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ASSESSMENT OF GENETIC DIVERSITY AMONG BARLEY VARIETIES WITH DIFFERENT ORIGIN USING SIMPLE SEQUENCE REPEAT (SSR) MARKERS

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Abstract

Barley (*Hordeum vulgare* L.) is one of the principal cereal crops in the world, because it is used as raw material in beer production, animal feed and human consumption. The propose of this research was to determine the genetic diversity among barley genotypes developed in different counties using the SSR marker. Twenty-one two row barley varieties with different origin were used as an experimental material. Three varieties and two promising lines were Macedonian, two varieties were Serbian, two varieties had Croatian origin and the other twelve varieties were developed in Bulgaria. Eighteen SSR markers were used to characterize the genetic diversity among the barley varieties and two of them had high polymorphisms (MGB402 and MGB318). The calculated polymorphism information content (PIC) values range from 0.163 to 0.574, which shows the importance of the markers for future diversity analysis of barley. Primer MGB318 showed the highest PIC and for MGB391 was obtained the lowest PIC. Using Unweight Pair Group Method, dendrogram tree was constructed and all studied genotypes were divided in three main groups, branched into sun-subgroup. These results can be useful for barley germplasm management and design of new crosses for future breeding propose.

Key words: *barley*, *simple sequence repeat*, *genetic diversity*, *polymorphism information content*, *varieties*

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most economic and important cereal crops in the world, because it is used as raw material in beer production, animal feed and human consumption. By area and production, barley is ranked as the fourth most produced variety, followed by wheat, rice and corn (Ferreira et al., 2016). It is considered one of the best adapted cereals due to it is tolerance to salinity, low temperatures, limited water and nutrients requirements.

The primary goal in the breeding programme is crop yield, whose significance is valuated through the economic benefit for the producers. In any breeding programme as a basis requisite, it is determining the distinguish among varieties of crop plants and establish their purity (Matus & Hayes, 2002). Assessment of the genetic variability within cultivated varieties has major role in plant breeding and conservation of genetic resources (Petersen et al., 1994). In order for plant improvement those information's provides right decision regarding selection of parental combinations in further hybridizations (Matus & Hayes, 2002). Actually, the study of the genetic diversity within barley germplasm has been based on morphological and physiological traits (Massood et al., 2003). Often morphological characters and their variability are influenced by environmental conditions. For several years, seed storage protein markers and isozymes have been used as tool to enhance barley variety

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identification capabilities (Liu et al., 2000; Yin et al., 2003). Molecular markers which evaluate genetic diversity at molecular level and assess the variability are not affected by plant's phenotype and the environmental factors which make them more powerful in genetic studies. Various DNA profiling methods have been established and currently are available. Plant breeders usually use multi locus techniques based on PCR and DNA molecular markers as tools for directly evaluating the genetic variation among related varieties without effect of environmental factors. In addition, DNA techniques allow the assessment of unlimited number of polymorphic marker loci (Nguyen et al., 2004). The most informative polymorphic available markers are microsatellite or simple sequence repeats (SSR). The other molecular markers used to evaluate the extent of genetic diversity among the varieties are Random Amplified Polymorphic DNA (Eshghi & Akhundova, 2010), Amplified Fragment Length Polymorphism (Assefa et al., 2007), Single Nucleotide Polymorphism (Soleimani et al., 2007), Inter Simple Sequence Repeats (Rashal et al., 2004) and Simple Sequence Repeats (Matus et al., 2002; Chaabane et al., 2009; Jaiswal et al., 2010; Sípahí, 2011). SSR markers are the choice in many studies because of their multi allelic nature, high levels of polymorphism, codominant inheritance (Deric et al., 2005; Oliveira et al., 2006), highly variable and able to distinguishes closely related plant cultivars, easily assayed by PCR (Ramsey et al., 2000).

The purpose of the present research was to determine the genetic diversity among twenty-one two row barley genotypes developed in different countries using the SSR markers.

