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Genetic diversity among *Puccinia melanocephala* isolates from Brazil assessed using simple sequence repeat markers

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ABSTRACT. Brown rust (causal agent *Puccinia melanocephala*) is an important sugarcane disease that is responsible for large losses in yield worldwide. Despite its importance, little is known regarding the genetic diversity of this pathogen in the main Brazilian sugarcane cultivation areas. In this study, we characterized the genetic diversity of 34 *P. melanocephala* isolates from 4 Brazilian states using loci identified from an enriched simple sequence repeat (SSR) library. The aggressiveness of 3 isolates from major sugarcane cultivation areas was evaluated by inoculating an intermediately resistant and a susceptible cultivar. From the enriched library, 16 SSR-specific primers were developed, which produced scorable alleles. Of these, 4 loci were polymorphic and 12 were monomorphic for all isolates evaluated. The molecular

characterization of the 34 isolates of *P. melanocephala* conducted using 16 SSR loci revealed the existence of low genetic variability among the isolates. The average estimated genetic distance was 0.12. Phenetic analysis based on Nei's genetic distance clustered the isolates into 2 major groups. Groups I and II included 18 and 14 isolates, respectively, and both groups contained isolates from all 4 geographic regions studied. Two isolates did not cluster with these groups. It was not possible to obtain clusters according to location or state of origin. Analysis of disease severity data revealed that the isolates did not show significant differences in aggressiveness between regions.

Key words: Brown rust; Genetic diversity; Microsatellite; Sugarcane; *Puccinia melanocephala*; Simple sequence repeat

INTRODUCTION

Sugarcane brown rust, caused by the basidiomycete *Puccinia melanocephala* H. & P. Sydow, is an important sugarcane (*Saccharum* spp) disease that is responsible for large yield losses worldwide. This pathogen now occurs in all regions where sugarcane is cultivated worldwide, but it was only recently introduced to the Americas (Whittle and Holder, 1980). Its ability to produce large numbers of infectious spores (uredinospores) asexually, which are adapted for aerial transport, is responsible for determining severe losses in susceptible cultivars. Sexual recombination is recognized in other *Puccinia* species, although the sexual stage has not been described for *P. melanocephala*. In July 1978, *P. melanocephala* was first identified in the Dominican Republic, marking the beginning of the brown rust epidemic in the Americas (Purdy et al., 1983). *P. melanocephala* is thought to have reached the Americas via wind currents from Cameroon, West Africa, which is the nearest source of inoculum. Until then, this pathogen was present in scattered localized areas in Africa and Asia and did not cause significant damage. Only after introduction into the Americas, the disease became economically important (Purdy et al., 1983). In the same year, brown rust was also detected in Queensland, Australia, and rapidly spread to all areas that cultivated sugarcane, severely affecting the main cultivated varieties (Taylor, 1992). The introduction of *P. melanocephala* in Australia can be attributed to the monsoon winds that are able to carry the spores from a close source across the Indian Ocean (Whittle and Holder, 1980). In Brazil, brown rust was first detected in 1986 near Capivari in São Paulo State in the largest producing region (Amorim et al., 1987). In a short period of time, the disease was detected in Pernambuco and Alagoas states, more than 2000 km away from São Paulo.

The existence of *P. melanocephala* races has not been reported in Brazil, but races have been identified in other regions. For instance, 4 races of *P. melanocephala* were identified in Lousiana, USA (Shine et al., 2005), but no race has been detected in Australia (Taylor, 1992).

In countries, where sugarcane is widely cultivated, and, therefore, subject to large *P. melanocephala* populations and edapho-climatic conditions, it is imperative to understand the range of the genetic diversity of this pathogen. This information may provide significant information related to the pathosystem. Thus, understanding the genetic diversity of the pathogen in the main cultivation areas, together with processes that affect diversity, are required for de-

veloping breeding strategies. Because of the inherent biological characteristics of rust spores and disease epidemics, many rust pathogens are recognized for their long-distance dispersal, following either a single aerial transport event or stepwise range expansion from a source into new regions (Isard et al., 2011).

