

GENETIC DIVERSITY IN MAIZE FLINT LANDRACES ASSESSED BY MORPHOLOGICAL AND SSR MARKERS

Danijela Ristic, Marija Kostadinovic, Natalija Kravic, Violeta Andjelkovic,

Jelena Vancetovic, Dragana Ignjatovic-Micic

Maize Research Institute "Zemun Polje", Slobodana Bajica 1, 11185 Belgrade, Serbia

Abstract

Maize landraces represent potential sources of favorable traits for current and future breeding programs.. A comparative characterization of twenty-one flint maize landraces from Maize Research Institute "Zemun Polje" gene bank was achieved by using morphological traits and SSR markers. Applied marker systems revealed high level of genetic heterogeneity between landraces. Cluster analysis of the tested genotypes was based on average values of eighteen observed phenotypic traits. Ten SSR probes revealed total of 68 alleles. Genetic distance between pairs of landraces ranged from 0.05 up to 0.98. Cluster analysis of morphological and SSR markers distances did not exhibited the same grouping of accessions. Individual application of marker systems did not confirm differentiation of certain landraces into a particular agro-ecological group, as was expected according to their origin data. The best way for achieving the most accurate assessment of genetic diversity should comprise parallel application of both approaches.

Key words: gene bank, genetic distance, landraces, phenotypic diversity, SSR, *Zea mays* L.

1. INTRODUCTION

The diversity of several important crops, including maize, is threatened by rapid urbanization and human population growth (Lobell *et al.*, 2008), habitat erosion, as well as by the unpredictable and extreme climatic events (Cairns *et al.*, 2012). Despite these challenges, maize has enormous variation in morphological traits and extensive polymorphism in DNA sequences (Matsuoka *et al.*, 2002) that offers amazing opportunities for genetic enhancement. There is no lack of favourable alleles in the global maize germplasm that contribute to higher yield, abiotic stress tolerance, disease and pests resistance or nutritional quality improvement. However, these desirable alleles are often scattered over a wide array of landraces or populations. Our ability to broaden the genetic base of maize and to breed climate-resilient and high yielding cultivars adaptable to diverse agro-ecologies where maize is grown will undoubtedly depend on the efficient and rapid identification and introgression of novel and/or favourable alleles (Prasanna, 2010).

Narrow genetic base of commercial maize varieties emphasize the necessity for conservation, characterization and utilization of germplasm stored within gene banks (Carena *et al.*, 2009). Maize Research Institute „Zemun Polje“ gene bank maintains the collection of 2217 maize local landraces, collected from different agro-ecological sites of the former Yugoslavian territories and the collection of 3258 introduced accessions (inbred lines, synthetics, composites, and landraces) from about 40 countries worldwide. Local landraces, classified into 18 agro-ecological groups (Pavlicic and Trifunovic, 1966), are considered to be the most significant genotypes, since they represent the original biological material created by the process of natural selection and adapted to local growing conditions, thus offering the great opportunities for different breeding purposes and contributing to yield increase (Bracco *et al.*, 2009; Mladenovic Drinic *et al.*, 2011; Cömertpay *et al.*, 2012).

Many methodologies exist for the assessment of genetic diversity in maize. Since morphological markers are influenced by the environment, they often do not accurately portray genetic diversity. Moreover, the morphological differences are usually determined by a small number of genes and may not be representative of genetic divergence in the entire genome (Brown-Guedira *et al.*, 2000). Evaluation based on these traits is also expensive and time consuming. Nevertheless, the most important traits that indicate the total variance in the morphological analysis of maize genotypes are those related to the size of plants, tassel architecture and grain (Hartings *et al.*, 2008).

The classification methods based on phenotypic traits were enhanced with the use of DNA markers in the last two decades. Compared with morphological variation, molecular polymorphism is generally considered to be independent of the environment (Ghebru *et al.*, 2002). Molecular genetic techniques can be applied for the evaluation of genetic diversity as a complementary strategy to traditional approaches in the conservation and

utilization of plant genetic resources (Bardosa-Neto *et al.*, 1997). Besides providing useful information on genetic diversity and relationships between accessions, advantages of DNA-based markers include independence from environmental and pleiotropic effects, a potentially unlimited number of available markers and selectively neutral nature of many molecular markers (Spooner *et al.*, 2005). These advantages do not imply that other more traditional data used to characterize biodiversity are not valuable. On the contrary, morphological, ecological and other traditional data will continue to provide practical and often critical information needed to characterize genetic resources (Ignjatovic-Micic *et al.*, 2013).

