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# A SIMPLE SSR ANALYSIS FOR GENETIC DIVERSITY ESTIMATION OF MAIZE LANDRACES

Dragana IGNJATOVIC-MICIC*, Danijela RISTIC, Vojka BABIC, Violeta ANDJELKOVIC and Jelena VANCETOVIC

Maize Research Institute Zemun Polje, Belgrade, Serbia

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A collection of 2217 landraces from western Balkan (former Yugoslavia) is maintained at Maize Research Institute Zemun Polje gene bank. Nine flint and nine dent accessions from six agro-ecological groups (races), chosen on the basis of diverse pedigrees, were analyzed for genetic relatedness using phenotypic and simple sequence repeat (SSR) markers. One of the aims was to establish a reliable set of SSR markers for a rapid diversity analysis using polyacrilamide gels and ethidium bromide staining. In the principal component analysis (PCA) the first three principal components accounted for 80.86% of total variation and separated most of the flint from dent landraces. Ten SSR primers revealed a total of 56 and 63 alleles in flint and dent landraces, respectively, with low stuttering and good allele resolution on the gels. High average PIC value (0.822) also supports informativeness and utility of the markers used in this study. Higher genetic variation was observed among flint genotypes, as genetic distances between flint landraces covered a larger range of values (0.11- 0.38) than between dent (0.22 – 0.33) genotypes. Both phenotypic and SSR analyses distinguished flint and dent landraces, but neither of them could abstract agro-ecological groups. The SSR method used gave clear, easy to read band patterns that could be used for reliable allele frequency determination. Genetic diversity revealed for both markers indicated that the landraces were highly adapted to specific environmental conditions and purposes and could be valuable sources of genetic variability.

*Key words*: accessions, genetic diversity, phenotype, SSR, *Zea mays* L.

## INTRODUCTION

Maize is the most diverse crop containing huge variation in morphological traits and extensive polymorphism in DNA sequences (MATSUOKA *et al*., 2002). However, it was

***Corresponding author:*** Dragana Ignjatovic-Micic, Maize Research Institute Zemun Polje, Slobodana Bajica 1, Belgrade, Serbia, phone: +381113756704, fax: +381113756707, e-mail: idragana@mrizp.rs

approximated that today only about 5% of maize germplasm is in commercial use (CARENA *et al*., 2009). Even though this germplasm enables production of high yielding hybrids, its narrow genetic base is adverse for maize adaptation to different stresses and in this context inadequate to meet the contemporary and future major challenges - population growth and climate changes. One of the solutions to this problem lies in the utilization of diversity preserved in maize gene banks (landraces), which represent the raw material that farmers and breeders can use to improve its quality and productivity.

The collection of landraces from western Balkan (former Yugoslavia) was created in 1960-ies (PAVLIČIĆ and TRIFUNOVIĆ, 1968). Eighteen agro-ecological groups were established upon the natural classification method of ANDERSON and CUTLER (1942)**.** A re-classification, conducted by a concurrent analysis of the widest range of morpho-biological traits of all populations in one year, confirmed the validity of the original classification (RADOVIĆ *et al*., 2000). These agro-ecological groups encompass early introduced flint types grown on small and isolated areas, later introduced dent types that spread on wide areas of crop production in river valleys and plains, as well as types created through hybridization between flint and dent genotypes. Today, Maize Research Institute Zemun Polje gene bank maintains a collection of 2217 landraces.

The classification methods based on phenotypic traits were improved with the use of DNA markers in the last 15-odd years, with a variety of different techniques for genetic variation analysis (SPOONER *et al*., 2005). Due to their high allelic diversity and genetically codominant nature, simple sequence repeats (SSR) loci have been used successfully for genetic diversity studies and population structure of maize (DUBREUIL *et al.,* 2006; SHARMA *et al.,* 2010**;** CÖMERTPAY *et al.,* 2012). However, although DNA markers provide many advantages over phenotypic traits (e.g. independence from environmental and pleiotropic effects) morphological and other 'traditional' data will still continue to provide practical and often critical information needed to characterize genetic resources.

The main difficulty with SSR analysis is the complexity of band patterns (stutter bands), which is the consequence of *Taq* polymerase slippage during DNA amplification. This can especially be an obstacle when analyzing bulk samples of a population. Using a DNA sequencer, fluorescent dye-labeled SSR markers and accompanying softwares enhances the efficiency and precision of genotyping (CÖMERTPAY *et al*., 2012). However, this method is still too expensive and unavailable to many researches. Simpler and attainable methods, but reliable in predicting genetic relationships, are thus welcome to enable quality research of genetic diversity to the wider research community.

