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Genetic variability among elite popcorn lines based on molecular and morphoagronomic characteristics

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Genet. Mol. Res. 16 (2): gmr16029243
Received September 12, 2016
Accepted March 8, 2017
Published June 29, 2017
DOI http://dx.doi.org/10.4238/gmr16029243

ABSTRACT. Knowledge of genetic diversity among genotypes and relationships among elite lines is of great importance for the development of breeding programs. Therefore, the objective of this study was to evaluate genetic variability based on the morphoagronomic and molecular characterization of 18 elite popcorn (*Zea mays* var. *everta*) lines to be used by Universidade Estadual de Maringá breeding programs. We used 31 microsatellite primers (widely distributed in the genome), and 16 morphological descriptors (including the resistance to maize white spot, common rust, polysora rust of maize, cercospora and leaf blights). The molecular data revealed variability among the lines, which were divided into four groups that were partially concordant with unweighted pair group method with arithmetic mean (UPMGA) and Bayesian clusters. The lines G3, G4, G11, and G13 exhibited favorable morphological characters and low disease incidence rates. The four

groups were confirmed using the Gower distance in the UPGMA cluster; however, there was no association with the dissimilarity patterns obtained using the molecular data. The absence of a correlation suggests that both characterizations (morphoagronomic and molecular) are important for discriminating among elite popcorn lines.

Key words: *Zea mays* var. *everta*; Genetic diversity; Germplasm characterization

INTRODUCTION

Popcorn (*Zea mays* var. *everta*) is a popular snack food with Brazilian consumers, and is sold in a variety of commercial establishments throughout the country (Amaral Júnior et al., 2013; Gonçalves et al., 2014). The main limitations to the cultivation of popcorn in Brazil are product distribution, few cultivars with favorable agronomic traits, and high popping expansion volumes (de Carvalho et al., 2013; Vittorazzi et al., 2013; Gonçalves et al., 2014). According to data from the Agriculture Livestock and Food Supply Ministry (MAPA), there are 48 registered cultivars; however, most of them belong to packing companies that establish restricted-use access to the company's partner growers (Gonçalves et al., 2014).

The development of popcorn breeding programs aimed at obtaining improved populations and/or hybrids that are adapted to Brazilian conditions is extremely important. In hybrid breeding, a knowledge of the heterotic patterns among genetically different groups is crucial for the maximum exploration of heterosis (Reif et al., 2003; Hallauer et al., 2010). Melchinger and Gumber (1998) define heterotic groups as groups of related or non-related genotypes of the same or different populations that have the same combination capacities and heterotic responses when crossed with genotypes from other genetically distinct germplasm groups.

Heterotic groups are not well defined in popcorn. Santacruz-Varela et al. (2004) determined the genetic relationships among 56 popcorn populations from the US and nine from Latin American countries using morphological and molecular descriptors, and identified three main groups: i) the first group included the Yellow Pearl popcorn, which is commercially produced in the US; ii) the second group included the Pointed Rice popcorn, which may have given rise to the traditional popcorn races in Latin America; and iii) the third group included the North American Early, which revealed genetic traces of Northern flint corn. Working with Iowa State University popcorn germplasm, Blanco et al. (2005) identified three heterotic groups: Amber Pearl, South American, and Supergold.

Miranda et al. (2008) evaluated genetic variability and identified heterotic groups among Brazilian popcorn varieties using diallel analysis, and reported that they exhibit heterosis and have lower genetic variability for popping expansion than commercial cultivars. However, the authors concluded that it is possible to increase yield using local races, and suggested backcrossing Brazilian populations with North American heterotic groups to increase popping expansion, yield, and disease resistance (Miranda et al., 2008).

Genotype classification among heterotic groups can be conducted through genealogy, genetic-quantitative analysis, heterosis, and molecular data (Hallauer et al., 2010). Molecular markers have been widely used in the development of heterotic groups, because they provide reliable measures for genetic diversity, and, therefore, can be used for determining genealogy (Reid et al., 2011; Semagn et al., 2012; Wende et al., 2013; Akinwale et al., 2014). Among

the different types of molecular marker, simple sequence repeats (SSRs) exhibit several advantageous characteristics, such as co-dominance, a multi-allelic nature, reproducibility, relatively high abundance, and wide genome coverage (Varshney et al., 2005).

