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Genetic Diversity and Molecular Characterization of Brazilian Wheat Varieties Obtained by Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Author ALS made the DNA extraction and all the PCR reactions. Authors MBO and ESNV managed the Gene Mapper analysis. Authors VSM and FAF managed the literature searches. Author IS designed the study, analyzed the data and wright the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Evaluating the genetic diversity and molecular characterization of wheat varieties in Brazil, using microsatellite markers.
Study Design: Random sampling of seeds from 32 varieties, was done.
Place and Duration of Study: Biotechnology lab, Coodetec, BR 467, km 98. Cascavel, PR, Brazil, between July 2011 to July 2012.
Methodology: Thirty-two varieties were evaluated with 23 markers, using capillary gel electrophoresis. After DNA extraction, and gels scoring, the genetic distances were obtained, the clustering by UPGMA method, the frequency of each allele and Probability of Random Identity (PRI).

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Results: It was observed two to eight alleles by loci and genetic distances ranging from 0.31 to 0.90. The varieties were grouped into 11 groups. From the estimated PRI, 15 markers were identified that identify all 32 varieties with a maximum of 0.0001% PRI. High variability among wheat varieties was observed and also high efficiency in the identification of varieties with microsatellite markers.
Conclusion: This approach can be used in breeding programs and for the protection of intellectual property of wheat varieties breeders.

Keywords: Triticum aestivum; genetic variability; cluster analysis; probability of random identity; plants protection.

1. INTRODUCTION

Genetic diversity is the basis for plant breeding. Thus, knowledge of the genetic diversity of a species has a significant impact on the improvement of crop plants, especially the diversity among elite varieties. In this case the diversity can be used directly in the generation of breeding populations. Knowing the genetic diversity in specific germplasm, one can define how to increase variability in the future.

Molecular markers can be used to characterize genetic diversity in germplasm collections, certification of genetic purity of seeds, characterization of varieties, protection of intellectual property in addition to genetic mapping applications, marker assisted selection, prediction of hybrids between other applications [1,2].

Molecular markers can also be used to characterize varieties for protection of intellectual property [3,4,5]. From the observed allele frequencies of molecular markers loci, one can estimate the probability that two samples are of the same variety [2,6].

The Brazilian wheat production is 0.7% of world production that was just over 714 million tons in 2011 [7]. Despite an insignificant production globally, wheat is an important source of income for farmers in Southern Brazil [6]. In this region, wheat is grown annually in about 2 million hectares [8], and is the main option cultivation for the winter. Because of its regional importance, the genetic breeding programs of wheat in the country have obtained increasingly more productive varieties resistant to diseases, and with good grain quality for the industry.

Mostly wheat breeding programs in Brazil use traditional methods of selection through phenotypic evaluations. Rarely use the aid of molecular markers in germplasm characterization or marker assisted selection of plants or progenies.

This study aimed to accomplish a genetic diversity analysis and cluster in wheat varieties in Brazil, from the genetic distances obtained by microsatellite markers; and identifying a set of molecular markers for the accurate identification of varieties, from the estimated probability of random identity, using the allele frequencies observed for each locus of molecular markers.

2. MATERIALS AND METHODS

2.1 Plant Material and DNA Analysis

Thirty-two Brazilian wheat varieties were used in the analysis (Table 1). For DNA extraction, 200 seeds of each variety were ground, and 50 mg of ground powder was used for DNA extraction [9].

PCR reactions were performed using 23 microsatellite markers (Table 2) with the sense primer fluorescently labeled with 6FAM, PET, VIC or NED. In a final volume of 20 uL, PCR solution contained 75 ng DNA, 3 mM $MgCl_2$, 3.2 mM Tris, 8 mM KCl, 200 uM dNTPs, 0.8 uM of each primer (forward and reverse) and 1 unit of Taq DNA polymerase. Amplifications were performed on Thermo Hybaid thermocycler (Ashford, Middlesex, United Kingdom) set at a cycle of 94ºC for 3 minutes; 45 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 45 seconds; and a final cycle at 72ºC for 20 minutes.

