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STUDY OF POLYMORPHISM OF MAIZE USING DNA AND PROTEIN MARKERS

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ABSTRACT

In the present investigation 40 genotypes of maize from Czechoslovakia, Hungary, Poland, Union of Soviet Socialist Republics, Slovakia and Yugoslavia were analysed using 20 start codon targeted (SCoT) markers, 10 simple sequence repeat (SSR) markers, 13 random amplified polymorphic (RAPD) markers and using SDS-PAGE markers. Twenty SCoT primers produced 114 DNA fragments with an average of 5.7 bands per primer. Out of the total of 114 amplified fragments, 86 (76.43 %) were polymorphic, with an average of 4.30 polymorphic bands per primer. Ten SSR primers revealed a total of 65 alleles ranging from 4 (UMC1060) to 8 (UMC2002 and UMC1155) alleles per locus with a mean value of 6.50 alleles per locus. 20 SCoT primers produced total 114 fragments across 40 maize genotypes, of which 86 (76.43 %) were polymorphic with an average of 4.30 polymorphic fragments per primer and number of amplified fragments ranged from 2 (SCoT 45) to 8 (SCoT 28 and SCoT 63). The number of total scorable protein bands was twentythree as a result of SDS-PAGE technique but those that were not cosistent in reproducibility and showed occasional variation in sharpness and density were not considered. Based on these bands forty accessions of maize were screened. Out of twentythree polypeptide bands, 6 (31%) were commonly present in all accessions and considered as monomorphic, while 17 (65%) showed variations and considered as polymorphic. The dendrogram of 40 old maize genotypes based on SSR, SCoT, RAPD and SDS-PAGE markers using UGMA algorithm was constructed.

Keywords: RAPD; SSR; SDS-PAGE; SCoT; old maize; dendrogram

INTRODUCTION

With the advent of the first maize hybrids, in 1933 in the US and around 1950 in Europe, maize cultivation has undergone a complete change **(Gay, 1984; Dubreuil and Charcosset, 1998)**. Since 1990, random amplified polymorphic DNA (RAPD) markers have been successfully applied for identification of DNA polymorphism in various plant species **(Williams et al., 1990).** RAPD technique requires only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster **(Masojc´ et al., 2001).** Molecular markers based on polymerase chain reaction (PCR) methods, such as simple sequence repeats (SSRs) or microsatellites, have become important genetic markers in a wide range of crop species, including maize **(Elçi et al., 2015)**. SSRs markers have many advantages over other types of molecular markers, such as co-dominance, abundant in genomes, highly polymorphisms, locus specificity, good reproducibility and random distribution throughout the genome **(Sun et al., 2011)**. These features, coupled with their ease of detection, make them ideal for identifying and distinguishing between accessions that are genetically very similar **(Saker et al., 2005)**. Recently, a simple novel DNA marker technique namely start codon targeted (SCoT) polymorphism, was developed by **(Collard and Mackill, 2009)**. Primers for SCoT marker analysis were designed from the conserved region surrounding the translation initiation codon, ATG **(Sawant et al., 1999)**. Single 18-mer oligonucleotides were used as both forward and reverse primer for PCR, and the annealing temperature was set at 50 °C. Suitability of SCoT markers for the construction of genetic maps, fingerprinting and phylogenetic studies has been proved by many authors in many crops, such as tomato **(Shahlaei et al., 2014)**, citrus **(Mahjbi et al., 2015)**, date palm **(Al-Qurainy et al., 2015)**, castor **(Kallamadi et al., 2015)** and mango **(Gajera et al., 2014)**. Maize seed consists of two types of protein i.e., zein and non-zein protein. The term zein is used for prolamins in maize which is alcohol soluble protein and could be extracted with ethanol **(Lawton et al., 2006)**. Zein is major seed storage protein of maize and consists of one major and three minor classes and these four classes constitute approximately 50 – 70%