The objective of this study was to evaluate genetic diversity and relationships of flint and dent accessions belonging to different agro-ecological groups of former Yugoslavia landraces. Genetic diversity among a set of flint (IGNJATOVIC-MICIC *et al*., 2007; IGNJATOVIC-MICIC *et al*., 2008), as well as among a set of dent landraces (RISTIC *et al*., 2013) has been previously estimated. The flint and dent landraces were introduced in different periods and are of different ancestry (Central America and North America, respectively), but it is assumed that hybridization between the flint and dent genotypes occurred that created new authentic varieties. Genetic diversity and relatedness were analyzed with phenotypic traits and SSR markers. Additional objective was to establish a set of SSR markers appropriate for diversity analysis using simple detection system based on polyacrilamide gel electrophoresis and ethidium bromide staining.

## MATERIALS AND METHODS

*Genetic Material*

The 18 analyzed accessions from the Maize Research Institute gene bank belong to three flint and three dent agro-ecological groups (Table 1). Each of the group was presented with three landraces in this work.

*Table 1. Agro-ecological groups, landrace abbreviated name and country of collection of the analyzed maize landraces*

| Agro-ecological group | Group number | Landrace abbreviated name* | Country of collection |
|---|---|---|---|
| Montenegrin flints | I | FI1 | Montenegro |
| | | FI2 | Serbia |
| | | FI3 | Montenegro |
| Macedonian flints | IV | FIV1 | Montenegro |
| | | FIV2 | Macedonia |
| | | FIV3 | Serbia |
| Mediterranean flints | VII | FVII1 | Bosnia |
| | | FVII2 | Slovenia |
| | | FVII3 | Croatia |
| Dent type of USA Corn Belt dents | XIII | DXIII1 | Serbia |
| | | DXIII2 | - |
| | | DXIII3 | Serbia |
| Dent type of Southern areas of USA | XV | DXV1 | Croatia |
| | | DXV2 | Croatia |
| | | DXV3 | Croatia |
| Serbian dents | XVI | DXVI1 | Croatia |
| | | DXVI2 | Serbia |
| | | DXVI3 | Serbia |

* The abbreviations for each landrace consists of a letter indicating flint (F) or dent (D) affiliation, number of the agro-ecological group (given in Roman numerals) and number of the landrace from the particular agro-ecological group (1, 2 and 3).

*Phenotypic Analysis*

All accessions were sown in Zemun Polje in 2010, at two different sowing densities – 44640 and 64935 plants/ha. The experimental design applied was RCBD (Random Complete Block design) with two replicates, four rows per replicate and 20 plants per row. Two medium rows per population were used for trait measurements. For each plot 15 morphological traits (according to CIMMYT/IBPGR descriptors for maize) and anthesis-silking interval (ASI) were measured on 40 competitive plants per population. Kernel characteristics were measured on two kernels per ear. The traits are listed in Table 2.

Principal Component Analysis (PCA) was performed on the phenotypic correlation matrix of the adjusted means of the populations for the 16 traits from the descriptor. The matrix of distances between populations was calculated upon the standardized principal components

with eigenvalue higher than one. Common components coefficients, eigenvector values and cumulative proportions of the total variance expressed by single traits were calculated. Traits with a correlation >0.6 were considered as relevant for that component. All statistical analyses were performed using program package SPSS 15.0 (http://spss-for-windows-evaluation-version.software.informer.com/).

*SSR Analysis*

SSR analysis was carried out using a DNA bulk analysis with 30 plants per accession. Pooled-samples contained 0.1g of leaf tissue from which DNA was isolated by the modified method of SAGHAI-MAROOF *et al.* (1984). PCR amplification was initially performed with 50 SSR probes, but only ten were chosen for genetic diversity estimation.

