

Full Length Research Paper

Assessment of genetic diversity among maize accessions using inter simple sequence repeats (ISSR) markers

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The narrow genetic base of popcorn has been of concern to the breeders of this cultivar, hence the present research aimed at assessing popcorn variability using inter simple sequence repeats (ISSR) markers. From 52 accessions, nine groups were made at a cutoff value of 0.36 on the abscissa in the UPGMA dendrogram. Among the genotypes, the ancestors *Tripsacum* sp. and teosinte were the most divergent, which corroborates the efficiency of the ISSR technique. Teosinte was in more proximity with maize than *Tripsacum* sp. Although, belonging to different heterotic groups, the dent and flint types of the common maize assembled in the group I confirmed that the compared analysis of genetic diversity was more remarkable. In the third group, however, almost all the popcorn cultivars recommended for Brazil were gathered, besides popcorn wild materials, from different countries, thus providing a reason for breeders to be cautious and avoid selecting materials from genetically close parents in breeding programs.

Key words: *Zea mays*, *Tripsacum* sp., genetic narrowing, gene pool, DNA markers.

INTRODUCTION

The use of hybrid vigor or heterosis in maize culture is an outstanding successful example in applied genetics as it allowed a better understanding of the genetic phenomenon and plant breeding in general (Springer and Stupar, 2007). However, due to strong commercial pressure, breeders were forced to use a narrow band of the elite germplasm during the development of modern hybrids (Goodman, 2005; Hartings et al., 2008; Fan et al., 2010; Reif et al., 2010). This replacement of landraces by elite cultivars reduced the genetic variability within the gene pool for breeding and resulted in lower selection gains in a long run for yield and other traits, besides increased vulnerability to biotic and abiotic stress (Smith

et al., 2004; Reif et al., 2010). To fight this genetic narrowing process, it is therefore necessary to collect and preserve these varieties in germplasm banks, thus enabling availability in future breeding programs (Aguilar et al., 2008; Gonçalves et al., 2008). However, the value of a germplasm bank depends on the information employed to promote its use. In this sense, the characterization and evaluation of accessions of germplasm banks are vital, since better knowledge about them will allow detection of possible genotypes to be used in plant breeding programs and possible duplicates in germplasm banks (Gepts, 2006; Sudré et al., 2010).

Genotype characterization by molecular information has been increasingly used in plant breeding programs. Molecular markers are stable and detectable in all plant tissues regardless of the growth, differentiation, development or status of cells. Besides, they are not affected by

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environmental, pleiotropic and epistatic effects (Agarwal et al., 2008; Collard and Mackill, 2008; Moose and Mumm, 2008). In popcorn, the genetic basis is narrow as it is originated only from the dent type of common maize (Kantety et al., 1995), hence, investigation on genetic diversity of popcorn is extremely important (Munhoz et al., 2009; Leal et al., 2010) especially in comparison with wild forms of popcorn, common maize, sweet maize and ancestral species. For such, the use of DNA markers, compared to phenotypic aspects, becomes even more important. There are many markers available, among them are: the simple sequence repeats (SSR) markers (Tautz, 1989), randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990), inter simple sequence repeats (ISSR) (Zietkiewicz et al., 1994), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), single nucleotide polymorphisms (SNPs) (Chen and Sullivan, 2003) and diversity array technology (DArT) (Kilian et al., 2005). These different types of molecular markers have potential to differentiate and detect differences among genotypes, in relation to cost, easiness of use, consistency and repeatability of the results (Schlötterer, 2004; Schulman, 2007; Bernardo, 2008). One of these techniques, ISSR, involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multi-locus markers (Reddy et al., 2002; Oliveira et al., 2010). The technique is considered simple and fast like RAPD, but has more stringency than RAPD. Also, ISSR markers are highly polymorphic, which makes them useful for studies on genetic diversity, phylogeny, genetic coding, genomic mapping and evolutionary biology (Reddy et al., 2002). Therefore, the present research work was undertaken with the aim of assessing popcorn variability, in comparison with different forms of maize, using ISSR markers

MATERIAL AND METHODS

Plant material and DNA extraction

Fifty accessions of maize from different origins were evaluated (Table 1). Of these, 34 of them were of popcorn (including wild materials at breeding stage and seven recommended for use by farmers), 14 of common maize (including ancestral materials recommended for cultivation, totaling 5 types of the flint group and 9 of the dent group), one type of tetraploid maize and one specimen of sweet maize.

