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Molecular and phenotypic characterisation of diverse temperate maize inbred lines in Southeast Europe

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Abstract

Maize (*Zea mays* L.) displays large genetic diversity created during the history of introduction from its Mexican centre of origin to other parts of the world and adaptation to a range of diverse environments. Despite such diversity, maize breeders use only a small portion of the available maize germplasm to develop modern hybrids. Broadening diversity of breeding collections by the introduction of new germplasm, as a source of favourable traits, requires its characterisation and classification of new germplasm into heterotic groups. The aim of this study was to estimate genetic diversity of maize breeding material from the Institute of Field and Vegetable Crops in Serbia, including previously uncharacterised inbred lines, elite lines with known pedigrees and historically important inbred lines. Microsatellite-based cluster analysis and principal coordinate analysis separated 96 inbred lines into six clusters, Iowa Stiff Stalk Synthetic (BSSS), Lancaster Sure Crop (LSC), Iodent (IDT) heterotic group, a cluster with unrelated independent inbreds and two clusters of miscellaneous germplasm crossed with inbreds of BSSS and Lancaster background. The microsatellites *umc1035*, *bnlg666*, *dupssr23*, *umc1083* and *dupssr10* contributed most to the differentiation between the groups. The largest values of molecular diversity parameters were detected in the BSSS group, following by the Lancaster and then the other groups. An analysis of variance showed that almost all traits significantly varied among the groups and between the years. The investigated lines demonstrated sufficient variation in most of the analysed phenotypic traits, proving suitable for further genetic studies. A principal component analysis based on agronomic traits differentiated inbred lines from the BSSS and Lancaster pools, but failed to separate the other groups. The characterisation and classification of genetic resources using microsatellite markers may assist hybrid breeding by efficient exploitation of heterotic patterns.

Key words: heterotic groups, inbreds, microsatellites, simple sequence repeats, *Zea mays*.

Introduction

Maize (*Zea mays* L.), one of the most important cereal crops grown for food and feed globally, exhibits a large phenotypic and genotypic variability (Yan et al., 2009). A great diversity of agroecological environments for growing maize contributed to the development of divergent populations adapted to different edaphic and climatic conditions and biological factors, which ultimately manifested in a wide range of morphological, physiological, biochemical, agronomic and genetic traits.

Serbia belongs to the European Corn Belt, the world's second largest maize growing area. Its southeastern and central parts, encompassed by the Pannonian Basin and spread throughout Hungary, southwestern Slovakia, Slavonia, northern Serbia and western Romania, present one of the main maize production regions in Europe (Stojaković et al., 2015). In these specific agroecological conditions, during the introductions of Caribbean, Mexican, Andean, North American flints and American Corn Belt dents and following their successive crosses and adaptation, local maize varieties with distinct genetic backgrounds have been developed (Hadi, 2006). These diverse open-pollinated populations, adapted to various stress factors, such as drought, pathogen infections and pest infestations, and with high yield potential, were a valuable source of beneficial agronomic traits and used as initial breeding material in the 1960s in Southeast Europe (Mitrović et al., 2016). However, since the mid-twentieth century, the local germplasm began to gradually lose significance in regional breeding programmes, because of the global success of commercial maize hybrids. Nowadays, the narrow genetic base of modern hybrids is the main concern of maize breeders: only as few as six or seven inbred lines were identified to be founders of modern maize hybrids (Technow et al., 2014).

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The introduction of new germplasm into the existing breeding programmes is an essential step for broadening genetic diversity of working breeding material and could serve as an untapped source of favourable alleles. Maize breeders could enrich their working collections by introgressing a portion of local landraces, inbred lines developed from them, introduced accessions from gene banks and adapted exotic germplasm into elite inbred lines or into multiparental populations. Yet, such genetic material often has incomplete pedigree information and unknown heterotic response (El-Kassaby et al., 2011). To fully exploit the potential of such breeding material and phenomenon of heterosis, it is important to characterise it at molecular and phenotypic levels and assign newly developed inbred lines to heterotic groups. Traditionally, crosses are made between inbred lines with unknown heterotic response and inbred lines of different known heterotic groups and their F_1 offspring is observed for hybrid vigour, requiring at least two seasons and considerable land and labour resources.

Molecular markers, such as the most commonly used biallelic single nucleotide polymorphism (SNP) and multialelic simple sequence repeat (SSR), may facilitate characterisation and assignment of maize inbred lines to heterotic groups. The growing number of SNP-based maize genetic diversity studies due to decreasing costs per data point, high throughput application and large abundance of SNPs in the maize genome, do not necessarily presume the absolute supremacy of SNPs over SSRs. A recent study demonstrated the suitability of 1065 SNP markers to group 450 diverse maize inbreds according to their pedigrees using different multivariate methods, but it also showed their failure to clearly distinguish between two main maize heterotic groups (Semagn et al., 2012). Comparing the discriminating power of SSR and SNP markers in assessing maize diversity, Wu et al. (2016) indicated that the better genome coverage of SNPs contributed to their better performance. However, the authors pointed out that more SNPs were needed to compensate for highly polymorphic SSRs. Similarly, Yang et al. (2011) suggested that the moderate density of SSR markers was more informative than SNPs for maize diversity analyses.

The aim of this study was to assess adequacy of SSRs to estimate genetic diversity of maize breeding material from the Institute of Field and Vegetable Crops in Serbia, as a representative of Southeast European environment, to assign maize inbreds to heterotic groups and to analyse and compare molecular and phenotypic data of previously uncharacterised inbred lines to those whose pedigrees and heterotic groups are well-known in order to make the best used of available maize germplasm for breeding purposes.