DNA markers have been widely adopted for analyzing the dynamics of plant pathogen populations because of their high levels of precision and accuracy (Milgroom and Peever, 2003). Simple sequence repeats (SSR) or microsatellite sequences are relatively abundant and evenly distributed in eukaryote genomes (Weising et al., 1995). Notably, fungal genomes appear to contain fewer SSR sequences than other eukaryotes (Dutech et al., 2007); however, when polymorphic loci are available, they can be very useful for genome mapping and genetic diversity and population genetic studies, which warrant the search and development of such genetic markers. The objectives of this study were to develop SSR loci from a *P. melanocephala* enriched genomic library and explore their potential for evaluating the genetic diversity of *P. melanocephala* isolates from the main sugarcane production regions in Brazil. Additionally, the aggressiveness of isolates from 3 important sugarcane cultivation regions was evaluated by artificial inoculation.

MATERIAL AND METHODS

Sampling and identification of rust isolates

Sugarcane leaf segments exhibiting typical symptoms of brown rust were collected from 4 Brazilian States (São Paulo, Minas Gerais, Goiás, and Alagoas), which represent the main sugarcane cultivation regions in Brazil. A total of 34 isolates were obtained from 4 Brazilian states (Table 1). All leaf samples were examined under a microscope to evaluate uredinium and urediniospore structures that characterize *P. melanocephala* (Virtudazo et al., 2001a; Dixon et al., 2010).

Construction of SSR locus-enriched library

Genomic DNA was extracted from urediniospores from *P. melanocephala* isolated from the cultivar 'RB835486' collected at "Centro de Cana" at Ribeirão Preto, SP (Aljanabi et al., 1999). Fresh urediniospores were collected from pustules (uredinia) present on the abaxial leaf side using a vacuum pump. The SSR locus-enriched library was developed using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles (Creste et al., 2006). Briefly, 5 µg genomic DNA was digested with *Rsa*I, and a sample of 600 ng was ligated to adaptors (*Rsa*21: 5'-CTCTTGCTTACGCGTGGACTA-3'; *Rsa*25: 5'-TAGTCCACG CGTAAGCAAGAGCACA-3'). The ligated fragments were then amplified by polymerase chain reaction (PCR) and products were allowed to hybridize to biotinylated $I_5(CT)_8$ and $I_5(GT)_8$ probes, which were later recovered by Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison, WI, USA). The eluted fragments were re-amplified using the *Rsa*21 primer and cloned into the pGEM-T Easy vector (Promega). Plasmids were transformed into *Escherichia coli* XL1-Blue cells. Positive clones were sequenced using the M13 primer and BigDye terminator Cycle Sequencing Kit (Applied Biosystems; Foster City, CA, USA) in a 3100 DNA Analyzer. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0), and primer quality was evaluated using Netprimer (http://www.premierbiosoft.com/netprimer/netprimer.html).

Table 1. Sugarcane cultivars, regions (AL = Alagoas; GO = Goias; MG = Minas Gerais; SP = São Paulo) and years of isolation of *Puccinia melanocephala* isolates.

Isolate	Host	Origin	Year of isolation
P201	RB85 5486	Pontal, SP	2010
P204	SP79 1011	Santa Lúcia, SP	2010
P207	IAC84 2480	Mococa, SP	2010
P210	SP70 1143	Conchal, SP	2010
P213	IAC86 2480	São João da Boa Vista, SP	2010
P217	IACSP98 5024	Batatais, SP	2010
P219	RB83 5486	Pompeu, MG	2010
P222	CTC 11	Lençóis Paulistas, SP	2010
P225	IAC84 2480	Itápolis, SP	2010
P228	IACSP97 6682	São Joaquim da Barra, SP	2009
P231	RB83 5486	Patos de Minas, MG	2010
P234	IAC84 2480	Pindorama, SP	2010
P237	RB83 5486	Catanduva, SP	2010
P240	IACSP95 1218	Santa Lúcia, SP	2010
P243	SP79 1011	Olímpia, SP	2010
P246	RB 83 5486	Guaíra, SP	2010
P249	IAC87 3396	Patos de Minas, MG	2010
P252	RB83 5486	Itápolis, SP	2010
P255	IAC91 5155	Goianésia, GO	2010
P258	IAC91 5155	Jaú, SP	2011
P261	IAC97 6682	Goianésia, GO	2011
P264	SP79 1011	Goianésia, GO	2010
P267	SP70 1143	Goianésia, GO	2010
P270	IAC97 6682	Goianésia, GO	2010
P273	IAC86 2480	Jaú, SP	2011
P300	IAC86 2480	Jaú, SP	2010
P303	IAC87 3396	Ribeirão Preto, SP	2010
P306	IAC91 5155	Ribeirão Preto, SP	2011
P309	IAC86 2480	Ribeirão Preto, SP	2010
P312	IAC86 2480	Indiara, GO	2010
P315	SP71 6949	São José da Laje, AL	2010
P318	SP78 4264	São José da Laje, AL	2010
P321	SP86 42	Jaboticabal, SP	2010
P324	IACSP03 8145	Orindiuva, SP	2010

