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Conserving maize in gene banks: Changes in genetic diversity revealed by morphological and SSR markers

Violeta Andjelkovic[1*], Ana Nikolic[1], Dragan Kovacevic[1], Snezana Mladenovic-Drinic[1], Natalija Kravic[1], Vojka Babic[1], Mirjana Srebric[1], Mirjana Jankulovska[2], Sonja Ivanovska[2], and Dane Bosev[2]

[1]Maize Research Institute Zemun Polje, Slobodana Bajica 1, 11185 Belgrade, Serbia.
[*]Corresponding author (avioleta@mrizp.rs).
[2]University Ss Cyruk and Methodius, Faculty of Agricultural Sciences and Food, blvd. Aleksandar Makedonski bb, 1000 Skopje, Republic of Macedonia.

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ABSTRACT

In the second half of 20th century the awareness of importance of landraces for the future, led to organized collecting missions for numerous plant species. A total of 2217 maize (*Zea mays* L.) landraces, collected in the former Yugoslavia, are stored at Maize Research Institute (MRIZP) gene bank. During 2014, new collecting missions were organized in the eastern and western parts of Macedonia. According to collecting site and kernel type, 14 samples from the Faculty of Agricultural Sciences and Food, R. Macedonia were chosen for the comparison and identification of possible duplicates, through coupling with the 16 MRIZP gene bank accessions from the same area and kernel characteristics. Phenotypic characterization was done for 21 traits according to International Board for Plant Genetic Resources descriptors for maize. The Principal Component Analysis (PCA) identifies five PCs with Eigenvalue > 1, explaining 80% of the total phenotypic variation. The most discriminative traits with the strongest positive associations were tasseling and silking dates, plant height, leaf length and ear length. Compared to the *ex-situ* populations, the number of alleles and the number of specific alleles, showed a significant decrease in the *in situ* populations. Twelve unique alleles were detected in samples from MRIZP gene bank, and only four were found in new Macedonian samples. Cluster analysis of morphological and molecular markers distinguished groups of maize accessions with distinctive morphological traits and genetic profiles that will be useful for conservation, and management of gene bank collection, as well as for possible utilization in breeding.

Key words: Accessions, landraces, molecular markers, variability, *Zea mays*.

INTRODUCTION

Maize genetic resources are of high importance for agriculture, but breeding of modern hybrids in the second half of the 20th century, contributed to rapid replacement of traditional landraces in Europe. The need for preventing the loss of maize (*Zea mays* L.) genetic diversity led to organized collecting missions. Since landraces are a valuable source of potentially useful traits, there is necessity to properly conserve and characterize them, prevent from disappearing, and to keep for utilization in breeding.

Ex situ method is very useful for seed conservation in gene banks. In contrast, *in situ* conservation protects diversity and identifies varieties with useful and adaptive genes. Many maize gene banks were established in

different countries, and characterization of genetic diversity was carried out (Rebourg et al., 2001). Global Plan of Action for Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture (FAO, 1996) recommended increase efficiency in *ex situ* conservation by the development of core collections and reduction in duplicate accessions within and between collections.

However, the usefulness of applied morphological characterization of gene bank accessions became limited, since they are strongly affected by environment. In the last few decades, application of molecular markers for polymorphism evaluation at the DNA level has been very successful in genetic diversity studies (Ignjatovic-Micic et al., 2008; Drinic Mladenovic et al., 2012; Barcaccia et al., 2016). Since molecular characterization of accessions is becoming more important for identification and elimination of potential duplicates in collections (Reif et al., 2004), relationship between diversity on morphological and molecular level, could be of high importance for conservation in gene bank.

Replacement of local maize landraces with hybrid maize in the second half of the 20th century occurred in former Yugoslavia territory (western Balkan region, Andjelkovic and Ignjatovic-Micic, 2012). Collection, characterization, and natural classification in coming decades resulted in more than 2000 local maize landraces stored at Maize Research Institute Zemun Polje (MRIZP) gene bank, and out of them, 222 landraces collected in the Republic of Macedonia (Andjelkovic and Ignjatovic-Micic, 2012).

