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DIVERSITY OF MAIZE (*ZEA MAYS* L.) LANDRACES IN EASTERN SERBIA: MORPHOLOGICAL AND STORAGE PROTEIN CHARACTERIZATION

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ABSTRACT - Ten maize accessions (labelled as PF-ZM plus number) from Eastern Serbia were characterized by means of morphological and storage protein analysis. Estimation of 20 morphological traits was based on the IBP-GR Descriptors for maize. Zein composition was determined by the SDS gel electrophoresis. Variation of morphological characteristics among populations ranged from 4% for kernel width to 28% for kernel length. Accessions with the highest values of morphological traits were identified: PF-ZM 4 for ear diameter and kernel length, PF-ZM 5 for number of leaves and diameters of cob and rachis, PF-ZM 12 for leaf width and kernel thickness, PF-ZM 18 for number of kernels per row, PF-ZM 34 for number of ears per plant, venation index, number of rows per ear, and diameter of ear basis, and finally PF-ZM 37 for plant height, leaf length, and ear length.

The gel electrophoresis showed the presence of five different fractions of zein proteins, which were classified as α-zeins and γ-zeins. The α-zeins comprised on average 64 percent of the total registered zeins. Molecular weights of α-zein fractions identified on the gels were 17, 18, 21 and 24 kDa, and the only fraction identified as γ-zein was 27 kDa.

The assessment of morphological / productive traits of landraces, together with molecular markers, could represent a prerequisite for their protection. At the same time, such information could be of importance for both scientists and breeders.

KEY WORDS: Maize (*Zea mays*); Local varieties; Germoplasm Characterization and conservation; Zein proteins.

INTRODUCTION

Maize landraces are valuable resources because of their high genetic diversity. The evolutionary value of landraces is immense, especially when having in mind the constant diminishment of crop genetic variability fostered by the development of intensive agriculture. Furthermore, since the landraces are most often related to the traditional agricultural practices, their preservation is interconnected (LUCCHIN *et al.*, 2003).

Maize landraces cultivation in Serbia has a long tradition, since it has been practiced there for centuries. According to EDWARDS and LENG (1965), maize landraces in south-eastern Europe have different origins than those in the other parts of Europe. On the other hand, LENG *et al.* (1962) concluded that the origin of maize in south-eastern Europe is generally unclear, but suggested a common origin for all maize landraces present in Europe. The same authors have claimed that maize had been first introduced into Balkan by the Turks during expansion of their empire. This presumption has been supported by the similarity of the names used for the maize by all the nations in the region. However, as GOUESNARD *et al.* (2005) suggested, it is necessary to conduct more exhaustive survey of the diversity among the eastern European maize landraces, since most of the data used in the existing studies have been related to the western European maize populations. This could be important from a global point of view, since such a survey would enable the establishment of a core collection that could be representative for the whole Europe (GOUESNARD *et al.*, 2005).

During the last decades, commercial maize varieties became widely accepted in agriculture and their expansion suppressed cultivation of the landraces. Commercial hybrids were readily accepted because of a higher yield over a short time perspective. The same process also occurred in Serbia, and

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the areas where the landraces were traditionally cultivated have severely diminished. The concern is that *in situ* conservation alone will not be enough to preserve the genetic diversity that exists within the landrace populations. PERALES *et al.* (2003) stated that the introduction of new varieties and new agricultural practices led to evolutionary changes and even to a loss of landraces.

Eastern Serbia is situated on the border of the Carpathian Mountains, and it remains both geographically and socio-economically a relatively isolated and underdeveloped region, quite suitable for the survival of some native agricultural landraces. Constant evolution and adaptation to the environment, both natural and human-made, resulted over time in stable phenotypes. One of the important characteristics of eastern Serbia climate, particularly regarding the agricultural production, is soil drought induced by an arid climate that inevitably limits plant growth and reduces production (DODIG *et al.*, 2005). This region possibly represents a refuge area for maize landraces, where their traditional cultivation is still being practiced.

