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SUMMARY

Sustaining and developing of genetic diversity in winter wheat germplasm is one of the main prerequisite for success in future winter wheat breeding programs. Selection of diverse parents is essential for creation of superior new varieties. Danger of genetic erosion specially exists in smaller breeding programs and selection for limited production area with similar growing conditions. Croatian winter wheat breeding has a long tradition but it is relatively small compared to countries with bigger growing areas. Use of molecular markers in evaluating genetic diversity of winter wheat varieties is limited in Croatian breeding programs. Therefore, aim of this study was to evaluate genetic diversity of Croatian winter wheat varieties using SSR and AFLP markers as a powerful tool for assessing genetic diversity. Forty winter wheat varieties, from three Croatian breeding centres and foreign centres were included in the study. A set of 26 microsatellite primers were used, covering three wheat genomes and 42 chromosomes by 0.66 average genetic distance. The largest calculated distance was between the varieties Zlatna Dolina and Lela (d_{ij}=0.98), while most similar varieties were Super Žitarka and Barbara with the distance value of d_{ij}=0.21. Four AFLP marker combinations generated 108 polymorphic bands with average of 34 specific bands per primer combination. On the average 27 polymorphic bands were generated with average PIC value of 0.34. Specific polymorphic bands were discriminant for the three varieties Žitarka, Super Žitarka and Barbara, which, therefore, can be used for their identification. Grouping of varieties was in accordance with their origin (breeding centre) and pedigree data.

Key-words: wheat, genetic diversity, SSR, AFLP

INTRODUCTION

Genetic diversity relies on very complex statistical approach that involves variance among alleles at single gene loci, among several loci, between individuals within population and among populations (Smale, 1997). Nevertheless genetic base of crop plants population is also associated with responses to selection pressures. So plant breeding is based on detection and application of genetic variation.

Analysis of genetic diversity among elite germplasms can offer predictive estimates of genetic variability between segregating progeny for pure line cultivar development (Manjarrez-Sandoval et al., 1997). There are several methods to estimate genetic diversity: morphological, agronomical, economical, pedigree, biochemical and molecular. The use of molecular markers is now broadly used in breeding programs because of their subjectivity exclusion. In many studies combination of several diversity criteria for more accurate estimation is used (Solemani et al., 2002; Marić et al., 2004; Stodart et al., 2005). In bread wheat two marker systems were extensively used in recent years for mapping and diversity estimates, the microsatellites or simple sequence repeat (SSR) and amplified fragment length polymorphisms (AFLPs). Microsatellites are ubiquitous in plants (Powell et al., 1996), and have very high level of polymorphism and ability to distinguish close related genotypes (Morgante and Olivieri, 1993; Plaschke et al., 1995). AFLP method (Vos et al., 1995) has also proved to

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be effective and reproducible (Barett and Kidwell, 1998; Buerstmayr et al., 2002); it also gives a very large number of scorable bands which enhances the power to detect polymorphism. Combination of techniques can increase the information content gained to a point where large amounts of beneficial data may be acquired for population containing many individuals (Manifesto et al., 2001; Stodart et al., 2005). SSR markers offer high resolution in population structure while AFLPs offer high resolution in genetic relationships at the individual level (Maccafery et al., 2007; Karsai et al. 2011). The aims of this study were a) to evaluate application of microsatellites and AFLP markers in diversity estimation and b) to evaluate genetic diversity of Croatian winter wheat varieties.

MATERIAL AND METHODS

Plant material and DNA extraction

Total of 40 winter bread wheat varieties, registered in Croatia from 1931 to 2008, were used in this study. Varieties originate from Croatia (CR), Austria (AU), France (FR), Italy (IT) and Russia (RU) (Table 1). Varieties of Croatian origins came from three breeding centres Agricultural Institute Osijek (AIO), BC Institute in Zagreb (BC) and Jošt seed Ltd. in Križevci (JS). Twenty plants per genotype were grown in a greenhouse during 20 days. DNA was isolated by CTAB method (Doyle and Doyle, 1987) modified by Grljušić, (2003). DNA concentration was measured using Thermo Scientific NanoDrop 2000® spectrophotometer, while DNA quality was determined by electrophoresis with standard λ-DNA. Two methods were used to estimate genetic diversity of the tested varieties (a) SSR (Simple Sequence Repeat) and (b) AFLP (Amplified Fragment Length Polymorphism).