Besides these accessions, two ancestors were included (Teosinte and *Tripsacum* sp.), totaling 52 accessions.

The total cellular DNA was extracted according to Doyle and Doyle (1987), with modifications suggested by Daher et al. (2002). After extraction, the DNA was quantified on agarose gels at 1.0%, with the use of high molecular weight DNA ladder (Invitrogen, USA). The gel was stained with a mixture of Blue juice 6X (0.4 ml TAE 10X, 0.5 M; 0.2 ml SDS 10%; 0.2 ml of bromophenol blue; 7.0 ml of glycerol; 1.7 ml of sterile water) with GelRed 5X (1 µL of GelRed 10.000X in DMSO 0.5 ml; 2 ml of ultrapure water), at the proportion of 1:1. The image was captured and analyzed using MiniBis Pro photodocumentation.

ISSR amplification

ISSR analysis was carried out with 15 ISSR primers which were selected (Tables 2). The amplification reactions were carried out according to the protocol of Zietkiewicz et al. (1994), with modifications done by Oliveira et al. (2010), in a final volume of 20 µL. The reaction mixture contained: 2 µL of buffer 10X (500 mM KCl, 100 mM Tris-HCL in pH 8.4), 2 µL of 25 mM MgCl₂, 1.6 µL of 2mM dNTPs, 1 µL of DMSO (dimethyl sulfoxide), 1.8 µL of 0.5 mM of primer, 0.12 µL of 5 U of Taq DNA polymerase and 2 µL of 5 ng of genomic DNA. The final volume was supplemented with ultrapure water. The PCR reactions for the optimization of the optimum temperature for annealing (T_a) were carried out in an Eppendorf gradient thermocycler, in compliance with the following program: 4 min of denaturation at 94°C, followed by 42 amplification cycles [94°C at 1 min, 1 min with T_m (temperature of melting) ranging every 2°C in a spectrum of up to 14°C for each primer, from the average temperature achieved by the Equation: $T_m = (G + C)4 + (A + T)2$, and 72°C for 3 min]. The DNA fragments were separated by electrophoresis on 2% agarose gel with TAE 1X as buffer and run at 100 V for 2 h and later stained with loading buffer 6X with 'Gel Red', at the proportion of 1:1. The 250 base pair DNA Ladder was used to determine the size of the fragments generated. The gels were photo-documented using "Mini Bis Pro" equipment (BioAmérica).

Data analysis

For ISSR marker analysis, the gels were scored for the presence and absence of bands, generating a binary matrix. To estimate the genetic distances among the accessions, Jaccard's similarity coefficient was estimated. Later, the representation of the genetic distances among the accessions was performed by the Unweighted Pair-Group Method Using Arithmetic Average (UPGMA). All the analyses were carried out using the R software system (<http://www.r-project.org>).

RESULTS AND DISCUSSION

In ISSR analysis, using fifteen primers, 137 bands were generated (Table 2) of which 122 were polymorphic (89.05%) and 15 were monomorphic (10.95%). The number of polymorphic bands ranged from 4, for the primer (GA)₆CC, to 11 bands for the primers (GA)₈T, (GA)₈YC and (CTC)₅RC. The level of polymorphism observed in the present work is in accordance with Carvalho et al. (2002), who had reported 75.8% polymorphism with ISSR markers in maize.

The analysis of the distribution of the dissimilarity frequencies of the 1326 combinations for pairs of accessions revealed predominance of dissimilarity coefficient of 0.30 to 0.39 with 59.40% of the combinations, followed by frequency of 0.40 to 0.49 and 0.20 to 0.29, with 23.80 and 11.90% of the combinations, respectively (Figure 1). The average distance was 0.38 (± 0.077). The accessions PR 023 and TATU 2 of popcorn were the closest, with a dissimilarity value of 0.15; while the highest distance estimate, 0.69 was observed between the ancestor germplasm, *Tripsacum* sp., and a popcorn accession, VALL 429, originated in Colombia. At a cutoff value of 0.36 in the dendrogram, nine groups were distinguished (Figure 2). The ancestors *Tripsacum* sp.