Simple sequence repeat (SSR) markers are highly informative, and easy detectable with PCR. They occur frequently in plant genomes, showing an extensive variation in different individuals and accessions (Sharama *et al.*, 2010; Cömertpay *et al.*, 2012). As SSR markers are co-dominant, multi-allelic, highly polymorphic and randomly distributed throughout the genome (Morgante *et al.*, 2002; Barcaccia *et al.*, 2006), they are widely used for assessing maize genetic diversity (Nguyen *et al.*, 2012; Babu *et al.*, 2012).

The objective of this study was assessment of genetic diversity and relationships between flint maize accessions from different agro-ecological groups using morphological and SSR markers in order to i) determine agreement with existing classification of agro-ecological groups and ii) compare results based on morphological and SSR markers.

2. MATERIALS AND METHODS

2.1. Plant material

A set of 21 flint maize landraces was analyzed, with three landraces per the following agro-ecological group: Montenegrin flints (MOF), Bosnian early dents (BD), Kosmet flinty dents (KFD), Macedonian flints (MAF), Drived flints (DF), Mediterranean flints (MEF) and Small-kernelled flints (SF). Bosnian early dents belong to semi-flint kernel type, but they bear their name from time when only flints were known.

2.2. Field Experiment and Morphological Traits

Chosen genotypes were tested in four-replicate trial, set up according to Randomised Complete Block Design (RCBD). Each genotype was sown in four rows per replica, with 20 plants per row. Eighteen morphological and agronomical traits were observed on twenty randomly selected plants per replica. Phenotypic characterization of accessions included measurements of plant height (PH), ear height (EH), leaf number up to the ear insertion (LNEI), ear leaf length (ELL), tassel length (TL), length of tassel main axis (TSMA), number of primary tassel branches (NPTB), length of the branched part of the tassel (LBPT), ear length (EL), kernel row number (KRN), number of kernels per row (NKR), ear diameter (ED), kernel length (KL), kernel width (KW), kernel thickness (KT), grain yield (Y), 1000 kernels weight (KWE) and anthesis silking interval (ASI).

2.3. Molecular Analysis

2.3.1. DNA isolation

Genomic DNA was isolated from seed bulk using the CTAB procedure according to Saghai-Marooft *et al.* (1984). Bulks were prepared by pooling an equal amount of flour obtained by grinding 30 seeds per landrace. Seeds were taken from 30 different plants.

2.3.1. SSR Analysis

For each landrace, SSR analysis was done on bulked DNA samples, using 15 primer pairs. Due to absence of amplification product (5 primers), a set of 10 primers which gave scorable bands were chosen for the analysis. Polymerase chain reaction was carried out in 25µL reaction volume containing: DreamTaq™ Green PCR Master Mix (2X), 0.5µM primers and 50ng of DNA. Amplifications were performed in thermocycler Biometra TProfessional Standard 96 with the following touch-down program: an initial denaturation at 95°C/5min, by 15 cycles each of denaturation at 95°C /30 s, annealing at 63.5°C/1min (-0.5°C/cycle) and extension at 72 °C /1min; another 22 cycles of 95 °C /30 s, 56°C/1min and 72°C/1min were performed. Final elongation was at 72°C for 4min. The amplified fragments were resolved by electrophoresis on 8% polyacrylamide gel, with 100bp ladder as a marker. Gels were run on small format (7.3x10cm) vertical gel system (Mini Protean Tetra-Cell BioRad) at 40mA for 1.5h. After staining with 0.5µg/µL ethidium bromide they were photographed under UV light on BioDocAnalyse Biometra.

2.4. Statistical analysis

Analyses of variance were performed on each morphological trait to test the significance of variation between landraces. Coefficients of variation (CVs) were calculated to determine variations in different traits. Morphological data matrix was constructed according to average values of 18 observed morphological traits and their standard deviations (SD). Cluster analysis was conducted using square Euclidean distance and complete linkage method. Principal Component Analysis (PCA) was performed based on the phenotypic correlation matrix of the adjusted means of the landraces for the 18 morphological traits using SPSS 16.0 (<http://spss-for-windows-evaluation-version.software.informer.com/>).

The matrix of distances between maize landraces was calculated upon the standardized principal components with eigenvalue higher than one. Traits with a correlation > 0.7 were considered as relevant for that component.