SSR and AFLP procedure

Twenty six microsatellite primer pairs created by Röder et al. (1998) were used. PCR amplification was carried out according to Röder et al. (1998) modified for LI-COR® Biosciences 4200 DNA Analyzer, while GeneAmp® Thermocycler 9700 was used for PCR reaction. PCR conditions for SSR analysis were: step 1: 5 min at 94°C; step 2: 5 cycles of 45 sec at 95°C, 5 min at 68°C (-2°C by cycle) and 1 min at 72°C; step 3: 5 cycles of 45 sec at 95°C, 2 min at 58°C (-2°C by cycle) and 1 min at 72°C; step 4: 27 cycles of 45 sek at 95°C, 75 sec at 45°C and 1 min at 72°C and final step 5: 10 min at 72°C. For AFLP procedure genomic DNA was digested using restriction enzymes *Sse8371* and *MseI*. Adapters with no selective nucleotides were used in pre-selective PCR reaction. Next step was amplification with primer pairs labelled with IRDye® (700 and 800CW) and each with two selective nucleotides (Buerstmayr et al. 2002) with PCR conditions as follow: step 1: 2 min at 94°C; step 2: 10 cycles of 30 sec at 94°C, 30 sec at 63°C (-1°C per cycle till 54°C) and 2 min at 72°C, and final step 3: 23 cycles of 30 sec at 94°C, 30 sec at 54°C and 2 min at 72°C. PCR products were separated on 6% acrylamide gel using a LI-COR® Biosciences 4200 DNA Analyser. Four AFLP reactions were used in the wheat collection, which are designated with the abbreviations of the four selective nucleotides (TCAT, GCAT, TCGA, and GCGA). Fragment

Table 1. Name, origin and pedigree of the tested varieties

Tablica 1. Ime, podrijetlo i pedigre ispitivanih sorata

Variety *Sorta*	Origin *Podrijetlo*	Pedigree *Pedigre*	Variety *Sorta*	Origin *Podrijetlo*	Pedigree *Pedigre*
U1	CR (AIO)	Carlotta strampeli/Marquis	Janica	CR (AIO)	Osk. 5.36-9-91/Srpanjka
Os.crvenka	CR (AIO)	Libellula/Bezostaja	Barbara	CR (AIO)	GO 3135/Žitarka
Os.20	CR (AIO)	Osk. 6.9-1-64/V-188-M	Katarina	CR (AIO)	Osk.5.B.4-1-94/Osk. 5.140-22-91
Slavonija	CR (AIO)	Osječka 20/Osk.4.216-2-76	Alka	CR(AIO)	Osk. 5.140-22-91/Sana
Zitarka	CR (AIO)	Osk. 6.30-20/Slavonka/3/Eph. M68/Osk. 154-19/Kavkaz	Seka	CR (AIO)	Srpanjka/Demetra
Srpanjka	CR (AIO)	Osk. 4.50-1-77/Zg 2696	Lela	CR (AIO)	Srpanjka/Osk. 5.136-8-90
Demetra	CR (AIO)	Osk. 4.216-2-76/Zg 2877-74	Sana	CR (BC)	Mura/CI14123//Zg241372
Su.zitarka	CR (AIO)	GO 3135/Žitarka	Adriana	CR (BC)	ZG 1758-70/TpR-349
Lucija	CR (AIO)	Srpanjka/Kutjevčanka	Divana	CR (JS)	Favorit/5/Cipriz/4/J.Kwang/2/Atlas66/ Comanc./3/Velvet
Renata	CR (AIO)	Žitarka /2/Osk.7.5-4-82/KB160-86/3/ Srpanjka	Libellula	IT	Tevere/Giuliari//San Past.
Aida	CR (AIO)	Srpanjka/Rialto	Bezostaja	RU	Skorospelka2/Lutescens17
Pipi	CR (AIO)	Soissons/Osk. 6.83-5-91	BC Patria	CR (BC)	Odesskaya-51/ZG-IPK-8210/2/GK-32-82
Ilirija	CR (AIO)	Osk.14.294-16-95/Soissons	BC Elvira	CR (BC)	Bc 2377-79/MV-C2-33//Irena
Felix	CR (AIO)	Srpanjka/K160/86	Soissons	FR	Iena/HN 35
Zlata	CR (AIO)	Srpanjka/Demetra	Valerius	AU	Carolus//Monopol/Karat// Ekspert/Severin
Anđelka	CR (AIO)	Srpanjka/Demetra	Antonius	AU	Pokal/Karat//Ekspert/Severin
Mihaela	CR (AIO)	Srpanjka/Osk. 5.136-11-90	Bastide	FR	Fertil/Arche
Ružica	CR (AIO)	Osk. 5.36-9-91/Srpanjka//Brea	Edison	AU	Agron/Regent//Capo
Zl.dolina	CR (BC)	Zg 414-57/Leonardo	Eurofit	AU	Pegassos/Kontrasst
Golubica	CR(AIO)	Slavonija/Gemini	Ludwig	AU	Ares/Farmer