Characterization of *P. melanocephala* isolates using SSRs

DNA was extracted from a suspension of urediniospores from 1 single pustule from the *P. melanocephala* isolates (Virtudazo et al., 2001b). Approximately 300-400 urediniospores were collected and ground using a glass stick in a tube containing 200 mL buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.0; 1.5 mM $MgCl_2$; 0.01% proteinase K). Next, the suspension was incubated at 37°C for 1 h followed by 95°C for 15 min and subsequently stored at 4°C to be used later for SSR amplification. The amplification reactions contained 1 mL urediniospores suspension in a 20-mL final reaction volume, containing buffer (100 mM KCl; 20 mM Tris-HCl, pH 7.4; 1 mM DTT; 0.1 mM EDTA; 200 mg/mL bovine serum albumin), 1.5 mM $MgCl_2$, 0.05 mg/mL casein, 200 mM dNTP, 1 mM of each primer, and 1 U Phire Hot Start DNA Polymerase (Finnzymes Oy; Espoo, Finland). Amplifications were performed in a MyCycle thermocycler (Bio-Rad; Hercules, CA, USA) using the touchdown PCR program, comprised of an initial step of 3 min at 95°C, followed by 9 cycles of 40 s at 94°C, 40 s at annealing temperature decreased from 60°-50°C by 1°C every cycle, and 60 s at 72°C, followed by 30 identical cycles, except for the annealing temperature at 50°C for 40 s.

SSR locus data analysis

Amplified SSR fragments were scored in terms of loci and alleles. Relationships among the isolates were evaluated using phenetic cluster analysis with unweighted pair-grouping and arithmetic average clustering using Nei's unbiased (Nei, 1973) genetic distance. The results were plotted in a dendrogram using NTSYS-PC version 2.0j (Exeter Software, Setauket, NY, USA). Bootstrap analysis (Efron, 1981) was performed using the Winboot program (Yap and Nelson, 1996) with 1000 repetitive samplings of SSR data to compute bootstrap P values. The polymorphism information content (PIC) was estimated for each locus by $PIC = 1 - SPi^2$, where *Pi* was the frequency of allele *i* within the population (Anderson et al., 1993). The coefficient of correlation (r) was computed between genetic and geographic distances, considering all pairs of locations. The significance of r was found using the Mantel Z test (Mantel and Valand, 1970).

Evaluation of aggressiveness of *P. melanocephala*

The aggressiveness of *P. melanocephala* was evaluated using 3 isolates from the main sugarcane Brazilian cultivation regions. Urediniospores P261 (Goianésia, GO), P273 (Jaú, SP), and P306 (Ribeirão Preto, SP) were maintained and multiplied in leaves of the susceptible cultivar 'SP701143' under greenhouse conditions. Individual buds were planted in 3-L pots containing a 3:1:1 mixture of soil, sand, and substrate (pine and coconut bark-Tropstrato) and grown in a greenhouse until inoculation. Initially, 30-day-old plants were kept in a humid growth chamber for 12 h. Subsequently, the abaxial face of leaves was sprayed with a solution of 2×10^4 urediniospores/mL from each of the 3 regions (Garcia et al., 2007). After inoculation, samples were kept in humid growth chamber for 24 h. Next, plants remained protected in a chamber, separated by regions to enable spore maintenance and multiplication.

