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Original scientific paper

MOLECULAR CHARACTERIZATION OF BARLEY (*Hordeum vulgare* L.) ACCESSIONS OF THE SERBIAN GENEBANK BY SSR FINGERPRINTING

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Molecular diversity of 145 barley (*Hordeum vulgare* subsp. *vulgare* L.) accessions from the Serbian GenBank was assessed by single sequence repeats (SSR) markers. A set of 15 SSRs, covering all chromosomes of the diploid barley genome with 2-3 SSR markers per chromosome, with a range of 4-18 alleles per locus were used. In total, 15 loci and 119 alleles were detected, with an average of 7.93 alleles per locus. The Polymorphic information content value ranged from 0.220 to 0.782 with a mean value of 0.534. Regarding the growth habit and row type groups, gene diversity was comparatively higher for the spring (0.616) and six-rowed accessions (0.616) than for the winter and two-rowed accessions (0.322 and 0.478, respectively). Analysis of molecular variance showed that all sources of variation were significant ($P < 0.01$), but the between-

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group component was predominant (76.85%) for growth habit and 89.45% for row type. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis based on the shared allele distance (D_{SA}) matrix estimated on the SSR data assigned the genotypes into two clusters - the first smaller consisting of the six 6-rowed spring cultivars and the second comprising six subclusters. Genotype MBR1012 was separated from all other genotypes that constitute UPGMA tree. The associations of genotypes belonging to different growth habit and row type groups were assessed using Principal Coordinate Analysis revealing separation of winter growth habit group from facultative one. The use of the STRUCTURE clustering algorithm allowed the identification of 2 subpopulations of genotypes.

Key words: barley diversity, Serbian Gene Bank, SSRs, population structure

INTRODUCTION

Barley is one of the main grain crops, and is used for animal feed, brewing, and human consumption (HAYES *et al.*, 2003). According to the World Information and Early Warning System of the FAO (WIEWS), as of May 2011, there were around 470 000 *Hordeum* spp. accessions in the world, of which around 149 000 are stored in Europe held in 34 countries (KNÜPFFER *et al.*, 2011). Knowledge regarding the amount of genetic variation in germplasm arrays and genetic relationships between genotypes are important considerations for efficient conservation and utilization of germplasm resources in the changing climate (MATUS and HAYES 2002, DODIG *et al.,* 2008; DODIG *et al.*, 2012). Crop diversity has important implications for the genetic vulnerability and the potential for crop improvement. Recent developments in plant molecular biology and emerging molecular markers have allowed for the detection of diversity and differences among and within cultivars at the DNA level (AHSYEE *et al.,* 2013, PAVLOVIĆ *et al.,* 2012)

Microsatellite markers, also known as simple sequence repeats (SSR), have proven to be one of the most suitable type of molecular markers for estimating genetic diversity in barley, genome mapping and marker assisted breeding, due to multi-allelic nature, chromosome specificity, high polymorphism rates, random distribution throughout the genome, cost-effectiveness and good reproducibility (VARSHNEY *et al.*, 2010). Thirty-six SSR were used to profile 96 wheat genotypes from the collection of genetic resources at the Institute of Field and Vegetable Crops, Novi Sad, Serbia (DODIG *et al.*, 2010). New trends in germplasm management include development of core subsets from the larger collection and using these collections for association mapping. Core genotype collections are being established as tools for germplasm study and resources for breeders when sources of new alleles are needed. The objectives of the study were to: (i) screen germplasm of 145 barley genotypes with a set of 15 EST SSRs equally distributed along the seven chromosomes; (ii) study the genetic diversity among the barley germplasm at molecular levels.

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MATERIALS AND METHODS

One hundred forty and five barley (*Hordeum vulgare* subsp. *vulgare* L.) accessions from the collection of genetic resources of the Serbian GeneBank, Belgrade, Serbia and breeding

lines from five institutes/companies were used in this study (Table 1). Accessions named with MBR don't have complete passport information and country of origin.