analysis of SSR and AFLP amplification were carried out on 6% polyacrylamide gel using LI-COR 4300 analyser in 10× TBE buffer. Fragments of known size were used as standards, for microsatellites IRDye® 350CW, and for AFLP fragments IRDye® 700CW and IRDye® 800CW. Gel analysis of SSR allele sizes were carried out in SAGA^{GT} genotyping software program ver 3.2. (LI-COR® Biosciences Saga unix 1.0) while for AFLP fragments in Kodak® 1D v.3.6.4 Scientific imaging system. All SSR alleles and all polymorphic AFLP alleles were scored for presence ('1') and absence ('0') in each accession, and the data were entered in binary matrices.

Data analysis

Genetic diversity and polymorphism of microsatellites were analysed based on total and average allele number per marker (N_a). Polymorphic information content (PIC) was estimated for each microsatellite loci and each AFLP marker combination using this formula: $PIC = 1 - \sum_{i=1}^{I} p_i{}^2 - 2\sum_{i=j+1}^{I}\sum_{j=1}^{I-1} p_i{}^2 p_j{}^2$ where p_i is the frequency of the i^{th} allele or locus, and calculated with Powermarker. Due to different marker types (SSR are codominant markers and AFLPS are dominant markers) different genetic distance measure methods were used. Binary matrices of allelic frequencies based on SSR data were used to calculate Rogers distance $d_{ij} = \frac{\sum_{s=1}^{S}\sqrt{\frac{1}{2}\sum_{g=1}^{n_{s-1}}(y_{isg}-y_{jsg})^2}}{S}$, where the y_{isg} and y_{jsg} are frequencies of the allele g on marker locus s for variates *i* and *j*, while S is total number of loci. For AFLP data, similarity coefficient (S_{ij}) is used according to Dice: $S_{ij} = \frac{2N_{ij}}{N_i + N_j}$, where N_{ij} is the number of detected fragments in genotypes *i* and *j*, N*i* is the number of fragments in the *i* genotype, while N_j is the number of fragmemnts in the *j* genotype. Distance and similarity matrices are then used to make dendrogram using Unweighted Pair Group Method Using Arithmetic Average (UPGMA). Calculations were carried out in NTSYS ver.2.2.

RESULTS AND DISCUSSION

Microsatellite analysis

Set of 26 microsatellite primer pairs were used to profile 40 varieties. The SSR markers yielded total of 108 different alleles with 3.88 alleles per locus on the average, ranging from 2 (gwm311_1) to 11 (gwm609). PIC value for 26 microsatellites ranged from 0.222 to 0.787 with an average of 0.503 (Table 2). These results are consistent with data achieved by Ahmad (2002) and Christiansen et al. (2002), and lower than those achieved by Khlestkina et al. (2004) and Landjeva et al. (2006). Quality of plant material is one of the main factors which affect the allele number per locus, it is higher when seeds from gen banks are used (Stępien et al., 2007).

