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GENETIC DIVERSITY OF MAIZE INBRED LINES AS INFERRED FROM SSR MARKERS

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Creating new maize hybrids with greater yield potential is a permanent goal of breeding programs all over the world. Long-time existing and new problems related to different biotic and abiotic stresses and the growing needs of the world market require constant work on finding new ways for advancing maize production. Molecular marker technology is one of the fastest developing fields and its implementation has already given results in solving different problems related to maize breeding improvement. The aim of the study presented herein was characterization and genetic similarity assessment of twenty-nine maize inbred lines from Maize Research Institute collection using *Simple Sequence Repeats* (SSR) markers. The analysis was done using 20 pairs of SSR primers with clearly visible and reproducible results. A total of 119 alleles were detected with a mean of 5.8 per locus. PIC (*Polymorphism Information Content*) values were in the range from 0.45 to 0.92 (average 0.74). Genetic similarities calculated using *Jaccard's* coefficient ranged from 0.27 to 0.99. Cluster and *Principal Component Analysis* (PCA) analysis were done using matrices of similarity in the NTSYSpc software, version 2.1. Results of both classifications were moderately in agreement with the pedigree data of analysed genotypes. The information about genetic diversity of maize inbred lines revealed by SSR markers could be useful in planning strategies for future maize breeding programs.

Key words: genetic relatedness, inbred lines, maize, SSR markers

INTRODUCTION

Challenges of modern agriculture such as yield reduction due to climate change (severe abiotic stress, extreme weather conditions, etc.) and growing needs caused by constant increase of

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the human population impose demand for finding new solutions for mitigation or overcoming these problems (ARAUS *et al*., 2008). Classical breeding, in spite of its indispensable role, reached the limitations in achieving significant progress regarding yield increasing and its stability. With the advent and development of molecular genetic techniques a new field of tremendous possibilities has been opened.

Maize, as one of the most exploited cultivated plants worldwide has been the object of diverse extensive studies over the time (COE, 2009). The aim of these studies was to improve the understanding of genetic basis of different traits and processes (developmental, physiological, morphological) in maize plants and to describe genetic relationships among different genotypes, since broadening and upgrading the knowledge about these topics could help in creating new strategies for achieving the maximum outcome under the existing conditions.

Enigma of heterosis, an increased performance of the progeny relative to its parents (SPRINGER and STUPAR, 2007) on which relies production of maize hybrids, has not been resolved yet. It has been shown in many cases that genetic distance is positively correlated with heterosis. Thus, heterosis is proportional to the genetic distance between maize parental lines. Nowadays, the prevailing means for testing the existence of the relationship is the application of molecular genetics methods (LEGESSE *et al*., 2006; DRINIĆ *et al*., 2012; FRASCAROLI *et al.,* 2013).

In spite of constant and rapid development of new techniques for molecular characterization of plants (for example SNP markers), some of the long-time existing and used methods persist as reliable tools for getting the valuable information in many topics. One such technique is using SSR markers, which are valued as reproducible, highly polymorphic, abundant and co-dominant (HECKENBERGER *et al*., 2002). SSR markers proved to be useful in various analysis like genetic diversity studies (VAZ PATTO *et al*., 2004; HILDAGO *et al.,* 2013), evolutionary studies (MATSUOKA *et al*., 2002; ZHENG *et al*., 2013) and genetic map construction (SA *et al*., 2012). These markers were frequently applied in different molecular genetic studies in maize: for genetic structure and diversity characterization of maize inbred lines (SRDIC *et al*., 2008; JAMBROVIC *et al*., 2008; SHEHATA *et al*., 2009; SUTEU *et al*., 2013) and landraces (QI-LUN *et al* 2008; MLADENOVIC *et al*. 2011; IGNJATOVIC MICIC *et al*. 2013; MOLIN *et al*., 2013).

In this study 29 inbred lines form Maize Research Institute collection were characterized using SSR markers. The aim of the study was determination of genetic similarities and relationships among the chosen maize genotypes. The results of the analysis could be used for future breeding planning - choosing the best combinations of parental lines which will exhibit good yield potential.

MATERIALS AND METHODS

Plant material

Twenty-nine maize inbred lines from Maize Research Institute collection were selected for genetic diversity determination. These genotypes were already used in breeding programs and their pedigree data are shown in Table 1. Characterization of maize inbreds was done using 25 SSR markers chosen according to good amplification results shown in previous studies.