MATERIAL AND METHODS

Plant material and field experiment

Twenty-one two row barley genotypes from different origin were carried out in this study. Three varieties and two promising lines are Macedonian (Hit, Izvor, Egej, Line 1 and Line 2), two varieties are Croatian (Zlatko and Rex), two varieties are Serbian (NS 525 and NS 565) and the other twelve varieties are with Bulgarian origin (Obzor, Rerun, Emon, Lardeya, Orfej, Imeon, Zagorec, Asparuh, Kuber, Sayra, Deviniya and Odisej) (Tab. 1).

Table 1. Barley varieties used in the experiment, their origin and end use.

No.	Name of genotype	Origin	End use
1	Hit	Macedonia	Fodder
2	Izvor	Macedonia	Fodder
3	Egej	Macedonia	Fodder
4	Line 1	Macedonia	Fodder
5	Line 2	Macedonia	Fodder
6	Zlatko	Croatia	Fodder
7	Rex	Croatia	Fodder
8	NS 525	Serbia	Malt
9	NS 565	Serbia	Malt
10	Obzor	Bulgaria	Malt
11	Perun	Bulgaria	Malt
12	Emon	Bulgaria	Malt
13	Lardeya	Bulgaria	Malt
14	Orfej	Bulgaria	Malt
15	Imeon	Bulgaria	Malt
16	Zagorec	Bulgaria	Malt
17	Asapruh	Bulgaria	Malt
18	Kuber	Bulgaria	Malt
19	Sayra	Bulgaria	Malt
20	Deviniya	Bulgaria	Malt
21	Odisej	Bulgaria	Malt

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DNA extraction

After germination and seedlings emergence, fresh leaves were taken from a single plant from each genotype in order to extract deoxyribonucleic acid (DNA). Young leaves of the seedlings were transferred to 2 ml Eppendorf tubes containing stainless steel beads and immediately frozen in liquid nitrogen. DNA was extracted by CTAB method described by (Doyle & Doyle, 1987). The extraction buffer was composed of 20 mM EDTA (pH 8.0), 100 mM Tris-HCL (pH 8.0), 1.4 mM NaCl, 2% CTAB, 0.5% Na Bisulfite, 1% β mercaptoethanol. The quantitative and qualitative assessment of the DNA was done by spectrophotometer. Electrophoresis was applied at the agarose gel with concentration of 0.8% to know the quality of the used DNA.

Microsatellite (SSR) amplification

Eighteen microsatellite primers pairs were selected on the basis of their chromosomal location. Their names, nucleotide sequences and chromosomal locations are given in Table 2. Polymerase chain reaction (PCR) reactions were performed in 25 µl reaction mixture, each containing a final concentration of 75 ng DNA, 2.5 µl of 1X Taq Reaction Buffer, 2 µl of 1.5 mM $MgCl_2$, 0.125 µl of 5 Units of Taq of DNA Polymerase, 0.5 µl of 0.2 mM dNTPs and 1µl of 1µM of each primer. The amplifications were carried out in an Eppendorf Master cycler. Depending of the different primer combinations used, DNA amplifications were performed using the following cycling parameters: one cycle of 95°C for 3 min, 35 cycles of 1 min denaturing step at 94°C, 1 min annealing temperatures between 52 and 56°C (optimized individually for each SSR, Tab. 2) and 2 min extension at 72°C. After the final cycle, samples were incubated at 72°C for 10 min to ensure the complete extension.

Gel electrophoresis

The amplified PCR products were separated by electrophoresis using 3% agarose gel, 1xTBA buffer, then stained with ethidium bromide (10mg/ml) and visualized under UV transilluminator. A DNA ladder was injected to determine the molecular weight of the resulting bands.

Statistical analysis

The results of the amplification process were collected based on the comparation of the presence or absence of DNA bands. The cluster analysis was done using Unweighted Pair Group Method (UPGMA) (Tamura et al., 2011) and the values of the polymorphism information content (PIC) were calculated. PIC is strong coefficient which give an estimation to discriminate a group of genotypes studied, by taking not only the number of alleles, but also the relative frequencies of each allele (Smith et al., 2000).

