological, physiological and biochemical variability among the isolates when grown in dextrose potato agar. Tinline (1961) proposed that mutation, heterokaryose and hybridization would increase the possibility of variability among the isolates.

Jaiswal et al. (2007) studied monoconidial cultures of *B. sorokiniana* isolated from wheat-growing regions in India and showed high morphological variability between the isolates, which formed distinct morphological groups; these researchers also observed more polymorphic than monomorphic fragments in the RAPD assay. Similarly, in the present study, high variability was observed in the amplification profiles and a greater number of polymorphic than monomorphic fragments were obtained. Although the amplification products of some of the monosporic isolate groups showed the same profiles, the presence of variable patterns was more common. One explanation for this variability might be the multinucleated conditions of *B. sorokiniana* mycelial cells and conidia, in which heterokaryosis can lead to mitotic recombination that may contribute to the DNA polymorphisms detected in this pathogen (Müller et al. 2005). When we analyzed some of the resulting groupings among isolates obtained with primers URP-38F and URP-4R, some clustering among isolates from Brazil and Mexico was apparent. So, we can suggest that these two primers can be used to access genetic diversity. This information is applicable to epidemiologic studies examining the mechanism of introduction or

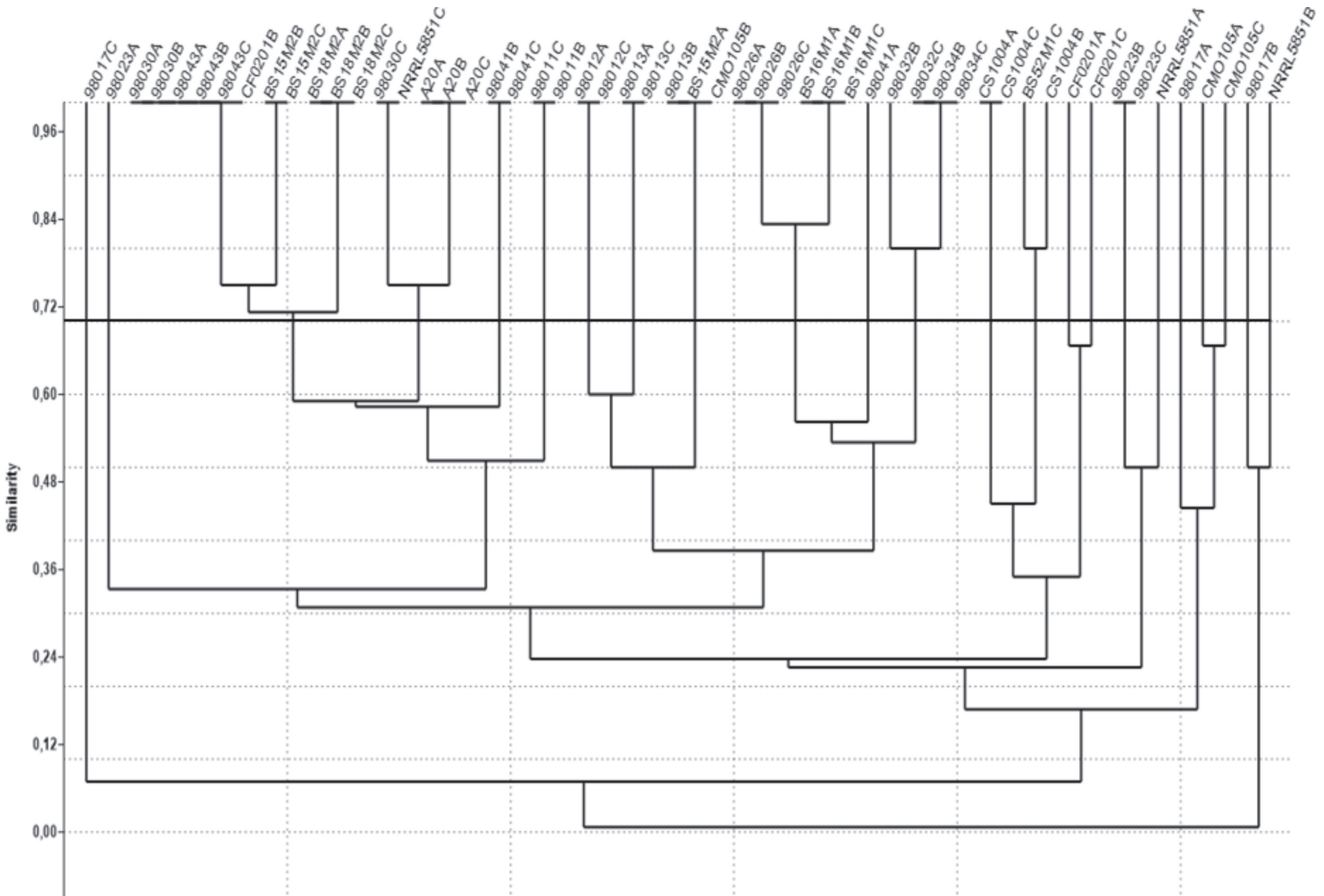

FIGURE 2 - Dendrogram of *Bipolaris sorokiniana* strains amplified with primer URP-38F with a cut off above 70% of similarity. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. From these data, a similarity matrix was constructed with PAST (Paleontological Data Analysis) using Jaccard's coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