Genomic DNA was extracted from leaves of 14-day-old plants according to STEIN *et al*. (2001). The concentration and quality of DNA was determined using the NanoDrop ND-100 spectrophotometer (PeQLab, Erlangen, Germany) and gel electrophoresis. All samples were adjusted to a final concentration of 20 ng/µl. PCR was performed in a volume of 10 µl, containing 1µl of 10x buffer, 1 µl of 25 mM $MgCl_2$, 0.2 µl of each 10 mM dNTPs, forward primer (1.0 pmol/µl) and reverse primer (10.0 pmol/µl), 0.08 µl 5U Hot FIREPol®DNA polymerase (Solis BioDyne, Tartu, Estonia), 6.12 µl HPLC gradient grade water (Carl Roth, Karlsruhe, Germany) and 1µl template DNA. For SSR amplification, M13 tailed forward primers were used according to (PEROVIC *et al*., 2013), so that 0.1 µl of 'M13' primer (10.0 pmol/µl) labeled with 5' fluorescent dyes was added to the reaction mix. DNA amplification was performed in a Gene Amp® PCR System 9700 (Applied Biosystems, Darmstadt, Germany). The following PCR conditions were used for all primers: 94°C for 5 min followed by a touchdown PCR with 12 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C; and then 35 cycles with 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and a final extension at 72°C for 10 min.

Detection of allele size for the SSR marker was conducted using a capillary electrophoresis ABI PRISM® 3100 genetic analyzer (Applied Biosystems, Darmstadt, Germany). The output from the sequencer was analyzed with Genescan software (PE Applied Biosystems, Foster City, CA, USA) to measure the molecular size of each SSR allele. A set of 15 EST SSR markers, with 2-3 SSR markers per chromosome, with a range of 4-18 alleles per locus were used to characterize the genetic diversity of 145 barley accessions (Table 2).

Microsatellite diversity statistics was estimated using genetic data analysis (GDA) (LEWIS and ZAYKIN, 2001) and PopGene software. In addition, unbiased estimates of gene diversity-allelic richness were determined using FSTAT (GOUDET, 2002) software. Microsat software was used to produce a distance matrix (D_{SA}) among pairs of barley accessions based on the proportion of shared alleles. The D_{SA} matrix was submitted to analysis of molecular variance (AMOVA) using Arlequin software (SCHNEIDER *et al*., 2000). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was constructed using MEGA software (TAMURA *et al*., 2007), based on the shared allele distance (D_{SA}) matrix among barley accessions. The substructure of the accessions was analyzed using the Bayesian model based clustering approach provided in STRUCTURE software (PRITCHARD *et al*., 2000) with the length of burn-in period of 10,000 steps followed by 100,000 Monte Carlo Markov Chain replicates and a model allowing for admixture and correlated allele frequencies (FALUSH *et al*., 2003).

RESULTS

In total, 15 loci and 119 alleles were detected, with an average of 7.93 alleles per locus (Table 2). Frequencies of the major (most numerate) alleles of the 15 SSR loci ranged from 0.326 (GBM1029) to 0.875 (GBM1043) with an average of 0.527 (Table 2). The polymorphism information content (PIC) ranged from 0.220 (GBM1043) to 0.782 (GBM1021) with a mean PIC of 0.534 (Table 2). Four microsatellites (GBM1029, GBM1031, GBM1021 and GBM1047), all with more than 8 alleles, had PIC higher than 0.70 indicating that wide genetic variability had been accumulated among these genotypes (Table 2).

Table 1a Name, country of origin, growth habit and row type of the 145 barley accessions used in the study derived from the Serbian Genbank