The amplification reaction was carried out in 25 µl reaction volume containing 2X DreamTaq™ Green PCR Master Mix (Fermentas), 0.5 µM primers (LKB) and 50 ng of DNA. The amplification was performed in TProfessional Thermocycler (Biometra) with the following profiles: an initial denaturation at $95^0$C/5 min, followed by 15 cycles each of denaturation at 95°C/30 s, annealing at 63.5°C/1 min (–0.5°C/cycle) and extension at 72°C/1 min; another 22 cycles of 95°C/30 s, 56°C/1 min and 72°C/1 min were performed. Amplified fragments were separated on 8% polyacrylamide gels (Mini-PROTEAN® Tetra System, BioRad) and stained with ethidium bromide (0.5 µg $ml^{-1}$). The gels were photographed with Nikon D60 camera under UV light and SSR profiles for each primer were scored.

Genetic distance (GD) estimation was done using Rogers' Distance coefficient (A, 1972) based on allele frequencies. The probes used detected single loci and each detected band was assumed to be an allele. Allele frequency was scored as pixel total percentage of individual bands within the sample, using UN-SCAN–IT gel 6.1 program package.

The information content of each marker and locus was calculated using the polymorphism information content – PIC (LYNCH and WALSH, 1998). PIC values were calculated as PIC = 1- $\Sigma f_i^2$, where $f_i^2$ is the frequency of the $i^{th}$ allele.

Cluster analysis was performed with unweighted pairgroup method (UPGMA) and relationships between landraces were visualized as dendrograms. All statistical analyses were done by NTSYSpc2.1 program package.

## RESULTS

*Phenotypic analysis*

The 18 analyzed landraces displayed great variation for most traits, but flint landraces were more diverse. Relationships between the traits were estimated using PCA (Table 2). The first three principal components accounted for 80.86% of the total variation. In the first PC (57.10%) PH, EH, LN, HLL, NTPB, ERN, NKR, ED, KL and ASI were the most important traits. In the second PC (16.53%) predominant traits were TL, CSTL, BPTL and KW. The third PC (7.23%) described variation in EL and KT.

The analyzed landraces were plotted in the area defined by the first three PC (Figure 1). Flint and dent landraces were clearly separated. Majority of flint landrace (except FIV3 and FVII1) had negative PC1 value, while all dent landraces except DXIII3 had positive PC1 value – flint and dent landrace separation was yet achieved by the PC1 alone. Although secluded from the rest of the flint landraces, FIV3 and FVII1 positions in the plot were distinctive from the dent landraces.

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

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| Plant height (PH) | .878 | .376 | .078 |
| Ear height (EH) | .890 | .300 | .114 |
| Leaf number (LN) | .908 | .262 | .008 |
| Husk leaf length (HLL) | .838 | .482 | .108 |
| Tassel length (TL) | .488 | .733 | .101 |
| Central spike/tassel length (CSTL) | .301 | .791 | -.065 |
| Number of tassel primary branches (NTPB) | .874 | .065 | .080 |
| Branched part/tassel length (BPTL) | .162 | .629 | .467 |
| Ear length (EL) | .487 | .361 | .717 |
| Ear row number (ERN) | .854 | -.338 | -.007 |
| Ear kernels/row (NKR) | .716 | .396 | .238 |
| Ear diameter (ED) | .843 | .255 | -.077 |
| Kernel length (KL) | .776 | .362 | -.280 |
| Kernel width (KW) | -.130 | .820 | .069 |
| Kernal thickness (KT) | -.475 | -.065 | .698 |
| Anthesis-silking interval (ASI) | .885 | -.081 | -.348 |
| Eigenvalue | 9.14 | 2.65 | 1.16 |
| Accumulated variation (%) | 57.10 | 73.63 | 80.86 |

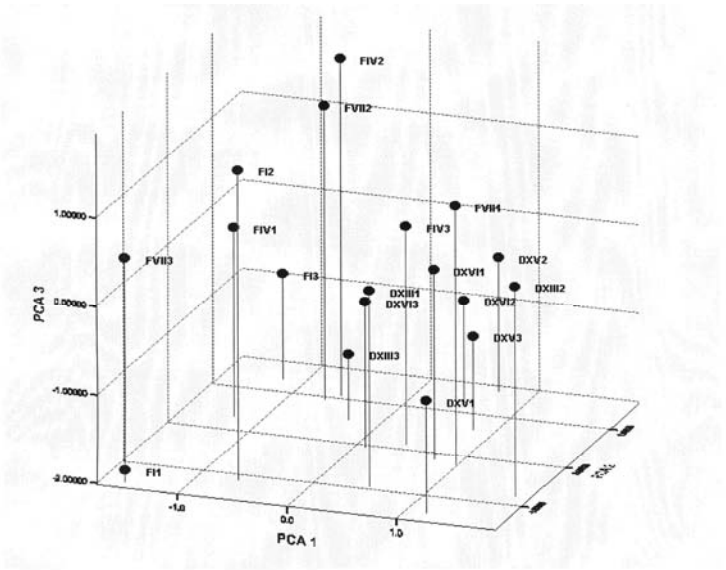


Fig. 1. Distribution of the 18 maize landraces on the first three principal components PC1, PC2 and PC3 of the PCA performed for phenotypic data.