of maize endosperm **(Freitas et al., 2005; Vasal, 1999)**. The non-zein protein consists of globulins (3%), glutelins (34%) and albumins (3%). Zein is specific to maize endosperm and not present in any other part of plant. Proteins are primary gene products of active structural genes; their size and amino acids sequence are the direct results of nucleotide sequences of the genes; hence, any observed variation in protein systems induced by any mutagen is considered a mirror for genetic variations **(Prasanna et al., 2001; Hamoud et al., 2005)**. Determination of protein molecular weight (MW) via polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) is a universally used method in biomedical research; concluded that electrophoresis (SDS-PAGE) of proteins can be economically used to assess genetic variation and relation in germplasm and also to differentiate mutants from their parent genotypes **(Ranjan et al., 2013)**. Some studies used SDS-PAGE for detection of alterations in protein profiles occurring during exposure to electric field **(Hanafy et al., 2006; Dymek et al., 2012)**. For the analysis of genetic diversity of maize genotypes were used several dominant molecular markers: amplified fragment length polymorphism (AFLP) **(Roy and Kim, 2016)**, random amplified polymorphic DNA (RAPD) **(Balážová et al., 2016)**, start codon targeted (SCoT) **(Vivodík et al., 2016)**, inter-simple sequence repeat (ISSR) **(Idris et al., 2012; Žiarovská et al., 2013)** and sequence-related amplified polymorphism (SRAP) **(Abd El-Azeem et al., 2015)**. And codominant molecular markers were also used for the analysis of maize genotypes: simple sequence repeat (SSR) **(Shiri et al., 2014)**, expressed sequence tag (EST)-SSR **(Galvão et al., 2015)**, single nucleotide polymorphism (SNP) **(Sa et al., 2012)** and using protein markers (SDS-PAGE) **(Vivodík et al., 2016)**. The polymerase chain reaction (PCR) has been used by many authors, such as **Žiarovská et. al., (2015)**; **Kanti et. al., (2015)**; **Vyhnánek et. al., (2015)**; **Bošeľová and Žiarovská (2016)**; **Ražná et. al., (2016)**; **Žiarovská et. al., (2017)**; **Simões et. al., (2017)**.

Scientific hypothesis

The present study aimed to examine the genetic variability within and among old 40 maize genotypes cultivated in the Europe, using SSR, SCoT, RAPD and SDS-PAGE markers. The data collected will contribute to identification, rational exploitation and conservation of germplasms of maize genotypes.

MATERIAL AND METHODOLOGY

Maize genotypes (40) were obtained from the Gene Bank VURV Praha-Ruzine (Czech Republic) and from the Gene Bank in Piesťany, the Slovak Republic. DNA of 40 genotypes of maize was extracted from leaves of 10 day old seedlings using the Gene JET Plant Genomic DNA Purification Mini Kit.

SSR analysis: Amplification of SSR fragments was performed according to **Elçi et al. (2015)** (Table 1). Polymerase chain reaction (PCR) were performed in 20 µl of a mixture containing 7.5 µl H_2O, 10.0 µl Master Mix (Genei, Bangalore, India), 0.75 µl of each primer (10 pmol) and 1 µl DNA (100 ng). Amplification was performed in a programmed thermocycler (Biometra, Germany) and amplification program consisted of an initial denaturing step at 94 °C for 2 min, followed by 35 cycles of amplification [95 °C (30 s), 1 min at the 55 °C, 72 °C (30 s)] and a final elongation step at 72 °C for 10 min. Amplification products were confirmed by electrophoresis in 7% denaturing polyacrylamide gels and silver stained and documented using gel documentation system Grab-It 1D for Windows. Data analysis: For the assessment of the polymorphism between maize genotypes and usability of SSR markers in their differentiation diversity index (DI) **(Weir, 1990)**, the probability of identity (PI) **(Paetkau et al., 1995)** and polymorphic information content (PIC) **(Weber, 1990)** were used.

SCoT analysis: A total of 20 SCoT primers developed by **Collard and Mackill (2009),** were selected for the present study (Table 2). Each 15-µL amplification reaction consisted of 1.5 µL (100 ng) template DNA, 7.5 µL Master Mix (Genei, Bangalore, India), 1.5 µL 10 pmol primer, and 4.5 µL distilled water. Amplification was performed in a programmed thermocycler (Biometra, Germany) using the following program: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; a final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1 × TBE buffer. For the assessment of the polymorphism between genotypes maize and usability SCoT markers in their differentiation we used polymorphic information content (PIC) **(Weber, 1990)**.