Genotype (origin[a])	Growth type	Row type	Genotype (origin[a])	Growth type	Row type
Jagodinac (SER-II)	Winter	2	J-29 (SER-I)	Winter	6
Maksa (SER-II)	Winter	2	J-16 (SER-I)	Winter	6
Rekord (SER-II)	Winter	2	J-21 (SER-I)	Winter	6
NS[b]587 (SER-I)	Winter	2	Nectaria (FRA)	Winter	2
NS293 (SER-I)	Winter	2	Boreale (FRA)	Winter	2
NS595 (SER-I)	Winter	2	Nektar (SER-IV)	Winter	2
NS519 (SER-I)	Winter	2	Vanessa (DEU)	Winter	2
NS565 (SER-I)	Winter	2	Kristal (SER-III)	Winter	2
NS183 (SER-I)	Winter	2	Bingo (CRO)	Winter	2
NS525 (SER-I)	Winter	2	34/II (SER-II)	Winter	6
NS589 (SER-I)	Winter	2	12/I (SER-II)	Winter	2
NS593 (SER-I)	Winter	2	154/II (SER-II)	Winter	6
J-110 (SER-I)	Winter	2	33/II-5 (SER-II)	Winter	6
J-90 (SER-I)	Winter	2	IBSP/04-22 (SER-II)	Winter	2
J-96 (SER-I)	Winter	2	PKB Pivan (SER-V)	Winter	2
J-82 (SER-I)	Winter	2	MBR500[c]	Spring	2
J-103 (SER-I)	Winter	2	MBR505	Spring	2
J-176 (SER-I)	Winter	2	MBR506	Spring	2
J-81 (SER-I)	Winter	2	MBR513	Spring	2
J-104 (SER-I)	Winter	2	MBR514	Facultative	2
Grand (SER-II)	Winter	6	MBR515	Spring	2
NS313 (SER-I)	Winter	6	MBR520	Spring	2
Ozren (SER-I)	Winter	6	MBR526	Spring	2
Somborac (SER-I)	Winter	6	MBR527	Spring	2
Sremac (SER-I)	Winter	6	MBR530	Spring	6
Atlas (SER-I)	Winter	6	MBR532	Spring	6
Leotar (SER-I)	Winter	6	MBR533	Spring	2
NS773 (SER-i)	Winter	6	MBR540	Facultative	6
Nonius (SER-I)	Winter	6	MBR541	Spring	2
NS737 (SER-I)	Winter	6	MBR548	Spring	2
Javor (SER-I)	Winter	6	MBR552	Spring	2
J-26 (SER-I)	Winter	6	MBR553	Spring	2
J-32 (SER-I)	Winter	6	MBR556	Spring	2
J-24 (SER-I)	Winter	6	MBR557	Spring	2
J-9 (SER-I)	Winter	6	MBR560	Spring	2
J-33 (SER-I)	Winter	6	MBR562	Spring	2
J-27 (SER-I)	Winter	6	MBR566	Spring	2

Table 1b Name, country of origin, growth habit and row type of the 145 barley accessions used in the study derived from the Serbian Genbank

Genotype (origin[a])	Growth type	Row type	Genotype(origin[a])	Growth type	Row type
MBR568	Spring	2	MBR1035	Facultative	6
MBR569	Spring	2	MBR1038	Spring	6
MBR574	Spring	2	MBR1039	Spring	6
MBR576	Spring	2	MBR1043	Spring	6
MBR580	Spring	2	MBR1046	Spring	6
MBR862	Spring	6	MBR1055	Spring	6
MBR901	Spring	6	MBR1056	Spring	6
MBR934	Spring	6	MBR1058	Spring	6
MBR941	Spring	6	MBR1061	Spring	6
MBR959	Spring	6	MBR1089	Spring	6
MBR970	Spring	6	MBR1092	Spring	6
MBR974	Spring	6	MBR1094	Spring	6
MBR980	Spring	6	MBR1104	Spring	6
MBR984	Spring	6	MBR1127	Spring	6
MBR991	Spring	6	MBR1129	Facultative	2
MBR992	Spring	6	MBR1140	Facultative	2
MBR995	Spring	6	MBR1163	Spring	2
MBR997	Spring	6	MBR1170	Spring	2
MBR998	Spring	6	MBR1183	Spring	2
MBR1001	Spring	6	MBR1189	Spring	2
MBR1003	Spring	6	MBR1190	Spring	2
MBR1011	Spring	6	MBR1197	Spring	2
MBR1012	Spring	6	MBR1205	facult.	2
MBR1014	Spring	6	MBR1209	Spring	6
MBR1015	Spring	6	MBR1210	Spring	6
MBR1016	Spring	6	MBR1211	Spring	2
MBR1017	Spring	6	MBR1213	Spring	6
MBR1023	Spring	6	MBR1215	Spring	6
MBR1024	Spring	6	MBR1216	Spring	6
MBR1025	Spring	6	MBR1218	Spring	6
MBR1026	Spring	6	MBR1248	Spring	6
MBR1027	Facultative	6	MBR1249	Spring	6
MBR1028	Spring	6	MBR1251	Spring	6
MBR1029	Spring	6	MBR1252	Spring	2
MBR1030	Spring	6	MBR1253	Spring	2
MBR1033	Spring	6			

[a]SER-I Institute of Field and Vegetable Crops Novi Sad, Serbia; SER-II Small Grains Research centre, Kragujevac; SERIII-Center for Agricultural and Technological Research; Zajecar; SER-IV Maize Research Institute, Zemun Polje, , SER-V Institute Science application in Agriculture, Belgrade; FRA-France; Deu Gemany;Cro Croatia; [b] Novosadski; [c]MBR number in gen bank of Yugoslavia/Serbia.