Binary matrix of SSR data was used to calculate Rogers distance (d_{ij}). Average genetic distance was 0.66. The largest calculated distance was between varieties Zlatna Dolina and Lela (d_{ij}=0.98), while the most similar varieties were Super Žitarka and Barbara with distance value of d_{ij}=0.21.The dendrograms based on genetic distance confirmed high level of genetic diversity within the varieties tested (Figure 1). All varieties could be discriminated and are in line with origin and pedigree information. The most similar varieties, except the above mentioned, were Slavonija and Golubica (d_{ij}=0.27). Five major groups can be distinguished in the dendrogram. The first group consists of 10 varieties which can be further branched in two subgroups (1a and 1b). The subgroup 1a consists of 7 varieties from BC Institute and two from PIO (Alka and Katarina). Three closely related varieties are in the subgroup 1b: Žitarka, Super Žitarka and Barbara. The last two originate from crossing 'GO 3135/ Žitarka'. Similar results were also obtained by Dvojković (2009). Most of the tested varieties (20) are in the second group. This group consists of merely Croatian varieties with two exceptions, Italian variety Libellula and Russian Bezostaja, which are in background pedigrees of many new and old not only Croatian but also European varieties. Clustering of varieties in the subgroup 2a is influenced by variety Osječka 20 as one of the parents in varieties Slavonija and Golubica.

Table 2. Polymorphic information content (PIC), genome location, amplified alleles and primer designation of 26 microsatellites

Tablica 2. Mjesto lokusa na kromosomima pšenice, broj alela (N_a) i polimorfizam (PIC) za 26 mikrosatelitnih markera

Microsatellite primer *Mikrosatelitna početnica*	Locus *Lokus*	N_a	PIC
gwm2_1	3A	3	0.368
gwm2_2	3D	3	0.522
gwm11	1B	6	0.632
gwm55	6D	2	0.372
gwm68_1	5B	3	0.548
gwm68_2	7B	4	0.551
gwm121_1	5D	2	0.372
gwm121_2	7D	3	0.574
gwm135	1A	4	0.649
gwm149	4B	5	0.381
gwm169	6A	6	0.677
gwm186	5A	4	0.662
gwm257	2B	3	0.402
gwm261	2D	4	0.440
gwm311_1	2A	2	0.222
gwm311_2	2D	3	0.282
gwm458	1D	3	0.571
gwm497_1	1A	4	0.564
gwm497_2	2A	6	0.657
gwm497_3	3D	2	0.359
gwm573_1	7A	3	0.585
gwm573_2	7B	5	0.519
gwm609	4D	11	0.787
gwm610	4A	3	0.553
gwm626	6B	4	0.365
gwm642	1D	3	0.455
	Average	3.88	0.503

Certain authors explain that direction and intensity of selection can cause significant deviations from the assumption that progeny share 50% of genetic background with their parents, whereas breeder is the one that modifies selection which further changes frequencies of certain alleles in favour of one of the parents (Corbellini et al. 2002; Zhang et al., 2002). You et al. (2004) elaborates that 550 microsatellite markers is required to make reliable dendrogram in which all genetic relations between varieties with different genetic background and geographic origin can be detected. All Austrian varieties are in the fourth group except Croatian varieties Divana (because of its foreign pedigree) and Ilirija. Old variety U1 belongs to the final fifth group. Distinction of the genotypes with small genetic distance (Super Žitarka and Barbara) justifies the proper selection of DNA samples and high polymorphism of used microsatellites in the analysis of genetic diversity of related varieties, which is in accordance with the results published by Morgante and Olivieri (1993), Plaschke et al. (1995) and Bánayi et al. (2006).