Table 1. Accessions evaluated and classified according to the type of grains, cariopsis and origin.

Genotype	Type of grain	Cariopsis		Origin
		Pericarp	Endosperm	
VALL 429	Popcorn	Yellow	Orange	Colombia
UFVM2-Barão Viçosa	Popcorn	Incolor	Orange	Brazil
Argentina	Popcorn	Colorless	Yellow/orange	Argentina
SE 013	Popcorn	Colorless	White/yellow	Brazil
UNB-2U-C1	Popcorn	Colorless	Orange	Brazil
URUG 298	Popcorn	Red	Orange	Uruguay
IAC-125	Popcorn	Colorless	Orange	Brazil
RS-20	Popcorn	Colorless	Orange	Brazil
TATU 2	Popcorn	Colorless	White	Brazil
UNB-2U-C0	Popcorn	Colorless	Orange	Brazil
PAZM 130703	Popcorn	Colorless	White	Brazil
SAM	Popcorn	Colorless	Orange	EUA
BRS Angela	Popcorn	Colorless	White	Brazil
IAC-112	Popcorn	Colorless	Orange	Brazil
Viçosa	Popcorn	Yellow	Orange	Brazil
Compuesto	Popcorn	Colorless	White/yellow	-
TATU 1	Popcorn	Colorless	Black/purple	Brazil
PA 091	Popcorn	Colorless	Yellow	Brazil
ARZM 13 050	Popcorn	Colorless	Orange/yellow	Argentina
Beija-Flor	Popcorn	Colorless	Orange	Brazil
BOYA 462	Popcorn	Colorless	Colorless and black/purple	Colombia
PARA 172	Popcorn	Colorless	White/yellow	Brazil
ARZM 07 049	Popcorn	Colorless	Orange	Argentina
BOZM 260	Popcorn	Colorless	Branco	Bolivia
ARZM 05 083	Popcorn	Colorless	Branca	Argentina
CHZM 13 0134	Popcorn	Brown	Yellow/orange	Chile
UNB-2U-C3	Popcorn	Colorless	Orange	Brazil
PR-023	Popcorn	Colorless	Orange	Brazil
Zélia	Popcorn	Colorless	Orange	Brazil
UNB-2U-C4	Popcorn	Colorless	Orange	Brazil
PARA 170	Popcorn	Colorless and Brown	White	Brazil
Colombiana	Popcorn	Colorless	Orange	Colombia
Jade	Popcorn	Colorless	Orange	Brazil
UNB-2U-C2	Popcorn	Colorless	Orange	Brazil
Doce de Cuba	Sweet corn	Colorless	Yellow	Brazil
Composto F. N.	Flint corn	Colorless	Orange	Brazil
Cateto Sete Lagoas	Flint corn	Colorless	Orange	Brasil
Cateto Colombia	Flint corn	Colorless	Yellow/orange	Colombia
CIMMYT – 11	Flint corn	Colorless	Yellow/orange	Brazil
Cateto Roxo	Flint corn	Brown	Yellow/orange	Brazil
Maya	Dent corn	Brown	Yellow/orange	Brazil
Centralmex	Dent corn	Colorless	Yellow/orange	Brazil
Cateto Amarelo	Dent corn	Colorless	Yellow	Brazil
Piranão 11	Dent corn	Colorless	Yellow	Brazil
Dente de Burro	Dent corn	Colorless	Yellow	Brazil

Table 1. Continue

Azteca	Dent corn	Colorless	Yellow/orange	Brazil
Maya Antigo	Dent corn	Colorless	Yellow/orange	Brazil
Saracura	Dent corn	Colorless	Yellow/orange	Brazil
BR 106	Dent corn	Colorless	Yellow/orange	Brazil
Tetraplóide	-	Colorless	Yellow	Brazil
Teosinte	Ancestors	-	-	Brazil
<i>Tripsacum</i> sp.	Ancestors	-	-	Brazil

Table 2. Number of polymorphic and monomorphic bands obtained with ISSR markers in 52 accessions, 49 of which are accessions of maize, 2 are ancestors (teosinte and *Tripsacum* sp.) and one, a tetraploid common maize.