SSR profiles were scored as presence/absence of fragments in each sample and the data were assembled into a binary matrix. Genetic distances between populations were evaluated by Nei and Li (1979). Unweighted Pair Group Method with Arithmetic mean (UPGMA) was applied for cluster analysis. All marker data analyses were performed using statistical NTSYSpc2 program package (Rohlf, 2000).

3. RESULTS AND DISCUSSION

Both types of markers, morphological and molecular, are necessary for effective conservation and development of genetic resources. Morphological markers are technically simple and they are helpful in preliminary evaluation of diversity (Beyene *et al.*, 2005).

Phenotypic analysis exhibited high level of heterogeneity between landraces. Tassel and kernel related traits, ear height, ASI and yield revealed wide variation between landraces. Coefficients of variation for these traits ranged from 16% to 29%. The narrowest variation was found for leaf number up to the ear insertion trait, with CV value of 9%, i.e. tassel length and kernel thickness, with CV of 10% (Table 1).

Table 1. Range and the average values of the measured phenotypic traits of the analyzed flint landraces

Phenotypic traits	Abbrev.	Mean $\pm$ SD	Range		CV (%)
			Min	Max	
Plant height (cm)	PH	187.3 $\pm$ 23.9	121.9	222.7	13
Ear height (cm)	EH	64.1 $\pm$ 14.2	31.7	86.1	22
Leaf number up to the ear insertion (cm)	LNEI	11.6 $\pm$ 1	9.2	13.1	9
Ear leaf length (cm)	ELL	70.1 $\pm$ 8.1	51.1	83.8	12
Tassel length (cm)	TL	44.4 $\pm$ 4.2	36.3	49.9	10
Length of tassel main axis (cm)	TSMA	24.1 $\pm$ 2.7	18.4	28.6	11
Number of primary tassel branches	NPTB	13.9 $\pm$ 2.9	7.8	19.3	21
Length of the branched part of the tassel (cm)	LBPT	9.5 $\pm$ 1.8	5.9	12.6	19
Ear length (cm)	EL	14.1 $\pm$ 1.9	7.9	17.8	13
Kernel row number	KRN	12.1 $\pm$ 2	8.9	15.8	17
Number of kernels per row	NKR	28.9 $\pm$ 4.7	15.4	35.6	16
Ear diameter	ED	3.6 $\pm$ 0.4	3.1	4.3	11
Kernel length (cm)	KL	0.8 $\pm$ 0.09	0.7	1.0	11
Kernel width (cm)	KW	0.8 $\pm$ 0.09	0.6	1.0	11
Kernel thickness (cm)	KT	0.4 $\pm$ 0.04	0.4	0.6	10
Anthesis silking interval (days)	ASI	4.5 $\pm$ 1.3	2	7.5	29
Yield (t/ha)	Y	3.1 $\pm$ 0.7	1.3	4.1	23
1000 kernels weight (g)	KW	261.130.7	152.5	270.4	12

*SD - standard deviation; CV - coefficients of variation; Abbrev. - abbreviations

Clustering and classification of the accessions based on morphological traits was performed using PCA and cluster analysis. Relationships between the traits were estimated using principal component analysis (Table 2).

Table 2. Eigenvectors, eigenvalues and accumulated variation of the first four principal components (PC) from the correlation matrix based on maize population means

Phenotypic traits	Abbrev.	PC1	PC2	PC3	PC4
Plant height (cm)	PH	0.805	0.502	0.099	0.103
Ear height (cm)	EH	0.923	0.221	0.053	0.146
Leaf number up to the ear insertion (cm)	LNEI	0.813	0.089	-0.087	0.082
Ear leaf length (cm)	ELL	0.803	0.532	-0.061	-0.046
Tassel length (cm)	TL	0.330	0.843	0.206	0.126
Length of tassel main axis (cm)	TSMA	0.127	0.890	0.017	0.191
Number of primary tassel branches	NPTB	0.860	-0.205	-0.074	0.053
Length of the branched part of the tassel (cm)	LBPT	0.639	0.305	0.289	-0.016
Ear length (cm)	EL	0.714	0.242	0.170	0.028
Kernel row number	KRN	0.327	-0.066	-0.870	-0.231
Number of kernels per row	NKR	0.563	0.373	-0.009	0.629
Ear diameter	ED	0.737	0.241	-0.216	-0.365
Kernel length (cm)	KL	0.401	0.507	0.307	0.423
Kernel width (cm)	KW	0.172	0.268	0.900	-0.120
Kernel thickness (cm)	KT	0.005	-0.164	0.172	-0.937
Anthesis silking interval (days)	ASI	0.088	0.349	-0.620	0.111
Yield (t/ha)	Y	0.823	0.209	0.112	0.324
1000 kernels weight (g)	KW	0.350	0.284	0.841	-0.135
% of Variance		45.897	16.927	11.512	6.519
Cumulative %		45.897	62.824	74.336	80.856