PCR reactions were performed in singleplex, and the electrophoresis in multiplex. Multiplex was set up by combining markers containing four different fluorescence, in a single sample, by combining 5 uL of each individual amplification product. This initial mixture was withdrawn and diluted with 1uL to 50 uL of ultrapure water. Then 1 ul was transferred to another PCR plate and then added 8.85 uL hi-di formamide and 0.15 uL

molecular weight standard 500 LIZ orange staining (Applied Biosystems). The amplified fragments were separated by capillary gel electrophoresis, using the automatic sequencer ABI 3130xl, according to the manufacturer's recommendation. The data were coded with the aid of Gene Mapper program (Applied Biosystems).

2.2 Data Analysis

The genetic relationships among varieties were evaluated by means of a similarity matrix obtained using the complement of the similarity index for codominant and multi-allelic data with the help of Genes software [10]. This ratio is obtained by dividing the total number of common alleles by the total number of alleles evaluated in each individual.

The varieties were grouped using hierarchical method UPGMA (Unweighted Pair-Group Mean Average) using STATISTICA software [11]. Genetic informativeness of each marker was assessed by the PIC (Polymorphism Index Content):

$$PIC = 1 - \sum_{j=1}^{n} p_{ij}^{2}$$

where p_{ij} is the frequency of the j^{th} allele for the i^{th} primer. Allele frequencies were estimated by dividing the number of times each allele appeared in a population of wheat varieties by the total number of alleles observed for each marker. Although wheat is a hexaploid species, it is an allohexaploid, and segregates as a diploid, and there are two alleles at each locus. For homozygotes, the alleles were counted as

Table 1. Wheat varieties used in the study of genetic diversity and their genealogies.

Variety	Genealogy
AVANTE	PF 89232/2*OR 1
BRS 179	BR 35/PF 8596/3/ PF 772003*2/PF 813//PF 83899
BRS 210	CPAC 89118/3/BR 23//CEP 19/PF 85490
BRS 220	EMBRAPA 16/TB 108
CD 101	AURORA/UP 301//OCEPAR 12
CD 102	IAC 5/ALDAN"S"//CEP 7780
CD 103	PG 864/OCEPAR 14
CD 104	PFAU"S" / IAPAR 17
CD 105	PFAU"S"/2*OCEPAR 14//IAPAR 41
CD 106	PG 864/GENARO
CD 107	COC 75*2/BR 23//BR 35
CD 108	TAM 200 / TURACO
CD 109	MUNIA/BAGULA
CD 110	ANAHUAC 75 / EMBRAPA 27
CD 111	EMBRAPA 27/OCEPAR 18//ANAHUAC 75
CD 112	IOC 905 / PG 877
CD 113	EMBRAPA 27 / OC 946
CD 114	PF 89232 / OC 938
CD 115	OC 926 / OC 935
CD 116	MILAN / MUNIA
CD 117	PF 87373 / OC 938
CD 118	VEERY "S"/KOEL//SIREN/3/ARIVCH 92
CD 119	BRS 49 / CDI 0303
CD 120	RUBI / CD105
CD 150	CD 104 / CD 108
FRONTANA	FRONTEIRA/MENTANA
FUNDACEP 46	CEP 88132/PG 876/3/BR 34//CORRIDON
FUNDACEP 50	CEP 88132/PG 876/3/BR 34//CORRIDON
FUNDACEP 52	CEP 88132/PG 876/3/BR 34//CORRIDON
IPR 85	IAPAR 30/BR 18
ONIX	CEP 24/RUBI"S"
VANGUARDA	OR 1/PARANÁ(RM)

two copies, and heterozygous loci each allele was counted once. The total number of alleles corresponds to the number of varieties multiplied by two, since each individual has two alleles at each locus.

The probability of random identity (PRI) was calculated as described by Schuster et al. [2]:

$$PRI = \left(\prod_{j=1}^{n} P_{ij} \right) x100$$

where P_{ij} is the frequency of i^{th} allele in j^{th} loci and *n* is the number of locus evaluated. The product of the frequencies of alleles is multiplied by 100 to obtain the probability as a percentage. The estimate of PRI was performed for each variety, using the frequencies of alleles observed in each variety at all loci of molecular markers.