The objective of this study was to evaluate genetic variability through the morphoagronomic and molecular characterization of 18 popcorn lines that were to be used in breeding programs, as well to compare the two characterization methods.

MATERIAL AND METHODS

Eighteen popcorn lines from the Universidade Estadual de Maringá corn breeding program were evaluated (Table 1). For the molecular evaluation, the lines were sown in plastic pots in a greenhouse. Young leaves from six to seven individuals from each line (totaling 120 plants) were collected 15 days after sowing for the extraction of genomic DNA, according to the procedure described by Hoisington et al. (1994).

Table 1. Characterization of elite popcorn lines.

Line	Type	Name	Origin
G1	Commercial	Zaeli	Unknown
G2	Variety	BRS Angela	Embrapa White Composite CMS 43
G3	Simple Hybrid	IAC 125	Unknown
G4	Simple Hybrid	IAC 125	Unknown
G5	Simple Hybrid	IAC 125	Unknown
G6	Triple Hybrid	Jade	Pioneer Hybrids- Endogamy level S_3
G7	Triple Hybrid	Zélia	Pioneer Hybrids- Unknown
G8	Simple Hybrids	IAC 125	Unknown
G9	Modified Simple Hybrid	IAC 112	Guarani and IAC 64
G10	Composite	Common Corn	Cross between local populations and North American hybrids
G11	Modified Simple Hybrid	IAC 112	Guarani and IAC 64
G12	Unknown	Unknown	Unknown
G13	Unknown	Unknown	Unknown
G14	Unknown	Unknown	Unknown
G15	Composite	CMS42	Embrapa yellow composite
G16	Modified Simple Hybrid	IAC 112	Guarani and IAC 64
G17	Composite	Common corn	Cross between local populations and North American hybrids
G18	Unknown	Unknown	Unknown

For the selection of the SSR primers, 269 primers that had been previously mapped for corn in the MaizeGDB database (https://www.maizegdb.org/) were analyzed, 31 of which were selected due to their amplification quality and the presence of at least one primer for each of the 10 chromosomes (Table 2). A polymerase chain reaction (PCR) was conducted using a Techne™ TC-512 thermal cycler. Amplifications were performed using a touchdown PCR program (Don et al., 1991) with a 20 µL reaction volume that contained 25 ng of genomic DNA, 2.0 µL 10X reaction buffer, 2.5 mM $MgCl_2$, 0.1 µM of each dNTP, 1 U *Taq* DNA Polymerase (Invitrogen), and 0.4 µM each of the F- and R-specific primers.

Following amplification, 20 µL of each sample was separated by electrophoresis on 4% agarose gel [50% common agarose (Invitrogen) and 50% MetaPhor™ agarose (Cambrex Corp., NJ, USA)] with 0.5X TBE buffer (4.5 mM Tris, 44.5 mM boric acid, and 1 mM ethylenediaminetetraacetic acid) (Hoisington et al., 1994). All of the 120 samples that were amplified for each primer were subjected to electrophoresis at 60 V for 3 h. In order to ascertain the sizes of the amplified fragments, a 1-kb DNA Ladder (Invitrogen) was used. Gels were

Table 2. Characteristics of the primers used to estimate genetic diversity among 18 elite popcorn lines.