In the present paper, 14 pairs of maize landraces, collected in Republic of Macedonia in the second part of 20th century, and in 2014, were compared by morphological traits (International Board for Plant Genetic Resources; IBPGR, 2008) and microsatellite markers, with the aim to examine variation in genetic diversity of landraces in past 40 yrs. The results of the assessment will contribute to the more efficient *ex situ* conservation, management, and rationalization of gene bank collection, e.g. elimination of potential duplicate accessions.

MATERIALS AND METHODS

Field trails

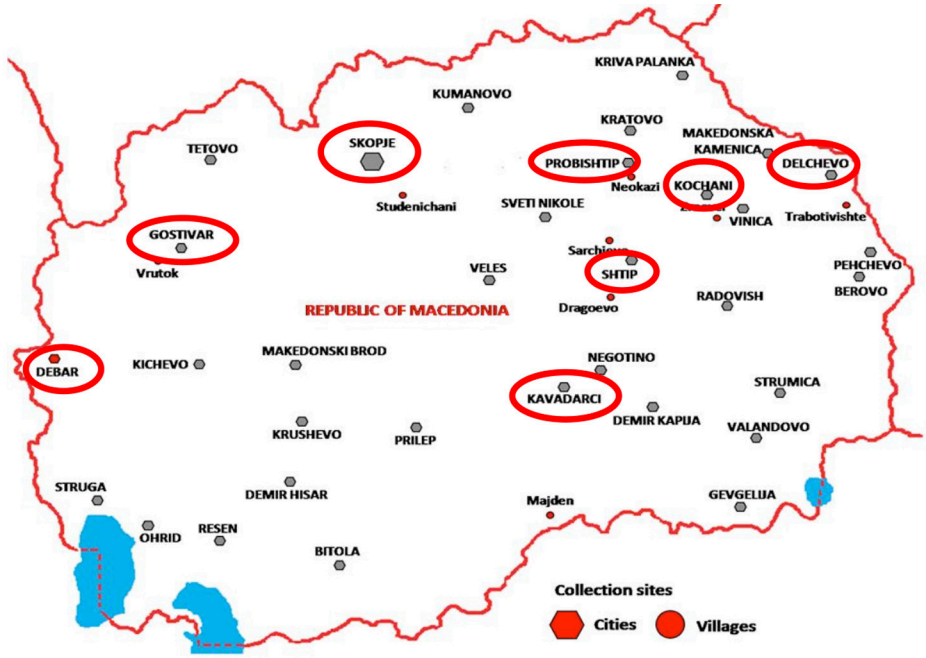
To analyze temporal changes in genetic diversity, 73 samples from the Faculty of Agricultural Sciences and Food, Republic of Macedonia (FASFMK) collection, and 222 accessions from MRIZP gene bank were paired, based on the collection sites, and kernel color and texture, since kernel color, type and texture are initial steps for monitoring accession identity, according to guidelines (http://cropgenebank.sgrp.cgiar.org/images/file/maize/Maize_ENG.pdf). After comparison, 16 accessions represented *ex-situ* accessions, collected in different years (1974, 1975, 1978, 1980, 1983, 1984, and 1989) and conserved at MRIZP gene bank, and 14 *in-situ* samples collected from farmers in 2014 from several locations in the Republic of Macedonia (Table 1, Figure 1), were chosen for evaluation.

For *ex-situ* samples, seeds were taken from regeneration stocks from MRIZP gene bank, after up to five times of multiplication (detailed characterization of landraces is available at https://www.mrizp.rs/emdb/default-en.htm), and from FASFMK collection, for *in-situ* samples, seeds of landraces collected in 2014, were used. The landraces were

Table 1. List of maize landraces accessions, gene bank identification document (GB ID), collecting site, acquisition year of maize landraces collected in the Republic of Macedonia.