Table 2 Chromosome location, number of alleles, frequency of major alleles and polymorphism information content (PIC) for 15 EST SSR loci

Chromosome location	SSR marker	Allele frequency	Allele number	Gene diversity	PIC
1H	GBM1007	0.550	13	0.599	0.538
1 H	GBM1029	0.326	14	0.749	0.710
2 H	GBM1218	0.442	7	0.615	0.538
2 H	GBM1047	0.403	9	0.770	0.744
3 H	GBM1031	0.414	9	0.746	0.714
3 H	GBM1043	0.875	7	0.229	0.220
4 H	GBM1020	0.664	8	0.472	0.396
4 H	GBM1003	0.464	7	0.586	0.499
5 H	GBM1026	0.563	4	0.531	0.433
5 H	GBM1054	0.536	5	0.617	0.559
5 H	GBM1064	0.697	4	0.445	0.377
6 H	GBM1021	0.349	18	0.802	0.782
6 H	GBM1075	0.386	4	0.717	0.667
7 H	GBM1060	0.696	4	0.443	0.373
7 H	GBM1516	0.541	6	0.547	0.453
Mean		0.527	7.93	0.591	0.534

To characterize genetic diversity of barley germplasm related to the growth habit and morphological classification based on row type, the total number of alleles, mean alleles per locus, number of group specific alleles (hereafter referred to as unique alleles, i.e. alleles present in only one group and absent in the others) and gene diversity were calculated and expressed as mean (Table 3). Total number of alleles (106) and mean alleles per locus (7.07) were the highest for the spring barley group and were similar to those in the 6-rowed barley (95 and 6.33, respectively). Accessions from the facultative group had the lowest total number of alleles (44) and mean alleles per locus (2.93). The 2-rowed barley group had less total allele number (82) and mean alleles per locus (5.46) than the 6-rowed group. Regarding the growth habit and row type groups, gene diversity was comparatively higher for the spring (0.616) and six-rowed accessions (0.616) than for the winter and two-rowed accessions (0.322 and 0.478, respectively). The results of AMOVA for the growth habit and the ear type effects are shown in Table 4. All sources of variation were significant ($P < 0.01$), but the between-group component was predominant (76.85%) for growth habit classification and 89.45% for row type classification.

The dendrogram derived from SSR data using distance-based UPGMA cluster analysis is shown in Fig 1. Genotype MBR1012 was separated from all other genotypes that constitute UPGMA tree. The UPGMA cluster analysis revealed two main clusters, one smaller consisting of 6 genotypes (I) and the second (II) divided in (II_a) and (II_b) subclusters. The subcluster II_b included four distinct cluster groups: III_a (11 genotypes); III_b (30 genotypes); III_c (5 genotypes) and III_d. Cluster III_d consisted of two subclasters: IV_a (30 genotypes) and IV_b (52 genotypes).

Table 3 Genetic diversity statistics related to the growth habit and morphological classification

Group	N_g	N_a	N_{al}	N_{pa}	GD	PIC
	growth habit classification					
spring	86	106	7.07	55	0.616	0.559
winter	52	53	3.53	8	0.322	0.286
facultative	7	44	2.93	5	0.494	0.439
	morphological classification					
two-row	65	82	5.46	27	0.478	0.431
six-row	80	95	6.33	37	0.616	0.557

N_g - number of genotypes; N_a - number of alleles; N_{al} - number of alleles per loci; N_{pa} - number of private alleles, GD - gene diversity; PIC - polymorphic information content

Table 4 Analysis of molecular variance for the investigated barley germplasm related to the growth habit and morphological classification

Source of variation	df	Variance of component	%	φ_{st}	P
		growth habit classification			
Among groups	2	0.07706	23.15	0.2315	0.000
Between groups	142	0.25584	76.85	–	–
		morphological classification			
Among groups	1	0.03299	10.55	0.1055	0.000
Between groups	143	0.27964	89.45	–	-