*SSR Analysis*

Most of the 50 SSR primers used for PCR amplification could not be applied for landrace analysis due to the absence of amplification product (two primers), poor amplification (15 primers) or complex band pattern (28 primers). The list of 10 informative primers chosen for statistical analysis and their characteristics is given in Table 3. Only one of the ten primers was a di-nucleotide probe. The PIC values were in the range from 0.704 (umc1109) to 0.919 (phi087), with the average value of 0.822.

The genetic structure was determined with respect to all 18 landraces, as well as to flint and dent landraces separately (Table 3). Total number of alleles found for all landraces was 63, varying from 3 (umc1418) to 9 (umc1274 and umc1492) and with the average value of 6.3. Total number of alleles for dent landraces was 62, with the average value of 6.2. Flint landraces had lower number of alleles (56), with the average value of 5.6.

Average number of alleles per population was very similar for all 18 (flint and dent), flint and dent landraces – 3.4, 3.4 and 3.5, respectively. The biggest differences between flint and dent landraces was found for umc1418 (1.2 and 2.8 alleles, respectively) and umc1827 (3.7 and 2.3 alleles, respectively) probes.

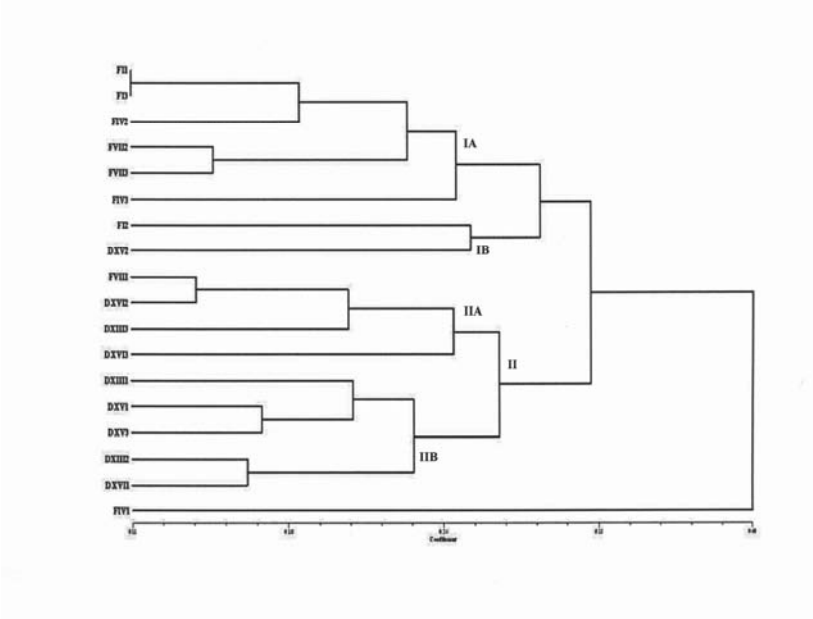


Fig. 2. Dendrogram of the 18 maize landraces (bulk samples) constructed using UPGMA cluster analysis of Roger's distance values obtained by SSR frequency analysis.

GD values were in the range from 0.11 (FI1 and FI3) to 0.52 (FIV1 and DXVI3). Most of the analyzed landraces from the same agro-ecological group had lower average GD compared to those obtained with landraces from other groups, meaning that they were genetically more similar among themselves. Montenegrin flints were genetically the most similar, i.e., showed the lowest GD values (0.21 for FI1 – FI2, 0.11 for FI1 – FI3 and 0.21 for FI2 – FI3). Macedonian

flints had the highest GD values, i.e., 0.27 for FIV1 – FIV2, 0.38 for FIV1 - FIV3 and 0.25 for FIV2 – FIV3. On average, higher GD were observed within dent (0.31, 0.28 and 0.24 for DXIII, DXV and DXI respectively) than flint agro-ecological groups (0.18 and 0.22 for FI and FVII), with the exception of Macedonian flints (0.30).