Nr	GB ID	GB	Collecting site	Acquisition year	Nr	Bank ID	GB	Collecting site	Acquisition year
1	MK1	MK	Stip	2014	15	MK18	MK	Gostivar	2014
2	ZP 1818	SRB	Stip	1982	16	ZP 1086	SRB	Gostivar	1973
3	MK37	MK	Probistip	2014	17	MK60	MK	Delcevo	2014
4	ZP 1819	SRB	Probistip	1982	18	ZP 2223	SRB	Delcevo	1989
5	ZP 1820	SRB	Probistip	1982	19	MK30	MK	Skopje	2014
6	MK36	MK	Probistip	2014	20	ZP 1642	SRB	Skopje	1978
7	ZP1822	SRB	Probistip	1982	21	ZP 1643	SRB	Skopje	1978
8	MK52	MK	Stip	2014	22	ZP 1645	SRB	Skopje	1978
9	MK6	MK	Kavadarci	2014	23	MK31	MK	Debar	2014
10	ZP 1682	SRB	Kavadarci	1978	24	ZP 1090	SRB	Debar	1973
11	MK10	MK	Kocani	2014	25	ZP 1091	SRB	Debar	1973
12	ZP 1969	SRB	Kocani	1984	26	ZP 1093	SRB	Debar	1973
13	MK58	MK	Stip	2014	27	MK50	MK	Stip	2014
14	ZP 1172	SRB	Stip	1974	28	ZP 1141	SRB	Stip	1973

Figure 1. Collecting sites for the maize samples.



grown in 2015 at two locations: Zemun Polje (44°52'1" N, 20°19'16" E, 80 m a.s.l.), Serbia, and Kumanovo (42°05' N, 21°40' E, 340 m a.s.l.), Republic of Macedonia, in randomized complete block design with two replicates. In Zemun Polje the soil was Mollisols and in Kumanovo the soil was Vertisol. At both locations, the usual cultivation practice for maize was applied. Plants were sown in single row plots with 15 hills per row and spaced 0.75 m apart with intra-row spacing of 0.4 m between hills. Plots were overplanted and thinned to two plants per hill after seedling establishment. Two landraces from FASFMK were with very low germination rate, and were excluded from morphological evaluation, and in total, 28 accessions were studied.

Data collection and statistical analysis

Twenty-one morphological traits were recorded for each entry using maize IBPGR descriptor (IBPGR, 2008). Plant traits were measured on 10 randomly chosen plants per plot and averaged at both locations: Plant height (PH), ear height (EH), leaf width (LW), leaf length (LL), number of leaves above the uppermost ear including ear leaf (NoL). Number of days from planting to tasseling (DT) and silking (DS) were calculated, too. Ear leaf length was measured in cm after pollination; also, the width of the same leaf was measured at the widest point, in cm (LW, Tollenar et al., 2004). Ear traits were measured on 10 ears after harvesting and drying to 14% of moisture content: Ear length (EL), number of rows per ear (NRE), number of kernels per row (NKR), ear diameter (ED), cob diameter (CD). Kernel characters were measured on 10 kernels selected from the central part of each evaluated ear: Kernel length (KL), kernel width (KW), kernel thickness (KT), 1000 kernels weight (TKW), kernel type (Ktyp), and kernel color (Kcol). Following traits were visually evaluated: Foliage rating (F), tassel type (TM), ear shape (ES), and row arrangement (RA). Leaf samples for SSR analysis were collected from 15 plants per replicate, in Zemun Polje, after flowering.

Principal components analysis (PCA) was performed on the correlation matrix of 28 landraces and 21 response variables. Components were extracted until the Eigen value > 1. Eigen vectors were used to identify traits that best differentiated landraces. The first two PC scores, PC1 and PC2 that accounted for maximum variability of the tested parameters, were used to group the landraces. The standardized values (mean = 0, sd = 1) of all analyzed traits were utilized for estimating the Euclidian distance between the accessions. The obtained distance matrix was clustered with the unweighted pair-group method with arithmetic averages (UPGMA) for creation of a dendrogram. All analyses were performed using R 3.3.1 statistical software (R Foundation for Statistical Computing, Vienna, Austria).

DNA extraction and SSR analysis

At the flowering stage, leaf tissue from each plant was collected. Isolation of DNA was done using 'population DNA bulk strategy' (Rebourg et al., 2001; 2003; Wasala and Prasanna, 2013). DNA samples from each of the 28 accessions were isolated by pooling an equal amount of leaf material from 15 individuals per population. Harvested leaves were kept frozen at -30 °C and powdered with liquid nitrogen. Total genomic DNA was extracted and purified from 0.1 g of the powder by a CTAB method (Saghai-Maroof et al., 1984). The DNA was quantified and assessed for purity spectrophotometrically, and diluted to a working concentration of 50 ng μL^{-1}.

