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Research Article

Microsatellite polymorphism in wheat from Brazilian cultivars; inter- and intra-varietal studies

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Abstract

Eleven samples of wheat (*Triticum aestivum*) from different Brazilian cultivars and six American varieties were compared for polymorphism, using primers for nine different STR loci. STR analysis of DNA from single grains of the Brazilian varieties showed that for most loci there was very little intra-cultivar polymorphism. The polymorphic variation observed for Brazilian cultivars was similar to that seen in the American varieties. For the Brazilian cultivars PCR analysis could be performed on only one half of a grain. The American samples required more seeds for analysis. The nucleotide sequences of five amplified microsatellites selected at random from the Brazilian samples were also determined and compared to those of the Chinese Spring variety. Although generally the dinucleotide sequence repeat was preserved for most loci, there were significant differences in sequences interspersed within the repeat domain. This result suggested that it may be possible to unequivocally identify the geographical origin of the cultivar by inspection of the DNA sequences of the repeat region.

Keywords: wheat, Brazilian, cultivars, DNA, STR, polymorphism.

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Introduction

Genomic polymorphism between individuals can arise through several different mechanisms, which include single nucleotide changes, deletions and insertions and above all, through variable numbers of simple sequence repeats. As somatically stable, co-dominant, locus specific Mendelian markers, microsatellites have been extensively used for DNA profiling, aiming primarily at the determination of the number and types of repeats. These parameters constitute tags that are informative within the context of genetic identity of individuals and to a certain degree, as a measure of geographical dispersion.

PCR-based genotyping is favored over other techniques because it is fast, does not require radioactive labeling and can be carried out with very small amounts of genomic DNA. PCR-based genomic polymorphism has been detected in cultivated hexaploid wheat *Triticum aestivum* L. (Ma *et al*., 1996). This study and others have shown that even though the genome of bread wheat is hexaploid and extremely large, the microsatellite allelic patterns generated through PCR are capable of individualizing cultivars. This should not come as a surprise since more than 80% of the wheat genome is composed of repetitive DNA (Stein *et al*., 2001; Hayden and Sharp, 2001a).

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Brazil is not a major producer of wheat and therefore, data on the regional polymorphism of this agronomically important plant is scarce, if non-existent. In an attempt to study genome diversity within and between cultivated biotypes from several stocks, we have carried out a preliminary set of experiments using previously described primers (Plaschke *et al*., 1995; Röder *et al.*, 1995). The results obtained with the microsatellites represent a first effort with Brazilian varieties that may be expanded to include genes. A more detailed study focusing on targeted regions of specific wheat genes may be important for grain grading, *i.e.*, finding polymorphic genes related to a particular trait of economic importance.

Materials and Methods

Wheat samples

Samples from Brazilian wheat cultivars were supplied by Dr. Sergio Dotto, EMBRAPA, Paraná, Brazil. These were B1 (BR-18), B2 (BR-35), B3 (BRS-49), B4 (BRS-120), B5 (BRS-192), B6 (BRS-193), B7 (CEP-24), B8 (IAPAR-53), B9 (PF-940384), B10 (WT-96061) and B11 (WT-96063). American wheat samples were obtained from commercial cultivars (US Wheat Associates) and were a kind gift from Moinho Vera Cruz, Minas Gerais, Brazil. The American cultivars were E1 (Hard Red Winter), E2 (Hard Red Spring), E3 (Soft Red Winter), E4 (Hard Durázio), E5 (Hard White) and E6 Soft White.