There is an apparent lack of studies dealing with the above discussed issues. It is important to focus the research on regions where modern varieties have not yet been widely adopted and where landraces production still has an important role in the rural society. Such research activities are important for monitoring threats to maize diversity, as well as for a better understanding of social, cultural and economic factors that have impact on maize diversity (VAZ PATTO *et al.*, 2007). Furthermore, they are important for evaluating and documenting genetic diversity of existing collections of maize landraces in the whole country. According to LUCCHIN *et al.* (2003), the research of distinctive and characteristic traits could be used to create core populations for basic maintenance nucleus. Studies of the morphological and agronomic traits, together with molecular markers fingerprinting, could be a basis for the recognition of geographic indication of the landraces and, therefore, used for their protection. The above mentioned activities are important prerequisite for the definition of the effective conservation measures and management of maize landraces.

The main objective of this study was to evaluate the genetic diversity of maize landrace accessions collected in eastern Serbia, using both morphological and protein characterization methods. We hypothesized that the significant differences observed among traits would reflect the diversity on a wider scale. Available landrace descriptors and their potential in the evaluation and description of the maize landraces were also evaluated.

MATERIAL AND METHODS

Plant material

Ten maize landrace accessions collected from eastern Serbia (maintained in the seed collection at the Faculty of Agriculture, Belgrade University) were used for setting up the field experiment. Although the origin of this collection is from eastern Serbia (more precisely, from the Homolje region: Latitude between 44°05' and 44°22'N, Longitude between 21°31' and 21°50'E), the exact locations and the time of collection are unknown. It is believed that the material was collected from villages Zagubica, Laznica, Krepoljin, Suvi do and Osanica during the last two decades of the 20th century, when social and economic turmoil were the probable reason for poor documentation. The samples tested were: PF-ZM 4, PF-ZM 5, PF-ZM 10, PF-ZM 12, PF-ZM 18, PF-ZM 19, PF-ZM 26, PF-ZM 34, PF-ZM 37 and PF-ZM 39.

Field experiment

In order to characterize the accessions, a field experiment was set up within the Institute for Vegetable Crops in Smederevska Palanka (Latitude: 44°21'N, Longitude: 20°57'E). Sowing was conducted on 29 April 2008. Each accession was sown in a separate row and the distance between sowing points was 40 cm. In each sowing point, four accession seeds were sown and, when the first two leaves were formed on each of the four sprigs, two sprigs were removed. The distance between rows was 70 cm.

The isolation of each accession was provided by placing a paper bag on each tassel and plastic bags on each ear before silking. Following the tasseling, the plastic bags were briefly removed and the paper bags, containing the pollen from tassels, were placed over the silk of the uppermost ear of the same plant, thus providing the self-fertilization of each plant within the accession.

Morphological characteristics were measured on randomly chosen ten plants per accession. The characterization of the vegetative parts of the plants was performed on 28 and 29 July 2008, and the harvest was conducted on 12 September 2008, with further characterization being performed after the drying of the harvested material.

Morphological and storage protein characterization

The plants were characterized for morphological and productive traits following the guidelines of the Descriptors for maize (IBPGR, 1991). The morphological and agronomical characteristics measured were: plant height, ear height, number of leaves above the uppermost ear, total number of leaves per plant, number of ears per plant, leaf length, leaf width, venation index, number of kernel rows, number of kernels per row (one and two), ear length, ear diameter (top, middle, basis), cob diameter, rachis diameter, kernel length, kernel width, and kernel thickness.

Following the characterization of the harvested material, eight kernels from each accession were randomly selected and

characterised for zein proteins by gel electrophoresis. Zeins represent the main storage proteins in maize kernels and account for more than 50 percent of all proteins in the endosperm (YUNCHANG *et al.*, 2005). The kernels were soaked in water and incubated for an hour at 65°C, to enable easy removal of a pericarp and germ. Afterwards, the endosperm was dried at 65°C for 16 hours, according to the method described by GUIMARÃES *et al.* (1995), and 100 mg of dried kernel endosperm was pulverized in a ball mill.