A total of 25 microsatellite markers were chosen from the maize database of public SSRs (http://www.maizegdb.org). Markers were selected based on amplification size and quality, and whole genome coverage. Primers were excluded from the study when banding patterns were difficult to score accurately on acrylamide gels, or when they failed to amplify consistently in all genotypes. A final set of 20 SSR primers were applied in the analysis of the genotypes according to the method of Senior et al. (1998).

The amplification reaction was carried out in 25 µL reaction volume containing 1x enzyme buffer, 2.4 mM MgCl, 200 µM dNTP, 0.5 µM primers, 1xBSA, 1 U Taq polymerase and 50 ng DNA template. Touch-down amplifying program was applied (thermocycler TProfessional Standard 96, Biometra GmbH, Göttingen, Germany) as follows: Initial denaturation at 95 °C for 5 min, 15 cycles of denaturation at 95 °C for 30 s, annealing at 63.5 °C for 1 min (-0.5 °C $cycle^{-1}$) and extension at 72 °C for 1 min, then 22 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min and finally elongation at 72 °C for 4 min. The amplified DNA fragments were separated using 8% polyacrylamide gel electrophoresis for 1.5 h at 80 mA. After staining with ethidium-bromide for 30 min, gels were photographed under UV light using BioDocAnalyze Live gel documentation system (Biometra, Analytik Jena AG, Jena, Germany). The alleles were scored manually and their sizes were estimated in comparison with 20 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts, USA).

The number of alleles per locus, Polymorphism Information Content (PIC) and number of unique alleles for *in situ* and *ex situ* populations and the whole set of data were estimated using the software PowerMarker 3.25 (Liu and Muse, 2005). Genetic distances were estimated using Roger's coefficient (Rogers, 1972) according to allele frequencies and the obtained distance matrix was used in cluster analysis. UPGMA method was applied to obtain the dendrogram.

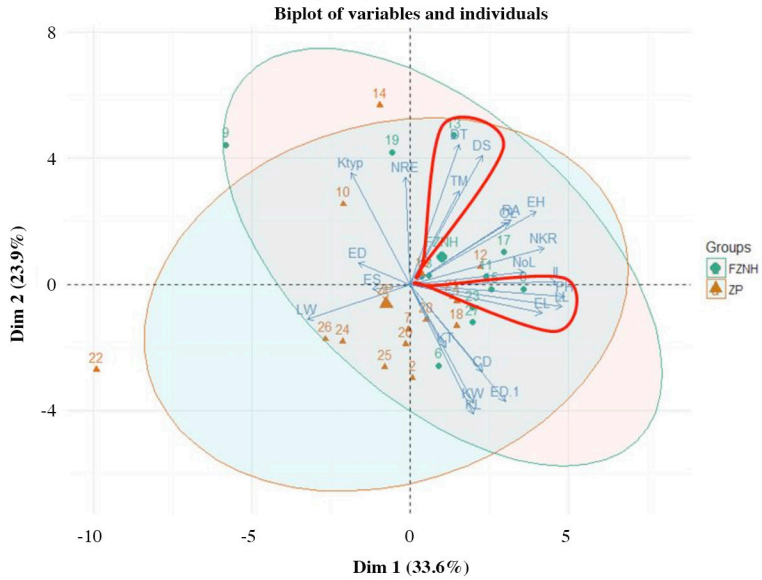
RESULTS

Phenotypic variability

This study evaluated phenotypic and molecular diversity of maize landraces collected in the Republic of Macedonia, in 40 yr period. The PCA (Figure 2) identifies five PCs with Eigen value > 1, which explained 80% of the total phenotypic variation. In the first PC, explained 34% of the total variability, the most important traits were PH, LL, NKR, and EL. The second PC was associated mostly with DT, DS, Ktyp and NRE, encompassing 24% of the total variance. This component was negatively correlated with cob and kernel traits. The third PC, accounted 10% of the total variation, was determined by ED and LW, while fourth and fifth PCs explained 7% and 6% of the total variation, accordingly. Biplot of PC1 *vs*. PC2 gave both, the information on association among traits and separation of landraces based on their morphological diversity and/or similarity. Distance of landrace from the biplot measures the difference from a hypothetical 'average' genotype that has an average level for all traits (Yan and Fregeau-Reid, 2008). Large distance of the landraces 9, 10, 13, 14 and 19 indicate that they have extreme values for one or more traits. Besides, relation among traits could be indicated by the angle between trait vectors (Yan and Kang, 2003). The strongest positive association, indicated by acute angles, was determined between DT and DS, and between PH, LL and EL. The landraces showed the greatest variability for plant height and ear length, and the lowest difference for flowering dates.