RAPD analysis: Amplification of RAPD fragments was performed according to **Gajeraa et al. (2010)** (Table 3) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA). Polymerase chain reactions (PCR) were carried out in 25 µl of following mixture: 10.25 µl deionized water, 12.5 µl Master Mix (Promega, USA), 1.25 µl of genomic DNA, 1 µl of 10 pmol of primer. Amplification was performed in a thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system Grab-It 1D pre Windows. For the assessment of the polymorphism between genotypes maize and usability RAPD markers in their differentiation we used diversity index (DI) **(Weir, 1990)**, the probability of identity (PI) **(Paetkau et al., 1995)** and polymorphic information content (PIC) **(Weber, 1990)**.

SDS-PAGE analysis: SDS-PAGE was carried out according to the standard reference ISTA method **(Wrigley, 1992)**. Storage proteins were extracted from individually ground seeds using extracting using a buffer composed of 6.25 mL Tris (1.0 mol L-1, pH = 6.8), 10 mL glycerol, 12.05 mL H_2O and 2.0 g SDS, diluted with mercaptoethanol and H_2O in a 17:3:40 (v/v) proportion. The buffer was added to flour in a 1:25 (w/v) proportion. Extraction was performed at room temperature overnight and heating in boiled water for 5 minutes, centrifugation at 5000 x g for 5 min. 10 µL of extracts were applied to the sample wells. The gel (1.0 mm thick) consists of two parts: stacking gel (3.5% acrylamide, pH = 6.8 acrylamide) and resolution gel (10 % acrylamide, pH = 6.8). Staining of gels was performed in a solution of Coomassie Brilliant

Table 1 List of SSR primers of maize.

SSR markers	F primer	R primer
UMC1363	AAAGGCATTATGCTCACGTTGATT	TCTCCCTCCCCTGTACATGAATTA
UMC1004	CTGGGCATACAAAGCTCACA	TGCATAAACCGTTTCCACAA
UMC2002	TGACCTCAACTCAGAATGCTGTTG	CACAAAATCCTCGAGTTCTTGATTG
UMC1117	AATTCTAGTCCTGGGTCGGAACTC	CGTGGCCGTGGAGTCTACTACT
UMC1587	ATGCGTCTTTCACAAAGCATTACA	AGGTGCAGTTCATAGACTTCCTGG
UMC1060	ACAGGATTTGAGCTTCTGGACATT	GGCCTCTCCTTCATCCTATTCAA
UMC1155	TCTTTTATTGTGCCCGTTGAGATT	CCTGAGGGTGATTTGTCTGTCTCT
UMC1072	GAGGAGACCGCCTCTGGTTC	CTTCGGGTTCCTGGACCTTCT
UMC1133	ATTCGATCTAGGGTTTGGGTTCAG	GATGCAGTAGCATGCTGGATGTAG
UMC1413	CATACACCAAGAGTGCAGCAAGAG	GGAGGTCTGGAATTCTCCTCTGTT

Table 2 SCoT markers used in maize**.**

SCoT Primers	Primer sequence (5´-3´)
SCoT 6	CAACAATGGCTACCACGC
SCoT 8	CAACAATGGCTACCACGT
SCoT 9	CAACAATGGCTACCAGCA
SCoT 12	ACGACATGGCGACCAACG
SCoT 23	CACCATGGCTACCACCAG
SCoT 26	ACCATGGCTACCACCGTC
SCoT 28	CCATGGCTACCACCGCCA
SCoT 29	CCATGGCTACCACCGGCC
SCoT 30	CCATGGCTACCACCGGCG
SCoT 36	GCAACAATGGCTACCACC
SCoT 40	CAATGGCTACCACTACAG
SCoT 44	CAATGGCTACCATTAGCC
SCoT 45	ACAATGGCTACCACTGAC
SCoT 54	ACAATGGCTACCACCAGC
SCoT 59	ACAATGGCTACCACCATC
SCoT 60	ACAATGGCTACCACCACA
SCoT 61	CAACAATGGCTACCACCG
SCoT 62	ACCATGGCTACCACGGAG
SCoT 63	ACCATGGCTACCACGGGC
SCoT 65	ACCATGGCTACCACGGCA

Table 3 List of RAPD primers and their sequences.