Total extraction of proteins was performed according to the protocol described by WALLACE *et al.* (1990). The extraction buffer was added to each endosperm sample, followed by incubation with shaking at the room temperature and centrifugation. Following the centrifugation, ethanol was added to the first supernatant fraction to a final concentration of 70 percent, in order to precipitate non-zein proteins. The supernatant obtained after another centrifugation comprised the total zein fraction. It was transferred to a new tube and the zein proteins concentration in the fractions was determined by absorbance at 280 nm using the NanoDrop ND-1000 Spectrophotometer.

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol described in FLING and GREGERSON (1986). Following the electrophoresis, the gels were stained in Coomassie Brilliant Blue G-250 at the room temperature with agitation and destained afterwards, prior to the further analysis. The gels were digitalized and the densitometry was performed using the ImageJ software (RASBAND, 2008). Zein fractions were quantified through the software analysis. In order to compensate for the differences between gels due to various factors, such as inconsistency of the staining process (BURSTIN *et al.*, 1993), and in order to enable gel comparison, a scaling procedure was applied according to CONSOLI and DAMERVAL (2001) and LEYMARIE *et al.* (1996). Following the densitometry, the integrated density of the band i on the gel j was multiplied by the scaling factor (F). The scaling factor was calculated according to equation:

$$F_{ij} = m/m_j \quad (1)$$

where m represents the mean integrated density of bands of the calibration set for all the gels, while m_j is a mean integrated density of bands of the calibration set for the gel j.

Statistical analysis

All statistical analyses were performed with the SPSS software (Version 15.0, SPSS Inc., 2006). Mean values and variation coefficients were calculated for morphological / productive characteristics. Similar statistical methods were applied to the results from both morphological and protein analysis. Distribution of variables values were evaluated using the Kolmogorov-Smirnov test for normality. Since the data obtained from morphological characterisation of plants generally suggested a normal distribution ($p>0.05$), the parametric tests were employed. However, the dataset obtained by electrophoresis was not normally distributed; therefore, the data were log-transformed prior to applying the parametric tests. The ANOVA and the sequential Bonferroni correction for multiple comparisons were employed for the comparison of each pair of accessions. In order to assess the differentiation of accessions based on all variables that were measured, the canonical discriminant analysis was applied. Relationships among different variables of the protein analysis were tested by means of Pearson's correlation test.

RESULTS AND DISCUSSION

The analyzed morphological characteristics of maize are presented in Table 1. Some measurements were not performed for the accession PF-ZM 26, due to a high percentage of lodged plants which have not produced ears; evidently, this landrace is not adapted to high nitrogen conditions that were present in experiment setup.

Based on the results of ANOVA, the accessions differed in most characteristics. By the comparison of between and within group variability, the most selective morphological traits were plant height ($F=14.146$) and ear height (9.751), leaf length (8.056) and width (4.386), ear length (4.567) and kernel length (5.627), as well as the diameter at ear top (6.717), middle (4.737) and basis (4.779; $p<0.001$ for all characteristics). Mean values of all measured characteristics are presented in Table 1. The most stable characteristics were kernel width (CV: 4%) and venation index (5%), while the most variable characteristic was kernel length (28%). Although the most variability was between groups, some variables revealed a comparable variability within groups.

The ten studied accessions belonged to dent, flint and semi-flint types of kernel endosperm. This finding is supported by DALLARD (2007), who claimed that all European maize landraces had either flint or dent kernel type. Most of the accessions we studied could be identified as belonging to the group of "derived flints" mentioned by GERIĆ *et al.* (1989) and LENG *et al.* (1962), which were found to be the result of hybridization between the Mediterranean flints and the other flint races. Distribution of this group was estimated to be wide and morphologically heterogeneous.