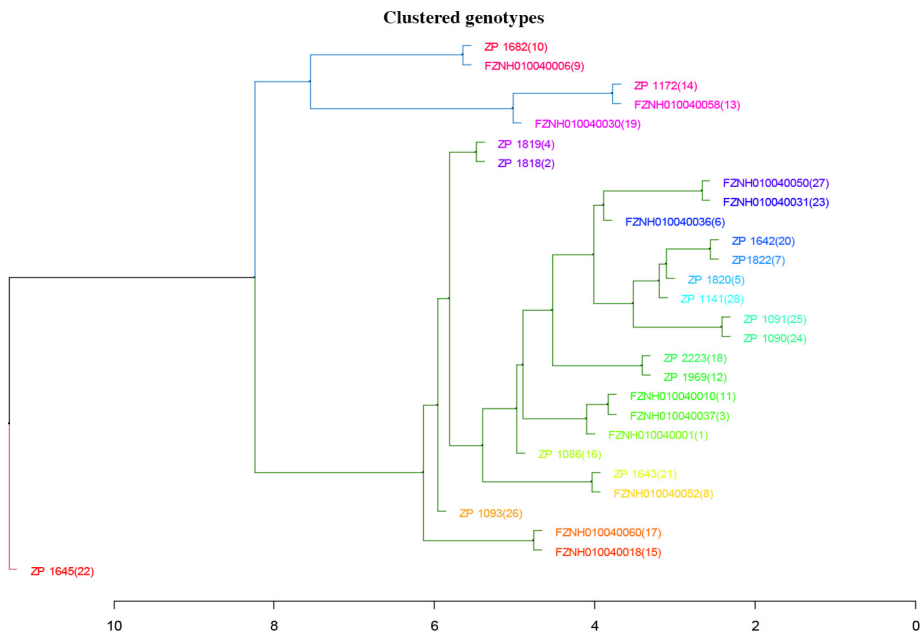
Dendrogram for morphological traits grouped landraces in two sub-clusters (Figure 3), predominantly based on collecting time. There was one outlier (ZP 1645-landraces 22), solely separated and the most distanced from the others, due to the shortest PH, LL, and EL, and the smallest number of days from planting to flowering, that did not belong to any cluster. Smaller cluster consists of landraces 9, 10, 13, 14 and 19, separated from the others due to

Figure 2. Biplot of morphological traits of the maize landraces. Analysis explained 57.5% of observed field variability. The most discriminative traits are rounded in red color.



extreme value of traits, like it was previously shown on bi-plot (Figure 2). These sub-clusters consisted of pairs of accessions from different gene banks, as being with pop-corn kernel type. Within second cluster, recently collected landraces from MKGB have greater plant height, and number of days from planting to flowering, whereas accessions from the MRIZP gene bank, have shorter plants (< 2 m) and number of days from planting to flowering less than 75 d. Pairs of samples from the same gene bank formed small sub-clusters according to collecting time and site.

Figure 3. Classification of maize landraces as revealed by UPGMA cluster analysis based on morphological traits.