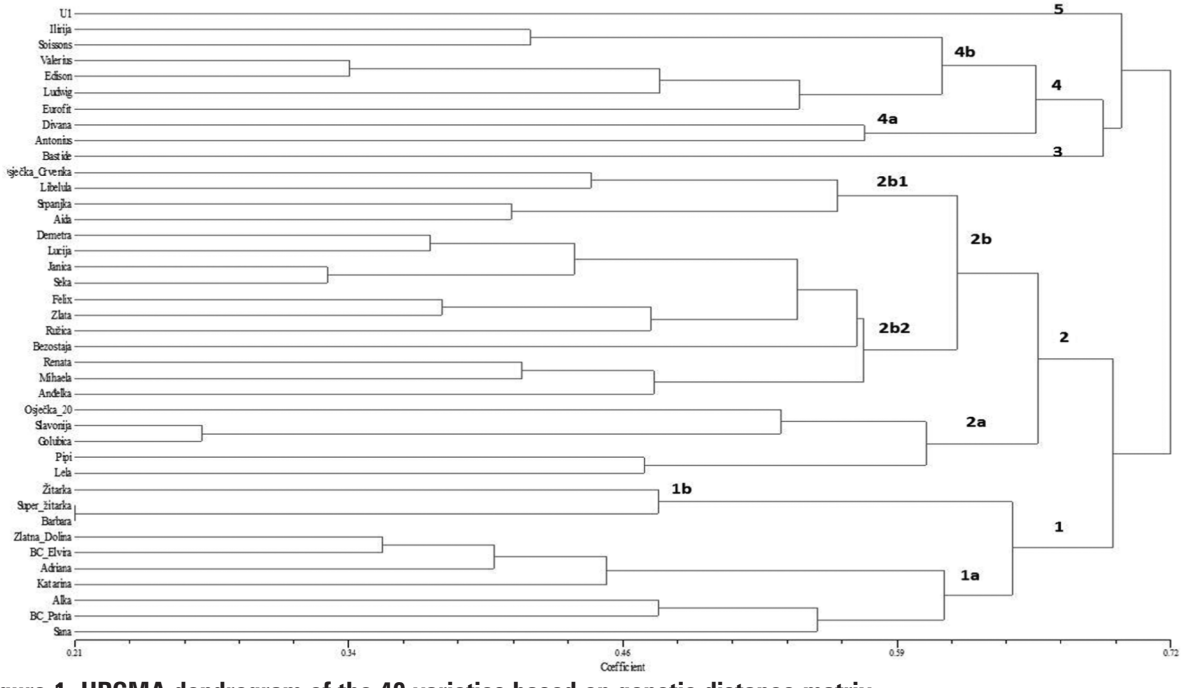

Figure 1. UPGMA dendrogram of the 40 varieties based on genetic distance matrix

Slika 1. UPGMA dendrogram za 40 genotipova temeljem matrice genetske udaljenosti

AFLP analysis

The four AFLP primer combinations produced 108 polymorphic bands. Primer combination SseTC/MseGA produced the highest number of polymorphic bands (33), while the first combination (SseTC/MseAT) produced the lowest number of twenty polymorphic bands (Table 3). On the average 27 polymorphic bands were generated with average PIC value of 0.34. Similar results were recorded by Manifesto et al. (2001) with 4 and Martos et al. (2005) with 14 primer combinations. Higher number of polymorphic bands was recorded by Stodard et al. (2005) with 133 polymorphic bands per primer combination and Vieira et al. (2007) with 44 polymorphic bands on 19 wheat accessions. The highest PIC was generated with the third primer combination (0.35). Specific AFLP bands were determined for some varieties. Primer combinations SseGC/MseAT with allele size of 215 bp, SseTC/MseGA with allele size of 530bp and SseGC/MseGA (186 bp) were determined for three varieties Žitarka, Super Žitarka and Barbara, which therefore can be used for their identification. This collaborates that specific bands can be very effective in identification of closely related lines or varieties (Macaferri et al., 2007). Binary matrix based on AFLP data was used to calculate genetic similarity between varieties using Dice coefficient (S_{ij}). All the tested varieties could be differentiated (Figure 2). Sorting of the varieties based on the AFLP data was different than the dendrogram based on the SSRs.