DNA extraction

DNA was extracted from single seeds unless otherwise stated according to the method described by Plaschke *et. al* (Plaschke *et al*., 1995), using slight modifications. Briefly, each seed was sliced and the half corresponding to the germ pole was wrapped in laminated paper and crushed with a hammer at room temperature. The resulting powder was incubated in 0.5 mL of a buffer consisting of 100 mM Tris-HCl pH 8.0, containing 500 mM NaCl, 50 mM EDTA, 1.25% SDS (V/V) and 2.5 µL of 2-mercaptoethanol. The buffer was used fresh for every extraction. Samples were then incubated at 65 °C for 40 min with occasional gentle shaking. After the incubation, 0.5 mL of a mixture of chloroform : isoamylic alcohol (24:1 v:v) was added and the suspension was centrifuged at 3500 g for 10 min in a microcentrifuge. 0.4 µL of the aqueous phase were then transferred to a new tube to which 2 volumes of ice cold absolute ethanol were added. The DNA was spooled out and washed once in 70% ethanol. The pellet was air dried and dissolved in 50 µL of milli-Q water. A 0.5 µL aliquot was withdrawn for agarose gel electrophoresis. For the American wheat samples, 10 seeds were used for DNA extraction, carried out as above.

PCR reaction

The sequences of the primers and the PCR conditions were essentially those described by Röder et al (Röder *et al*., 1995). The primers used in the present study corresponded to the following loci: WMS-2, WMS-3, WMS-5, WMS-6, WMS-11, WMS-18, WMS-43, WMS-44 and WMS-46 from the Cheyenne cultivar of *T. aestivum* and are indicated in the legends to the figures.

Denaturing polyacrylamide sequencing gels

Amplified DNA samples were fractionated in 0.4 mm, 6% polyacrylamide gels containing 7 M urea, using a 0.5 X Tris-borate-EDTA buffer (TBE) pH 8.0. After separation the DNA bands were visualized by silver staining (Sanguinetti *et al*., 1994).

DNA sequencing

The PCR-amplified DNA was sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB), according to the manufacturer's instructions. [α-^{33}P] ddNTPs 16 MB/mL were obtained from Amersham-Pharmacia.

Results

Polymorphism of Brazilian cultivars

The PCR results using primers for loci WMS-44 and WMS-46 are shown in Figure 1. These results were obtained with Brazilian cultivars and correspond to the amplification of single seeds. It can be seen that the amplicons

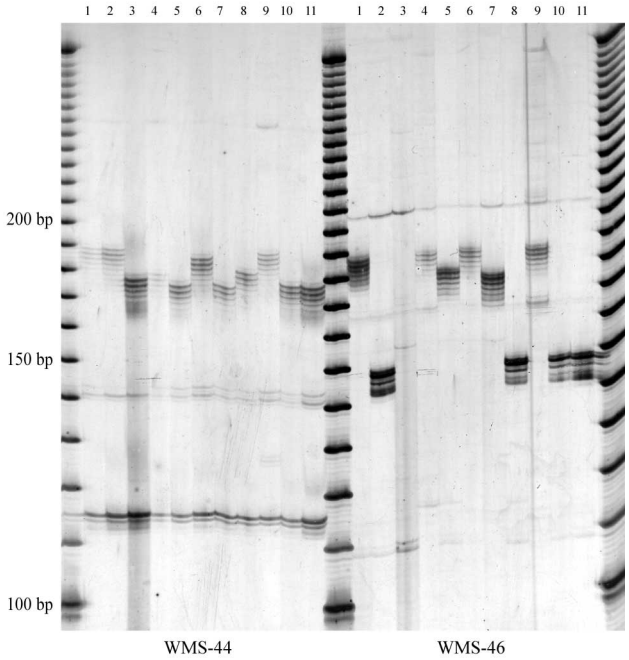

Figure 1 - Inter-cultivar polymorphism of Brazilian wheat for loci WMS-44 and WMS-46. DNA from half a seed was extracted, amplified with primers WMS-44 and 46, respectively, and fractionated on a denaturing polyacrylamide gel as described in methods. For each locus, the numbers on top of each lane represent the cultivars B1, B2, B3, B4 B5, B6, B7, B8, B9, B10 and B11, respectively. The molecular weight standard consisted of a 10 bp ladder.

