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Genetic characterization of maize (*Zea mays* L.) landraces grown in Kosovo assessed by MITE-Hbr markers

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Genetic characterization of maize *(Zea mays* L.) landraces grown in Kosovo assessed by MITE-Hbr markers

Abstract: The aim of this study was to examine and describe genetic structure on autochthonous maize germplasm (flint types) from different localities in Kosovo using Hbr markers. The genetic characterization of 6-8 individual seedlings per each of 20 landraces was conducted by Hbr display calculated per selective base, the most efficient genetic diversity estimator to distinguish between landraces was primer combination Hbr-Int5-F/MseI+T. The strongest genetic relatedness (r = 55.57) had landrace ACC4 having orange colored seeds, showing the highest genetic uniformity when compared to other accessions. Clustering analysis using the Bayesian approach generated two genetic clusters for observed landraces. As a measure of population structure influenced by genetic drift and migration, Fst values for each genetic cluster were obtained. Higher Fst (0.4027) was calculated within the first genetic group comparing to the second one (0.2001), reflecting a higher levels of out-crossing and conservation between landraces from the first genetic cluster. A similar distribution of genetic linkages was observed from dendrogram, constructed using Dice coefficient and neighbour-joining (NJ) algorithm with minor deviations for landraces ACC6 and ACC28. Genotypes of ACCmk landrace reveal the highest genetic distinction compared to other genotypes, reflecting the highest number of bands (241) and the highest number of private bands (10) as the number of bands unique to a single population, respectively.

Key words: genetic variability; heartbreaker family markers; maize

Genetska karakterizacija lokalnih sort koruze (*Zea mays* L.) gojenih na Kosovu ovrednotena z MITE-Hbr označevalci

Izvleček: Namen raziskave je bil preučiti in opisati genetsko strukturo avtohtone dednine trdinke iz različnih lokalitet Kosova s Hbr označevalci. Genetska karakterizacija 6-8 sejank od vsake lokalne sorte je bila izvedena s prikazom nabora Hbr profilov, izračunanega na osnovi selektivne baze. Najučinkovitejši določitelj genetske raznovrstnosti za razločevanje med lokalnimi sortami je bila kombinacija primerjev Hbr-Int5-F/MseI+T. Najmočnejšo genetsko povezanost med genotipi (r = 55.57) je imela lokalna sorta ACC4 z oranžnimi zrni, ki je izkazovala največjo genetsko izenačenost v primerjavi z drugimi akcesijami. Klasterska analiza z uporabo modela aposteriorne verjetnosti (Bayesian approach) je za vključene lokalne sorte oblikovala dve genetski skupini. Kot merilo za analizo populacijske structure, na katero vplivata genetski zdrs in migracija, so bile izračunane vrednosti Fst za obe genetski skupini. Večja vrednost Fst (0,4027) je bila izračunana znotraj prve genetske skupine v primerjavi z drugo (0,2001), kar kaže na večji delež navskrižnega križanja in ohranjanja raznolikosti med lokalnimi sortami prve genetske skupine grozda. Podobna porazdelitev genetskih povezav je bila določena na dendrogramu, izdelanem z uporabo Dice-ovega koeficienta podobnosti in algoritma razvrščanja po metodi združevanja najbližjih sosedov (NJ) z manjšim odstopanjem za akcesiji ACC6 in ACC28. Genotipi akcesije ACCmk so se genetsko najbolj razlikovali od drugih na osnovi največjega števila prisotnih namnožkov (241) in največjega števila prisotnih "privatnih namnožkov" (10) in v številu namnožkov, ki so bili omejeni samo na eno populacijo.