Locus	Nucleotide sequence (F and R)	Replication	No. of alleles	Chromosome
Umc2118	F: CGTCTCCGTCTGCAGTCACTATTA	$(CTTT)_4$	3	3
	R: TATGGTCCTCGGAGTTTGTTTGTT			
Umc1178	F: AGGCTTACCTCCTGAGAAGCAGTT	$(GGC)_6$	4	6
	R: AGGCTTAGCATCGGTGGAGAG			
Umc2226	F: AGCTTCACGCTCTTCTAGACCAAA	$(TGG)_6$	5	1
	R: TGCTGTGCAGTTCTTGCTTCTTAC			
Umc1407	F: AGGCTTACCTCCTGAGAAGCAGTT	$(GGC)_6$	2	7
	R: AGGCTTAGCATCGGTGGAGAG			
Bnlg2295	F: CGGAGGAGTGGTTCTTGAAA	$AG_{(30)}$	4	1
	R: GGTTAGTGAAAGGGTTGCCA			
Umc1287	F: ATGGGATGATCAGTCGTTTCAGTC	$(CCGTGC)_4$	3	8
	R: AGAAGGAGGCCCACTACGAGAG			
Umc1418	F: TCACACACACACTACACTCGCAAT	$(GGAAG)_4$	3	4
	R: GAGCCAAGAGCCAGAGCAAAG			
Umc1015	F: TCCGACTCCAAGAAGAGGAGAA	$(GA)_{45}$	5	7
	R: CAGACACAAGCAGCAAAGCAAG			
Bnlg1451	F: TGATCGATGGCTCAATCAGT	$AG_{(34)}$	3	10
	R: ATCTGGAACACCGTCGTCTC			
Um2132	F: GAGGCCTCAAATCGCTCTCTG	$(CGCT)_5$	4	5
	R: GGTCGTCAGTGAGCTGGTCC			
Bnlg1194	F: GCGTTATTAAGGCAAGCTGC	$AG_{(33)}$	4	8
	R: ACGTGAAGCAGAGGATCCAT			
Umc2059	F: GGAAAAGGAGGAACAGTGTAAGCA	$(CAG)_8$	4	6
	R: AGCGTGATCAGACGTACAATGCTA			
Umc2363	F: TTGACTCGAAAGACTTGTGAGCTG	$(ATGT)_4$	2	2
	R: GTGTGTCAGAAGGAGGACTGATGA			
Umc2245	F: GCCCTGTTATTGGAACAGTTTACG	$(CAA)_7$	3	2
	R: CGTCGTCTTCGACATGTACTTCAC			
Umc2389	F: TACCACCGCTGTGCCTAATAATTG	$(GCTA)_4$	2	6
	R: GCTCCCTCCACATCAAGAACC			
Umc1636	F: GTACTGGTACAGGTCGTCGCTCTT	$(ACTGC)_4$	4	9
	R: CATATCAGTCGTTCGTCCAGCTAA			
Bnlg1598	F: GGCAAGATTCGGACCAGG	$(GGC)_6$	3	6
	R: CGGTTAGGAGCAGTACGTCA			
Bnlg1367	F: CGACGGCGTACAGAGAGAG	$AG_{(42)}$	4	7
	R: GGTCGCCACCCCACCT			
Umc1118	F: ATCAGATTCCGAAGGGTCCATAAT	$(GAGCA)_4$	2	1
	R: GTAGTGAAATGAATCGTGAGAGCG			
Umc2350	F: CGAATCGAGGATGGTTTGTTTTT	$(GGCCGT)_4$	2	10
	R: AGTAGCGACTCCTCTGCGTGA			
Umc2205	F: CATGATCATTTGGCGATGGTAAT	$(CTCTCTCTCT)_4$	4	2
	R: ATGGTGAGCGAGTGAAAGAGAGAT			
Umc2257	F: AAAAAGGCAAACTCGACCCC	$(CTCCT)_4$	3	3
	R: GTCGTCATCTGCAAACCCTAGC			
Umc1904	F: CAGCCACTCGTTTATGGAGGTTTA	$(TAAGC)_5$	3	8
	R: TGTTACTAGTCGATCTGATGCCCA			
Umc1068	F:AGTCGTTTTCAAAGGCTGCTGATATG	$(GAAAA)_6(GAA)_2$	4	7
	R: AGTCACCTCATTTCTTCTGGTTC			
Umc2121	F: AAAAGTGGGCGCTAGTACAAGATG	$(AGCG)_4$	3	9
	R: GTGGTGTTCTCCTTTTGATACGCT			
Umc2025	F:CGCCGTAGTATTTGGTAGCAGAAG	$(AGCT)_4$	2	1
	R: TCTACCGCTCCTTCGTCCAGTA			
Bnlg1318	F: TTATGTGTGCAGAACGACTCG	$AG_{(28)}$	3	4
	R: AGCATGGCAGAGAAGGTGAT			
Bnlg1371	F: ACGACCGGTGTGGTTACATT	$AG_{(22)}$	4	6
	R: TTGCCGATAAGAACCAAACA			
Mmc0501	F: TGCTGAACACTCTAAGCAATAC	$(GA)_{36}$	7	10
	R: ATTACTCTACTCGCTGCCTG			
Bnlg1175	F: ACTTGCACGGTCTCGCTTAT	$AG_{(38)}$	3	2
	R: GCACTCCATCGCTATCTTCC			
Bnlg1250	F: CCATATATTGCCGTGGAAGG	$AG_{(30)}$	5	10
	R: TTCTTCATGCACACAGTTGC			