exhibited a molecular mass in the range of 170-190 bp for WMS-44 and 150-190 bp for locus WMS-46, respectively. Estimation of the MW of the amplimer was always based on the band displaying the highest mass because as a rule, amplification of repeats consisting of dinucleotides generates an array of bands (Hayden and Sharp, 2001a; Hayden and Sharp, 2001b;Brian *et al*., 1997; Rodriguez and Zapata, 2002). The polymorphisms observed in Figure 1 are fairly typical of the pattern obtained for most loci, except for locus WMS-43. In this particular case, the pattern obtained for both, Brazilian and American cultivars was essentially monomorphic. Although this locus would not be informative in the context of polymorphic analysis, it would still be useful as internal marker for electrophoresis, similarly to the monomorphic k562 marker used in human genotyping (Inman and Rudin, 1997).

Polymorphism of American cultivars

The polymorphism of the wheat from the American cultivars is shown in Figure 2, A and B, for the same loci as in Figure 1. Although in some samples the PCR products had a MW below 150 bp, as shown for sample 4 in locus WMS-44, the overall pattern was similar to that of the Brazilian cultivars.

Since the polymorphism analysis of the Brazilian cultivars shown in Figure 1 was carried out with single seeds, the possibility of intra-cultivar variation had to be

considered. Thus, experiments were carried out in order to detect variations of several seeds from the same cultivar. To that end, 11 seeds from cultivar B3 (lane 3, Figure 1) were amplified with locus WMS-44 and fractionated as described in methods. Apart from the fact that locus WMS-44 consistently generated a reproducible pattern, there was no other criterion for the selection of this particular cultivar. The results are shown in Figure 3. It can be seen that most samples generated the same pattern. The only possible exception might have been the pattern obtained for the PCR products of the DNA from the seed fractionated in lane 8. However, the heterogeneous behavior of this sample seems to be due mainly to a difference in the intensity of the band bearing the highest MW. The other bands of the array co-migrated with the DNA from the adjoining lanes and for all practical purposes, the sample amplified in lane 8 did not display an electrophoretic migration exhibiting the same degree of variation as the samples in Figures 1 and 2. Therefore, these patterns obtained indicated that there was no intra-cultivar polymorphism, a result which could be used to evaluate the genetic homogeneity of seeds in batches of grains. There was no attempt to determine intra-cultivar polymorphism in the American cultivars, since we could not obtain enough material from single grains.

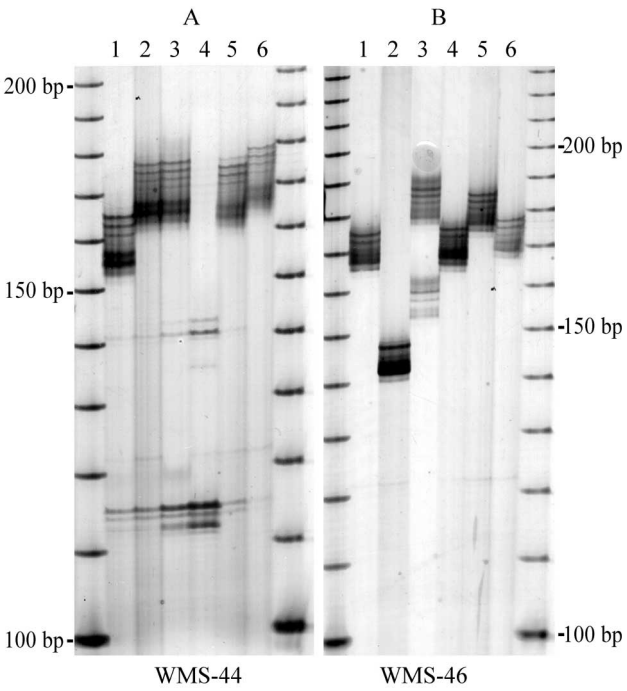

Figure 2 - Inter-cultivar polymorphism of American wheat for *locus* WMS-44 (A) and locus WMS-46 (B). DNA from 10 seeds was extracted, amplified with primers WMS-44 and WMS-46, respectively, and fractionated on a denaturing polyacrylamide gel as described in methods. For each locus, the numbers on top of each lane represent cultivars E1, E2, E3, E4, E5 and E6. The molecular weight standards consisted of a 10 bp ladder.