To evaluate differences in aggressiveness among *P. melanocephala* isolates, inoculations were conducted on excised leaves (Garcia et al., 2007). The abaxial side of a 20-cm segment of the +1 leaf of cultivars 'SP701143' (susceptible) and 'RB835486' (intermediately resistant) was sprayed with a solution of 2×10^4 viable urediniospores/mL. Inoculated leaf segments were then transferred to 25-cm width test tubes, filled with 3/4 volume distilled water, and sealed. Tubes for each treatment were kept at 21°C under a 12-h photoperiod for 15 days. Symptom severity was evaluated 14 days after inoculation using a diagrammatic scale (Amorim et al., 1987). The experimental design consisted of 4 treatments (3 isolates plus a control) in a completely randomized design with 5 replicates (test tube containing 1 inoculated leaf segment). The control treatment consisted of leaf segments inoculated with sterile distilled water. For statistical analysis, the original data were transformed by $\sqrt{(x + 0.5)}$, where X = % infected leaf area, and subjected to analysis of variance and the Tukey test for mean comparisons.

RESULTS

SSR locus-enriched library

A library containing 384 clones was obtained for *P. melanocephala* and enriched for 2 types of SSR motifs (CT and GT). Of the 178 clones sequenced, 52.2% were found to con-

tain SSRs, of which 15.1% had more than 1 SSR within the same region, resulting in a total of 135 SSR loci. Of the SSR loci identified, the most frequent dinucleotide motif observed was GT/TG (20.7%), followed by CA/AC (15.5%), GA/AG (5.2%), TA/AT (3.0%), and CT/TC (2.2%). Trinucleotide repeat motifs were identified and were found to account for 19.2% of the total. Tetranucleotide (TAAT and TTTA; 1.5%), pentanucleotide (TTGGG; 0.8%), and hexanucleotide (ACACGC; 0.8%) motifs were also observed. Only 4 sequences showed SSR motifs interrupted with non-repeated motifs. Mononucleotides were identified with only A- or T-repeat motifs ranging from 8-15 times, but these were not included in the primer design because mononucleotide SSRs generally show low polymorphism (Tóth et al., 2000). SSRs with dinucleotide motifs were observed ranging from 8 repeats (set as the lowest cutoff) to 37 repeats, whereas tri- and tetranucleotide motifs ranged from 4-13 and 6-8 repeats, respectively.

Characterization of the SSR loci

A set of 21 pairs of primers was developed from the enriched SSR library. Sixteen (72.6%) produced well-defined discrete fragments, whereas 5 failed to show amplification. Of the 16 primer pairs producing scorable products, 4 (25%) were polymorphic and 12 (75%) were monomorphic for all isolates tested (Table 2). The number of alleles per locus ranged from 1-5, with a total of 34 alleles and an average of 2.1 alleles per locus. Of the 4 loci containing polymorphisms, a total of 18 alleles were obtained, with an average of 4.5 alleles per polymorphic locus (Table 2). The PIC of the 16 loci ranged from 0-0.64, with an average of 0.45. Loci *mPmIAC101*, *mPmIAC103*, *mPmIAC107*, and *mPmIAC111* were characterized as informative loci (PIC > 0.5); *mPmIAC105*, *mPmIAC108*, *mPmIAC112*, *mPmIAC114*, *mPmIAC116*, and *mPmIAC120* were considered to be moderately informative (0.25 > PIC > 0.5), and *mPmIAC109*, *mPmIAC110*, *mPmIAC113*, *mPmIAC115*, *mPmIAC118*, and *mPmIAC119* were considered to be not informative (PIC < 0.25) (Botstein et al., 1980). The amplified loci displayed alleles of expected fragment sizes, and the greatest allele amplitude was observed for *mPmIAC105*, with alleles ranging from 188-246 bp.