Materials and methods

A collection of 96 diverse maize inbred lines, developed at the Institute of Field and Vegetable Crops in Novi Sad, Serbia, was selected for molecular and phenotypic characterisation. They comprised several historically relevant inbred lines and the majority of elite inbred lines from Iowa Stiff Stalk Synthetic (BSSS), Lancaster Sure Crop (LSC) and Iodent (IDT) heterotic groups, unrelated independent (IND) heterotic group, inbred lines with mixed origin, including adapted non-tropical exotic germplasm, and local Serbian maize varieties, with limited information about their full pedigrees.

For molecular characterisation, genomic DNA was extracted from the bulk of approximately 10 seedlings for each maize inbred line using the CTAB (cetyl trimethylammonium bromide) method. Forty fluorescently labelled SSRs (Table 1) were used for polymerase chain reactions (PCR). Total PCR mix consisted of 25 ng of genomic DNA, 0.2 mM dNTP, 1 × Taq buffer with KCl, 2 mM $MgCl_2$, 1U Taq polymerase and 0.5 pmol of each primer (Applied Biosystems, USA). The first step of PCR started with DNA denaturation at 94°C for 5 min, followed by 38 cycles for 30 s at 94°C, 45 s at the primer specific annealing temperature (Table 1), 45 s at 72°C and one cycle of the final extension for 7 min at 72°C, performed on Veriti Thermal Cycler (Applied Biosystems, USA). The 10 µL reaction volume for fragment analysis contained 2 µL mixtures of differently labelled PCR products, 0.2 µL GeneScan500 LIZ size standard and 7.8 µL Hi-Di formamide (Applied

Table 1. Names, chromosome locations, primer sequences, annealing temperatures, expected allele size and repeat motifs of forty analysed microsatellite markers

Locus	Bin	Forward and reverse primers	Annealing temperature °C	Allele range bp	Repeat motif
1	2	3	4	5	6
bnlg1067	8.03	5'-GGCTTGCTTTTGCTTCACTT-3' 5'-CTCATCCCATTCGTTCCACT-3'	63	116–130	AG(26)
bnlg1209	9.04	5'-GTCCCGGGCAGAATAATACC-3' 5'-TTCCTCCTTGAAGTGCTCGT-3'	53	164–196	AG(12)
bnlg1237	5.05	5'-TGGCGCGATTTTCTTCATAT-3' 5'-AAAGAGCAACCTTCAACGGA-3'	58	151–185	AG(29)
bnlg125	2.02	5'-GGGACAAAAGAAGAAGCAGAG-3' 5'-GAAATGGGACAGAGACAGACAAT-3'	53	164–196	unknown
bnlg1360	10.07	5'-TCTGCTCATCCACAACTTGC-3' 5'-AGAACGTGAAGCTGAGCGTT-3'	58	105–139	AG(25)
bnlg1451	10.02	5'-TGATCGATGGCTCAATCAGT-3' 5'-ATCTGGAACACCGTCGTCTC-3'	58	164–190	AG(34)
bnlg1520	2.09	5'-TCCTCTTGCTCTCCATGTCC-3' 5'-ACAGCTGCGTAGCTTCTTCC-3'	53	165–195	AG(22)
bnlg1523	3.03	5'-GAGCACAGCTAGGCAAAAGG-3' 5'-CTCGCACGCTCTCTCTTCTT-3'	53	176–236	AG(17)