Table 2. Barley SSR primers, their sequence, the annealing temperature used in the PCR reaction and the chromosomal location (Von Korff et al., 2004).

Primer	Sequence	Annealing temperature (°C)	Chromosome location
MGB391	For 5' –AGCTCCTTTCCTCCCTTCC-3' Rev 5'- CCAACATCTCCTCCTCCTGA-3'	54	2 (2H)
HVITR1	For 5' –CCACTTGCCAAACACTAGACCC-3' Rev 5'- TTCATGCAGATCGGGCCAC-3'	55	3 (3H)
Bmag13	For 5'-AAGGGGAATCAAAATGGGaG-3' Rev 5'- TCGAATAGGTCTCCGAAGAAA-3'	54	3 (3H)
HV13GEIII	For 5' –AGGAACCCTACGCCTTACGAG-3' Rev 5'- AGGACCGAGAGTGGTGGTGG-3'	56	3 (3H)
HVB23D	For 5' –GGTAGCAGACCGATGGATGT-3' Rev 5'- ACTCTGACACGCACGAACAC-3'	54	4 (4H)
MGB396	For 5' –CGCTAGCTTGTTTCTCGTTTG-3' Rev 5'- TCGCATGGCATCAACTACAG-3'	-	4 (4H)
MGB402	For 5' –CAAGCAAGCAAGCAGAGAGA-3' Rev 5'- AACTTGTGGCTCTGCGACTC-3'	55	5 (1H)

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Bmag 149	For 5' –CAAGCCAACAGGGTAGTC-3' Rev 5'- ATTCGGTTTCTAGAGGAAGAA-3'	-	5 (1H)
HVGLUEND	For 5' –TTCGCCTCCATCCCACAAAG-3' Rev 5'- GCAGAACGAAAGCGACATGC-3'	-	5 (1H)
MGB371	For 5' –CACCAAGTTCACCTCGTCCT-3' Rev 5'- TTATTCAGGCAGCACCATTG-3'	56	6 (6H)
MGB 356	For 5' –TGGTCTGGAGCTCTCAACAG-3' Rev 5'- AAGCCACATTGAAGGAGCAC-3'	-	6 (6H)
EBmac624	For 5' –AAAAGCATTCAACTTCATAAGA-3' Rev 5'- CAACGCCATCACGTAATA-3'	54	6 (6H)
Bmag 210	For 5' –ACCTACAGTTCAATAGCTAGTACC-3' Rev 5'- GCACAAAACGATTACATCATA-3'	-	6 (6H)
MGB 384	For 5' –CTGCTGTTGCTGTTGTCGTT-3' Rev 5'- ACTCGGGGTCCTTGAGTATG-3'	-	7 (5H)
BMS02	For 5' –AGAGTAGTGGAAAGAAAGTT-3' Rev 5'- TGGTAGTGAGATGAGGTGAC-3'	-	7 (5H)
MGB318	For 5' –CGGCTCAAGGTCTCTTCTTC-3' Rev 5'- TATCTCAGATGCCCCTTTCC-3'	55	7 (5H)
MGB357	For 5' –GCTCCAGGGCTCCTCTTC-3' Rev 5'- AGCTCTCTCTGCACGTCCTT-3'	52	7 (5H)
GMS1	For 5' –CTGACCCTTTGCTTAACATGC-3' Rev 5'- TCAGCGACAAACAATAAAGG-3'	55	7 (5H)

RESULTS AND DISCUSSION

An assessment of genetic diversity among varieties with SSR markers in different crop, including barley is a subject of work in a lot of researches. SSR markers are used to determinate the degree of genetic variability between and within barley populations, primary due to the high annealing temperatures during PCR amplification (Jaiswal et al., 2010). By applying SSR markers in the researches of Baek et al. (2003), Koebner et al. (2003) and Malysheva-Otto et al. (2006) were determinate a significant difference between the studied barley genotypes. Greater allelic diversity has been detected in wild compared in cultivated barley (Ledovskoy et al., 2010; Nandha & Singh, 2014).