The first four principal components with eigenvalue higher than one, accounted for 80.855% of the total phenotypic variation. In the first PC, which explained 45.897% of the total variation, the most important characteristics were plant growth and yield related traits. In the second PC, which explained 16.921% of the total variation, predominant were tassel related traits. The third PC (11.366%) and fourth PC (6.519%) described variation in kernel traits. These results indicated that the traits related to the size of the plant and yield are traits that contribute most to the phenotypic variability between maize flint landraces. Our findings were in accordance to those on French maize populations (Gouesnard *et al.*, 1997) and on Italian maize landraces (Hartings *et al.*, 2008), indicating that traits related to the size of plants contributed the most to the phenotypic variability between the accessions.

The analyzed landraces are displayed in the graph, which is defined by PC1 and PC2 (Figure 1). Two landraces from MEF (MEF1 and MEF2), MAF (MAF2 and MAF3) and BD agro-ecological group (BD1 and BD3) had positive, while MEF3, MAF1 and BD2 had negative PC1 values. On the contrary, two landraces from KFD (KFD1 and KFD3), DF (DF1 and DF2), SF (SF2 i SF3) and MOF (MOF2 and MOF3) had negative, while KFD2, DF3, SF1 and MOF1 had positive PC1 values. Principal component analysis has not resulted in clear division of agro-ecological groups.

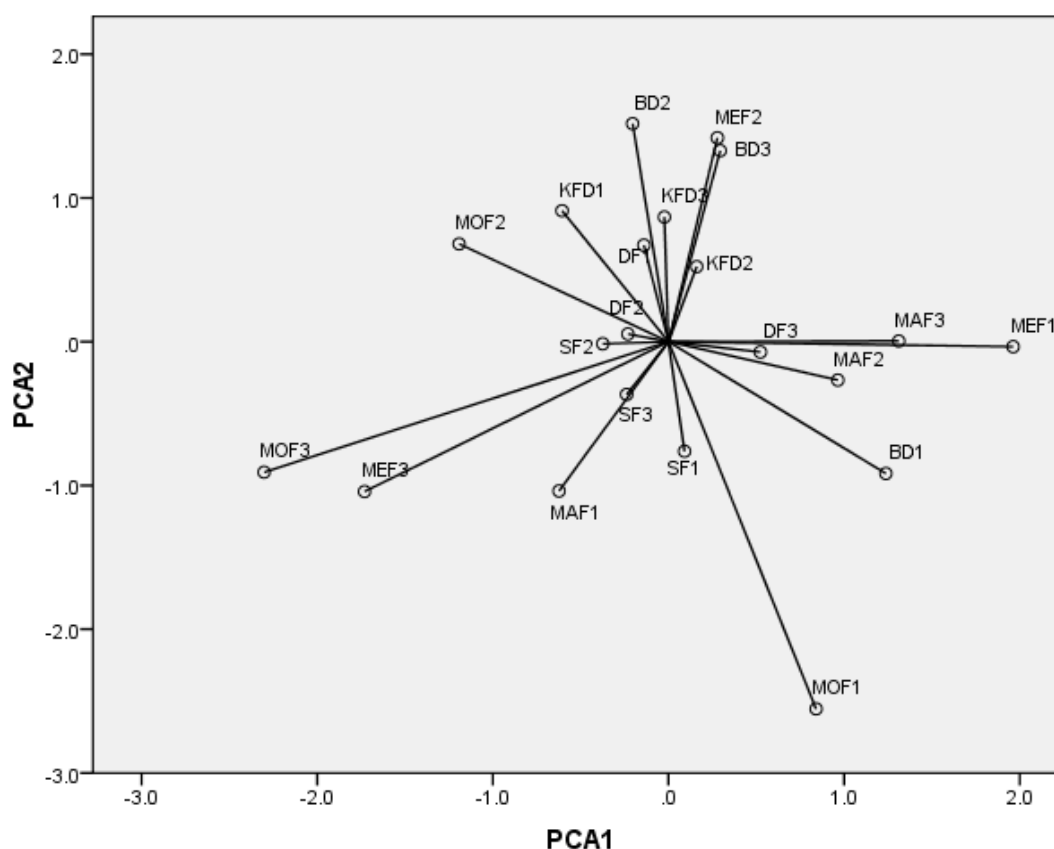


Figure1. Distribution of the 21 maize flint landraces on the first two principal components PC1 and PC2 of the PCA performed for phenotypic data.