**P < 0.01

The first two PCoA axes explained a higher amount of variation (26.9 and 17.1%, respectively) (Fig. 2). Based on the projection of accessions in the Cartesian two-dimensional plane (Fig 2a) the winter growth habit group is separated from the facultative one conspicuously and from most accessions belonging to the spring growth habit group. Grouping of the accessions belonging to the different row type groups was not obvious neither along PCoA1 nor PCoA2 axes (Fig 2b). The estimated population structure adopting K = 2 for the model-based cluster analysis is presented in Fig 3. Using this approach, 120 accessions were assigned to the corresponding A-B sub-populations, accounting for 38.89, and 44.44% of the germplasm investigated.

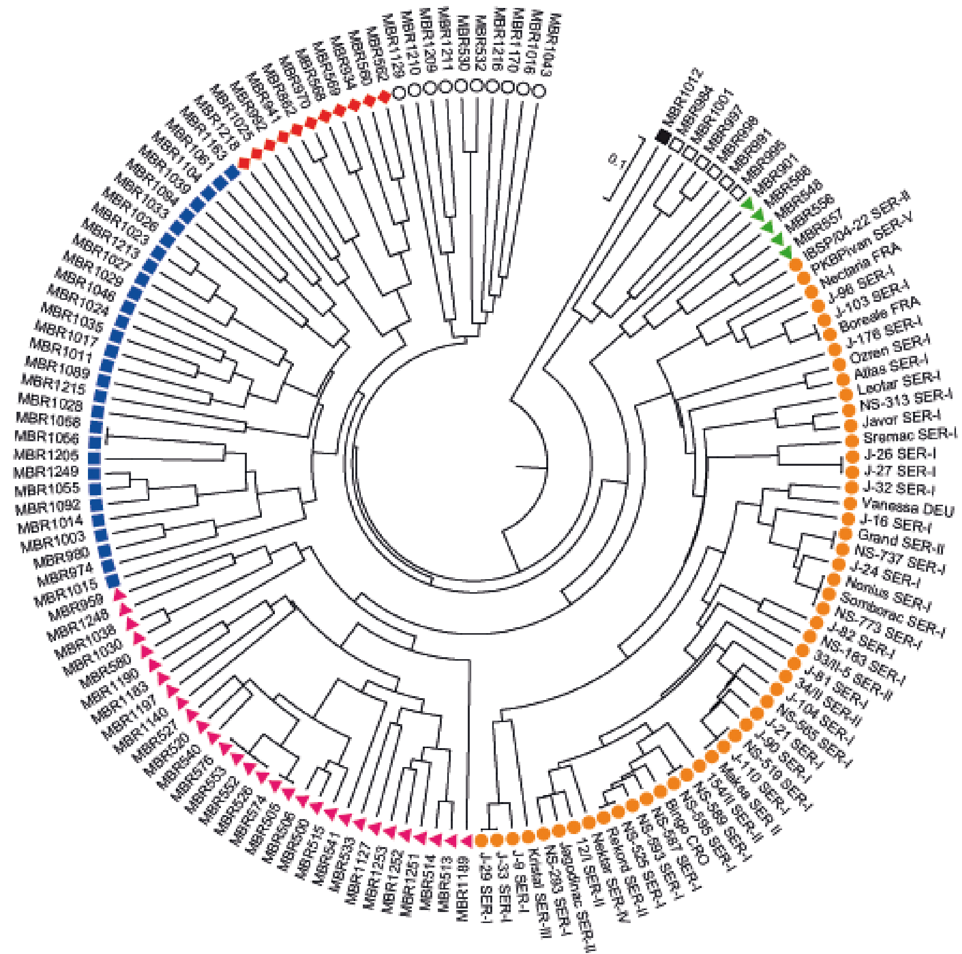

Fig 1 Unweighted pair group method with arithmetic mean cluster dendrogram of 145 barley accessions based on shared allele distance (D_{SA}) matrix

