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Development of Genomic SSR Markers and Molecular Characterization of *Magnaporthe oryzae* Isolates from Wheat in Brazil

Jorge Fernando Pereira · Luciano Consoli · Estevon Alexandre de Souza Bombonatto · Ana Lídia Variani Bonato · João Leodato Nunes Maciel

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Abstract *Magnaporthe oryzae*, the causal agent of wheat blast, was characterized on a molecular level with 38 newly isolated genomic SSR loci. Among the 31 wheat isolates analyzed, 15 polymorphic loci were detected, with an average of 1.7 alleles per locus, 28.9% of them being highly or reasonably informative. The number of polymorphic loci was higher in isolates from Londrina in the Brazilian state of Paraná and Coromandel in Minas Gerais compared with Goiânia in Goiás and São Borja in Rio Grande do Sul. The rice isolate was clearly different from the wheat isolates, and the size difference in polymorphic SSR loci between one isolate from wheat and one isolate from rice was associated with the number of repeats. Some isolates collected from different states and in different years demonstrated similarities of 100%. The markers developed here are useful for the genetic analysis of *M. oryzae* isolated from wheat, and isolates representing the variability detected in the field can be used to search for better wheat blast resistance.

Keywords Genetic variability · *Magnaporthe oryzae* · Microsatellite · Wheat blast

Introduction

Magnaporthe oryzae B. Couch (anamorph. *Pyricularia oryzae* Cavara) is the causal agent of blast disease in a number of gramineous plants and is a well-known pathogen of rice that is responsible for severe worldwide losses in yield. The species was described based on a phylogenetic analysis of *Magnaporthe* isolates that

J. F. Pereira · L. Consoli · A. L. V. Bonato · J. L. N. Maciel (✉)
Embrapa Trigo, Passo Fundo, RS 99001-970, Brazil
e-mail: joao.nunes-maciel@embrapa.br

E. A. de Souza Bombonatto
Universidade Luterana do Brasil, Carazinho, RS 99500-000, Brazil

classified the species *Magnaporthe grisea* (Hebert) Barr. into two distinct clades: *M. oryzae*, which is associated with cultivated cereals, and *M. grisea*, which is associated with the grass genus *Digitaria* (Couch and Kohn 2002). Although rice is the major host for *M. oryzae*, this pathogen also causes important damage in wheat (*Triticum aestivum*), mainly in the Brazilian states of Paraná, Mato Grosso do Sul, Goiás, São Paulo, and the Distrito Federal (Igarashi et al. 1986; Goulart and Paiva 1990, 2000; Igarashi 1990; Prabhu et al. 1992; Anjos et al. 1996). Since the mid-1980s, when it was first reported in Brazil (Igarashi et al. 1986), wheat blast has represented a serious problem for wheat production (Kohli et al. 2011; Maciel 2011). Yield losses observed for different cultivars, locations, and growing seasons in Brazil were reported ranging from 10 to 51% (Goulart and Paiva 1990, 1993, 2000; Goulart et al. 2007; Urashima et al. 2009). In addition, during the wheat-growing season of 2009, particularly in the Cerrado Region and north of Paraná, severe yield losses were reported (Duveiller et al. 2010). Wheat blast infections have also been observed in Argentina, Bolivia, Paraguay, and Uruguay (Duveiller et al. 2010; Kohli et al. 2011).

Even if some integrated management practices could reduce yield loss (Mehta et al. 1992), other practices such as chemical control have limited effectiveness (Urashima and Kato 1994; Urashima et al. 2009; Maciel 2011). The use of resistant wheat genotypes is also restricted since only moderate resistance was found in a range of wheat genotypes tested (Barros et al. 1989; Goulart and Paiva 1992, 1993; Urashima et al. 2004; Prestes et al. 2007; Cruz et al. 2010), even though more recent papers have reported high levels of resistance for some wheat genotypes (Cruz et al. 2011; Kohli et al. 2011) and have already identified resistance and defense genes (Zhan et al. 2008; Tufan et al. 2012). In this particular context, blast resistance of wheat genotypes is reportedly under the influence of the natural genetic variability of *M. oryzae* present in the field. For instance, the reaction of a specific wheat genotype in a geographic region under field conditions is not always confirmed in other regions (Urashima et al. 2004), and some wheat genotypes showed different reactions according to the wheat *M. oryzae* isolate used in inoculation procedures, a situation that was observed for seedlings as well as the heading stage (Urashima et al. 2004; Cruz et al. 2009, 2010).

Considering that *M. oryzae* genetic variability is one of the components for wheat blast development in different fields, several research groups have studied the genotypic and phenotypic variability of the wheat pathogen using a probe homolog to the MGR583 retrotransposon (Urashima et al. 1999), restriction fragment-length polymorphism (Lebrun et al. 1990; Valent and Chumley 1994), virulence analysis (Urashima et al. 2004; Cruz et al. 2009), pulsed field gel electrophoresis (Orbach et al. 1996), random amplified polymorphic DNA, and vegetative compatibility tests (Busso et al. 2007). Alternatively, simple sequence repeats (SSR), or microsatellites, were characterized for *M. oryzae* from rice (Brondani et al. 2000; Kim et al. 2000; Kaye et al. 2003; Wang et al. 2005; Adreit et al. 2007; Zheng et al. 2008). Different from the molecular techniques used in previously reported studies of wheat *M. oryzae* genetic variability, the microsatellite markers are multiallelic, easily detectable by PCR, relatively abundant, and spread throughout the genome; moreover, they require minimal DNA for genetic analysis (Saghai Maroof et al.

1994; Oliveira et al. 2006). Some SSR loci previously developed for *M. oryzae* that were isolated from rice (Brondani et al. 2000) have been used to analyze isolates from wheat. Only one wheat *M. oryzae* isolate was analyzed with three microsatellites (Brondani et al. 2000), and our group has already amplified nine SSR loci (also described by Brondani et al. 2000) to study the genetic variability of 18 isolates of *M. oryzae* from wheat (Cruz et al. 2009). We found 1.9 alleles per locus, and 3 of the 9 SSR loci analyzed were monomorphic.

In this study, we have characterized 38 new SSR markers from an enriched microsatellite library constructed from *M. oryzae* isolated from wheat. These markers were used to characterize molecularly 31 wheat *M. oryzae* isolates from different areas of four Brazilian states. Our main goal was to increase the number of polymorphic SSR markers available to study wheat *M. oryzae* isolates so that the analysis of its population in Brazil could be improved.

Materials and Methods

Magnaporthe oryzae Strains, Culture Conditions, and DNA Extraction

We used the same 18 strains of *M. oryzae* obtained by Cruz et al. (2009), and in order to increase the number of samples, we evaluated another 13 strains isolated from wheat-infected leaves and spikes showing blast symptoms (Table 1). One strain of *M. oryzae* isolated from infected rice leaves (Py 003.3.1) was kindly provided by Dr. Gustavo Daltrozo Funck from IRGA (Rio Grande do Sul Rice Institute). Cultures of all 32 strains were maintained on oat-extract agar in Petri dishes at 25°C. From each strain, five 7-mm discs containing mycelia and spores were grown in 50 ml of complete medium (Pontecorvo et al. 1953 modified by Azevedo and Costa 1973) for five days at 25°C and 150 rpm. Afterward, the mycelia were collected by filtration, washed in distilled water, frozen in liquid nitrogen, and ground to a powder using a mortar and pestle. DNA was extracted using the protocol described by Specht et al. (1982) with modifications as follows: ground mycelium (200–300 mg) was resuspended in extraction buffer (2% SDS, 0.05 M Tris–HCl, pH 8.0; 0.02 M EDTA, pH 8.0; and 10 μg proteinase K) and incubated at 70°C for 30 min. After the addition of 5 M potassium acetate, the samples were placed on ice for 30 min and the supernatant was recovered by centrifugation. Two deproteinization steps, using phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1), were performed. The total DNA was precipitated with isopropanol and ammonium acetate, resuspended in 50 μl of TE (containing RNase A), and quantified by gel electrophoresis after an incubation step at 37°C for 30 min.