SSR analysis

Maize genomic DNA was extracted from fresh leaves according to modified method of SAGHAI-MAROOF *et al*. (1984). The PCR reaction mix was carried out in a 25 µl volume. The mixture contained 1 x reaction buffer, 2.4 mM $MgCl_2$, 0.8mM dNTP, 0.5 µl primers, 1 U *Taq*

polymerase and 50ng DNA template. Touch-down amplifying program was applied (thermocycler Biometra TProfessional Standard 96) as follows: initial denaturation at 95□C for 5 minutes, 15 cycles of denaturation at 95°C for 30 seconds, annealing at 63.5°C for one minute (-0.5°C per cycle) and extension at 72°C, then 22 cycles of denaturation at 95□C for 30 seconds, annealing at 56°C for one minute and extension at 72°C for one minute and final elongation at 72° for four minutes. Electrophoretic separation of the amplified products was performed on 8% polyacrylamide gels for 1.5 hours at 80mA. After staining with ethidium-bromide for 30 minutes, gels were photographed under UV light using Biometra BioDocAnalyze Live gel documentation system.

Table 1. List of maize inbred lines and their pedigree

Line number	Pedigree
L1	Lancaster
L2	Iowa dent x Lancaster
L3	WF-9
L4	Iowa dent x BSSS
L5	Iowa dent x BSSS
L6	Iowa dent x BSSS
L7	Iowa dent x unrelated
L8	Iowa dent x BSSS
L9	Iowa dent
L10	Iowa dent x BSSS
L11	BSSS x Iowa dent
L12	BSSS x Iowa dent
L13	Lancaster
L14	Lancaster
L15	BSSS
L16	BSSS x Iowa dent
L17	unrelated
L18	BSSS x Iowa dent
L19	Ohio
L20	BSSS x Iowa dent
L21	Iowa dent x Lancaster
L22	BSSS
L23	Lancaster
L24	BSSS
L25	BSSS
L26	BSSS
L27	BSSS x domestic
L28	Lancaster x unrelated
L29	BSSS x Iowa dent x unrelated

Statistical analysis

Presence/absence of DNA bands was transformed into binary data (1 and 0 respectively). Polymorphism Information Content (PIC) for each primer pair was calculated according to formula PIC = 1 - Σ Pi^2, (i = 1 to *n*) where Pi is the frequency of *i-th* allele at a locus (SMITH *et al.,* 1997). Genetic similarity matrices were generated using *Jaccard*'s coefficient. Matrix was used for cluster UPGMA (Unweighted Pair-Grouping Method Analysis) and PCA analysis. Cophenetic correlation coefficient was calculated for testing the significance of association between genetic similarity matrix and dendrogram. All the calculations were done using NTSYSpc software, version 2.1.

RESULTS AND DISCUSSION

Twenty out of 25 SSR markers used were informative, producing clear and reproducible profiles. SSR marker names, sequences and map positions, as well as PIC values are presented in Table 2. A total of 119 alleles were detected, out of which 115 were polymorphic (96.94%). Number of alleles was in the range from 1 (phi033, umc1265) to 15 (bnlg1443), with the average number of 5.95 per primer. Lower values for the average number of alleles per marker (~5) were presented in the studies of WARBURTON *et al.* (2002) and CHOUKAN *et al.* (2006). In our study, the highest number of alleles was detected for the marker with a dinucleotide type of repeat (bnlg1443). Also, number of alleles amplified with the dinucleotide SSR umc1633 was very high (13). It is possible that the higher average number of alleles detected in this study compared to WARBURTON *et al.* (2002) and CHOUKAN *et al*. (2006) is ~~a~~ the consequence of a large number of dinucleotide markers used (50%). It was that high number of alleles is generally detected with dinucleotide SSR markers (XIA *et al.,* 2004; CHOUKAN *et al.,* 2006). PIC values were in the range from 0.64 to 0.92. The mean PIC was 0.74, which is higher than 0.60 given in LI *et al*. (2000) and XU *et al*. (2004) and could be explained by the small number of samples and high number of dinucleotide SSR primers.

Genetic similarity ranged from 0.27 (between L2 and L3) to 0.99 (between L7 and L8) with an average of 0.45. Most of the values fell between 0.40 and 0.50 (43.60%). The calculated average genetic similarity indicates high level of diversity among the analysed inbreds. Very high value for genetic similarity was detected between lines L7 and L8, suggesting that SSR markers could be successfully used for distinguishing closely related genotypes.