Primer	Polymorphic band	Monomorphic band	% of polymorphism
(CT)8RC	8	0	100.00
(CT)8TG	7	0	100.00
(GA)8T	11	1	91.66
(GA)8YC	11	1	91.66
(CTC)5RC	11	4	73.33
(GTC)6	7	1	87.50
(GA)6CC	4	2	66.66
(GT)6CC	7	0	100.00
(CAC)3GC	7	1	87.50
(AG)8YT	8	2	80.00
(AC)8CT	7	1	87.50
(AC)8YG	9	0	100.00
(CT)8RG	8	1	88.88
(GGAT)3GA	9	1	90.00
(GAA)6AA	8	0	100.00
Total	122	15	

and teosinte (groups IX and VIII, respectively) were the most divergent in relation to the other accessions studied, Teosinte was in close proximity with maize as compared to *Tripsacum* sp. This result corroborates the evidences of archaeological (Goloubinoff et al., 1993) and molecular (Eubanks, 1997; Poggio et al., 2000; Koo and Jiang, 2008; Fu et al., 2010) research works.

Of the group I estimated 18 accessions, nine, five and four accessions belonged to groups of dent, flint and popcorn, respectively. It must be emphasized that for all

the accessions studied, those of the group flint and dent were placed in the respective group. The accessions Dente de Burro, Maya, Maya Antigo and Azteca reported by Fidelis et al. (2005) as old forms of common maize, which went through breeding, were launched in the market by 1960 and multiplied by small farmers. As for the materials of the breed Cateto, two varieties of flint grain - Cateto Colômbia and Cateto Sete Lagoas - were placed close to each other. Cateto Roxo and Cateto Amarelo were separated from the other catetos. Cateto