Construction of SSR-Enriched Genomic Library

Total DNA from the monosporic isolate Py 05001, one of the isolates of our first collection (its virulence was evaluated by Cruz et al. 2009), was used to construct a wheat *M. oryzae* SSR-enriched library based on a protocol described by Kijas et al.

Table 1 *Magnaporthe oryzae* isolated from wheat and rice analyzed in this study

Identification	Origin[a]	Wheat cultivar	Year of isolation
Py 05001*	São Borja (RS)	BRS Angico	2005
Py 05002*	São Borja (RS)	BRS Angico	2005
Py 05003	Londrina (PR)	BRS 248	2005
Py 05005*	Londrina (PR)	BRS 248	2005
Py 05012*	Londrina (PR)	BRS 229	2005
Py 05015	Londrina (PR)	BRS 229	2005
Py 05017*	Londrina (PR)	BRS 193	2005
Py 05020*	Londrina (PR)	BR 18	2005
Py 05021*	Londrina (PR)	BR 18	2005
Py 05022	Londrina (PR)	BR 18	2005
Py 05025*	Londrina (PR)	BRS 249	2005
Py 05029*	Londrina (PR)	CD 105	2005
Py 05031	Londrina (PR)	CD 105	2005
Py 05034	Londrina (PR)	BRS 220	2005
Py 05038*	Londrina (PR)	BRS 220	2005
Py 05039*	Londrina (PR)	BRS 208	2005
Py 05042	Londrina (PR)	BRS 208	2005
Py 05047	Londrina (PR)	BRS 208	2005
Py 06001*[,b]	Coromandel (MG)	BRS 208	2006
Py 06008*[,b]	Coromandel (MG)	BRS 208	2006
Py 06010*[,b]	Coromandel (MG)	BRS 208	2006
Py 06012*	Coromandel (MG)	BRS 208	2006
Py 06015	Coromandel (MG)	BRS 208	2006
Py 06018*	Coromandel (MG)	BRS 208	2006
Py 06025*[,b]	Coromandel (MG)	BRS 208	2006
Py 06028	Goiânia (GO)	BRS 208	2006
Py 06030*	Goiânia (GO)	BRS 208	2006
Py 06032	Goiânia (GO)	BRS 208	2006
Py 06041	Goiânia (GO)	BRS 208	2006
Py 06044	Goiânia (GO)	BRS 208	2006
Py 06048	Goiânia (GO)	BRS 208	2006
Py 003.3.1[c]	Cachoeirinha (RS)	Dular	1996

Asterisks (*) indicate the 18 isolates analyzed by Cruz et al. (2009). Isolates were obtained from different plants collected in experimental plot (Londrina) or farmer fields (São Borja, Coromandel, and Goiânia)

[a] Brazilian state: RS, Rio Grande do Sul; PR, Paraná; MG, Minas Gerais; GO, Goiás

[b] These isolates were obtained from leaf lesions, the others from spikes

[c] The isolate Py 003.3.1 was obtained from a leaf lesion of the rice cultivar Dular

(1994) and modified by Oliveira et al. (2005). Total DNA was digested with *Sau*3AI and ligated to adapters (CTCTTGCTTACGCGTGGACTA and GATCTAGTC CACGCGTAAGCAAGAG). A pre-amplification step was performed using the

adapters and the previously digested DNA (95°C for 4 min followed by 20 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min). The pre-amplified DNA was purified and enriched by hybridization with biotinylated $(TC)_{13}$ oligonucleotides, followed by incubation with magnetic beads linked to streptavidin (Promega). Enriched SSR DNA fragments were amplified using the same adapters and the following program: 95°C for 1 min followed by 20 cycles of 94°C for 40 s, 60°C for 1 min, and 72°C for 2 min. After a purification step, the PCR fragments were cloned into the pGEM-T-Easy vector and transformed into *E. coli* JM109 cells (Promega), according to standard protocols. We recovered 348 white colonies, and after plasmid isolation by alkaline lysis mini-prep, all clones were sequenced using M13 and SP6 primers and Big Dye Terminator version 3.1 on an ABI 3100 Sequence Analyzer (Applied Biosystems).

SSR Detection, Design of Primers, and Genotyping of *M. oryzae* Isolates

Contigs were identified using the Staden sequencing analysis package (Staden 1996), and microsatellite loci were screened using the WebTroll software (http://wsmartins.net/webtroll/troll.html) (Castelo et al. 2002; Martins et al. 2006). Based on the Primer3 software (Rozen and Skaletsky 2000), primers were designed to anneal at the flanking regions of the SSR sequences containing at least three repeats. Reverse primers were normally synthesized, but forward primers were synthesized with an additional M13 tail (TGTAAAACGACGGCCAGT). These primers were used in a PCR reaction along with an M13 primer labeled with a fluorescent dye (FAM, NED, PET, or VIC), as described in Schuelke (2000). Each SSR primer was tested for amplification using a reaction mixture containing 100 ng total DNA, 1× buffer, 0.2 μM of reverse primer, 0.02 μM of forward primer, 0.2 μM of labeled M13 primer, and the following four concentrations of $MgCl_2$, dNTPs, and *Taq* polymerase: mix A (1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of *Taq* polymerase), mix B (1.5 mM $MgCl_2$, 0.35 mM of each dNTP, 0.75 U of *Taq* polymerase), mix C (2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.75 U of *Taq* polymerase), and mix D (2.5 mM $MgCl_2$, 0.35 mM of each dNTP, 0.75 U of *Taq* polymerase). For each SSR primer, only one reaction mixture was chosen (Table 2). All amplification reactions were performed in a PTC-100 Thermal Cycler (MJ Research) programmed as follows: 94°C for 3 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; followed by four cycles at decreasing annealing temperatures in decrements of 1°C per cycle; then 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 3 min. After amplification, reactions were diluted in water, mixed with Hi-Di formamide and GeneScan 500 LIZ size standard (Applied Biosystems), denatured, and run on an ABI 3100 Sequence Analyzer containing a 36 cm capillary array with POP6 polymer. The program GeneMapper version 3.5 was used to analyze the data.