Table 2. Microsatellite markers used in the characterization of 32 wheat varieties

Marker[1]	Core SSR[2]	Staining	Linkage group.[3]
DuPw115	ACG	VIC	5B
DuPw205	AAG	6FAM	5B
DuPw217	AAG	NED	3A, 3B
DuPw167	AAGCAT	6FAM	6A
Xbarc12	TAA	6FAM	3A
Xbarc343	No information	PET	4A
Xgwm003	CA	VIC	3D
Xgwm102	CT	NED	2D
Xgwm135	GA	6FAM	1A
Xgwm149	GA	PET	4B, 4D
Xgwm155	CT	VIC	3A
Xgwm160	GA	PET	4A
Xgwm161	CT	NED	3D
Xgwm164	CT	6FAM	1A
Xgwm165	GA	PET	4A,4B,4D
Xgwm219	GA	PET	6B
Xgwm232	GA	GA	1D
Xgwm247	GA	VIC	3A,3B,6R
Xgwm257	GT	6FAM	2B
Xgwm304	CT	VIC	5A
Xgwm413	GA	6FAM	1B
Xgwm44	GA	VIC	7D
Xgwm526	CT	NED	2A,2B,7A, 7B

[1] The sequences of the primers are available in http://wheat.pw.usda.gov/GG2/index.shtml
[2] Repeating unit of the microsatellite
[3] Source: USDA (http://wheat.pw.usda.gov/GG2/index.shtml)

For rare alleles (very low frequency), the information was used conservatively, replacing the frequency of rare alleles for 5/2*n*, where *n* is the number of varieties [12].

A minimum set of markers was selected for the characterization of each variety and all varieties at the same time. For each variety to be characterized with the least possible number of markers, the markers that showed the lower frequency alleles in the variety were selected. The minimum number of primers needed for the characterization of a variety is a set of primers needed to obtain PRI equal or less than 0.0001%, that means a different variety may display the same set of alleles at this marker in less than 0.0001% of cases.

3. RESULTS AND DISCUSSION

Twenty-one of the 23 evaluated markers were polymorphic. In these 21 loci, 90 alleles were observed, ranging from 2 to 8 alleles per marker (Table 3), with a mean of 4.29. The PIC values ranged from 0.219 (Xgwm165) to 0.842 (Xgwm44), averaging 0.593. Only seven of the 21 polymorphic markers had PIC value below 0.5. The values obtained for PIC are high considering the number of samples and the samples composed only by elite germplasm, where the variability tends to be lower than in germplasm collections.

The genetic distances ranged from 0.31 (between varieties CD 110 and CD 117) to 0.90 (between varieties CD 108 and CD 109), averaging 0.61. Most of the estimated genetic distance was in the range between 0.50 and 0.70, but there was a high frequency distances within a range from 0.30 to 0.40 (Fig. 1).

Estimates of genetic distance between wheat varieties are similar or slightly higher than those reported in the literature. Salem et al. [13], using 48 microsatellite markers in wheat, obtained distance values ranging from 0.42 to 0.63. Ahmad [14] analyzing 13 wheat genotypes from different sources obtained genetic distances ranging from 0.10 to 0.70 using 43 microsatellite markers. Using 36 Brazilian varieties of wheat, Schuster et al. [2] obtained distances ranging from 0.10 to 0.88.

The cluster analysis obtained by the UPGMA method is shown Fig. 2. Cluster analysis using hierarchical methods such as UPGMA, is not intended to obtain groups of individuals, but

provide a preview of the existing nesting of the individuals analyzed, and the formation of some "clusters" of individuals. Even so, it is possible to make cuts in the dendrogram regions that allow separation of individual clusters, each cluster considering a group. By performing a cut in the distance 0.54, eleven groups were obtained. Five of these groups contain a single variety. The largest group has nine varieties. It can be seen in Fig. 2, that there is a large genetic variability in the level of microsatellite markers in this set of varieties, even considering that it is a set of elite varieties where it is expected that genetic variability is lower, due to selection exerted by breeding.

