Assessment of genetic diversity in maize (*Zea mays* L.) landraces using inter simple sequence repeat (ISSR) markers

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ABSTRACT

Inter Simple Sequence Repeat (ISSR) markers were used to assess the genetic relationships among 79 landraces and two improved varieties of maize cultivated in Brazil. Nine primers comprising dinucleotides $(GA)_9$, $(AT)_9$, trinucleotides $(GTG)_6$, $(TAG)_6$, and the tetranucleotides $(GATA)_4$, $(GACA)_4$, $(CCTA)_4$, and $(GGTA)_4$ were used for PCR amplifications. From a total of 153 DNA fragments produced, 116 (75.8%) were found to be polymorphics. The dinucleotide motifs $(GA)_9T$ and $(GA)_9C$ combined with other di-, tri- and tetra-nucleotides produced a greater number of DNA fragments, which suggests a high frequency of the poly GA microsatellite motifs in the maize genome. The UPGMA clustering algorithm associated the varieties into three major groups that were correlated to the endosperm colors and fourteen sub clusters that were mostly related to the flowering time. The results revealed that ISSR markers could be efficiently used to quickly access the genetic variation available in the maize germplasm. The information on genetic similarity among varieties will be useful for selecting the accessions to establish a germplasm bank of maize landraces and to develop breeding programs.

KEY WORDS: Genetic diversity, ISSR markers, maize landraces.

INTRODUCTION

The maize germplasms in Brazil are essentially represented by local or landrace varieties adapted to local conditions. These varieties are derived from a) intercrossing among indigenous races cultivated by local tribes before colonization, b) old races cultivated by civilized peoples after colonization, c) recent commercial races developed by crossing between local races and introduced varieties followed by selection, and d) exotic commercial maize recently introduced from well known varieties cultivated in other countries (Paterniani et al., 2000).

The maize landraces are valuable sources of genetic variability and have been intensively used in breeding programs (Udry and Duarte, 2000). The knowledge of the genetic relationship among accessions is very important for applications in organizing the germplasm (Thormann et al., 1994), planning crosses to develop lines for heterotic groups, and even protecting cultivars (Pejic et al., 1998). Therefore, understanding the genetic relationships among maize landraces could maximize the rational use of the genetic resources of the maize landraces.

Genetic studies of maize germplasms based on molecular markers were first performed using isozymes (Prince et al., 1986; Lamkey et al., 1987) and RFLP (Helentjaris et al., 1986; Helentjaris, 1987, 1991; Burr et al., 1988; Lee et al., 1989; Godshalk et al., 1990; Bernardo, 1994; Sourdille et al., 1996;). The RFLP technique was used for mapping the maize genome (Beaumont et al., 1996). PCR based techniques, such as RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeats), or even microsatellites, have also been described for genetic studies of the maize genome (Helentjaris et al., 1988; Heun and Helentjaris, 1993, Gupta et al., 1994; Wang et al., 1994; Chin et al., 1996; Taramino and Tingey, 1996; Senior et al., 1998).

The SSR is a powerful technique used to obtain molecular markers derived from PCR amplification of tandemly repeated sequences. SSR or microsatellites are very polymorphic and widespread in plant genomes and they have, therefore, become an alternative for genetic analyses in several species (Akkaya et al., 1992; Lagercrantz, 1993; Morgante and Olivieri, 1993). In SSR, the number of repeat units determines the polymorphism for fragment lengths and the heterozigote for different fragments in diploid genomes can usually be distinguished.

Individual loci corresponding to specific primer pairs are then co-dominant and can be multi-allelic. The products generated have been found to be highly reproducible (Jones et al., 1997).

Zietkiewics et al. (1994) and Kantety et al. (1995) described a marker system named Inter Simple Sequence Repeat (ISSR) amplification. The ISSR analysis involves the PCR amplification of regions between adjacent, inversely oriented microsatellites by using a single simple sequence repeat (SSR) containing primer. The technique can be applied to any species that contains a sufficient number and distribution of SSR motifs, and has the advantage that sequence data is not required (Gupta et al., 1994; Goodwin et al., 1997). The primers used in ISSR can be based on any di-, tri-, tetra- or penta-nucleotide SSR motifs found at microsatellite loci, giving a wide array of possible amplification products (Blair et al., 1999).

The ISSR technique is more reliable than RAPD and generates larger numbers of polymorphisms per primer because variable regions in the genome are targeted (Hantula et al., 1996). The potential use of ISSR markers depends on the variety and frequency of microsatellites, which changes with the species and with the targeted SSR motifs (Morgante and Olivieri, 1993). The number of bands produced by an ISSR primer with a given microsatellite repeat should reflect the relative frequency of that motif in the genome and would provide an estimate of the motif's abundance as an alternative for library hybridization (Blair, et al., 1999).

ISSR markers have been used to assess genetic diversity in maize inbred lines (Kantety, et al., 1995), to investigate the organization, frequency, and the level of polymorphism of different SSR motifs in rice (Beverley et al., 1997; Parsons et al., 1997; Akagi et al., 1997; Blair, et al., 1999) and to study genetic diversity in wheat (Nagaoka and Ogihara 1997), millet (Salimath et al., 1995), and sorghum (Yang et al., 1996). The genetic relationships were evaluated using ISSR markers in *Vigna* (Ajibade et al., 2000), *Phaseolus* (Hamann et al., 1995), and many other plant species (Gupta et al., 1994; Kantety et al., 1995; McGregor et al., 2000). The GATA and GACA repeats were used for QTL mapping in tomatoes (Grandillo and Tanskley, 1996) and for the genetic characterization of *Lycopersicom esculentum* cultivars (Vosmam and Arens, 1997).