Primers	Primer sequence (5´-3´)
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-13	CAGCACCCAC
OPB-08	GTCCACACGG
OPD-02	GGACCCAACC
OPD-07	TTGGCACGGG
OPD-08	GTGTGCCCCA
OPD-13	GGGGTGACGA
OPE-07	AGATGCAGCC
OPF-14	TGCTGCAGGT
SIGMA-D-01	AAACGCCGCC
SIGMA-D-14	TCTCGCTCCA
SIGMA-D-P	TGGACCGGTG

Blue R250 dissolved in acetic acid and methanol solution. Gel was scanned with densitometer GS 800 (Bio-Rad) and evaluated with Quantity One-1D Analysis Software.

Statisic analysis

The SSR, SCoT, RAPD and SDS-PAGE bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed.

RESULTS AND DISCUSSION

SSR analysis: Ten maize SSR primers were used for identification and estimation of the genetic relations among 40 old European maize genotypes. All 10 SSR primers generated clear banding patterns with high polymorphism. Ten SSR primers revealed a total of 65 alleles ranging from 4 (UMC1060) to 8 (UMC2002 and UMC1155) alleles per locus with a mean value of 6.50 alleles per locus (Table 4). Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. The PIC values ranged from 0.713 (UMC1060) to 0.842 (UMC2002) with an average value of 0.810 and the DI value ranged from 0.734 (UMC1060) to 0.848 (UMC2002) with an average value of 0.819 (Table 4). 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis.

Similar results were detected by other authors (**Kanagarasu et al., 2013; Molin et al., 2013; Al-Badeiry et al., 2014; Shiri et al., 2014; Efendi et al., 2015; Ignjatovic-Micic et al., 2015; Salami et al., 2016)** and these results presented a high level of polymorphism of old maize genotypes detected by SSR markers. **Kanagarasu et al. (2013)** used 10 SSR molecular markers to analysis of 27 maize inbred lines. Ten SSR markers produced 23 polymorphic alleles with an average of 2.3 alleles per locus and mean polymorphic information content (PIC) of 0.45. The aim of **Molin et al. (2013)** was study the genetic diversity across 48 varieties of maize landraces cultivated at different locations in the States of Rio Grande do Sul and Paraná by 47 simple sequence repeat (SSR) markers. SSR analysis resulted in amplification of 105 polymorphic fragments and a polymorphic index of 78.3%. **Al-Badeiry et al. (2014)** detected 41 alleles among the tested maize varieties using 10 Simple Sequence Repeat (SSR).The molecular size of bands obtained from amplification of SSR products ranged from 91 to 288 bp. Alleles ranged from one in umc1653 to ten in bnlg1189 loci. **Shiri et al. (2014)** study genetic diversity of 38 maize

hybrids using 12 SSR primers. The total number of PCR-amplified products was 40 bands, all of them polymorphic. Primer Phi031 generated the highest number of bands (6). Among the studied primers, UMC2359, PHI031 and UMC1862 showed the maximum polymorphism information content (PIC) and the greatest diversity. The aim of **Efendi et al. (2015)** was to select homozygosity and analyze genetic diversity of 51 maize inbreds using 36 SSRs markers. The research was aimed to select among 51 maize inbreds with high homozygosity and to investigate the genetic diversity using 36 SSRs markers. The result shows that there were 30 inbreds indicating homozygosity level of more than 80%. **Ignjatovic-Micic et al. (2015)** analyzed nine flint and nine dent accessions from six agro-ecological groups, chosen on the basis of diverse pedigrees. Ten SSR primers revealed a total of 63 alleles. High average PIC value (0.822) also supports informativeness and utility of the markers used in this study. The aim of study **Salami et al. (2016)** was to evaluate the genetic diversity of Benin's maize accessions by SSR marker. Thus, 187 maize accessions from three areas were analyzed using three SSR markers. A total of 227 polymorphic bands were produced and showed high genetic diversity. The polymorphic information content (PIC) values for the SSR loci ranged from 0.58 to 0.81, with an average of 0.71.