Ključne besede: genetska raznolikost; družina "Heartbreaker" označevalcev; koruza

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1 INTRODUCTION

Maize (Zea mays L.) is one of the most genetically diverse and widespread crops in South-East Europe (Đalović et al., 2015; Ignjatović-Micić et al., 2015). Maize landraces have been largely replaced by commercial maize hybrids in Kosovo. The share of hybrids was 4 % in the 1960's and already at 90 % in the early 2000's due to their high yield potential. Studies of specific combining abilities of inbred lines and physiological traits of some hybrids were already performed in agroecological conditions of Kosovo (Aliu et al., 2008, 2010). Maize became widely investigated plant species regarding different applications; its water use efficiency (Wang et al., 2013), morphological, physiological and biochemical response to SiO_2 nanoparticles (Sharifi-Rad et al., 2016), different irrigation regimes and planting methods (Singh Brar *et al.*, 2016). There are also different marker systems, applied to assess genetic characterization of maize germplasm, including RAPD (Random Amplified Polymorphic DNA) (Srdić et al., 2007), SSR (Simple Sequence Repeats) (Ignjatović-Micić et al., 2015) and MITE-Hbr (Miniature Inverted Repeat Transposable Element from the family Heartbreaker) (Casa et al. (2000; 2002) and Kavar et al. (2007)). MITE-Hbr markers have been firstly exploited and developed as a new marker system (modification of the AFLP-Amplified Fragment Length Polymorphism procedure) for evaluation of the maize genome (Casa et al. (2000; 2002)). Kavar et al. (2007) used Hbr markers to evaluate the genetic diversity of Slovenian maize germplasm, originating from Western Balkan (former Yugoslavia). Related to those three reports, MITE-Hbr markers were proven to be stable, highly polymorphic, cost-effective, easily mapped and evenly distributed throughout the maize genome.

SSRs are highly applicable markers in our genetic studies of different plant species, among other grapevines (Rusjan etal., 2012, 2015) sweet potato (Pipan et al., 2016), brassicas (Pipan et al., 2011, 2013), and beans (Maras et al., 2015). In a study by Ignjatović-Micić et al. (2015), using SSR markers, they reported that higher genetic variation was observed among flint genotypes, comparing to dent ones. They also suggest that landraces from Western Balkans are highly adapted to specific environmental conditions and uses and therefore could be a valuable source of genetic variability. The adaptation to diverse agro-ecological conditions is a result of natural and selection by farmers.

The aim of this study was to examine and describe genetic structure of autochthonous maize germplasm from Kosovo using Hbr marker system and to employ Hbr display to show genetic differences and associations between and within observed flint landraces of maize collected in different localities of Kosovo, with a possibility to detect conservation of gene flow into the maize genome. The knowledge of genetic characteristics and landrace-specific background of maize germplasm would be of a benefit for future breeding and germplasm improvement programmes in Kosovo.

2 MATERIALS AND METHODS

Twenty maize landraces, collected from different locations in Kosovo (Table 1), were screened using MITE-Hbr markers. DNA from 6 - 8 individuals of each landrace was extracted from each individual seedling using BioSprint 15 DNA Plant Kit (Qiagen) and MagMax Express Magnetic Particle Processor (Life Technologies, Grand Island, NY) following manufacturer's instructions. Hbr display with some modifications was performed as described by Casa et al. (2000, 2002) and Kavar et al. (2007). 600 ng of genomic DNA was digested for 3 h at 65 °C in 20 µl of 10x Tango buffer containing MseI (Fermentas). Adaptors (5'gacgatgagtcctgag and 5'tactcaggactcat) to the digested DNAs and aliquots of the restriction/ligation reactions were visualized on 0.9 % agarose gels to check the quality of DNA digestion. Pre-selective amplification was performed using primers Hbr-Int5-E (5'gattctccccacagccagattc) and MseI+0 (5'gacgatgagtcctgagtaa). Selective amplification was performed with each of the three selective primer combinations (MseI+C, MseI+G and MseI+T) with a fluorescently labeled Hbr internal primer (5'-6FAM-agccagattttcagaaaagctg). Fragment analysis was performed on the 3130XL Genetic Analyzer (Applied Biosystems), and sizing of fluorescent fragments/bands was determined by comparison with size standard GeneScan-500 ROX (Applied Biosystems) using GeneMapper 4.0 (Applied Biosystems). A binary matrix was constructed by scoring fragments as either present (1) or absent (0) in each DNA sample.