According to Paterniani (2000), the maize landraces were developed from intercross among multiples races. Therefore, the knowledge of the genetic relationships among these varieties is important to avoid the use of genetically close materials, allowing for a more efficient use of the genetic heterogeneity. In this study the ISSR markers were used to assess the genetic relationships among maize landraces.

MATERIAL AND METHODS

The 81 accessions of maize used in this study consist of 79 landraces and the two improved varieties. These accessions were obtained from the Assessoria e Serviços a Projetos em Agricultura Alternativa (AS-PTA), a non-governmental organization that coordinates a program that aiming at the characterization and preservation of the maize landrace genetic resources maintained by small-scale farmers. The producers in this network have kept the maize varieties that were analyzed in this study in reproductive isolation by long period, by means of repeated planting of seeds derived from the same population. The two improved varieties were developed at the research institution Instituto Agronômico do Paraná (IAPAR). The 81 varieties form a collection containing varieties that are characterized by four different seed colors, namely yellow, white, red and blue. The yellow and white colors are caused by the color of the endosperm, while the red and blue are conditioned by color of the aleurone. Some varieties are segregating for endosperm colors (Table 1). In addition, the maize landraces are adapted to local environmental conditions (Soares et al., 1998) and to rusticity of the cultural techniques adopted by small farmers. IAPAR-50 and IAPAR-51 are improved varieties developed at the IAPAR research institute and highly cultivated in the state of Paraná. IAPAR 50 has a tall architecture (282 cm), a good prolificacy, and a productivity of 6,800 Kg/ha. This variety is also recommended for silage due to its good vegetative growth. IAPAR 51 has a short architecture (234 cm) and a mean productivity of 6,791 Kg/ha. It shows a yellowish color with occasional white seeds, since it resulted from crossings between varieties with white and yellow seeds. IAPAR 51 is recommended for cultivation in high fertility soils (Instituto Agronômico do Paraná, 1993).

DNA extraction and amplification

Seeds of the 81 accessions were arranged on filter

Table 1. Collection number, endosperm color, grain type, average flowering time (FT), altitude, and origin of 79 maize landraces and two improved maize varieties.

Variety	Endosperm color	Grain type	FT (Average)	City / State	Altitude (meters)
1 - Asteca	Yellow	Dent	74 days	Rio Azul/PR	856
2 - Asteca antigo do Prestupa	Yellow	Dent	78 days	Bituruna/PR	900
3 - Asteca Baixo Sabugo Fino	Yellow	Dent	73 days	Porto União/SC	752
4 - Asteca Sabugo Fino	Yellow	Dent	76 days	São João do Triunfo/PR	800
5 - Astecão Antigo	Yellow	Dent	75 days	Bituruna/PR	900
6 - BR 473[6]	Yellow	Semi dent	69 days	Porto União/SC	752
7 - BR106[7]	Yellow	Semi dent	73 days	Bituruna/PR	900
8 - Cabo Roxo[4]	Yellow	Dent	74 days	São João do Triunfo/PR	800
9 - Caiano	Yellow	Dent	78 days	Bituruna/PR	900
10 - C 408 x AG[8]	Yellow	Dent	71 days	Rio Azul/PR	856
11 - Carioca	Yellow	Dent	75 days	Bituruna/PR	900
12 - Comum Antigo x Sabugo Fino	Yellow	Dent	73 days	Rio Azul/PR	856
13 - Cravinho do Prestupa	Yellow	Dent	76 days	Bituruna/PR	900
14 - Cravinho Sabugo Grosso	Yellow	Dent	78 days	Cruz Machado/PR	950
15 - Cunha Amarelo	Yellow	Dent	73 days	Rio Azul/PR	856
16 - Dente de Cotia	Yellow	Dent	76 days	Cruz Machado/PR	950
17 - Ivo Agostiniak	Yellow	Dent	76 days	Cruz Machado/PR	950
18 - Macaco	Yellow	Dent	75 days	Porto União/SC	752
19 - Maia	Yellow	Dent	76 days	Cruz Machado/PR	950
20 - Milho Faxinal	Yellow	Dent	73 days	São Mateus do Sul/PR	760
21 - Milho Sem Nome	Yellow	Semi dent	74 days	Palmeira/PR	864
22 - Ouro Verde	Yellow	Dent	73 days	Iratí/PR	812
23 - Palha Roxa	Yellow	Dent	74 days	Porto União/SC	752
24 - Palha Roxa	Yellow	Dent	73 days	São João do Triunfo/PR	800
25 - Sete Variedades	Yellow	Dent	73 days	Porto União/SC	752
26 - Sol da Manhã	Yellow	Semi dent	69 days	Palmeira/PR	864
27 - Azcril	Segregant	Dent	76 days	Cruz Machado/PR	950
28 - Cabo Roxo[5]	Segregant	Dent	76 days	São João do Triunfo/PR	800
29 - Pintado	Segregant	Dent	72 days	Porto União/SC	752
30 - Sangue do Adão[2]	Yellow	Dent	75 days	Bituruna/PR	900
31 - IAPAR 51[1]	Yellow	Dent	73 days	IAPAR, Londrina /PR	
32 - Amarelão Antigo	Yellow	Dent	77 days	Porto União/SC	752
33 - Amarelão Bazonni	Yellow	Dent	80 days	Porto União/SC	752
34 - Amarelão Diwietz	Yellow	Dent	74 days	Porto União/SC	752
35 - Amarelo Antigo do Valdivino	Yellow	Dent	80 days	Bituruna/PR	900
36 - Amarelo do Tião	Yellow	Dent	79 days	Rebouças/PR	778
37 - Amarelo Graudo	Yellow	Dent	77 days	Rio Azul/PR	856
38 - Amarelo Taguari	Yellow	Dent	79 days	Rio Azul/PR	856
39 - Antigo 30 anos	Yellow	Dent	82 days	Iratí/PR	812
40 - Antigo Linha 5	Yellow	Dent	80 days	Iratí/PR	812
41 - Cravinho do Zeno	Yellow	Dent	79 days	Cruz Machado/PR	950
42 - Dente de Rato	Yellow	Dent	80 days	Iratí/PR	812
43 - Encantilado	Yellow	Dent	83 days	Cruz Machado/PR	950
44 - Linha Paraná	Yellow	Dent	81 days	Cruz Machado/PR	950
45 - Milho Antigo	Yellow	Dent	78 days	Palmeira/PR	864
46 - Milho Antônios I	Yellow	Dent	80 days	Iratí/PR	812
47 - Milho Caxoeira	Yellow	Dent	78 days	São João do Triunfo/PR	800
48 - Milho Fabrício Darci	Yellow	Dent	78 days	São João do Triunfo/PR	800