browned using a solution containing 0.5 μg/mL ethidium bromide, and images were captured using the L-PIX-HE system (Loccus Biotecnologia). The number of alleles per locus was determined based on their relative positions on the gel.

For the morphological characterization, the lines were sown in the field and the following descriptors proposed by MAPA were evaluated: i) pigmentation of the coleoptile by anthocyanin; ii) plumule pigmentation; iii) foliar blade behavior above the superior ear; v) number of leaves below the superior ear; vi) main stem length; vii) angle between the main stem and the lateral ramification; viii) stigma coloration by anthocyanin; ix) disease occurrence [white spot (*Phaeosphaeria maydis*/*P. ananas*), common rust (*Puccinia sorghi*), polysora rust (*Puccinia polysora*), *Cercospora* (*C. zeae-maydis*), and *Helminthosporium turcicum*]; and x) grain coloration.

The molecular parameters analyzed were allelic frequency, observed and expected mean heterozygosity (H_O and H_E), mean number of alleles per locus (N_A), and number of effective alleles (N_E). Nei and Feldman (1972)'s distance and the unweighted pair group method with arithmetic mean (UPGMA) were used. The STRUCTURE v.2.3.2 program (Pritchard et al., 2000) was used to generate a group separation model and assign individuals to a K number of groups based on the Bayesian estimate. A coefficient association graph of the genotypes and individuals was generated using the DISTRUCT program (Rosenberg, 2004). The morphoagronomic descriptors were analyzed using the Gower (1971) algorithm and the UPGMA grouping. Associations between the morphological and molecular distance matrix data were investigated using the Mantel test with 1000 permutations. The statistical analyses were conducted in R (http://www.r-project.org).

RESULTS

The number of alleles per locus varied from 2 to 7 (Table 2) and 107 alleles were identified, with the average number of alleles per polymorphic locus equal to 3.45.

Of the 31 SSR loci studied, only 22.22% (six) were formed by dinucleotide replications; the average number of alleles at these loci was 3.3. The other loci (25), which were formed by replications greater than two nucleotides, contributed 95 alleles, with an average of 3.8 alleles per locus; therefore, a greater number of alleles were produced than by dinucleotide replication.

Exclusive alleles were observed in *Umc2059*, *Bnlg1194*, *Umc2257*, *Bnlg1451*, and *Umc1636* in the G13, G14, G17, G1, and G9 lines, respectively. In the G9 line, the *Umc1636* allele was exclusive and fixed. The observed heterozygosity varied from 0.0839 in the G4 line to 0.2710 in the G1 line (Table 3).

Only locus *Umc2132* did not have a fixed allele in any of the lines studied, followed by *Mmc0501*, with only one fixed allele in line G2. The other loci had fixed alleles in a minimum of four (*Umc1178*) and maximum of 15 (*Umc2350*) lines.