The result of cluster analysis is presented in the form of dendrogram (Figure 2). Two clusters were formed, with most of the flint landraces (seven out of nine) being in cluster I and most of the dent landraces (eight out of nine) in cluster II. Each cluster was divided into two sub-clusters. Sub-cluster IA included two landraces from each of the flint groups, while sub-cluster IB consisted of one flint (FI2) and one dent (DXV2) landrace. Sub-cluster IIA grouped together one flint (FVII1) with three dent landraces and sub-cluster IIB grouped five dent landraces. One Macedonian landrace (FIV1) was most distinctive as it did not group with any of the analyzed landraces.

## DISCUSSION

The accessions analyzed in the presented experiment are of different origin. Montenegrin, Macedonian and Mediterranean flint landraces are among the earliest maize types introduced to the region of former Yugoslavia. They were produced from the earliest introduced landraces of Central and Southern America, and are characterized by early maturity, shorter plants, shorter ear and small grain weight (PAVLICIC and JELENIC, 1977). On the other hand, dent types are the most recently introduced types that were the varieties used for development of hybrids. They are medium late and late maturity groups, with high leafy plants, medium long ears and greater kernel weight.

The results of flint and dent landraces' genetic assessment showed large heterogeneity and genetic diversity revealed by both phenotypic and molecular traits indicated that the landraces were highly adapted to specific environmental conditions and purposes through a long period of selection and local adaptation (from XVI century to the present).

Phenotypic analysis showed great variation for most traits. Flint landraces were more diverse. The same results were obtained SSR marker analysis. Higher genetic variation was observed among flint genotypes, as GD between pairs of flint landraces covered a larger range of values than GD between pairs of dent genotypes. It was previously shown that the diversity of flint types is greater compared to dent types, due to their earlier and predominant introduction into this region. Flints grown in the former Yugoslavia were among the ones with the greatest variability in Europe (BRANDOLINI, 1968).

PCA analysis of morphological traits could separate most flint and dent types, but not agro-ecological groups. It could be due the small number of landraces per agro-ecological group analyzed and/or the fact that morphological parameters are not the most reliable indicators of genetic relationships, especially when heterogeneous open-pollinated populations are considered. It was stated in REBOURG *et al*. (2001) that highly heritable traits can be easily modified by several cycles of selection and that morphological analysis does not seem fully appropriate for the classification of maize populations according to their genetic origin.

Genetic structure of the landraces analyzed in this research was determined with a set of ten SSR primers. The average number of alleles per locus was similar to the average number of alleles found in the analyses of different maize landraces presented in REIF *et al*. (2005) and CÖMERTPAY *et al*. (2012). Higher average number was found in DUBREUIL *et al*. (2006), MATSOUKA *et al*. (2002) and SHARMA *et al*. (2010). Different numbers of alleles detected in

different studies can be attributed to several factors, such as the type of SSR loci and whether individual plants or bulked samples were used. Three and more nucleotide repeat motifs limit the stuttering often associated with di-nucleotide SSR loci, which leads to complex band patterns and interferes with allele identification. However, tri-nucleotide or higher repeat motifs are less polymorphic and thus reveal lower number of alleles (VIGOUROUX *et al*., 2002). Additionally, bulk approach might discard some alleles with frequencies < 0.02 (REIF *et al*., 2005). Also, the more genotypes analyzed larger is the possibility of finding different alleles.

Cluster analysis did not group the landraces precisely with their affiliation to agro-ecological groups, with mostly two landraces from the same agro-ecological group linked together. Also, one flint landrace joined dent and one dent joined flint landraces. A high level of compliance was detected between distribution of landraces in the dendrogram and their origin. Macedonian landraces are significantly distant from Montenegrin and Mediterranean landraces (PAVLICIC, 1973) and the position of FIV1 (separated from all the analyzed accessions) and FIV3 (joined with IA sub-cluster) confirms these findings. On the other hand, all groups of dents developed in west Balkan regions were affected by Dent type of USA Corn Belt dents and Dent type of Southern areas of USA. Serbian dents have traits that are intermediate between these two dent types. It was assumed that they originated from Dent type of Southern areas of USA or Mexican dent that had been crossed with flints existing in Serbia at that time (PAVLICIC *et al*., 1976). In this context, clustering together of some flint and dent landraces could be expected.