The results of cluster analysis are presented as a dendrogram (Figure 1). Cophenetic value of 0.80 confirmed good correspondence between the genetic similarity matrix and the dendrogram. Cluster analysis clearly distinguished analysed maize genotypes. Twenty – eight inbred lines were clustered into two main clusters (A and B). The line L3 was not grouped into any of the clusters, suggesting its loose relationship with the other inbreds, which is in accordance with the pedigree data - unlike the other analysed inbred lines, L3 was of independent origin. Cluster A consisted of L1, L2 and L4 lines, which mutually had at least one pedigree component in common. Within much larger cluster B comprising the remaining maize genotypes, subclusters I and II were clearly defined. Each of those was divided into the groups– subcluster I in groups Ia and Ib and subcluster II in groups IIa and IIb. Group Ia included inbreds of the same pedigree. BSSS pedigree component was the same for all the genotypes grouped in Ib, except for the L23 which was of Lancaster origin. Two groups of genotypes of different origin were assigned to group IIa.

Table 2 List of SSR primer names, their chromosome positions, sequences, types of repeat, number of alleles and PIC values

Name	Chromosome position	Type of repeat	Number of alleles	PIC
umc1265	2.02	TCAC(4)	1	0
bnlg1633	2.07	AG(16)	13	0.89
umc2129	2.07	CGC(5)	4	0.65
bnlg198	2.08	**-**	14	0.92
umc1643	4.09	CGACGC(4)	8	0.85
phi087	5.06	ACC	2	0.48
umc1019	5.06	CT(17)	5	0.78
bnlg1443	6.05	AG(25)	15	0.90
phi102	6.05	AT	2	0
umc1350	6.07	GCT(5)	3	0.66
umc1695	7.00	CA(8)	4	0.75
phi057	7.01	GCC	5	0.64
phi112	7.01	AG	2	0.45
umc1782	7.04	GAC(4)	3	0.61
umc1799	7.04 -7.06	TG(12)	5	0.75
bnlg2235	8.02	AG(23)	9	0.87
umc2014	8.07	**-**	10	0.84
phi033	9.01	AAG	1	0
umc1040	9.01	CT(11)	4	0.73
bnlg1526	10.04	AG(15)	9	0.86
average			**5.95**	**0.74**

The larger group consisted of two subgroups: inbreds with Iowa dent pedigree component in common were organized in the same group, while the lines L13 and L14 of Lancaster origin allotted to the other. The smaller group was composed of tree lines each of various origins. Group IIb consisted of two lines with white maize germplasm (data not shown) as a pedigree component. Inbreds related in pedigree in most cases grouped together.

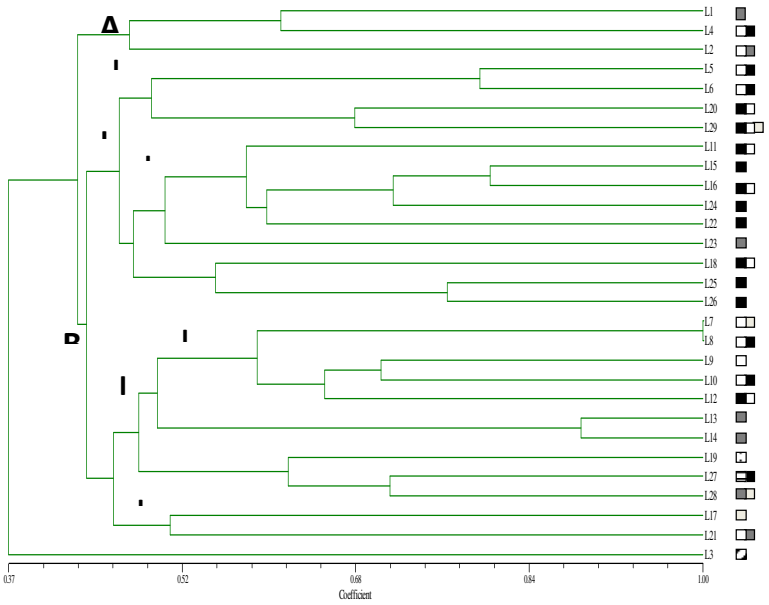

Figure 1 Cluster analysis of 29 maize inbred lines according to genetic similarity calculated using SSR marker data (*Jaccard* coefficient). Pedigree is presented with boxes: black – BSSS, white – Iowa dent, dark grey – Lancaster, light grey – unrelated, diagonal lines – line L3, independent origin dots – Ohio, horizontal lines – domestic

PCA showed somewhat different classification of the genotypes (Figure 2) compared to cluster analysis. This analysis determined that the first two principal components (PC) explained 23.87% of the total variation (13.66% PC1 and 10.21% PC2 respectively). Moderate agreement was detected between cluster and PCA analysis. On the positive side of PC1 axis genotypes grouped mainly in subcluster II were positioned, while inbreds assigned to subcluster I were predominantly on the negative side. In the lower right plot side genotypes with Iowa dent pedigree component were grouped. The group on the lower left plot side was formed of inbreds with BSSS pedigree component. In the upper left and the upper right plot side maize lines were not grouped according to pedigree data.