Table 1 continued

1	2	3	4	5	6
bnlg1525	9.07	5’-AGGAATTGCGAGTCTTCCAA-3’ 5’-CAACCCCCAAAATGAACAAA-3’	56	156–200	AG(25)
bnlg1556	1.07	5’-ACCGACCTAAGCTATGGGCT-3’ 5’-CCGGTTATAAACACAGCCGT-3’	53	150–184	AG(18)
bnlg162	8.05	5’-ACTAGCAGCAGTAAAACCTAATAAAGGA-3’ 5’-CAAGTAGCTAGCAGTCATTTGCAGTGT-3’	56	214–260	unknown
bnlg1792	7.02	5’-CGGGAATGAATAAGCCAAGA-3’ 5’-GCGCTCCTTCACCTTCTTTA-3’	58	107–139	AG(16)
bnlg2291	4.06	5’-CCTCTCGATGTTCTGAAGCC-3’ 5’-GTCATAACCTTGCCTCCCAA-3’	53	153–197	AG(17)
bnlg238	6.00	5’-CTTATTGCTTTCGTCATACACACACATCAT-3’ 5’-GAGCATGAGCTTGCATATTTCTTGTGG-3’	58	135–179	unknown
bnlg430	9.03	5’-CTTACTGAGCATCTTCCTTCTCTCC-3’ 5’-TCCGGTGATGCTCCAGCGAC-3’	58	99–111	unknown
bnlg666	8.05	5’-AAAAGGCAAGTAGCTAGCATGCATTGCAG-3’ 5’-GGCTCACGTCCGTATCCAAACCAACA-3’	58	111–158	unknown
dupssr10	5.04	5’-AGAAAATGGTGAGGCAGG-3’ 5’-TATGAAATCTGCATCTAGAAATTG-3’	53	156–198	AC(22)
dupssr23	3.06	5’-TGATCATCATAAGCACACCG-3’ 5’-CCAATGTGAAGCAAGAGAGAA-3’	56	64–118	(GA)2TA(GA)19
dupssr26	1.04	5’-GTCGGAGCACTCCAAGAC-3’ 5’-CTTCTCGCTCATCAGCTTAAA-3’	53	112–142	(GA)23
nc005	4.05	5’-CCTCTACTCGCCAGTCGC-3’ 5’-TTTGGTCAGATTTGAGCACG-3’	56	120–152	CT
phi027	9.03	5’-CACAGCACGTTGCGGATTTCTCT-3’ 5’-GCGTACGTACGACGAAGACAC-3’	58	141–156	GCGCT
phi034	7.02	5’-TAGCGACAGGATGGCCTCTTCT-3’ 5’-GGGGAGCACGCCTTCGTTCT-3’	58	118–149	CCT
phi053	3.05	5’-CTGCCTCTCAGATTCAGAGATTGAC-3’ 5’-AACCCAACGTACTCCGGCAG-3’	53	150–190	ATAC
phi059	10.02	5’-AAGCTAATTAAGGCCGGTCATCCC-3’ 5’-TCCGTGTACTCGGCGGACTC-3’	58	139–154	ACC
phi083	2.04	5’-CAAACATCAGCCAGAGACAAGGAC-3’ 5’-ATTCATCGACGCGTCACAGTCTACT-3’	56	121–137	AGCT
phi093	4.08	5’-AGTGCGTCAGCTTCATCGCCTACAAG-3’ 5’-AGGCCATGCATGCTTGCAACATGGATACA-3’	58	280–296	AGCT
umc1014	6.04	5’-GAAAGTCGATCGAGAGACCCTG-3’ 5’-CCCTCTCTTCACCCCTTCCTT-3’	58	113–141	(GA)12
umc1022	4.01	5’-AACAAGTTTTGTTTGACAAGCCG-3’ 5’-ATGATCACCCCGTCAGCG-3’	53	65–97	(CA)9
umc1025	3.04	5’-GCTCCACTTCCACCCTGATATG-3’ 5’-CGCTAATGTCCCCATTGATGAT-3’	56	101–117	(CT)11
umc1035	1.06	5’-CTGGCATGATCACGCTATGTATG-3’ 5’-TAACATCAGCAGGTTTGCTCATTC-3’	58	110–212	(CT)19
umc1075	8.01	5’-GAGAGATGACAGACACATCCTTGG-3’ 5’-ACATTTATGATACCGGGAGTTGGA-3’	56	136–146	(ATTGC)5
umc1083	6.02	5’-CTTTCCTCTCTGGAGCGTGTATTG-3’ 5’-ATATGTTGCAGAACCATCCAGGTC-3’	56	90–128	(GA)16
umc1109	4.01	5’-GCAACACAGGACCAAATCATCTCT-3’ 5’-GTTCGGTCCGTAGAAGAACTCTCA-3’	56	103–115	(ACG)4
umc1122	1.06	5’-CACAACTCCATCAGAGGACAGAGA-3’ 5’-CTGCTACGACATACGCAAGGC-3’	58	141–168	(CGT)7
umc1221	5.04	5’-GCAACAGCAACTGGCAACAG-3’ 5’-AAACAGGCACAAAGCATGGATAG-3’	56	69–95	(CT)7
umc1360	8.02	5’-GCTAGTTGAGTTCGACACCAGGTT-3’ 5’-TGACTGTGACTGTGACTATGACCG-3’	56	139–160	(ACA)4
umc1792	5.08	5’-CATGGGACAGCAAGAGACACAG-3’ 5’-ACCTTCATCACCTGCAACTACGAC-3’	58	113–128	(CGG)5
umc1944	7.04	5’-GAAGAAGGATCGCACACATGG-3’ 5’-AGACTGTCGCGCTGTACTATACCC-3’	56	117–145	unknown
umc2003	10.04	5’-CTCATCGGTTAGCAGCAGCAG-3’ 5’-GTTCTTAATCGGCACTCCTCGTC-3’	58	71–91	unknown
umc2176	4.03	5’-ATAGATCTTTGTCGCGTGTTCTGC-3’ 5’-CTCAAGAACACCACCAGACGAGTT-3’	58	130–154	(TGC)4

Biosystems, USA). The PCR products were separated by capillary electrophoresis on ABI Prism 3130 and their sizes were determined and visualised with software *Gene Mapper*, version 4.0 (Applied Biosystems, USA).

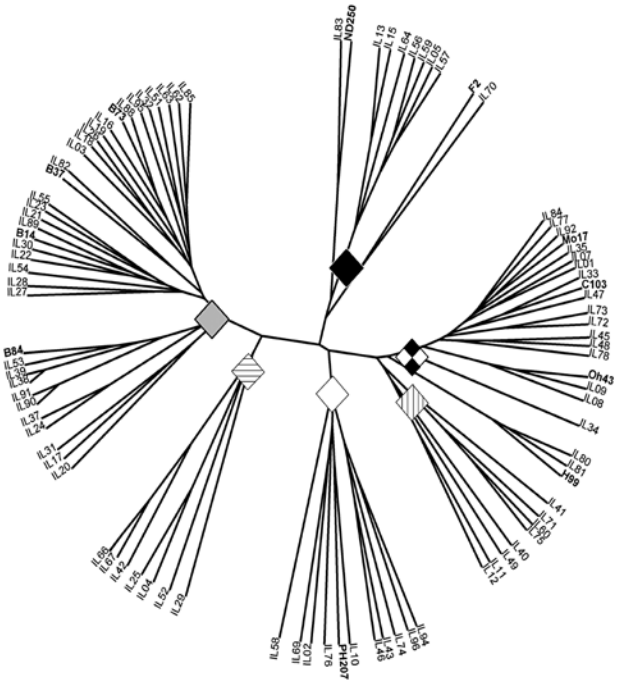
To classify maize inbred lines based on the data of SSR markers, a cluster analysis was performed with unweighted pair-group method using arithmetic average algorithm (UPGMA) to reconstruct the phylogeny from a Roger's frequency-based distance in the software *PowerMarker 3.25* (North Carolina State University, USA) and visualised in the software *Dendroscope 3* (Huson et al., 2007). The molecular data was analysed in *GenAlEx 6.5* (Peakall, Smouse, 2012) and presented with the following molecular diversity parameters: average number of different alleles, average number of different alleles excluding rare variant with minor allele frequency less than 5%, average number of effective alleles ($1 / (\Sigma p_i^2)$), Shannon's information index ($-1 \times \Sigma(p_i \times \ln(p_i))$), average number of alleles specific to each group, number of common alleles, observed heterozygosity and expected heterozygosity ($1 - \Sigma p_i^2$), where p_i is the frequency of the i^{th} allele and Σp_i^2 is the sum of the squared allele frequencies (Peakall, Smouse, 2012). The analysis of molecular variance (*AMOVA*) was applied to partition genetic variation among groups based on the codominant allelic distance matrix and fixation index (*Fst*) was calculated to measure the genetic differentiation among groups, as a proportion of the estimated variance among populations in the total variance. The principal coordinate analysis (PCoA) was used to visualise the patterns of genetic relationship among inbred lines via covariance matrix with data standardization in a trial version of software *XLSTAT 2016.1* (Addinsoft, USA).