Genetic diversity among *P. melanocephala* isolates

Molecular characterization of the 34 isolates of *P. melanocephala* conducted with 16 SSR loci revealed low genetic variability among the isolates. Based on phenetic analysis, we clustered the 34 isolates into 2 main distinct groups, from which several subgroups were derived (Figure 1). Group I consisted of 18 isolates, while group II contained 14 isolates, and both groups were composed of isolates from all 4 Brazilian states evaluated. The isolates P312 (Goianésia, GO) and P201 (Pontal, SP) were more distant from the others and were clustered as an out-group. The dendrogram shows that within group I, some isolates were genetically identical based on the loci evaluated, including P264 (Goianésia, GO) and P246 (Guaíra, SP); P217 (Batatais, SP) and P321 (Jaboticabal, SP); P210 (Conhal, SP) and P222 (Lençóis Paulista, SP). It was not possible to cluster the isolates according to location or state of original collection. The highest genetic distance observed was 0.31 between the isolates P309 (Ribeirão Preto, SP), P324 (Orindiuva, SP), P334 (Pindorama, SP), and P255 (Goianésia, GO). The average estimated genetic distance was 0.12. No association between genetic and geographic distances was observed (Figure 2).

Table 2. Characteristics of 21 *Puccinia melanocephala* SSR loci and their primer sequences; motif; annealing temperature; original allele size; allele size range; number of alleles per locos (N); and polymorphic information content (PIC).

Locus	Primer sequence (5'-3')	Repeat motif	T (°C)	Original cloned allele (bp)	Allele size range (bp)	N	PIC
mPmIAC101	F: CGTGTCAACCTATCAACCTG R: CTCCACAACACTACAACCTG	$(GT)_8$	55	248	232-248	5	0.64
mPmIAC102	F: GCACCATTCACAAACAGC R: CACACACCACACGAGAGTAA	$(AGA)_6$	55	173	NA	-	-
mPmIAC103	F: TACCGCCGACAGATTTAC R: ATACACAAGCGTGGGAGA	$(TC)_{17}$	55	195	185-198	4	0.59
mPmIAC104	F: ATAGACGCAATGGAAGAAAC R: ACACACACAAACGCTTACAA	$(AC)_{10}$	55	183	NA	-	-
mPmIAC105	F: GAGAAGGAGTGAGAAGGAAA R: TAACCGCGTCTTAGCAAC	$(AG)_9$	53	246	188-246	2	0.37
mPmIAC106	F: CGACCGCTTATGATAGAGTA R: GTCTGTGTGATTGAGATTGC	$(CA)_{23}$	53	227	NA	-	-
mPmIAC107	F: GGGCGTAATGAAACTCTG R: ATTCGGACGACCACAAAC	$(AC)_{14}$	55	250	246-252	4	0.51
mPmIAC108	F: GACGCCTGGAATGAGAGAAC R: GGCTACTTCCTGGTCCGATA	$(TC)_{18}$	60	161	155-161	2	0.37
mPmIAC109	F: TGCGTGTATTCGTGAGTTCG R: CGTCGCTTCCATCCGTTAT	$(GT)_7$	60	245	245	1	0.00
mPmIAC110	F: TCGATTGCTGTCCTGATTCC R: GCTCGCTCTCGGTAACTTTCT	$(AG)_{16}$	60	212	112	1	0.00
mPmIAC111	F: CGAGGTGAAACGATGGAAA R: AGGCGGGCATACAAAAATAG	$(AT)_8$	60	249	220-249	5	0.50
mPmIAC112	F: GAGGCAGGCAGTGAGAGA R: CTGGAGATGGTGATGCTATG	$(AG)_{17}$	57	246	225-246	2	0.37
mPmIAC113	F: AGTCATAAAAACCGTCCAAC R: CGTGTCAGTCCTTCAGAGA	$(CA)_{10}$	55	171	171	1	0.00
mPmIAC114	F: GACTTGCGACTGGTGAATGA R: GTGACGAGTGCTTGCTACGA	$(TG)_{29}$	60	175	178-210	2	0.37
mPmIAC115	F: GACGGACAGATAGACTTTTCAC R: ATCACTCTCCCCTTTCTCAC	$(GT)_{16}$	55	165	155	1	0.00
mPmIAC116	F: CGCGCTACACTACGTTTAAG R: ACACTGCTCACTCACACACC	$(AAT)_6$	56	136	136-138	2	0.37
mPmIAC117	F: GTGCAGACGAAATGATGGT R: CAGATTCGGATGATGATGG	$(GT)_9$	56	239	NA	-	-
mPmIAC118	F: AGCGATTTTTGTTGTTAGCC R: GTTCGGAGCATTCGTGTT	$(TAA)_5$	57	201	201	1	0.00
mPmIAC119	F: GCCCACGGATAAAATACC R: GCATAGGAGACGGGAAGA	$(CA)_{11}$	53	201	201	1	0.00
mPmIAC120	F: GAGATTGTGGCAGGTGAA R: AAAGGGAAAGCTCCAACA	$(AGA)_6$	55	176	162-176	2	0.37
mPmIAC121	F: GAGTGGTTTGTGTGTGTGTT R: GAGACCGTATCTTCTAAGTGC	$(AAT)_{13}$	53	180	NA	-	-

NA = no amplification.