Partial congruence in grouping of maize genotypes on the basis of SSR marker and pedigree data was found in different studies (LEGESSE *et al.* 2007; ZHENG *et al*., 2008). This study also showed that classification of genotypes using marker data, whether cluster or PCA analysis were applied, was not the same as grouping of inbreds taking into account their pedigree. Maize is cross-pollinated crop and the reason for this inconsistency could be selection drift during the process of inbred line creation (KUMARI *et al*., 2005) or errors in pedigree information.

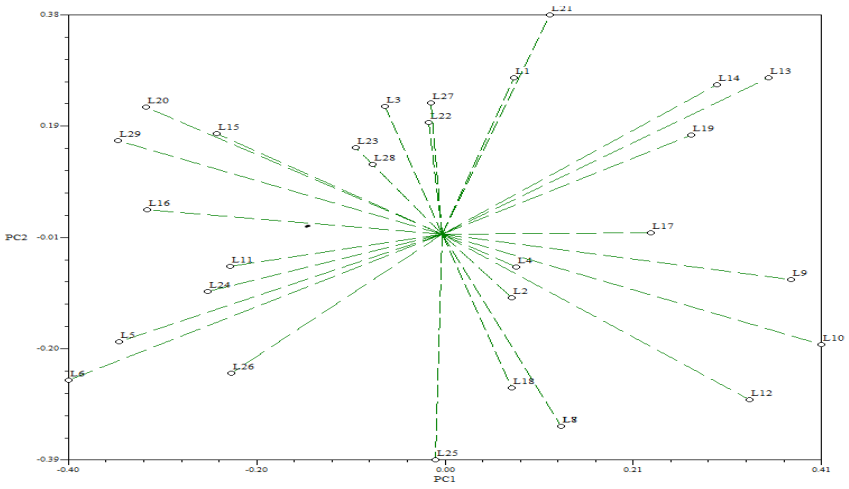

Fig. 2. PCA analysis

CONCLUSION

Most of the inbred lines could be discriminated with SSR markers used, indicating them as a valuable and powerful tool in genetic diversity assessment. The use of greater number of markers evenly distributed across genome could give more precise information about genetic relatedness among maize genotypes under study. These data could help in selection of the most suitable parental lines for creating new improved maize hybrids.

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PROCENA GENETIČKOG DIVERZITETA LINIJA KUKURUZA PRIMENOM SSR MARKERA

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Izvod

Stvaranje novih hibrida kukuruza sa većim potencijalom za prinos je stalna težnja programa oplemenjivanja svuda u svetu. Već postojeći i novi problemi koji se javljaju, a vezani su za različite biotičke i abiotičke stresove kao i rastuće potrebe svetskog tržišta zahtevaju stalan rad na pronalaženju novih rešenja za povećanje proizvodnje kukuruza. Tehnologija molekularnih markera je polje koje se brzo razvija, a primena dostignuća iz ove oblasti već je dala rezultate u rešavanju problema vezanih za unapređenje oplemenjivanja. Imajući u vidu navedeno, cilj ovog istraživanja bila je karakterizacija i utvrđivanje genetičke sličnosti 29 inbred linija kukuruza iz kolekcije Instituta za kukuruz primenom SSR markera. Analiza je urađena korišćenjem 20 parova SSR prajmera koji su dali jasno vidljive i reproducibilne rezultate. Detektovano je 119 alela, sa prosečnim brojem od 5.8 alela po lokusu. PIC (*Polymorphism Information Content*) vrednosti su bile u opsegu od 0.45 do 0.92 (srednja vrednost 0.74). Genetička sličnost između parova genotipova izračunata je korišćenjem *Jaccard* koeficijenta i kretala se u opsegu od 0.27 do 0.99. Grupisanje genotipova urađeno je primenom klaster i PCA (*Principal Component Analysis*) analize korišćenjem matrica genetičke sličnosti (NTSYS-pc softvera, verzija 2.1). Rezultati obe analize bili su u izvesnoj meri u saglasnosti sa pedigree podacima ispitivanih inbred linija kukuruza. Informacija o genetičkom diverzitetu inbred linija kukuruza detektovana primenom SSR markera može biti korisna u planiranju budućih strategija u programima oplemenjivanja.

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