The phenotypic evaluation of inbred lines was performed for eight traits, namely, number of days to pollination, plant height (cm), ear diameter (cm), ear length (cm), number of rows per ear, number of kernels per row, number of leaves per plant and grain yield per plant (g). The trial was conducted during 2011 and 2012 in a randomized complete block design with three replicates in two locations, Rimski šančevi (45°20′ N, 19°51′ E, 84 m a.s.l.) and Srbobran (19°09′ E, 45°46′ N, 88 m a.s.l.). This area has a continental semi-arid climate and *Chernozem* soil type. The average precipitation in 2011 and 2012 was 488 mm and 388 mm, respectively, while the average daily temperatures were 12°C and 12.8°C, for 2011 and 2012, respectively. More meteorological and climatic data for the tested locations is available on the official site of the Hydrometeorological Service of the Republic of Serbia (http://www.hidmet.sr.gov.rs). Standard cultivation technology adapted to local agro-ecological conditions was applied. The application of the mineral fertilisers was based on the soil chemical analyses and the timing and method of their application were the same for the tested sites. The plot size for each genotype was 6 m^2 and consisted of two rows, each 4 m long. The distance between rows was 0.75 and 0.22 m within rows, with a density of 60,600 plants ha^{-1}.

An analysis of variance (*ANOVA*) for phenotypic traits and Tukey's multiple comparisons of means test for 95% and 99% confidence levels were performed to test differences between mean values. A multivariate data analysis method, principal component analysis (PCA), was used in a trial version *XLSTAT 2016* (Addinsoft, USA) to visualise a pattern of relationship between inbred lines based on the phenotypic data.

Results and discussion

The cluster analysis performed with UPGMA algorithm based of Roger's distance from 40 microsatellites, distinguished inbred lines according to their affiliation to heterotic groups (Fig. 1). Out of the total 96 analysed inbred lines, 36 lines grouped in the BSSS cluster, including referent lines B14, B37, B73 and B84, developed in different cycles of recurrent selection from the BSSS population. The inbred lines B14 and B37 had the largest direct ancestral contributions to the BSSS heterotic group in the first pre-1950 era of maize breeding in North America, while B73 was the main contributor to this heterotic group in the second pre-1980 era (van Heerwaarden et al., 2012). Distinctive sub-clusters were formed within the BSSS cluster with each referent line as a representative of the sub-cluster. A separate cluster from the BSSS group, but close to it, was also differentiated. It was formed by inbred lines with predominant BSSS background mixed in different proportions with other germplasm, such as local, exotic and other genetic material of unknown origin. This cluster of mixed BSSS genetic constitution was denoted as mBSSS and



Note. Grey square presents maize inbred lines that belong to the Iowa Stiff Stalk Synthetic (BSSS) pool, horizontal lines square denotes BSSS inbred lines mixed with lines of other origins, black square marks inbred lines from various independent sources, white square represents Iodent (IDT) lines, vertical lines square denotes Lancaster Sure Crop (LSC) inbred lines mixed with lines from other groups and diamond square shows LSC inbred lines.

Figure 1. Radial dendrogram of 96 maize inbred lines based of Roger's allele frequency distance of 40 simple sequence repeat (SSR) markers

represented in Figure 1 with a horizontal line square. Twenty three inbreds were assembled in the LSC pool, encompassing C103, Mo17, H99 and Oh43. Two related inbred lines, C103 and Mo17, were grouped in the same sub-cluster, as expected, since Mo17 was developed from the CI187.2 × C103 cross. The inbred Oh43 also formed a separate sub-cluster. This inbred has Richey Lancaster, Minnesota 13 and Northwestern Dent in its pedigree. The line H99, developed from Illinois Synthetic 60C with Richey Lancaster background, was distant from the other referent lines in this group. Similarly to mBSSS, a group of inbreds of LSC origin mixed with germplasm from local maize populations, IDT and other various independent genetic material, was separated from the LSC group and was denoted as mLSC. Eleven inbred lines of IDH origin grouped together in a single cluster, with PH207 as a representative. The inbreds Mo17 and PH207 were the main representatives of the second historical era of maize breeding with the highest contributions to the non-BSSS (or traditionally designated Lancaster) and Iodent genepools, respectively (var Heerwaarden et al., 2012). Eleven lines with independent heterotic response formed a cluster encompassing adapted exotic inbred lines of Argentinean origin, the lines containing local Serbian germplasm, European flints and other independent sources, with F2 and ND250 as references. The remaining inbred lines with a considerable portion of referent lines' genetic background in their pedigrees and some inbreds with undisclosed pedigrees were grouped by genetic similarity into the corresponding sub-clusters. Similar patterns of clustering, represented by the main referent historical inbred lines, were also identified in other studies. Wu et al. (2015) revealed clustering patterns of 1857 maize accessions from around the world represented by Mo17, B73, 207, Oh43 and A634 (containing 87.5% of B14 germplasm), several Chinese lines, Reid Yellow Dent, tropical and subtropical germplasm. The B73, Mo17, Oh43, PH207 and A321 maize subpopulations were identified with SSR and SNP markers by Schaefer and Bernardo (2013). Pedigree information and shared allele frequencies of the inbred lines developed at the Institute of Field and Vegetable Crops, Serbia and the referent historical lines, such as B73, Mo17, B14 and PH207, showed a considerable contribution of these ancestor-inbreds to in the lineage of the modern inbred lines and indicated the importance in maize breeding in the southeast Europe.