Characterization of aggressiveness of *P. melanocephala*

The evaluation of symptom severity of *P. melanocephala* isolates verified with cultivars 'SP701143' (susceptible) and 'RB835486' (intermediately resistant) showed no significant variation when artificially inoculated with isolates P261 (Goianésia, GO), P273 (Jaú, SP), or P306 (Ribeirão Preto, SP), although there were differences in the severity of symptoms among the assessed cultivars (Figure 3). For 'SP701143', the mean infected leaf area was 2.8%, while it was 0.73% for 'RB835486'.

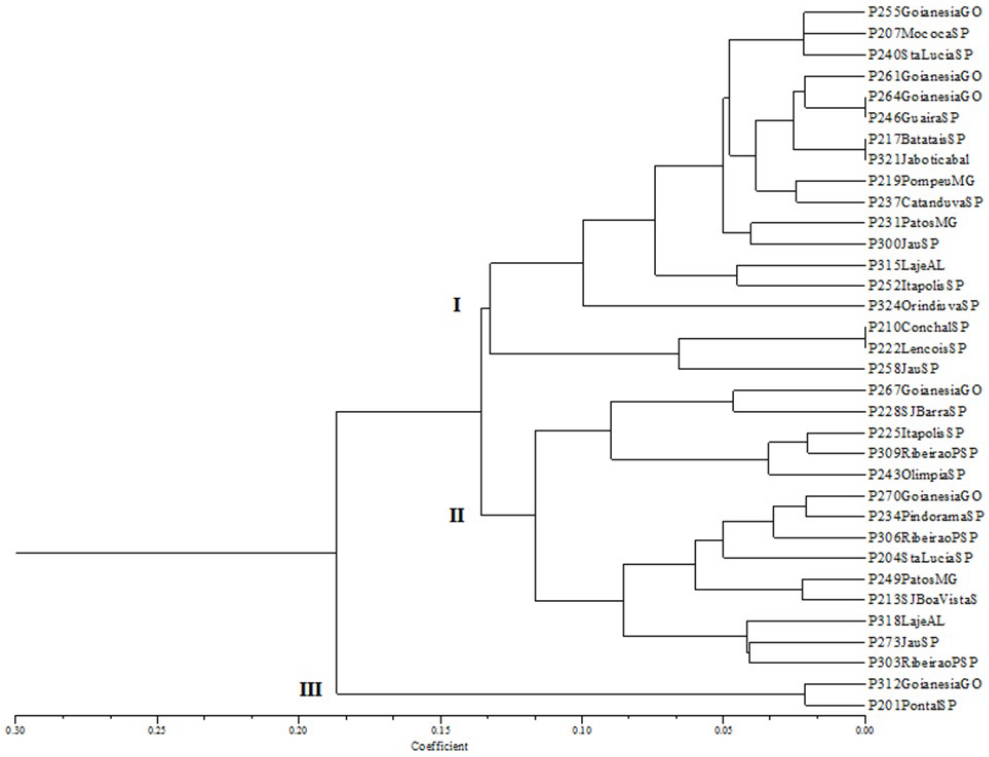

Figure 1. Estimates of genetic diversity based on Nei's unbiased genetic distance among *Puccinia melanocephala* isolates collected from 4 Brazilian states based on SSR polymorphisms.