Table 4 List of SSR primers, total number of bands and the statistical characteristics of the SSR markers used in maize.

Marker name	Number of alleles	DI	PIC	PI
UMC1363	7	0.808	0.799	0.011
UMC1004	6	0.830	0.823	0.005
UMC2002	8	0.848	0.842	0.005
UMC1117	5	0.794	0.780	0.010
UMC1587	7	0.835	0.827	0.006
UMC1060	4	0.734	0.713	0.022
UMC1155	8	0.835	0.830	0.007
UMC1072	7	0.845	0.839	0.004
UMC1133	6	0.818	0.808	0.007
UMC1413	7	0.846	0.841	0.005
Average	**6.50**	**0.819**	**0. 810**	**0.008**

Note: DI – diversity index, PIC – polymorphic information content, PI – probability of identity.

SCoT analysis

In this work, all 20 SCoT primers used for analysis of 40 European old maize genotypes produced amplification products and all resulted in polymorphic fingerprint patterns. Twenty primers produced 114 DNA fragments with an average of 5.7 bands per primer (Table 5). Out of the total of 114 amplified fragments, 86 (76.43 %) were polymorphic, with an average of 4.30 polymorphic bands per primer. From these twenty primers, primers SCoT 28 and SCoT 63, respectively, were the most polymorphic, where 8 polymorphic amplification products were detected. The lowest number of amplified polymorphic fragments (2) was detected by primer SCoT 45. To determine the level of polymorphism in the analysed group of maize genotypes, polymorphic information content (PIC) was calculated (Table 5). The polymorphic information content (PIC) value ranged from 0.374 (SCoT 45) to 0.846 (SCoT 28) with an average of 0.739.

Similar values of PIC were detected by other authors and these values presented a high level of polymorphism of genotypes detected by SCoT markers. **Huang et al. (2014)** assessed the genetic diversity of six Hemarthria cultivars using seven SCoT primers, which together amplified 105 bands with an average of 15 bands per sample. **Fang-Yonga et al. (2014)** assessed the genetic diversity of 31 germplasm resources of Myrica rubra from Zhejiang Province, the major gathering site and the largest producer of M. rubra in China using start codon-targeted polymorphism (SCoT) markers. Authors used 38 primers to perform PCR amplification of 31 genotypes, from which 298 reproducible bands were obtained, including 251 polymorphic bands (84.23%). **Satya et al. (2015)** used 24 start codon targeted (SCoT) markers to assess genetic diversity and population structure of indigenous, introduced and domesticated ramie (*Boehmeria nivea* L. Gaudich.). **Jiang et al. (2014)** used start codon-targeted (SCoT) markers to analyze the diversity and genetic relationships among 95 orchardgrass accessions. In total, 273 polymorphic bands were detected with an average of 11.4 bands per primer. In the study **Zhang et al. (2015)**, used SCoT markers to study the genetic diversity and relationships among 53 *Elymus sibiricus* accessions.

Table 5 Statistical characteristics of the SCoT markers used in maize.

SCoT Primers	TNoB	NoPB	PoPB	PIC
SCoT 6	5	4	80.00	0.729
SCoT 8	4	4	100.00	0.652
SCoT 9	6	4	66.66	0.780
SCoT 12	7	5	71.43	0.715
SCoT 23	7	5	71.43	0.816
SCoT 26	5	4	80.00	0.714
SCoT 28	8	5	62.50	0.846
SCoT 29	6	4	66.66	0.810
SCoT 30	7	6	85.71	0.825
SCoT 36	7	7	100.00	0.812
SCoT 40	6	5	83.33	0.731
SCoT 44	4	2	50.00	0.710
SCoT 45	2	2	100.00	0.374
SCoT 54	5	3	60.00	0.717
SCoT 59	6	3	50.00	0.794
SCoT 60	6	3	50.00	0.790
SCoT 61	6	5	83.33	0.808
SCoT 62	4	4	100.00	0.618
SCoT 63	8	7	87.50	0.832
SCoT 65	5	4	80.00	0.697
Average	**5.70**	**4.30**	**76.43**	**0.739**
Total	**114**	**86**	**-**	**-**

Note: TNoB-Total number of bands, NoPB- Number of polymorphic bands, PoPB- Percentage of polymorphic bands (%), PIC- Polymorphic information content.