Identity values among the 18 lines varied between 0.3153 (between the G1 and G18 lines) and 0.8148 (between the G3 and G4 lines). The hierarchical UPGMA grouping based on Nei and Feldman (1972)'s genetic distance identified four groups (Figure 1A). The first group was formed by the G1, G2, G3, G4, G5, G8, G11, G12, G13, G15, and G16 lines, the second was composed of the G14 and G17 lines, the third by the G6 and G7 lines (both originated from Pioneer hybrids), and the fourth by the G9, G10, and G18 lines.

Table 3. Parameter estimates for different elite popcorn lines.

Line	N (pl)	%P	A	H_O	H_E
G1	18	58.06	1.5618	0.2710	0.2703
G2	15	48.39	1.4903	0.2258	0.2361
G3	10	32.26	1.2355	0.1419	0.1316
G4	6	19.35	1.1481	0.0839	0.0806
G5	14	45.16	1.3962	0.1419	0.2065
G6	10	32.26	1.3268	0.1419	0.1542
G7	7	22.58	1.2049	0.0903	0.0987
G8	15	48.39	1.3082	0.1484	0.1813
G9	7	22.58	1.2387	0.0903	0.1097
G10	20	64.52	1.6205	0.1613	0.2858
G11	11	35.48	1.2876	0.1032	0.1418
G12	21	67.74	1.5573	0.2323	0.2903
G13	23	74.19	1.6704	0.1742	0.3284
G14	26	83.87	1.7718	0.1226	0.3761
G15	26	83.87	1.6570	0.2516	0.3381
G16	18	58.06	1.5337	0.1806	0.2652
G17	22	70.97	1.6984	0.1806	0.3252
G18	11	35.48	1.3474	0.1871	0.1671

N (pl), number of polymorphic loci; %P, percentage of polymorphic loci; A, effective number of alleles; H_O, average heterozygosity; H_E, Nei's expected average heterozygosity.

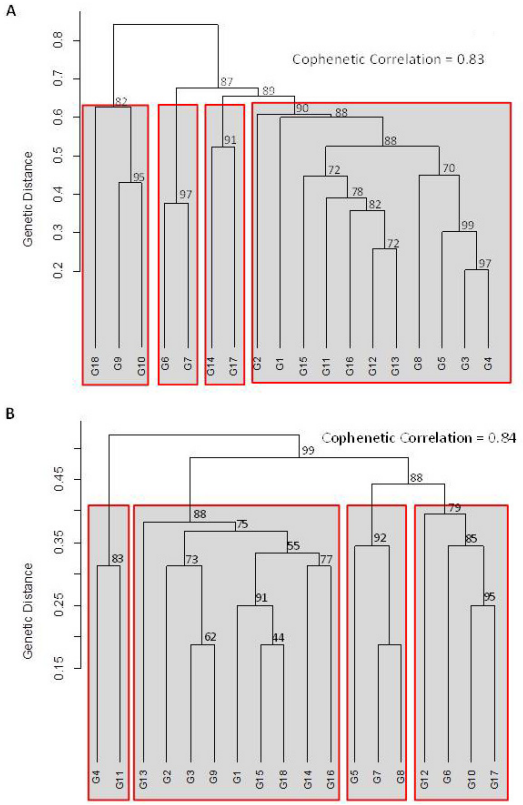

Figure 1. Dendrogram based on Nei and Feldman (1972)'s genetic distance – molecular data (**A**) and Gower (1971) - morphoagronomic data (**B**) for the 18 corn lines, using the UPMGA method. Bootstrap values are given at the corresponding knot for each cluster.

According to the Gower matrix (Figure 1B), lines G3 x G10 and G13 x G10 were the most distant with a distance of 0.75, while lines G9 x G3, G8 x G7, and G18 x G15 were the most similar, with a distance of 0.19, and the average distance observed between the lines was 0.44 ± 0.12SD. There was a cut-off at the 0.40 distance, and the abrupt change promoted the development of four possible groups: Group I contained lines G4 and G11, Group II contained lines G1, G2, G3, G9, G13, G14, G15, G16, and G18, Group III contained lines G5, G7, and G8, while Group IV contained lines G6, G10, G12, and G17.