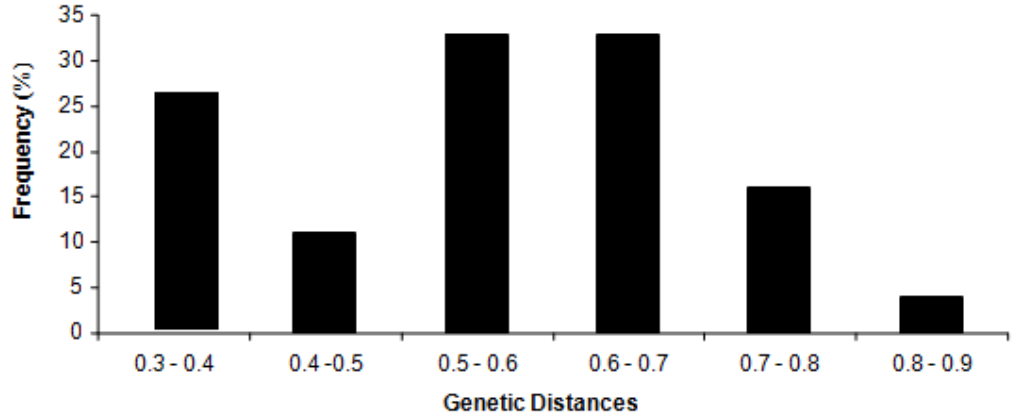

Fig. 1. Frequency distribution of genetic distances between 32 wheat varieties, obtained with 21 microsatellite markers

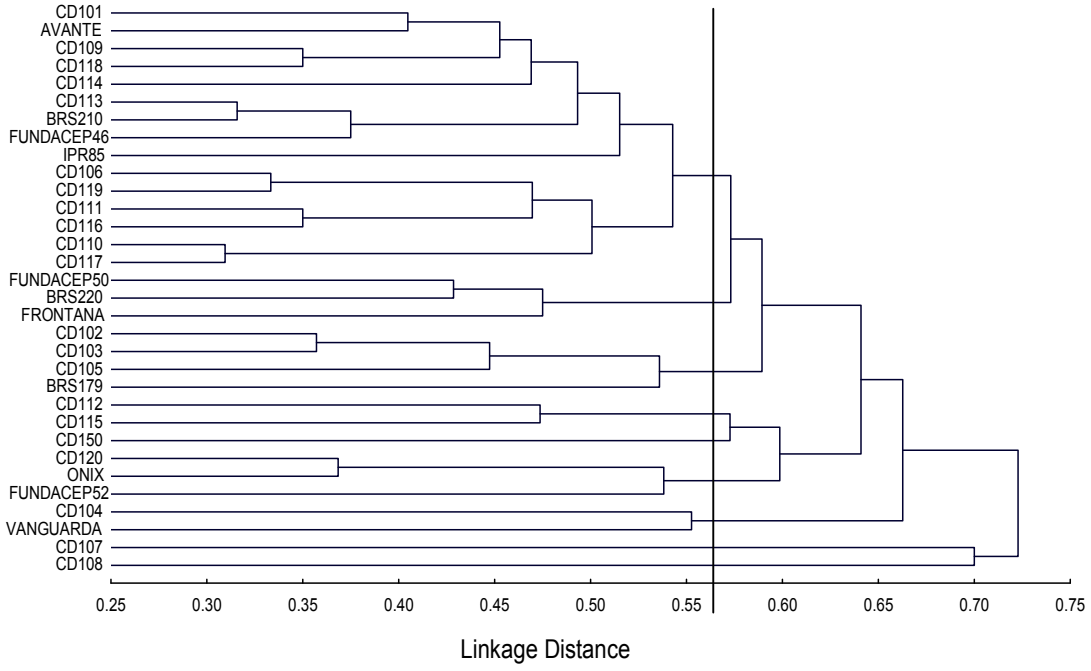

Fig. 2. Cluster analysis of 32 wheat varieties by UPGMA method, based on genetic distances obtained by the analysis of 21 microsatellite markers. The vertical line indicates the cut to form groups