■-MBR 1012; □-I cluster; ○-II_a; ♦-III_a; ■-III_b; ▼-III_c; ▼-IV_a; ●-IV_b

For the countries codes see Table1

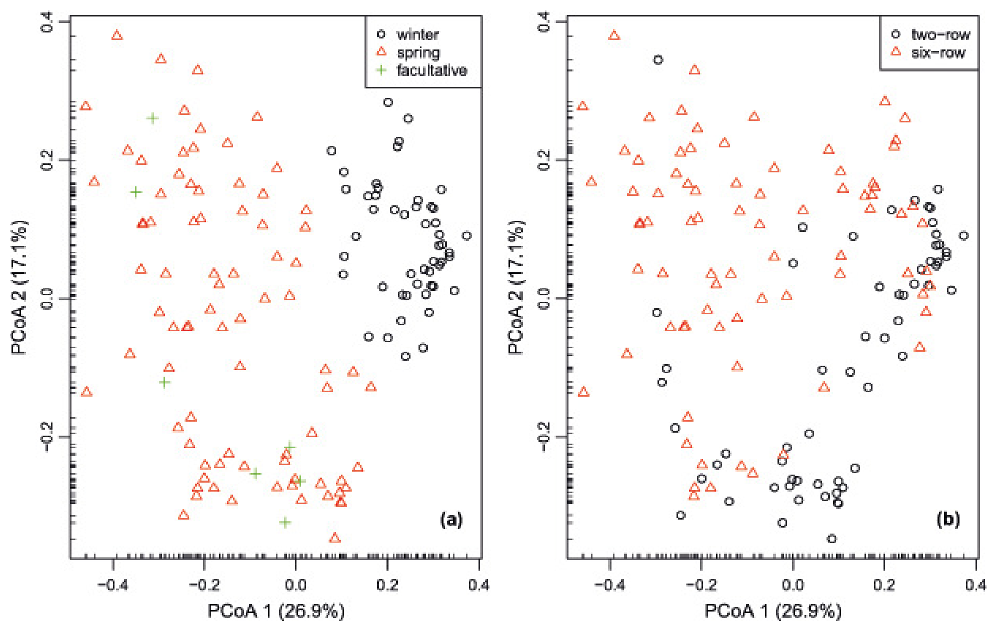

Fig 2 Plot of the first two principal coordinates (PCoA) based on shared allele distance (D_{SA}) matrix for 145 barley accessions with respect to: growth habit grouping of accessions (a), and morphological grouping of accessions based on row type (b)

Fig 3 Bar plot of estimated population structure membership of 145 barley accessions. Each accession is represented by a thin vertical segment which is partitioned into *K* colored segments that represent the individual estimated membership to the *K* cluster

DISCUSSION

To this date molecular assessment of diversity of barley genotypes from the Serbian GenBank has not been conducted. The average number of alleles per SSR indicated a greater magnitude of diversity among the barley accessions included in this study. Much lower values for average number of alleles per locus were obtained for the 15 and 16 SSR primers, screened over 26 and 69 barley accessions in the reported studies, respectively (HAMZA *et al*., 2004; JAISWAL *et al*., 2010). While in a worldwide set of 953 barley accessions, on average 16.7 alleles/marker were found, in the subset of 565 European accessions released over the century 11.3 alleles per locus were reported (MALYSHEVA-OTTO *et al*., 2007). In the six rowed accessions the average number of alleles per locus was greater than in the two rowed accessions what is in accordance with the study of ORDON *et al*., (2005). The number of unique alleles was the highest for the spring growth type. Therefore, these barley accessions can be considered as an important genetic reservoir for mainstream spring barley breeding in Serbia.

PIC value represented more than 50% of polymorphism, which confirms that SSR markers used in this study were highly informative and are generally extremely useful in distinguishing the polymorphic rate of a marker at a specific locus. PIC is also an estimate of the discriminatory power of an SSR marker locus (HAMZA *et al*., 2004). Our PIC values were higher than the previously reported PIC values by HAMZA *et al*., (2004) and PANDEY *et al*., (2006) and lower than those in the study of VARSNEY *et al*., (2010). Higher PIC values were obtained in the six-rowed group than in the two-rowed group in our study. The descending order of growth habit groups regarding PIC value in our study is spring, facultative and winter group. PIC showed strong significant correlation with the number of alleles per loci (r = 0.580) (not shown) but PANDEY *et al*., (2006) reported stronger correlation (r = 0.760) obtained in the study of barley genetic diversity.