To be continued...

...continuation.

Table 1. Collection number, endosperm color, grain type, average flowering time (FT), altitude, and origin of 79 maize landraces and two improved maize varieties.

Variety	Endosperm color	Grain type	FT (Average)	City / State[10]	Altitude (meters)
49 - Milho Ferrinho	Yellow	Dent	77 days	União da Vitória/PR	752
50 - Milho Gropires	Yellow	Dent	78 days	Palmeira/PR	864
51 - Milho Pires	Yellow	Dent	78 days	Cruz Machado/PR	950
52 - Palha Roxa Alicheski	Yellow	Dent	78 days	São João do Triunfo/PR	800
53 - Pirulim do Tadeu	Yellow	Dent	80 days	Bituruna/PR	900
54 - Indígena[3]	Yellow	Dent	83 days	Cruz Machado/PR	950
55 - IAPAR 50[1]	Yellow	Dent	78 days	IAPAR, Londrina/PR	
56 - Antigo	Segregant	Dent	78 days	Rio Azul/PR	856
57 - Antigo Venglarek	Segregant	Dent	75 days	São Mateus do Sul/PR	760
58 - Asteca Branco Sabugo Fino	White	Dent	75 days	São João do Triunfo/PR	800
59 - BR 451 (QPM) [9]	White	Dent	71 days	Rebouças/PR	778
60 - Branco Comum	White	Dent	75 days	Rio Azul/PR	856
61 - Branco do Ferraz	White	Dent	78 days	São Mateus do Sul/PR	760
62 - Bromado	Segregant	Dent	71 days	São João do Triunfo/PR	800
63 - Bugre Branco	White	Dent	75 days	Rio Azul/PR	856
64 - Casano	Segregant	Dent	79 days	Rio Azul/PR	856
65 - Cinquentinha	Segregant	Dent	75 days	Cruz Machado/PR	950
66 - Milho Branco do Vicente Huk	White	Dent	73 days	Rebouças/PR	778
67 - Oito Carreiras	Segregant	Dent	76 days	Cruz Machado/PR	950
68 - Tostão Oito Carreiras	White	Dent	75 days	Rio Azul/PR	856
69 - Asteca Branco	White	Dent	81 days	Rio Azul/PR	856
70 - Astecão Branco	White	Dent	80 days	São João do Triunfo/PR	800
71 - Branco	White	Dent	84 days	Rio Azul/PR	856
72 - Branco de Cercado	White	Dent	83 days	Palmeira/PR	864
73 - Branco do Norte	White	Dent	81 days	Iratí/PR	812
74 - Branco dos Borges	Segregant	Dent	83 days	Rebouças/PR	778
75 - Branco Mexicano	Segregant	Dent	82 days	Palmeira/PR	864
76 - Branco Lastek Dinart	White	Dent	83 days	São João do Triunfo/PR	800
77 - Cunha Branco	White	Dent	83 days	Iratí/PR	812
78 - Maizena	White	Dent	80 days	Cruz Machado/PR	950
79 - Milho Branco Palha Roxa	Segregant	Dent	81 days	Cruz Machado/PR	950
80 - Milho Mexicano	Segregant	Dent	81 days	Cruz Machado/PR	950
81 - Tostão	White	Dent	79 days	Rebouças/PR	778

[1] Improved variety developed at Instituto Agronômico do Paraná (IAPAR); [2] Variety with red aleurone; [4, 5] Varieties with the same name, cultivated in same city, but planted by different local communities; [6, 7, 8, 9] Commercial varieties submitted to massal selection by local farmer for several years; [10] Cities located in regions with a subtropical humid climate.

paper and placed in a germination chamber at a controlled temperature of 25°C and 95% humidity. After seven days, equal quantities of leaves were collected from 15 plants of each variety and mixed to form a bulk. The DNA was extracted from each bulk according to the protocol described by Ferreira and Grattapaglia (1996) with CTAB (cationic hexadecyl trimethyl ammonium bromide) extraction buffer consisting of 2.0% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1.0% PVP and 0.2% β-mercaptoethanol. The extracted DNA was diluted in 100 mL TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA), quantified in a fluorometer (DyNA Quant 200, Höefer-Pharmacia) and diluted to 10ng/mL for the PCR amplification reactions.