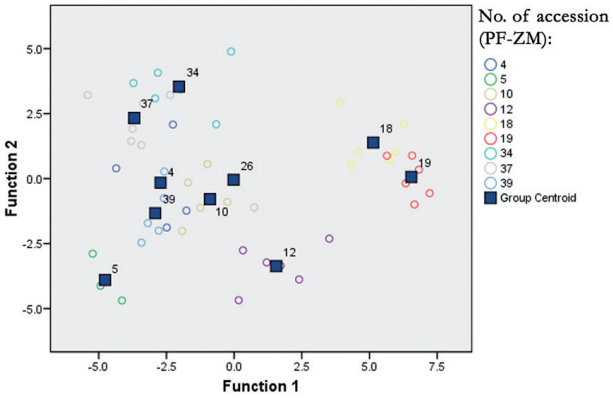

FIGURE 1 - Canonical discriminant analysis of maize accessions by morphological characteristics.

TABLE 1 - *Mean values of measured characteristics (according to the Maize descriptors, IBPGR 1991) in ten maize landraces.*

Characteristic*		Accession number										Mean	CV (%)
		PF-ZM 4	PF-ZM 5	PF-ZM 10	PF-ZM 12	PF-ZM 18	PF-ZM 19	PF-ZM 26	PF-ZM 34	PF-ZM 37	PF-ZM 39		
Plant related traits	HP	159.3	188.0	184.2	169.5	151.0	139.3	138.1	178.5	193.3	187.9	168.9	12
	HE	98.8	125.0	125.6	106.5	86.0	81.4	83.1	111.5	118.3	128.6	106.5	17
	NLA	4.7	5.8	5.0	4.9	5.2	5.1	5.0	5.1	4.8	4.9	5.1	6
	NLT	11.2	13.3	12.7	11.2	10.8	11.0	11.4	11.9	12.3	11.6	11.7	7
	NE	1.5	1.4	2.0	1.4	1.9	2.3	2.3	2.3	1.7	2.1	1.9	20
	LL	78.8	82.7	90.3	75.1	68.8	69.9	67.9	77.1	95.9	92.9	79.9	13
	WL	10.9	8.4	10.7	11.1	10.0	9.5	9.3	8.9	10.2	9.4	9.8	9
	IV	2.2	2.5	2.3	2.3	2.4	2.4	2.5	2.6	2.3	2.4	2.4	5
Ear related traits	NR	15.0	14.7	14.3	11.7	12.3	11.3	/	15.3	14.3	13.2	13.6	11
	NKR1	25.0	27.3	26.0	23.5	28.4	24.7	/	24.8	16.7	23.4	24.4	14
	NKR2	24.5	26.3	27.0	23.7	27.7	22.8	/	23.8	16.2	23.6	24.0	14
	LE	21.3	16.4	18.9	18.4	16.9	14.9	/	19.1	21.9	14.8	18.1	14
	DET	4.1	3.7	3.4	3.5	3.2	3.0	/	3.8	3.7	3.5	3.5	9
	DEM	4.8	4.5	4.2	4.6	3.9	3.7	/	4.6	4.2	4.1	4.3	8
	DEB	4.5	4.0	4.2	4.1	3.7	3.5	/	4.5	4.1	3.9	4.1	8
	DC	2.8	3.0	2.5	2.8	2.3	2.3	/	2.7	2.8	2.6	2.6	9
	DR	1.5	2.0	1.7	1.9	1.5	1.5	/	1.6	1.6	1.7	1.7	11
Kernel related traits	LK	1.97	0.93	1.06	0.90	1.05	0.98	0.94	1.18	1.08	1.04	1.11	28
	WK	0.89	0.88	0.80	0.90	0.86	0.89	0.86	0.90	0.91	0.94	0.88	4
	TK	0.52	0.56	0.52	0.70	0.51	0.55	0.64	0.50	0.62	0.48	0.56	13

* HP - Plant height, HE - Ear height, NLA - Number of leaves above the uppermost ear, NLT - Total number of leaves per plant, NE - Number of ears per plant, LL - Leaf length, WL - Leaf width, IV - Venation index, NR - Number of kernel rows, NKR1 - Number of kernels per row (1), NKR2 - Number of kernels per row (2), LE - Ear length, DET - Ear diameter – top, DEM - Ear diameter – middle, DEB - Ear diameter – basis, DC - Cob diameter, DR - Rachis diameter, LK - Kernel length, WK - Kernel width, TK - Kernel thickness.