Table 3. Alleles, allelic frequencies Polymorphism Index Content (PIC), estimated to 21 microsatellites loci from 32 wheat varieties

Marker	Num alleles	Allele	Frequency	PIC	Marker	Num alleles	Allele	Frequency	PIC
Dupw205	2	163	0.344	0.451	Xgwm149	2	156	0.258	0.383
		166	0.656				166	0.742	
Xgwm165	2	187	0.125	0.219	Dupw115	3	184	0.484	0.612
		193	0.875				187	0.156	
							190	0.359	
Dupw167	3	230	0.094	0.498	Xgwm135	3	96	0.250	0.398
		242	0.656				110	0.016	
		244	0.250				166	0.734	
Xgwm257	3	168	0.031	0.506	Xgwm164	4	118	0.703	0.468
		194	0.375				122	0.172	
		196	0.594				124	0.063	
							126	0.063	
Xgwm102	4	146	0.552	0.587	Xgwm247	4	148	0.115	0.388
		148	0.310				156	0.038	
		154	0.103				162	0.769	
		214	0.034				176	0.077	
Xgwm161	4	150	0.484	0.659	Dupw217	4	212	0.156	0.665
		152	0.266				221	0.422	
		154	0.078				224	0.359	
		178	0.172				269	0.063	
Xbarc343	4	149	0.484	0.675	Xbarc12	5	159	0.281	0.748
		158	0.188				183	0.250	
		164	0.188				189	0.313	
		203	0.141				201	0.063	
							207	0.094	
Xgwm155	5	125	0.031	0.739	Xgwm160	5	150	0.063	0.702
		141	0.141				180	0.391	
		145	0.313				184	0.250	
		147	0.328				186	0.281	
		151	0.188				190	0.016	
Xgwm526	5	122	0.115	0.751	Xgwm304	6	199	0.438	0.676
		130	0.192				201	0.344	
		136	0.269				203	0.094	
		156	0.346				207	0.031	
		160	0.077				217	0.031	
							221	0.063	
Xgwm413	6	87	0.047	0.667	Xgwm219	7	102	0.016	0.814
		89	0.313				150	0.219	
		97	0.031				156	0.031	
		99	0.469				168	0.203	
		103	0.031				180	0.156	
		105	0.109				184	0.203	
							192	0.172	
Xgwm44	8	169	0.031	0.842					
		173	0.078						
		175	0.141						
		177	0.266		-	-	-	-	-
		179	0.109						
		183	0.094						
		185	0.125						
		187	0.156						

Among the varieties with some degree of kinship, some were in the same group. This is the case of the varieties CD 103 and CD 105, which have in common, the variety Ocepar 14 in the genealogy, and grouped in the same group. The varieties CD 110 and CD 111 have in common the varieties Embrapa 27 and Anahuac 75 in their genealogy and also grouped in the same group.

However, the most closely related varieties were in different groups. Varieties Fundacep 46, Fundacep 50 and Fundacep 52 have the same genealogy and were classified into three different groups. The variety CD 120 is descended from the variety CD 105, and these two varieties were in separate groups. The pairs of varieties CD 120 and Onyx, CD 104 and CD 105, Avante and Vanguarda, CD 114 and CD 117, CD 103 and CD 106, BR 210 and CD 107, CD 111 and CD 113 have common parents in the genealogy, and all these pairs of varieties grouped in different groups.

Salem et al. [13] obtained consistent clusters when comparing the clustering of wheat varieties based on the genetic distances obtained by microsatellite markers and genealogy. Bertini et al. [15] also obtained consistent data between the grouping data from microsatellite markers in cotton, with the genealogy available. Priolli et al. [16] obtained consistent grouping of soybean varieties, using molecular data and the genealogy of the varieties. However, for some varieties, the cluster analysis based on data of molecular markers and genealogy do not coincided. Bonato et al. [17] using AFLP markers found no correlation between the dissimilarity estimates obtained by molecular markers and the genealogy of soybean varieties.