Molecular analysis among the three growth habit groups showed that genetic diversity was higher in the spring growth habit group than in the facultative and winter group, and can be possibly related to adaptation of spring growth habit accessions to a broad range of environmental conditions. The lower level of polymorphism in the winter growth habit group may be attributed to the narrower genetic diversity of this group, as 48 of 52 originated from the same country (Serbia). MIKEL and KOLB (2008) showed that genetic diversity estimated with coefficient of parentage was substantially lower for six-row malting barley versus two row malting barley, but among feed types a greater diversity was present within six-rowed than within two-rowed types. For German winter barley cultivars, a slight decrease of genetic diversity over time was found for six-rowed cultivars; whereas in two-rowed cultivars a considerable increase was detected according to ORDON *et al.* (2005). Contrary to our results MALYSHEVA-OTTO *et al.* (2007) between-group component accounted only for 19.48% and 16.98% of the total variation for row type and growth habit groups, respectively. Also SOLEIMANI *et al*. (2005) reported for barley grown in the USA and Canada, a 22.6% and 23.2% variance component between two-rowed versus six-rowed and spring versus winter cultivars.

Subcluster III_a and III_c included only spring types both 2-rowed and 6-rowed, IV_b included only winter types both 6- rowed and 2-rowed; II_a, III_b and IV_a included spring and facultative types both 6- rowed and 2-rowed. According to HAMZA *et al.* (2004) two-rowed barley cultivars were clustered together and formed a distinct group. Cluster separation of North American two-row barley and six-row barley accessions were reported by SUN *et al*., (2011). The associations of genotypes belonging to different growth habit and row type groups were assessed

using PCoA revealing separation of the winter growth habit group from the facultative group. Acording to PILLEN *et al*., (2000) the first principal coordinate clearly differentiated winter and spring barley forms. Model-based estimates hold an advantage over distance-based estimates because structural membership proportions are quantitatively assigned, which account for admixture in individuals that may be assigned to more than one sub-population. Of the 145 accessions 83.33% were assigned to one of the two sub-populations, the remainder was of mixed-origin. This procedure has been used for clustering of collections of barley genotypes and for evidence of population substructure (ORDON *et al*., 2005; PANDEY *et al*., 2006; SUN *et al*., 2011).

From the study conducted it was concluded, that barley accessions fingerprinting with 15 EST SSR primers were sufficient enough to distinguish between 145 genotypes from the Serbian barley GenBank. Marker based identification and selection of the diverse genotypes could be helpful both for the development of new barley varieties. Usability of this collection regarding detection of novel resistance genes (PEROVIC *et al*., 2001; KÖNIG *et al*., 2012) was already confirmed but current results enable more efficient screening procedures in the future. Although some of the accessions named MBR contains very useful genes, their passport data could not be found in the GeneBank documentation.

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MOLEKULARNA KARAKTERIZACIJA GENOTIPOVA JEČMA (*Hordeum vulgare* L.) IZ GEN BANKE SRBIJE SSR MARKERIMA

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Izvod

Molekularni diverzitet 145 genotipova ječma (*Hordeum vulgare* subsp. *vulgare* L.) iz Gen Banke Srbije je procenjen SSR markerima. Korišćeno je SSR markera sa svih hromozoma diploidnog genoma ječma u proseku 2-3 markera po hromozomu i sa 4-18 alela po lokusu. Utvrđeno je 15 lokusa i 119 alela sa prosekom od 7.93 alela po lokusu. PIC je bio u opsegu od 0.220 do 0.782 sa prosekom 0.534. Genski diverzitet je bio veći za jare (0.616) i šestorede genotipove (0.616) u odnosu na ozime i dvorede (0.322 i 0.478). Analizom molekularne varijanse utvrđena je značajnost svih izvora variranja ($P < 0.01$), ali je međugrupna komponenta dominirala. Primenom metoda UPGMA analize zasnovane na zajedničkoj distanci alela (D_{SA}) na osnovu SSR podataka dobijeno je grupisanje genotipove u dva klastera-jedan manji koji sadrži šest šestoredih jarih genotipova ječma i drugi koji je obuhvatao šest podklastera. Genotip MBR1012 je bio odvojen od svih ostalih genotipova. Korišćenjem metoda Osnovna Analiza Koordinata dobijeno je razdvajanje ozimih genotipova ječma od fakultativnih. Utvrđeno je postojanje dve podpopulacije genotipova primenom algoritma grupisanja STRUCTURE.

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