Data Analysis

The SSR loci of the 32 genotypes were used to build a matrix, and the data were analyzed using the PowerMaker program (Liu and Muse 2005). The unweighted

Table 2 Primers and characteristics for 38 SSR markers identified in wheat isolates of *Magnaporthe oryzae* from Brazil

Primer	Forward[a] sequence	Reverse sequence	Dye	PCR program[b]	Repeat motif[b]	Expected size[c] (bp)	Observed size[c] (bp)	Alelle size range (bp)	Number of alleles[d]	PIC
cnpt_mg_p1a10	GGAAGTGGAAGTGGTAGTGG	TGTTCGGGTTGTGTTTGAGA	FAM	TD60-56	(GCGGAA)7	167	179	179	1	0.00
cnpt_mg_p1b3	CAAACATCACTGGCTGCG	GCATCAACACCAGAAAGGAGA	NED	TD60-56	(TC)5	116	132	99 and 132	2	0.12
cnpt_mg_p1e10	AGGCTAAAGAGGGTGTGGGT	GCATTGAGCATACACGGTTG	PET	TD60-56	(TC)10	343	362	362	1	0.00
cnpt_mg_p1e11	AACAATCAAAGGGCTCAACG	AAATAACGGGAGTGGTGCAT	FAM	TD60-56	(TC)14	377	391	391 to 415	8[e]	0.76
cnpt_mg_p1f5	CATTTCGCCTGTGAGTTGG	TCGAGTCCACTGCCCATTA	FAM	TD60-56	(AG)12	324	341	341	1	0.00
cnpt_mg_p1b8	CAACTGCGAATCTCTCCACA	CGGGTACAACGTAAATTGGG	NED	TD60-56	(AG)10	350	364	364	1	0.00
cnpt_mg_c069	GATCAACGGATGGATGATAC	CGTAGAGTATGTGTGCCAATCA	PET	TD60-56	(AG)9	246	266	266	1	0.00
cnpt_mg_p1h10	GATCACAATTCAAGCCCACC	CTGCCGACCGTGGTAATAGT	VIC	TD60-56	(GA)22	296	315	315 to 317	4[e]	0.69
cnpt_mg_c018	AGGGACAGACAGGATGGAATAA	ATTTCAGCGGATACTTTTCGTC	FAM	TD60-56	(TC)10	177	196	173 and 196	2	0.27
cnpt_mg_c019	AGAGCATGTCGGTTAGGATAGG	TGGAAATCATCATCAGGTCAAG	NED	TD60-56	(A)12	284	300	300	1	0.00
cnpt_mg_c031	ATCAATCTGCACTGTCGTGTG	TGAGCATCAGGGTATGTTTACG	PET	TD60-56	(TACC)3	133	152	152	1	0.00
cnpt_mg_c035	AGGGAGGGGAAGAAACAATATC	CTGAATGAGTCCGTCCACCTA	VIC	TD60-56	(AAT)4	113	128	128	1	0.00
cnpt_mg_c041	TAGCCCTCTCTCCCTCTCTCT	CGGTGGTAGAACTCTTCGTTGT	FAM	TD60-56	(TC)7	153	169	169	1	0.00
cnpt_mg_c047	AGGTTGGTGACGACGAAAAC	CATTGTACGCCCACATAGCTT	NED	TD60-56	(GACG)3	196	215	215 and 216	2	0.27
cnpt_mg_c049	CCTGTTCTTCTCGCTATTGTCC	TATCATCCTGAAATCGACAGCA	PET	TD60-56	(CT)8	218	244	244 and 245	2	0.49
cnpt_mg_c053	CGAGACACGTTCAATCAATCA	ATATGGAGATTTGGTGGGGTTT	VIC	TD60-56	(ACGA)2	169	185	185	1	0.00
cnpt_mg_c059	CTTTGGCTGTTGTTTACCCACT	AGCCTATTGTGATTTTGGTCGT	FAM	TD60-56	(CATA)5	191	208	208 to 216	3	0.50
cnpt_mg_c060	CCGGTTGGCTTTCGTCAT	GTCCAAAGTGCCCCTGACTATC	NED	TD60-56	(AG)14	136	153	145 to 153	3	0.18
cnpt_mg_c065	ACCTCCAGCACTAATACTCCCA	AAAAGCACACATACAGACGCC	PET	TD60-56	(GA)7	155	175	175	1	0.00
cnpt_mg_c066	CGGTAAATCTTGTCGCTTCAC	CATGTTCATACCATTCCAGCC	VIC	TD60-56	(GCT)4	130	147	147	1	0.00
cnpt_mg_c068	ATGAACGACATGGACTCGAATA	GAATCCTCTCCAATGCAACTTC	FAM	TD60-56	(TC)9	179	196	196	1	0.00
cnpt_mg_c077	GTACCTAGAAATGCACGTCGG	CCGTTCCTTGAGTGTCTGTTTT	NED	TD60-56	(A)27	173	187	187 to 189	3	0.53
cnpt_mg_c091	AACACTAAGGGGTCGGCTTT	AGTCAATCCCATCACTCTGGTC	PET	TD60-56	(TC)28	119	138	99 to 138	3	0.65
cnpt_mg_c092	GTCACTACTGTGCGTATTTGC	GATCAATCAAGCTGGCTG	VIC	TD60-56	(CTAC)3	206	224	224	1	0.00
cnpt_mg_p1e7	CTGTCTCCCGGCTTACACA	TAACAGGTTGAATGCCCGA	FAM	TD60-56	(TC)8	147	164	162 and 164	2	0.12
cnpt_mg_c013Tri	CACTCTGCCCACAGGATTTT	AAATTGTATGCCAAGGTGGAAC	NED	TD60-56	(GCC)5	221	240	240	1	0.00
cnpt_mg_c147	ACTTTGGTGGAGTCATCGTCTT	AGGAACCACGCTAAAATCAGAG	PET	TD60-56	(TC)6	354	370	370	1	0.00
cnpt_mg_c129	CCGGAGAAAAGGTATTTTAGGG	GATCACATCTACAAGCGAAACCT	VIC	TD60-56	(AG)15	216	236	236	1	0.00

Table 2 continued

Primer	Forward[a] sequence	Reverse sequence	Dye	PCR program[b]	Repeat motif[b]	Expected size[c] (bp)	Observed size[c] (bp)	Alelle size range (bp)	Number of alleles[d]	PIC
cnpt_mg_c108	CTCCGAATGACTCTGCCAAT	TTGGGATGGAACCTCGATATAC	FAM	TD60-56	(GA)10	179	202	202 and 203	2	0.17
cnpt_mg_c133	CCTCGATTTGCTCCACTTAATC	ATGCGACTGCCTACCTACCTAC	NED	TD60-56	(GTAG)3	146	164	164	1	0.00
cnpt_mg_c159	GATCATAGGGGACATTGGGTTA	TCTCTCTCTTCTTAATTCGCCC	PET	TD60-56	(GAA)4	397	415	415	1	0.00
cnpt_mg_c160	GTGGCGTAAAGAACTCTGTGAA	GAGGCTTGTCTGGAAGAATTTG	VIC	TD60-56	(CCGG)2	343	356	355 and 356	2	0.28
cnpt_mg_c168	CGTATGCGAGTGACAGAAGAAA	TCCTGGGCGAGATATTAAAAGA	FAM	TD60-56	(TCC)4	234	253	253	1	0.00
cnpt_mg_c146	CACCACATCTTTTGTTTCTCCA	ATTCTCGTATGCCTTCTGGCT	NED	TD60-56	(CTCC)3	145	162	162	1	0.00
cnpt_mg_c233	ACGAGTGAGCTGGGATGTTATT	TACGGCGCAACCTTTCAC	PET	TD60-56	(GA)9	102	122	122	1	0.00
cnpt_mg_c183	GACCGCCCGTGTTTCTACTAC	CTGACTTCGGCAACAGCATATT	VIC	TD60-56	(GCTG)3	173	191	191	1	0.00
cnpt_mg_c211	GATCACGCAGGGGAGCAT	GTTGCCTCTGTAGTCTTGTACCCTT	NED	TD60-56	(AG)9	218	235	235 and 243	2	0.27
cnpt_mg_c248	GCGGAATCTTTCTTTATCCTGA	AAGCTCCCATATTTGCTTTGAC	VIC	TD60-56	(GA)6	119	136	134 and 136	2	0.35