The lack of agreement between the cluster analysis from molecular data and the genealogy can be related to the selection practiced in the populations of breeding programs. Consider the case of any one variety by genealogy analysis, this variety must have half the genome of each parent. However, the selection can lead to a large proportion of the bias in the contribution of each parental genotype in descending variety. By genealogy analysis, this cannot be observed. But the analysis of molecular markers performs a sampling of the genome of plants, so that the input bias can be perfectly detected. Thus, the cluster analysis, when performed from markers that widely sample the genome of the varieties, reflects the genetic relationship between them better than genealogy.

Wheat is an autogamous species, and all the plants of a variety are homozygous. However, in several microsatellite loci, two alleles in the same variety were observed. In these cases, the variety is observed as heterozygous genotype, but is a result of mixing seeds homozygous for different alleles. In all 32 varieties, the observed heterozygosity ranged from zero to 29%, more frequently at 5% (Fig. 3).

This variation observed at the molecular level in some varieties is due to the production process of genetic seeds of each variety. Typically, the genetic seeds of wheat varieties are obtained from a bulk of plants, generally in the generation F8. This means that varieties may be composed of a mixture of very similar inbred lines visually indistinguishable by morphological descriptors. DNA analysis detects existing variations at the molecular level between these inbred lines which compose the variety.

These variations are probably not related to characteristics expressed by the plants, since field-level phenotypic differences are not observed between the plants of these varieties. However, it is necessary to consider the existence of internal variability in wheat varieties, when using molecular markers to distinguish varieties of wheat. The simple observation of one molecular difference may not be sufficient to declare two samples as being of different varieties. It is necessary to estimate the probability of identity of these samples from the allele frequencies obtained from a reference population of wheat varieties.

The information on the frequency of each allele in each loci is presented in Table 3. Few papers that study the genetic variability in crop plants present the frequencies of observed alleles. In Brazil, Priolli et al. [16] and Oliveira et al. [18] showed allele frequencies of microsatellite markers for soybean and Schuster et al. [6] showed allele frequencies of microsatellite markers in wheat. With the frequency information of alleles at each locus, one can calculate the probability of random identity (PRI) for each variety.

PRI is the probability that two different varieties present the same alleles in a set of markers, and this identity is due to chance [2]. The probability of exclusion of identity at random (PE) is the complement of the PRI, and is the probability that two varieties had the same alleles at a set of markers not by chance, but because the two samples are the same variety.

Using the PRI, one can define a set of molecular markers to be used in identifying each variety. When the PRI is equal or less than 0.0001%, it can be stated that a second sample will only contain the same alleles in this set of markers, if it is the same variety. That is, if another sample has the same alleles in this set of markers, the probability that the two samples are the same variety (PE) is equal to 99.9999%.

The probability of random identity may be used in cases where it is not possible to distinguish varieties based on morphological descriptors. In these cases, there are always doubts about how many molecular markers need to be used to declare that two samples are the same variety, when no differences are observed between the loci of molecular markers. Using the information of allele frequencies, we could identify a minimum set of markers to characterize each one of the 32 varieties, and a single set that simultaneously identifies all 32 varieties. In evaluating each individual variety, the number of markers required for the PRI 0.0001% ranged from five to 12 markers (data not shown). A set of 15 markers (Xgwm44, Xgwm102, Xgwm155, Xgwm160, Xgwm219, Xgwm257, Xgwm304, Xgwm526, Dupw115, Dupw167, Dupw205, Dupw217, Xbarc12, Xbarc343, Xbarc413) is able to identify all 32 varieties with PRI less than 0.0001% for all varieties.

In cases of loci with two alleles (observed heterozygosity), the sum of the frequency of both alleles was used to estimate PRI. This procedure was used because in the case of evaluation of genetic identity between samples, the presence of either of the two alleles cannot rule out the identity of the samples. Using the same approach, Oliveira et al. [18] identified a set of 11 microsatellite markers to identify 32 soybean varieties in Brazil with PRI less than or equal to 0.0001%.