The STRUCTURE program was used as a second method of classifying the genotypes according to the molecular data. The highest ΔK value (10.65) was obtained with the formation of the four groups. Figure 2 suggests that there were isolated groups among the 18 groups, and not all of the groups shared alleles with the four groups identified.

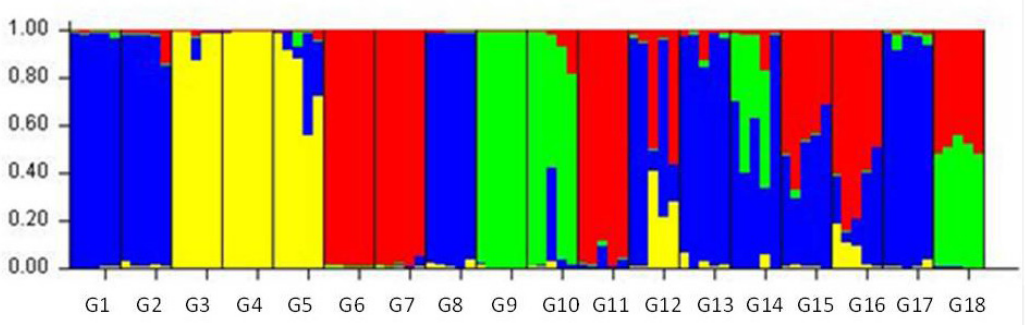

Figure 2. Bar plot based on 31 microsatellite loci for the 18 popcorn lines, showing their distribution within the four groups. Lines and individuals are represented horizontally and each genetic grouping is represented by a different color.

Group I (represented as blue in Figure 2) contained lines G1, G2, G8, G13, G14, and G17, Group II (yellow) contained lines G3, G4, and G5, Group III (red) contained lines G6, G7, G11, G15, G16, and G18, and Group IV (green) contained lines G9, G10, and G18. Regarding the morphological descriptors, the lines did not have coleoptile pigmentation, except for G7. Lines G6 and G10 had average plumule pigmentation; the other lines had less pigmentation. Regarding foliar blade behavior (above the superior ear), lines G5, G7, and G8 had a straight foliar blade, G1, G2, G6, G10, G12, and G17 had a strongly recurved foliar blade, and the recurved characteristic was also observed in the other lines. The number of leaves above the superior ear varied from three to five, while the number below the superior ear varied from four to seven.

Regarding the main stem, lines G3, G8, and G11 had short stems, lines G13 and G15 had long stems, while the others had average-length stems. In regards to the angle between the main stem and the lateral ramification, most lines had acute angles (55.5%), followed by average (27.7%), and large (16.7%) angles. Regarding stigma coloration by anthocyanin, 61.1% had no anthocyanin and 38.9% did have anthocyanin.

With regard to the descriptors related to the occurrence of disease, most lines were classified as grade 2 for white spot (G3, G4, G5, G6, G11, G13, G14, G16, and G18), followed by grades 3 (G2, G7, G8, G9, G12, and G17), 4 (G1 and G10), and 5 (G15). Regarding common and polysora rusts, most lines were also classified as grade 2, except G4 and G11

(with grade 3 for both diseases), G12 (grade 3 for common rust), G14 (grade 3 for polysora rust), and G10 (grade 4 for both diseases). In relation to *Cercospora*, all of the lines were classified as grade 2, while for leaf blights, six lines were classified as grade 2 (G2, G3, G4, G9, G11, and G13), eight as grade 3 (G1, G5, G6, G7, G8, G10, G12, and G17), three as grade 4 (G14, G15, and G16), and one as grade 5 (G18). For grain coloration, most lines had an orange color, except for G3 (yellow color) and G2 and G14 (white color).

DISCUSSION

Popcorn belongs to the same species of corn as common corn; however, studies that have compared genetic diversity in SSR loci have reported that popcorn has a lower number of alleles per locus and average heterozygosity than common corn (Liu et al., 2003). According to Erwin (1949) and Brunson (1955), the most probable origin of popcorn is Central and South America, and it might have originated from a flint corn mutation. This may explain why popcorn has less genetic diversity than common corn.