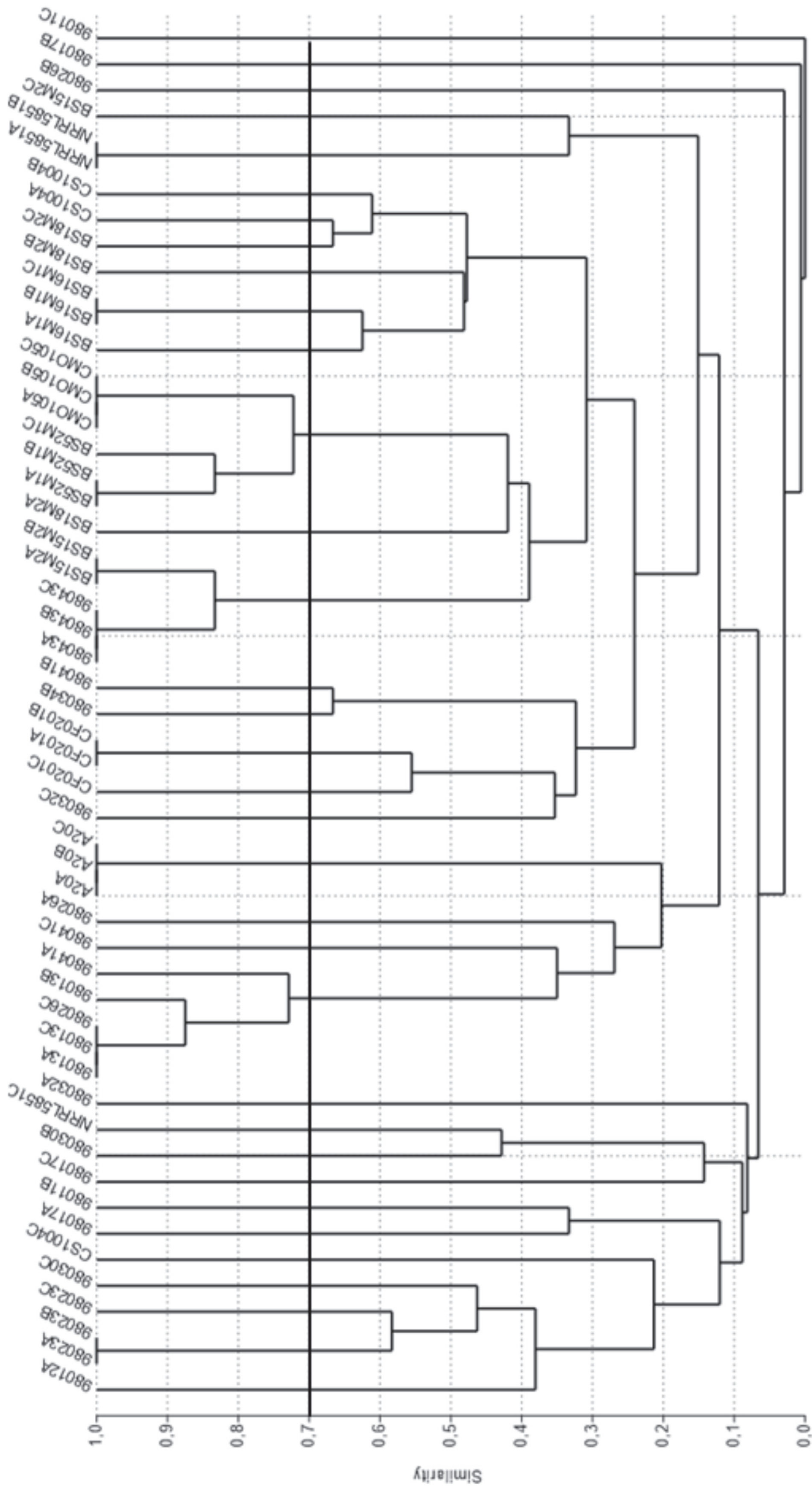

FIGURE 3 - Dendrogram of *Bipolaris sorokiniana* strains amplified with primer URP-4R with a cut off above 70% of similarity. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. From these data, a similarity matrix was constructed with PAST (Paleontological Data Analysis) using Jaccard's coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

gene flow of *B. sorokiniana* among distinct geographical regions.

The same 12 URP-PCR primers as used in the present study were used to characterize of *B. sorokiniana* from India; only two of these primers failed to produce amplification products and one primer produced monomorphic fragments that could be used as molecular markers (Aggarwal et al., 2010). Here, we observed that isolates from Brazil were more efficiently amplified with URP primers and different behavior was observed in isolates from other countries. It was not possible to group the isolates according to geographical region. Using RAPD, Oliveira et al. (2002) and Müller et al. (2005) observed high levels of genetic variability among isolates from Brazil, suggesting high rates of polymorphism. Nascimento & Van Der Sand (2008) analyzed polymorphism of ITS regions of *B. sorokiniana* rDNA using PCR-RFLP, and observed great diversity among isolates from different regions and countries. In that study, the authors were able to separate isolates from Brazil from those of the other countries, but isolates could not be geographically separated at within-country scales (Nascimento & Van Der Sand, 2008). Zhong & Steffenson (2001) assessed the genetic diversity of *Cochhliobolus sativus* based on virulence and AFLP markers and found no correlation between genetic similarity and geographic origin.

In contrast, Aggarwal et al. (2010) correlated URP-PCR data with the geographic origin of the isolates. In our studies, URP primers were also shown to be conserved in fungi, enabling detection of intraspecific variation among sets of single-spore isolates derived from the same polysporic strain. We observed that Brazilian isolates showed higher genetic diversity than isolates from other countries, which had little genetic similarity to those from Brazil. A similar