RAPD analysis

Our study dealt with detection of genetic polymorphism in maize cultivars using RAPD markers. For the differentiation of forty maize genotypes thirteen RAPD markers (Table 6) were chosen according to **Gajeraa et al. (2010)**.

Table 6 Statistical characteristics of the RAPD markers.

Primers	Number of alleles	DI	PIC	PI
OPA-02	5	0.768	0.755	0.041
OPA-03	7	0.826	0.820	0.007
OPA-13	10	0.874	0.872	0.006
OPB-08	5	0.718	0.709	0.032
OPD-02	6	0.765	0.751	0.049
OPD-07	5	0.725	0.723	0.026
OPD-08	8	0.834	0.829	0.006
OPD-13	9	0.856	0.849	0.005
OPE-07	7	0.835	0.829	0.006
OPF-14	8	0.865	0.862	0.003
SIGMA-D-P	7	0.839	0.833	0.005
SIGMA-D-01	8	0.854	0.849	0.004
SIGMA-D-14	7	0.741	0.728	0.023
Average	**7.08**	**0.808**	**0.801**	**0.016**

Note: SDS-PAGE analysis.

Chosen primers amplified DNA fragments across 40 maize genotypes studied, with the number of amplified fragments ranged from 5 (OPA-02, OPB-08, OPD-07) to 10 (OPA-13). The polymorphic information content (PIC) values varied from 0.709 (OPB-08) to 0.872 (OPA-13), with an average of 0.801 and index diversity (DI) value varied from 0.718 (OPB-08) to 0.874 (OPA-13) with an average of 0.808 (Table 6). Similar values of DI and the PIC were detected by other authors and these values presented a high level of polymorphism of maize genotypes detected by RAPD markers. **Osipova et al. (2003)** used RAPD markers to analyse the genetic divergence between the regenerated plants derived from callus cultures and the original maize line A188. Specific polymorphism revealed with random primers was completely confirmed using five SCAR markers. **De Vasconcelos et al. (2008)** used the RAPD technique to evaluate somaclonal variation in maize plants derived from tissue culture from the maize inbred line L48. Forty seven different decamer oligonucleotide primers generated 221 amplification products, 130 of them being polymorphic. **Mukharib et al. (2010)** used RAPD markers to fingerprint 20 varieties of maize. The primers of the most interest of this purpose were those that produced more variety specific DNA profiles, such as OPD-03, OPE-18, OPF-05, OPL-11 and OPX-04. Much higher number of alleles (7) compared to **Al-Badeiry et al. (2013)**, who detected only one allele, can be caused by diverse set of maize varieties used for analysis. **Mrutu et al. (2014)** assessed the genetic diversity of maize hybrids grown in Southern highlands of Tanzania by using RAPD markers. Twelve maize samples were collected and used in this study. The aim of **Molin et al. (2013)** was to estimate the genetic diversity across 48 varieties of maize landraces cultivated at different locations in the States of Rio Grande do Sul and Paraná by means of different marker system including random amplified polymorphic DNA. Maize landrace accessions were genotyped using the 30 RAPD primers. Similar level of polymorphism (84.44%) obtained also **Bruel et al. (2007)**.

The number of total scorable protein bands was twentythree as a result of SDS-PAGE technique but those that were not cosistent in reproducibility and showed occasional variation in sharpness and density were not considered. Based on these bands forty accessions of maize were screened. Out of twentythree polypeptide bands, 6 (31%) were commonly present in all accessions and considered as monomorphic, while 17 (65%) showed variations and considered as polymorphic. The size of the protein bands obtained through SDS-PAGE ranged from 20 to 140 kDa. On the basis of banding profiles of proteins of different kDa, gel was divided into zones A, B and C. The major protein bands were lied in zones A and B, while minor bands were present in zones C. It was noted that different accessions of maize showed more diversity in seed storage proteins in minor bands in comparison to major bands. In zone A out of 10 protein bands, 1 were monomorphic and 9 were polymorphic. In zone B out of 8 protein bands, 3 was monomorphic and 5 was polymorphic and in zone C out of 5 protein bands, 2 were monomorphic whereas 3 polymorphic. By considering these facts zone A and B were more polymorphic.