Table 3. AFLP primer combinations, number of polymorphic and specific bands, and PIC

Tablica 3. Kombinacije početnica za AFLP analizu, broj polimorfnih i specifičnih fragmenata i PIC

AFLP primer combination *Kombinacije AFLP početnica*	No. polymorphic bands (np_i) *Br. polimorfnih fragmenata* (np_i)	No. specific bands *Br. specifičnih fragmenata*	PIC
SseTC/MseAT	20	1	0,34
SseGC/MseAT	29	4	0,33
SseTC/MseGA	33	3	0,35
SseGC/MseGA	26	5	0,34

The varieties can be clustered in 7 groups. The sixth and the seventh group consisted of two Croatian varieties Aida and Osječka 20 with the lowest Dice coefficient of 0.20 with average genetic similarity in relation to the remained varieties of 0.44 being less than the average similarity coefficient among all varieties tested (S_{ij}=0.54). Very similar results were determined by Barrett et al. (1998) and Manifesto et al. (2001). The fifth group consisted of varieties Lela and Divana, three Croatian and one Austrian variety clustered in the third, while variety BC Elvira had singled out as the fourth group. Clustering of the second group was most influenced by variety Srpanjka, whereas varieties Lucija, Renata, Felix, Anđelka, Alka and Seka had Srpanjka in their genetic background. Most similar varieties were in the first group. Varieties in the subgroup 1b shared common genetic background, Slavonija and Golubica, which relates to the dendrogram based on the microsatellites and the results by Marić et al. (2004) who established great similarity between these two varieties in just a few RAPD bands. The highest Dice coefficient (0.81) was calculated in the subgroup 1a1 among wheat varieties Žitarka, Super Žitarka and Barbara, which also coincides with the SSR data. The mentioned varieties were also segregated with specific polymorphic AFLP bands, but it is not known if these "anonymous" bands are connected with trait loci of interest (Barett and Kidwell, 1998). AFLPs are monogenic dominant markers, so the tested varieties were not clustered according to pedigree information probably because of insufficient wheat genome coverage with these four primer combinations. SSRs are codominant markers and by that enabled better and higher resolution among the tested varieties clustering them by its origin and pedigree. Similar discrepancies in the studies were found by Manifesto et al. (2001), and Stodard et al. (2005) reported that these deviations are linked with differences between these two marker systems.