Dendrogram based on morphological traits, presented in Figure 2, can be divided into two main clusters (I and II), with cluster I containing two subclusters (Ia and Ib). Furthermore, subcluster Ib contains two groups Ib1 and Ib2. BD and KFD landraces fell into one group Ib1, i.e. Ib2, respectively, and they were the most similar within themselves. MAF landraces were grouped in cluster I (subcluster Ia and Ib). Other landraces were distributed in both clusters. DF landraces, distributed in subcluster Ia (DF2), subcluster Ib1 (DF31) and cluster II (DF1), was the most diverse agro-ecological group. Cluster analysis indicated wide variability among the studied landraces.

The cluster analysis and PCA could not clearly separate landraces based on morpho-agronomical traits, indicating the limitations of these analyses in the assessment of genetic variability, especially among the heterogeneous maize landraces (Rebourg *et al.*, 2001; Sharma *et al.*, 2010). Despite that, morpho-agronomical traits of landraces are important in genetic diversity studies and they are useful in preliminary characterization. Landraces from Ib subcluster of the dendrogram obtained by morphological data were plants with high mean values of plant height, ear height and ear leaf length. On the contrary, landraces from cluster II had the lowest 1000 kernels weight.

Disadvantages of morphological markers can be surpassed by the use of molecular markers, in order to detect variation on the DNA level (Dubreuil *et al.*, 2006) and SSR markers are becoming the marker of choice for fingerprinting and genetic diversity studies for a wide range of plants (Gupta and Varshney, 2000). In our work, total number of alleles detected with ten SSR markers in observed 21 flint maize landraces was 68. All ten SSR loci were polymorphic. Average number of alleles was 6.8 per primer pair. A similar number of alleles (6.99) was reported in the work of Barcaccia *et al.* (2003), while the lower value of the average number of alleles (4.9) was reported by Beyene *et al.* (2006). In contrast, larger value of the average number of alleles (7.8) was achieved on landraces from different regions of Europe and the United States (Dubreuil *et al.*, 2006). In our

study, the range of alleles richness varied from three, for umc1418 to 11 for umc1274. Genetic distance between landraces determined by SSR analysis ranged from 0.05 (MEF3 and BD2) to 0.98 (MAF1 and BD1), while the average value of genetic distance between flint maize landraces was 0.40.

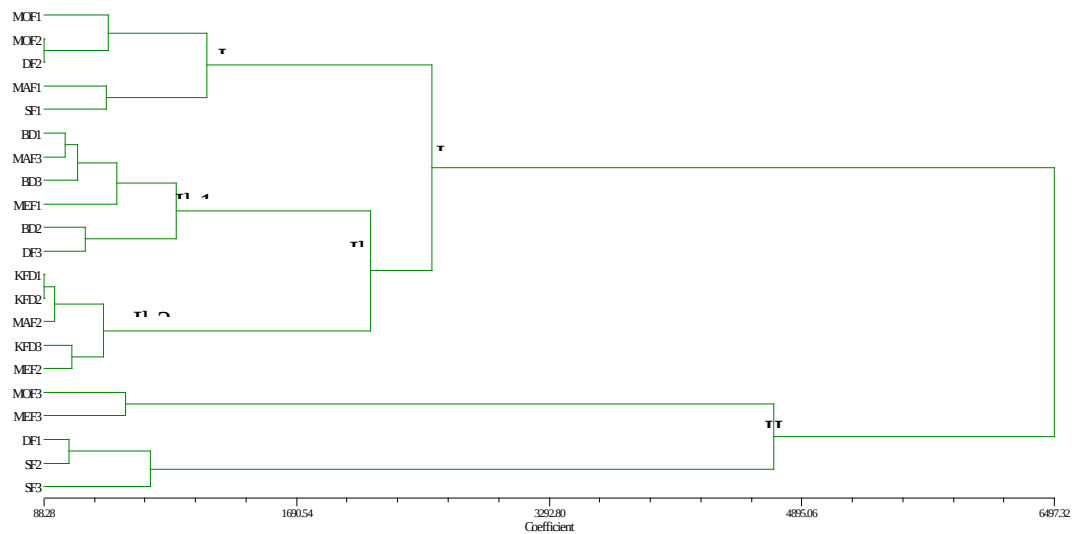


Figure 2. Dendrogram of the 21 maize flint landraces constructed using UPGMA cluster analysis of Euclidean distance values obtained by morphological data.