An analysis of molecular variance showed that genetic variation was much higher within the groups (93%), while variance among six groups was around 7% (Table 2). The genetic differentiation among the groups was significant, and pair-wise fixation index ranged from 0.024 between the LSC and the IDT subpopulations to 0.191 between the mBSSS and the IDT subpopulations. The average *Fst* value was 0.069, larger than in breeding programs of the Corn Belt (Romay et al., 2013), between tropical and temperate lines (Liu et al., 2015) and between elite Chinese lines and public US lines (Jiao et al., 2012). However, obtained *Fst* value in this study was smaller compared to *Fst* = 0.165 in a diverse panel of 284 maize inbreds (Schaefer, Bernardo, 2013).

Table 2. Analysis of molecular variance among and between six groups of maize inbred lines

Source	Degrees of freedom	Sum of squares	Mean square	Estimated variance	Estimated variance %	*Fst* value	*P* value
Among groups	5	186.839	37.368	0.874	6.9	0.069	0.001
Within groups	186	2186.843	11.757	11.757	93.1		
Total	191	2373.682		12.631	100.0		

Comparison of different parameters of genetic diversity among six maize groups, showed that the largest average number of alleles (6.78), number of rare alleles (4.58), effective number of alleles (3.77), Shannon's information index (1.45) and number of private alleles per locus (1.53) were detected in the BSSS group, followed by the LSC and then the other groups (Table 3). Moreover, the BSSS group had the fewest alleles shared with other groups (0.42); whereas LSC inbred lines had the largest average number of common alleles (1.33). Expected heterozygosity for all the analysed inbred lines was 0.68, with the smallest values observed in the mLSC and the largest in the LSC group. The number of alleles and expected heterozygosity depended on the type and number of markers and the diversity of germplasm used. Microsatellites, comparing to other marker systems, excel at high discrimination power due to multiple alleles (Olmos et al., 2014). The genetic diversity assessed by SSRs in this study was similar or larger compared to other maize studies with the same marker type. Jones et al. (2007) found on average 5.1 alleles and expected heterozygosity of 0.62 among 58 inbred lines characterised by 80 SSRs. Much lower number of alleles

Table 3. Parameters of genetic diversity in maize heterotic groups obtained with microsatellites

Allelic parameter/group	ITD	IND	LSC	mLSC	BSSS	mBSSS	Total
Average number of alleles	3.81	3.47	4.89	3.25	6.78	3.11	8.25
Average number of alleles with ≥5%	3.47	3.39	3.80	3.05	4.58	3.11	5.93
Number of effective alleles	2.64	2.63	3.20	2.60	3.77	2.33	3.57
Shannon's Information Index	1.07	1.03	1.24	1.00	1.45	0.86	1.45
Average number of private alleles	0.08	0.11	0.56	0.27	1.53	0.22	–
Average number of common alleles	0.83	0.73	1.33	0.53	0.43	0.55	–
Expected heterozygosity	0.64	0.58	0.69	0.48	0.59	0.57	0.68

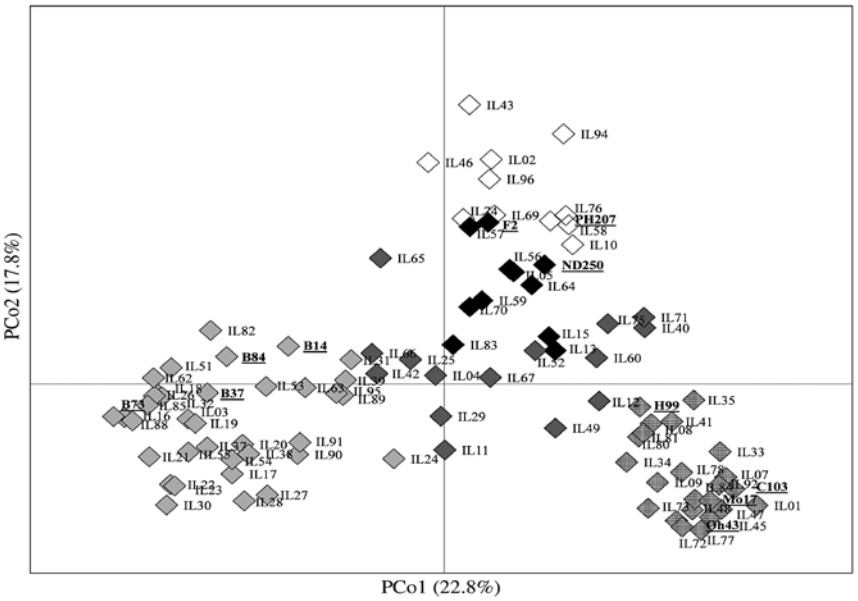
IDT – Iodent, IND – independent, LSC – Lancaster Sure Crop, mLSC – mixed Lancaster Sure Crop, BSSS – Iowa Stiff Stalk Synthetic, mBSSS – mixed Iowa Stiff Stalk Synthetic

per locus and expected heterozygosity of the LSC, the BSSS and other groups were observed in the study of Zheng et al. (2008), while the values of these genetic parameters in the study of Yang et al. (2011) were similar to our findings.