Similarly the present study of genetic variability in the seed storage polypeptide determined by SDS-PAGE technique proved that it is fruitful to identify genetic diversity among accessions of maize. Similar results were detected by other authors (**Osman et al., 2013; Iqbal et al., 2014; Iqbal et al., 2014; Khan et al., 2014; AL-Huqail et al., 2015**) and these results presented a high level of polymorphism of old maize genotypes detected by SDS-PAGE. **Osman et al. (2013)** study genetic relationship between some species of *Zea mays* using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of seed proteins. Autors identified 78 bands across the studied species. The number of bands varies from 17 bands in sample number 5 to 6 in sample number 6. **Iqbal et al. (2014)** analyzed 73 genotypes of maize from China, Japan and Pakistan for the total seed storage proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 18 protein bands were recorded. Among these 7 (39%) were monomorphic and 11 (61%) polymorphic, with molecular weight varied from 10 kDa to 122 kDa. The aim of **Iqbal et al. (2014)** was to estimate the genetic diversity across 83 genotypes of maize of Pakistan and Japanese origin using SDS-PAGE. A total of 18 protein subunits were noted out of which 7 (39%) were monomorphic and 11 (61%) were polymorphic, with molecular weight ranging from 10 to 122 kDa. Coefficients of similarity among the accessions ranged between 0.89 and 1.00. The dendrogram obtained through UPGMA clustering method showed two main clusters: 1 and 2. First cluster contained 9 genotypes, while second cluster contained 74 genotypes. **Khan et al. (2014)** study the variation of zein fraction of seed storage protein in maize by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