Dendrogram generated on genetic distance values based on SSR data, grouped the landraces in four-clusters, with two subclusters in the first cluster (Ia and Ib), whereas landraces KDF3(c) and BD1(d) were loosely linked to the dendrogram (Figure 3). MOF were grouped in the subcluster Ia and they were the most similar to each other. MAF were located in subcluster Iib (MAF2 and MAF3) and cluster III (MAF1). SF landraces were grouped in subcluster Ia (accession SF3) and subcluster Ib (SF1 landrace) respectively, while SF2 was allocated to cluster II. KFD and BD are the most heterogeneous groups of accessions, because they were divided into two clusters and loosely linked branches c, i.e. d.

Most of the agro-ecological groups showed different grouping comparing morphological data and SSR analysis. BD and KFD were grouped within the same subcluster based on morphological markers, while based on SSR markers were found within the different clusters and branches.

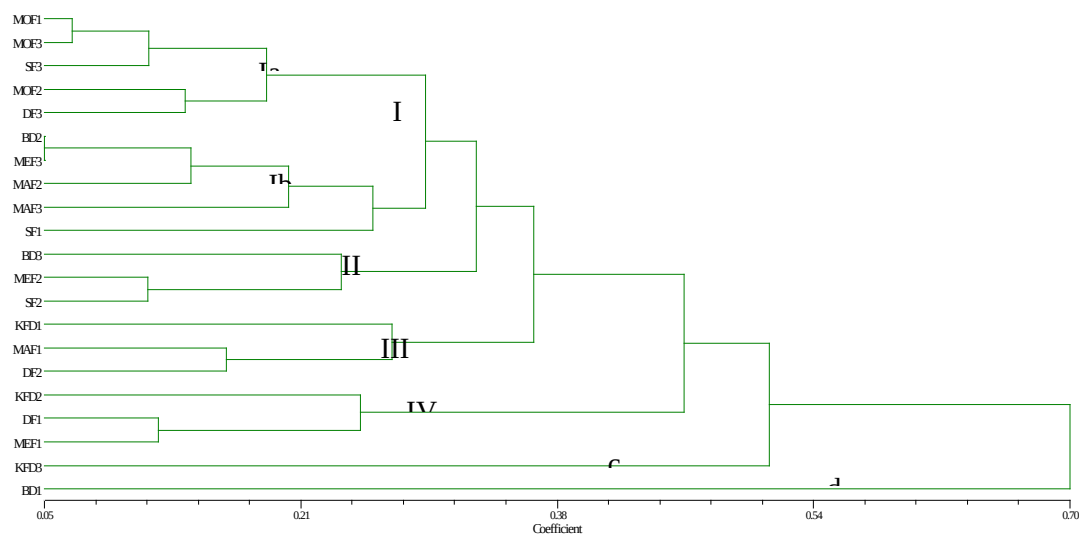


Figure 3. Dendrogram of 21 flint maize landraces constructed using UPGMA cluster analysis of genetic distance values (Nei and Li, 1979) obtained from SSR data.

4. CONCLUSIONS

Investigation of variability within chosen flint maize landraces, based on results obtained by application of morphological and SSR markers, showed high degree of phenotypic and genetic diversity, which could be explained by adaptation to specific environmental conditions and purposes through a long period of selection and local adaptation. Results obtained from PC analysis indicated that flint maize landraces could be characterized by traits such as plant growth, tassel characteristics and yield, which contribute the most to the phenotypic variation between landraces. In this work neither PCA nor cluster analysis could ~~not~~ make a clear distinction between different flint maize landraces, based on morphological and agronomic traits. Also, significant matching of landraces origin data with any of applied marker systems was not obtained. Results revealed with SSR markers can be explained by a small number of analyzed populations per agro-ecological group, as well as number of markers (ten), which cover a small part of the genome. Genetically distinct landraces may be similar by phenotype, which indicates the advantage of analysis by molecular markers in the study of maize landraces diversity.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, through the project TR31028 "Exploitation of maize diversity to improve grain quality and drought tolerance".

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