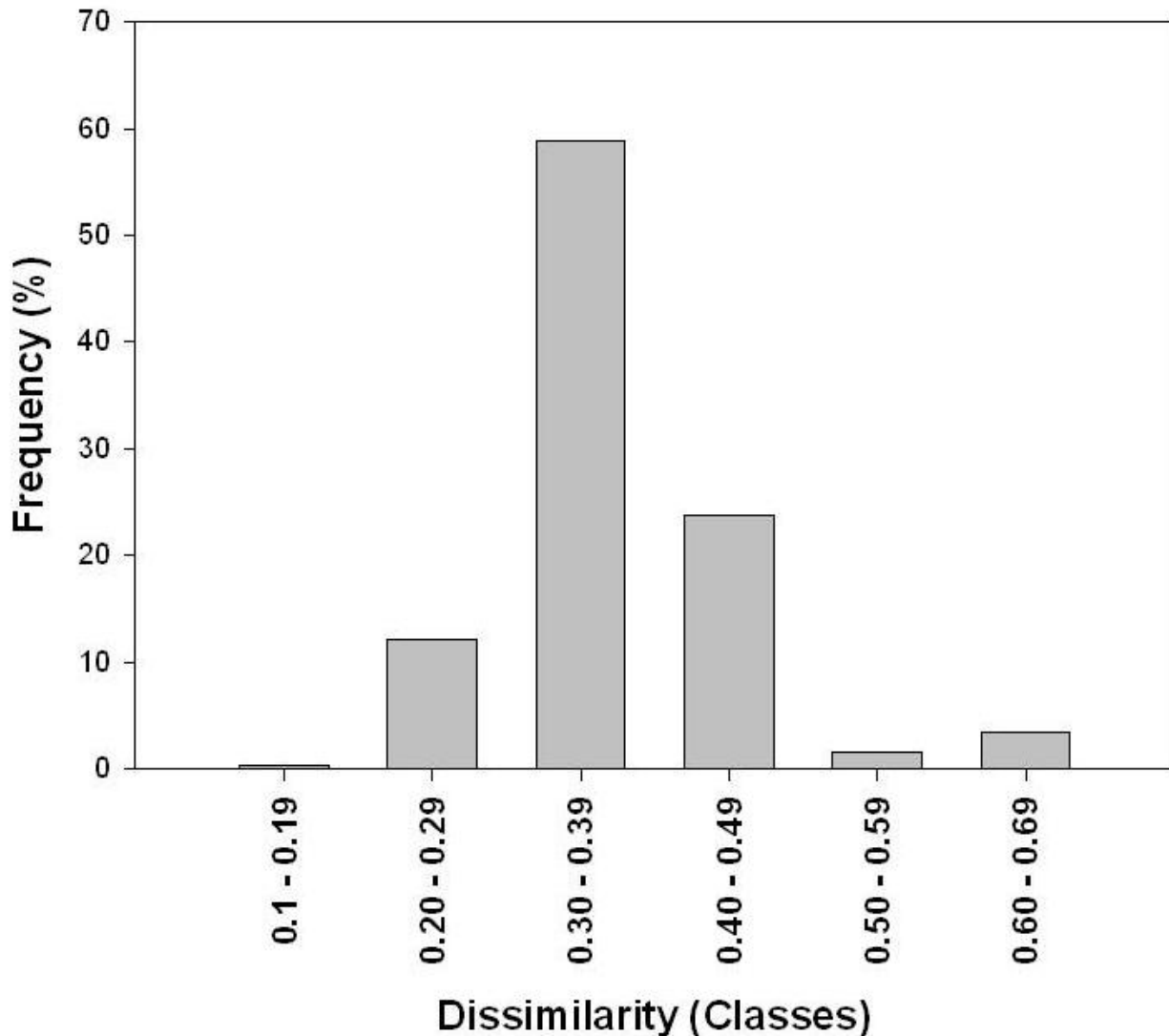


Figure 1. Distribution of dissimilarity frequency from the ISSR marker among 52 accessions, 49 of which are accessions of maize, 2 are ancestors (teosinte and *Tripsacum* sp.) and one, a tetraploid common maize.

Roxo is a type of semi-flint, but its pericarp is brown, while the other catetos have colorless pericarp. Cateto Amarelo is the only one of the dented type. The following accessions of the popcorn group were joined in the group I: Argentina, Boya 462, ARZM 13050 and CHZM 130134, all of them from other countries (Table 1). Moreover, the accessions ARZM 05083, SE 013 and PA 091 were placed in the group II. The first was from Argentina and the others were from Brazil.

Furthermore, the group III contained 18 accessions, most of which were Brazilian commercial materials namely, IAC 112, IAC 125, UFVM2 Barão de Viçosa, RS20, Jade and Zélia. Among the commercial materials, only BRS Angela from Brazil did not join the other accessions of the third group. The assembly of almost all the

cultivars recommended in the country was in the third group, which was in agreement with other works on molecular and morpho-agronomic markers (Munhoz et al., 2009; Silva et al., 2009). This should however, provide a warning to the few breeders dealing with popcorn in Brazil, demonstrating the need to increase the genetic diversity in breeding programs developed by public and private institutions. In the analysis of the UNB populations (UNB-2U C0, UNB-2U C1, UNB-2U C2, observed, in relation to the other UNB populations. Such distance is explained by the strategy of employing the S₁ progeny selection to form the C2 cycle. Popcorn is more fragile than common maize and the effects of the inbreeding depression is also strong, thus favoring abrupt changes in the allele frequencies and consequently, in