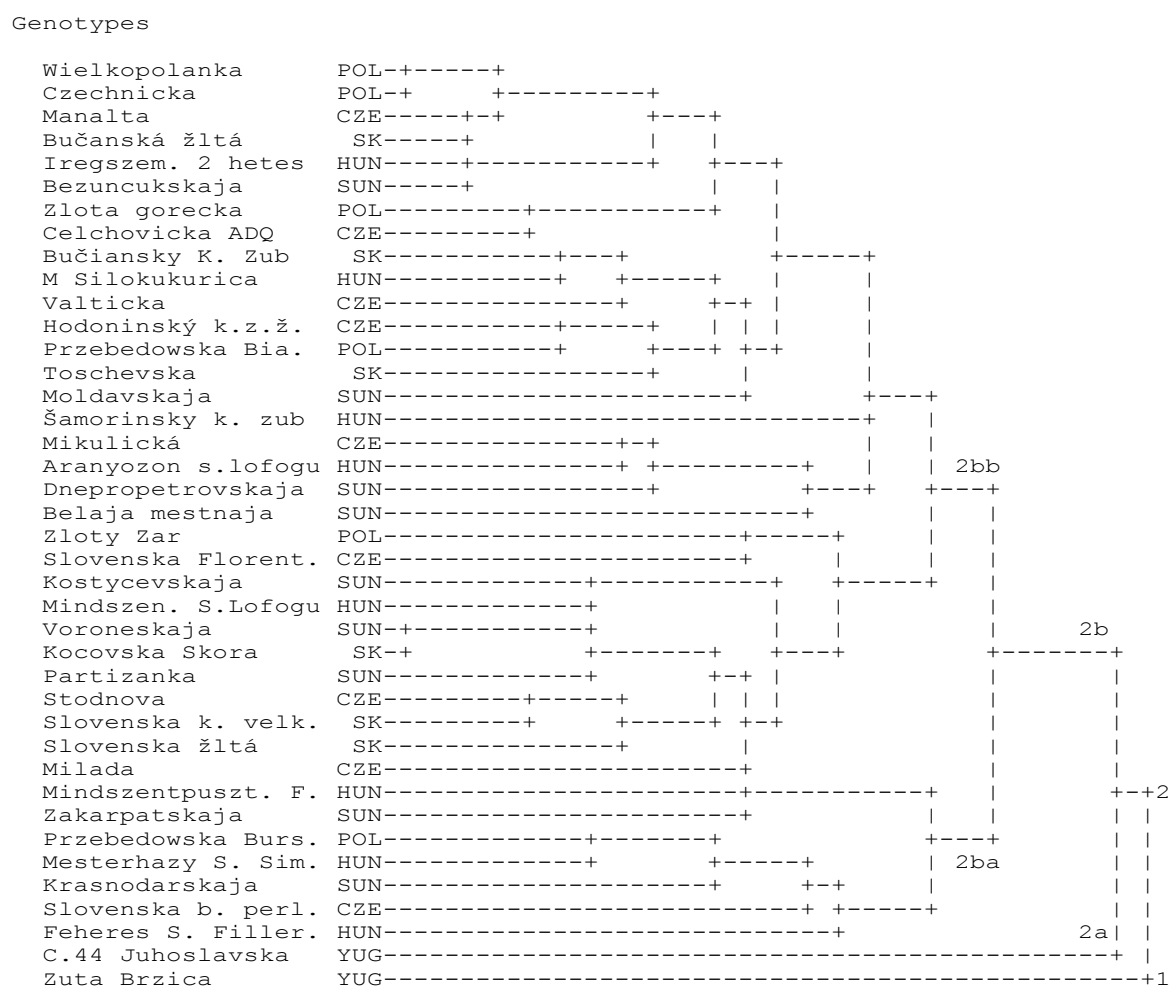

Figure 1 Dendrogram of 40 maize genotypes prepared based on SSR, SCoT, RAPD and SDS-PAGE markers. CZE – Czechoslovakia, HUN – Hungary, POL – Poland, SUN – Union of Soviet Socialist Republics, SK – Slovakia, YUG – Yugoslavia.

Variation in terms of absence and presence, intensity and molecular size was observed in zein polypeptides. **AL-Huqail et al. (2015)** used SDS-PAGE to detection of 46 polypeptides bands with different molecular weights ranging from 186.20 to 36.00 KDa. It generated distinctive polymorphism value of 84.62%.

The dendrogram of 40 maize genotypes based on SSR, SCoT, RAPD and SDS-PAGE markers using UGMA algorithm was constructed (Figure 1). The hierarchical cluster analysis divided maize genotypes into two main clusters. Unique maize genotype Zuta Brzica, originated from Yugoslavia (cluster 1), separated from others. Cluster 2 containing 39 genotypes was divided into two main subclusters (2a and 2b). Subcluster 2a contained one Yugoslavian genotype Juhoslavanska and subcluster 2b was divided in two subclusters 2ba and 2bb. In the subcluster 2ba were grouped 7 genotypes from Hungary (42.87%), Poland (14.29%), Czechoslovakia (14.29%) and Union of Soviet Socialist Republics (28.58%). Subcluster 2bb of 31 genotypes included genotypes of Polish origin (16.15%), Union of Soviet Socialist Republics origin (22.61%), Slovakia origin (19.38%), Czechoslovak origin (25.84%) and Hungarian origin (16.15%). Two genotypes of 2bb subcluster (Czechnicka and Wielkopolanka) from Poland and two genotypes (Voroneskaja and Kocovska Skora) from Union of Soviet Socialist Republics and Slovakia, respectively, were genetically the closest. We can assume that they have close genetic background.

CONCLUSION

The present study indicates the validity of PCR technique for estimating genetic diversity among old maize genotypes. The current data will enhance the breeding efficiency and will add the strength of Marker Assisted Selection (MAS). In the light of information about the genetic diversity in 40 European maize genotypes, it is suggested that the breeding programs with the help of DNA fingerprinting technology will be helpful to utilize the genotypes to produce cultivars/varieties by crossing them with different elite. Genetic diversity plays a key role in crop improvement. In conclusion, a high level of genetic diversity exists among the old maize accessions analyzed. A SSR, SCoT, RAPD and SDS-PAGE markers system are a rapid and reliable method for cultivar identification that might also be used in quality control in certified seed production programs, to identify sources of seed contamination, and to maintain pure germplasm collections. The present study shows effectiveness of employing SCoT, RAPD, SSR and SDS-PAGE markers in analysis of maize, and would be useful for further studies in population genetics, conservation genetics and genotypes improvement.

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