Principal Coordinate Analysis (PCoA), Analysis of Molecular Variance (AMOVA), number of different alleles (Na), number of effective alleles (Ne) and Shannon's information index (I) across landraces for each selective base was calculated in GenAlEx v.6.4 (Peakal and Smousse, 2006). Genetic similarities were calculated on the basis of a binary matrix using the Dice similarity index (Dice, 1945). These coefficients were used to construct the clustering using the neighbur-joining (NJ) algorithm by 100 bootstraps in FreeTree (Pavliček et al., 1999) and visualized using TreeView (Page, 1996) software. Genetic diversity parameters between and within landraces including AMOVA, band patterns (number of bands, number of private bands, number of locally common bands alleles occurring in 50 % or fewer landraces, expected heterozygosity) and mean within landrace pairwise values (r) were conducted using GenAlEx v.6.4 (Peakal and Smousse, 2006). Structure 2.3.3 software (Pritchard et al., 2009) was employed for inferring landrace structure using a Bayesian approach. Ten independent runs for each K (from1 to 7) in the case of admixture model were performed and burning period of 10,000 followed by 100,000 Markov Chain Monte Carlo repeats was used. The ideal K-value was selected based on the increases in likelihood ratios between runs using Evanno's delta K statistic (Evanno et al., 2005) implemented in a Structure Harvester (Earl and von Holdt, 2011). The estimation of flowering time was made in the same year at different locations, which are presented in Table 1.

3 RESULTS

Kosovo landraces included in our study are all of a flint type with white kernel (fruit) color, except for a landrace ACC4, which kernels are orange (Table 1).

Genetic characterization of 6-8 individual seedlings per each landrace was conducted using Hbr display. A total of 498 markers, ranging in size from 60-500 bp, were generated using three primer combinations: Hbr-Int5-F/MseI+T, Hbr-Int5-F/MseI+C, and Hbr-Int5-F/MseI+G. Regarding genetic diversity estimators (PCoA, AMOVA, Na, Ne and I) calculated per selective base (T, C, G), the most efficient primer combination to distinguish between landraces from Kosovo, was Hbr-Int5-F/MseI+T, and Hbr-Int5-F/MseI+C respectively (Table 2). First three axes in PCoA (via covariance distance matrix) cumulatively explained 70 % of genetic variability for Hbr-Int5-F/MseI+T; 67 % for Hbr-Int5-F/MseI+C and 59 % for Hbr-Int5-F/MseI+G (Table 2). Percent of molecular variability among landraces in Hbr screening varied from 10 (Hbr-Int5-F/MseI+C) to 18 (Hbr-Int5-F/MseI+T), depending on selective primer applied (Table 2). Additionally, the highest values of Na (7.400), Ne (1.067) and I (0.074) were calculated for Hbr-Int5-F/MseI+T (Table 2).

Landrace-specific genetic diversity was estimated by applying different algorithms to compare a genetic composition among and within autochthonous landraces from Kosovo. The summary of mean within landrace pairwise values, based on genetic distance, is presented in Figure 1. The lowest mean r value was calculated