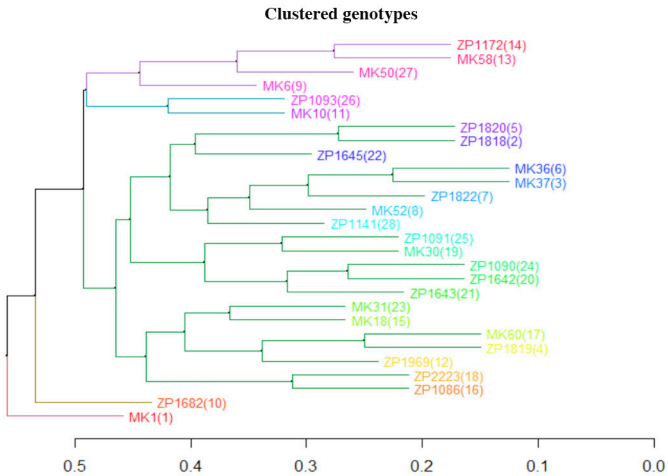
SSR based variability

With 20 SSR loci distributed uniformly over all 10 maize chromosomes, in total 173 alleles with an average of 6.18 alleles per locus were detected among 28 landraces. The number of alleles per primer was in a range from two to 13. PIC values were in range from 0.32 to 0.80, with average value of 0.67. In total, 16 unique alleles were detected, four in *in situ* and 12 *in ex situ* populations. The genetic analysis of maize accessions based on SSR polymorphism classified landraces into the three gene pools (Figure 4).

The accessions were grouped according to collecting sites: In the first sub-cluster were four accessions collected in the vicinity of town Stip. The second sub-cluster consists of two samples, from both gene banks. Although they were collected from different sites (Kocani and Debar), and differ in kernel type, they share high similarity at molecular level (0.781). The third group consists of 13 previously collected landraces (MRIZP gene bank), and seven landraces from FASFMK collection. They could be grouped in small cluster of eight samples collected in the vicinity of Stip. Another two sub-clusters had nine samples collected in the Western Macedonia region (Gostivar and Debar), and three from the Eastern part (Delcevo, Probistip and Kicevo).

The only samples grouped together for both, morphological and SSR markers, were ZP 1172 and FZNH 06 (landraces 13 and 14), with coefficient of similarity of 0.844.

Figure 4. Classification of maize landraces based on SSR marker analysis.



DISCUSSION

The phenotypic characterization of plant genetic resources is necessary for optimal exploitation by breeders. However, the usefulness of morphological characterization is rather limited, because of the significant environmental impact on expression of characters. Application of DNA markers, unaffected by environment, successfully discriminate closely related genotypes and possible duplicate accessions in many crops (Börner et al., 2000; Lund et al., 2003). Morphological and genetic variations are important to estimate variability, potential loss of diversity and the best accessions for conservation and possible utilization in breeding (Beyene et al., 2005).

Redundant duplication increased costs for storage and multiplication, without widening of genetic diversity. Efficient gene bank management comprehended increased number of samples and reduction of financial resources for their maintenance, by identification and removal of redundant germplasm (Hintum et al., 1996). Different authors distinguish the term 'duplicate accession' between historical, genetic or biological duplicates. Historical duplicates could be treated as genetic duplicates, if passport data together with genetic composition of the accessions have high similarity (Hintum et al., 1996; Willner et al., 1998; van Treuren et al., 2001). However, handling, multiplications and maintenance within gene bank, could lead to diversifications of accession from their original samples, and they are very seldom completely genetically identical. The level of genetic diversity between potential duplicates is

not clearly defined, particularly for allogamous species (Lund et al., 2003). Identification of absolutely genetically identical samples is possible by comparison of complete genome, which is very expensive and not necessary for samples considered as duplicates in gene bank (van Treuren et al., 2001). In practice, samples from the same outcrossing population will not be completely identical, but will have a similar genetic origin.

In the present study, morphological characterization for 41 descriptors (IBPGR, 2008) was performed. The effect of flowering influenced grouping of landraces, i.e. number of days from planting to silking and tasseling, as being the most stable traits, with the lowest variation. It was obvious that newly collected samples were with greater number of days from planting to flowering, compared to previously collected accessions conserved in MRIZP gene bank. Apparently, in seed multiplication process, either in gene banks or on farmers' fields, pollination (natural or artificial) occurs among plants flowering simultaneously. Important role of earliness/flowering time on morphological variation and differentiation was reviled in the analysis of traditional European maize populations (Rebourg et al., 2001) and in the studies of local maize landraces in Turkey (Cömertpay et al., 2012).