After a preliminary test to check the capacity to amplify repeatable and analyzable fragments, 9 ISSR primers (Life Technologies BRL) including di, tri- and tetra-nucleotides motif, were selected and used in the DNA

Table 2. Primers repeats selected for ISSR analysis of 79 maize landraces and two improved maize varieties, total number of fragments, number of polymorphic fragments, percent of polymorphism produced, and annealing temperature.

Primers	Number of fragments	Number of polymorphic fragments	Polymorphic fragments (%)	Annealing temperature (ºC)
$(GA)_9T/(GA)_9C$	17	14	82.3	51.4
$(GA)_9T/(GACA)_4$	13	10	76.9	51.4
$(GA)_9T/(TAG)_6$	11	10	90.9	51.4
$(CCTA)_4$	10	10	100	50
$(GACA)_4/(CCTA)_4$	10	9	90	50
$(GA)_9C$	11	8	72.7	54.2
$(GA)_9T$	9	8	88.8	54.2
$(GA)_9T/(AT)_9$	9	8	88.8	51.4
$(GA)_9C/(AT)_9$	9	8	88.8	51.4
$(GA)_9T/(GGTA)_4$	10	6	60	52.5
$(GATA)_4$	7	5	71.4	50
$(GA)_9T/(CCTA)_4$	6	5	83.3	52.5
$(GA)_9C/(GATA)_9$	9	4	44.4	52.5
$(GATA)_4/(GACA)_4$	7	4	57.1	50
$(GACA)_4$	5	4	80	50
$(GTG)_6$	10	3	30	56.4
Total	153	116	75.8	

amplification reaction of the 81 varieties. The selected primers were used individually and in pairwise combinations in the same reaction (Table 2).

The ISSR amplification reactions were carried out in a 15mL volume, containing buffer 1x (75 mM Tris-HCl pH 9.0, 50 mM KCl, and 2.0 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.3 mM each of dNTP (dCTP, dGTP, dATP, dTTP), 0.4 mM primer, 0.7 unit of *Taq* DNA polymerase (Biotools) and 20 ng of template DNA. The amplifications were performed in a MJ Research PTC-200 thermocycler programmed for an initial denaturation of 4 min at 94 ºC, followed by 30 cycles consisting of 1 min at 94 ºC, 1.30 min between 50 ºC and 56.4 ºC depending on the primer (Table 2), 1 min at 72 ºC, and a extension of 7 min at 72ºC. The amplification products were loaded in 1.3% agarose gel stained with ethidium bromide. The amplified fragments were separated by electrophoresis in TAE buffer (Tris-acetate 0.04 M and EDTA 0.01 M pH 7.5) at 100 volts for two hours. Then, the amplification products were visualized in ultraviolet light and the gel images were transferred to a microcomputer for future analysis.

Data analysis

The PCR products were scored for the presence (1) and absence (0) of amplified fragments. The data were then used to analyze the genetic associations among the varieties. A similarity matrix was constructed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis for personal computers) software, version 2.1 (Rohlf, 2000) for all pairwise comparisons according to Jaccard's similarity coefficient. A dendrogram was constructed from the similarity matrix using the UPGMA method (unweighted pair-group method with arithmetical averages) and the SAHN subprogram (Sequential, Agglomerative, Hierarchical and Nested clustering). The PCA (Principal Coordinate Analysis) was applied using the presence (1) and the absence (0) of amplified fragments. The first two principal coordinates were used to describe the varieties dispersion in a bidimensional graph.

The bootstrap procedure was applied to calculate the coefficient of variance of the genetic similarities obtained from the markers. The analysis was

obtained from 1,000 bootstraps using the simple matching coefficient (Gover, 1985). The Dboot software, 1.1 version (Coelho, 2001), was used for the calculations.

RESULTS AND DISCUSSION

ISSR marker analysis

Nine primers were selected based on the preliminary tests for their capacity to amplify polymorphic and repeatable fragments. Three of them contained the dinucleotides $(AT)_9$ and $(GA)_9$ anchored with the T or C nucleotides at the 3´end; two were composed of trinucleotide $(GTG)_6$ and $(TAG)_6$, and four were the tetranucleotides $(GATA)_4$, $(GACA)_4$, $(GGTA)_4$, and $(CCTA)_4$ (Table 2). The amplified PCR products were easily distinguished in 1.3 % agarose gel (Figure 1).

A total of 153 DNA fragments were scored with an average of 9.5 fragments per primer, 116 of which (75.8%) were polymorphic. Primers based on the dinucleotide sequences $(GA)_9$ produced the greatest number of fragments (Table 2). Figure 1 shows a standard example of amplified fragments by the primer $(GA)_9T$.