Table 1: Characteristics of maize landraces from Kosovo

Landrace label	Locality	Latitude [° ‘ “]	Longitude [° ‘ “]	Altitude [m]	Vernacular name	Landscape	Kernel type	Kernel Color	Kernel shape	Flowering time [days]
ACC2	Ferizaj	42.25.21	21.09.06	555	Bardhosh	Flat	Flint	White	Oval	69
ACC4	Shtime	42.26.40	21.42.96	642	Kolomboq	Mountain	Flint	Orange	Oval	72
ACC6	Skenderaj	42.44.39	20.48.04	603	Miser	Valley	Flint	White	Oval	71
ACC8	Skenderaj	42.44.39	20.47.39	597	Miser	Valley	Flint	White	Oval	75
ACC12	Skenderaj	42.45.00	20.48.23	623	Miser	Flat	Flint	White	Oval	76
ACC14	Drenas	42.39.30	20.42.46	565	Kolomboq	Flat	Flint	White	Oval	76
ACC16	Drenas	42.39.21	20.42.32	586	Kolomboq	Flat	Flint	White	Oval	75
ACC26	Vushtrri	42.48.38	20.58.30	518	Kolomboq	Flat	Flint	White	Oval	72
ACC28	Suharekë	42.21.45	20.49.02	388	Miser	Valley	Flint	White	Oval	77
ACC30	Vushtrri	42.50.46	20.59.26	557	Kolomboq	Flat	Flint	White	Oval	71
ACC32	Drenas	42.34.50	20.54.06	585	Kolomboq	Flat	Flint	White	Oval	71
ACC34	Podujevë	42.53.39	21.12.12	598	Kolomboq	Mountain	Flint	White	Oval	75
ACCmk	Lipjan	42.31.45	21.07.20	551	Miser	Flat	Flint	White	Oval	69
ACC38	Kamenicë	42.33.56	21.31.32	812	Kolomboq	Mountain	Flint	White	Longi	67
ACC40	Kamenicë	42.34.16	21.31.32	766	Kolomboq	Mountain	Flint	White	Oval	72
ACC42	Prishtinë	42.35.35	21.20.40	824	Kolomboq	Mountain	Flint	White	Oval	65
ACC44	Drenas	42.41.21	20.45.31	691	Kolomboq	Flat	Flint	White	Oval	71
ACC46	Malisheve	42.27.56	20.43.22	576	Miser	Mountain	Flint	White	Oval	72
ACC48	Malisheve	42.28.01	20.44.04	562	Miser	Mountain	Flint	White	Oval	70
ACC50	Drenas	42.41.50	20.44.43	567	Kolomboq	Flat	Flint	White	Oval	76

Table 2: Analysis of genetic diversity among landraces by selective bases

	Cumulative % in PCoA (first 3 axes)	AMOVA (% among landraces)	Na	Ne	I
Hbr-Int5-F/MseI+T	70	18	7.400	1.067	0.074
Hbr-Int5-F/MseI+C	67	10	6.850	1.066	0.072
Hbr-Int5-F/MseI+G	59	16	7.100	1.065	0.069

Notes: Na-The number of alleles; Ne- The number of detected effective alleles; I-Shannon's information index

across ACC50 (39.03) where r was also outside U and L limits (Figure 1) reflecting the weakest genetic relatedness of genotypes within ACC50 landrace. The strongest genetic relatedness (r = 55.57) was reached within orange colored seeds of ACC4 landrace (Figure 1) showing the highest genetic uniformity of included genotypes within ACC4 compared to other landraces. The genetic structure of observed landraces, described by Bayesian clustering approach in Figure 2 shows higher uniformity of ACC4, with 98.1 % probability that landrace ACC4 belongs to the first (red) genetic cluster and lower genetic uniformity within ACC50 with 79.9 % probability that genotypes from ACC50 belong to the second genetic cluster (green) which is also confirmed by r value (Figure 1).

In general, clustering analysis using the Bayesian method generated two genetic clusters (ideal K, conducted using Structure Harvester) for observed landraces (Figure 2).