In this study, for molecular characterization, eighteen pair of simple sequence repeats (SSR) technique were applied, and the number of total and polymorphic alleles are given in Table 3. Based on the obtained results from the SSR markers, the genetic divergence and closeness among all genotypes, was calculated. The largest number of polymorphic alleles was obtained from primer combinations MGB402 and MGB318 (Tab. 3). The primer combination MGB402 in Chaabane at al. (2009) research also proved to be suitable for obtaining the largest number of polymorphic alleles. The degree of polymorphism is expressed through PIC (Polymorphism Information Content). In Table 3 are given the PIC values for each SSR marker. The highest PIC was obtained for primer combination MGB318 (0,574) and the lowest for primer combination MGB391 (0,163).

Figures 1, 2 and 3 showed the polymorphic alleles using primers MGB318, MGB391 and MGB402.

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Table 3. Used primers, total and polymorphic amplified fragments for primer pairs and polymorphism information content (PIC) values.

Primer	Sequence	Amplified fragments		PIC
		Total alleles	Polymorphic alleles	
MGB391	For 5' –AGCTCCTTTCCTCCCTTCC-3' Rev 5'- CCAACATCTCCTCCTCCTGA-3'	1	1	0,163
HVITR1	For 5' –CCACTTGCCAAACACTAGACCC-3' Rev 5'- TTCATGCAGATCGGGCCAC-3'	1	1	0,172
Bmag13	For 5'-AAGGGGAATCAAAATGGGaG-3' Rev 5'- TCGAATAGGTCTCCGAAGAAA-3'	1	1	0,170
HV13GEIII	For 5' –AGGAACCCTACGCCTTACGAG-3' Rev 5'- AGGACCGAGAGTGGTGGTGG-3'	1	1	0,183
HVB23D	For 5' –GGTAGCAGACCGATGGATGT-3' Rev 5'- ACTCTGACACGCACGAACAC-3'	1	1	0,174
MGB396	For 5' –CGCTAGCTTGTTTCTCGTTTG-3' Rev 5'- TCGCATGGCATCAACTACAG-3'	1	1	0,175
MGB402	For 5' –CAAGCAAGCAAGCAGAGAGA-3' Rev 5'- AACTTGTGGCTCTGCGACTC-3'	3	3	0,402
Bmag 149	For 5' –CAAGCCAACAGGGTAGTC-3' Rev 5'- ATTCGGTTTCTAGAGGAAGAA-3'	2	1	0,192
HVGLUEND	For 5' –TTCGCCTCCATCCCACAAAG-3' Rev 5'- GCAGAACGAAAGCGACATGC-3'	1	1	0,189
MGB371	For 5' –CACCAAGTTCACCTCGTCCT-3' Rev 5'- TTATTCAGGCAGCACCATTG-3'	1	1	0,182
MGB 356	For 5' –TGGTCTGGAGCTCTCAACAG-3' Rev 5'- AAGCCACATTGAAGGAGCAC-3'	1	1	0,171
EBmac624	For 5' –AAAAGCATTCAACTTCATAAGA-3' Rev 5'- CAACGCCATCACGTAATA-3'	2	1	0,195
Bmag 210	For 5' –ACCTACAGTTCAATAGCTAGTACC-3' Rev 5'- GCACAAAACGATTACATCATA-3'	1	1	0,188
MGB 384	For 5' –CTGCTGTTGCTGTTGTCGTT-3' Rev 5'- ACTCGGGGTCCTTGAGTATG-3'	1	1	0,179
BMS02	For 5' –AGAGTAGTGGAAAGAAAGTT-3' Rev 5'- TGGTAGTGAGATGAGGTGAC-3'	1	1	0,184
MGB318	For 5' –CGGCTCAAGGTCTCTTCTTC-3' Rev 5'- TATCTCAGATGCCCCTTTCC-3'	4	3	0,574
MGB357	For 5' –GCTCCAGGGCTCCTCTTC-3' Rev 5'- AGCTCTCTCTGCACGTCCTT-3'	2	1	0,269
GMS1	For 5' –CTGACCCTTTGCTTAACATGC-3' Rev 5'- TCAGCGACAAACAATAAAGG-3'	1	1	0,189
Average				