[a] Forward primer contains a M13 sequence (TGTAAAACGACGGCCAGT) at the 5′ end

[b] The PCR program contains a touchdown step (see Materials and Methods)

[c] For the isolate Py 05001 used in the SSR-enriched genomic library sequencing

[d] Number of alleles considering only the *M. oryzae* isolates from wheat. Null allele was considered for primers p1e11 and p1h10

pair-group method using arithmetic average (UPGMA) algorithm was used to construct a dendrogram using the shared allele distance for each pair of individuals. The polymorphism information content (PIC) value of each polymorphic marker was calculated according to Anderson et al. (1993) using the formula:

$$\mathrm{PIC}_i = 1 - \sum_{j=1}^{n} P^2 ij$$

where P_{ij} is the frequency of the jth allele for marker i, and summation extends over n alleles. Only the allelic frequencies obtained from the wheat *M. oryzae* isolates were considered.

Sequencing of SSR Loci

The 38 primers (Table 2) were used to amplify total DNA from the Py 05001 and Py 003.3.1 isolates. For this experiment, the M13 primer was not linked to a fluorescent dye. After thermocycling, the samples were precipitated and quantified on a 0.8% agarose gel. The samples were then mixed with primer F or R (3.2 μM), sequencing buffer, and Big Dye Terminator version 3.1 and incubated in a PTC-100 Thermal Cycler (MJ Research) programmed as follows: 96°C for 1 min, then 35 cycles at 96°C for 20 s, 56°C for 20 s, 60°C for 2 min and 45 s. Samples were precipitated again, resuspended in Hi-Di formamide, denatured, and run on an ABI 3100 Sequence Analyzer. The Sequencing Analysis Software version 5.1.1 and the Staden sequencing analysis package (Staden 1996) were used to analyze the sequences.

Results

Development of the SSR Markers

Of the 384 recombinant plasmids sequenced, 220 contigs were recovered, and 39 of them (17.7% based on the number of contigs) had an adequate size and position that allowed primers to be designed. After testing these primers in four reaction mixtures, one of them (cnpt_mg_c131) did not show a good amplification pattern. The remaining 38 primers used in this study are listed in Table 2. Two primers were designed based on SSR loci containing mononucleotide repeats, 21 on dinucleotide repeats, five on trinucleotides, nine on tetranucleotides, and one on heptanucleotides. There were 18 loci with 8 or more repeats and 20 with 3–7 repeats. Two primers (cnpt_mg_c053 and cnpt_mg_c160) were initially designed based on sequences containing three repeats, but when the PCR products were resequenced to check the fragment size and number of repeats, these primers were found to contain only two repeats.

Analysis of the SSR Markers

Of the 38 useful SSR primers, 25 amplified polymorphic DNA fragments in the 32 tested isolates. Among those 25 polymorphic SSR markers, 10 were monomorphic for the wheat isolates but amplified a different fragment for the rice isolate, and 15 showed polymorphisms among the wheat isolates (Table 2). Considering only the polymorphisms presented by wheat isolates, the number of alleles per locus ranged from 2 to 8, with an average of 2.8. However, the analysis of all markers (including polymorphic and monomorphic ones) for only the wheat isolates resulted in an average of 1.7 alleles per locus. Considering just the same 18 isolates that Cruz et al. (2009) analyzed with SSR markers described for *M. oryzae* from rice, an average of 1.6 alleles per locus was found with the SSR markers described in this study, which was lower than the 1.9 alleles per locus found by Cruz et al. (2009). Using the criteria proposed by Botstein et al. (1980), four (10.5% based on the 38 primers analyzed) of the polymorphic markers showed a PIC greater than 0.50 and were classified as highly informative, seven (18.4%) presented a PIC of 0.25–0.50 and were reasonably informative, and four (10.5%) were slightly informative, presenting a PIC less than 0.25. The remaining 60.5% represented the 23 SSR markers that amplified a monomorphic fragment (PIC = 0) among the 31 wheat *M. oryzae* isolates. Three (20%) of 15 polymorphic SSR markers were tetranucleotides, 11 (73.3%) were dinucleotides, and one (6.6%) was a mononucleotide. None of the trinucleotide or heptanucleotide markers was polymorphic. In the Py 05001 isolate that was used to construct the SSR library, the observed size of the SSR markers was always higher than the expected size (the higher and lower differences were 26 and 12 bp, respectively, with an average difference of 17.6 bp), but the difference is related to the addition of the M13 tail and the fluorophores used in the electrophoresis (Table 2). Only two primers (cnpt_mg_p1h10 and cnpt_mg_p1e11) were unable to amplify PCR products in 11 and 12 isolates, respectively, and those loci were considered null alleles. This lack of amplification could be a consequence of mutations, deletions, or insertions in the primer annealing site; recombination events; or even insertion of DNA sequences between the primer annealing sites.

Genotyping of *M. oryzae* Isolates

Genetic variation was identified among the isolates (Fig. 1). The rice isolate (Py 003.3.1) was easily distinguished from the wheat isolates. It was found to amplify a specific fragment (a fragment different from all fragments amplified in the wheat isolates) in 18 primers (data not shown). Thus, 47.4% of the primers developed in this study were able to separate the rice isolate (Py 003.3.1) from the wheat isolates. In addition, a comparison of SSR sequences from the rice isolate and the wheat isolate Py 05001 (used to construct the library) showed that the size difference in all 21 polymorphic SSR loci was related to differences in the number of repeats (Table 3), although the size difference for the locus cnpt_mg_c059 was found in a second motif, which was not the most repeated one. For the 17 monomorphic SSR loci between Py 003.3.1 and Py 05001, 16 presented the same number of repeats,