Similary colorored segments represents the estimated membership to the genetic cluster. The first genetic cluster (red) posses 0.0778 of expected heterozygosity between genotypes and 0.0925 was calculated for the second one (green), respectively. Regarding their genetic structure, landraces ACC6, ACC12, ACC14, ACC34,

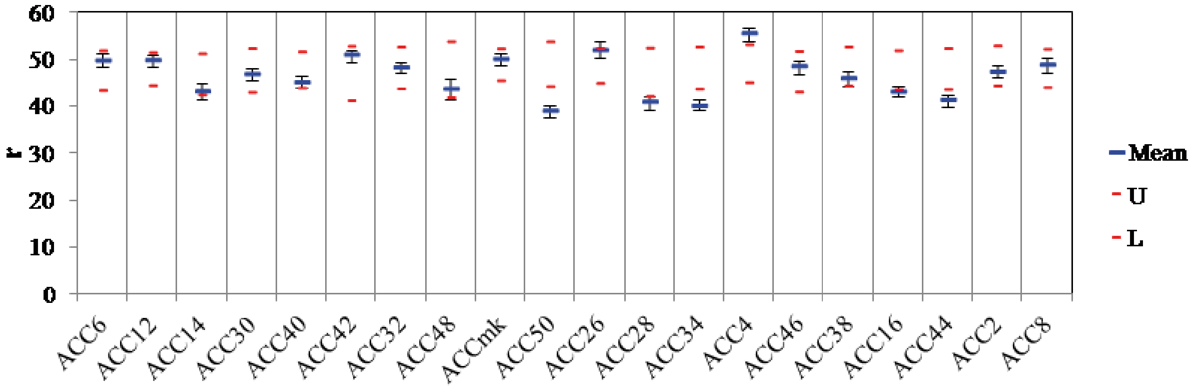

Figure 1: Mean within landrace pairwise values (r) according to genetic distance. Upper (U) and lower (L) confidence limits bound the 95 % confidence interval about the null hypothesis of 'No difference ' across the landraces as determined by permutation (99)

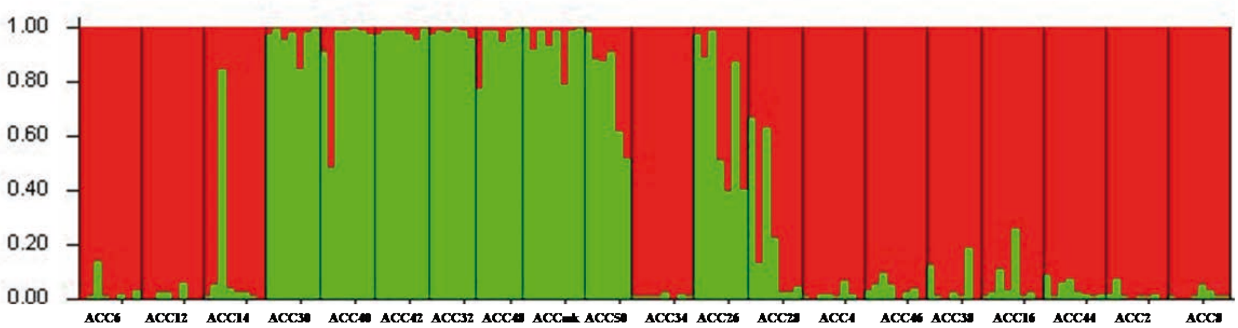

Figure 2: Structure plot of maize landraces from Kosovo

ACC28, ACC4, ACC46, ACC38, ACC16, ACC44, ACC2, and ACC8 belong to the first cluster of landraces (red); meanwhile landraces ACC30, ACC40, ACC42, ACC32, ACC48, ACCmk, ACC50, and ACC26 comprise the second genetic cluster (green) (Figure 2). A similar distribution of genetic relations between landraces was observed in a dendrogram, constructed using Dice coefficient (Dice, 1945) and the NJ algorithm with minor deviations for landraces ACC6 and ACC28 (Figure 3).