The microsatellite markers *umc1035*, *bnlg666*, *dupssr23*, *umc1083* and *dupssr10* most efficiently differentiated between the heterotic groups, as they had the largest number of private alleles (6 to 11). Additionally, these five markers had different alleles present in at least 4 heterotic groups, demonstrating their high discriminatory power. Within each group, the percent of private alleles was the highest for the BSSS (22.6%) and LSC (11.5%) pools. The highest values of genetic diversity parameter in BSSS and LSC are not surprising, since these are two most important heterotic groups and the genetic distance between them increased gradually during selection for yield (Cooper et al., 2009). The results also indicate the importance of the most diverse BSSS group in common BSSS-Lancaster and BSSS-Iodent heterotic patterns in breeding programmes, as well as the positive effects of previous introduction of new germplasm and reselection in existing inbred lines collections. The lower percentage of group-specific alleles in the other groups could be due to the smaller number of contributing inbred lines. Different number of lines in the groups reflects the actual participation of each group in a real breeding programme. The similar portions of private alleles in different groups and subgroups were found in the research of Yang et al. (2010). The microsatellites revealed considerable genetic diversity of investigated inbred lines, which may be used in diversity-based QTL analyses (Holland, 2007), as a source of mining of beneficial alleles for introgressing traits of interest or heterotic group partitioning of allelic combinations for planning crosses to exploit hybrid vigour (Guo et al., 2014; Olmos et al., 2014).

A principal coordinate analysis (PCoA) was performed to visualise relative genetic relationship between maize inbred lines and to determine if there was agreement in grouping inbreds using PCoA and UPGMA clustering method. The percentage of the total variation explained by the first two coordinates was 22.8% and 17.8%, respectively (Fig. 2). The first coordinate separated the inbred lines that belonged to the BSSS heterotic group from the rest, while the second coordinate differentiated the LSC lines. The IDT inbreds were clearly distinguished from the BSSS and LSC groups. The IND lines clustered together coherently to some extent in the centre of the biplot, with certain overlaps with the IDT, mBSSS and mLSC lines. This is understandable as the most of the inbreds from this cluster are not related and have quite different genetic background, which was reflected in their positioning on the plot towards the adjacent groups. Likewise, some mBSSS and mLSC inbred lines inclined towards BSSS, LSC or the independent cluster, since a portion of these clusters play a certain role in their genetic makeup. Our results were in accordance with PCoA of 266 elite Texas lines based on SNP markers by Smith et al. (2015). The first principal coordinate in their study separated the BSSS from the rest, while the second coordinate differentiated other non-Stiff Stalks, Texas cluster, tropical lines and Iodent group.

The PCoA results showed a good agreement with the UPGMA analysis. Although UPGMA provided clearer perspective in distinguishing clusters and sub-clusters of inbred lines according to their pedigrees, PCoA allowed more insight into relationship between the lines, especially those of mixed origin. It is noteworthy to point out that mBSSS and mLSC groups, although denoted as separate clusters from BSSS and LSC, respectively, do not represent distinct heterotic groups. Moreover, they belong to the BSSS and LSC heterotic groups, as the largest part of their genetic background is constituted of those two heterotic pools. Their separation into distinct clusters served to analyse the effects of introduction of various germplasm into two most popular and widely used



Note. Vertical lines – LSC inbred lines mixed with lines from other groups, horizontal lines – BSSS inbred lines mixed with those of other origins; grey – maize inbred lines belonging to BSSS pool, black – inbred lines from various independent sources, white – IDT lines, diamond – LSC inbred lines.

Figure 2. Principal coordinate analysis (PCoA) of 96 maize inbred lines based on microsatellite data

heterotic groups in breeding programmes. Thus, our results demonstrated the efficiency of SSR markers to accurately classify inbreds in heterotic groups and subgroups and also support the argument that the differences among heterotic groups are more the outcome of modern breeding than the result of differentiation of genetically diverse founders (van Heerwaarden et al., 2012).

The analysis of variance of different phenotypic traits (days to pollination, plant height, number of leaves, ear length, ear diameter, number of rows per ear, number of kernels per ear row and grain yield) of the six groups of maize inbred lines is showed in Table 4. Almost all traits significantly varied among the groups and between the trial years. There were no significant differences in yield mean values between the maize groups. This is not surprising as the inbred lines included in the study have undergone the selection process for good performances, especially high yield *per se* for hybrid seed production. All the traits, except leaf number, significantly differed in two years of the experiment, while the location significantly affected variation of only two traits, namely, days to pollination and number of kernels per ear row. Significantly lower values of traits in 2012, as compared to 2011, can be attributed to severe drought period during the flowering, fertilisation and grain filling stages. Most of the interactions between maize groups, years and locations were not significant, save those between years and locations for flowering time, leaf number and number of kernels per row. The inbred lines from the IDT and IND groups had shorter flowering time. The BSSS inbreds had significantly larger number of leaves, shorter ears, larger number of rows per ear, larger ear diameter

Table 4. Analysis of variance and comparison of means for eight phenotypic traits of six groups of maize inbred lines