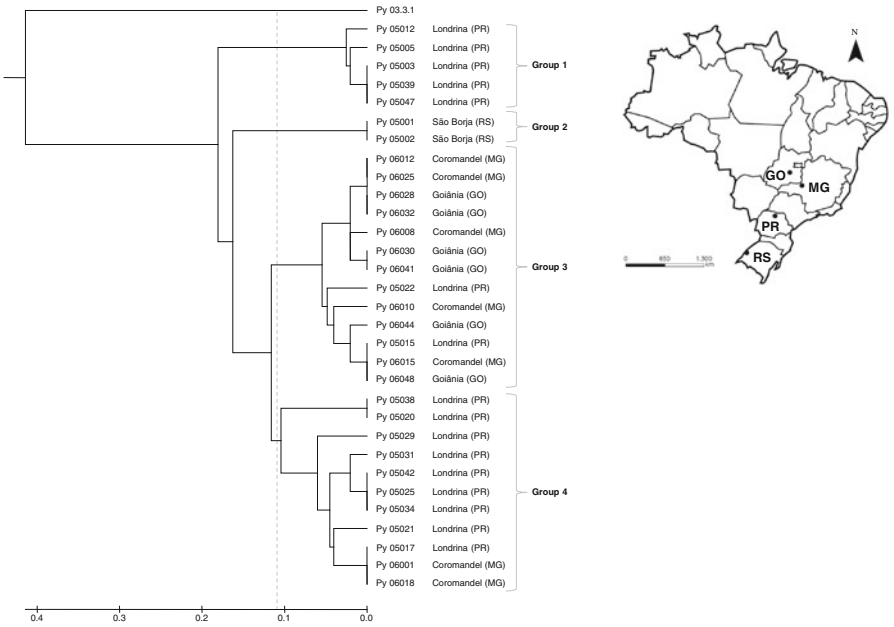

Fig. 1 Hierarchical analysis of the 32 *M. oryzae* isolates with the SSR markers developed in this study. (*Left*) Clustering dendrogram based on the alleles detected in the wheat and rice isolates. The dotted line represents the distance used to separate the four groups. (*Right*) Collection sites of isolates within Brazil. Dots represent collection sites in the cities of (GO) Goiânia in the state of Goiás, (MG) Coromandel in Minas Gerais, (PR) Londrina in Paraná, and (RS) São Borja in Rio Grande do Sul. The distances between sites (based on latitude and longitude coordinates), starting from GO, are 310 km to MG, 815 km to PR, and 1633 km to RS; distances starting from MG are 725 km to PR and 1580 km to RS; and the distance between PR and RS is 854 km

and only one (cnpt_mg_c049) showed a different number of repeats that was compensated by differences in a second motif inside the same locus.

In the hierarchical analysis, a threshold of 1.1 was selected for establishing a division into four groups by considering only the 31 *M. oryzae* isolated from wheat. In some of those groups, a distinguishable separation was found concerning the location where the isolates were collected (Fig. 1). Five isolates from Londrina (in the state of Paraná) were clustered into group 1, while group 2 represented the two isolates from São Borja (in Rio Grande do Sul). Group 4 is represented by 11 isolates, of which nine are from Londrina and two are from Coromandel (in Minas Gerais). Thus, from the 16 isolates from Londrina, 14 were clustered into group 1 or 4. Moreover, group 3 represented isolates from three states. All six isolates from Goiânia (in Goiás) were grouped in this cluster, and two of them were identical to two isolates from Coromandel (Py 06028, Py 06032, Py 06012, and Py 06025). It is interesting that the isolates Py 05015, Py 06015, and Py 06048, which were isolated in different years and belong to three different states, were found to be identical. This result was also found in group 4, where one isolate from Londrina (Py 05017) was identical to two isolates from Coromandel (Py 06001 and Py 06018). Five out

Table 3 Repeats of 38 SSR loci in *M. oryzae* isolated from wheat (Py 05001) and rice (Py 003.3.1)

SSR locus	Visualized size			Motif and number of repeats		
	Py 05001 (bp)	Py 003.3.1 (bp)	Dif.[a] (bp)	Py 05001	Py 003.3.1	Dif.[b] (bp)
cnpt_mg_p1a10	179	179	–	$(GCGGAA)_7$	$(GCGGAA)_7$	–
cnpt_mg_p1b3	132	99	33	$(TC)_5$	$(TC)_3$	4
cnpt_mg_p1e10	362	359	3	$(TC)_{10}$	$(TC)_9$	2
cnpt_mg_p1e11	391	397	6	$(TC)_{14}$	$(TC)_{15}$	2
cnpt_mg_p1f5	341	341	–	$(AG)_{12}$	$(AG)_{12}$	–
cnpt_mg_p1b8	364	362	2	$(AG)_{10}$	$(AG)_9$	2
cnpt_mg_c069	266	263	3	$(AG)_9$	$(AG)_8$	2
cnpt_mg_p1h10	315	299	16	$(GA)_{22}$	$(GA)_{18}$	8
cnpt_mg_c018	196	180	16	$(TC)_{10}$	$(TC)_4$	12
cnpt_mg_c019	300	298	2	$(A)_{12}$	$(A)_{10}$	2
cnpt_mg_c031	152	152	–	$(TACC)_3$	$(TACC)_3$	–
cnpt_mg_c035	128	128	–	$(AAT)_4$	$(AAT)_4$	–
cnpt_mg_c041	169	173	4	$(TC)_7$	$(TC)_8$	2
cnpt_mg_c047	215	215	–	$(GAGC)_3$	$(GAGC)_3$	–
cnpt_mg_c049[c]	244	244	–	$(CT)_8/(T)_{22}$	$(CT)_9/(T)_{20}$	2
cnpt_mg_c053	185	185	–	$(ACGA)_2$	$(ACGA)_2$	–
cnpt_mg_c059[c]	208	212	4	$(CATA)_5/(CATC)_2$	$(CATA)_5/(CATC)_3$	4
cnpt_mg_c060	153	165	12	$(AG)_{14}$	$(AG)_{17}$	6
cnpt_mg_c065	175	177	2	$(GA)_7$	$(GA)_{10}$	6
cnpt_mg_c066	147	147	–	$(GCT)_4$	$(GCT)_4$	–
cnpt_mg_c068	196	192	4	$(TC)_9$	$(TC)_6$	6
cnpt_mg_c077	187	168	19	$(A)_{27}$	$(A)_{10}$	17
cnpt_mg_c091	138	97	41	$(TC)_{28}$	$(TC)_{10}$	36

Table 3 continued

SSR locus	Visualized size			Motif and number of repeats		
	Py 05001 (bp)	Py 003.3.1 (bp)	Dif.[a] (bp)	Py 05001	Py 003.3.1	Dif.[b] (bp)
cnpt_mg_c092	224	224	–	$(CTAC)_3$	$(CTAC)_3$	–
cnpt_mg_p1e7	164	164	–	$(TC)_8$	$(TC)_8$	–
cnpt_mg_c013Tri	240	240	–	$(GCC)_5$	$(GCC)_5$	–
cnpt_mg_c147	370	379	9	$(TC)_6$	$(TC)_{10}$	8
cnpt_mg_c129	236	218	18	$(AG)_{15}$	$(AG)_6$	18
cnpt_mg_c108	202	184	18	$(GA)_{10}$	$(GA)_8$	4
cnpt_mg_c133	164	164	–	$(GTAG)_3$	$(GTAG)_3$	–
cnpt_mg_c159	415	415	–	$(GAA)_4$	$(GAA)_4$	–
cnpt_mg_c160	356	356	–	$(CCGG)_2$	$(CCGG)_2$	–
cnpt_mg_c168	253	253	–	$(TCC)_4$	$(TCC)_4$	–
cnpt_mg_c146	162	162	–	$(CTCC)_3$	$(CTCC)_3$	–
cnpt_mg_c233	122	118	4	$(GA)_9$	$(GA)_8$	2
cnpt_mg_c183	191	191	–	$(GCTC)_3$	$(GCTG)_3$	–
cnpt_mg_c211	235	243	8	$(AG)_9$	$(AG)_{13}$	8
cnpt_mg_c248	136	135	1	$(GA)_6$	$(GA)_2$	8

[a] Size difference (in bp) of the SSR locus between the wheat and rice isolate of *M. oryzae*

[b] Size difference (in bp) of the SSR number of repeats between the wheat and rice isolate of *M. oryzae*