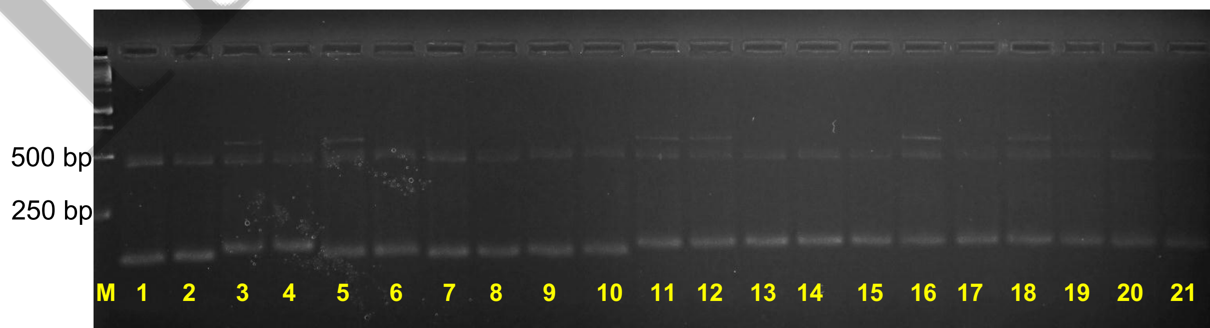

Figure 1. Agarose gel observe the polymorphism resulting from using MGB318 marker in all studied barley genotypes.

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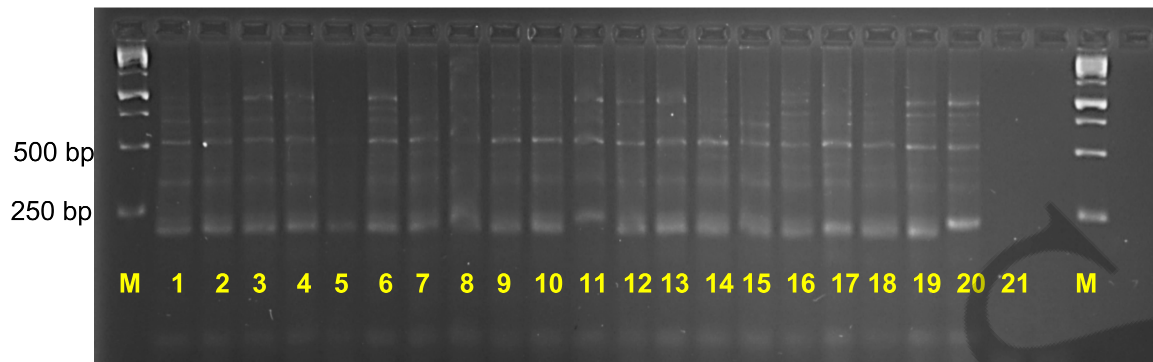

Figure 2. Agarose gel observe the polymorphism resulting from using MGB391 marker in all studied barley genotypes.