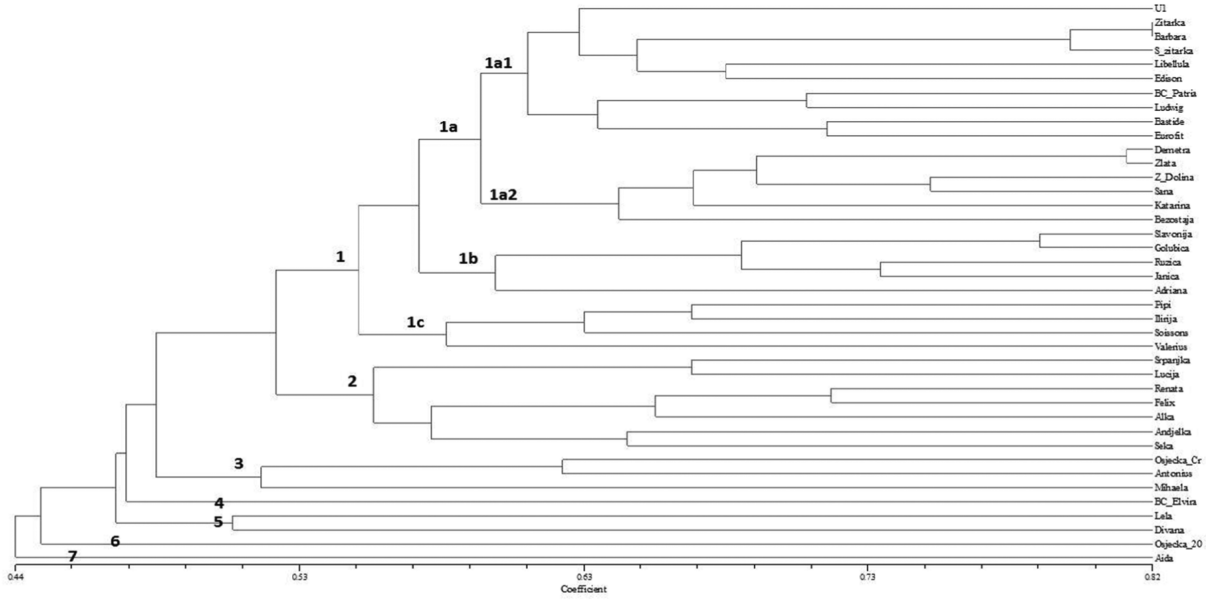

Figure 2. UPGMA dendrogram of the 40 wheat varieties based on genetic similarity matrix

Slika 2. UPGMA dendrogram za 40 genotipova temeljem matrice genetske sličnosti

Microsatellite markers are more efficient in determination of population structure, grouping the varieties almost perfectly according to pedigree, while AFLP are more adjusted to detect genetic relation at individual level (Neigel et al., 1997; Schut et al., 1997). Mészáros et al. (1996) stated that the application of markers with unknown location, such as AFLPs, rule of some chromosome regions would be overestimated. On the other hand studies by Roy et al. (2004) reported that AFLP markers showed very high effectiveness in relation to morphological and SSR markers, and that utilisation of different types of marker combination can give different genetic diversity assessments.

CONCLUSION

These results confirm significant genetic diversity estimated for the tested Croatian and foreign varieties in this study. Distinction of genotypes with small genetic distance (Super Žitarka and Barbara) justifies the proper selection of DNA samples and high polymorphism of the used microsatellites and AFLP primer combinations in the analysis of genetic diversity. Utilisation of SSR and AFLP methods was proven to be very effective in estimation of genetic diversity among the tested varieties. These results could be useful indicators and potentially valuable source for selecting parents which can be used in future crossings, and by that create new and broader genetic base in wheat breeding programs.

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PROCJENA GENETSKE RAZLIČITOSTI HRVATSKIH SORATA PŠENICE UPOTREBOM SSR I AFLP MARKERA

SAŽETAK

Razvoj i održivost genetske različitosti germplazme ozime pšenice jedan je od osnovnih uvjeta za uspjeh u budućim oplemenjivačkim programima. Odabir divergentnih i genetski različitih roditelja najvažniji je korak u stvaranju novih superiornih sorata. Opasnost od genetske erozije je pogotovo prisutna u manjim oplemenjivačkim programima koji imaju manji ograničenih proizvodnih površina u kojima se selekcija odvija u sličnim uvjetima uzgoja. Upotreba molekularnih markera u procjeni genetske različitosti sorata ozime pšenice je ograničena u hrvatskim oplemenjivačkim programima. Cilj ovoga istraživanja je bio procijeniti genetsku različitost hrvatske sorata ozime pšenice koristeći SSR i AFLP markere. U istraživanje je bilo uključeni 40 sorata ozime pšenice iz hrvatskih i stranih oplemenjivačkih centara. Korišten je set od 26 mikrosatelitnih početnica s kojima se nastojao pokriti genom pšenice sa 42 kromosoma. Prosječna udaljenost iznosila je 0,66. Najveća udaljenost zabilježena je između sorata Zlatna dolina i Lela (d_{ij}=0,98), dok su najsličnije sorte bila Super Žitarka i Barbara s genetskom udaljenosti od d_{ij}=0,21. Pomoću četiri kombinacije AFLP početnica proizvedeno je ukupno 108 polimorfnih fragmenata sa prosjekom od 34 fragmenta po kombinaciji. Prosječno je utvrđeno 27 polimorfnih alela po kombinaciji i prosječnom vrijednosti PIC od 0,34. Specifični AFLP fragmenti uspješno su razlučili tri sorte pšenice: Žitarku, Super Žitarku i Barbaru te se stoga mogu koristiti za njihovu identifikaciju. Grupiranje sorata bilo je u skladu s njihovim podrijetlom i podacima o pedigreu.

Ključne riječi: pšenica, genetska različitost, SSR, AFLP

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