The canonical discriminant analysis of the traits is presented in Fig. 1. The first two canonical functions described 69.9 percent of the existing variance. Plant height had the strongest influence in the Function 1, followed by the ear height, ear top diameter and cob diameter, while the Function 2 was mostly influenced by the kernel length, followed by rachis diameter, number of ears per plant and ear length (Table 2). The accessions PF-ZM 18 and PF-ZM 19 were separated from the other accessions along Function 1, while the accessions PF-ZM 5 and PF-ZM 12 as one group, and PF-ZM 34 and PF-ZM 37 as the second group were separated from the other accessions along Function 2.

It is an important task to identify maize characteristics that are most suitable for the characterization of seed collections. Our findings are similar to those presented by Ilarslan *et al.* (2002). Furthermore, Abu-Alrub *et al.* (2004) recommended kernel and ear traits as the best descriptors for Peruvian highland maize germplasm, while Ortiz *et al.* (2008) showed that it is feasible to classify the same germplasm using vegetative traits, such as plant height, ear height and leaf number. Galarreta and Alvarez (2001) and Rebourg *et al.* (2001) suggested that the traits that have a high heritability, such as ear related characteristics (length, diameter, row number) and vegetative characteristics (days to flowering, plant and ear height), are more appropriate for characterization and classification of maize landraces. However, Sánchez *et al.* (1993) implied that there was a lack of knowledge regarding the most suitable characters for the study of racial differences among maize populations, and that some of the characters have not been tested to determine the relative importance of the influences of the genotype, environment and their interactions.

Zeins usually account for 50-70 percent of the proteins in kernels (Azvedo *et al.*, 2004). Specifically, zeins are classified into the following four

TABLE 2 - *Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions; canonical discriminant analysis performed on morphological characteristics of studied maize accessions (Fig. 1).*

Characteristic*	Function 1	Function 2
HP	-.369	.026
HE	-.289	-.045
DET	-.229	.060
DC	-.206	-.108
LK	-.056	.377
DR	-.083	-.177
NE	.043	.156
LE	-.129	.130
LL	-.183	.007
DEM	-.168	.022
DEB	-.157	.084
NR	-.144	.123
NLT	-.087	.031
NKR1	.053	-.045
WK	-.039	-.032
NKR2	.029	-.062
NLA	.023	-.014
IV	.013	.104
TK	.008	-.088
WL	-.007	-.068

* See Table 1 for abbreviation description.

TABLE 3 - *Analysis of the variance of zein protein fractions among different accessions.*

Zein fractions		Mean Square	F-value
27 kDa	Between accessions	0.109	8.8*
	Within accessions	0.012	
24 kDa	Between accessions	0.014	0.9
	Within accessions	0.016	
21 kDa	Between accessions	0.037	2.9*
	Within accessions	0.013	
18 kDa	Between accessions	1.383	73.1*
	Within accessions	0.019	
17 kDa	Between accessions	0.667	33.2*
	Within accessions	0.020	

* $p<0.01$.

groups: α-, β-, γ- and δ-zeins, according to their genetic characteristics and amino acid sequences (Rodriguez-Nogales *et al.*, 2006). The analysis of endosperm proteins in this study showed the presence of five different fractions of zein proteins, which were classified as α-zeins and γ-zeins (Zhu *et al.*, 2007; Azvedo *et al.*, 2004). The α-zeins comprised on average 64 percent of the total zeins. In their studies, Landry *et al.* (2001) and Jimenez-Flores and Bleck (1996) determined that the α-zein group accounted for 70 percent of the total zeins in a mature kernel. Studies of Landry *et al.* (2004) and Mestres and Matencio (1996) found that the zein content in maize kernels had a strong correlation with the vitreous endosperm and kernel hardness.