According to Morgante and Olivieri (1993) the potential of ISSR markers to generate genetic information through polymorphic fragments depends on the microsatellite frequency and distribution in the genome of the species. In this study, we found that the polymorphism index varied from 30% to 100% for different primers with a mean of 75.8% (Table 2). The primer containing the tetranucleotide $(CCTA)_4$ was the most polymorphic (100%) while primers containing the dinucleotide $(GA)_9$ also produced high levels of polymorphisms when combined with other primers as observed with the $(GA)_9/(AT)_9$ combination (Table 2). These results are in line with a *Zea mays* DNA report where the most commonly detected sequence was the $(AT)_n$ containing repeats (Wang et al., 1994). The abundance of $(AT)_n$ repeats was reported in sequence studies of the rice genome (Akagi et al., 1997) and in the analysis of tree genomes (Condit and Hubbell, 1991). Although less frequent, the dinucleotide $(AG/CT)_n$ and trinucleotide $(AAG/CTT)_n$, $(ATG/CAT)_n$ and $(GTG/CAC)_n$ repeats were detected in the *Arabdopsis thaliana* genotype (Depeiges et al., 1995).

Regions containing (GA) repeats were more frequent in this study. Sequence studies reported in maize revealed that seven out of 34 sequences analyzed had regions containing the dinucleotide $(GA)_n$ and other sequences such as $(TA)_n$, $(AG)_n$ and $TG)_n$ have also been detected in mapping studies of maize (Taramino

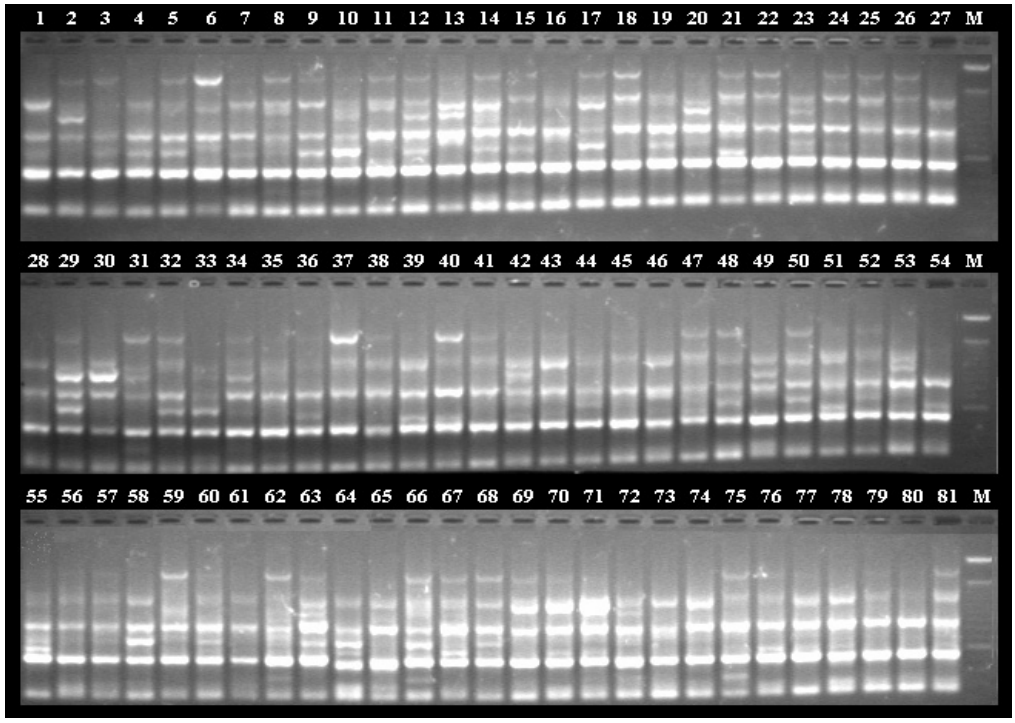

Figure 1. ISSR profile generated by primer $(GA)_9T$ with 79 maize landraces and 2 improved varieties. Lines 1 through 81 refer to collection numbers listed in Table 1. M is 100bp DNA size marker (Gibco BRL).

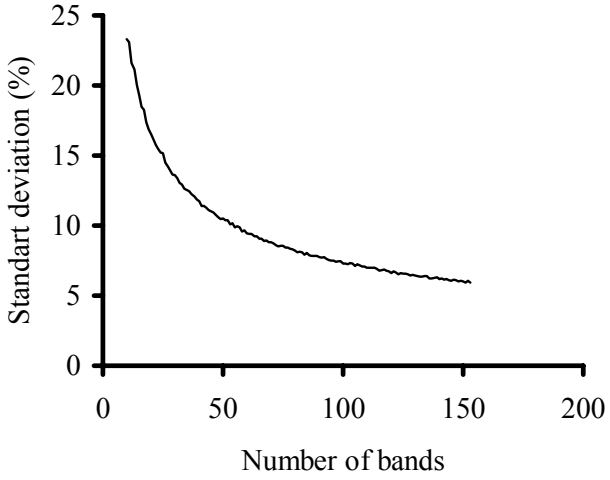

Figure 2. Sample variance of genetic similarity estimation for 79 maize landraces and two improved varieties depicted as the relationship between the mean coefficient of variation (%) and the number of bands derived from the bootstrap procedure.

and Tingey, 1996). In rice varieties, fragments amplified by primers containing the polymotif sequence (GA) were more common and more polymorphic. However, there was no fragment amplification of DNA from rice (*Oriza sativa* L) for primers containing the anchored tetranucleotide sequences (GATA) and (GACA) (Blair et al., 1999). Regions containing (GA) repeats were also more frequent in *Vigna* (Ajubade et al., 2000), in diploid, tetraploid and hexaploid wheat (Nagaoka and Ogihara, 1997), and in *Quercus macrocarpa* (Dow et al., 1995).