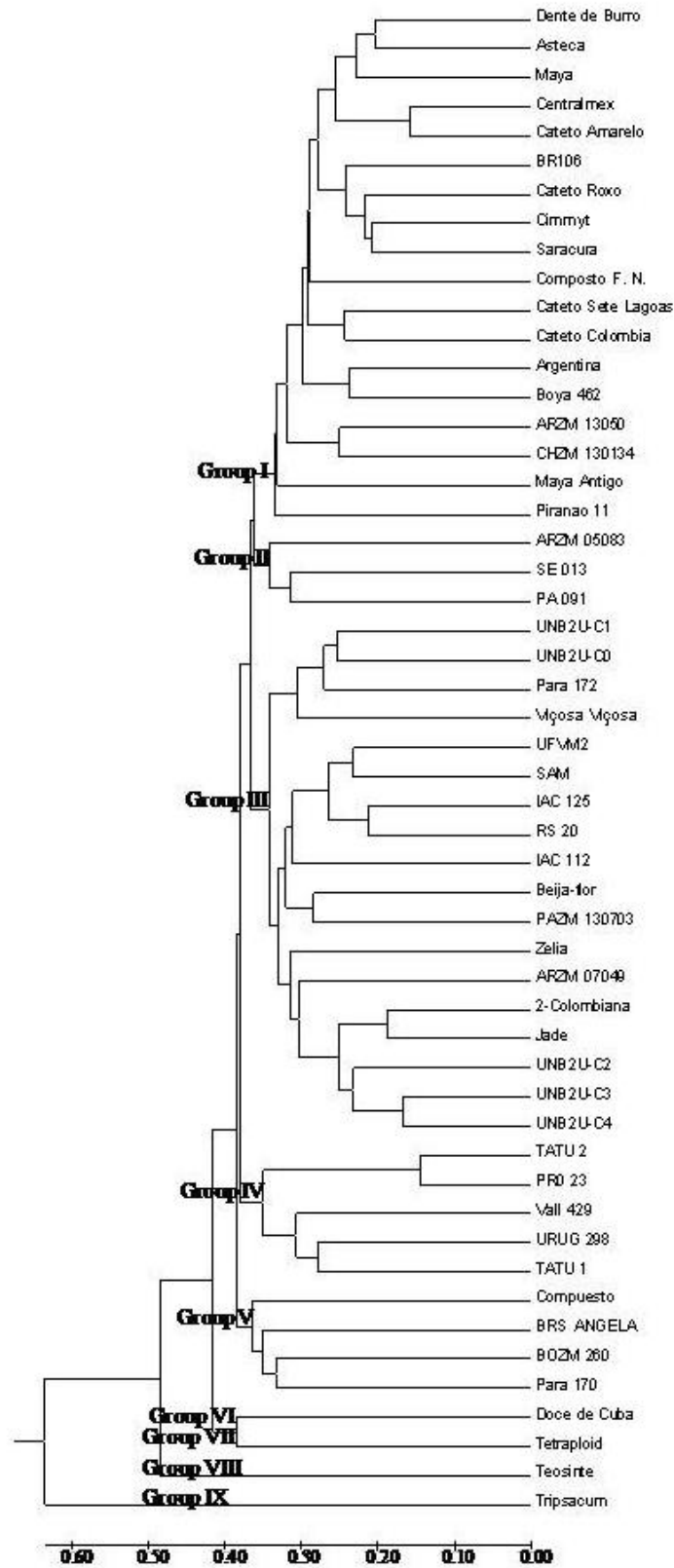


Figure 2. Dendrogram achieved by the UPGMA grouping based on the Jaccard's dissimilarity matrix, with 52 accessions, 49 of which are accessions of maize, 2 are ancestors (teosinte and *Tripsacum* sp.) and one tetraploid common maize.

the behavior of the population under selection.

The group IV was formed by the accessions Tatu 2, PR 023, Vall 429, URUG 298 and Tatu 2. The accessions Tatu 1 and Tatu are from the Universidade Estadual de Maringá (UEM), and were procured from the western region of Paraná. PR 023 is a variety derived from North-American hybrids, developed in the northwest of Paraná. The accessions URUG 298 and Vall 429 are from Uruguay and Colombia, respectively. Moreover, the group V contained accessions like Compuesto, BRS Angela, BOZM 260 and Para 170, all of them with white endosperm. BRS Angela is a variety developed by the Embrapa Milho e Sorgo, by means of six cycles of intrapopulation recurrent selection, by crossing the variety CMS-43 and the population Angela (BRA 065901). The accession Compuesto provided by the International Maize and Wheat Improvement Center (CIMMYT), is of an unknown origin, while BOZM 260 and UNB-2U C3 and UNB-2U C4) of the plant breeding program of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), a longer distance between the populations UNB-2U C0 and UNB-2U C1 is Para 170 are from Bolivia and Brazil, respectively. Finally, the groups VI and VII comprised of just one accession each; Doce de Cuba, a sweet type of maize was placed in the group VI, while the tetraploid common maize was placed in the group VII.

These results demonstrate the efficiency of ISSR molecular markers in the separation and identification of the variability of the accessions of maize evaluated in the present work. It is concluded that popcorn breeders in Brazil are using genotypes of narrow diversity, and therefore greater attention should be focused on the selection of more distinct genotypes among the breeding programs carried out by public and private institutions in the country.

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