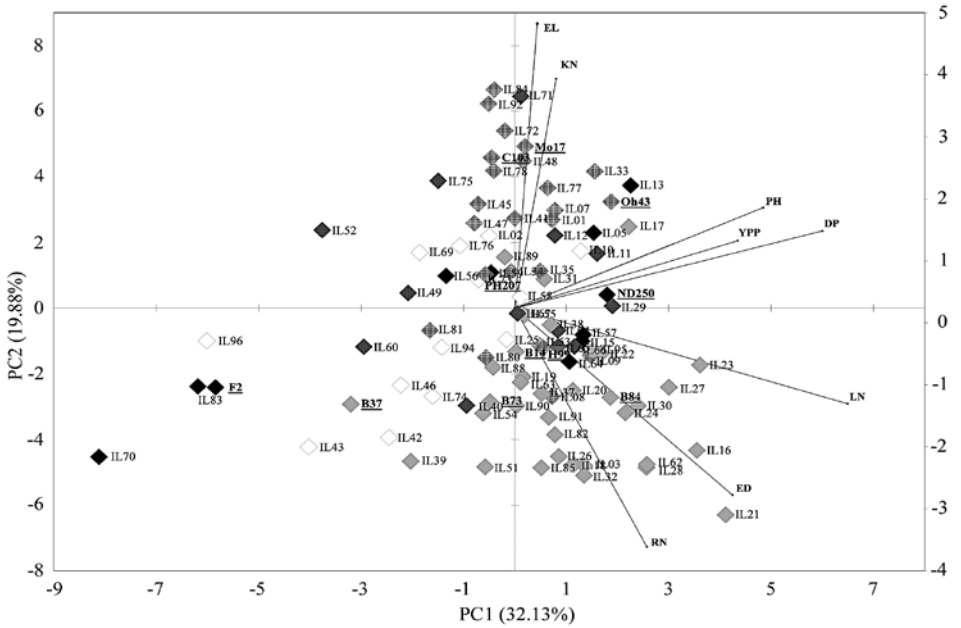
Group	Days to pollination	Plant height cm	Leaf number	Ear length cm	Ear diameter cm	Number of rows per ear	Number of kernels per ear row	Yield per plant g
IDT	77.4 a	168.6 a	11.7 a	14.8 abc	3.8 a	13.7 a	23.5 a	96.9 a
IND	78.7 a	174.8 ab	11.8 a	14.4 ab	3.7 a	13.9 a	24.2 ab	96.7 a
LSC	82.1 b	183.1 b	12.4 b	15.3 bc	3.7 a	12.9 b	25.9 bc	96.8 a
mLSC	82.4 b	168.2 a	12.0 ab	15.8 c	3.7 a	13.9 a	27.4 c	104.1 a
BSSS	81.5 b	173.0 a	13.2 c	13.5 d	4.1 b	15.5 c	22.7 a	99.3 a
mBSSS	81.4 b	171.4 a	12.4 b	13.9 ad	3.8 a	14.4 a	23.6 ab	103.2 a
Average	80.6	173.2	12.3	14.6	3.8	14.1	24.5	99.5
2011	77.8 a	188.2 a	12.3 a	15.4 a	3.9 a	14.4 a	27.0 a	117.6 a
2012	83.5 b	158.2 b	12.2 a	13.8 b	3.6 b	13.8 b	22.1 b	81.3 b
Rimski šančevi	83.2 a	172.0 a	12.2 a	14.5 a	3.8 a	14.2 a	23.9 a	97.8 a
Srbobran	78.0 b	174.4 a	12.3 a	14.7 a	3.8 a	14.1 a	25.2 b	101.1 a
Group (G)	**	**	**	**	**	**	**	ns
Year (Y)	**	**	ns	**	**	**	**	**
Location (L)	**	ns	ns	ns	ns	ns	*	ns
G × L	ns	ns	ns	ns	ns	ns	ns	ns
G × Y	ns	ns	ns	ns	ns	ns	ns	ns
Y × L	**	ns	*	ns	ns	ns	*	ns
G × Y × L	ns	ns	ns	ns	ns	ns	ns	ns

Note. IDT – Iodent, IND – independent, LSC – Lancaster Sure Crop, mLSC – mixed Lancaster Sure Crop, BSSS – Iowa Stiff Stalk Synthetic, mBSSS – mixed Iowa Stiff Stalk Synthetic; means in the same column with different superscript letters are significantly different; a-d – $P < 0.05$; ** – significance at 0.01 probability level, * – significance at 0.05 probability level; ns – non significant.

and smaller number of kernels per row compared to the LSC, mLSC and most of the other groups (Table 4). The results show that all the groups had significant genetic variability and are suitable for further genetic analyses.

The relationship between inbred lines based on the phenotypic data was depicted on the biplot of PCA (Fig. 3). The first PC interpreted 32.1% of total variation, whereas the second PC accounted for 19.9%, explaining together 52.0% of the total variation. Days to pollination, plant height and leaf number contributed most to the PC1, while ear length, number of rows per ear and number of kernels per row contributed most to the PC2. The PCA biplot showed positive correlations between yield, plant height and earliness (days to pollination), which form acute angles among each other. Similarly, positive correlations were found between ear length and number of kernels per ear row and between number of rows per ear and ear diameter. These two pairs of variables were negatively correlated, as the angles between their vectors were obtuse.

Most of the LSC inbred lines clustered on the upper part of the biplot, that is, with positive values of PC2, around the vectors for ear length and number of kernels per ear row (Fig. 3). Contrarily, the majority of BSSS lines were positioned on the lower part of the biplot, around vectors for number of rows, ear diameter and number of leaves. This depicted the main ear architecture characteristics of the two maize groups, in general, and was in keeping with the results obtained from *ANOVA* and means comparison tests. It also demonstrated the efficiency of PCA in differentiation between the BSSS and LSC pools, despite some overlap. However, PCA failed to distinctly separate the other four groups, indicating that the phenotypic traits chosen for



Note. Vertical lines – LSC inbred lines mixed with lines from other groups, horizontal lines – BSSS inbred lines mixed with those of other origins; grey – maize inbred lines belonging to BSSS pool, black – inbred lines from various independent sources, white – IDT lines; diamond – LSC inbred lines; EL – ear length, KN – number of kernels per ear row, PH – plant height, YPP – yield per plant, DP – days to pollination, LN – leaf number, ED – ear diameter, RN – number of rows per ear.