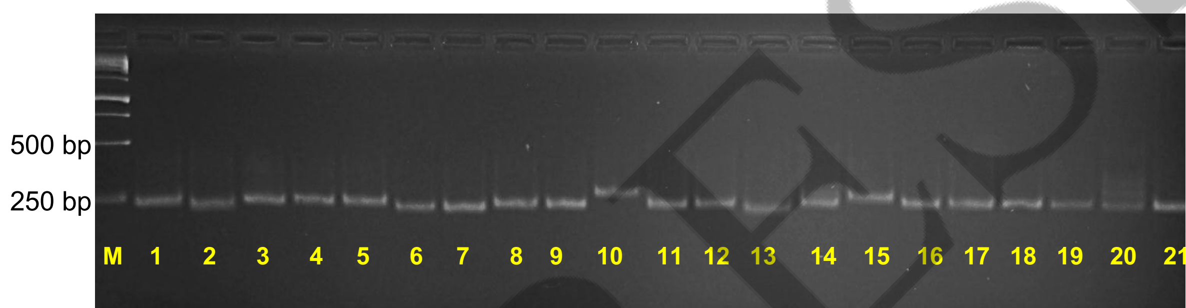

Figure 3. Agarose gel observe the polymorphism resulting from using MGB402 marker in all studied barley genotypes

Legend: M. Marker; *1. Hit*; 2. *Izvor*; 3. *Egej*; 4. *Line 1*; 5. *Line 2*; 6. *Zlatko*; 7. *Rex*; 8. *NS 525*; 9. *NS 565*; 10. *Obzor*; 11. *Perun*; 12. *Emon*; 13. *Lardeya*; 14. *Orfej*; 15. *Imeon*; 16. *Zagorec*; 17. *Asparuh*; 18. *Kuber*; 19. *Sajra*; 20. *Devinija*; 21. *Odisej.*

Cluster analysis of the studied barley genotypes resulting from the use of SSR technology allows to divide the examinated varieties into groups reflecting the degree of genetic kinship between them and genotypes may be grouped into one group based on their original habitat, trait and origin. A cluster analysis of the obtained results was conducted with the aim of creating a genetic kinship tree to determinate the degree of genetic kinship and drawing a dendrogram between the studied genotypes.

The studied genotypes were divided into three main clusters and within each of them there were smaller divisions of the genotypes into smaller groups (Fig. 4.). The first cluster includes eight genotypes (Izvor, NS 565, Obzor, Zlatko, Egej, Rex, NS 525 and Perun), the second was composed by two genotypes (Orfej and Zagorec) and the third cluster was made up from the eleven genotypes (Odisej, Emon, Imeon, Kuber, Line 2, Asparuh, Sajra, Devinija, Lardeya, Line 1 and Hit).

According to Al-Khalaf et al. (2020), the examinated barley varieties were divided in two main clusters, branched into sub-clusters.

The genotypes from the first cluster have different origin. Within this cluster, two smaller groups were separated, one between NS 565 and Zlatko, while the second group was consisted of Rex and Perun. The genotypes NS 565 and Zlatko were related and belong to the same cluster also in cluster from Principal Component Analysis (PCA), based on yield components and grain yield (Markova Ruzdik et al., 2015). Genetically the most distant from the first cluster were Izvor and Perun.

The second cluster was consisted of two genotypes (Orfej and Zagorec) which have Bulgarian origin and showed similar values for PCA (Markova Ruzdik et al., 2015).

The genotypes from the third cluster were domestic and introduced from Bulgaria. Whitin this cluster, the Bulgarian varieties Devinija and Imeon were separated like sub-cluster (group). The other genotypes from this cluster showed greater genetic distance and the most distant were Hit and Odisej (Fig. 4).