Grouping of the landraces in the PCA analysis was similar. FVII1 grouped with dent landraces, the same as in cluster analysis with SSR markers. FIV1, detached from the other flint accessions in Ia subcluster, was positioned with dents in the PCA analysis, while one dent landrace grouped with flints. Differences between PCA and cluster analysis could be explained by different type of markers used, where morphological traits were influenced by environment.

The main intention in this study was to establish a set of SSR markers appropriate for diversity analysis using polyacrilamide gel electrophoresis and ethidium bromide staining. The results of ten chosen SSR primers, in combination with bulked samples and polyacrylamide gel electrophoresis, indicated that they could be successfully used in maize population genetic diversity studies. BRACCO *et al*. (2009) showed that ten SSR primers are adequate for genetic diversity estimations. The main advantage of this marker set is that the stuttering was significantly reduced and thus allele reading was more accurate. Additionally, high average PIC value supports informativeness and utility of the markers used in this study. Also, allele resolution on polyacrylamide gels was sufficient for reducing reading errors to an eligible level. Even eventual problems with detecting alleles present in low frequencies should not have affected the final estimations because detection of rare alleles is not the most important aim of a genetic characterization. REIF *et al*. (2005) showed that the effects of the most common alleles were sufficient to differentiate between maize populations. Although more sophisticated methods for SSR analysis are available, the equipment and chemicals are still too expensive for many research institutes. Thus, simpler but reliable methods are required and the presented results in our experiment affirmed this postulate.

In conclusion, both morphological and SSR analyses distinguished most flint and dent landraces, but neither of them could abstract agro-ecological groups. The ten SSR primer set showed to be an adequate option for timely and cost effective evaluation of maize landraces' genetic diversity. The results revealed a large genetic heterogeneity indicating that the analyzed landraces could be valuable sources of genetic variability. Certain incongruities between the

results of morphological and molecular analyses suggest that both phenotypic and genetic studies are necessary for achieving the most accurate assessment of genetic diversity.

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## JEDNOSTAVNA SSR METODA ZA UTVRĐIVANJE GENETIČKOG DIVRZITETA LOKALNIH POPULACIJA

Dragana IGNJATOVIC-MICIC*, Danijela RISTIC, Vojka BABIC, Violeta ANDJELKOVIC, Jelena VANCETOVIC

Institut za kukuruz Zemun Polje, Beograd, Srbija

Izvod

Institut za kukuruz Zemun Polje čuva i održava kolekciju od 2217 lokalnih populacija sakupljenih na teritoriji bivše Jugoslavije. Analizirana je genetička srodnost devet uzoraka tvrdunaca i devet uzoraka zubana različitog porekla iz šest agro-ekoloških grupa, pomoću fenotipskih i SSR markera. Jedan od ciljeva ovog rada je bio da se uspostavi pouzdan set SSR markera za brzu analizu diverziteta, korišćenjem poliakrilamidnih gelova i bojenja etidijum-bromidom. U analizi principalnih komponenti (PCA) prve tri komponente su iznosile 80.86% ukupne varijabilnosti i razdvojile su većinu populacija tvrdunaca od populacija zubana. Deset SSR prajmera je dalo ukupno 56 i 63 alela u populacijama tvrdunaca, odnosno zubana, sa malim stepenom pojeve *stutter* traka i visokom rezolucijom alela na gelu. Visoke prosečne PIC vrednosti (0.822) takođe podupiru informativnost i upotrebljivost korišćenih markera. Veća genetička varijabilnost je nađena kod tvrdunaca, kod kojih su genetičke distance bile u opsegu od 0.11 do 0.38, dok se kod zubana taj opseg kretao od 0.22 do 0.33. I fenotipski i molekularni markeri su razlikovali tvrdunce i zubane, ali nisu mogli da razlikuju agro-ekološke grupe. Izabrani SSR markeri su dali jasne, lake za čitanje trake i mogu da se koriste za pouzdano utvrđivanje alelnih frekvencija. Rezultati analize genetičkog diverziteta ukazuju na visok stepen adaptiranosti ovih lokalnih populacija na specifične uslove spoljne sredine i specifične namene, tako da mogu biti dobar izvor genetičke varijabilnosti.

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