Figure 3. Principal component analysis of 96 maize inbred lines based of phenotypic data

the multivariate analysis were not discriminatory enough. Similar results were obtained by Babić et al. (2008), who used 30 traits from International Union for the Protection of New Varieties of Plants (UPOV) descriptor to classify 45 inbred lines with cluster and discriminant analyses. The authors clearly distinguished two clusters of inbreds, the one with complete or partial BSSS background and the other containing completely or partially LSC line, but the IDT and early French lines were includes in both clusters and did not group according to their heterotic group or origin.

In our study, the IDT and particularly IND inbreds were spread out along the vectors for plant height, yield and days to pollination, displaying large variation for these traits. The reason for such variation can be explained by heterogeneity of the IND group consisting of inbreds from rather different origin and by wider genetic bases of the IDT pool. Besides, the main selection criteria during inbred lines development, namely good combining abilities and the overall fitness, the driving force for creating divergent heterotic groups, were much better reflected on a molecular level. Molecular markers were more efficient in differentiating inbred lines according to their genetic backgrounds (Figs. 1 and 2) than the analysis based on phenotypic data. Babić et al. (2012) estimated values of correlations of morphological and molecular similarities for different maize inbred lines to range from 0.47 to 0.75. The supremacy of PCoA and methods based on molecular markers in elucidating genetic relationship among maize inbred lines and reconstructing pedigrees were also reported in previous studies (Nelson et al., 2008; Van Inghelandt et al., 2010; Lorenz, Hoegemeyer, 2013). The information obtained from molecular analyses may facilitate breeders to better characterise and classify genetic resources in heterotic groups and exploit heterotic patterns for superior hybrid development.

Conclusions

1. Microsatellite-based cluster analysis and principal coordinate analysis (PCoA) assigned 96 inbred lines to six clusters, namely, Iowa Stiff Stalk Synthetic (BSSS), Lancaster Sure Crop (LSC), Iodent (IDT) heterotic group, a cluster with unrelated independent inbreds and two clusters of miscellaneous germplasm crossed with inbreds of BSSS and Lancaster origin.

2. The microsatellites revealed considerable level of genetic diversity of the investigated groups of maize inbred lines, reflecting the importance and prevalence of selection and improvement of the BSSS and Lancaster pools during maize breeding.

3. The investigated inbred lines demonstrated sufficient variation in most of the analysed phenotypic traits, which enables their further use for various genetic studies, such as association mapping for traits of interest.

4. Marker-based methods were more efficient in assigning inbred lines to their corresponding heterotic groups and elucidating their genetic relationships than the analysis based on phenotypic data. The molecular characterisation and classification of genetic resources may assist hybrid breeding by efficient exploitation of heterotic patterns.

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Paprastojo kukurūzo inbredinių linijų molekulinis ir fenotipinis įvertinimas Pietryčių Europoje

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Santrauka

Paprastajam kukurūzui (*Zea mays* L.) būdinga genetinė įvairovė, istoriškai susiformavusi nuo jo introdukavimo iš kilmės centro Meksikoje į kitas pasaulio vietas, ir prisitaikymas prie įvairių auginimo sąlygų. Nepaisant šios įvairovės, vykdant paprastojo kukurūzo selekciją ir kuriant hibridus panaudojama tik maža dalis turimos genetinės medžiagos. Siekiant selekcinių kolekcijų įvairovę praplėsti naujais genotipais kaip tinkamų požymių šaltiniu, reikia ją apibūdinti ir suklasifikuoti į heterozines grupes.

Tyrimo tikslas – įvertinti Serbijos lauko augalų ir daržovių instituto turimos paprastojo kukurūzo selekcinės medžiagos genetinę įvairovę, taip pat ir anksčiau neapibūdintas inbredines linijas, žinomos kilmės elitines linijas ir istoriškai svarbias inbredines linijas. Mikrosatelitų metodu atlikus klasterinę ir principinių koordinačių analizes 96 inbredinės linijos buvo suskirstytos į šešias grupes: *Iowa Stiff Stalk Synthetic* (BSSS), *Lancaster Sure Crop* (LSC), *Iodent* (IDT) heterozinė grupė, negiminingų inbredų grupė ir dvi grupės genetinės medžiagos, sukryžmintos su BSSS ir *Lancaster* inbredais. Į grupes skirstyta pagal mikrosatelitus *umc1035*, *bnlg666*, *dupssr23*, *umc1083* ir *dupssr10*. Molekulinės įvairovės didžiausios parametrų vertės nustatytos BSSS grupėje, po to *Lancaster* ir kitose grupėse. Dispersinė analizė parodė, kad beveik visi požymiai reikšmingai varijavo tarp grupių ir metų. Tirtosios linijos pasižymėjo pakankama daugelio analizuotų fenotipinių požymių variacija ir pasirodė tinkamos tolesniems genetiniams tyrimams. Principinė komponentinė analizė pagal agronominius požymius inbredines linijas atskyrė nuo BSSS ir *Lancaster* genetinių grupių, tačiau taikant šią analizę nepavyko identifikuoti kitų grupių. Genetinių išteklių apibūdinimas ir klasifikavimas pagal mikrosatelitinius žymeklius gali padėti kuriant hibridus ir veiksmingai panaudojant heterozinius modelius.

Reikšminiai žodžiai: heterozinės grupės, inbredai, mikrosatelitai, paprastos pasikartojančios sekos, *Zea mays*.