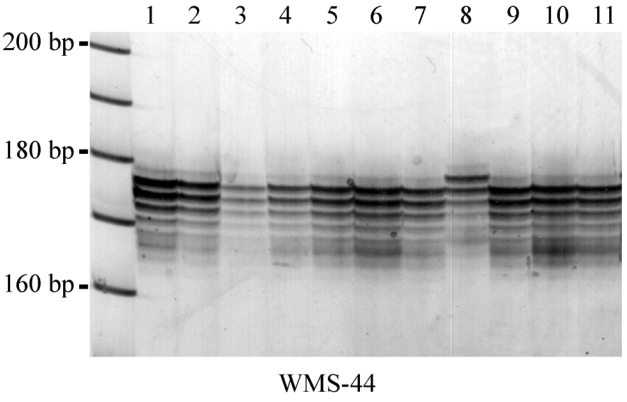

Figure 3 - Intra-cultivar polymorphism of Brazilian wheat for locus WMS-44. DNA was extracted from 11 different seeds from cultivar B3 (lane 3 from Figure 1) and each was amplified with primers WMS-44 and fractionated on a denaturing polyacrylamide gel, as described in methods. The numbers on top of each lane represent individual seeds. The molecular weight standard consisted of a 10 bp ladder.

DNA sequences of the microsatellites

Finally, we addressed the question whether the sequences of the microsatellites of the Brazilian cultivars were identical to those previously reported (Röder *et al*., 1995). The results in Table 1 summarize the comparisons between the samples from some Brazilian cultivars and those published for the Chinese Spring variety using five different loci (Röder *et al*., 1995). Again, the selection of cultivars to be sequenced and compared to the American cultivars obeyed no specific criteria.

Except for locus WMS-11 that generated identical sequences, there were significant variations of the basic motif for the other loci. Although the same repeats were present in samples of both cultivars, the Brazilian samples displayed unique sequences interspersed within the microsatellite region. These variations were consistent and therefore, might constitute a marker for the cultivar. Admittedly, the present study concentrated on a relatively small sample. However, a more extensive profiling of the amplicon pool might eventually permit the geographical identification of the cultivars, based on both their microsatellites pattern and the DNA sequences.

Discussion

Dinucleotide repeats are particularly informative because they are evenly spread throughout the genomes and typically are highly polymorphic. In the present study we confirmed such polymorphism on samples obtained from Brazilian cultivars. At a first glance, judging by the molecular mass of the PCR products alone, inspection of the patterns generated by PCR amplification of known loci in samples of wheat from Brazilian cultivars did not reveal any distinguishing features when compared to polymorphism of the American varieties and others previously reported (Röder *et al*., 1995). However, the DNA sequence of the microsatellite region showed that the wheat from Brazilian cultivars was indeed different from those reported for the same loci. This should not be surprising since the tracts

Table 1 - omparison of DNA sequences of wheat from Brazilian cultivars with *T. aestivum* cultivar Chinese Spring.