[c] For those primers, differences in a second motif inside the SSR locus were observed

Table 4 SSR markers amplified from wheat isolates of *M. oryzae* originating from Brazilian locations using the primers developed in this study

Location	Number of wheat cultivars[a]	Number of isolates	Number of alleles	Alleles per locus	Polymorphic locus
Goiânia (GO)	1	6	42	1.1	3
Coromandel (MG)	1	7	46	1.2	7
Londrina (PR)	8	16	57	1.5	12
São Borja (RS)	1	2	38	1.0	0
Total	9	31	65	1.7	15

[a] Isolates were collected from cultivars listed in Table 1

of seven isolates from Coromandel were sorted into group 3, two being identical (Py 06012 and Py 06025), and two were grouped into group 4, also with two identical isolates (Py 06001 and Py 06018). Although we were not able to analyze the same number of isolates for all locations, an analysis of isolates from the same region revealed that the average number of alleles was 1.0 in São Borja, 1.5 in Londrina, 1.2 in Coromandel, and 1.1 in Goiânia, and the number of polymorphic loci was higher in Londrina (12) and Coromandel (7) compared with Goiânia (3) and São Borja (0) (Table 4). All isolates from the southern region of Brazil (Londrina and São Borja) were separated among all four groups of the hierarchical analysis (Fig. 1), with 88.9% of them distributed into groups 1, 2, and 4. Moreover, 84.6% of the 13 isolates from the central area of Brazil (Goiânia and Coromandel) were sorted into group 3. The only exceptions were two isolates from Londrina (Py 05015 and Py 05022) that clustered in group 3 with isolates from central Brazil and two isolates from Coromandel (Py 06001 and Py 06018) that sorted into group 4 together with isolates from southern Brazil.

Discussion

Although other groups have already developed SSR markers for *M. oryzae* isolated from rice (Brondani et al. 2000; Kim et al. 2000; Kaye et al. 2003; Wang et al. 2005; Adreit et al. 2007; Zheng et al. 2008), this is, to our knowledge, the first report of SSR markers isolated from an enriched library constructed from a *M. oryzae* isolated from wheat. Our main goal was to increase the number of polymorphic SSR loci available for population studies of *M. oryzae* isolated from wheat. We were able to develop 38 SSR markers, averaging 1.7 alleles per locus, which is slightly lower than the average of 1.9 obtained by Cruz et al. (2009) with the analysis of wheat *M. oryzae* isolates using SSR markers described for rice isolates. Nevertheless, in our study, 15 (39.5%) of the newly developed SSR markers were polymorphic in a sample of 31 wheat *M. oryzae* isolates from four different Brazilian states. The 39.5% of polymorphic loci in our study is reasonably lower than the mean value of polymorphic loci (53.2 ± 7.4%) described from 17 fungal species (Dutech et al.

2007). In addition, the percentage of sequences suitable for primer design, which was 17.7% (39 of 220 contigs), was also lower than the mean value (28.6 ± 3.9%) described by those same authors, not taking into account that we stipulated a minimum of three repeats instead of the five perfect repeats considered by Dutech et al. (2007). Other authors have also reported a higher percentage of polymorphic loci in *M. oryzae* isolated from rice (73%, Kaye et al. 2003; 73.6%, Zheng et al. 2008). In addition, 10.5% of the SSR markers isolated in this study were highly informative, and 18.4% were reasonably informative. Thus, we were not able to isolate a good number of polymorphic markers in *M. oryzae* isolated from wheat. The explanation for that is not likely to be related to the use of $(TC)_{13}$ to enrich the SSR library, as the most abundant dinucleotide in the rice *M. oryzae* genome is AG (Lim et al. 2004). Among the loci with eight or more repeats, 55.5% were polymorphic, whereas only 25% of the loci with two to seven repeats were polymorphic, indicating that a higher proportion of polymorphisms originated from sequences with a higher number of repeats. In fungal genomes, the number of repeats or the length of SSR sequences have been reported to be positively correlated with the number of SSR polymorphisms (Zheng et al. 2008), and a motif repeated at least eight times appears to be desirable for predicting polymorphisms (Lim et al. 2004). However, in our study, one locus (cnpt_mg_c047) with three repeats and one (cnpt_mg_c160) with only two repeats were also polymorphic (PIC = 0.27 and 0.28, respectively). According to Dutech et al. (2007), the number of polymorphisms of long SSR loci in 17 fungal genomes was high for some species and low for others, such that a significant effect of the number of repeats on the number of alleles was also detected. Those authors also reported that 88% of the SSR polymorphic loci in fungi were dinucleotides; in our study, 55.3% of the isolated loci were dinucleotides.

Although wheat blast is a serious threat to wheat production, not only in Brazil but also in some neighboring countries, little is known about the population structure of its pathogen. Because of the severe impact of the disease, an understanding of the evolution of *M. oryzae* from wheat is important for decisions about selecting resistant plants, cloning resistance genes, and mapping QTLs, among other actions. For instance, different levels of wheat resistance to blast can be observed depending on the geographic regions and the *M. oryzae* isolates inoculated (Urashima et al. 2004; Cruz et al. 2009, 2010), which means that *M. oryzae* genetic variability plays an important role in the search for wheat blast resistance. Recently, some wheat genotypes were classified as highly resistant to wheat blast (Cruz et al. 2011; Kohli et al. 2011). Although this finding is a significant step toward achieving better control over the disease, it is important to point out that only one *M. oryzae* isolate was used by Cruz et al. (2011). Based on the results of our survey and on other reports (Urashima et al. 2004; Cruz et al. 2009, 2010), the screening for wheat blast resistance needs to take into account the genetic variability of *M. oryzae* found in the field.

According to phenotypic studies, differences in virulence, mating type, and vegetative compatibility groups have been reported for *M. oryzae* from wheat (Bruno and Urashima 2001; Urashima et al. 2004; Busso et al. 2007; Galbieri and Urashima 2008; Cruz et al. 2009). In addition, some efforts have been made to study

M. oryzae genetic variability based on different molecular techniques. Some reports revealed a narrow genetic variability in wheat *M. oryzae* isolates, such as an analysis of five *M. oryzae* isolates from wheat using RAPD that revealed a discrete genetic variation (Busso et al. 2007). Chromosomal studies employed by PFGE in seven *M. oryzae* isolates from wheat collected at several Brazilian locations over a period of three years showed remarkable karyotype conservation (Orbach et al. 1996). On the other hand, using a probe homologous to the MGR583 retrotransposon, Urashima et al. (1999) reported a high degree of diversity in wheat *M. oryzae* isolates from major regions in Brazil. In addition, diversity of about 25–50% in 18 isolates of *M. oryzae* from wheat was observed by Cruz et al. (2009) using eight SSR markers previously described for *M. oryzae* in rice. In our study, the analysis of 31 isolates of wheat *M. oryzae* revealed that isolates from Londrina and Coromandel presented more polymorphic markers than isolates from Goiânia and São Borja (Table 4). In addition, isolates from Londrina were sorted into three out of the four groups detected, and isolates from Coromandel were sorted into two of them, while isolates from São Borja and Goiânia were sorted into just one group (groups 2 and 3, respectively). Our analysis also revealed that some isolates from Goiânia and Coromandel (separated by approximately 310 km), Londrina and Coromandel (725 km apart), and Londrina and Goiânia (815 km apart) were identical, even though, in some cases, they were collected in different years. Identities through RFLP markers and virulence patterns among isolates separated by large geographic distances have already been reported for *M. oryzae* from wheat (Urashima et al. 1999, 2004). It has been proposed that their identity might be a consequence of the movement of wheat seeds infected with a specific *M. oryzae* isolate between different regions (Urashima et al. 2004). Unfortunately, it is not possible, with our results, to determine whether such a migration occurred or in which direction.