In Brazil, the protection of the right of plant breeding companies is guaranteed by the Plant Variety Protection, provided by the SNPC (*Serviço Nacional de Proteção de Cultivares* - National Plant Protection Service). Although plant varieties cannot be patented in Brazil, the Plant Variety Protection guarantees to breeding companies the exclusive marketing or licensing of seeds of protected varieties, during the period of protection. In the case of unauthorized use of seed of a protected plant variety, one must provide evidence of genetic identity between the protected plant and misused variety. This evidence can be provided by PRI. The set of 15 selected molecular markers can be used in cases of intellectual property protection, when there is a suspicion of misuse of seeds. Furthermore, these markers can be used to evaluate the genetic purity of commercial seeds of wheat varieties.

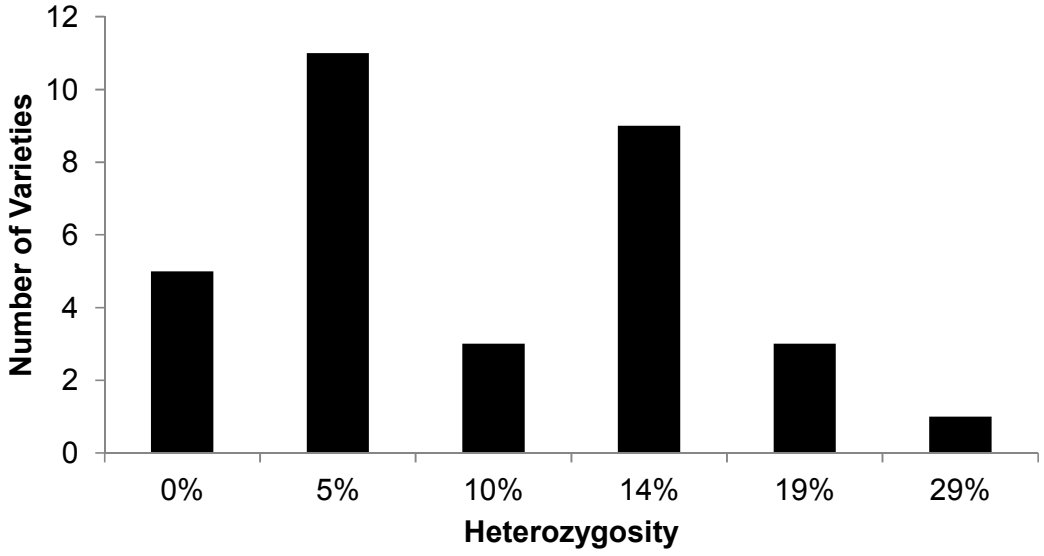

Fig. 3. Heterozygosity percent (loci with two alleles) observed in 32 varieties of wheat

In this work, an analysis of grouping a set of Brazilian wheat varieties were performed, demonstrating high variability of the sample investigated (Fig. 2). This variability can also be observed from the genealogy of the varieties (Table 1), which have few parents in common. It was also observed that most of the evaluated wheat varieties showed some heterozygosity, indicating at the molecular level there is a variability between inbred lines composing the varieties (Fig. 3). From the genetic frequencies of alleles observed at each locus of molecular marker, PRI of each variety was estimated, and obtained a set of 15 markers that can identify with 99.9999% probability in all 32 varieties.

The data obtained in this work can be used for the protection of intellectual property and genetic purity certification of these varieties. The cluster analysis results can still be used by wheat breeders to guide crossings and to evaluate the need to incorporate greater genetic variability in their breeding programs.

4. CONCLUSION

Commercial Brazilian wheat varieties have a high genetic variability. Using PRI, it is possible to identify with 99.9999% of certainty, all of the 32 evaluated varieties, and maybe, all of the Brazilian varieties. Microsatellite markers can be used for Plant Variety Protection with high level of confidence.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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