Cultivar	*Locus*	DNA sequence of Brazilian cultivars	DNA sequence Chinese spring	Allele size (bp)
B5-A	WMS-5	$C(TC)_{31}T(GT)_{14}(GA)_9GCACCC$	$(CT)_{23}(TT)_2(GT)_{12}(GA)_{10}$	179
B6-A		$C(TC)_{>29}TTT(GT)_{11}(GA)_{10}CACCC$		179
B7-A		$C(TC)_{>29}[C/T]T[G/T]T(GT)_{11}(GA)_9CACCCAG$		173
B8-A		$C(TC)_{>31}[C/T]TT[G/T]T(GT)_{11}(GA)_{10}CACCC[A/C][C/G]C$		179
B5-A	WMS-11	$A(TA)_4CATA(CA)_{17}(TA)_8CA$	$(TA)_6CATA(CA)_{19}(TA)_6$	198
B6-A		$(TA)_3CATA(CA)_{17}(TA)_8CA$		208
B7-A		$(TA)_6CATA(CA)_{17}(TA)_8$		206
B3-A	WMS-2	$[C/T]A(CA)_9[A/G]CGAGCG[A/G](CA)_2CT$	$(CA)_{18}$	126
B4-A		$A[C/T]A(CA)_7C[A/G]C[A/G](CA)_4TAA[A/C]A$		122
B3-A	WMS-44	$C(G)_5\ (AG)_{>34}$	$(GA)_{28}$	177
B1-A	WMS-18	$TT(AC)_{>37}$	$(CA)_{17}(GA)(TA)_4$	189

of mono-, di- and trinucleotide repeats that make up the microsatellites are known to constitute mutation hot spots (da Silva and Reha-Krantz, 2000). The PCR products themselves suggest that this is the case. Figures 1 and 2 show that, except for locus WMS-43, invariably an array of bands was produced, a result which clearly demonstrates that slippage of DNA polymerase occurred. This phenomenon has been described in several other reports (Hayden and Sharp, 2001a; Hayden and Sharp, 2001b; Brian *et al*., 1997). Although the accepted view on the origin of such arrays is that they are generated by polymerase "slippage", we were able to show that irrespective of its size, each of the bands within the same array was able to generate all the others when used as a template for re-amplification under the same conditions (results not shown).

Although the polymerase stutter might favor the mis-sizing of rare alleles differing by a single repeat unit, in the present study this did not prevent comparisons between separate cultivars. Interestingly, WMS-43, the only monomorphic locus, was also the only one generating a single band of 146 bp (results not shown). This microsatellite is composed of simple CA repeats and has, therefore, the potential to create the same array of bands observed for the other loci. The reason for this anomalous behavior remains unexplained.

Taken together, the results obtained for the other loci suggest that due to the hypermutability caused by dinucleotide repeats, individual DNA microsatellite sequences may be expected in any isolated wheat cultivar. It is thus conceivable that the random changes in the frequencies of the alleles over several generations in the cultivar will inevitably give rise to distinct sequences. Such a phenomenon might allow the geographical identification of a particular cultivar, although this hypothesis must be tested in a broader survey. The fact that no intra-cultivar variations were found indicated also that the population within each cultivar is genetically homogeneous, a feature that might be exploited by end-users interested in checking the purity of a certain batch of grains. As an added bonus, examination of the polymorphic pattern may be helpful in determining the geographical origin of a particular batch of seeds.

Brazil produces only a small fraction of the wheat it consumes. Until recently Brazil ranked as the fifth biggest importer of wheat in the world. As a rule the Brazilian mills resort to rheological tests to grade grain. These tests designed to determine the physical dough properties are time consuming and often require a relatively high amount of grains in order to prepare enough dough for the farinographs, viscographs, etc. The results presented here pave the way towards alternatives to rheological methods. Genotyping of wheat genes, rather than the non-coding genome may afford a quicker and more economic approach to grain grading. The physical properties of the dough are phenotype-associated and derived mainly from the structures of the proline and glutamine rich prolamins glutenine and gliadin, these genes are also polymorphic (Colot *et al*., 1989; Shewry *et al*., 1988). Indeed, the codons for proline and glutamine, CCA and CAA, respectively, would produce long tracts of dinucleotide repeats in the genomic sequences of these genes and thus render them amenable to slippage.

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