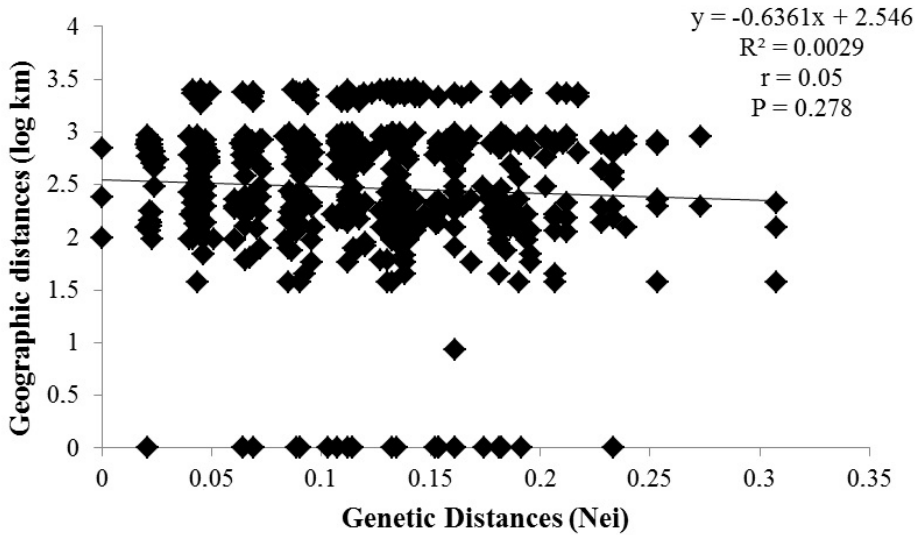

Figure 2. Correlation between Nei's genetic distances in *Puccinia melanocephala* isolates and geographic distances considering all pairs of locations.

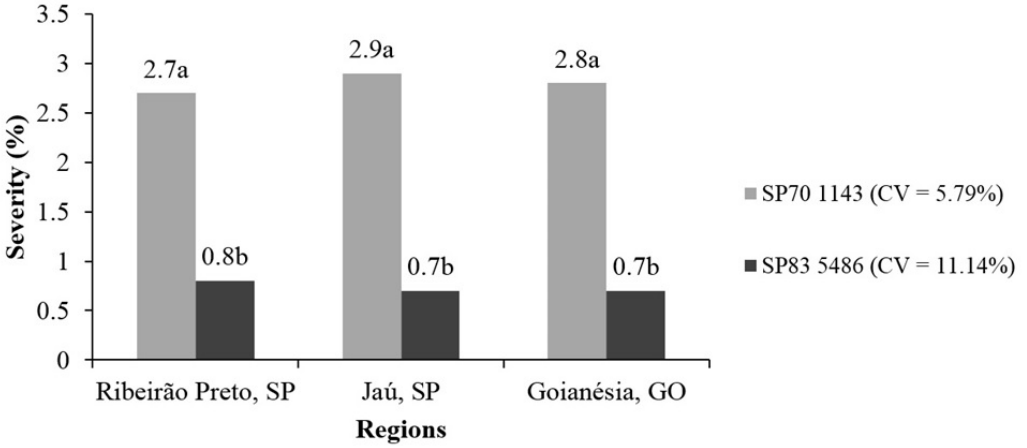

Figure 3. Brown rust severity (percentage of leaf area affected) generated by populations of *Puccinia melanocephala* of different locations in 2 sugarcane cultivars (same letters on the bars indicate no significant differences among according to the Tukey test at 5% probability).

DISCUSSION

Although fungal genomes show a low frequency of SSRs compared to other eukaryotes (Morgante et al., 2002), the enriched genomic library of *P. melanocephala* developed in this study successfully allowed the identification of polymorphic loci. The percent of polymorphic loci obtained (25%) agreed with values reported for other *Puccinia* genomic SSRs, ranging from 25-50% (Enjalbert et al., 2002; Szabo and Kolmer, 2007), and were higher than those described for expressed sequence tag-SSRs (10.2%) (Wang et al., 2010). The number of alleles per locus detected using our SSRs was similar to those reported for expressed sequence tag-SSRs (Wang et al., 2010) and genomic SSRs (Duan et al., 2003), both for *Puccinia triticina*. The most abundant dinucleotide motif for *P. melanocephala* was GT/TG (20.7%) and CA/AC (15.5%). These repeats were expected because of the probes used in this study. The observation that the dinucleotide GT/TG was the most frequent in the library suggests that this motif is more abundant in the *P. melanocephala* genome.