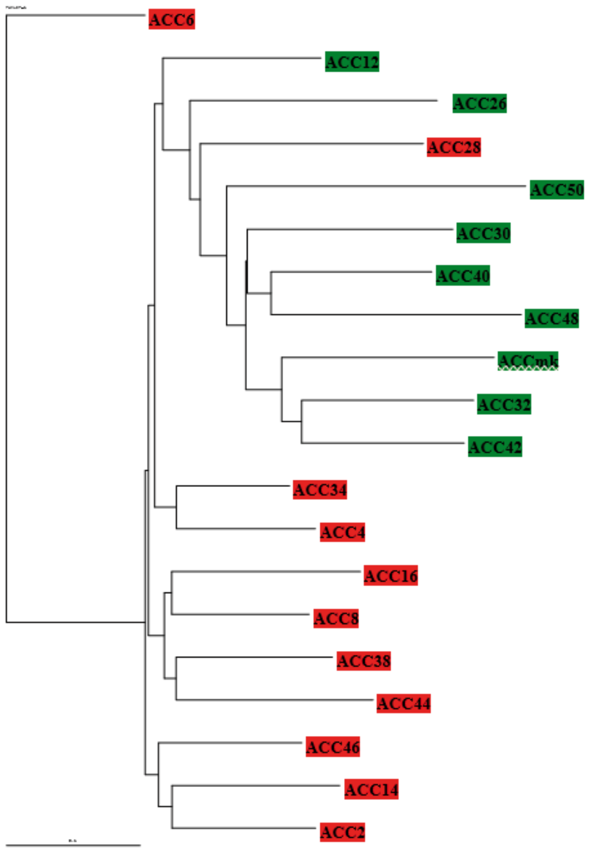

Figure 3: Genetic linkages of observed landraces from Kosovo using Dice coefficient and neighbour-joining (NJ) algorithm. (Green and red colours assign cluster colours from structure plot on Figure 2)

We have calculated landrace-specific parameters of genetic diversity (Figure 4) to compare genetic characteristics between all maize landraces collected, applying Hbr display. Genotypes of landrace ACCmk reveal the highest genetic distinction compared to genotypes within other landraces, reflecting the highest number of bands (241) and the highest number of private bands (10) (Figure 4), respectively.

A number of common bands with a frequency of > 5 %, which are found in the 50 % assessed landraces, reached the highest values for ACC40 (115), ACC32 (114) and ACCmk (111), respectively (Figure 4).

Evaluation of genetic differentiation within and between landraces, applying Hbr markers, provided useful information about genetic the structure, relatedness and genetic diversity of autochthonous maize germplasm from Kosovo. Genetic uniformity of genotypes within landraces is high, regardless to low values of expected heterozygosity (max He = 0.067, data not shown) as a measure of genetic diversity within landraces. Genotypes within the second (green) cluster reveal lower genetic diversity (Fst = 0.2001) compared to the first (red) cluster (Fst = 0.4027).

4 DISCUSSION

Results presented in Table 2 indicate that Hbr-Int5-F/MseI+T is the most informative selective primer provided by Hbr display to distinguish twenty maize landraces collected from different locations in Kosovo. In the study by Kavar et al. (2007) evaluating Slovenian maize landraces, the most informative primer combination was Hbr-Int5-F/MseI+G, revealing the highest number of loci (103) scored using Hbr display. On the other hand, calculated genetic diversity values for maize landraces from Kosovo are similar using Hbr display. This was as well the case evaluating Slovenian accessions by Kavar et al. (2007), where similar values of scored loci (73-103) were obtained applying different selective primers. Cluster analysis using Bayesian approach revealed no genetic relatedness (regarding their genetic