Even though we have analyzed only two isolates from São Borja, the lack of polymorphism in those isolates could be related to the temperate climate and high humidity of that region during the wheat-growing season, which is unfavorable to wheat blast development. According to that theory, low variability in those isolates would be expected, although Cruz et al. (2009) found substantial differences in their virulence spectrum. Counterclockwise, the high temperatures and the low humidity during the wheat-growing season in Londrina, Coromandel, and Goiânia are much more favorable for wheat blast establishment, and thus, in those areas, the pathogen is more likely to show variability. Some authors have proposed a role for the parasexual and sexual cycles in the genetic variability of *M. oryzae* isolated from wheat (Urashima et al. 1993; Bruno and Urashima 2001; Busso et al. 2007), although more recent findings revealed an apparently low possibility of sexual crosses in wheat fields (Galbieri and Urashima 2008). It is important to point out that the experiments showing sexual crosses among wheat *M. oryzae* isolates were conducted in laboratory conditions, and the role of that stage in nature has not been documented. Nevertheless, genotypic variability can be observed among Brazilian isolates of *M. oryzae* from wheat (Fig. 1). For instance, in this study, we were able to find differences in some isolates that Cruz et al. (2009) found to be identical.

It has been proposed that pathogen populations may establish some level of selectivity among hosts, and based on that, plant cultivars could have long-term

effects on the composition of the population (Kistler 1997). The possible effect of the plant cultivar on fungal genetic diversity has been reported (Baturo-Ciesniewska 2011), although in our study, the number of isolates from each cultivar (Table 1) did not allow us to evaluate that effect.

The rice isolate (Py 003.3.1) used in this study was clearly distinct from the 31 isolates of wheat. In all 21 polymorphic SSR markers between the rice isolate and one wheat isolate, variation in the number of repeats was associated with the allele size difference (Table 3). Nevertheless, 18 of 38 primers developed in this study amplified a specific fragment in the rice isolate. In this case, the SSR markers gave a clear result when compared to the host specificity. This result increases the amount of evidence suggesting that *M. oryzae* isolated from rice and wheat possess molecular differences. For instance, RFLP analysis revealed differences in the copy number of a MGR583 homolog probe (Urashima et al. 1999), PFGE showed different karyotypes among Brazilian isolates from wheat and rice (Orbach et al. 1996), and a five-fold difference in the number of AG repeats for one SSR marker was identified between one *M. oryzae* isolate from rice and one from wheat (Brondani et al. 2000).

The newly polymorphic SSR markers developed in this study are useful for evaluating the genetic variability of *M. oryzae* from wheat and for understanding the population structure of that pathogen. The population structure (i.e., the amount of genetic and phenotypic variation) can vary through time and space as the pathogens evolve or adapt in response to environmental conditions (McDonald and Linde 2002), and hence a constant characterization of new isolates from different locations and different wheat-growing seasons is required. Our group is currently analyzing a larger collection of *M. oryzae* collected through different wheat-growing regions in our country.

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References

Adreit H, Andriantsimialona DS, Utami W, Notteghem JL, Lebrun MH, Tharreau D (2007) Microsatellite markers for population studies of the rice blast fungus, *Magnaporthe grisea*. Mol Ecol Notes 7:667–670

Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrells ME (1993) Optimizing parental selection for genetic linkage maps. Genome 36:181–186

Anjos JRN, da Silva DB, Charchar MJD, Rodrigues GC (1996) Ocorrência de brusone (*Pyricularia grisea*) em trigo e centeio na região dos cerrados do Brasil Central. Pesq Agropec Bras 31:79–82

Azevedo JL, Costa SOP (1973) Exercícios práticos de genética. Editora Nacional, São Paulo

Barros BC, Felício JC, Camargo CEO, Ferreira Filho AWP (1989) Reação de cultivares de trigo a brusone em condições de campo. Summa Phytopathol 15:21 (Abstract)

Baturo-Ciesniewska A (2011) Genetic variability and pathogenicity among polish isolates of *Bipolaris sorokiniana* from spring barley. J Plant Pathol 93:291–302

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331

Brondani C, Brondani RPV, Garrido LR, Ferreira ME (2000) Development of microsatellite markers for the genetic analysis of *Magnaporthe grisea*. Genet Mol Biol 23:753–762

Bruno AC, Urashima AS (2001) Inter-relação sexual de *Magnaporthe grisea* do trigo com a brusone de outros hospedeiros. Fitopatol Bras 26:21–26

Busso C, Kaneshima EN, Franco FA, Castro-Prado MAA (2007) Genetic and molecular characterization of pathogenic isolates of *Pyricularia grisea* from wheat (*Triticum aestivum* Lam.) and triticale (*x Triticosecale* Wittmack) in the state of Paraná. Brazil. Rev Iberoam Micol 24:167–170

Castelo AT, Martins W, Gao GR (2002) TROLL-tandem repeat occurrence locator. Bioinformatics 18:634–636

Couch BC, Kohn LM (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. Mycologia 94:683–693

Cruz MF, Maciel JLN, Prestes AM, Bombonatto EAS, Pereira JF, Consoli L (2009) Caracterização genética e fenotípica de isolados de *Pyricularia grisea* do trigo. Trop Plant Pathol 34:393–401

Cruz MF, Prestes AM, Maciel JLN, Scheeren PL (2010) Resistência parcial à brusone de genótipos de trigo comum e sintético nos estádios de planta jovem e de planta adulta. Trop Plant Pathol 35:024–031

Cruz CD, Bockus W, Pedley K, Peterson G, Stack J, Tang X, Valent B (2011) Resistant among U.S. wheat (*Triticum aestivum*) cultivars to the wheat pathotype of *Magnaporthe oryzae*. Phytopathology 101(Suppl.):s220

Dutech C, Enjalbert J, Fournier E, Delmotte F, Barrès B, Carlier J, Tharreau D, Giraud T (2007) Challenges of microsatellite isolation in fungi. Fungal Genet Biol 44:933–949

Duveiller E, Hodson D, Tiedmann A (2010) Wheat blast caused by *Magnaporthe grisea*: a reality and new challenge for wheat research. In: 8th International Wheat Conference, 2010, Saint Petersburg, Russia. 8th International Wheat Conference - Abstracts. Saint Petersburg: Vavilov Research Institute of Plant Industry. pp 247–248

Galbieri R, Urashima AS (2008) Caracterização, compatibilidade e ocorrência de reprodução sexual entre isolados de *Pyricularia grisea* de diferentes hospedeiros. Summa Phytopathol 34:22–28

Goulart ACP, Paiva FA (1990) Perdas em trigo (*Triticum aestivum*) acusadas por *Pyricularia oryzae*. Fitopatol Bras 15:120

Goulart ACP, Paiva FA (1992) Incidência da brusone (*Pyricularia oryzae*) em diferentes cultivares de trigo (*Triticum aestivum*) em condições de campo. Fitopatol Bras 17:321–325