Of the total of 16 loci for which amplification products were observed, 4 (25%) were polymorphic and able to discriminate 28 of the 36 (82.3%) isolates of *P. melanocephala* evaluated. This efficiency was higher than that obtained for *Grosmannia clavigera*, in which only 8 (13.3%) of the 60 SSR loci tested in isolate characterization were polymorphic (Tsui et al., 2009). The failure in amplifications of 5 loci may be attributed to the presence of introns or mutations in the target region of primer annealing region (Dutech et al., 2007).

A low level of genetic diversity was observed among the isolates from 4 Brazilian states evaluated. This may be explained by dispersion mechanisms and fungus biology. Because no sexual recombination occurred, the most probable cause of the genetic diversity levels observed in this study includes constant sources of exogenous introduction of exotic, genetically distinct isolates (Virtudazo et al., 2001b), followed by mutational events. Additionally, it was not possible to cluster the isolates according to location or state of original collection. This can be explained in part by the strong flow of infected plants within the major

sugarcane producing regions in Brazil, as well as by the long-distance dispersion ability of urediniospores, either following single aerial transport events or stepwise range expansion into new regions (Isard et al., 2011). The genetic diversity of *P. melanocephala* has been examined in several countries where sugarcane is cultivated. In Argentina, amplified fragment length polymorphism markers were used to evaluate 30 *P. melanocephala* isolates from 3 regions and a high level of genetic diversity was observed (Pocovi et al., 2010). In contrast, genetic characterization of *Punninia kuehnii* with rDNA regions revealed a low level of variability among Australian isolates, while significant differences were observed among isolates from Papua New Guinea, Indonesia, and China (Braithwaite et al., 2009).

We analyzed the severity of brown rust in 3 sugarcane isolates from the main area in which sugarcane is grown in Brazil using excised leaves and found no differences among isolates, although symptom severity varied according to the cultivar assessed. Therefore, according to the diagrammatic scale used (Amorim et al., 1987), cultivar SP70-1143 was clearly susceptible to brown rust, whereas RB835486 showed intermediate resistance for all 3 isolates observed. These results suggest that the populations of brown rust studied from Brazil were not distinct races.

The existence of *P. melanocephala* races is poorly defined. In Brazil, *P. melanocephala* isolates from São Paulo State showed variability in aggressiveness and severity, suggesting the existence of brown rust races (Rago, 2005). In the USA, the existence of *P. melanocephala* races was described based on changes in the disease reaction in a set of resistant cultivars a few years after their release, indicating the existence of at least 4 races of brown rust (Shine et al., 2005). However, in Australia, no physiological race was identified with 12 differential cultivars when isolates from 4 regions of Queensland were inoculated on excised leaves (Taylor, 1992). The constant variations in the severity of brown rust in Australia have been attributed to changes in climatic conditions and not to the development of new races. Climatic conditions are known to be important for the initiation and intensity of epidemics caused by rust (Taylor, 1992). However, resistant clones may show increased susceptibility over time because of the variability of pathogen populations. This indicates that races of this pathogen and the resistance currently exhibited by the cultivars may change in the future (Braithwaite et al., 2009). Some studies have demonstrated that the heritability of brown rust resistance in sugarcane is high and is controlled by one or a few major effect genes (Asnaghi et al., 2001). However, other studies have shown that additional minor effect genes may also be important in rust resistance (Ramdoyal et al., 2000). Thus, rust resistance in sugarcane may be conferred through the integrated action of these genes, with major effect genes having a stronger influence or effect on resistance.

Investigating brown rust genetic variability and identifying regions with favorable climatic conditions for disease development are extremely important for the success of sugarcane breeding programs, mainly over the long term and aiding the development of new cultivars. Our results suggest that the populations of brown rust from Brazil studied are not distinct races. Therefore, genetic breeding for resistance to brown rust may be conducted in locations where climatic conditions are highly favorable to pathogen occurrence or even in controlled environments through artificial inoculation; these results are likely representative for other regions. Additionally, management strategies involving the cultivation of no more than 20% of the area with cultivars with similar reaction behavior (susceptible and intermediate) can circumvent brown rust outbreaks. The adoption of such procedures will prevent problems derived from the release of cultivars that may show changes in resistance to brown rust after its release.

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