The relative abundance of different repetitive sequences of dinucleotide and trinucleotide motifs such as $(AG/CT)_n$, $(CCT/GGA)_n$, and $(CCG/GGC)_n$ were described in improved maize lines (Chin et al., 1996). Repetitive $(CT)_n$ sequences were also described in mapping studies of maize lines (Senior and Heum, 1993). The results obtained in this study, as well as those reported in the literature, suggest that the dinucleotide repeats $(GA)_n$ are very common in the genome of plants belonging to the Gramineae group.

According to Sneath and Sokal (1973) the number of traits required to stabilize genotype classification is very important in studies of genetic relationship studies. The bootstrap procedure applied to the 153 markers showed a variation coefficient of 5.9% suggesting that the number of bands was sufficient to stabilize the genotype classification (Figure 2).

Cluster analysis

The UPGMA clustering algorithm grouped the 81 maize varieties into three clusters designated as 1, 2 and 3 (Figure 3). Grain color was considered a very strong trait for genetic differentiation between varieties of the two main clusters - 1 and 2. These two groups were also supported by the principal coordinate analysis (Figure 4). Grain color is a very important trait for farmers because it is related to maize use. The varieties with white endosperm are used for human consumption and flour manufacturing while the flowering time is an important character for breeding programs (Paterniani, 2000).

Fifty-three varieties were grouped in cluster 1, and 48 of these varieties have yellow seeds, 44 have a dent grain type and four have a semi-dent grain type, three are segregant to endosperm color, and two varieties (Sangue do Adão and Indígena) show red and blue seeds, respectively, which are conditioned by the aleurone color. Both have a dent grain type (Figure 3, Table 1).

The varieties in cluster 1 were subdivided into 10 sub clusters that were mostly related to the flowering time (Figure 3, Table 1). In this cluster, the mean flowering time was 73.5 days for the earlier (sub cluster 7) and 79 days for the latter (sub cluster 9). The earliest variety flowered up to 69 days (variety BR 473) and the latest flowered at 83 days (varieties Encantilado and Pirulim do Tadeu) (Table 1). Two varieties (Asteca and Asteca Antigo do Prestupa) bearing a yellow endosperm appeared as sub cluster that was relatively isolated from the other sub clusters, showing only 67.5% of genetic similarity (Figure 3). These results suggest a high genetic variability within cluster 1. The Asteca and Antigo do Prestupa varieties flowered at 76 days on average. Sub clusters 2 and 3 include three and 13 varieties, respectively, which flowered at 76.3 (sub cluster 2) and 74.3 (sub cluster 3) days, on average. Sub cluster 4 consisted of seven varieties that, except for varieties Linha Paraná and Milho Antônios I - which flowered at 80.5 days -, present an average flowering time of 74.3 days. Sub cluster seven was formed by four varieties that flowered at 73.5 days. Two of these varieties (Azcril and Cabo Roxo) are segregants for endosperm and aleurone colors. The longest flowering time (79.6 days in average) in cluster 1 was found in the varieties arranged in the sub clusters 5, 8, 9, and 10. In these sub clusters, the varieties Sangue do Adão (sub cluster 5) and Pirulim do Tadeu (sub cluster 9) presented red and blue seed colors, respectively. The Cravinho do

Prestupa variety, with a yellow endosperm, was arranged between sub clusters 9 and 10. However, it presented only 70% of genetic similarity with all other varieties within cluster 1.

Twenty-six varieties grouped in cluster 2, 16 of which have white seeds and 10 are segregant to endosperm color. In this group, all varieties have dent grain type (Table 1). Cluster 2 was divided into four sub clusters, which were mostly related to the flowering time (Figure 3, Table 1). The flowering time average was 71.6 days for the early varieties (sub cluster 4) and 82 days for the late varieties (sub cluster 3). In these clusters, the varieties segregating for endosperm were more closely related to the varieties with white endosperm than to those with yellow endosperm. Segregating varieties were distributed into the four