Goulart ACP, Paiva FA (1993) Response of wheat cultivars and breeding lines to blast (*Pyricularia grisea*) under field conditions, 1991. Ann Wheat Newsl 9:109–112

Goulart ACP, Paiva FA (2000) Perdas no rendimento de grãos de trigo causada por *Pyricularia grisea,* nos anos de 1991 e 1992, no Mato Grosso do Sul. Summa Phytopathol 26:279–282

Goulart ACP, Sousa PG, Urashima AS (2007) Danos em trigo causados pela infecção de *Pyricularia grisea*. Summa Phytopathol 33:358–363

Igarashi S (1990) Update on wheat blast (*Pyricularia oryzae*) in Brazil. In: Saunders DA (Ed) A proceeding of the International Conference - wheat for nontraditional warm areas. CIMMYT, Mexico. p. 480–483

Igarashi S, Utiamada CM, Igarashi LC, Kazuma AH, Lopes RS (1986) *Pyricularia* em trigo. 1. Ocorrência de *Pyricularia sp*. no estado do Paraná. Fitopatol Bras 11:351–352

Kaye C, Milazzoa J, Rozenfeldb S, Lebrunc MH, Tharreau D (2003) The development of simple sequence repeat markers for *Magnaporthe grisea* and their integration into an established genetic linkage map. Fungal Genet Biol 40:207–214

Kijas JM, Fowler JC, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16:656–662

Kim NS, Park NI, Kim SH, Kim ST, Han SS, Kang KY (2000) Isolation of TC/AG repeat microsatellite sequences for fingerprinting rice blast fungus and their possible horizontal transfer to plant species. Mol Cells 10:127–134

Kistler HC (1997) Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology 87:474–479

Kohli MM, Mehta YR, Guzman E, de Viedma L, Cubilla LE (2011) Pyricularia blast: a threat to wheat cultivation. Czech J Genet Plant Brred 47:s130–s134

Lebrun MH, Capy MP, Garcia N, Dutertre M, Brygoo Y, Notteghem JL, Valès M (1990) Biology and genetics of *Pyricularia oryzae* and *P. grisea* populations: current situation and development of RFLP markers. In: Rice Genetics Conference, Los Banos, IRRI, pp 1–12

Lim S, Notley-McRobb L, Lim M, Carter DA (2004) A comparison of the nature and abundance of microsatellites in 14 fungal genomes. Fungal Genet Biol 41:1025–1036

Liu K, Muse SV (2005) PowerMarker: integrated analysis environment for genetic marker data. Bioinformatics 21:2128–2129

Maciel JLN (2011) *Magnaporthe oryzae*, the blast pathogen: current status and options for its control. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 6:1–8

Martins W, de Sousa D, Proite K, Guimaraes P, Moretzsohn M, Bertioli D (2006) New softwares for automated microsatellite marker development. Nucl Acids Res 34:e31

McDonald BA, Linde C (2002) The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica 124:163–180

Mehta YR, Riede CR, Campos LAC, Kohli MM (1992) Integrated management of major wheat diseases in Brazil: an example for the Southern Cone region of Latin America. Crop Prot 11:517–524

Oliveira EJ, Pádua JG, Zucchi MI, Camargo LEA, Fungaro MHP, Vieira MLC (2005) Development and characterization of microsatellite markers from the yellow passion fruit (*Passiflora edulis* f. *flavicarpa*). Mol Ecol Notes 5:331–333

Oliveira EJ, Pádua JG, Zucchi MI, Vencovsky R, Vieira MLC (2006) Origin, evolution and genome distribution of microsatellites. Genet Mol Biol 29:294–307

Orbach MJ, Chumley FG, Valent B (1996) Electrophoretic karyotypes of *Magnaporthe grisea* pathogens of diverse grasses. Mol Plant Microbe Interact 9:261–271

Pontecorvo G, Roper JA, Hemmons LM, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141–238

Prabhu AS, Filippi MC, Castro N (1992) Pathogenic variation among isolate of *Pyricularia oryzae* infecting rice, wheat and grasses in Brazil. Trop Pest Manag 38:367–371

Prestes AM, Arendt PF, Fernandes JMC, Scheeren PL (2007) Resistance to *Magnaporthe grise*a among Brazilian wheat genotypes. In: Buck HT, Nisi JE, Salomón N (eds) Wheat production in stressed environment. Springer, Dordrecht, pp 119–123

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, pp 365–386

Saghai Maroof MA, Biyashev RM, Yang G, Zhang Q, Allard RW (1994) Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. Proc Natl Acad Sci USA 91:5466–5470

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Specht CA, Dirusso CC, Novotny CP, Ullrich RC (1982) A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. Anal Biochem 119:158–163

Staden R (1996) The Staden sequence analysis package. Mol Biotechnol 5:233–241

Tufan HA, McGrann GRD, MacCormack R, Boyd LA (2012) *TaWIR1* contributes to post-penetration resistance to *Magnaporthe oryzae*, but not *Blumeria graminis* f. sp. *tritici*, in wheat. Mol Plant Pathol 13:653–665

Urashima AS, Kato H (1994) Varietal resistance and chemical control of wheat blast fungus. Summa Phytopathol 20:107–112

Urashima AS, Igarashi S, Kato H (1993) Host range, mating type and fertility of *Pyricularia grisea* from wheat in Brazil. Plant Dis 77:1211–1216

Urashima AS, Hashimoto Y, Don LD, Kusaba M, Tosa Y, Nakayashiki H, Mayama S (1999) Molecular analysis of the wheat blast population in Brazil with a homolog of retrotransposon MGR583. Ann Phytopathol Soc Jpn 65:429–436

Urashima AS, Lavorent NA, Goulart ACP, Mehta YR (2004) Resistance spectra of wheat cultivars and virulence diversity of *Magnaporthe grisea* isolates in Brazil. Fitopatol Bras 29:511–518

Urashima AS, Grosso CRF, Stabili A, Freitas EG, Silva CP, Netto DCS, Franco I, Mérola Bottan JH (2009) Effect of *Magnaporthe grisea* on seed germination, yield and quality of wheat. In: Wang GL, Valent B (eds) Advances in Genetics. Springer, Genomics and Control of Rice Blast Disease, pp 267–277

Valent B, Chumley FG (1994) Avirulence genes and mechanisms of genetic instability in the rice blast fungus. In: Zeigler RS, Leong S, Teng PS (eds) Rice Blast Disease. CAB International, UK, pp 111–134

Wang Y, Kaye C, Bordat A, Adreit H, Milazzo J, Zheng X, Shen Y, Thearreau D (2005) Construction of genetic linkage map and location of avirulence genes from cross CH63 and TH16 of *Magnaporthe grisea*. Chinese J Rice Sci 19:160–166

Zhan SW, Mayama S, Tosa Y (2008) Identification of two genes for resistance to *Triticum* isolates of *Magnaporthe oryzae* in wheat. Genome 51:216–221

Zheng Y, Zhang G, Lin F, Wang Z, Jin G, Yang L, Wang Y, Chen X, Xu Z, Zhao X, Wang H, Lu J, Lu G, Wu W (2008) Development of microsatellite markers and construction of genetic map in rice blast pathogen *Magnaporthe grisea*. Fungal Genet Biol 45:1340–1347