Some authors have analyzed mutations in SSR corn loci and reported different rates and types of mutations in these loci, as well as in their flanking regions. According to Vigouroux et al. (2002) and Liu et al. (2003), high genetic diversity values estimated for SSR loci are found when the largest proportion of the loci analyzed is formed by dinucleotides. This was not found in the present study, in which of the 31 SSR loci studied, only 22.22% (six) were formed by dinucleotide replications; the average number of alleles at these loci was 3.3. The other loci (25), which were formed by more replications than two nucleotides, had 95 alleles, with an average value of 3.8 alleles per locus. Therefore, they had a greater number of alleles per locus than those produced by dinucleotide replications.

The average heterozygosity was low (H_O = 0.1627), and varied from 0.0839 (G4) to 0.2710 (G1), which is what was to be expected from lines in the process of inbreeding or being selected for characteristics of interest in popcorn breeding programs. The differences observed in allelic frequencies among the popcorn lines could have been due to the alleles' different frequencies in the genotypes from which the lines originated (Table 1), or to the fact that the microsatellite markers could have been related to loci undergoing artificial selection and therefore subject to selection pressure. Only *Umc2132* did not have a fixed allele in any of the lines studied, and *Mmc0501* had only one fixed allele in line G2; the other loci had fixed alleles in up to 15 lines (*Umc2350*). Although SSR loci have been considered evolutionarily neutral DNA markers (Schlötterer and Wiehe, 1999; Schlötterer, 2000), there is evidence of functional significance for simple sequences replicated in the genome (Li et al., 2002, 2004; Subirana and Messeguer, 2008). Polymorphisms in SSR loci in the gene for cellulose synthase, for example, are associated with the growth and properties of *Populus tomentosa* (Du et al., 2013).

Our study also used the STRUCTURE program to classify genotypes based on molecular data. This program is superior to methods based on genetic distance, mainly because the parameters corresponding to each group are inferred based on each individual the group (Pritchard et al., 2000). The dendrogram (Figure 1) and the bar plot generated by the STRUCTURE program (Figure 2) were in agreement for most of the groups. Lines G3, G4, and G5, which were grouped in the same cluster in the dendrogram, were also together in the group generated by the Bayesian approach (Group II). Lines G6 and G7 were grouped together in the dendrogram and belonged to Group III (red color in the bar plot). In addition, G9 and G10 were grouped in the same dendrogram cluster and were together in the bar plot

(Group IV). Lines G12 and G16 had high heterogeneity (a mixture of colors in the bar plot), suggesting that significant allele-sharing had occurred.

A wide genetic variability towards resistance to most diseases was observed in the lines, which favors their use in breeding programs. The identification of lines resistant and/or tolerant to particular diseases is of great importance for popcorn growing, due to the high susceptibility of this crop to certain diseases, such as leaf blight (Miranda et al., 2003). Vieira et al. (2009) evaluated the performance of novel, simple popcorn hybrids, and reported the potential of these lines in relation to resistance to polysora rust, leaf blight, and white spot. In this study, a relatively high (grade 20) tolerance to black spot, both types of rust, and *Cercospora* was detected in most lines, and six lines had the same grade for leaf blight.

All of the lines had orange grains, except G3 (yellow grains) and G2 and G14 (white grains). This favors their use in breeding programs, because grain color is an important commercial characteristic: yellow or orange grains are the most popular with the Brazilian public.

We found distinct dissimilarities among the matrices generated by the morphological and molecular data (e.g., for color, $P = 0.1203$). While the SSR markers covered a large proportion of the genome, including non-coding regions, the phenotypic markers only included coding regions. Consequently, the molecular profile may have had regions that were not correlated with the phenotypic characteristics. The absence of a significant correlation suggests that both characterization methods (morphoagronomic and molecular) are important for discriminating among popcorn lines.

CONCLUSIONS

Molecular and morphoagronomic characterizations are equally important, and are the best methods to discriminate among popcorn lines.

Conflicts of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária of Paraná State.

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