finding was reported by Nascimento & Van Der Sand (2008), who examined genotypic variability of *B. sorokiniana* by PCR-RFLP analysis using rDNA from polysporic strains. Kang et al. (2003) demonstrated that eight URP primers could be used to detect inter- and intraspecific polymorphisms among 25 isolates of six *Alternaria* species, and obtained results that allowed the isolates to be grouped according to their geographical regions.

In the present study, the URP primers 13R, 25F, and 32F amplified smaller number of genomic DNA fragments in monosporic isolates. According to Aggarwal et al. (2008), these same primers failed to amplify the genomic DNA of *Chaetomium* spp., suggesting low genetic homology with this pathogen. The genetic variability could be attributed to interactions between genes and environmental conditions such as climatic differences among the geographic areas from where isolates are collected. In this study the diversity observed among single spore *B. sorokiniana* isolates using the URP primers might be a result of many factors such as population dynamics, gene flow, mutations, and the multinuclear nature of conidia, which can lead to high polymorphism. Gene flow is especially important in relation to plant pathogens in agroecosystems because it is the process that introduces new genes into agricultural fields distant from the site of the original mutation what could be one of the reasons to explain why some isolates from Brazil cluster with isolates from Mexico. Further work must be done on the genetic characterization of *B. sorokiniana* isolates and then the identification of the pathogen will be more fast and accurate.

The results obtained with URP-PCR in the present study enabled a fast and efficient variability analysis for *B. sorokiniana*. The monosporic isolates examined in this study showed very high genomic diversity. The profiles generated from the URP-PCR results difference among the monosporic isolates from each other and also among the isolates derived from the same polysporic strains. The dendrogram analysis showed higher diversity for isolates from Brazil, compared to isolates from international collections.

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