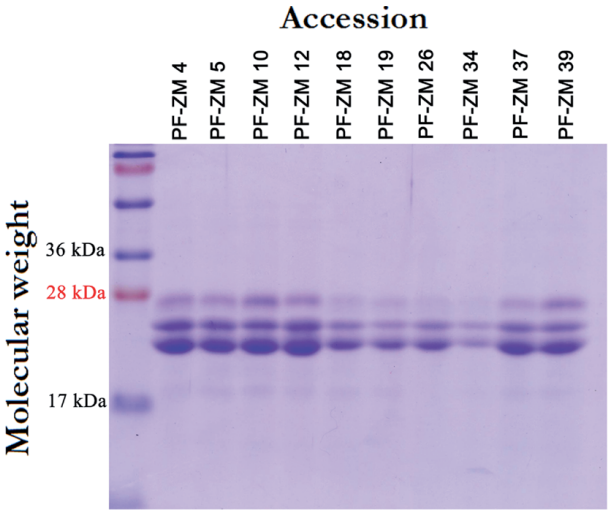

FIGURE 2 - SDS-PAGE gel comprising α- and γ-zein fractions from all ten analyzed maize accessions.

Molecular weights of α-zein fractions identified on the gels were 17, 18, 21 and 24 kDa, and the only fraction identified as γ-zein was 27 kDa (Fig. 2). The accessions PF-ZM 4, PF-ZM 5, PF-ZM 10, PF-ZM 12, PF-ZM 18, PF-ZM 19, PF-ZM 37 and PF-ZM 39 comprised all five fractions of zein proteins, while the accession PF-ZM 26 comprised only the α-zein fractions of 24 and 21 kDa and the γ-zein fraction, while the accession PF-ZM 34 did not comprise the α-zein fraction of 18 kDa.

Except for the 24 kDa fraction, the detected zein fractions showed significant differences among the different accessions (see Table 3). The Bonferroni correction for multiple comparisons indicated that the accession PF-ZM 26 was different from all other accessions based on the γ-zein fraction of 27 kDa and α-zein fractions of 18 and 17 kDa. Similarly, the accession PF-ZM 34 was distinguished from the other accessions by 27 and 18 kDa fractions. This was most likely due to the absence of the 17 and 18 kDa fractions in the accession PF-ZM 26 and the fraction 18 kDa in the accession PF-ZM 34. The α-zein fraction of 17 kDa also significantly influenced

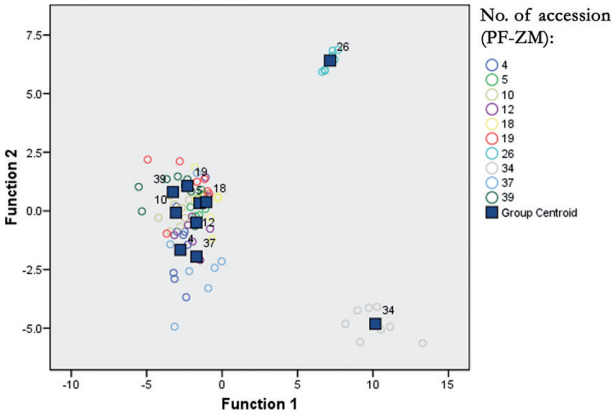

FIGURE 3 - Canonical discriminant analysis of maize accessions by zein fractions.

TABLE 4 - *Pearson correlations of zein fractions.*

	27 kDa	24 kDa	21 kDa	18 kDa	17 kDa
27 kDa					
24 kDa	0.416**				
21 kDa	0.556**	0.732**			
18 kDa	-0.518**	0.127	-0.241*		
17 kDa	-0.271*	0.018	-0.063	0.662**	

** $p<0.01$
* $p<0.05$

TABLE 5 - *Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions; canonical discriminant analysis performed on zein fractions of studied maize accessions (Fig. 3).*

Zein fractions	Function 1	Function 2
z18 kDa	.638	.170
z17 kDa	.223	.610
z27 kDa	-.196	-.101
z20 kDa	-.101	-.053
z24 kDa	-.003	-.081

the differentiation of the accession PF-ZM 37 from PF-ZM 5 and 18.