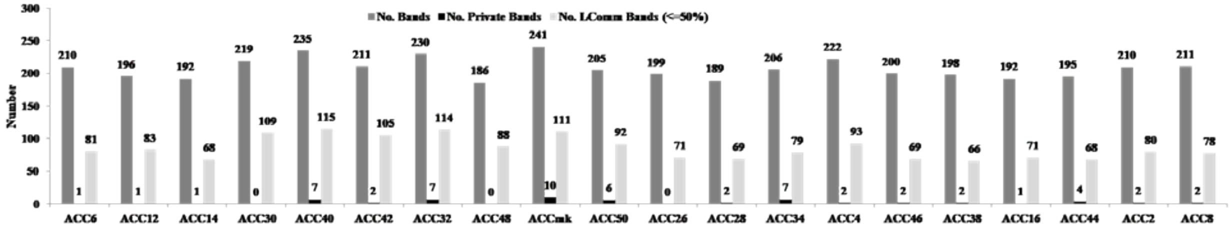

Figure 4: Band patterns across maize landraces from Kosovo

structure) between landraces from the same geographic origin (locality and landscape), a name of landrace and kernel shape (no difference for ACC38 which is oval), respectively. Structure plot also shows that individual plants from landraces ACC14 (87.3 % red, 12.7 % green), ACC50 (20.1 % red; 79.9 % green), ACC26 (28.0 % red, 72.0 % green), and ACC28 (75.0 % red, 25.0 % green) are sharing on average higher proportion of germplasm belonging to both genetic clusters (Figure 2). According to the data in Table 1, four landraces named Kolomboq (vernacular name or local name), originating from flat landscape have the longest flowering period of more than 72 days. There is one exception in this cluster, landrace ACC28 Miser (local name), originating from the valley (Suharekë) with the longest flowering period (77 days). A difference in landrace distributions for genetic clustering, when comparing the structure plot (Figure 2) and dendrogram (Figure 3) could be a logical consequence of different algorithms/approaches applied for specific purpose, required for genetic diversity assessment.

According to the landrace-specific parameters of genetic diversity (Figure 4), landrace ACCmk represents a potentially interesting source for further genetic studies and germplasm improvement. A high number of private bands actually represents unique copies of *Hbr* transposons that ACCmk landrace harbors compared to private alleles observed in other studies using SSR markers. Private alleles in those cases could reflect accumulation and conservation of introduced genes via out-crossing along generations to the plant genome (Pipan et al., 2013). Calculated number of common bands with a frequency > 5 % (Figure 4) for landraces ACC40, ACC32 and even ACCmk, are sharing the highest number of scored bands with other landraces, even though that the three landraces belong to the second (green) genetic cluster/group (Figures 2 and 3). Evaluation of maize landraces from Kosovo was successfully assessed by a rare type of marker system, miniature inverted repeat transposable element - Hbr marker, using three selective primer combinations. To distinguish genotypes between and within different landraces, application of only one selective marker could be sufficient, as reported Kavar et al. (2007). Application of SSR markers as a codominant marker system is also used in genetic diversity studies of maize landraces (Ignjatović-Micić et al., 2015). According to the results presented, there are strong genetic relations between different landraces, comprising two genetic groups, which could be assigned to the two general micro centers of diversity in Kosovo. As a measure of a population structure influenced by genetic drift and migration, Fst values for each genetic cluster generated using Bayesian cluster analysis, were obtained. Higher Fst (0.4027) was calculated within the first (red) genetic group compared to the second (green) one (0.2001), reflecting higher levels of out-crossing and conservation between landraces from the first genetic cluster. This result could be also a consequence of simultaneous cultivation and uncontrolled gene flow between observed maize landraces listed in Table 1 within the same production area of Kosovo in the past.

5 CONCLUSIONS

Related to mean r value, there is 29.7 % of a variable genetic part, which is dispersed along included landraces from Kosovo. It is important to point out that landraces evaluated, originated from different localities with a diverse landscape (flat, mountain, and valley) and from various production areas. According to the results, there are strong genetic relations between different landraces, comprising from two genetic groups, which could indicate on two micro diversification locations of flint type in Kosovo. The results provided, we can conclude that MITE-Hbr markers are highly applicable and cost-effective tool for maize genetic diversity studies and as in this case for a genetic distinction between and within landraces collected in Kosovo.

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