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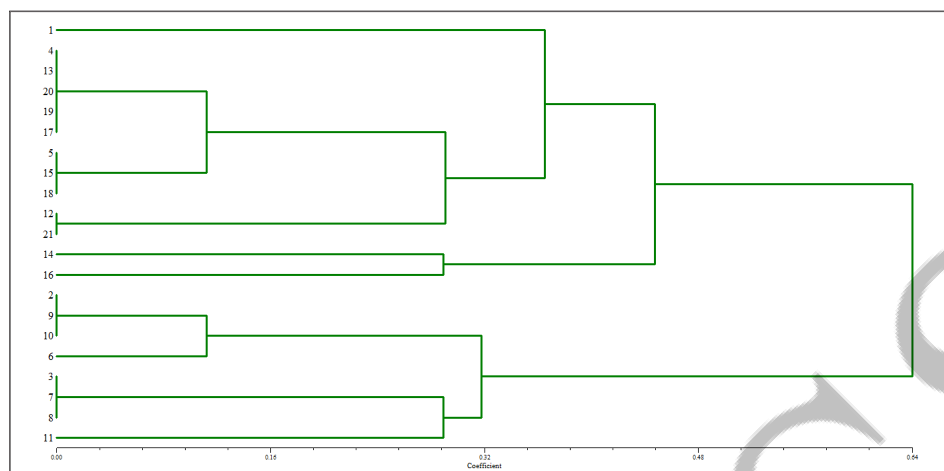

Figure 4. Cluster analysis of studied barley genotypes using SSR technique

Legend: *1. Hit*; 2. *Izvor*; 3. *Egej*; 4. *Line 1*; 5. *Line 2*; 6. *Zlatko*; 7. *Rex*; 8. *NS 525*; 9. *NS 565*; 10. *Obzor*; 11. *Perun*; 12. *Emon*; 13. *Lardeya*; 14. *Orfej*; 15. *Imeon*; 16. *Zagorec*; 17. *Asparuh*; 18. *Kuber*; 19. *Sajra*; 20. *Devinija*; 21. *Odisej*.

CONCLUDING REMARKS

The used SSR technique showed to be effective in distinguishing among twenty-one studied barley genotypes. Eighteen SSR primers were applied had have different polymorphism. The highest value for polymorphism information content was obtained for primer MGB318, which had the largest number of polymorphic alleles, followed by primer combination MGB402. The constructed dendrogram tree divided barley genotypes according the degree of genetic kinship into three main clusters, branched into sub-cluster. The presence of a genetic diversity between the genotypes used in this study makes them more suitable for breeding and genetic improvement programmes.

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ПРОЦЕНА НА ГЕНЕТСКАТА РАЗНОВИДНОСТ ПОМЕЃУ СОРТИ ОД ЈАЧМЕН КОИ ИМААТ РАЗЛИЧНО ПОТЕКЛО СО КОРИСТЕЊЕ НА ЕДНОСТАВНИ ПОВТОРУВАЧКИ СЕКВЕНЦИ (SSR МАРКЕРИ)

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Резиме

Јачменот (*Hordeum vulgare* L.) претставува една од главните житни култури во светот бидејќи се користи како суровина во производството на пиво, како храна за животните и во исхраната на луѓето. Целта на ова истражување беше да се утврди генетската разновидност помеѓу сортите од јачмен кои имаат различно потекло со користење на молекуларни маркери (SSR). Како експериментален материјал беа користени вкупно дваесет и една сорта од двореден јачмен, од кои три сорти и две линии се македонски, две сорти се хрватски, две сорти се српски, а дванаесет сорти имаат бугарско потекло. Осумнаесет SSR маркери беа употребени за процена на генетската разновидност помеѓу испитуваните генотипови, а два од нив покажаа висок степен на полиморфност (MGB402 и MGB318). Пресметаните вредности за полиморфната генетска содржина (PIC) се движеа од 0,163 до 0,574 што покажува на важноста на користењето на маркерите за идна анализа во генетската разновидност кај јачменот. Прајмерот MGB318 покажа највисока вредност за полиморфната генетска содржина, додека најмала беше добиена за прајмерната комбинација MGB391. Користејќи го методот на парови беше конструиран дендрограм, со кој сите испитувани генотипови беа поделени во три главни кластери (групи) и подгрупи во рамките на секој кластер. Овие резултати можат да бидат корисни за одржување на гермплазмата на јачменот, но и за создавање нови крстоски во идните селекциски програми.

Клучни зборови: *јачмен, едноставна повторувачка секценца, генетска разновидност, полиморфна генетска содржина, сорти.*

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