The strongest correlation among the observed zein protein fractions was observed between the fractions of 24 and 21 kDa (Table 4). These two fractions of α-zeins are typically the most abundant in the maize endosperm and they on average comprised 54 percent of the total α-zeins. This has also been confirmed by other authors, such as ZHU *et al.* (2007) and LIU and RUBENSTEIN (1993).

The canonical discriminant analysis revealed a high differentiation of two accessions, PF-ZM 26 and 34 (Fig. 3). Two canonical functions accounted for 95.6 percent of the total heterogeneity (the Function 1 accounted for 69.6% and the Function 2 for 25.9%). The most influential variable for the Function 1 was the zein fraction of 18 kDa, followed by the 17 kDa zein fraction, while the strongest influence in the Function 2 was of the 17 kDa fraction (Table 5).

In this study, within accessions variability was lower than between the accessions, for both morphological and storage protein characteristics. Similar findings were recorded by other authors, such as REBOURG *et al.* (2001) and BEYENE *et al.* (2006), who used both morphological and genetic analyses. These findings imply that ten studied accessions were significantly diverse, even though they came from a relatively small area. On the other hand, some studies (e.g., LUCCHIN *et al.*, 2003) found that the variation of certain Italian maize landraces was much higher within than between populations, a finding explained by the long practice of seed exchange among farmers and pollen dispersion between the fields. The proteins fingerprinting indicated that the accessions PF-ZM 26 and 34 were closer to each other and differentiated from the other accessions. Such similarity is often caused by the proximity of collection sites or a crosspollination during the previous regenerations of accessions (VIGOUROUX *et al.*, 2008). One of the shortcomings of this study was the fact that the records regarding the exact location of the collected accessions and their on-farm cultivation history were missing due to the bad management of the seed collection in the past, probably because of the social and economic circumstances in the country. This is unfortunate, since many other studies have successfully employed analysis that integrated morphological and geographical differences of maize populations to express the maize landrace's integrity – its place of origin (CORRAL *et al.*, 2008; MAGOROKOSHO *et al.*, 2005; REBOURG *et al.*, 2001; GOUESNARD *et al.*, 1997).

ROBUTTI *et al.* (2000) addressed the problem of the ear and kernel phenotypes being affected by the environment, and proposed that the molecular analysis would be more representative for racial classification and characterization. Other authors (CARVALHO *et al.*, 2004; REBOURG *et al.*, 2001; SANCHEZ *et al.*, 2000) have also suggested that the genetic analyses could represent an attractive method for

evaluation of polymorphism among the accessions in the seed collection, and that they would perform even better if they were used in combination with morphological characterization. This study could not compare the results obtained by the analyses of morphological and protein data, since the maize kernels used in protein analysis were taken from a bulked seed material. However, it would be of advantage if these different types of analysis could be used at the same time to generate complex picture of the relationships among the seed collection material in general. ROBUTTI *et al.* (2000) implied that the association of storage proteins, such as zeins in maize, together with the genotype, could be a useful molecular marker for future analysis. However, DENIĆ (1983) stated that the storage proteins, due to their role in the crop ontogeny, are not under the strict genetic control as other proteins, e.g. enzymes.

CONCLUSIONS

Both the morphological and storage protein comparison of the ten maize accessions, assessed in the present study, revealed a significant differentiation. Some of the accessions were shown to be similar, which might imply the same origin and/or possible proximity of their collection sites. The study also indicated morphological traits that express the greatest variability among the accessions. In line with the findings of the other authors, this study suggests that the most selective morphological traits within the group of vegetative characteristics were plant and ear height, and leaf length and width. Within the group of ear related characteristics, the most selective traits were ear length and diameter, and kernel length. The analysis of the storage proteins (zeins), performed in this study, could be a suitable method to scrutinize variability among maize accessions. In combination with morphological characterization, proteins fingerprinting could have a high potential to be used for future assessments of the origin and diversity of the accessions within collections.

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