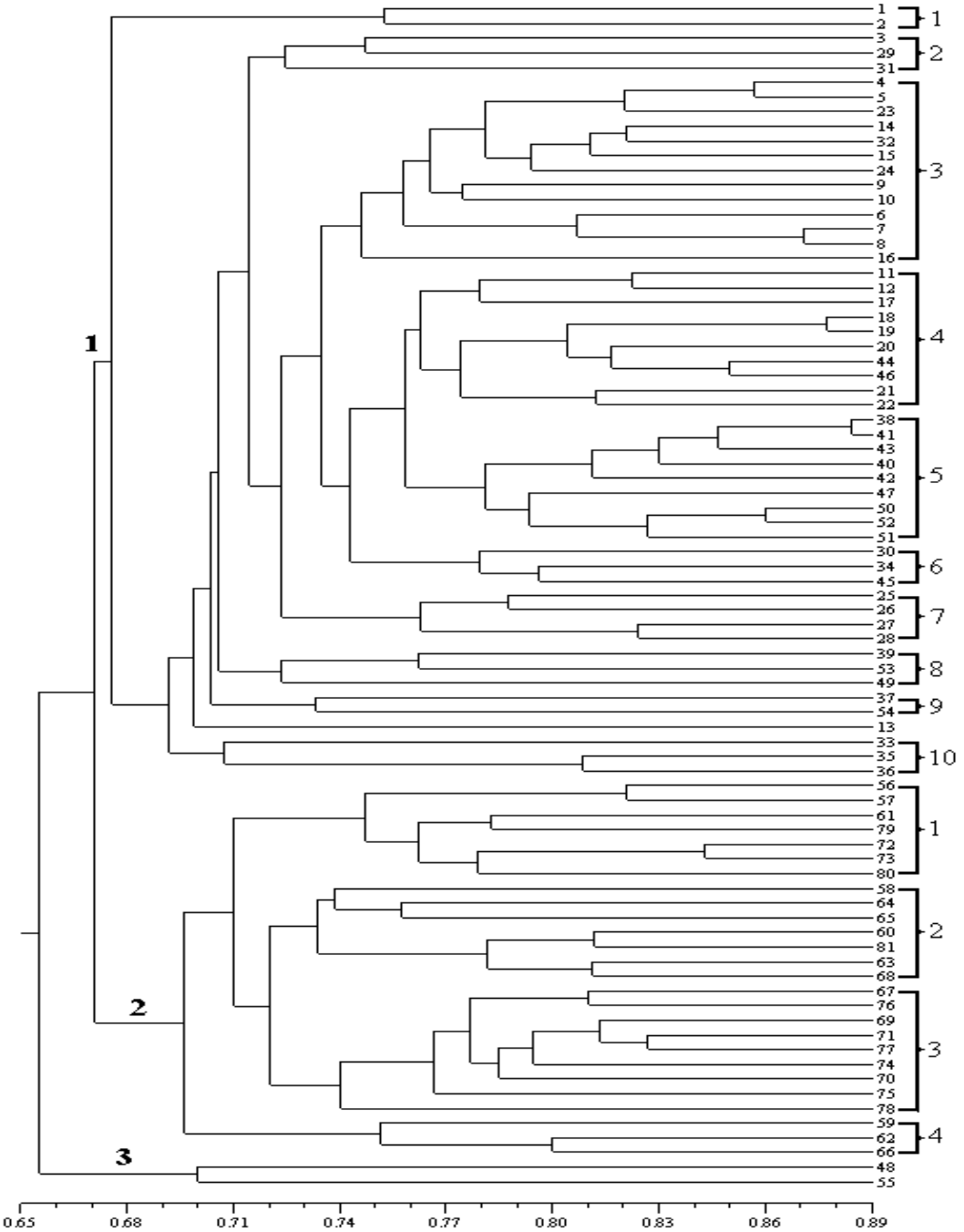

Figure 3. Genetic relationship among 79 maize landraces and two improved varieties, based on 153 ISSR markers. The numbers 1 to 81 correspond to the varieties listed in Table 1. The numbers 1, 2, and 3 in the left correspond to three clusters. The numbers 1 to 10, and 1 to 4 in the right correspond to the sub clusters of the clusters one and two, respectively.

sub clusters. The flowering time for varieties of cluster 1 varied on average from 71.6 days (sub clusters 4) to 82 days (sub cluster 3).

Flowering time is a very important trait in breeding programs. The cluster analysis (Figure 3) showed that the maize landraces classified as latest or earliest varieties within each cluster also exhibited the lowest genetic similarity. For instance, the more contrasting varieties BR 473 and Pirulim do Tadeu exhibited a flowering time of 70 and 83 days. These varieties were grouped in sub clusters 3 and 9, respectively, and the genetic similarity between them was only (66%). The same conclusions were achieved for cluster 2, where the earliest and latest varieties Milho Branco do Vicente Hulk and Branco also exhibited very low genetic similarity (65%). These results suggested that molecular data could be used in breeding programs.

In cluster 3 (Figure 3), only two varieties (IAPAR-50 and Milho Fabrício Darci) were grouped, however, they were associated with only 70% of genetic similarity. The variety IAPAR-50 is a commercial hybrid and therefore, its isolation from the other maize landraces is justified.

The principal coordinate analysis (Figure 4) was performed using the Jaccard's similarity matrix. Although the first and second principal coordinates explain only 9.7% and 5.3% of the total variation, respectively, this procedure was effective for the partition of the maize varieties into two major groups, formed by varieties bearing yellow and white endosperm, respectively. Therefore the principal coordinate analysis strengthen the results observed in the dendrogram for determining the genetic relationships among the maize landraces.

In this study, the use of ISSR markers revealed high levels of polymorphism among the 81 varieties of maize analyzed. A mean genetic similarity was 70% with the values ranging from 54% between varieties Asteca and Maizena to 88% between the varieties Amarelo Taguari and Cravinho do Zeno (data not shown). These results are in line with the probable origin of the maize landraces. Paterniani (2000) suggested that these varieties may have derived from old commercial races, such as Cristal and Cateto maize and other recent commercial varieties, originally cultivated by indigenous peoples and later adopted by the civilized man. The materials were