The cluster analysis based on morphological traits was appropriate considering length of vegetation season, plant height, and leaf length. However, landraces were not distinguished according to the collecting site, rather based on collecting time. Apparently, during nearly 50 yr (between first and last collecting missions) migration and seed exchange between farmers, contributed to adaptation to different agro-climatic conditions, which further influenced the changes in phenotypic characteristics of landraces. Previously collecting, *ex-situ* accessions were characterized by higher plants, longer leaves and greater number of kernels per row. Limitation of morphological data for discrimination of landraces according to geographical origin was revealed in different studies on heterogeneous maize landraces (Sharma et al., 2010; Cömertpay et al., 2012). Nevertheless, morphological variation in maize was of high importance during evaluation in different environments, when expression of particular trait could be valuable for utilization in breeding.

For efficient genetic resources conservation, it is important to know whether phenotypically similar genotypes share similarity in gene combinations. Microsatellite markers are appropriate for the assessment of genetic diversity given their informativeness, co-dominance, high polymorphism, repeatability, and simplicity (Reif et al., 2005; Bourguiba et al., 2010). In numerous diversity studies, application of SSRs was appropriate for grouping accessions according to their geographical origin, e.g. in bean (Sharma et al., 2013), tomato, (Mercati et al., 2015), maize (Cömertpay et al., 2012). Based on SSR markers, maize landraces from the same small area were in the group with genetically similar accessions (Figure 4). The average number of alleles per locus is a measure of genotypic diversity. In our study it was 6.18, very similar to reported numerous studies on maize landraces diversity (Qi-Lun et al., 2008; Cömertpay et al., 2012). This value indicates a great level of diversity, since the set of used SSR primers was based on specificity and informativeness.

Usually, reports about great phenotypic variability of traditional landraces indicated minor genetic differences between or within populations grown in the same region. In the present study, examined landraces showed considerable degree of variation in morphological traits. Genetic difference of landraces from *ex situ* and *in situ* conservation was due to regeneration and/or conservation in 40 yr period. Only two samples (landraces 13 and 14, as *in situ* and *ex situ* conserved accessions, respectively) expressed the highest similarity coefficient, clustered together for both morphological and molecular markers, and shared very similar genetic background (0.844). Thus, the *in situ* accession could be considered as duplicate sample and will not be introduced to MRIZP collection, since redundant germplasm decrease the efficiency of every genetic resource management.

There is evidence that *ex situ* conservation preserve alleles that have been lost in populations under *in situ* conservation. *Ex situ* samples of American Indian Hopi maize landraces, compared by morphological and SSR markers, differed significantly from their *in situ* accessions (Soleri and Smith, 1995). Similar level of genetic diversity of *ex situ* and *in situ* samples of Jala maize could be addressed to good maintenance practice in gene bank (Rice et al., 2006).

The main factor affecting diversity is selection by farmers. Numerous studies reported various factors, such as biological, socio, and economical that influenced farmers to choose and maintain, or eliminate particular landraces for further use (Jarvis et al., 2000). During planting, growing and harvesting, farmers also made a kind of selection that might cause the narrowing of genetic variability. Advances in agricultural practice and decrease of rural

development over 40 yr resulted in more careful farmers' attention towards varieties selection and seed production. On the other side, multiplication and regeneration of accessions in gene banks are aimed to keep genetic variability and diversity of original sample. Assessment of the accessions within gene bank and identification of variation on morphological and molecular level are also, very valuable for development of core collections and utilization in breeding, as well as for rationalization and elimination of redundant germplasm.

CONCLUSIONS

Efficient collection, conservation and utilization of plant genetic resources include periodical comparison of *ex situ* and *in situ* samples. Application of morphological and molecular markers (e.g. appropriate set of SSR markers) could be efficient for identification of possible duplicates. In the present study, greater total number of alleles and unique alleles were found in *ex situ* than *in situ* populations, indicating that farmer activities reduced allelic richness in recently collected landraces. Compared to *ex situ* gene bank accessions, newly collected landraces lost some alleles, and changed some traits, due to local cultivation practice, climatic and geographical conditions, and artificial selection by farmers. Obtained results are in line with general consensus that the most effective strategy for crop germplasm conservation is integration of both, *in situ* and *ex situ* concept. Through adequate management, *ex situ* MRIZP gene bank collection successfully preserve genetic diversity for future needs. In addition, landraces that clustered together either by morphological or molecular markers, could be used for development of core collections for breeding purposes.

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