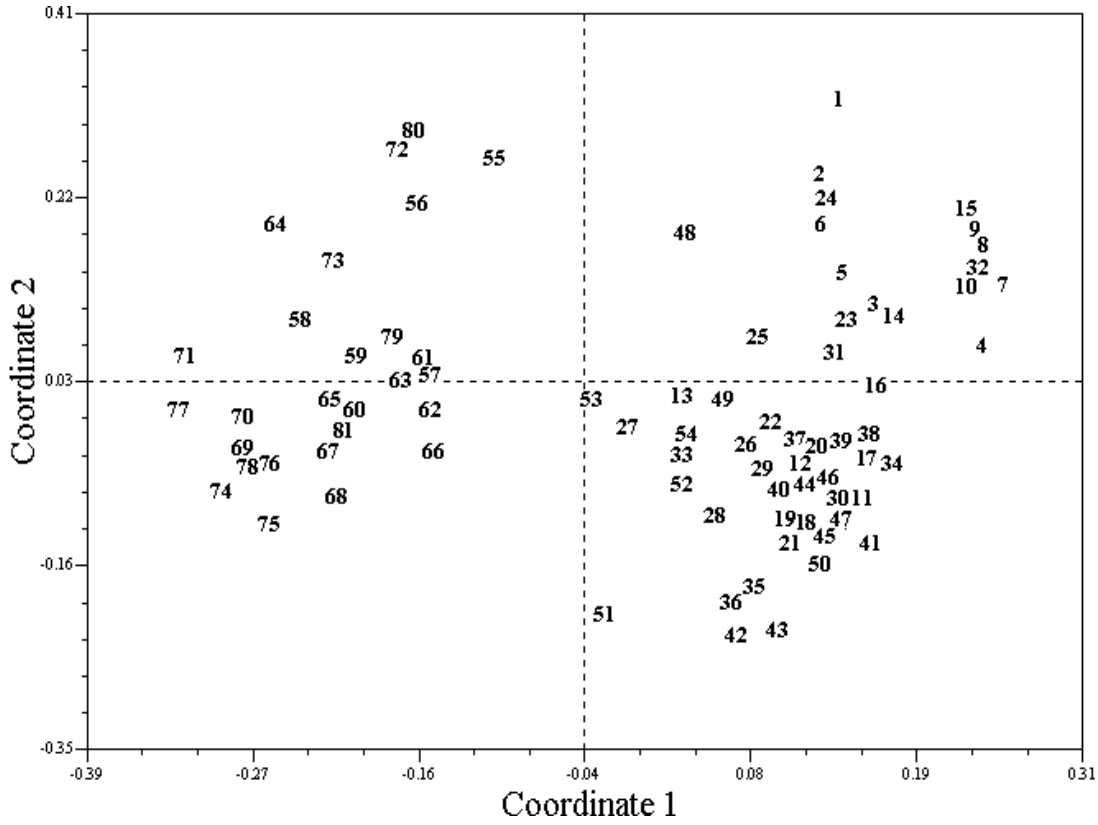

Figure 4. Principal coordinate analysis of the 81 maize accessions. The associations are based on Jacard's similarity coefficient calculated from 153 ISSR markers. Numbers 1 through 81 refer to collection numbers listed in Table 1. The first and second coordinate explains 9.7% and 5.3% of the genetic variation, respectively.

subjected to selection by farmers and crossed to local maize, such as Dente Riograndense, Dente Paulista, Dente Branco, Cravos, and exotic commercial races recently introduced from maize cultivated outside the country. The introductions include maize from Southern USA, Mexico, and Caribbean. Therefore, the high levels of genetic variability detected in the studied collection are justified by the multiple origin of the varieties associated with cultivation in different localities for several years.

While molecular data may be unsuitable for determination of heterotic groups in maize breeding programs (Parentoni et al., 2001), they can be applied for pedigree analysis and to define phylogenetic relationships among varieties (Senior et al., 1998). Molecular markers are therefore, important tools for preservation and for management of the existing genetic variability.

ACKNOWLEDGEMENTS

The authors would like to thank the farming families and the Assessoria e Serviços a Projetos em Agricultura Alternativa (AS-PTA) for supplying the samples of the landrace varieties. We also thank the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" - CAPES and Universidade Estadual de Londrina for funding.

RESUMO

Análise da Diversidade Genética em Variedades Crioulas de Milho *zea mays* (l.) Usando Marcadores de Inter-Simple Sequence Repeat (ISSR)

Marcadores de Inter Simple Sequence Repeat (ISSR) foram usados para analisar a variabilidade genética entre 79 variedades de milho crioulo e duas variedades melhoradas cultivadas no Brasil. Nove primers constituídos de dinucleotídios $(GA)_9$, $(AT)_9$, trinucleotídios $(GTG)_6$, $(TAG)_6$ e tetranucleotídios $(GATA)_4$, $(GACA)_4$ e $(GGTA)_4$ foram usados nas reações de PCR em que foram amplificados 153 fragmentos, dos quais 116 (75,8%) polimórficos. Nas reações que foram usados os dinucleotídos $(GA)_9T$ e $(GA)_9C$, combinados com outros di, tri e tetra-nucleotídios, foi obtido o maior número de fragmentos sugerindo a ocorrência, com alta frequência, de microssatélites poli GA no genoma do milho. Usando o método de agrupamento UPGMA, as variedades foram agrupadas em três grupos e 14 subgrupos, concordantes com as características cor do endosperma e dias para o florescimento, respectivamente. Os resultados mostram que marcadores ISSR podem ser efetivos para acessar a variabilidade genética do germoplasma do milho crioulo. As informações sobre a similaridade genética, entre as variedades, poderão ser utilizadas na seleção de acessos para formar bancos de germoplasma de milho crioulo e em programas de melhoramento genético.

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Received: September 09, 2002;

Accepted: October 29, 2002.